Characterization of the DNA methylation patterns of chemosensitive and chemoresistant human cancer cells: Biological and clinical impact

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CHARACTERIZATION OF THE DNA METHYLATION PATTERNS OF CHEMOSENSITIVE AND CHEMORESISTANT HUMAN CANCER CELLS: BIOLOGICAL AND CLINICAL IMPACT

Memoria Tesis Doctoral

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DONE…

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CONTENTS

ABBREVIATIONS

5-AZA	5-Aza-2′-deoxycytidine
5-FU	5-fluororacil
A	
ADN	acido desoxirribonucleico
AFP	alfa-fetoprotein
AKT	v-akt murine thymoma viral oncogene homolog 1
AMBP	alpha-1-microglobulin/bikunin precursor
APC	adenomatous polyposis coli
ATP	adenosine tri-phosphate
ATP6V1G1	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G1
ATP7	ATPase, Cu^{++} transporting member A
ATPase	$sodium^{+}$, potassium- adenylpyrophosphatase, ATP monophosphatase, triphosphatase, SV40 T-antigen, adenosine 5'-triphosphatase, ATP hydrolase, complex V (mitochondrial electron transport), ($Calcium^{2+}$ + $Magnesium^{2+}$)-ATPase, $HCO3^{-}$-ATPase, adenosine triphosphatase
B	
BAX	BCL2-associated X
BCL-2	B-cell CLL/lymphoma 2
BCL-XL	BCL-2, BCL2-like 1
BEP	bleomycin+etoposide+cisplatin
BRAF	v-raf murine sarcoma viral oncogene homolog B
BRCA1	breast cancer 1, early onset
β-hCG	beta subunit human chorionic gonadotropin
C	
CAV1	caveolin 1, caveolae protein
C-FLIP	CASP8 and FADD-like apoptosis regulator
CG	Cytosine/guanine
CGH	hibridación genómica comparada
CpG	Cytosine-phosphate-Guanine
D	
DNA	Deoxyribonucleic acid
DNMT	DNA (cytosine-5-)-methyltransferase
E	
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERBB	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog
F	
FAM111A	family with sequence similarity 111, member A
FAM84A	family with sequence similarity 84, member A
FAP	Familiar Adenomatous Polyposis
FAS	Fas cell surface death receptor
FOLFIRI	irinotecan+5-FU+ leucovorin
FOLFOX	oxaliplatin+5-FU+ leucovorin
FOLFOXIRI	5-FU+leucovorin+oxaliplatin+irinotecan
G	
GC	guanine-citosine
GCS	glucosyl ceramide synthethase
H	
HDAC	histone deacetylases
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
K	
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KRAS	Kirsten rat sarcoma viral oncogene homolog [Homo sapiens
L	
LDH	lactate dehydrogenase
M	

MBD	methylated CpGs, the methyl CpG binding proteins
MGMT	O^6-methylguanine DNA methyltransferase
MLH1	mutL homolog 1
MMR	mismatch repair
MSI	microsatellite instability
MTIC	5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide
MSP	reacción en cadena de la polimerasa, especifica de metilación; methylation specifc PCR; methyl-specific polymerase chain reaction
N	
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
O	
O	Oxigeno; Oxigen
P	
PAPPA	pregnancy-associated plasma protein A, pappalysin 1
PDGFR	platelet-derived growth factor receptor
PIK3A	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
POLE3	polymerase (DNA directed), epsilon 3, accessory subunit
R	
RNA	ribonucleic acid
S	
CTR1/2	solute carrier family 31 (copper transporter), member 1 and 2
SRBC	Protein kinase C, delta binding protein
STAT	signal transducer and activator of transcription
T	
TNFSF10	tumor necrosis factor receptor superfamily
TP53	tumor protein p53

RESUMEN GLOBAL

INTRODUCCIÓN: La resistencia a los tratamientos oncológicos es un factor importante que limita la eficacia de los mismos; siendo posiblemente el problema clínico más significativo en el tratamiento del paciente oncológico. Pueden definirse dos tipos diferentes de resistencia a la quimioterapia: (i), *la resistencia intrínseca*, que se da en pacientes con tumores refractarios ya en el momento del diagnóstico, y (ii) la *resistencia adquirida*, que ocurre como consecuencia de los tratamientos de quimioterapia. Además, durante el proceso de adquisición de quimioresistencia el tumor puede hacerse resistente a diferentes fármacos, *resistencia cruzada*, lo que en última instancia conduce al fracaso del tratamiento. Los mecanimos de resistencia son complejos y de afectación multifactorial. Muchos agentes quimioterapéuticos destruyen las células tumorales. Así, la desregulación de los genes implicados en la activación o ejecución de dichos mecanismos puede asociarse con procesos de resistencia a la quimioterapia. Desregulación que puede darse por diferentes mecanismos, entre los que se encuentran los mecanimos epigenéticos. Estos mecanismos pueden desregular genes de forma individual por la hipermetilación del ácido desoxirribonucleico (ADN) de su promotor y/o global por la hipometilación. Mientras que la hipermetilación conlleva el silenciamiento de genes (ej. supresores tumorales), la hipometilación global produce la activación de aquellos genes que se requieren para las diferentes etapas del proceso de transformación neoplásica. Aunque parezcan mecanismos contradictorios, los dos tienen lugar en las células transformadas y les confieren ventajas selectivas.

OBJETIVOS GENERALES:

1. Comprobar si hay cambios en el perfil de metilación del ADN de los promotores de genes asociados con la adquisición de resistencia a la quimioterapia. Estudios que realizaremos en cáncer colorectal y en tumores germinales testiculares.
2. Evaluar si los cambios en los patrones de metilación juegan un papel en los procesos de adquisición de resistencia a dacarbazina y a agentes platinados, como el oxaliplatino y el cisplatino.
3. Evaluar el valor pronóstico y/o predictivo de respuesta a la quimioterapia de los nuevos potenciales marcadores identificados.

MATERIALES Y MÉTODOS: El gen estudiado en el primer artículo, *O^6-methylguanine DNA methyltransferase* (*MGMT*), un gen de reparación del ADN, ha sido seleccionado en base a previos datos publicados por nuestro grupo. En el segundo estudio, analizamos el perfil diferencial de metilación de promotores de genes en un modelo *in*

vitro de resistencia adquirida al oxaliplatino. Así lo analizaremos en la línea celular de cáncer de colon (LoVo-S) y su línea derivada, diez veces más resistente al oxaliplatino (LoVo-R). El análisis será realizado empleando el *array* de metilación *Human DNA Methylation 27K Illumina* cuyas sondas, distribuidas estratégicamente por todo el genoma, permiten detectar cuantitativamente el estado de metilación de 27.000 dinucleótidos citosina/guanina (CpG), distribuidos en 14.495 genes. Los genes candidatos fueron posteriormente validados por secuenciación genómica del ADN modificado por tratamiento con bisulfito, seleccionando el gen *protein kinase C delta binding protein* (*SRBC*), un gen supresor tumoral para estudios *in vitro* y en muestras de pacientes. En los tumores primarios de pacientes, el estado de metilación del promotor de los genes *MGMT* y *SRBC* ha sido analizado mediante una reacción en cadena de la polimerasa, especifica de metilación (MSP). El impacto clínico de la metilación de estos dos genes en pacientes con cáncer colorectal metastásico se evalujó por curvas de Kaplan-Meier, donde se estudio la posible correlación entre metilación y tasa de respuesta al tratamiento, tiempo a la progresión y supervivencia libre de progresión. Siguiendo la misma metodología, también investigamos el impacto clínico de la metilación del gen *MGMT* en pacientes con cáncer testicular de células germinales. Este gen fue seleccionado a partir de estudios epigenéticos diferenciales de modelos tumorales generados por implantación ortotópica de tumores primários en ratones atímicos (concocidos como *patient-derived xenografts* u *orthoxenografts*) y de su evaluación *in vivo* en modelos de resitencia adquirida al cisplatino. De forma adicional mediante estudios de hibridación genómica comparada (CGH) de los mismos modelos tumorales, seleccionamos un conjunto de genes diferencialmente alterados entre tumores sensibles y resistentes al cisplatino. Así, entre estos está incluido el gen *glucosyl ceramide synthethase* (*GCS)*, habiendo sido profundizado en este trabajo su importancia en la adquisición de resistencia.

RESULTADOS: Estudio 1: Se incluyeron 68 pacientes con cáncer colorectal metastásico y se evaluó la metilación del promotor del gen *MGMT*. Dos pacientes (2%) alcanzaron una respuesta parcial y ocho (12%) consiguieron la estabilización de la enfermedad. La tasa de control de enfermedad (respuesta parcial + enfermedad estable) se asoció significativamente con la metilación del promotor del gen *MGMT*.

Estudio 2: Identificamos que la resistencia adquirida al oxaliplatino en líneas celulares humanas de cáncer colorectal se asocia con la inactivación del gen *SRBC* por hipermetilación de su promotor. En las dos cohortes independientes de pacientes con cáncer colorectal metastásico analizadas (Serie 1: n=131; y Serie 2: n= 58), el

promotor del gen *SRBC* se encontraba metilado en un 30% de los tumores primarios. La hipermetilación se asoció con una supervivencia libre de progresión menor, siendo especialmente importante en los casos tratados con oxaliplatino para los que no estaba indicada la cirugía de las metástasis (p=0,01 y p=0,045 para ambas cohortes respectivamente).

Estudio 3: Mediante estudios comparativos en xenografts ortotópicos de tumores germinales tersticulares (no seminomas) sensibles *vs*. resistentes al cisplatino generados en ratones atímicos hemos identificado un grupo de genes potencialmente asociados con la adquisición de resistencia a esa droga. Así hemos identificado: *GCS, ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G1* (*ATP6V1G1)*, *alpha-1-microglobulin/bikunin precursor* (*AMBP*), *polymerase (DNA directed), epsilon 3, accessory subunit* (*POLE3*), *pregnancy-associated plasma protein A, pappalysin 1* (*PAPPA)* y *solute carrier family 31 (copper transporter), member 1* and *2* (*CTR1/2)*. Estudios funcionales en líneas celulares humanas de cáncer testicular demuestran la importancia del gen *GCS* en los procesos de adquisición de resistencia al cisplatino. Además identificamos al agente químico DL-treo-PDMP, un inhibidor específico de la enzima GCS, como un re-sensibilizador de los tumores no seminoma refractarios al cisplatino. Siendo un ejemplo de reposicionamiento de un fármaco utilizado para otras enfermedades o *drug repositioning*.

Estudio 4: Identificamos la hipermetilación del gen *MGMT* asociado con la resistencia al cisplatino en xenografts de tumores germinales testiculares del tipo no seminoma. Asociación que se confirmó en estudios en líneas celulares. La relevancia clínica fue establecida analizando el estado de metilación del *MGMT* en una serie clínica de pacientes con tumores germinales testiculares metastásicos (n=72). Serie que incluye casos refractarios a la quimioterapia. La metilación del promotor del gen *MGMT* se asoció con una supervivencia global (p=0,025). Estudios preclínicos en nuestros modelos de xenografts demuestran que la inactivación farmacológica del enzima MGMT con el inhibidor Oxigeno $(O)^6$- benzilguanina en los tumores resistentes resensibilzaba estos tumores nuevamente al cisplatino. Adicionalmente la adición de temozolomida mejoraba la respuesta. Nuestra aproximación dá las bases para el desarrollo de un ensayo clínico que permita evaluar la utilidad terapeútica de la temozolamida en casos refractarios al cisplatino en pacientes con inactivación por metilación del gen *MGMT*.

Discusión: El proceso de resistencia a los tratamientos de quimioterapia, ya sea endógena o adquirida, es complejo y multifactorial, involucrando tanto eventos genéticos y/o epigenéticos. La mayoría de estudios se han centrado en la genética, siendo menos los estudios que han investigado la contribución de la epigenética. La hipermetilación del promotor de *MGMT* es uno de los ejemplos mejor conocidos, siendo un biomarcador de respuesta al tratamiento con temozolomida en gliomas. Para otros tipos tumorales, como el cáncer de colon y los tumores germinales testiculares, son pocos los estudios desde este punto de vista, no habiéndose identificado biomarcadores epigenéticos de una forma tan clara. En esta tesis profundizamos en el aspecto epigenético de la quimioresistencia en estos tumores, identificando dos nuevos biomarcadores.

En cáncer colorectal, que representa la segunda causa más común de muerte por cáncer, encontramos que la hipermetilación del gen *MGMT* predice sensibilidad a la dacarbazina. Nuestras observaciones apoyan la hipótesis de que tumores colorectales metastásicos defectuosos en los mecanismos de reparación del ADN, son más susceptibles a este tipo de agentes quimioterapéuticos. Hecho análogo a lo descrito en gliomas, en relación a la temozolomida, un análogo de la dacarbazina. Los tumores que expresan MGMT tienen un sistema específico para la reparación de aductos de O^6-metilo, el daño principal provocado por este tipio de agentes quimicos. Los tumores con inactivación/no expresión del gen presentan una mayor probabilidad de respuesta. Aunque la tasa de respuesta global en este estudio fue baja, 2%, no nos podemos olvidar que son pacientes para los cuales no existe alternativa terapéutica. Este es una posible opción terapeútica para el subgrupo de tumores seleccionados en base al biomarcador, debido a que los tumores que respondieran a la dacarbazina fueron los que presentaban hipermetilación del promotor del gen *MGMT*. Además, la baja respuesta observada podría estar condicionada por el hecho de que los pacientes del estudio habían sido previamente tratados con varias líneas de quimioterapia, y posiblemente tuvieran comprometida la capacidad hepática, necesaria para activar la dacarbazina. En este sentido, el uso de la temozolomida, un agente alquilante análogo y que se activa de forma higado- independiente, podría ser una buena opción terapeúitca para el desarrollo de un nuevo ensayo clínico, estratificando en base del biomarcador.

En un segundo estudio identificamos la metilación del promotor del gen *SRBC* en tumores colorectales asociada con la quimoresistencia al oxaliplatino, uno de los fármacos claves del tratamiento de este tipo de cáncer, tanto en adyuvancia como en

el cáncer avanzado. Este gen ha sido previamente descrito como un supresor tumoral. *In vitro* las células humanas de colon de cancer resistentes al oxaliplatino tambien presentavan hipermetilación del promotor de *SRBC*, así como su expresión disminuida. En estudios en series de pacientes con cáncer colorectal avanzado, estadio IV, la metilación predijo una supervivencia libre de progresión más corta. Nuestros datos basados en el análisis de series tumorales y en estudios *in vitro* sugieren que la metilación del promotor del gen *SRBC* sería más importante en la adquisición de resistencia al oxaliplatino, que en la resistencia propiamente intrínseca. Desde un punto de vista funcional el papel de SRBC con respecto a la sensibilidad al oxaliplatino puede justificarse por su interacción con la proteína breast cancer 1, early onset (BRCA1), elemento clave de la maquinaria de reparación del ADN. Esta proteína ejerce un papel importante en la reparación del ADN de cadena doble, por lo que su deficiencia puede poner en peligro la capacidad de las células cancerosas en reparar los daños producidos en el ADN tras la quimioterapia. El platino, y por analogía sus derivados como el carboplatino y el oxaliplatino ejercen su acción tras unirse al surco mayor del ADN. El papel de la proteína SRBC, en este complejo mecanismo es desconocido. SRBC, ha sido identificada también interaccionando con caveolin 1, caveolae protein (CAV1), hecho que podría afectar el tráfico vesicular, y por tanto el transporte y procesamiento intracelular del fármaco.

Aunque los tumors germinales testiculares son muy sensibles a las terapias basadas en el cisplatino (85%), incluyendo los pacientes metastásicos, todavía existe un porcentaje de pacientes jovenes con enfermedad diseminada (15%) que no se curan y fallecen a consecuencia de la enfermedad. Con nuestro trabajo basado en la generación de los únicos modelos u *orthoxenografts* que existen actualmente para el estudio de esta enfermedad hemos identificado dos genes que potencialmente se pueden regular farmacológicamente, con el fin de revertir la resistencia al cisplatino: *GCS* y *MGMT.* GCS es una proteína importante en la síntesis de glucosilceramidas, lo que permite a una célula escapar de la muerte inducida por ceramidas. Estudios publicados relacionan la alta expresión de esta proteína con el incremento de la resistencia celular a la doxorrubicina y a la daunorrubicina. Con nuestra investigación relacionamos también su elevada expresión con la aparición de resistencia al cisplatino en tumors testiculares tipo no seminoma. Respecto al gen *MGMT*, lo encontramos hipermetilado en los tumores no seminoma, sensibles al cisplatino. Ambas proteínas, GCS y MGMT posean inhibidores farmacológicos que se han propuesto o se están utilizando en el tratamiento de otras enfermedades/tumores. Los inhibidores de la síntesis de los glicoesfingolípidos han surgido como un nuevo

enfoque para el tratamiento de enfermedades metabólicas como Gaucher, Niemam-Pick y la diabetes. Siendo el enzima clave de esta vía la GCS, existen ya inhibidores que están o bien en uso clínico o en diferentes fases de desarrollo, incluyendo Migustat, DL-treo-PDMP, EXEL-0346, etc. Nuestros resultados preclínicos demuestran que DL-treo-PDMP podría ser un importante candidato al desarrollo de un ensayo clínico con el objetivo de intentar resensibilizar al cispaltino pacientes con tumores testiculares de células germinales refractarios. Otra opción terapeútica sería la selección de pacientes refractarios con hipermetilación/inactivación del gen *MGMT*, o su bloqueo farmacológico con los inhibidores tipo el O^6-benzylguanina, seguidos del tratamiento con temozolomida más cisplatino. Tanto la O^6-benzylguanina como la temozolomida han sido ya utilizados en diferentes fases de la clínica. Ambas en ensayos clínicos fase I y fase II. La temozolamida también en la clínica para el tratamiento de melanoma y glioma. Así, ambos tratamientos propuestos para el cáncer de testículo refractario se basarían en estrategias de reposicionamiento de fármacos o *drug repositioning*.

CONCLUSIÓNES:

1. Existen cambios en el perfil de metilación de los promotores de genes, en los modelos estudiados de quimioresistencia a la dacarbazina, oxaliplatino y cisplatino.

2. El estado de metilación de los promotores de los genes *MGMT* y *SRBC* influye en la quimosensibilidad de los tumores, a diferentes agentes antineoplásicos.
   2.1. La metilación del promotor del gen *SRBC* se asocia con la adquisición de resistencia al oxaliplatino en cáncer colorectal avanzado.
   2.2. La hipermetilación del gen *MGMT* se asocia con sensibilidad a la dacarbazina en pacientes con cáncer colorectal metastásico; y al cisplatino en tumores germinales testiculares metástasico tipo no seminoma.

3. El estado de metilación del promotor del gen *SRBC* es un buen candidato a biomarcador predictivo de resistencia al oxaliplatino, en pacientes con cáncer colorectal metastásico, para los cuales no es indicada la resección de las metástasis. Por otra parte la metilación del promotor del gen *MGMT* podría ser utilizada como biomarcador predictivo de respuesta a la dacarbazina también en cáncer colorectal metastasico y al cisplatino, en cáncer testicular de células germinales.

4. Los estudios realizados en base a las líneas celulares y a los *orthoxenografts* de tumores germinales testiculares identifica dos genes (GCS y MGMT) como base para el desarrollo de nuevas aproximaciones terapéuticas resensibilizantes al tratameinto con cisplatino.

GENERAL INTRODUCTION

1. Cancer

Cancer is a disturbance of one or more cellular activities that are crucial for the development and the maintenance of multicellular organisms, namely: growth, differentiation, programmed cell death, and tissue integrity. It is a malignant disease because frequently cancer cells invade into neighboring tissues and survive in this ectopic site. These cells that invade beyond the constraints of the normal tissue, from which they originate, enter into the circulation from where they can reach distant organs and eventually form secondary tumors, called metastases [1].

To perform a cancer diagnosis several parameters need to be taken in consideration; the site of the tumor, the histological type of the cancer, its grade of differentiation and its extent of growth and invasion. Attention is also paid to the host cell reaction evidenced by the stroma, blood vessels and leukocytes. Because cancers are known to metastasize, clinically it is mandatory to search for secondary tumors in the lymph nodes and in distant organs [1].

Qualitative and quantitative criteria are used to stage and grade cancers for therapeutic and prognostic purposes. Staging of tumors is done following the volume of the primary tumor and its depth of invasion (T stage), the number of lymph nodes with invasion (N stage) and the presence of distant metastases (M stage)-TNM system [2].

The above mentioned biological and clinical observations indicate that cancer is a disease caused by the accumulation of modified cells, disturbing differentiation and in most cases, causing loss of structure and function of the tissue and organ, leading to cancer cell invasion and cancer cell survival in an ectopic environment [3].

In order to get cell and tissue alterations a series of genetic and epigenetic changes occur in cancer cells (**Figure 1**). These genomic alterations occur in oncogenes and in tumor suppressor genes. In oncogenes, one allele is activated leading to gain of function, while in tumor suppressor genes, both alleles need to be inactivated leading to loss of function. Activation mechanisms of oncogenes implicate mutation, gene amplification, and promoter activation. Mechanisms of tumor suppressor genes inactivation are exemplified by loss of heterozygosity plus silencing of the second allele genetically, through mutation; or epigenetically, through deoxyribonucleic acid (DNA) methylation [4].

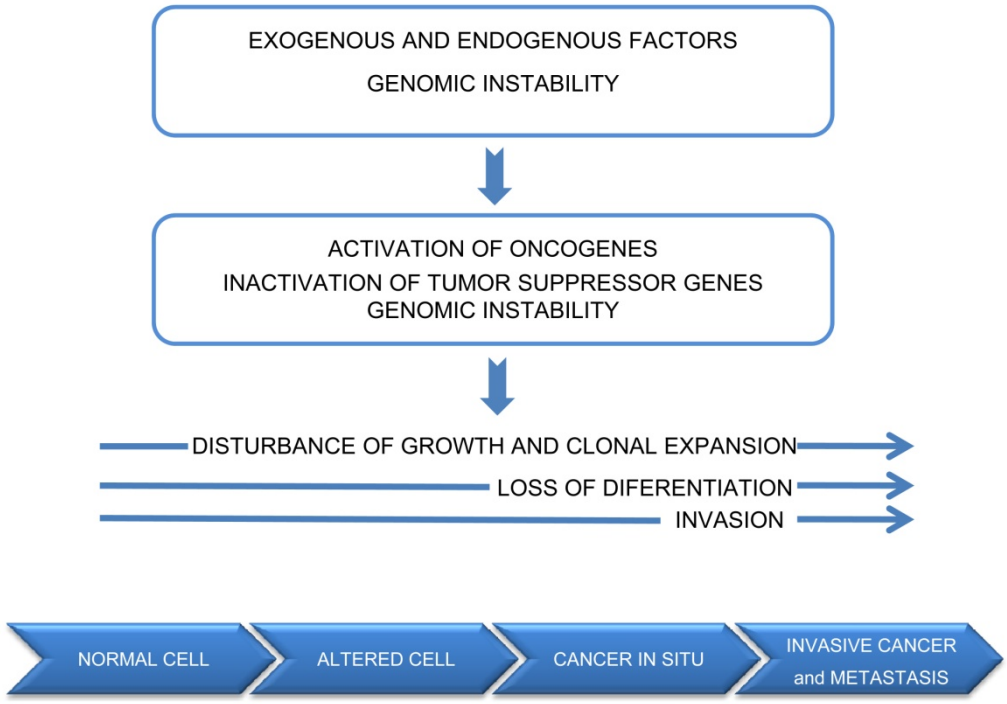

Figure 1: **Schematic Representation of Genetic, Epigenetic, and Phenotypic Aspects of Cancer Development** . *Adapted from Mareel, et al. 2003.*

These molecular alterations arise mainly as a consequence of individual's exposure to carcinogenic events that can be external factors (e.g. tobacco, infectious organisms, chemicals and radiation) or internal (e.g. inherited mutations, hormones, immune conditions and mutations that occur from metabolism). These causal factors may act together, or in sequence to initiate or promote the development of cancer [5].

1.1. Colorectal Cancer

Worldwide, every year, more than 1 million of individuals will develop colorectal cancer. In the developed world the disease-specific mortality rate is nearly 33% [6]. Most of the tumors are sporadic (**Figure 2A**); fewer than 5% are hereditary, being Familiar Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) the two recognized inherited forms (**Figure 2B**) [7]. The risk factors for developing colorectal cancer include a family history of this type of cancer, development of polyps, inflammatory bowel disease (e.g., ulcerative colitis), obesity, tobacco and alcohol abuse, high stress, and factors associated with the Western diet [8].

Colorectal cancer development is a multistep process and the vast majority of tumors are adenocarcinomas, which arise from preexisting adenomatous polyps that develop

in the normal colonic mucosa. This adenoma-carcinoma sequence is well characterized, and several molecular events that underlie the initiation and progression of colon cancer have been identified (**Figure 2**)[9, 10]. Traditionally colorectal cancer is explained by two pathways:

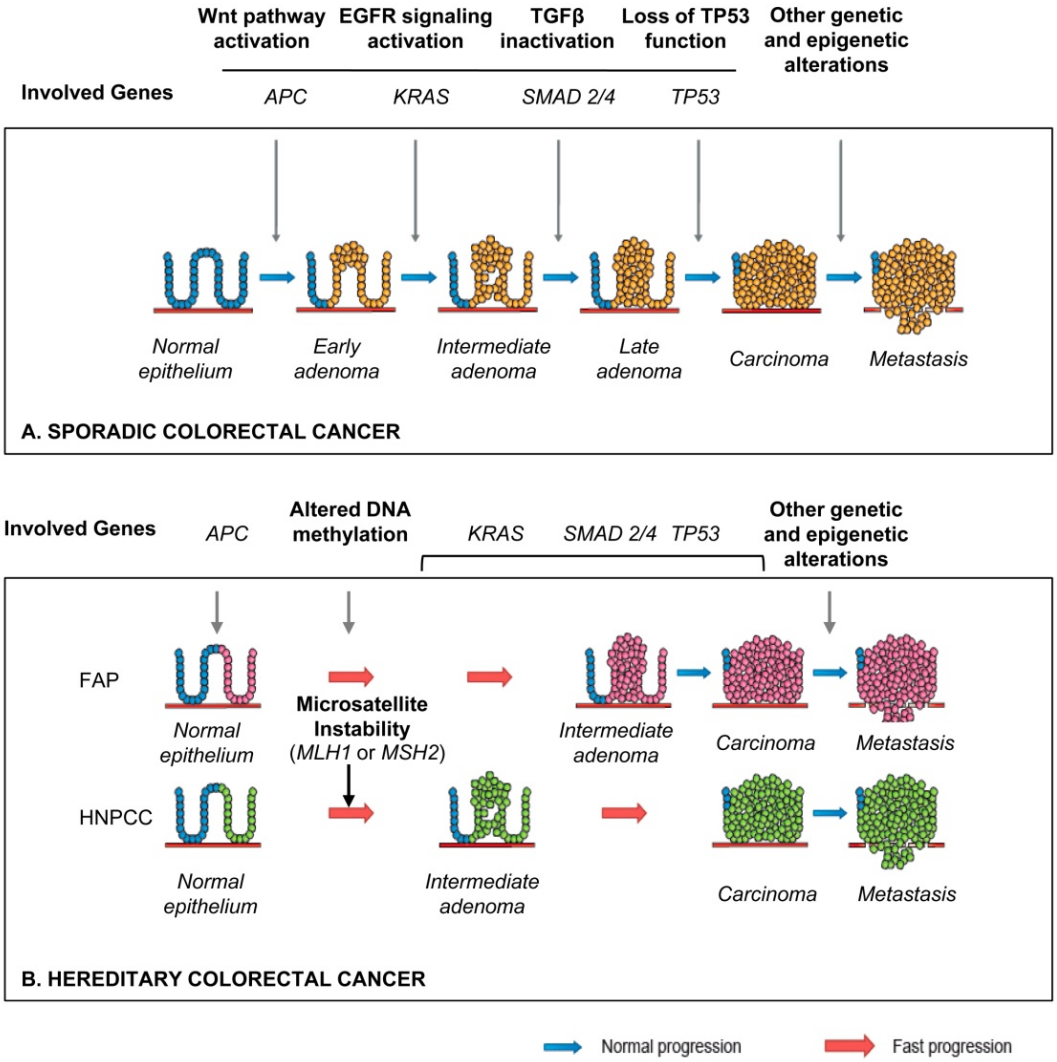

Figure 2: **Common Colorectal Cancer Development Pathways.** Progression from normal epithelium through adenoma to colorectal carcinoma is characterized by accumulated abnormalities of particular genes. As consequence there is activation/inactivation of different mechanisms (bold). **A. Sporadic colorectal cancer.** Around 85% of the colorectal tumors appear due to the represented alterations. **B. Hereditary colorectal cancer.** This type of tumors are characterized by germline alterations. Familiar Adenomatous Polyposis (FAP) syndrome is characterized by the inactivation of one *APC* allele. Hereditary Nonpolyposis Colorectal Cancer (HNPCC) tumors present the inactivation of mismatch repair genes like *MSH2* or *MLH1*. *Adapted from Davies, et al. 2005*

i. The gatekeeper is responsible for about 85% of sporadic colorectal cancer and FAP syndrome. Mutation of the tumor suppressor gene *adenomatous polyposis coli (APC)* is one of the key steps in this pathway. This alteration interferes in

the regulation of cell proliferation via β-catenin. Many other tumor suppressor genes (eg. *deleted in colorectal carcinoma* and *tumor protein p53* (*TP53)*) and oncogenes (eg. *kirsten rat sarcoma viral oncogene homolog* (*KRAS)* and *v-myc avian myelocytomatosis viral oncogene homolog*) are also involved [11]. These types of tumors have a molecular profile characterized by specific chromosomal amplifications and transformations, aneuploidy, and loss of heterozygosity [12].

ii. The caretaker pathway is responsible for 15% of sporadic colorectal cancer and the hereditary HNPCC syndrome. It is characterized by mutations or epigenetic changes of genes that maintain genetic stability like mismatch repair (MMR) genes (eg. *mutL homolog 1* (*MLH1)* and *mutS homolog 2*). The aberrant function of these genes, results in microsatellite instability (MSI) phenotype. MSI is characterized by somatic alterations in the size of simple repeat microsatellite nucleotide sequences, common throughout the genome. As a consequence, genes containing simple repeat sequences, such as *transforming growth factor beta receptor II*, *epidermal growth factor receptor* (*EGFR)*, or *BCL2-associated X* (BAX) [13], are often mutated in these tumors [12]. Colorectal malignancies demonstrating MSI have a very heterogeneous histological appearance and better prognosis [14].

In fact the two pathways might not be completely separated and additional pathways could exist. For example, the serrated, flat and depressed colorectal neoplasms cases [15, 16]. Loss of imprinting and histone acetylation, as well as modifier genes, such as *prostaglandin-endoperoxide synthase 2* and *peroxisome proliferator-activated receptor gamma,* also seem to be involved in the genesis of colorectal cancer [17].

If colorectal cancer diagnosis is made early when the disease is localized on the bowel mucosa, generally it is curable with over 90% probability of survival at 5 years [18]. However, a majority of the cases will eventually develop liver metastases, being this organ the most common metastasic target (50-60% of cases) [19]. Close to one third of patients have liver metastases either at the time of diagnosis (synchronous cases) or during the disease course (metachronous cases). Patients with metastasic colorectal cancer (stage IV), present a 5-year survival rate of 8% [20].

1.2. Testicular Germ Cell Cancer

Testicular cancers, 95% of which are type II germ cell tumors, are the most common solid malignancies affecting males between 15 and 35 years, although it accounts for

only 2% of all cancers in men [21]. In addition, the worldwide incidence of these tumors has more than doubled in the past 40 years [22]. West and North Europe present the higher incidence rates in contrast to Asia and Africa. In Spain every year, 400-500 new cases are diagnosed [23].

Germ cell tumors arise from embryonic germ cells that fail to properly differentiate and instead, undergo malignant transformation [24]. This type of cancer occur in testis, but also in ovary and occasionally in extra-gonadal primary sites [25]. As we can see in **Table I,** they can be divided in five singular types, with different localization, phenotype and origin [24, 25]. Although testicular germ cell tumors are represented in three types of germ cell tumors (I, II and II), since we focus our posterior studies in testicular germ cell type II tumors (seminoma and non-seminoma tumors), from now on we will use testicular germ cell tumors, to refer only them.

Seminomas are well circumscribed solid tumors that appear in patients between 30-40 years old and correspond to 40% of the testicular germ cell tumors [26]. Non-seminomas tumors appear in patients between 20 and 30 years, representing 50% of type II of testicular germ cell tumors. This last type of tumors presents different cell types, being classified in several sub-types like: embryonic cell carcinoma, choriocarcinoma, yolk sac tumor, and teratoma. Teratomas are considered to be either mature or immature, depending on whether adult-type differential cell types or partial somatic differentiation, similar to that present in the fetus are found [25].

Testicular germ cell tumors that present one predominant histological pattern are rare and normally a mixture of multiple histological types is represented [26]. As we can see in **Figure 3** this can be due to the capability of pre-germinal cells to be reprogrammed [27].

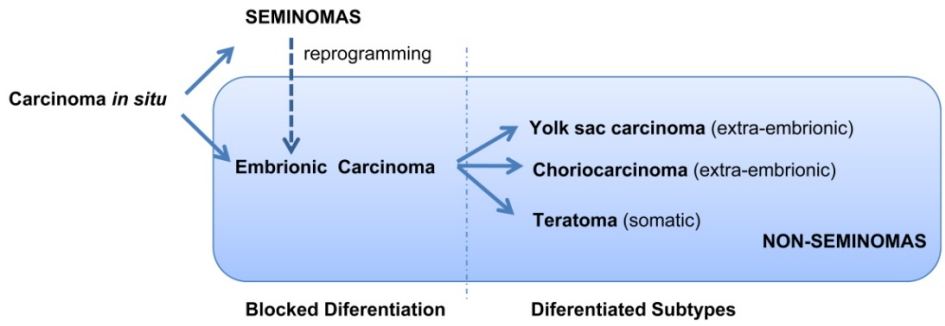

Figure 3: **Type II Testicular Germ Cell Tumors.** Seminomas can be reprogamated to non-seminomas. Normally this last type of tumor is composed by different subtypes: yolk sac carcinoma, choriocarcinoma and teratoma. *Addapted from de van de Gejin, et al. 2009*

For treatment purposes, two broad categories are recognized: pure seminomas and all others, which together are termed non-seminoma. When both elements are present in a tumor, treatment is made as if it was a non-seminoma tumor, since this type is more aggressive [26].

Table I: The five types of germ cell tumor

Type	Anatomical site	Phenotype	Age	Originating cell
I	Testis/ ovary/ sacral region/ retroperitoneum/ mediastinum/ neck/ midline brain/ other rare sites	(Immature) teratoma/yolk-sac tumor	Neonates and children	Early PGC/ gonocyte
II	Testis	Seminoma /non-seminoma	>15 years (median age 35 and 25 years)	PGC/ gnonocyte
	Ovary	Dysgerminoma/non-seminoma	>4 years	PGC/ gnonocyte
	Dysgenetic gonad	Dysgerminoma/non-seminoma	Congenital	PGC/ gnonocyte
	Anterior mediastinum (thymus)	Seminoma/non-seminoma	Adolescents	PGC/ gnonocyte
	Midline brain/(pineal gland/hypothalamus	Germinoma/non-seminoma	Children (median age 13 years)	PGC/ gonocyte
III	Testis	Spermatocytic seminoma	>50 years	Spermatogonium/spermatocyte
IV	Ovary	Dermoid cyst	Children/adults	Oogonia/oocyte
V	Placenta/ uterus	Hydatidiform mole	Fertile period	Empty ovum/spermatozoa

PGS: Primordial Germ Cell

Most of the testicular germ cell tumors are sporadic, although familiar clustering has been observed (≅ 2%), particularly among siblings [28]. Several risk factors have been identified, including: positive testicular germ cell tumor family history [29], cryptorchidism or testicular dysgenesis [30], Klinefelter syndrome, the presence of a contralateral tumor [31] or testicular intraepithelial neoplasia and infertility [25, 32].

At molecular level little is known about these tumors but many of them have complex karyotypes, with hipo or hyper-triploid index [33]. The most common chromosomal abnormalities identified are an ishochromosome of the short arm of chromosome 12 in 90% of cases [34] and the amplification of chromosome 17q in 70% [35]. Other genetic alterations that have been described are a deletion of 1.6 mega bases (designated gr/gr) in chromosome Y [36], a point mutation in *V-kit Hardy-Zuckerman 4 feline*

sarcoma viral oncogene homolog (*KIT*) [37], hipomethylation of *LINE1 retrotransposable element 1* [38], over-expression of micro RNA-371-3 [39] and the familiar segregation for genes found in the locus Xq27 [40]. Other factors related with the increased risk of appearance of testicular germ cell tumors are reduced levels of androgen hormone during teenage period [41], pre-birth hypertension or infections with Epstein-Bar or cytomegalovirus [42, 43] or even the environment [44].

Tumors markers like α-fetoprotein (AFP), beta subunit human chorionic gonadotropin (β-hCG) and lactate dehydrogenase (LDH) have an established role in the management of testicular cancer. AFP is the major serum protein of the fetus. It is not found in adults, with exception for some hepatic disorders or in young males with a malignant teratoma. β-hCG is a placental hormone. Its detection in males implies the presence of testicular germ cell tumors that presents throphoblastic elements. LDH is an enzyme that is expressed in cardiac and skeletal muscle as well as in other organs, although it is often found in testicular germ cell tumors [45].

2. Cancer Treatment

Therapeutic procedures for cancer patients still remain largely empirical. Treatment depends upon a variety of individual factors, which may include the specific pathological and molecular characteristics of the tumor, its location, extent of disease and the health status of the patient. The ultimate objective is to destroy all cancer cells whilst inflicting minimal damage on the normal tissue. This can be achieved in a number of ways, either directly or indirectly by depriving cancer cells of signals needed for cellular proliferation or by stimulation of the immune response [46].

There are several types of treatment, which may be used alone or in combination, either simultaneously or sequentially: surgical removal (resection), radiation exposure (radiotherapy) and use of antineoplasic agents. Cancer resection is the first choice, being most of times curative for patients with tumors in early stage. The use of antineoplasic agents involves molecules of different origin, mainly chemical drugs (chemotherapy) and biological agents (e.g. antibodies, small molecules, immunotherapy and gene therapy) [46]. Treatment with antineoplasic agents and/or radiotherapy can be applied after tumor resection, in order to kill some possible remaining cells (adjuvant or postoperative regimen) or as a first approach, in order to reduce tumor size with the intent of after proceed to its resection (neoadjuvant regimen). After neoadjuvant setting there are some patients that still not fill the surgery

criteria. In order to try an improvement of its quality of life it is applied the palliative treatment [47].
The concepts: response rate, overall survival, progression free survival, and time to progression, will be now introduced, since they will be crucial in the next points. They will be explained in the chemotherapy treatment context [47].

Response Rate is the percentage of patients whose tumor shrinks or disappears when they are under a treatment.

Overall Survival is the percentage of patients who are still alive after a certain period of time, since their cancer diagnosis. The overall survival is often stated as a five-year survival rate, which is the percentage of people in a study or treatment group that lives five years after their diagnosis. It can be also called survival rate.

Progression Free Survival is the time-span from diagnosis until tumor progression takes place. In a clinical trial, measuring the progression free survival or time to progression is one way to see how well a new treatment works.

Time to Progression is the length of time in which the disease is present but it does not get worse.

Because of tumors heterogeneity (site of origin, stage, and other molecular characteristics) and different adaptation of tumor cells to therapy with antineoplasic agents, evaluation of successful regimens and improvement of the rate response to treatment are complicated and sometimes deceptive. Reduction or stabilization of tumor mass, decline of symptoms, and decrease levels of specific tumor biomarkers in serum, rather than 5-year overall survival, represent the main goals in treating and monitoring patients outcome to different treatment regimens [48].

2.1. Chemotherapy

Chemotherapy can be defined as the treatment of cancer with chemical drugs. The first example of its use in clinical practice involved the treatment of a malignant lymphoma with nitrogen mustard [49]. Medical intervention in cancer continues to rely heavily on chemotherapy, being used in all clinical settings – from adjuvant treatment to palliation.

The excessively active growth-signaling pathways in cancer cells makes them susceptible to a wide range of drugs which target growth-signaling molecules and/or

processes involved in cellular replication and gene expression. However, these processes also happen in normal cells, particular in the bone marrow constituents and those of the intestinal lining, being the drugs effect in cancer cells preferential but not exclusive, which results in the undesired side-effects. The relatively wide spectrum of activity of cytotoxic drugs makes them a rather harsh and non-specific form of treatment that can only be tolerated for short periods. Indeed the effects of the treatment may sometimes cause more distress than the disease. These side-effects include dry flaky skin, loss of hair, nausea and vomiting, changes in taste and appetite, blood clotting problems, fatigue, depressed immune system and possible sterility. Most side-effects subside after the treatment is over, but sometimes there is permanent damage to the kidneys, heart, lungs or reproductive system. In general, however benefits outweigh the disadvantages, chemotherapy is the commonest form of cancer therapy [46].

Chemotherapy drugs can be divided into three major groups on the basis of their mode and site of action: **genotoxic agents**; **antimetabolites** and **mitotic spindle inhibitors (Figure 4)** [46, 50].

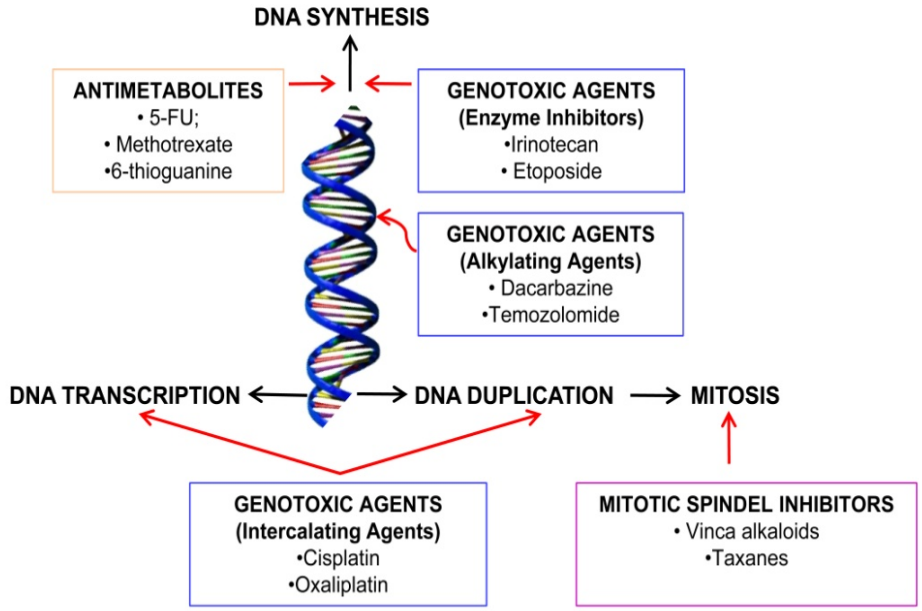

Figure 4. Major Groups of Chemotherapy Compounds. Antimetabolites (orange box) are chemical compounds that inhibit processes critical for DNA synthesis. Genotoxic agents (blue boxes) interfere directly over the DNA strands (alkylating and intercalating agents) or also in DNA synthesis, due to enzymes inhibition (enzyme inhibitors). After DNA duplication, occurs the mitosis, that can be blocked by spindles inhibitors (pink box).

Genotoxic agents either bind to DNA or indirectly damage it by affecting enzymes involved in replication, which leads to cell death induction. This class of drugs may be subdivided into three different groups. 1) Alkylating agents modify DNA bases leading

to mutations and bases cross-linking, interfering with replication and transcription (e.g. dacarbazine, temozolomide); 2) intercalating agents, chemicals that bind to DNA, interfering with polymerase activity during replication/transcription (e.g. cisplatin and oxaliplatin) and 3) enzyme inhibitors, agents that block replication by inhibiting enzymes, such as topoisomerases (e.g. etoposide and irinotecan) [46].

Antimetabolites include: 1) folate antagonists or antifolates are inhibitors of the folates, co-enzymes required for methylation and necessary for the formation of purines (e.g. methotrexate and pemetrexed); 2) pyrimidine antagonists block pyrimidine nucleotide formation or cause premature termination by themselves being incorporated into newly synthesized DNA (e.g. 5-fluororacil (5-FU) and gemcitabine); and 3) purine antagonists inhibit adenine and guanine synthesis (e.g. 6-mercaptopurine and 6-thioguanine) [46].

Mitotic spindle inhibitors disrupt mitosis by affecting the formation/function of spindle microtubule fibers required for chromosome alignment. They prevent the polymerization of tubulin monomers and act in a cell cycle-dependent manner. They also affect normal cells but to a much lesser extent, due to the lower frequency of cell division (e.g. plant-derived vinca alkaloids and taxanes) [46].

Since the projects that came out from this thesis are related with specific genotoxic agents, like dacarbazine, temozolomide, oxaliplatin and cisplatin, these drugs will be described in a more detail.

2.1.1. Dacarbazine

Dacarbazine is a synthetic analog of a naturally occurring purine precursor (**Figure 5A**). After intravenous administration this drug is enzymatically activated in the liver. The mechanism of action is not well understood, but appears to exert cytotoxic effects via its action as an alkylating agent (e.g. DNA damage). Other theories include DNA synthesis inhibition by its action as a purine analog. As with other alkylating agents, cells in all phases of the cell cycle are susceptible to dacarbazine. It is the most active agent used in metastatic melanoma and can be also combined with doxorubicin and other agents in the treatment of different sarcomas and Hodgkin's disease [51, 52].

2.1.2. Temozolomide

Temozolomide is a cytotoxic pro-drug and an imidazotetrazinone derivate of dacarbazine that, when hydrolyzed, inhibits DNA replication by methylation or

alkylation of nucleotide bases (**Figure 5B**). O^6 position from guanine base is the preferred target for temozolomide action (70% of adducts) [53]. Clinical response to temozolomide is closely linked to the activity of MGMT, a DNA repair protein that removes O^6-alkylguanine adducts from DNA [54]. Both *in vitro* and *in vivo* preclinical studies have shown that temozolomide is active against a variety of tumor types. Of particular interest is its clinical efficacy in patients with malignant glioma or malignant melanoma and its ability to enhance health related quality life [55, 56].

2.1.3. Cisplatin

Cisplatin (**Figure 5C**) is a platinum analog that has demonstrated efficacy against several tumor types. It is highly effective in the treatment of testicular and ovarian cancers and is also employed for treating bladder, cervical, head and neck, esophageal, and small cell lung cancer [57]. However tumors such as colorectal cancer have intrinsic resistance to it, while others develop resistance after initial treatment [58].

Once cisplatin has been intravenously administrated to a patient, it is rapidly diffused into the tissues and highly bond to plasma proteins [59]. The mechanism by which it crosses the cellular membrane is still unclear. At first, it was believed that cisplatin entered the cell by passive diffusion [60]. However several transporters, including the *sodium$^+$, potassium- adenylpyrophosphatase, ATP monophosphatase, triphosphatase, SV40 T-antigen, adenosine 5'-triphosphatase, ATP hydrolase, complex V (mitochondrial electron transport), (Calcium^{2+} + Magnesium^{2+})-ATPase, HCO3$^-$-ATPase, adenosine triphosphatase* (ATPase) and members of solute carrier transporters (CTR1 and CTR2) have been implicated in facilitating the entry of this compound into the cells [61, 62]. The organic cationic transporters, *solute carrier family 22* proteins had also been shown to participate in cisplatin influx [63]. Thus, cisplatin can enter cells by passive or facilitated diffusion and by active transport.

The biochemical mechanism of cisplatin cytotoxicity involves its binding to DNA but also to non-DNA targets (e.g. proteins and ribonucleic acid (RNA)) with subsequent induction of cell death through apoptosis, necrosis or both. The damage induced upon cisplatin binding to genomic DNA may interfere with normal transcription and/or DNA replication mechanism [60]. Genomic DNA adducts are more frequent in guanine residues, especially the ones located in the major groove of the double helix, since they are the most accessible and reactive nucleophilic sites for platinum compounds (mono-adducts). Beside this it can be produced other types of DNA adducts like: intra- and inter-strand cross-links and protein-DNA cross-link [64]. Cisplatin DNA-adducts are

usually repaired by nucleotide excision repair, MMR and DNA-dependent protein kinase pathways [65].

Besides lesions in genomic DNA, it is also known that cisplatin forms a high amount of adducts with mitochondrial DNA. So it should not be ruled out the possibility that mitochondrial DNA may also be an important pharmacological target for cisplatin [66]. Hence, of interest is the observation that only 5% of covalently bond cell associated cisplatin is found in DNA fraction, whereas 75-85% of the drug binds to protein and other cellular constituents. The resulting inactive forms, involving small thiol molecules such as glutathione, cysteine or methionine, then participate in cisplatin detoxication [67]. What concerns to cisplatin efflux, the *adenosine tri-phosphate (ATP) dependent glutathione-conjugated efflux pump* and copper (Cu) transporters like *ATPase, Cu^{++} transporting* (ATP7) A and ATP7B have been implicated [63].

2.1.4. Oxaliplatin

It has been used as an anticancer medication since 1999, together with 5-FU, for the treatment of colorectal cancer [68]. This chemical drug is a third generation platinum compound, which acts similarly to cisplatin, but it has activity in cisplatin-refractory tumor types [69]. The difference with its analog is the possession of a bulky diaminocyclohexane moiety and the presence of an oxaliplatin *leaving group* (**Figure 5D**). Due to this fact, oxaliplatin and cisplatin adducts have biological properties slightly different, not showing full cross-resistance and being the first drug more efficient in the DNA synthesis inhibition. Differences also have been described in intracellular cascades induced by DNA damage [70]. Different from cisplatin, oxaliplatin-DNA adducts are not able to activate MMR system and, for this reason, this drug is effective in MMR deficient tumors, such as colorectal cancer. Oxaliplatin DNA-adducts are mainly repaired by nucleotide excision repair system [69].

Passive diffusion is believed to be the main mechanism in oxaliplatin cellular uptake [70]. Once inside the cell, a variety of mechanisms of action are triggered. Induction of DNA lesions, like it happens with cisplatin, seems to be the main cytotoxic effect, leading to cell cycle arrest and cell death [71]. However other mechanisms were described. For example the synthesis of messenger RNA is blocked by platinum-DNA adducts which either bind to transcription factors or inhibit RNA polymerase [72]. Furthermore oxaliplatin seems to induce immunogenic signals on the surface of cancer cells before apoptosis, triggering interferon gamma production and interaction with toll-like receptor 4 on the dendritic cells, resulting in the immunogenic death of cancer cells

[73]. Oxaliplatin-adducts have been also detected in proteins and other macromolecules (e.g. RNA and glutathione) [70].

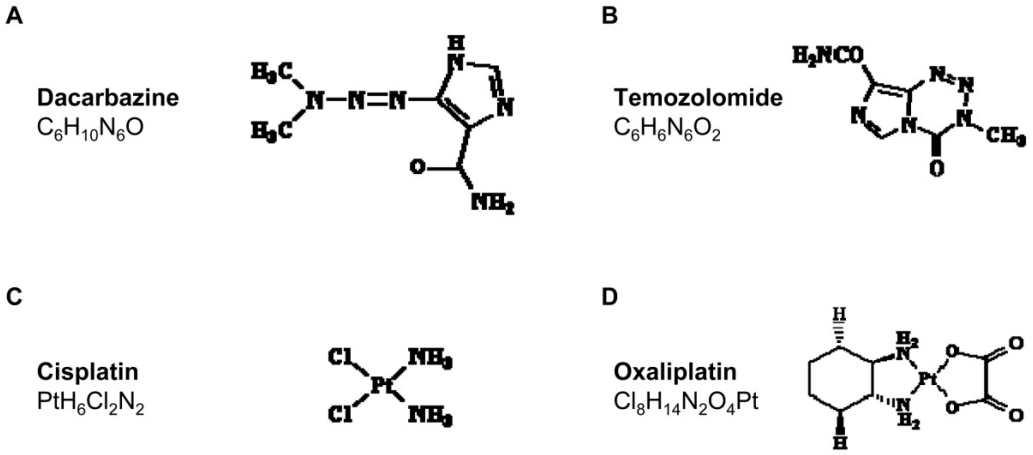

Figure 5. Chemical Formulas. Dacarbazine (**A**) and its analogue temozolomide (**B**). Platinium compounds: cisplatin (**C**) and oxaliplatin (**D**).

2.2. Antineoplasic Agents in Metastasic Colorectal Cancer Therapy

In general, antineoplasic agents based therapy given to colorectal stage IV patients, metastasic patients, pretends an increase in survival and a life quality improvement [74]. There are some patients with metastasis that can go under surgery (**Figure 6**), and others that cannot, depending this on metastases number, size, and/or sites of involvement [19, 75]. Surgery of liver metastasis for which the margins of the removed tissue are free from cancer cells, is related with 30-40% of overall survival at 5 years. Important refer that 15% of liver metastasis that cannot be removed at diagnosis time, can be removed after neoadjuvant therapy [76].

Over thirthy five years, the only agent available to treat metastasic colorectal cancer was 5-FU, followed by 5-FU regimens in combination with leucovorin in the 1990s [77]. However, in the last decade, three cytotoxic agents (irinotecan, oxaliplatin, and capecitabine) and two biologic agents (bevacizumab and cetuximab) have been approved for treatment of metastasic colorectal cancer [77].

Irinotecan was initially introduced as therapy for metastasic colorectal cancer refractory patients to 5-FU+leucovorin, being implemented as a second line treatment [78]. Clinical trials using irinotecan+5-FU+leucovorin (FOLFIRI) showed an improvement of response rate, median overall survival and progression free survival. Thus this combination replaced 5-FU+leucovorin as the standard therapy for metastasic

colorectal cancer [79]. Lately, the same results were observed when oxaliplatin was combined with 5-FU+leucovorin (FOLFOX). Because of this, FOLFOX also became a standard of care in metastasic colorectal cancer [79]. Clinical studies comparing the application order of the chemotherapy schemes (FOLFIRI+FOLFOX or FOLFOX+FOLFIRI) in first and second line treatment did not show significant benefit differences [75]. Actually they are being used without any specific order.

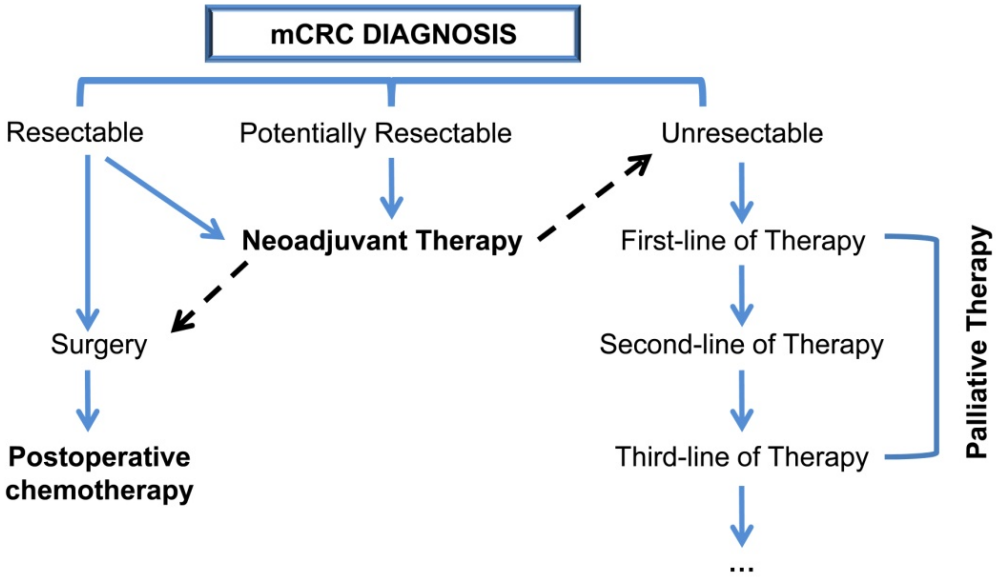

Figure 6: Management of metastasic colorectal cancer (mCRC). Patients with metastasic disease may present metastasis that can be removed by surgery. Sometimes to this patients is applied a previous chemotherapy treatment (neoadjuvant therapy), as it happens with patients that present potential resectable metastasis. Patients that pass trough surgery, posterior are submitted to an adjuvant or postoperative treatment. To the ones that do not fulfill surgery criteria, is applied the palliative treatment in order to try an improvement of its quality of life and outcome.

On the other hand, triple combination of 5-FU+leucovorin, oxaliplatin, and irinotecan, (FOLFOXIRI) was also compared with FOLFIRI in the first-line setting. Although FOLFOXIRI conferred significant benefit in progression free survival, overall survival and response rate, it was more toxic [80]. Given this fact and the lack of consensus on its superiority over standard therapy, this regimen is sparingly used in practice.

In 2005 capecitabine, an oral fluoropirimidine, analogue of 5-FU, was introduced in metastasic colorectal cancer treatment. New capecitabine based scheme seemed to be as effective as the ones based in 5-FU, previously described [81].

Other agents beside chemotherapeutic drugs were developed for the treatment of metastasic colorectal cancer. The synergic role of the biological agents plus the traditional chemotherapy is actually accepted, being they used in addition. They are biological agents like, cetuximab, panitumumab and bevacizumab.

Cetuximab and panitumumab are monoclonal antibodies that block epidermal growth factor (EGF) binding site, inactivating EGFR [18]. EGFR is a trans-membrane receptor that belongs to a family of four related proteins (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog (ERBB) 2, ERBB3, and ERBB4). After ligand activation, EGFR receptor forms a dimer that signals within the cell by autophosphorylation, through tyrosine kinase activity. This triggers a series of intracellular pathways that may result in cell proliferation, block of apoptosis, invasion and/or angiogenesis [82].These biological agents have shown clinical activity, either alone or in combination with irinotecan, in pre-treated metastasic colorectal cancer patients, generating interest for their use in first-line [83]. Although based in results from different clinical trials the use of anti-EGFR agents for metastasic colorectal cancer treatment should be limited to those patients with *KRAS* wild type [84, 85]. Moreover, it should be remembered that, also in this case, other mechanisms of escape, such as *v-raf murine sarcoma viral oncogene homolog B* (*BRAF)* gene mutations, or deregulation of the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3A*)/ phosphatase and tensin homolog / v-akt murine thymoma viral oncogene homolog 1 (*AKT*) pathway, may bypass the EGFR inhibition by these agents [86, 87]. The combination of these agents with 5-FU plus irinotecan have been proven to be effective at first-line and second-line of metastasic colorectal cancer treatment, contrary to the observed in oxaliplatin-based combinations [88] .

Bevacizumab is a humanized monoclonal antibody that binds to and sequesters vascular epidermal growth factor avoiding it binding with its receptor and consequent inactivation [18]. It has been approved in United States and Europe for the first-line treatment of patients with metastasic colorectal cancer [89]. The vascular epidermal growth factor receptor pathway plays a crucial role in tumor angiogenesis, and its blockage has been intensely pursued as a therapeutic target. Although the absence of benefit as a single-agent, it has been evaluated it effect also in combination with the standard chemotherapy regimens mentioned above [90]. The first trial showing a benefit for the use of bevacizumab in first-line of treatment was the comparison of 5FU+leucovorin+bevacizumab *versus* 5FU+leucovorin+placebo. The first combination led to an improvement in overall survival and in progression free survival [91]. Further,

bevacizumab was approved to be combined with standard schedules established for metastasic colorectal cancer treatment.

At the present time, to patients with metastasic colorectal cancer is typically administered a first-line chemotherapy regimen that is continued until documented disease progression. At progression, treatment is switched to a regimen with demonstrated activity in the refractory disease setting. This pattern of treatment until progression and then switching to non-crossresistant therapies continues until the patient has received all five active classes of agents. At that point, patients may be referred for Phase I clinical trials or provided with symptom-directed care [75]. It has been shown that patients that received different lines of treatment, in which there is a variability of drugs compounds, present an increase survival [92].

2.3. Metastatasic Non-seminoma Testicular Germ Cell Cancer Therapy

Nowadays, management of testicular germinal cell tumors is tailored using a universal applicable schema that was drawn up by the International Germ Cell Cancer Collaborative Group (IGCCCG) [93]. Non-seminoma patients based on this schema are divided into good, intermediate and poor prognosis. In the context of non-seminoma, AFP, β-hCG, and LDH levels are reliable markers of tumor burden, prognosis, and response to treatment [94, 95].

For 70-75% of non-seminoma patients that at diagnostic time present a clinical early stage disease, orchidectomy (resection) alone is curative [96]. Thirty percent of non-seminoma patients have already metastases at diagnosis. In this type of cancer the pattern of metastases is not predictable although the most common sites are retroperitoneal lymph nodes, mediastinal and supraclavicular nodes, lungs, brain and bone [97].

Cisplatin based combination chemotherapy, introduced in the 1978 has revolutionized the management of metastasic germinal cell tumors exceeding the cure rates the 80%. No patient with germ cell cancer, no matter how ill at diagnosis, should be considered beyond prospect of cure. Standard therapy for non-seminoma, including the metastasic cases, combines cisplatin with etoposide and bleomycin (BEP regimen) [98]. During chemotherapy, serum markers are checked regularly and should fall to normal levels as disease responds. When negative, surgery is an option to remove residual lesions [99]. While first chemotherapy treatment is curative for many, up to 50% in poor prognosis category will progress or relapse [99].

In contrast to initial therapy, second line treatment for non-seminoma is less well defined and presently there is not a well-established standard therapy. Nevertheless, treatment is still curative in approximately a third of the cases. Decision depends on different parameters such as the nature of the initial treatment and the subsequent response, the localization, and the time since treatment [96, 99]. Salvage conventional chemotherapy including ifosfamide, in combination with vinblastine or specially paclitaxel [100] associated with surgery of postchemotherapy residual masses when they are present is the most used strategy [101]. Moreover twenty to forty percent of patients who relapse after adjuvant treatment will achieve long-term survival with the use of platinum-containing standard-dose or high-dose salvage chemotherapy with autologous stem cell support [102, 103] (**Figure 7**). Patients who progress during or after salvage chemotherapy exhibit an extremely poor prognosis and long-term survival is achieved in less than 5% of patients [104, 105]. The identification of new active drugs remains a priority in these patients.

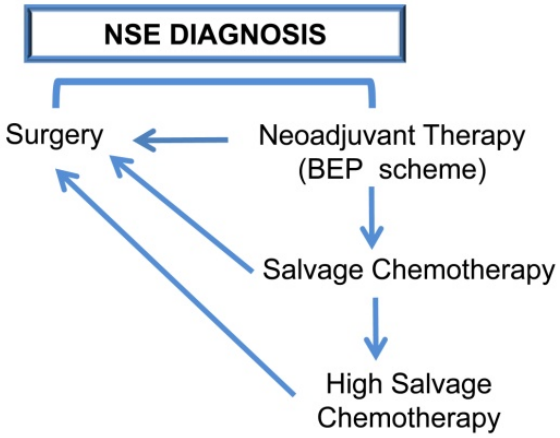

Figure 7: Management of Non-seminoma Germ Cell Tumors (NSE). The first choice to non-seminoma tumors patients is surgery. If the disease is in an advance stage, the patient is submit to neoadjuvant therapy, that consist in the conventional BEP scheme (cisplatin, etoposide an bleomycin). If the tumor responds, the patient can undergo surgery. If not, it is applied a salvage or even a high salvage chemotherapy. These last types of chemotherapy are more severe than the BEP scheme.

Like in many cancer types, target therapy has also been used in non-seminoma treatment. Sunitinib, an inhibitor of multiple receptor tyrosine kinases, was the first targeted substance to be tested in the treatment of cisplatin resistant non-seminoma, although the results were disappointing [106].

2.4. Chemoresistance

Although chemotherapy drugs often effectively suppress tumor growth in cancer patients, a significant proportion of tumors either do not respond (intrinsic or primary resistance), or later develop resistance to these chemotherapeutics, after primary therapy (acquired or secondary resistance) [107]. This leads to tumor progression, disease dissemination and ultimately patient mortality, which remains a major challenge for successful cancer treatments [48]. Therefore the identification and characterization of cellular genes responsible for chemotherapeutic drug resistance is critical for successful prognosis and treatment of cancer.

2.4.1. Potential Mechanisms of Chemoresistance

The problem of drug resistance is complex and some mechanisms were suggested as responsible for its appearance. Resistance can be due to different processes including alterations in the drug uptake, drug inactivation, alteration of the target molecules, enhanced DNA repair, replicative bypass, altered checkpoints, proliferative signals, and reduced cell death response (**Figure 8**) [107].

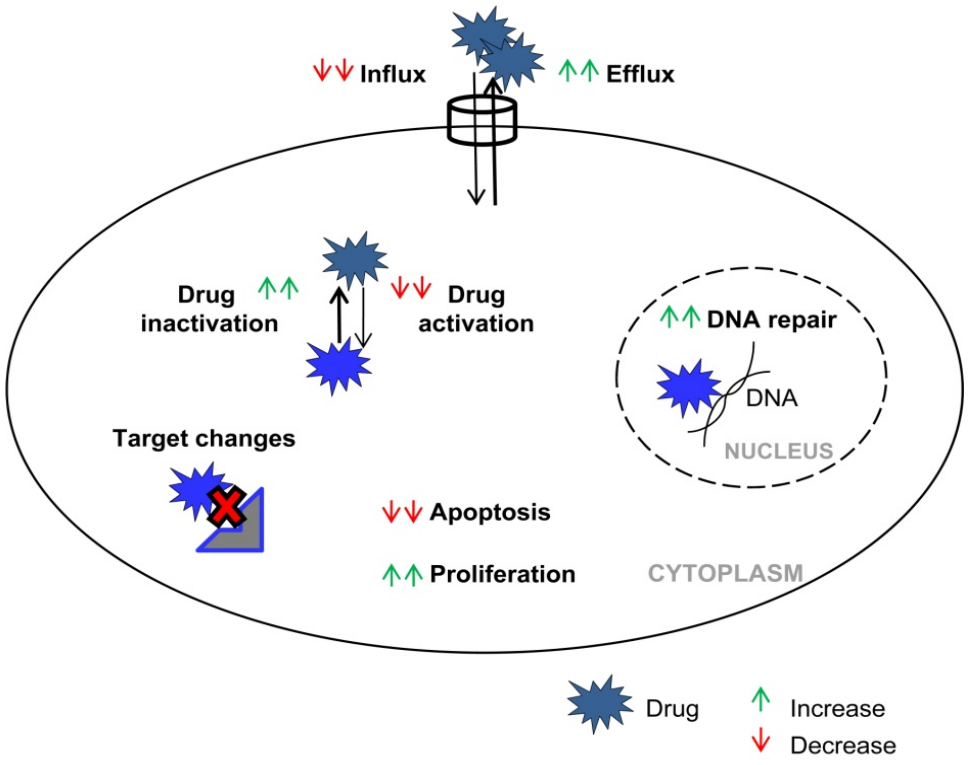

Figure 8. Examples of Cellular Mechanisms Responsible for Chemoresistance. Chemoresistance is a multifactor process. The turn off/on of different systems (e.g. apoptosis, proliferation, DNA repair) permits cells to escape death, induced by different chemical compounds.

2.4.1.1. Drug Uptake

Access of drugs to their intended site of action is a problem that is encountered and must be regarded as a form of resistance. The central portion of large tumors tends to have a poor blood supply and drugs will have limited access to this area. Therefore, chemotherapy is more effective for smaller tumors and becomes less effective as the tumor becomes larger. Treatment of brain tumors also faces problems posed by the blood-brain barrier. This is a dynamic network of vessels, which restrict movement of molecules into the central nervous system. Many drugs are ineffective because they cannot pass through this barrier. Others, which may initially have been able to penetrate inside, may later be blocked due to the dynamic nature of this resistance [108].

Other problem that can arise is the ineffective drug delivery to the cellular environment. Here resistance is achieved by hindering transport into, or by over-activation of transport efflux in the cell [107]. Alterations in transporter and metabolic enzymes are associated with differences in drug absorption, distribution, metabolism and excretion, and are considered to be the major determinants of inter-individual variability. For example, decreased uptake/increased efflux of cisplatin leads to lower intracellular concentrations of drug. Most *in vitro* models of acquired resistance to cisplatin exhibit a 2- and 4-fold decrease in platinium accumulation [109]. Recent reports describe that cisplatin resistant tumors over-express some members of the efflux family ABC-ATPase transporters, as ATP-binding cassette, sub-family C (CFTR/MRP), member 1 [110]. The members of this family have been associated with resistance to drugs of different origin, as oxaliplatin or temozolomide, leading also to multidrug resistance phenotype.

2.4.1.2. Drug Inactivation

Drug metabolism circumvents the cytotoxic nature of a drug typically by reducing availability of free drug to interact with its target. One way is the enhancement of hepatic drug elimination, with the decrease of drug concentration in the plasma. Another physiological response is the over-expression of drug metabolizing enzymes or carrier molecules. For example 5-FU is catabolised and inactivated by the cytosolic enzyme dihydropyrimidine dehydrogenase [111]. Over-expression of this protein both *in vitro* and *in vivo* is linked to 5-FU resistance [112]. Another example is the over-expression of glucoronidation enzymes UDP glucoronosyltransferase in irinotecan resistance [113]. Other mechanisms, as ubiquitinization, also contribute to inactivation of drugs by forming conjugates that are excreted [114].

By contrast, under-expression of drug-metabolizing enzymes can also reduce drug efficacy in situations where an administered inactive pro-drug has to undergo catalytic conversion to an active form. For instance, carboxilesterase lack of activity in the liver to convert irinotecan and capecitabine in their active metabolites has been related to resistance to these drugs [115, 116].

Platinium drugs such cisplatin and oxaliplatin are able to form conjugates with the antioxidant glutathione synthetase, resulting in the inactivation of these drugs [117]. This conjugated form is a substrate for ABC transporter proteins, thus, it is shuttled out of the cell [118]. Increased levels of glutathione synthetase have been identified in cancer cells resistant to platinum drugs [119]. Glutathione synthetase conjugation is catalysed by glutathione S-transferase kappa 1 enzyme family, with increased expression of the glutathione S-transferase pi 1 subgroup correlated with resistance to cisplatin in ovarian cancer cells and tumors [120]. Furthermore, repression of glutathione synthetase has reversed cisplatin resistance in breast cancer cells [121].

2.4.1.3. Altered Targets

Mutated drug targets or their over-expression can impact the efficacy of a drug. For example, the expression levels of thymidylate synthetase, the primary target molecule of 5-FU, regulates chemosensitivity to this drug [122]. It has been described some polymorphisms in the correspondent gene: two in the promoter region related to over-expression of this protein and one 6 base pair deletion in 3'untranslated region, related with messenger RNA stabilization. All of them have been associated with resistance to 5-FU [123, 124]. In another instance, decreased topoisomerase I or II activity due to reduced protein or mutations in the correspondent gene confers resistance to irinotecan or doxorubicin respectively [125, 126]. Topoisomerases are enzymes that regulate the overwinding or underwinding of DNA, for example during replication or transcription.

2.4.1.4. Enhanced DNA repair and Replicative bypass

Many chemotherapeutic regimes attempt to induce massive DNA damage, either directly (e.g. dacarbazine) or indirectly (e.g. doxorubicin). If plentiful enough, this damage should induce cell death. However, in some instances, the over-expression of DNA repair genes can efficiently reverse any acquired damage. For example, platinum agents result in bulky DNA adducts and are predominantly repaired by the nucleotide excision repair pathway [127]. Although this pathway involves multiple different genes,

over-expression of some few rate-limiting players (e.g. *excision repair cross-complementing rodent repair deficiency, complementation group 1* and *xeroderma pigmentosum, complementation group A*) is sufficient to induce platinum resistance. These genes are involved in the excision of the damaged strand and its over-expression is correlated with cisplatin resistance in clinical samples of numerous tumor types [128, 129].

Interestingly, cases of resistance are also reported in repair system deficient cells, like for some platinum drugs. Drug tolerance can be achieved without the need for DNA repair. For example in order for platinated DNA to be replicated, DNA polymerase must skip the platinum adduct, which is most commonly an intrastrand lesion. The classic DNA replication polymerases –α, δ, and ε – cannot bypass the lesion; however, several polymerases have been shown to bypass intrastrand crosslinks by translesion synthesis –namely, β, η, ζ, and ι. Over-expression of DNA polymerase β has been shown to lead to cisplatin resistance, while down-regulation using anti-sense RNA leads to sensitivity. Polymerase ζ has been shown in MMR deficient cells to play a role in DNA tolerance and bypass of lesions. The MMR system is critical for the maintenance of genomic stability as it scans newly synthesized DNA, excising single-base mismatches and insertion-deletion loops. The loss of MMR was linked to DNA methylation and MSI phenomena [130]. Cisplatin resistance has been attributed to defects in the MMR system arising from hypermethylation of the *MLH1* promoter [131]. Fascinatingly, cell lines which have been shown to be resistant to cisplatin due to MMR defects remain sensitive to oxaliplatin [132]. This observation has been attributed to the structural differences between the cisplatin DNA-adduct and the bulky oxaliplatin DNA-adduct, which are not recognized by the MMR system [133]. Furthermore, the loss of MMR coincided with increased translesion synthesis, suggesting the replicative bypass as a plausible mechanism which allows these cells to evade death [134].

2.4.1.5. Altered Checkpoints

To ensure that genetic integrity is maintained between generations, cells employ an elaborate system of checks and balances termed cell cycle checkpoints. In a normal system, cells induce apoptosis over proliferation. The master switch between DNA damage detection, cell cycle arrest and apoptosis is the TP53 protein [135]. Indeed, its importance is illustrated by the observation that *TP53* is mutated in up to 50% of cancers [136]. However, it appears that *TP53* gene mutations do not correlate with expression in 30-40% of cases [137]. Conflicting reports also exist about its role in drug resistance. For example, opposing outcomes (sensitizing and desensitizing) have been

reported on the effect of *TP53* mutations in cisplatin [138, 139] and 5-FU *in vitro* and *in vivo* studies [140, 141]. From a molecular perspective it is also difficult to deduce the effects of *TP53* on resistance. On one hand a lack of *TP53* may prevent a cell from inducing apoptosis while on the other hand; wild type or increased expression may increase the amount of time for DNA repair during cell cycle arrest. Either way, both result in a resistant phenotype. Interestingly, doxorubicin seems to have a more predictable outcome. Its sensitivity is dependent upon a wild type *TP53* function with mutated and null *TP53* leading to resistance [142].

2.4.1.6. Proliferative and Survival Signals

Cancer cells have developed various methods by which they can proliferate regardless of their environment. One method includes over-expression of protein kinases, like EGFR family proteins. Binding of growth factors such as EGF or tumor growth factor α, results in the activation of downstream pro-survival and proliferative pathways, such as *PIK3CA*/*AKT*, *mitogen-activated protein kinase* 3 and 1, *signal transducer and activator of transcription 3* (*STAT·3*) and *STAT5B* pathways [143]. Over-expression of EGFR and ERBB2 in glioblastomas has been shown to increase resistance to chemotherapy *in vitro* and poor prognosis in the clinical set [144, 145]. Published results show that targeting the protein kinase receptors can improve the effectiveness of commonly used chemotherapies [146]. As mentioned before, actually in the clinical, some treatment schedules already include besides chemotherapy, antibodies that inactivate these receptors. For example, the combination of trastuzumab (ERBB2 inhibitor) with chemotherapy in previously untreated patients has been shown to prolong time to progression, increase response rate and significantly improve survival in comparison with chemotherapy alone [147].

Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-kB) is a pro-inflammatory transcription factor which its aberrant activation has been proposed as an important cause of chemoresistance, through the activation of anti-apoptotic genes [148]. Different studies have reported an association between NF-kB inhibition and oxaliplatin activity [149, 150]. The pharmacological inhibition of NF-kB using specific inhibitors such as BAY 11-7082 or SC-514, sensitized human prostate cancer cells; parthenolyde, a natural inhibitor, could markedly enhance sensitivity of human lung cancer cells; quinacrine, an antimalarial drug, sensitizes human colon carcinoma cells and genistein, a natural isoflavonoid, sensitized resistant pancreatic cancer cells all to oxaliplatin [149, 151-153]. High levels of this protein were also detectable in multidrug resistant cells. The mechanism of this resistance was attributed to the activation of the

CCAAT enhancer binding protein family of transcription factors and induction of *ATP-binding cassette, sub-family B (MDR/TAP), member 1* gene expression [154]. What concerns the clinical stage, NF-kB serum level have been found elevated in cancer patients. Its relation with chemotherapy resistance comes from a study that described an NF-kB autocrine production in breast cancer cells that could promote resistance to chemotherapy, in contrast to cells that did not express it [155].

Besides, extracellular factors from the microenvironment were also linked to chemoresistance. Correlating chemosensitivity and stroma secreted proteins, in different tumors and different culture systems, it was found that elevated levels of acidic and basic fibroblast growth factors induced a broad-spectrum chemoresistance (paclitaxel, doxorubicin and mitomicin). Whereas, the known inhibitor of fibroblast growth factors suramin, was able to produce the reversion of this phenomenon [156, 157].

2.4.1.7. Failure of Cell Death Pathways

2.4.1.7.1. Apoptosis

Apoptosis is the death of a cell through a purposeful, mechanistic dismantling of the cellular machinery. To trigger apoptosis it is believed that cellular damage has to pass a certain threshold level. The malfunction of genes responsible for recognizing cellular damage can develop insensitive certain types of cancers to specific chemotherapeutic drugs. Upstream factors involved in the cellular response to damage mediate the induction of a network that transmits both pro- and anti-apoptotic signals. So, any interference that induces anti-apoptotic signal transduction, or abrogates pro-apoptotic pathways including transcriptional and translational response can be also a potential mechanism of drug resistance [158].

One apoptotic pathway, named *intrinsic pathway*, is regulated by mitochondrias, which are affected early in the apoptotic process and are known to act as central coordinators of cell death [159]. Several factors can induce mitochondrial-mediated apoptosis, including chemotherapy, ultra violet light, DNA damage, reactive oxygen species and growth factor withdrawal. Important players of this pathway are the B-cell CLL/lymphoma 2 (BCL-2) family of proteins. This family includes both pro-apoptotic (BCL2-associated agonist of cell death, BCL2-antagonist/killer 1 and *BAX*) and anti-apoptotic members (*BCL-2*, *BCL2-like 1* (*BCL-XL)* and *myeloid cell leukemia sequence 1*). Not surprisingly, there is a good correlation between the expression levels of the BCL-2 family of proteins and the response to a wide range of chemotherapeutic

agents. Specifically, down regulation of the anti-apoptotic members BCL2 and BCL-XL increase sensitivity to oxaliplatin, while loss of pro-apoptotic *BAX* decreases it [160]. In the clinical setting, several studies have shown that high BCL-2 expression correlates with a poor response to chemotherapy [161, 162]. What concerns to BAX, some clinical studies show a correlation between expression and response to chemotherapy, although other studies have not found a correlation [163, 164].

The extrinsic pathway is regulated by cell surface death receptors of TNF-receptor family, such as *Fas cell surface death receptor [13]*, *tumor necrosis factor receptor superfamily (*TNFSF10*), member 10a* and *member 10b.* Further, activation of proximal caspases in these patways leads to activation of downstream effector caspases, most importantly caspase 3 and 7. These executioner caspases cleave celular substrates to bring about morphological and biochemical changes that characterize apoptosis, including chromatin condensation and nuclear fragmentation, membrane blebbing, and cell shrinkage. *In vitro* studies have shown that targeting death receptors with recombinant death ligands or agonistic antibodies can induce apoptosis and/or enhance chemotherapy-induced apoptosis. In a clinical study from colorectal cancer patients, 5-FU treatment led to FAS over-expression, suggesting that this protein is an important mediator of response to chemotherapy. Apoptosis mediated by both FAS and TNFSF10a/TNFSF10b can also be inhibited by cytoplasmatic factors like CASP8 and FADD-like apoptosis regulator (C-FLIP). Inhibition of this protein dramatically sensitizes a panel of colon cancer cell lines to 5-FU, oxaliplatin, and capecitabine, suggesting an important role of C-FLIP in regulating colon cancer cell chemosensitivity. Interestingly C-FLIP has been found to be over-expressed in a high percentage of colonic and gastric carcinomas. Despite, it potencial as a predictive response biomarker was not studied. Survivin is other player in the inhibition of apoptosis. Over-expression of this protein has been shown to inhibit chemotherapy-induced apoptosis *in vitro*. Clinically, low levels of survivin have been correlated with better response to chemotherapy and improved prognosis in a range of cancers, suggesting that it may be a useful clinical marker [165, 166].

In somatic cells, the ends of chromosome (telomeres) shorten in each cell division. However, in tumor cells, telomere length is maintained, mainly thought activation of the reverse transcriptase enzyme telomerase. It has been reported that in cervical cancer cells sensitive to low doses of cisplatin may die through apoptosis as a consequence of cisplatin binding to telomerases and subsequent telomere loss. Therefore a putative

resistance mechanism to cisplatin might be related to telomerase hyperactivation and inability of the tumor cell to engage apoptosis [167].

2.4.1.7.2. Necrosis

Necrosis is considered a passive event in which the cell is irreversibly damaged by an environmental insult, leading to cell death. Several authors have reported the activation of the necrotic pathway in cells treated with oxaliplatin, especially in those harboring *TP53* mutations [168]. One protein related to this, is the glycogen synthase 3 β the inactivation of which induces cell death by caspase-independent necrototic process [169]. Its activation has been reported in colon carcinomas resistant to oxaliplatin. DNA damage induced by alkylating agents and ligation of death receptors, among others, were also described to be related with regulation of necrosis [170]. Oxaliplatin effectiveness was also associated with the production of oxygen reactive species, which in turn is a contributor to the execution of necrosis [171]. In addition, resistance to necrosis is also possible in cells over-treated with alkylating agents.

2.4.1.7.3. Autophagy

Autophagy is a critical catabolic process required for maintaining cellular homeostasis in health and pathological situations. It is typically observed in response to cellular stress, hypoxia, DNA damage or endoplasmic reticulum stress. Autophagy is activated in many tumors and its inhibition can lead to either increased cell death or increased survival, depending on several factors [171]. Its role in promoting chemoresistance or chemosensitivity is controversial. For instance, reducible high mobility group box 1 induces Beclin1 dependent autophagy and promotes tumor resistance to oxaliplatin [172]. In the same way down-regulation of autophagy related 5 proteins, enhanced sensitivity to oxaliplatin [173, 174].Other authors have reported that oxaliplatin treatment activates autophagy in hepatocellular carcinomas, and in cell lines and xenografts models of colon cancer, contributing to the tolerance of this drug by modulating oxygen reactive species generation, and as a consequence to chemoresistance. Contradictory examples are also described. Autophagy induction trought RAD001 (a potent activator of autophagy) in papillary thyroid cancer [175] or throught inhibition of oncomiR-21 in myeloid leukemia [176], enhanced the therapeutic response to cytotoxic chemotherapy.

2.4.1.7.4. Senescence

Cellular senescence can be induced through a multitude of internal/external pressures and in ideal situations acts as a selfprotecting mechanism [177]. It is a growth-arrest

program that prevents unlimited cell proliferation being linked to tumour suppression. However, if senescence is bypassed, cells can become immortalized and potentially undergo a malignant transformation [178]. Although there is little information about the conection between senescence and chemoresistance, researchers have already described that cancer cell lines can undergo senescence, when exposed to drugs like cisplatin, oxaliplatin or doxorubicin [179-181]. The same was reported in some *in vivo* cancer models [182] and also in the clinical. One of the earliest reports of treatment-induced senescence in patients, came from a neoadjuvant chemotherapy study in breast carcinoma in which approximately 42% of resected tumors stained positive for senescence markers [183]. This has been verified in later evaluations of lung cancer patients receiving neoadjuvant therapy [184] and in human prostate tumors that were monotorized before and after chemotherapy treatment with mitoxantrone, revealing an increase in senescent markers after treatment [185]. Sidi *et al*. concluded also that an induction of tumor cell senescence following neoadjuvant therapy was associated with a poor clinical outcome [186].

3. DNA Methylation

Epigenetics is the field of research devoted to those mechanisms affecting expression patterns without modifying the DNA sequence. This field of research includes studies of DNA methylation; histone tails modifications and non-coding RNAs (**Figure 9A**)[187]. These processes are not mutually exclusive and have an added role in the control of genome stability [188].

DNA methylation, the addition of a methyl group to the 5-carbon position of cytosine residues (5mC), is the most common covalent modification of human DNA (**Figure 9B**). It occurs almost exclusively at cytosine residues that are followed immediately by a guanine (so-called CpG dinucleotides) [189]. Cytosine methylation is observed at CpG dinucleotides that tend to cluster into islands containing more than 55% guanine-citosine (GC) content over a 500 base pair region [190]. The human genome contains roughly 29,000 CpG islands that are distributed in a non-random pattern, with a preference for the promoter and first exon regions of protein coding genes. These CpG islands are observed within the promoters of about more or less 72% of human genes [13].

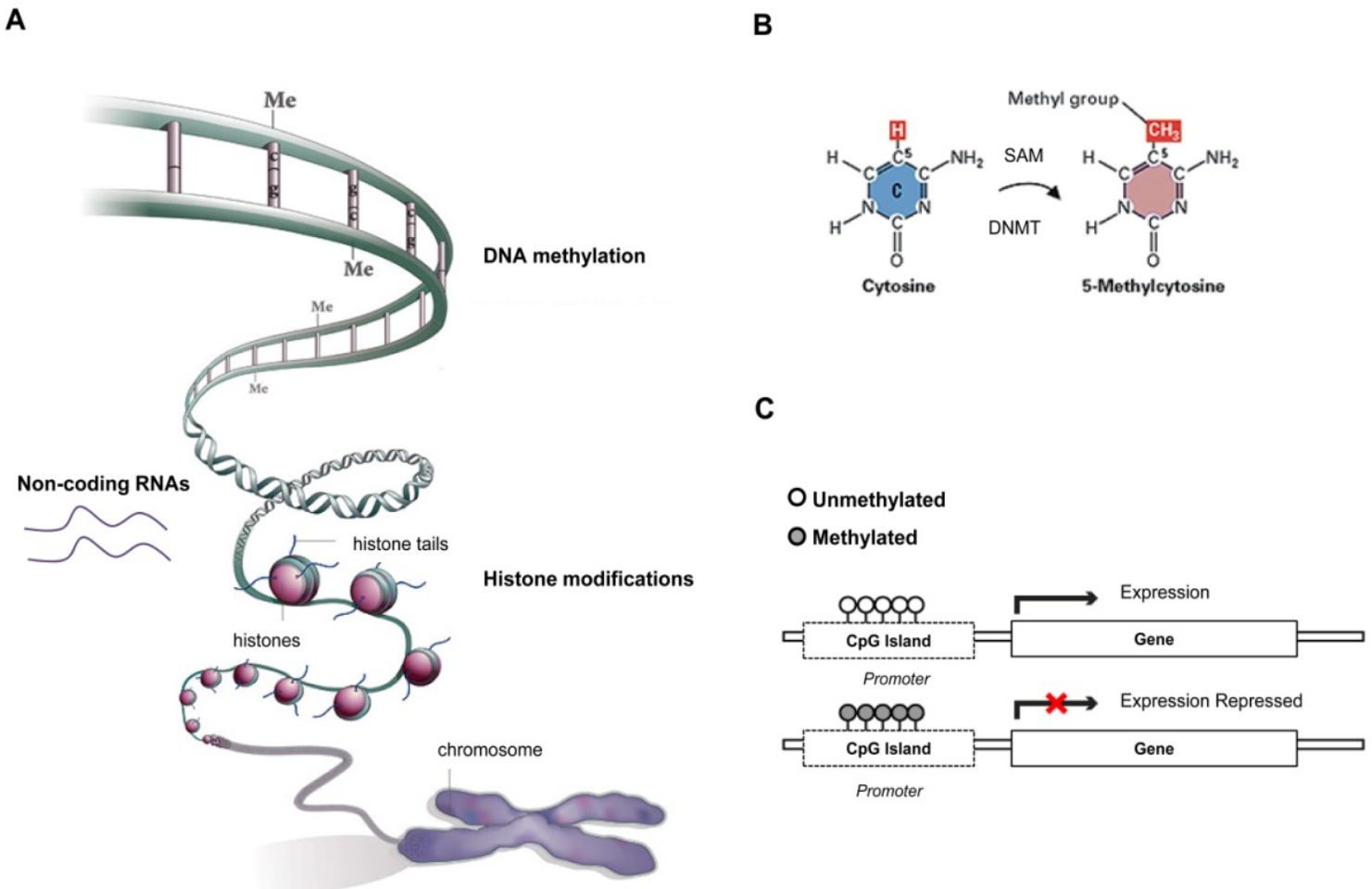

Figure 9: DNA Methylation. A. Main epigenetic mechanisms. DNA methylation: methyl marks (Me) are added to certain DNA bases for repress gene activity; non-coding RNAs; and histone modifications: a combination of different molecules can attach to the *tails* of proteins called histones. These alter the activity of the DNA wrapped around them. **B. DNA methylation in mammals**: addition of a methyl group from S-adenosyl methionine (SAM) to the 5-carbon position of cytosine residue, by DNA methyltransferase (DNMT). **C. Promoter methylation and gene expression.** CpG islands that remain free of methylation, are associated with transcriptional active genes in contrast to the one that are densely methylated (hypermethylated). *Adapted from Qiu, 2006*

The DNA methylation patterns are a stable and heritable epigenetic trait of mammalian genomes. There are three main proteins involved in establishing and maintaining DNA methylation marks within mammalian cells: DNA (cytosine-5-)-methyltransferase (DNMT) 1, DNMT3A, and DNMT3B [191]. DNMT1 is responsible for restoring methylation pattern on hemi-methylated DNA following replication [190]. In contrast DNMT3A and DNMT3B are responsible for *de novo* methylation. All of them appear to hold an indispensable function since mutant mice lacking these genes are not viable [192, 193]. Compared with other epigenetic mechanisms, DNA methylation was generally considered to be a relatively stable epigenetic modification. Actually the idea of an active DNA demethylation is taking strength. Ten-eleven translocation 1–3 proteins have recently been discovered in mammalian cells to be members of a family of DNA hydroxylases that possess enzymatic activity toward the methyl mark on the 5mC. Tet proteins can convert 5mC into 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine through consecutive oxidation reactions. These modified bases may represent new epigenetic states in genomic DNA or intermediates in the process of DNA demethylation [194].

In normal cells most CpG islands remain unmethylated and are associated with transcriptional active genes, predominantly the so called housekeeping, tumor suppressor and caretaker genes [195]. However there are certain CpG islands normally methylated, including those associated with imprinted and inactive X chromosome genes [196]. In general, there is an inverse correlation between promoter methylation status and genes expression (**Figure 9C**) [197]. Compared with their normal counterparts, cancer cells exhibit significant changes in DNA methylation patterns, which can generally be summarized as global hypomethylation of the genome accompanied by focal hypermethylation events [198]. The origin of these changes is largely unknown.

Much more is known about how changes in DNA methylation may lead to changes in gene expression that are implicated in carcinogenesis. The most emphasized implication of aberrant DNA methylation is the inactivation of tumor suppressor genes. The clear association between promoter hypermethylation and transcriptional inactivation has led to a revision of Knudson's two-hit hypothesis for tumor suppressor inactivation by adding a new pathway to gene inactivation [199]. In this respect, DNA methylation is functionally equivalent to genetic events. *De novo* DNA methylation occur early in tumor progression and lead to abnormal function of important cellular pathways, including those controlling cell cycle, apoptosis, and cell-to-cell growth

signaling [200]. Changes in DNA methylation may have many cellular consequences other than those affecting the transcriptional activity of tumor suppressor genes. For example methylation of cytosine strongly increases the rate of cytosine>timine transition mutations and is thought to be responsible for about one-third of all disease causing mutations in germline cells [201].

There are different models that explain the correlation between hypermethylation and gene transcriptional silencing. One of them is based on the fact that methylation of specific DNA sequences can prevent the binding of some ubiquitous transcription factors [200]. However this model can explain only a minority of cases, where methylation causes genes stable transcriptional silencing. An alternative model implicates changes in the architecture of the nucleosomal core as the repressive element. This model was reinforced by the identification of a family of proteins that preferentially bind to methylated CpGs, the methyl CpG binding proteins (MBDs) [202]. At least three of the five known members of this family (methyl CpG binding protein 2, MBD2 and MBD3) have been shown to be associated with large protein complex containing histone deacetylases (HDAC) 1 and 2 and chromatin-remodeling protein [203]. The action of these HDAC and chromatin remodeling activities catalyze the removal of acetyl groups from the core histones, converting the open, transcriptionally competent chromatin structure into a closed structure that can no longer be accessed by the basal transcriptional machinery. The linkage between MBDs, histone deacetylases and the chromatin remodeling machinery has provided a basis for understanding how DNA methylation may mediate a transcriptionally incompetent chromatin state [204].

Besides controlling gene expression, DNA methylation is complicit in suppressing parasitic DNA sequences such as transposonable elements and endogenous retroviruses [205]. Active transposable elements are highly mutagenic as they tend to insert within expressed genes disrupting its normal function and can cause illegitimate recombination events and genomic rearrangements [206]. Interestingly, global hypomethylation is a hallmark of all stages of tumor cells with a 20%-60% decrease in methylated cytosines. This decrease in methylated DNA coincides with the reactivation of transposable elements, mitotic recombination (leading to loss of heterozygosity) and aneuploidy [198, 200].

3.1. DNA Methylation and Chemoresistance

Chemoresistance can be the result of multiple genes expression alteration in different cellular pathways. Regulation by DNA methylation can have a large impact on gene expression [196]. A number of recent studies suggest a direct role for epigenetic inactivation of genes, in determining tumor chemosensitivity [207, 208]. Key genes involved in DNA damage response pathways, such as cell cycle control, apoptosis and DNA repair signaling can frequently become methylated and epigenetically silenced in tumors. This may lead to differences in intrinsic sensitivity of tumors to chemotherapy, depending on the specific function of the gene inactivated. Furthermore, it is proposed that chemotherapy itself can exert a selective pressure on epigenetically silenced drug sensitivity genes present in subpopulations of cells, leading to acquired chemoresistance [207].

In contrast to genetic alterations, epigenetic changes can be modified pharmacologically and the re-expression of epigenetically silenced genes may result in the suppression of tumor growth and in an increased sensitivity to anticancer drug. In fact, *5-Aza*-2′-deoxycytidine (5-AZA) is already used for the treatment of all subtypes of myelodysplastic syndrome [209]. This drug has hypomethylating activity and, possibly, exerts its action by reinducing expression of genes silenced by the hypermethylation of CpG islands in their promoters [210]. Since the end goal of all chemotherapeutic agents is to induce death, loss-of-function in any necessary member of cellular death pathway will be manifested as a resistant phenotype. For example the hypermethylation of the *apoptotic peptidase activating factor 1* promoter silences the activity of the gene and, thus, prevents apoptosis. Relief of this repression and increasing sensitivity of the cells was observed following treatment with the demethylating agent, 5-AZA [211].

In addition, DNA repair capacities can impact drug-sensitivity. Many drugs have their effect by causing DNA damage that if left unrepaired, is lethal [110]. An example is the hypermethylation of the DNA repair gene *MGMT.* This enzyme reverses the damage induced by DNA alkylating agents (e.g. temozolomide) being a strong prognostic tool for determining the treatment response of gliomas, i.e. methylated *MGMT* confers sensitivity to the drugs [54, 212].

Important to refer that it seems that the opposing processes of regional hypermethylation and global hypomethylation coexist in the same cells. Both confer a selective advantage upon cancer cells by targeting different sets of genes with

opposing roles in cellular transformation. Regional hypermethylation targets the silencing of genes which suppress tumorigenesis, while global hypomethylation probably targets activation of genes which are required for different stages of the transformation process [213].

4. Drug Resistance Predictive Biomarkers in Metastasic Colorectal and in Non-seminoma Cancer

Tumor chemoresistance is a multifactor process and a reality. A good way to fight against it is, apart from the combined treatments with different drugs, the identification of molecular markers that allow a personalized treatment, selecting the most suitable therapy for each patient. Until now there are a few examples for colorectal and non-seminoma cancers. At colorectal cancer *KRAS* mutational status has been established as a predictive factor of response to anti-EGFR agents, being the use of these only approved in KRAS wild-type tumors [214]. Concerning non-seminoma tumors, the only biomarker associated with cisplatin resistance is the presence of the *BRAF,* V600E mutation [215]. What concerns to epigenetic biomarkers, until now nothing is applied in the clinics for these two types of tumors.

AIMS

- Although chemotherapeutic drugs are widely used in order to improve the cancer outcome, intrinsic and acquired drug resistance remain the most unpredictable factor affecting chemotherapy and a major impediment to successful patient's treatment [107]. Understanding the cellular and molecular mechanisms leading to chemoresistance may dramatically impact on the way chemotherapeutic drugs are designed and used. Then, it would allow selecting the most suitable personalized therapy.

- It has become increasingly clear that many chemotherapeutic agents kill susceptible cells through the induction of the physiological cell death program. Accordingly, deregulation of any gene involved in the activation or execution of the death processes may be a major mechanism of chemoresistance [107]. Tumor suppressor and DNA repair genes were classified as important mediators of chemotherapeutic response [207]. While inactivation of tumor suppressor genes could lead to drug resistance, inactivation of DNA repair genes, drug metabolisms, and detoxification genes might lead to drug sensitivity. This can be due to different mechanisms like regional hypermethylation and/or global hypomethylation [213].

- The possibility that some genes conferring chemoresistance are reversibly switched on/off by DNA methylation is particularly important and may have relevant clinical implications. A very potent specific inhibitor of DNA methylation, 5-AZA, has been widely used as a demethylating agent *in vitro*, and is used clinically in the treatment of acute leukemia and myelodysplasia [216].

HYPOTESIS:

Taking all into account, the present Doctoral Thesis has been devoted to provide further knowledge about the cross-talk between genes promoters DNA methylation status and tumors chemosensitivity, on a more detailed understanding of the influence of its changes in resistance to dacarbazine and to platinum agents, such oxaliplatin and cisplatin. We also pretend to explore alternative therapies as an attempt for reverse tumors chemoresistance the main cause of patients dead. In order to address these goals, we studied two cancer models, colorectal and testicular germ cell tumors. For each one we defined specific aims.

AIMS

1. Determine if *MGMT* gene promoter methylation status influences rate response, progression free survival and/ or overall survival in metastasic colorectal cancer patients, treated with dacarbazine.
2. Discover genes responsible for oxaliplatin acquired resistance in a colorectal cancer *in vitro* model.
3. Uncover new predictive biomarkers for oxaliplatin based chemotherapy treated metastasic colorectal cancer patients.
4. Find candidate genes responsible for testicular germ cell tumors cisplatin acquired resistance.
5. Study the influence of *MGMT* promoter DNA methylation status in non-seminoma tumors chemoresistat to cisplatin.
6. Determine the potential role of novel therapeutic approaches for resensitize to cisplatin metastatic refractory non-seminoma tumors.

RESULTS

DIRECTORS REPORT

To who may concern, we authenticate that the PhD student CÁTIA ALEXANDRA MARTINS FREITAS MOUTINHO will present her PhD thesis by scientific publications. Her contribution for each publication will be next pointed out.

STUDY I

"Promoter CpG Island Hypermethylation of the DNA Repair Enzyme MGMT Predicts Clinical Response to Dacarbazine in a Phase II Study for Metastatic Colorectal Cancer"

Alessio Amatu, Andrea Sartore-Bianchi, **Catia Moutinho**, Alessandro Belotti, Katia Bencardino, Giuseppe Chirico, Andrea Cassingena, Francesca Rusconi, Anna Esposito, Michele Nichelatti, Manel Esteller, and Salvatore Siena

Contribution: Cátia Moutinho was the responsible for DNA extraction from paraffin embedded tumors and subsequent *MGMT* methylation analysis. Besides she participated in the analysis and interpretation of general data (e.g., statistical analysis, biostatistics, computational analysis), in the manuscript writing and revision.

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STUDY II

"Epigenetic Inactivation of the BRCA1 Interactor SRBC and Resistance to Oxaliplatin in Colorectal Cancer"

Catia Moutinho, Anna Martinez-Cardús, Cristina Santos, Valentin Navarro-Pérez, Eva Martínez-Balibrea, Eva Musulen, F. Javier Carmona, Andrea Sartore-Bianchi, Andrea Cassingena, Salvatore Siena, Elena Elez, Josep Tabernero, Ramon Salazar, Albert Abad, and Manel Esteller

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Contribution: In this paper Cátia Moutinho was the responsible of the experimental design, and execution, supervised by Dr. Esteller. She also performed the analysis and interpretation of the generated data together, with the manuscript writing and revision.

STUDY III

"Orthoxenografts of Testicular Germ Cell Tumors Enable Identification of Glucosylceramide Synthase as a Cisplatin Resensitizing Target"

Josep M. Piulats, August Vidal, Clara Muñoz, Francisco J, Gacía-Rodriguez, Marga Nadal, **Cátia Moutinho**, , María Martínez-Iniesta, Josefina Mora, Agnés Figueras, Elisabet Guinó, Veronica Davalos, Laura Padullés, Àlvaro Aytés, David G. Molleví, Sara Puertas, Wilmar Castillo, Victor Moreno, Purificación Muñoz, Ferrán Algaba, Jose Carlos Fernandez-Checa, Enric Condom, Francesc Viñals, Josep R. Germà, Manel Esteller, Gabriel Capella, Albert Morales, Julian Cerón, Xavier García-del-Muro and Alberto Villanueva

Journal: *Submitted to Cancer Cell*

Contribution: All the functional *in vitro* studies for *GCS* were designed and performed by Cátia Moutinho, under Dr. Villanueva supervision. She also participated in data analysis, scientific discussion and in manuscript writing.

STUDY IV

"Loss of MGMT Promoter Methylation and Resistance to Cisplatin in Non-Seminoma Testicular Germ Cell Tumors"

Cátia Moutinho, Xavier Garcia-del-Muro, Elisabet Guino, August Vidal, Sara Puertas, Clara Munoz, Josep M. Piulats, Alberto Villanueva and Manel Esteller

Contribution: Cátia Moutinho was in charge for the experimental design and experimental labor. She also performed the analysis and interpretation of the generated data, together with the manuscript writing. All the work was supervised by Dr. Villanueva and Dr. Esteller.

Journal: *In preparation*

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STUDY I

"Promoter CpG Island Hypermethylation of the DNA Repair Enzyme MGMT Predicts Clinical Response to dacarbazine in a Phase II Study for Metastatic Colorectal Cancer"

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Clinical Cancer Research. 2013 April 15; 19(8):2265-72. doi: 10.1158/1078-0432.CCR-12-3518. Epub 2013 Feb 19.
(Supplement I)

RESUMEN

Hipótesis: MGMT es una proteína de reparación del ADN que elimina los aductos generados por los diferentes fármacos en la posición O^6 de la guanina. Aproximadamente el 40 % de los cánceres colorectal tiene una deficiencia en esta proteína debido mayoritariamente a su silenciamiento por hipermetilación del promotor del gen. Agentes alquilantes tales como la dacarbazina, ejercen su actividad antitumoral por la metilación del ADN en la posición O^6 de las guaninas. Hecho que se asocia con un desfase entre los pares de bases, por lo tanto, la actividad de la dacarbazina puede ser más eficiente en tumores colorectal que carecen de MGMT que los repare. Hemos llevado a cabo un estudio del estatus de metilación del promotor del *MGMT* en el seno de un estudio clínico fase II (DETECT-01trial, EUDRACT número 2011-002080-21) donde los pacientes fueron randomizados a ser tratados con dacarbazina. Se incluyeron pacientes con cáncer colorectal que han fracasado con las terapias estándar (oxaliplatino, irinotecan, fluoropirimidinas; cetuximab, panitumumab y bevacizumab, si el *KRAS* no está mutado).

Diseño experimental: Todos los pacientes tenían tejido tumoral para evaluar, en un estudio blindado, la hipermetilación del promotor de *MGMT*. Los pacientes recibieron dacarbazina (250 mg/m^2) por vía intravenosa cada día, durante cuatro días consecutivos, cada 21 días, hasta progresión de la enfermedad o toxicidad intolerable. Se utilizó un diseño de *Simon two stage test* para determinar si la tasa de respuesta global sería de 10 % o más. Los objetivos secundarios incluyeron la asociación de la respuesta, progresión libre de enfermedad y la tasa de control de la enfermedad, con el estado de metilación del promotor de *MGMT*.

Resultados: Sesenta y ocho pacientes fueron incluidos en el estudio, desde Mayo de 2011 hasta Marzo de 2012. Los pacientes recibieron una media de tres ciclos de dacarbazina (intervalo 1-12). Grados 3 y 4 de toxicidad incluyeron: fatiga (41%), náuseas/vómitos (29%), estreñimiento (25%), disminución del recuento de plaquetas (19%), y anemia (18%). En general, dos pacientes (2%) alcanzaron una respuesta parcial y ocho pacientes (12%) han obtenido una enfermedad estable. La tasa de control de la enfermedad (respuesta parcial + enfermedad estable) se asoció significativamente con la hipermetilación del promotor del gen *MGMT,* en los tumores correspondientes.

Conclusión: Las respuestas clínicas objetivas a la dacarbazina en pacientes con cáncer colorectal metastásico se limitan a aquellos tumores que albergaban la inactivación epigenética de la enzima de reparación del ADN, MGMT. Sugiriendo nuestro estudio la realización de un nuevo estudio clínico estratificando los pacientes a ser tratados en función de la metilación de *MGMT*.

ABSTRACT

Purpose: *MGMT* is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA. Approximately 40% of colorectal tumors display MGMT deficiency due to the promoter hypermethylation leading to silencing of the gene. Alkylating agents, such as dacarbazine, exert their antitumor activity by DNA methylation at the O^6-guanine site, inducing base pair mismatch; therefore, activity of dacarbazine could be enhanced in colorectal tumors lacking MGMT. We conducted a phase II study with dacarbazine in colorectal tumors who had failed standard therapies (oxaliplatin, irinotecan, fluoropyrimidines, and cetuximab or panitumumab if *KRAS* wild-type).

Experimental Design: All patients had tumor tissue assessed for *MGMT* as promoter hypermethylation in double-blind for treatment outcome. Patients received dacarbazine 250 mg/m2 intravenously every day for four consecutive days, every 21 days, until progressive disease or intolerable toxicity. We used a Simon two-stage design to determine whether the overall response rate would be 10% or more. Secondary endpoints included association of response, progression-free survival, and disease control rate with *MGMT* status.

Results: Sixty-eight patients were enrolled from May 2011 to March 2012. Patients received a median of three cycles of dacarbazine (range 1–12). Grades 3 and 4 toxicities included: fatigue (41%), nausea/vomiting (29%), constipation (25%), platelet count decrease (19%), and anemia (18%). Overall, two patients 2% achieved partial response and eight patients (12%) had stable disease. Disease control rate (partial response + stable disease) was significantly associated with *MGMT* promoter hypermethylation in the corresponding tumors.

Conclusion: Objective clinical response to dacarbazine in patients with metastatic colorectal cancer is confined to those tumors harboring epigenetic inactivation of the DNA repair enzyme MGMT.

Introduction

Globally, nearly 1.25 million patients are diagnosed and more than 600,000 patients die from colorectal cancer each year (1). At least 50% of patients develop metastases (2), and most of these patients have unresectable tumors (2, 3).

In the last 10 years, thanks to a wider clinical use of a multidisciplinary approach, along with the introduction of new cytotoxic drugs and the addition of targeted therapies against the angiogenesis (bevacizumab and aflibercept), the EGFR pathway (cetuximab and panitumumab), or multiple receptor tyrosine kinases (regorafenib), the survival of patients with metastatic colorectal cancer has considerably been ameliorated (4–6). Nevertheless, prognosis remains poor and patients carrying *KRAS* mutations (35%–40% of colorectal cancers), which preclude responsiveness to cetuximab or panitumumab (6), have limited therapeutic options after failure of 2 lines of standard treatments, although a significant percentage of these patients retain a good performance status potentially allowing further therapies. There is therefore an unmet need of therapeutic options, based on specific molecular alterations that could prove their effectiveness also in the wide *KRAS*-mutated subgroup of colorectal cancers.

MGMT is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA. MGMT protects cells against these lesions, transferring the alkyl group from the O^6-guanine in DNA to an active cysteine within its own sequence. Such reaction inactivates one MGMT molecule for each lesion repaired (7). The inactivation of tumor suppressor genes by the presence of cytosine methylation encompassing the corresponding transcription start site located in a CpG island is gaining "momentum" in the management of oncology patients (8) and, in this regard, promoter CpG island hypermethylation leads to the transcriptional silencing of *MGMT* (9). The subsequent lack of repair of O^6-methylguanine adducts can result in a higher frequency of G:C>A:T transitions (10,11). It is known that approximately 40% of colorectal cancers have silencing of *MGMT*. Interestingly, in a retrospective analysis on 244 colorectal cancers samples, it has been found that 71% of tumors with G to A mutation in *KRAS* showed *MGMT* epigenetic inactivation, showing a strong association between the *MGMT* inactivation by promoter hypermethylation and the appearance of G to A mutations at *KRAS* (10). Furthermore, *MGMT* hypermethylation was also found in 35% of wild-type *KRAS* metastasic colorectal cancers. De Vogel and colleagues (12) found that *MGMT* hypermethylation is associated with G:C>A:T mutations in *KRAS*, but not in *APC*,

suggesting that *MGMT* hypermethylation may succeed *APC* mutations but it precedes *KRAS* mutations in colorectal carcinogenesis.

In cells, loss of MGMT expression leads to compromised DNA repair and may play a significant role in cancer progression and response to chemotherapy as it occurs in glioma (13–16). The mechanism of action of dacarbazine and temozolomide is DNA methylation at the O^6-guanine site, inducing base pair mismatch. The methyl group at O^6-site is removed by MGMT in a one step methyl transfer reaction. Therefore, we hypothesized that MGMT inactivation by hypermethylation may confer sensitivity to these agents (17). However, discrepant data about the clinical activity of these drugs in metastasic colorectal cancer are reported in the literature (18–21). A response rate of 19%, including one complete response, was reported in 26 fluoropyrimidine-resistant patients receiving cisplatin and dacarbazine (19). In another study, 48 patients refractory to fluoropyrimidine were treated with dacarbazine, irinotecan, and cisplatin obtaining a 33% of response rate (18). Temozolomide is an imidazotetrazine derivative of dacarbazine. The combination of lomeguatrib and temozolomide did not show activity in unselected metastasic colorectal cancer (20). In a pilot study including patients selected by tumor molecular profiling, temozolomide was effective in 2 patients with metastasic colorectal cancer exhibiting loss of MGMT expression (22). The latter finding was confirmed by a recent report by Shacham-Shmueli and colleagues (23) documenting objective response to temozolomide in 2 patients with MGMT-deficient metastasic colorectal cancer. On the basis of these findings, we designed a phase II trial aimed to assessing the antitumor activity of dacarbazine in patients with metastasic colorectal cancer with determined *MGMT* promoter methylation status and refractory to the standard therapies.

Materials and Methods

Trial design

The study was designed as a phase II trial (DETECT-01trial, EUDRACT number 2011-002080-21). Patients were treated with dacarbazine monotherapy until progression or unacceptable toxicity for 18 weeks (6 cycles). In case of partial response with clinical benefit, treatment was allowed until dose-limiting toxicity. Primary endpoint was to assess response rate to dacarbazine according to Response Evaluation Criteria in Solid Tumors (RECIST1.1) criteria. Secondary endpoints were to assess: disease control rate, progression free survival, identification of *KRAS*, and *MGMT* status in individual tumor samples as potential molecular biomarkers of response to dacarbazine. Written informed consent was obtained from each patient. The study

followed the Declaration of Helsinkiand good clinical practice, being approved by Ethic Committee of Ospedale Niguarda Ca' Granda (Milan, Italy).

Patients

All patients met the following inclusion criteria: age 18 years or more, Eastern Cooperative Oncology Group performance status of $\leq$ 1, histologically confirmed metastatic colorectal adenocarcinoma. A paraffin-embedded block from archival tumor tissue of primary and/or metastases for *MGMT* status analysis was requested. All patients had measurable disease (by RECIST criteria v1.1), and progressed on standard treatment with fluoropyrimidine, oxaliplatin, irinotecan, and cetuximab or panitumumab (the latter 2 drugs if *KRAS* wild-type). An adequate bone marrow, liver, and renal function was required.

Treatment schedules

Dacarbazine 250 mg/m^2 intravenously everyday for 4 consecutive days, every 21 days, was administered until progression, death, unacceptable toxicity, or patient with drawl of consemant. Antiemetic agents and supportive care were provided by treating physician as per standard clinical practice. In case of G3 hematologic toxicity (absolute neutrophil count <1.5x10^9/L and platelet count <100x10^9/L) dacarbazine was delayed by 1-week interval until recovery. Prophylactic use of colony-stimulating factors was allowed as per standard clinical practice.

Evaluation criteria

Patients were evaluated for primary overall response rate and secondary endpoint (disease control rate and progression free survival) according to RECIST criteria v1.1. Tumors were measured every 8 ± 1weeks through week 18 and then every 8 ± 1 weeks until the tumor progressed. Complete response was defined as disappearance of all target lesions. Any pathologic lymph nodes (whether target or non target) must have reduction in short axis to 10mm or less. An objective response (partial response) was defined as a reduction of at least 30 percent in the sum of all target lesions on computed tomography or magnetic resonance imaging scanning. Confirmed objective response were those for which a follow-up scan obtained at least 4 weeks later showed the persistence of the response. Progressive disease was defined as at least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also show an absolute increase of at least 5 mm. Stable disease was defined as shrinkage neither sufficient to qualify for partial

response nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum diameters while on study. Clinical investigators and radiologists were blinded as for *MGMT* status of the tumors.

Safety assessment

Safety assessments and blood biochemistry including complete blood counts were carried out at baseline and at the beginning of each treatment cycle. Any toxicity was assessed using the National Cancer Institute (NCI)-CTCAE version 4.0 and recorded at every visit until resolved.

Analysis of MGMT promoter methylation status

Loss of expression of MGMT was defined as promoter hypermethylation 25% or more as previously described (9). Tumor samples from patients' primary tumor were obtained from Pathology Department of the Ospedale Niguarda Ca' Granda or others Pathology Departments as referral. Formalin-fixed paraffin-embedded tumor blocks were reviewed for quality and tumor content. A single representative block, from either the primary tumor or metastasis, depending on availability, was selected for each case. White slides (2 cut of 10 μm, if from a tumor tissue paraffin block, or 3 cuts of 10 μm if from a biopsy) were sent to Bellvitge Biomedical Research Institute (IDIBELL; Barcelona, Spain) for DNA extraction and evaluation of *MGMT* promoter methylation status in blind as for clinical outcome. Genomic DNA was extracted from paraffin tissue samples following manufacturer's instructions (QIAamp DNA FFPE Tissue Kit). DNA was then subjected to bisulfate treatment using EZ DNA methylation kit (Zymo Research). Briefly, 1 μg of genomic DNA was denaturated by incubating with 0.2 mol/L NaOH. Aliquots of 10 mmol/L hydroquinone and 3 mol/L sodium bisulfate (pH 5.0) were added, and the solution was incubated at 50ºC for 16 hours. Treated DNA was purified, desulfonated with 0.3 mol/L NaOH, repurified on Zymo-Spin columns, and eluted with 25 μL water. *MGMT* promoter methylation status was analyzed by MSP. It was carried out in a 15 μL volume containing 1 μL of the sodium bisulfite-modified DNA. The characteristics of the MSP reactions and the primer sequence have been previously described (14). SW620 human colorectal cell line was used as a positive control for hypermethylated alleles of *MGMT* and DNA from RKO cell line used as a negative control (**Fig. 1**).

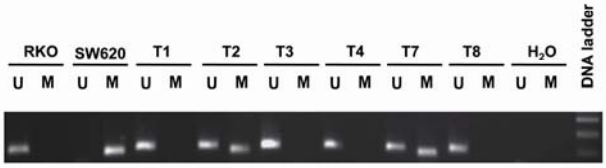

Figure 1. Methyl Specific PCR for *MGMT* promoter. Example of results obtained for 6 metastasic colorectal cancer primary tumours from the study cohort. Tumors T2 and T7 were methylated, and all the others unmetylated. U indicates unmethylated and M methylated tumor. RKO was the human colorectal cancer cell line used as negative control for methylation and SW620 the human colorectal cancer cell line used as positive one. H_2O is the experiment negative control.

Statistical analysis

According to clinical considerations and on the basis of the available literature, the efficacy of a treatment in this setting of metastasic colorectal cancer chemorefractory patients would be considered poor if the overall response rate is 3% or less, whereas it could be considered of clinical usefulness if the overall response rate is 10% or more. Assuming α= 0.05 and β=0.20, a Simon Optimal 2-stage design has been then chosen to test the null hypothesis that $P \leq 0.03$ versus the alternative that $P \geq 0.10$. According to this design, if at least 2 of the first 40 patients would have achieved an objective response, enrollment would have been extended by 28 patients. Overall, objective response rate of dacarbazine monotherapy would have been deemed unacceptable if objective response was 4 or less. The association between *MGMT* promoter methylation status and overall response rate and disease control rate was determined by 2-sided Student t-tests or Fisher exact test. Progression free survival was estimated by Kaplan–Meier product-limit method followed by log-rank test.

Results

Patients' characteristics

Sixty-eight patients were enrolled in our institution from May 2011 until March 2012. All patients had progressed on fluoropyrimidines, oxaliplatin, irinotecan, and cetuximab or panitumumab (the latter 2 drugs if *KRAS* wild-type). Eighty seven per cent of patients had received prior bevacizumab and 19% patient had received more than 4 lines of treatment. Twenty percent of patients received mitomycin C, 4% raltitrexed, and 12% previous experimental agents within clinical trials. Clinical characteristics of patients in this trial are reported in **Table 1**. Reasons for discontinuation of dacarbazine treatment included hematologic toxicity (1 patient), progression (61 patients), death (4 patients),

and withdrawal of consent (2 patients). Cause of death was recorded as metastasic colorectal cancer in all deceased patients.

Table 1. Patients characteristics

Demographics	Value (%)
Age, years	
Median	63,5
Range	29-81
Sex, No. (%)	
Male	47 (69)
Female	21 (31)
Clinical Charachteristics	**No. Of Patients (%)**
Performance status	
0	37 (54)
1	31 (46)
Tumor grade at diagnosis	
1	2 (3)
2	43 (63)
3	9 (13)
Not available	14 (21)
No. of prior treatments	
2	14 (20)
3	17 (25)
4	23 (35)
5	5 (7)
6	6 (9)
7	2 (3)

Clinical Characteristics	No. Of Patients (%)
Tumor KRAS status	
Wild type	35 (47)
Mutated	33 (49)
G12V	7
G12C	5
G12S	1
G12D	7
G12A	1
G13D	5
Codon not available	7
Tumor MGMT methylation status	
Hypermethylated	26 (38)
Unmethylated	39 (58)
Not assessable	3 (4)
No. of metastatic sites	
1	2 (3)
2	25 (37)
3	29 (43)
4	11 (16)
5	1 (1)
Patients previously treated with	
Bevacizumab	59 (87)
Mitomycin	17 (25)
Experimental drugs	8 (12)

Toxicity

Adverse events are listed in **Supplementary Table S1**. Hematologic toxicity was the most frequent adverse event reported and general toxicity was consistent with the known toxicity profile of dacarbazine. We observed 3 hematologic G4 adverse events (2 platelet count decreased and one neutrophil count decreased). Hepatic failure with increased bilirubin due to progression of disease was observed in 3 patients with extensive metastatic liver involvement.

Analysis of MGMT promoter hypermethylation

Sixty-five of 68 patients were tested for *MGMT* promoter CpG island methylation, as showed in **Table 1**. Overall, *MGMT* hypermethylation was found in 40% (26/65) of the colorectal neoplasms DNAs analyzed a similar frequency to the previously reported for

this tumor type (9). According to the location of the tumor, *MGMT* promoter status was assessed in 69% (45/65) in primary tumor, in 14% (9/65) in metastatic site, and in 17% (11/65) in both primary and metastatic site from the same patient. In the latter case, we observed concordance in 10 out of 11 pairs, with only one case showing a hypermethylated primary with unmethylated liver metastasis, and the result from liver metastasis was considered for the purpose of analysis. Sites of metastases were: liver 75% (15/20), 5% (1/20) ovary, 10% (2/20) lung, 5% (1/20) spleen, and 5% (1/20) cutaneous. *MGMT* hypermethylation was more frequent (61% and 31%, respectively) in tumors carrying *KRAS* mutation with G>A transition (G12D, G12V, or G13D), as previously described (10, 11), although the difference was not statistically significant due to the small size (only 26 patients were evaluable for both analysis; P=0.238).

Antitumor activity of dacarbazine

Overall response rate was 3%, with 2 partial responses. Stable disease was achieved in 8 of 68 patients (12%), accounting for a disease control rate (partial response + stable disease) of 15%. Median progression free survival was 57 days. Pre-planned analysis of secondary endpoints based on assessments of *MGMT* methylation and *KRAS* mutation status in individual tumors showed that objective response occurred only in patients displaying *MGMT*-methylated tumors (**Fig. 2A and Fig. 3**). In addition, we observed a significantly higher disease control rate (44.0% vs. 6%, P=0.012) in the *MGMT*-hypermethylated group (**Fig. 2**).

A trend toward better progression free survival [Hazard Ratio (HR) =0.66; 95% confidence interval 0.40–1.10; P=0.0982] was also found in the MGMT hypermethylated cases (**Fig. 4A**). A similar tendency was found between reduction of tumor volume following dacarbazine treatment and *MGMT* methylation status: tumor shrinkage of any size occurred more frequently in patients displaying *MGMT* hypermethylation (Fisher exact test, P=0.093). In contrast, *KRAS* status was not associated with progression free survival, disease control rate, and overall response rate (*KRAS* mutant vs. *KRAS* wild-type, P=0.735, 0.999, and 0.492, respectively; **Fig. 4B**).

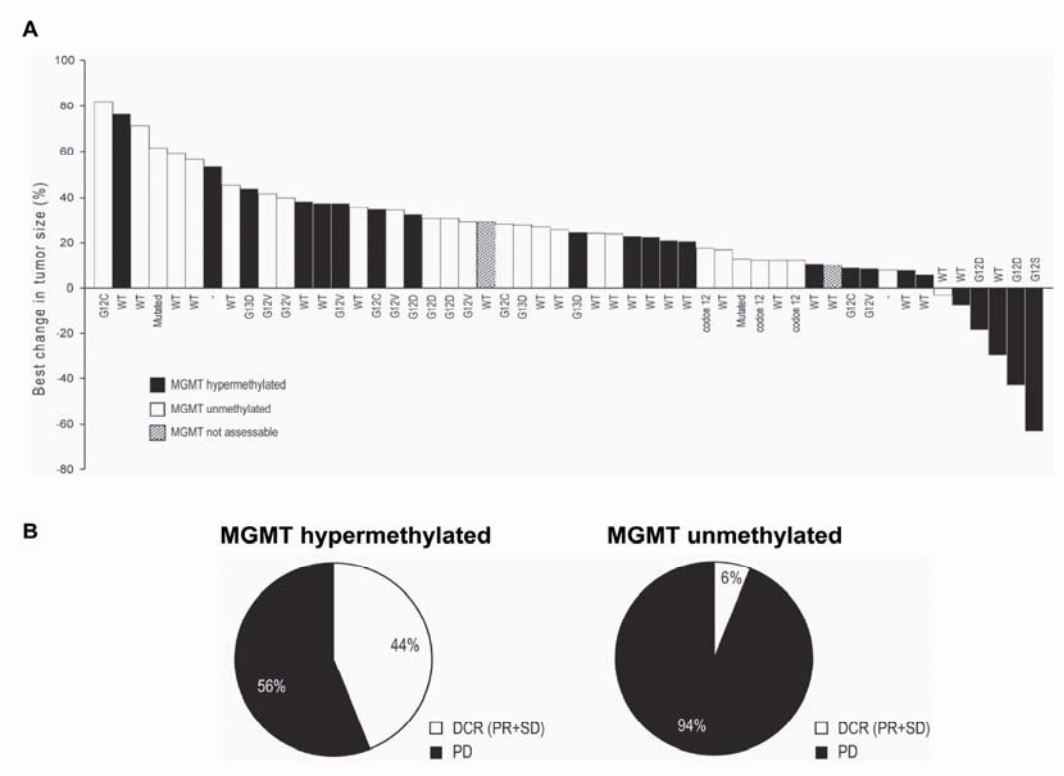

Figure 2. A, waterfall plot showing best change in tumor size (%) along with *MGMT* promoter methylation status (hypermethylated/unmethylated) and *KRAS* mutation status., if available. WT=*KRAS* wild-type, mutated= type of *KRAS* mutation not available. B, pie-charts showing disease control rate [DCR=partial response (PR) + stable disease (SD)] according to *MGMT* promoter methylation in individual colorectal tumors.

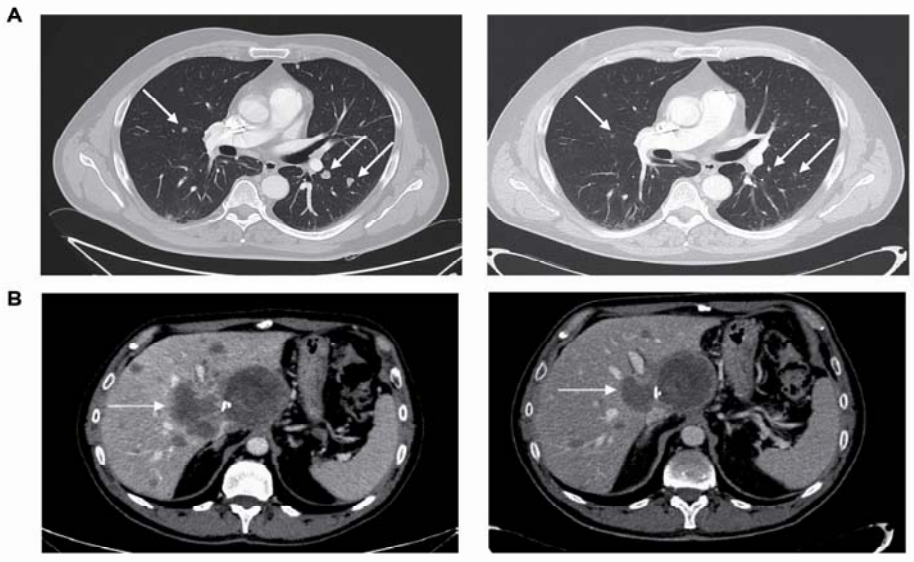

Figure 3. **Computed tomography.** Scan showing tumor shrinkage (white arrows) after treatment with dacarbazine in two patients, one with lung (A) and another with liver (B) metastases, both displaying MGMT promoter hypermethylation in primary tumor.

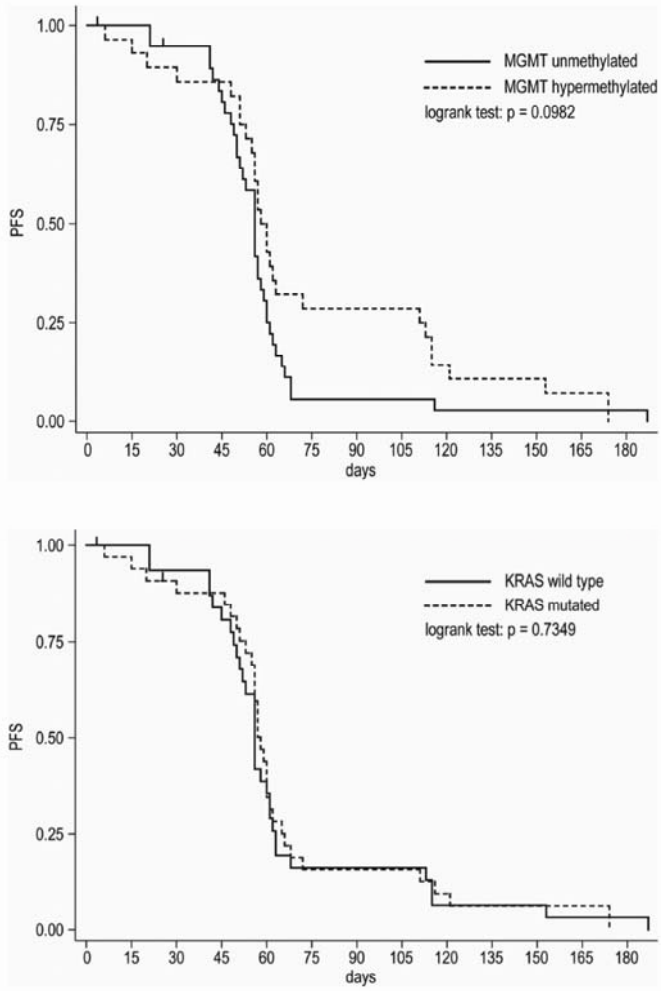

Figure 4. A, Kaplan-Meier progression free survival (PFS) analysis according to *MGMT* status in individual tumors. B, Kaplan-Meier progression free survival (PFS) analysis according to *KRAS* status in individual tumors.

Discussion

In this study, we document that dacarbazine is active after failure of standard therapies only in those patients with metastasic colorectal cancer whose tumor is harboring epigenetic inactivation of the DNA repair enzyme MGMT. Overall, we observed 2 objective response, accounting for 3% of overall response rate, and 8 stable diseases, accounting for 12% of the cases. The observation of a significant association between *MGMT* promoter hypermethylation and these clinical endpoints supports the hypothesis that DNA repair-defective metastasic colorectal cancer tumors are more susceptible to this chemotherapeutic agent. However, even in the case of *MGMT* hypermethylation, we observed that a fraction of 44% of patients achieved control of disease (stable disease + partial response), thus suggesting that a multiparametric signature including the DNA methylation associated silencing of MGMT together with other molecular traits

would improve the identification of colorectal cancer tumors with defects in DNA repair, susceptible to the action of dacarbazine.

The low response rate observed in the present cohort could be linked to the inclusion of heavily pretreated patients (median 4 lines of previous treatments). To interpret this clinical result in the context of therapy-resistant metastasic colorectal cancer, one should consider that second-line treatment with FOLFIRI or FOLFOX combination regimens induces overall response rate of 10% to 12% (24–26) and dramatically decreases in subsequent lines (6). It is also known that dacarbazine is activated in liver by CYP450 microsomial N-demethylation with formation of 5-[3-hydroxymethyl-3-methyl-triazen-lyl]-imidazole-4-carboxamide and 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC). Rapid decomposition of MTIC produces the major plasma and urine metabolite 5-amino-imidazole-4-carboxamide and the reactive species methane diazohydroxide, which produces molecular nitrogen and a methyl cation supposed to be the methylating species (27). It is therefore conceivable that the multiple (median 4) previous lines of cancer treatment as well as the high (79%) rate of liver involvement in the present study population may have exhausted the liver function capacity to activate dacarbazine.

It was our hypothesis that anticancer activity of dacarbazine could be enhanced by a specific defect in DNA repair system as evaluated by *MGMT* promoter hypermethylation in individual tumors. This epigenetic defect occurs in about 35% to 40% of metastasic colorectal cancers (9) and it is detected in more than 70% of *KRAS*-mutated tumors carrying the G>A transitions subtypes of mutation (10, 11), a subgroup of metastasic colorectal cancers with limited therapeutic options. Although the present trial was not designed, and thus, powered to assess a significant difference in progression free survival between *MGMT*-hypermethylated/unmethylated groups, we observed a trend toward better progression free survival in the MGMT hypermethylated group, together with a better disease control rate. The 2 patients displaying objective response were indeed carrying *MGMT*-hypermethylated tumors (**Fig. 2A**) and one of them showed a long-lasting maintenance of response of 6 months, which is uncommon in the advanced setting of metastasic colorectal cancer.

In conclusion, present data document that specific DNA repair defects can be associated with susceptibility to dacarbazine. The use of an alkylating agent that does not require hepatic activation may be preferable in heavily pre-treated patients with metastatic liver disease. In this regard, temozolomide is an alkylating agent whose

activity is also enhanced in tumors with MGMT loss (17) that is hydrolyzed in cells producing the active compound MTIC without requiring liver passage. A phase II trial with temozolomide has been designed and it is ongoing at our institution to assess the efficacy in patients with *MGMT* hypermethylated metastasic colorectal cancers after failure of standard therapies.

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SUPPLEMENTARY INFORMATION

Supplementary Table S1. Toxicities.

Non-hematological toxicities	All Grades		Grade 3-4	
	No. of patients	%	No. of patients	%
Fatigue	28	41	5	7
Nausea	20	29	5	7
Constipation	17	25	0	0
Blood bilirubin increased	11	16	0	0
Mucositis [217]	3	4	0	0
Flushing	2	3	0	0
Flu like symptoms	1	1	0	0
Dyspepsia	1	3	0	0
Rash	1	3	0	0
Arthralgia	1	3	0	0

Hematologic Toxicities	All Grades		Grade 3-4	
	No. of patients	%	No. of patients	%
Platelet count decrease	13	19	4	6
Anemia	12	18	1	1
White blood cell decrease	6	9	1	1
Febrile neutropenia	1	1	1	1

STUDY II

"Epigenetic Inactivation of the BRCA1 Interactor SRBC and Resistance to oxaliplatin in Colorectal Cancer"

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(Supplement II)

RESUMEN

Antecedentes: Uno de los problemas más importantes en el tratamiento de los pacientes de cáncer es la existencia de resistencia primaria (*tumores refractarios*) y/o la resistencia adquirida a los tratamientos de quimioterapia. Esto se asocia a diferentes defectos celulares, genéticos y epigenéticos.

Métodos: Para poder identificar posibles diferencias en los perfiles de metilación asociados con la adquisición de resistencia al oxalipaltino, líneas celulares pareadas sensibles vs. resistentes generadas *in vitro* se anlizaron con arrays de metilación. El gen *SRBC,* identificado diferencialmente metilado se validó mediante técnicas específicas de metilación de promotores y de expresión. Para evaluar el papel funcional de la expresión de esta proteína en la sensibilidad al oxaliplatino se realizaron experimentos *in vitro* tanto de sobre-expresión como de inhibición génica. La supervivencia libre de progresión de la enfermedad y la supervivencia global en pacientes con cáncer colorrectal metastásico se determinaron por curvas de Kaplan-Meier y con el análisis de regresión de Cox.

Resultados: En nuestro modelo celular se observó que la resistencia adquirida al oxaliplatino, depende, al menos en parte, de la metilación del promotor del gen *SRBC*, identificándose su inactivación por metilación con una mayor resistencia. La sobre-expresión *in vitro* de este gen o su inactivación conlleva un aumento en la sensibilidad o resistencia al oxaliplatino, respectivamente. En las dos cohortes independientes de pacientes con cáncer colorectal metastásico (n=131 y n= 58) el promotor del gen *SRBC* se encontraba metilado en un 30% de los tumores primarios. Este hecho se asoció con una menor supervivencia libre de progresión (Hazard Ratio (HR)=1,83; intervalo de confianza (IC) 95%=1.15-2.92; log-rank P=0.01). Siendo especialmente relevante en los casos tratados con oxaliplatino y para los que no estaba indicada la cirugía de las metástasis (HR=1.96, IC 95%=1.13-3.40; log-rank P=0.01 y HR=1.90, IC=1.01-3.60; log-rank P= 0,045).

Conclusiones: Nuestros resultados proporcionan una base para futuros estudios clínicos de validación de la hipermetilación del promotor del gen *SRBC*, como marcador predictivo de resistencia al oxaliplatino en el cáncer colorectal metastásico. Así la validación de este potencial marcador en ensayos clínicos estratificando en base a la metilación, puede permitir modificar el tratamiento de los pacientes con este cambio en su promotor.

ABSTRACT

Background: A major problem in cancer chemotherapy is the existence of primary resistance and/or the acquisition of secondary resistance. Many cellular defects contribute to chemoresistance, but epigenetic changes can also be a cause.

Methods: A DNA methylation microarray was used to identify epigenetic differences in oxaliplatin sensitive and resistant colon cancer cells. The candidate gene *SRBC* was validated by single locus DNA methylation and expression techniques. Transfection and short-hairpin experiments were used to assess oxaliplatin sensitivity. Progression free survival and overall survival in metastasic colorectal cancer patients were explored with Kaplan-Meier and Cox regression analyses. All statistical tests were two-sided.

Results: We found that oxaliplatin resistance in colon cancer cells depends on the DNA methylation-associated inactivation of the BRCA1 interactor *SRBC* gene. SRBC over-expression or depletion gives rise to sensitivity or resistance to oxaliplatin, respectively. *SRBC* epigenetic inactivation occurred in primary tumors from a discovery cohort of colorectal cancer patients (29.8%, 39 of 131), where it predicted shorter progression free survival (HR=1.83; 95% confidence interval (CI)=1.15-2.92; log-rank P=0.01), particularly in oxaliplatin-treated cases for which metastasis surgery was not indicated (HR=1.96; 95% CI=1.13-3.40; log-rank P=0.01). In a validation cohort of unresectable colorectal tumors treated with oxaliplatin (n=58), *SRBC* hypermethylation was also associated with shorter progression free survival (HR=1.90; CI=1.01-3.60; log-rank P=0.045).

Conclusions: These results provide a basis for future clinical studies to validate *SRBC* hypermethylation as a predictive marker for oxaliplatin resistance in colorectal cancer.

INTRODUCTION

Colorectal cancer is the second most common cause of cancer death in the western world (1). In metastasic colorectal cancer, polychemotherapy based on fluoropyrimidines plus oxaliplatin or irinotecan are the gold standard treatment, combined with biological agents such as cetuximab and panitumumab (2). Oxaliplatin forms intra-strand adducts that disrupt DNA replication and transcription (3,4). DNA damage induced by oxaliplatin is repaired in part by the nucleotide excision repair pathway (5), but the DNA double-strand breaks induced by the drug are also repaired by the *BRCA1* complex (6-8). In this regard, epigenetic inactivation of the BRCA1 gene by promoter CpG island methylation has been associated with increased sensitivity to cisplatin and carboplatin in breast and ovarian cancer (9,10).

Genes critical to colorectal tumor biology are frequently inactivated by hypermethylation of the CpG dinucleotides located in their 5'-CpG island regulatory regions (11-13). We wondered whether this epigenetic alteration was involved in the resistance to oxaliplatin in colorectal cancer, where treatment failure due to primary or acquired resistance remains a major obstacle to the management of the disease. Herein, we demonstrate that the epigenetic inactivation of the BRCA1 interactor *SRBC* gene by promoter CpG island hypermethylation is associated with poor outcome upon oxaliplatin treatment.

METHODS

Cell Lines

LoVo parental cell line (LoVo-S) and its derived 10-fold oxaliplatin resistant cells (LoVo-R)(14) were cultured at 37ºC in an atmosphere of 5% (v/v) carbon dioxide in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM-HAM's F12) medium supplemented with 20% (w/v) fetal bovine serum, 100U penicillin and 100µg/L streptomycin (Invitrogen, Carlsbad,CA).The HCT-116, SW48, SW480, SW620, RKO, Co115, and HCT-15 colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were authenticated by short tandem repeat profiling.

Determination of Drug Resistance

Oxaliplatin (5mg/ml) and 5-FU (50mg/ml) were obtained from TEVA (North Wales, PA, USA), and Accord Healthcare SLU (Barcelona, Spain), respectively. Cell viability was determined by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 1 x 10^3 cells were plated onto 96-well plates. Cells were treated

for 120 hours with different drugs concentration (oxaliplatin: 0-250μM and 5-FU: 0-35μM). MTT was added at a final concentration of 0.1%. After 2.5 hours incubations (37ºC, 5% carbon dioxide), the MTT metabolic product, formazan was dissolved in dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm. Prism Software (La Jolla, CA) was used to calculate drugs half maximal inhibitory concentration (IC_{50}).

DNA Methylation Analyses

DNA was subjected to bisulfite using EZ DNA methylation kit (Zymo Research, Orange, CA) as previously described (15). To perform the genome-wide DNA methylation profiling we used the Illumina Infinium HumanMethylation27 BeadChip® (Illumina, ,San Diego, CA, USA) microarray following the manufacturer's instructions (15).The Infinium assay quantifies DNA methylation levels at specific cytosine residues adjacent to guanine residues (CpG loci), by calculating the ratio (β-value) of intensities between locus-specific methylated and unmethylated bead-bound probes. The β-value is a continuous variable, ranging from 0 (unmethylated) to 1 (fully methylated). This microarray assesses the DNA methylation level of 27,578 CpG sites located at the promoter regions of 14 495 protein-coding genes. DNAs were processed on the same microarray to avoid batch effects. The array was scanned by a Bead Array Reader (Illumina), and intensity data analyzed using Genome Studio software (version 2011.1, Illumina). Further details are described in **Supplementary Methods**. The data is freely available at GeneExpressionOmnibus (http://www.ncbi.nlm.nih.gov/geo/) under GEO accession code GSE44446.

We established *SRBC* CpG island methylation status using three different polymerase chain reactions (PCR) based techniques: bisulfite genomic sequencing of multiple clones, MSP and pyrosequencing. Further technical details are described in **Supplementary Methods**. The used primer sequences are shown in **Supplementary Table 1.**

Messenger RNA and Protein Expression Analyses

Messenger RNA extraction, cDNA synthesis, conventional and quantitative real time PCR using Hs00376942_m1Taqman Gene Expression assay (Applied Bioystem, Madrid, Spain) were performed as previously described (16). Primer sequences are shown in **Supplementary Table 1.** Anti-SRBC (1/1000) from Cell Signaling and anti-β-actin-HRP antibody (1/20000) from Sigma (St. Louis, MO, USA) were used to develop the western blot analysis.

SRBC Transfection and Depletion Experiments

Human shRNAs and cDNA plasmids for SRBC were obtained from Origene (Rockville, MD, USA). After *E.coli* transformation, we preceded to plasmid DNA purification. Forty-eight hours after electroporation, cells transfected with shRNAs (TR317747, Origene) were grown in medium containing 0.8 or 0.6 μg/mL of puromycin (LoVo-S and HCT-116). Cells transfected with SRBC cDNA (SC320781, Origene) were grown with DMEM medium containing 0.8 or 0.6 mg/mL geneticin (G418, LoVo-R and HCT-15), to perform clonal selection. Once selected, clones were picked, grown and tested by western blot.

Patients

In our study we analyzed two independent Caucasian cohorts of stage IV colorectal cancer patients (17). In the discovery set 131 metastasic colorectal cancer primary tumors that received oxaliplatin plus fluoropirimidines based therapy, were retrospectively included. Formalin-fixed paraffin embedded tumors, obtained by surgical resection, came from three different hospitals (ICO-Hospitalet, ICO-Badalona and Niguarda Ca' Granda). Clinical features of the patients are showed in **Table 1**. From this cohort, 65 patients could undergo surgery to remove metastases. Following neoadjuvant regimen, 34 could be operated and 31 received palliative regimen. The rest of patients (n=66) showed unresectable metastases and directly underwent to palliative regimen. The greatest time of follow-up of this group was near 10 years. The validation cohort consisted in 58 stage IV colorectal cancer patients collected in Hospital Vall d'Hebron with a follow-up of near 3 years (**Table 1**). According to discovery set results, we selected patients with unresectable metastases, that received oxaliplatin plus fluoropirimidines based therapy in a neoadjuvant (n=20) or palliative regimen (n=38). The distribution of patients according to the different clinical features was similar in both cohorts. Signed informed consent was obtained from each patient and Clinical Research Ethical Committee from ICO-Hospitalet provided approval for the study. DNA from all cases was obtained from formalin-fixed paraffin-embedded tissue sections (10 μm) by xilol deparafination and digestion by proteinase K (Qiagen, Manchester, UK). Tumor specimens were composed of at least 70% carcinoma cells. DNA extraction was performed using a commercial kit (Qiagen), following manufacturer's instructions.

Table1. Clinical features of the discovery and validation cohorts of stage IV colorectal samples included in the study.*

Characteristic	DISCOVERY COHORT (n=131)							VALIDATION COHORT (n=58)						
	SBRC according to methylation status							SBRC according to methylation status						
	N	%	Unmethylated		Methylated		OR (95% CI)	N	%	Unmethylated		Methylated		OR (95% CI)
			N	**%**	**N**	**%**				**N**	**%**	**N**	**%**	
Gender														
Male	85	64.9%	61	71.7%	24	28.3%	1 (referent)	35	60.3%	29	82.8%	6	17.2%	1 (referent)
Female	46	35.1%	31	67.4%	15	32.6%	1.13 (0.85 - 1.47)	23	39.7%	15	65.2%	8	34.8%	0.60 (0.32 - 1.10)
Primary tumor														
Colon	102	77.8%	72	70.6%	30	29.4%	1 (referent)	41	70.7%	32	78.1%	9	21.9%	1 (referent)
Rectum	29	22.2%	20	68.9%	9	31.1%	0.94 (0.47 - 1.25)	17	28.3%	12	70.6%	5	29.4%	0.76 (0.33 - 1.79)
Metastatic site														
Liver	81	61.8%	52	64.2%	29	35.8%	1 (referent)	47	81.0%	35	74.5%	12	25.5%	1 (referent)
Lung	9	6.9%	5	55.5%	4	44.5%	0.72 (0.21 - 2.51)	3	5.2%	2	66.7%	1	33.3%	0.70 (0.07 - 7.12)
Others	18	13.7%	15	83.3%	3	16.7%	2.39 (0.74 - 7.66)	8	13.8%	7	87%	1	13%	2.10 (0.29 - 16.1)
Unknown	23	17.6%	20	86.9%	3	13.1%	-	0	0%	0	0%	0	0%	-
Chemotherapy schedule														
Oxaliplatin / 5-FU	107	81.7%	74	69.2%	33	30.8%	1 (referent)	41	70.7%	32	78.1%	9	21.9%	1 (referent)
Oxaliplatin / CAPE	10	7.6%	8	80.0%	2	20.0%	1.71 (0.38 - 7.64)	0	0%	0	0%	0	0%	-
Oxaliplatin / 5-FU / BA	13	9.9%	9	69.2%	4	30.8%	1.01 (0.33 - 3.05)	17	29.3%	12	70.6%	5	29.4%	0.76 (0.33 - 1.79)
Oxaliplatin / CAPE / BA	1	0.8%	1	100%	0	0%	-	0	0%	0	0%	0	0%	-
Chemotherapy regimen														
Neoadjuvant	65	49.6%	41	63.1%	24	36.9%	1(referent)	20	34.5%	15	75.0%	5	25.0%	1 (referent)
Palliative	66	50.4%	51	77.3%	15	22.7%	1.47 (0.95 - 2.27)	38	65.5%	29	76.3%	9	23.7%	1.02 (0.66 - 1.60)
Surgery of metastasis														
No	97	74.1%	72	74.3%	25	25.7%	1 (referent)	58	100%	44	75.9%	14	24.1%	-
Yes	34	25.9%	20	58.8%	14	41.2%	0.61 (0.34 - 1.07)	0	0%	0	0%	0	0%	-

Abbreviations: 5-FU = 5-fluorouracil; CAPE = capecitabine; BA= Biological agents

* None of the relationships were statistically significant after using the two-sided Chi-square test, considering $p<0.05$ as statistical significant threshold.

Statistical Analysis

In both independent cohorts we analyzed *SRBC* promoter methylation status and its association with response rate, progression free survival, and overall survival. The associations between categorical variables were assessed by χ^2 tests or Fisher exact test whenever required. Kaplan–Meier plots and log-rank test were used to estimate progression free survival and overall survival. The association between epigenetic variant and clinical parameters with progression free survival and overall survival was assessed through univariate and multivariable Cox proportional hazards regression models. The proportional hazards assumption for a Cox regression model was tested under R statistical software (Boston, MA) (cox.zph function). Statistical analysis was performed by using SPSS for Windows, (Armonk, NY), and *P* values less than .05 were considered statistically significant. All statistical tests were two-sided.

RESULTS

Identification of Epigenetics Changes Associated With oxaliplatin Resistance Using a DNA Methylation Microarray

To address in an unbiased manner whether epigenetic changes can be associated with oxaliplatin resistance, we adopted a whole genomic approach by comparing the DNA methylation status of 27 000 CpG sites (15) of an oxaliplatin-sensitive colon cancer cell line (LoVo-S) and an oxaliplatin-resistant clone (LoVo-R) that we derived by exposure to increasing concentrations of the drug (14).

This approach yielded only three differentially methylated target genes: *SRBC* (protein kinase C delta binding protein), *FAM111A* (family with sequence similarity 111, member A) and *FAM84A* (family with sequence similarity 84, member A) (**Supplementary Figure 1A**). The most noteworthy gene with the highest difference in DNA methylation was *SRBC*; thus, it was the logical option to pursue. However, we also studied initially the other two genes. For *FAM111A*, bisulfite genomic sequencing of multiple clones showed that indeed the CpG site included in the DNA methylation microarray was distinctly methylated in LoVo-S and LoVo-R cells; however, the remaining sites of the CpG island were unchanged (**Supplementary Figure 1B**). Thus, we excluded this gene from further experiments. For *FAM84A*, bisulfite genomic sequencing confirmed the differential methylation of the CpG island, but both conventional and quantitative real time PCR did not show any difference in gene expression (**Supplementary Figure 1, D and E**). Thus, we also excluded this second gene from further analyses. For the main target gene, *SRBC*, the DNA methylation microarray data showed that it had a CpG site located in its 5'-CpG island (−155 base

pair position) that was hypermethylated in LoVo-R but unmethylated in LoVo-S (**Supplementary Figure 1A**). Interestingly, *SRBC* CpG island methylation-associated silencing has already been found in cancer (18, 19), including colorectal tumors (20). From a functional standpoint, it is biologically plausible that *SRBC* is responsible for the different sensitivity to oxaliplatin because its protein interacts with the product of the *BRCA1* gene (18), which is widely accepted as being a mediator of response to DNA damage induced by platinum compounds (21).

To further demonstrate the presence of *SRBC* 5'-CpG island methylation in resistant cells, we undertook bisulfite genomic sequencing analyses. We found CpG island hypermethylation in LoVo-R but mostly an unmethylated CpG island in LoVo-S (**Figure 1A**). Importantly, SRBC expression was diminished in LoVo-R, showing CpG island methylation, whereas it was expressed in the unmethylated LoVo-S at the messenger RNA and protein levels (**Figure 1B**). SRBC re-expression was observed upon treatment with the DNA demethylating agent 5-AZA in LoVo-R cells (**Figure 1B**).

***SRBC* Epigenetic Inactivation and Oxaliplatin Resistance**

We next sought to demonstrate that the epigenetic inactivation of this gene functionally contributed to oxaliplatin resistance. We restored the expression of SRBC in LoVo-R by stably transfecting an exogenous expression vector (**Figure 1C**). Upon SRBC transfection, the cells proved to be statistically significantly more sensitive to the antiproliferative activity of oxaliplatin measured by the MTT assay (**Figure 1D**) than were the empty vector-transfected cells (LoVo-R + SRBC 1 and 2: P=0.02 and P< 0.001, respectively). In sharp contrast, we observed that SRBC stable down-regulation by the short hairpin RNA approach in SRBC-expressing and unmethylated sensitive cells (LoVo-S) (**Figure 1C**) had the opposite effect: a considerable enhancement of the resistance to the antiproliferative effect mediated by oxaliplatin (**Figure 1D**) (LoVo-S short hairpin SRBC A and B: P=0.04 and P<0 .001, respectively). The observed effects were specific for oxaliplatin because the in vitro depletion or enhancement of SRBC activity did not change the sensitivity to 5-FU (**Figure 1D**), other drug commonly used in colorectal cancer.

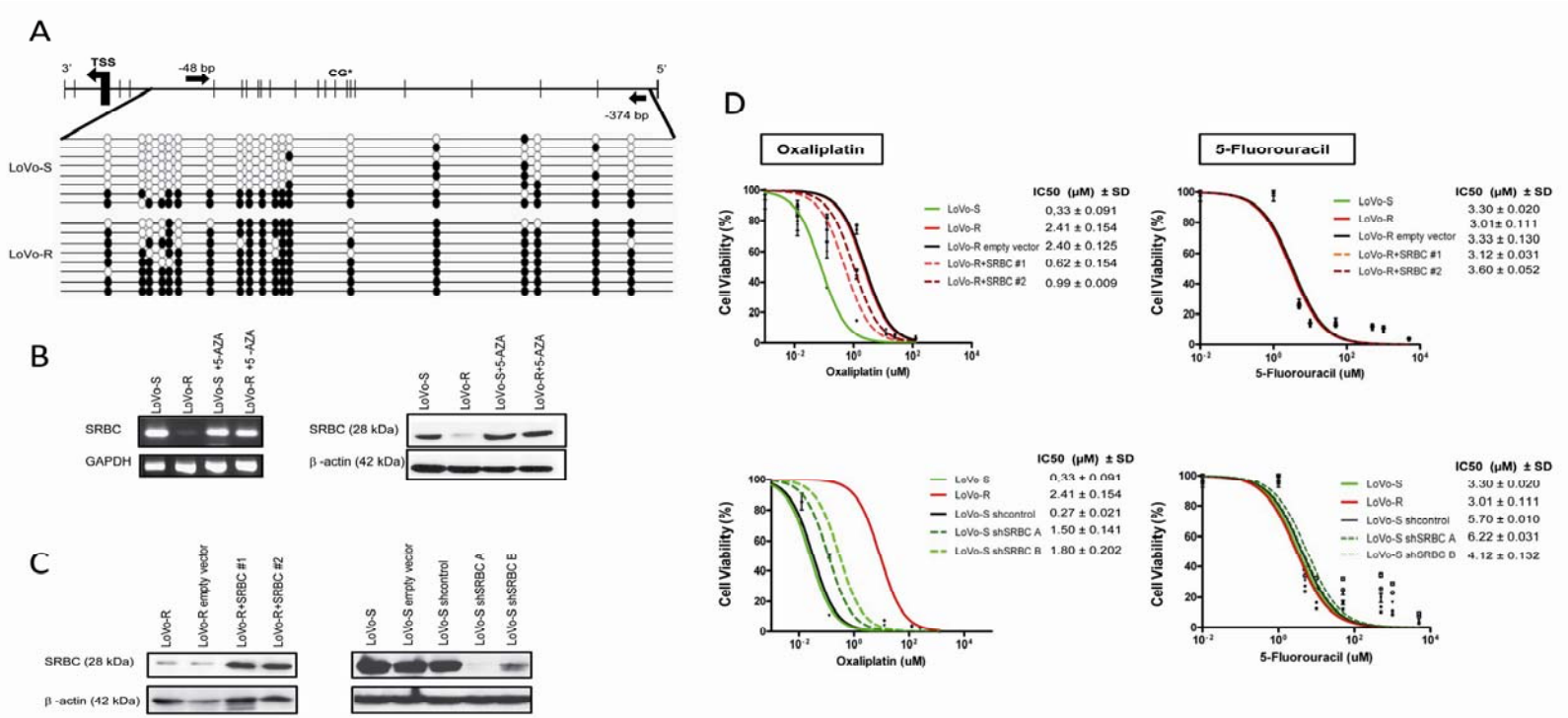


Figure 1. Epigenetic inactivation of SRBC is associated with resistance to oxaliplatin in colon cancer cells. A) Bisulfite genomic sequencing of eight individual clones in the SRBC promoter CpG island was used to determine DNA methylation status. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. Black arrows indicate the position of the bisulfite genomic sequencing primers. B) SRBC expression determined by semi-quantitative RT-PCR analyses (left) and western blot (right). GAPDH and B-actin were used as controls, respectively. The LoVo-R cell line (oxaliplatin-resistant) features a hypermethylated CpG island that is associated with the downregulation of the SRBC transcript and protein, in comparison with the SRBC-unmethylated and expressing LoVo-S cells (oxaliplatin-sensitive). Pharmacological treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine (5-AZA) restores SRBC expression. C) Western blot showing the in vitro enhancement (transfection in LoVo-R, left) or depletion (short hairpin RNA approach in LoVo-S, right) of the SRBC protein. D) Cell viability determined by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay upon use of oxaliplatin. External intervention by inducing SRBC overexpression (in LoVo-R cells) or depletion (in LoVo-S cells) gives rise to sensitivity or resistance to oxaliplatin, respectively (left panels). 5-fluorouracil sensitivity is not dependent on SRBC activity (right panels). The corresponding IC50 values are also shown.

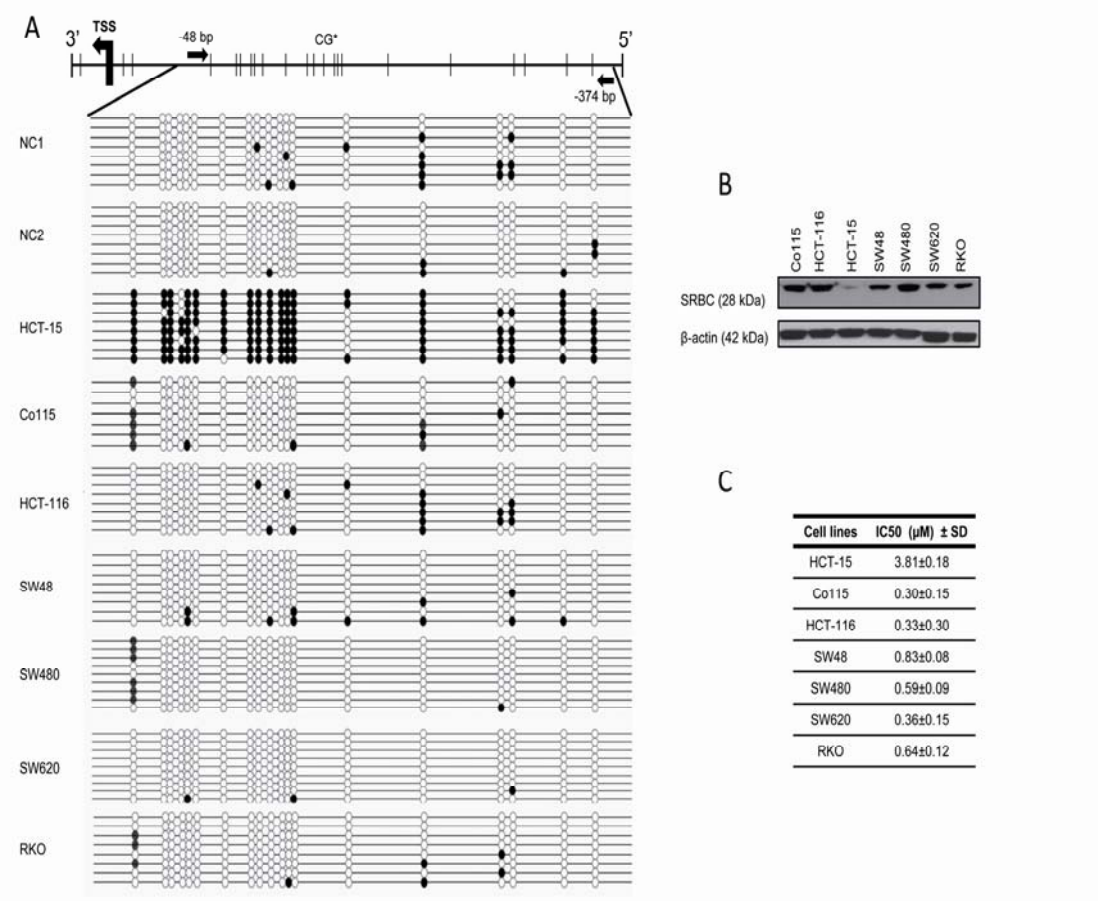
We extended our study to seven additional colon cancer cell lines (Co115, HCT-15, HCT-116, SW48, SW480, SW620, and RKO), in which we found *SRBC* promoter CpG island hypermethylation (**Figure 2A**) and the associated loss of expression only in HCT-15 cells (**Figure 2B**). Interestingly, these cells were the only ones showing resistance to oxaliplatin (IC_{50} ± standard deviation = 3.81 ± 0.18 μM); the remaining cells were sensitive to the drug (**Figure 2C**) (IC_{50} values ranging from 0.30 to 0.83 μM). As we did with LoVo-S and LoVo-R, we also sought to demonstrate that *SRBC* epigenetic inactivation functionally contributed to oxaliplatin resistance in these cells. We restored the expression of SRBC in the resistant cell line HCT-15 by stably transfecting an exogenous expression vector (**Supplementary Figure 2A**). Upon SRBC transfection, the cells proved to be statistically significantly more sensitive to the antiproliferative activity of oxaliplatin (HCT15+SRBC: *P*=0.02) (**Supplementary Figure 2B**). The opposite effect was observed with SRBC stable downregulation using the short hairpin RNA approach in SRBC-expressing and unmethylated sensitive cells (HCT-116): a noteworthy increase in the resistance to the antiproliferative effect mediated by oxaliplatin was found (**Supplementary Figure 2B**) (HCT-116 short hairpin SRBC A and B: *P*<0.001). The described effects were specific for oxaliplatin because the *in vitro* depletion or enhancement of SRBC activity did not change the sensitivity to 5-FU (**Supplementary Figure 2B**). Western blot analyses showed that the level of expression of the SRBC protein in the transfected clones was similar to that observed in unmethylated colon cancer cell lines (**Supplementary Figure 2A**).

SRBC Hypermethylation and Progression Free Survival in oxaliplatin-Treated Cases of Unresectable Colorectal Cancer

Given these in vitro findings that colon cancer cells with *SRBC* methylation-associated silencing were resistant to oxaliplatin, we wondered whether the same effect could be observed in clinical samples. The study of a first clinical cohort of 131 stage IV colorectal adenocarcinoma patients (termed *discovery cohort*) (**Table 1**), all of whom were treated with oxaliplatin in combination with a fluoropyrimidine, showed *SRBC* methylation in 29.8% (n=39 of 131) of the case patients analyzed by both methylation-specific PCR and pyrosequencing analyses (**Figure 3A; Supplementary Figure 3**). The described occurrence of *SRBC* hypermethylation in colorectal tumors was identical to the one available in the The Cancer Genome Atlas datasets (30.2%; n=70 of 232). Considering the whole population of studied advanced colorectal cancer case patients (n=131), we observed that *SRBC* hypermethylation was associated with progression free survival (HR=1.83; 95% CI=1.15 to 2.92; log-rank *P*=0.01) (**Figure 3B**). For the

105 case patients for whom overall survival information was available, *SRBC* hypermethylation was not associated with this variable (**Figure 3C**).

According to Cox regression multivariable test, surgery of metastases showed to be an independent progression free survival (HR=0.43; 95% CI=0.24 to 0.76; log-rank *P*= 0.004) and overall survival (HR=0.16; 95% CI=0.04 to 0.52; log-rank *P*=0.003) prognostic factor (**Supplementary Figure 4**). Taking this into account, our cohort was stratified in relation to this clinical feature and was divided into two groups: patients that underwent metastases resection (n=34) and patients with unresectable metastases (n=97). Subdividing the discovery cohort into these resectable or unresectable groups, *SRBC* hypermethylation did not have any predictive effect in progression free survival and overall survival for those case patients that received oxaliplatin as neoadjuvant therapy followed by the successful resection of the metastases (**Supplementary Figure 5**).



Cell lines	IC50 (μM) ± SD
HCT-15	3.81±0.18
Co115	0.30±0.15
HCT-116	0.33±0.30
SW48	0.83±0.08
SW480	0.59±0.09
SW620	0.36±0.15
RKO	0.64±0.12

Figure 2. Epigenetic inactivation of SRBC is associated with oxaliplatin resistance in colorectal cancer cell lines. A) Bisulfite genomic sequencing of 8 individual clones in the SRBC promoter CpG island was used to determine DNA methylation status. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. Black arrows indicate the position of the bisulfite genomic sequencing primers. HCT-15 cells are the only that present SRBC promoter CpG island hypermethylation. Normal colon mucosa samples (NC1 and NC2) are unmethylated. B) Western blot analyses for SRBC expression shows that the hypermethylated CpG island in HCT-15 cells is associated with loss protein in comparison with the remaining SRBC unmethylated and expressing colon cancer cell lines. C) IC50 values, determined by the MTT assay, upon use of oxaliplatin in the panel of colon cancer cell lines. All the studied cells are sensitive to oxaliplatin except the SRBC hypermethylated and silenced HCT-15 cell line.

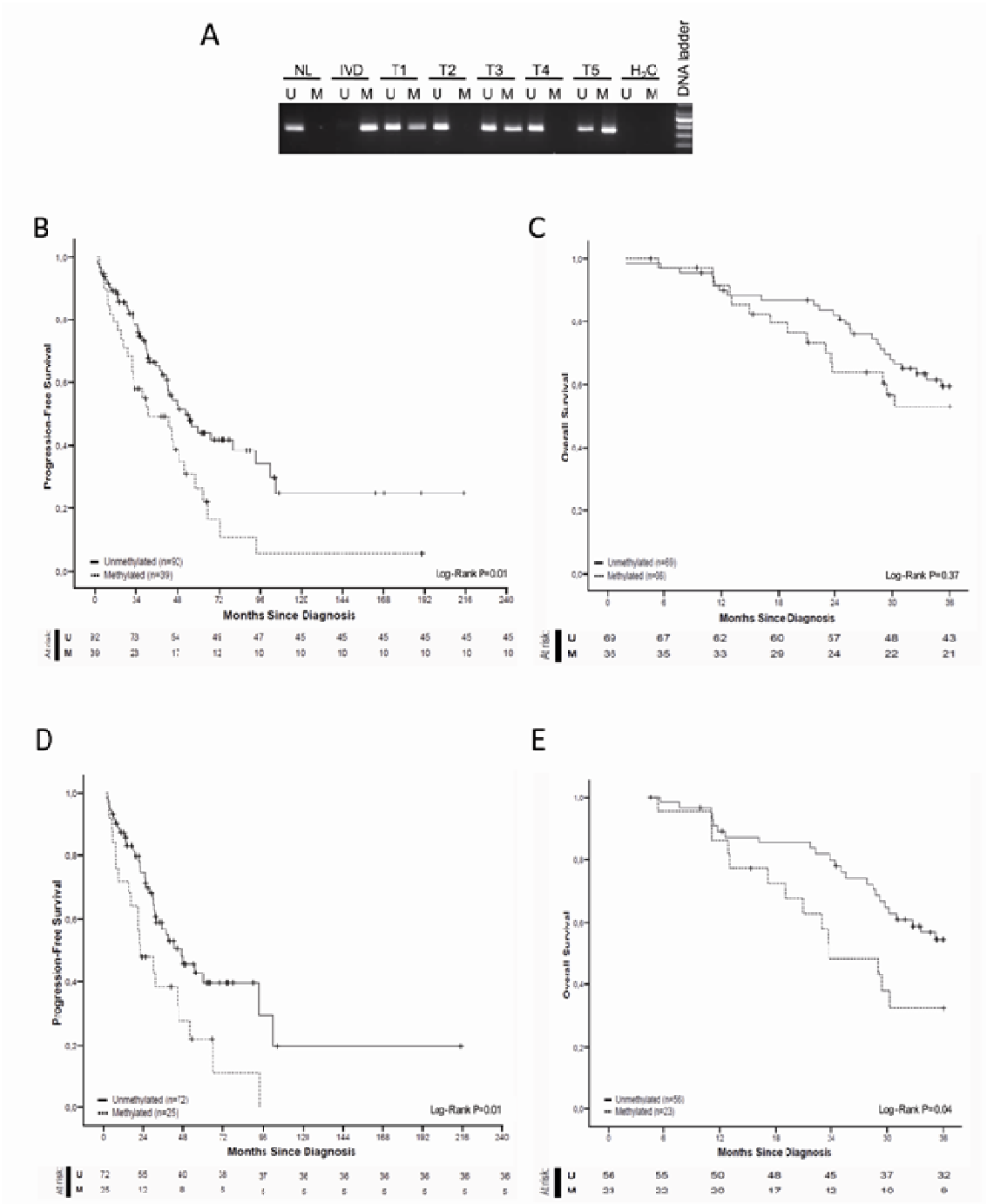


Figure 3. SRBC promoter hypermethylation occurs in primary tumors from colorectal cancer patients, where it predicts shorter progression-free survival in oxaliplatin-treated cases.A) Analysis by methylation-specific polymerase chain reaction (MSP) of the promoter region of SRBC in primary colorectal tumors. The presence of a visible PCR product in lanes marked U indicates unmethylated SRBC sequences; the presence of a product in lanes marked M indicates methylated sequences. In vitro-methylated DNA (IVD) was used as a positive control for methylated SRBC sequences. DNA from normal lymphocytes (NL) was used as a negative control for methylated SRBC sequences. MSP of SRBC in five colon cancer patients demonstrates SRBC promoter hypermethylation in Tumors 1, 3 and 5. B) Kaplan–Meier analysis of Progression-Free Survival (PFS) among the whole population of advanced colorectal cancer cases by SRBC methylation status. The p-value (P) corresponds to the Log-Rank test. Numbers of events (progression) are shown from 24 to 240 months in unmethylated (U) and methylated (M) groups. C) Kaplan–Meier analysis of Overall Survival (OS) among the whole population of advanced colorectal cancer cases by SRBC methylation status. The p-value (P) corresponds to the Log-Rank test. Numbers of events (exitus) are shown from 6 to 36 months in unmethylated (U) and methylated (M) groups. D) Kaplan–Meier analysis of PFS among the oxaliplatin-treated advanced colorectal cancer cases with unresectable metastases by SRBC methylation status. The p-value (P) corresponds to the Log-Rank test. Numbers of events are shown from 24 to 240 months in unmethylated (U) and methylated (M) groups. E) Kaplan–Meier analysis of OS among the oxaliplatin-treated advanced colorectal cancer cases with unresectable metastases by SRBC methylation status. The p-value (P)corresponds to the Log-Rank test. Numbers of events are shown from 6 to 36 months in unmethylated (U) and methylated (M) groups.

However, the scenario was completely different in the context of patients with colorectal adenocarcinomas with unresectable metastases who received oxaliplatin as neoadjuvant therapy and were subsequently not eligible for surgery (n=31) or patients with tumors that were originally classified as unresectable and were given oxaliplatin as palliative chemotherapy (n=66). For these 97 oxaliplatin-treated advanced colorectal cancer case patients with unresectable metastases, *SRBC* CpG island hypermethylation was statistically significantly associated with shorter progression free survival (HR=1.96; 95% CI=1.13 to 3.40; log-rank *P*=0.01) (**Figure 3D**). In this set of case patients, for whom overall survival data were available for 79 patients, we also observed that *SRBC* hypermethylation was statistically significantly associated with shorter overall survival (HR=2.01; 95% CI=1.13 to 3.40; log-rank *P*=0.04). These interesting results prompted us to study the *SRBC* methylation status in a second independent set of colorectal cancer patients with unresectable metastasis who also

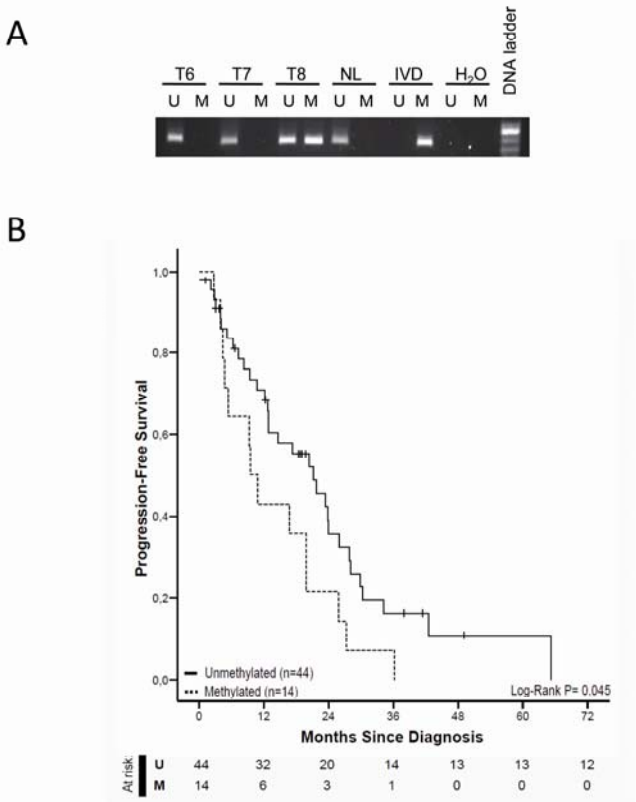


Figure 4. SRBC promoter hypermethylation in the validation cohort predicts shorter Progression-Free Survival in colon cancer with unresectable metastasis treated with oxaliplatin. A) Analysis by methylation-specific polymerase chain reaction (MSP) of the promoter region of SRBC in primary colorectal tumors. The presence of a visible PCR product in lanes marked U indicates unmethylated SRBC sequences; the presence of a product in lanes marked M indicates methylated sequences. In vitro-methylated DNA (IVD) was used as a positive control for methylated SRBC sequences. DNA from normal lymphocytes (NL) was used as a negative control for methylated SRBC sequences. MSP of SRBC in three colon cancer patients demonstrates SRBC promoter hypermethylation in Tumors 8. B) Kaplan–Meier analysis of PFS among the oxaliplatin-treated advanced colorectal cancer cases with unresectable metastases(n=58) by SRBC methylation status. The p-value (P) corresponds to the Log-Rank test. Numbers of events (progression) are shown from 12 to 72 months in unmethylated (U) and methylated (M) groups.

received oxaliplatin-based therapy (n=58) (**Table 1**). In this validation cohort, we confirmed that the presence of *SRBC* hypermethylation was associated with shorter progression free survival (HR=1.90; 95% CI=1.01 to 3.60; log-rank *P*=0.045) (**Figure 4**). Thus, the clinical data are similar to the results from the aforementioned cell cultures that suggest increased chemoresistance of *SRBC* hypermethylated colorectal tumors to oxaliplatin treatment.

DISCUSSION

The preexistence (primary resistance) or the de novo development (secondary resistance) of cellular mechanisms to escape the antitumoral effects mediated by the anticancer compounds probably involve a wide repertoire of genetic and epigenetic (22) events. From a genetics perspective in colorectal cancer, it has been described that the presence of *KRAS* mutations and gene amplification of the *EGFR* or *MET* genesis associated with resistance to overall anti-EGFR therapies (23,24,25). However, from an epigenetics perspective, very little is known. In spite of promising pharmacoepigenetics biomarkers, such as the example of *MGMT* hypermethylation and good response to temozolamide in gliomas (26), have been described for other tumor types, the examples in colorectal neoplasms are scarce, even more so if we just focus on resistance biomarkers. Herein, we provide an example to help fill this niche by showing that *SRBC* hypermethylation predicts resistance to the commonly used agent oxaliplatin in metastatic colorectal cancer, a disease stage that represents the second most common cause of death from cancer (1).

A role of SRBC in mediating different sensitivity to oxaliplatin can be clearly justified by its protein interaction with the product of the *BRCA1* gene (18). The BRCA1 protein exerts an important role in DNA double-strand break repair through homologous recombination 2, so its deficiencies can impair the capacity of cancer cells to repair DNA cross-links caused by chemotherapy drugs such as platinum derivatives (3–7).Two independent studies reported greater primary chemotherapy sensitivity to platinum based chemotherapy agents in patients with ovarian cancer who were carriers of *BRCA1* germline mutations (5,6). These observations have also been extended to *BRCA1* epigenetic silencing in sporadic breast and ovarian tumors, where it also predicts a good response to cisplatin and carboplatin (9,10,and 27). However, the biology of mammary tumors is very different from colorectal malignancies, and in all cases of colon cancer, the *BRCA1* promoter has always been found in an unmethylated status (28–30). Interestingly, in addition to its BRCA1-related roles, SRBC might have other functions related to the observed chemoresistance phenotype,

such as its interaction with caveolin 1, which may putatively affect intracellular vesicle traffic of the drug (31).

It is worth mentioning two possible avenues of further research. First, there is the possibility to detect *SRBC* hypermethylation by sensitive user-friendly techniques, such as methylation-specific PCR and pyrosequencing, which could be useful in the clinical setting. Instead of always requiring the use of the surgical tumor sample, stool or serum/plasma DNA could be useful alternative biological materials to predict oxaliplatin resistance in colorectal cancer patients. In this regard, DNA methylation changes are also amenable for the development of new powerful molecular techniques, such as those recently referred to as *liquid biopsies* (32). Second, our observation that sensitivity to oxaliplatin can be restored by the re-expression of the *SRBC* gene could represent a revival of the DNA demethylating agents in the therapy of solid tumors. With little therapeutic options against metastatic colorectal cancer once it has become insensitive to oxaliplatin, DNA methylation inhibitors, such as 5-AZA, could be used to resensitize these tumors to the oxaliplatin therapy. This idea has been recently explored in non–small cell lung carcinoma patients who had reached the last line of chemotherapy. The subsequent administration of 5-AZA was able to rescue previous chemosensitivity (33).

Limitations of our study to be addressed in further research include the lack of knowledge about the molecular mechanisms linking SRBC activity and DNA damage repair triggered by oxaliplatin, the use of non quantitative DNA methylation assays that will require transformation to quantitative DNA methylation tests to get specific cut offs for a future clinical application, and the extension of our colorectal cancer patient data source to stage II and III tumors and samples from other geographical origins.

In conclusion, we have demonstrated that DNA methylation-associated silencing of the BRCA1 interactor gene *SRBC* is associated with the acquisition of chemoresistance to the DNA damaging agent oxaliplatin in colorectal cancer both *in vitro* and *in vivo*. The validation of *SRBC* hypermethylation as a predictive marker will require further prospective studies. If successful, clinical trials would also be necessary to develop strategies to overcome or prevent the development of SRBC-mediated epigenetic resistance.

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SUPPLEMENTARY METHODS

Analysis of the DNA methylation microarray data

The methyl array data analysis was performed by GenomeStudiohttp://www.illumina.com/gsp/genomestudio_software.ilmn) and then processed using R (http://www.r-project.org/). All methylation values measured by microarray were presented as beta value, ranging from 0 to 1. Normalizations were performed using quantile normalization, on data previously adjusted by color balance between the two channels and corrected by background level.

We adopted multivariate outlier analysis to analyze our beta methylation data. We use the methylation data matrix as a main body made up of the mass none differentially methylated (NDM) CpGs "contaminated" with outliers constituting the few differential methylated (DM) CpGs. We model the former with a multivariate normal distribution and estimate its centre and covariance matrix with the Minimum Covariance Determinant estimator, and quantify the departure from the major NDM distribution with the robust Mahalanobis distance and a corresponding X^2 test p-value. The CpGs with small p-values are outliers to the majority of the data, and can be extracted as DM CpG. To adjust for the multiple test error, we used a False Discovery Rate (FDR) approach, according to Benjamini and Hochberg.

The Infinium HumanMethylation27 Bead Chip interrogates 27,578 CpG sites from 14,495 genes. The ratio of the average intensity of methylated probes to the average intensity of both methylated and unmethylated probes for a specific CpG provides an estimate of the fraction of DNA methylated that has been suggested to follow a beta distribution due to its potential bimodality. The differential methylation test uses a multivariate outlier analysis, assuming multivariate normal distribution, a generalization of the one-dimensional (univariate) normal distribution (which is reasonable when focusing on a two-group comparison on a single site or region) to higher dimensions.

DNA Methylation Analysis of Single Locus

We established SRBC CpG island methylation status using three different PCR based techniques. We used bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated cytosine. First, DNA methylation status was analyzed by bisulfite genomic sequencing of the SRBC CpG island using primers encompassing the transcription start site. Primer sequences are shown in **Supplementary Table 1**. Both DNA strands were sequenced and at least eight clones were analyzed per sequence. The second analysis used methylation-specific PCR

using primers specific for either the methylated or modified unmethylated DNA. Primer sequences are shown in **Supplementary Table 1**. The PCR annealing temperature was 59ºC and 36 cycles of PCR were performed. DNA from normal lymphocytes treated in vitro with SssI methyltransferase was used as a positive control for methylated alleles (IVD). DNA from normal lymphocytes (NL) and normal colon mucosa were used as a positive control for unmethylated alleles. Pyrosequencing was the third analysis performed. PCR was realized under standard conditions with biotinylated primers and the PyroMark Vacuum PrepTool (Biotage, Sweden) was used to prepare single-stranded PCR products according to manufacturer's instructions. Primer sequences are shown in **Supplementary Table 1**. PCR products were observed at 2% agarose gels before pyrosequencing. Reactions were performed in a PyroMark Q96 System version 2.0.6 (Qiagen) using appropriate reagents and protocols, and the methylation value was obtained from the average of the CpG dinucleotides included in the sequence analyzed.

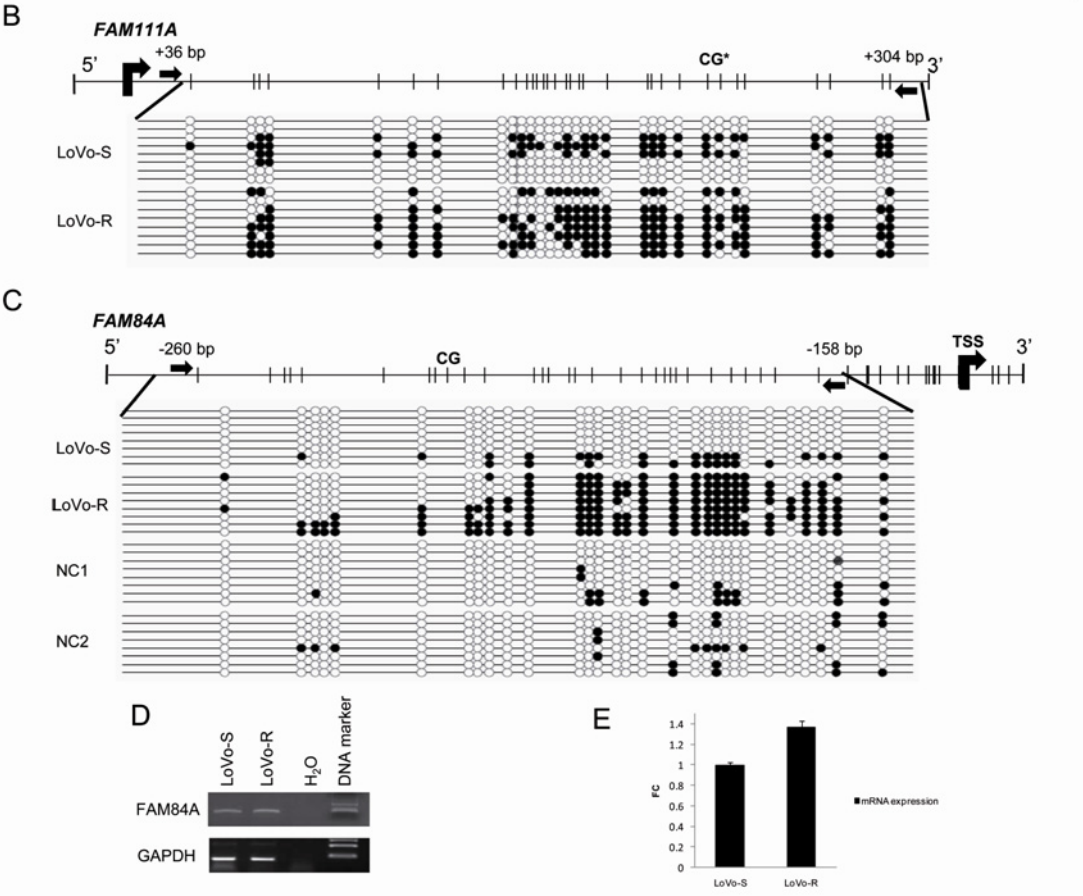
Supplementary Table 1: Primers Sequences

Primers	Sequence	Annealing temperature (ºC)	Product Size (bp)
Bisulfite Sequencing Primers			
SRBC_BS_F	AGTTTTAGTTGTGATTTAGGTAGG	56	327
SRBC_BS_R	CCCCTCTAATTATCTCTTTACC		
FAM111A_BS_F	TGTTTTTTTAGGGGTAAGGGTA	57	268
FAM111A_BS_R	AACAACCTTTTCCCAAAAAA		
FAM84A_BS_F	TTTTTTGTGYGTTTTGTTTT	58	103
FAM84A_BS_R	AATTTCTTCTCCATACCCAAAC		
Methylation Specific PCR			
SRBC_MSP_UF	TTTTTGAAAGTGTTTTGTTTTTT	59	187
SRBC_MSP_UR	TTCCATAACTCACCCTTTACAA		
SRBC_MSP_MF	TTGAAAGCGTTTCGTTTTTC	59	189
SRBC_MSP_MR	TCCGTAACTCGCCCTTTAC		
Semi-quantitative PCR			
SRBC_qPCR_F	GTTCTGCTCTTCAAGGAGGA	60	154
SRBC_qPCR_R	CTCTGTACCTTCTGCAATCC		
FAM84A_qPCR_F	GCGAGTTGCCCACAGGGGAC	60	136
FAM84A_qPCR_R	CGCTCTTGAGGCCCACCAGT		
Pyrosequencing primers			
BioPyro_SRBC_R	[Byn]ACCAACAAACTTCCCAAC	60	
Pyro_SRBC_F	AGGGYGTGGGGATGTTGGT		
PyroSeq_*SRBC* primer	AAGYGTTTYGTTTTTYGTTGTTTYGYGTYGGGGGTTTGT		

Supplementary Figure 1

A

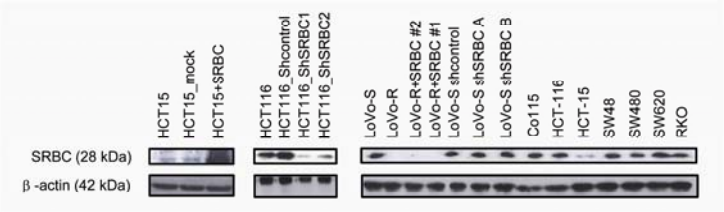
Gene Name	Gene ID	Function	LoVo-S	LoVo-R	Difference of Methylation	Chromosome	Distance to TSS	Strand	CpG island
SRBC	112464	Possible TSG	6.2%	97.2%	90.9 %	11	155	-	TRUE
FAM111A	63901	Catalytic activity	1.2%	76.1%	74.9 %	11	75	+	TRUE
FAM84A	151354	Possible DNA repair gene	3.2%	76.1%	72.8%	2	240	+	TRUE



Supplementary Figure1. A) Illumina Methylation microarray candidate genes. **B)** *FAM111A* BsSeq for LoVo *in vitro* model. There were not detected high methylation differences between both cell lines. **C)** *FAM84A* bisulfite sequencing (BsSeq) for LoVo-S, LoVo-R and two normal colon mucosa samples (NC). LoVo-R presents *FAM84A* promoter methylated when compared with sensitive cell line. **TSS**- Transcription Start Site. **CG**- CpG analyzed in the Illumina Methylation microarray. Bp- base pairs. Arrows and bp represent BsSeq primers localization. **D)** and **E)** FAM84A mRNA expression by conventional and quantitative real time PCR. Sensitive and resistant cell lines present the same amount of mRNA. **FC**-fold change.

Supplementary Figure 2

A

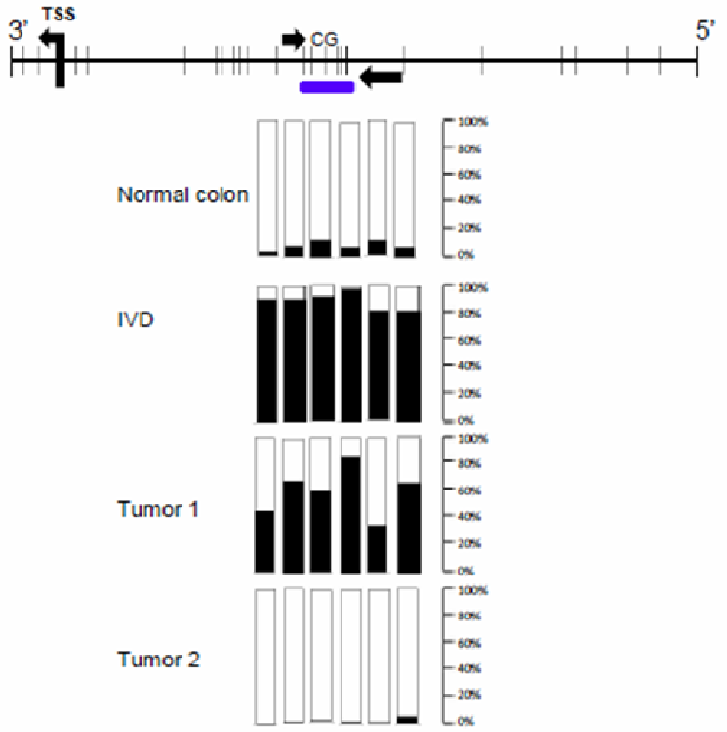


B

Cell line	Oxaliplatin IC50 (μM)	p-values
HCT-15 empty vector	3.91±0.03	**referent**
HCT15+SRBC	2.06±0.04	0.02
HCT-116 shcontrol	0.47±0.02	**referent**
HCT-116 shSRBC A	0.96±0.03	< 0.001
HCT-116 shSRBC B	1.58±0.02	< 0.001
Cell line	**5-Fluorouracil IC50 (μM)**	**p-values**
LoVo-S	3.30±0.02	0.43
LoVo-R	3.01±0.11	0.92
LoVo-R empty vector	3.33±0.13	**referent**
LoVo-R+SRBC #1	3.12±0.03	0.71
LoVo-R+SRBC #2	3.60±0.05	0.34
LoVoS shSRBC control	4.12±0.13	**referent**
LoVoS shSRBC A	5.70±0.01	0.89
LoVoS shSRBC B	6.22±0.03	0.04
HCT-15 empty vector	0.19±0.04	**referent**
HCT15+SRBC#1	0.18±0.02	0.21
HCT15+SRBC#2	0.17±0.01	0.32
HCT-116 shcontrol	1.29±0.20	**referent**
HCT-116 shSRBC A	1.39±0.04	0.92
HCT-116 shSRBC B	1.02±0.41	0.51

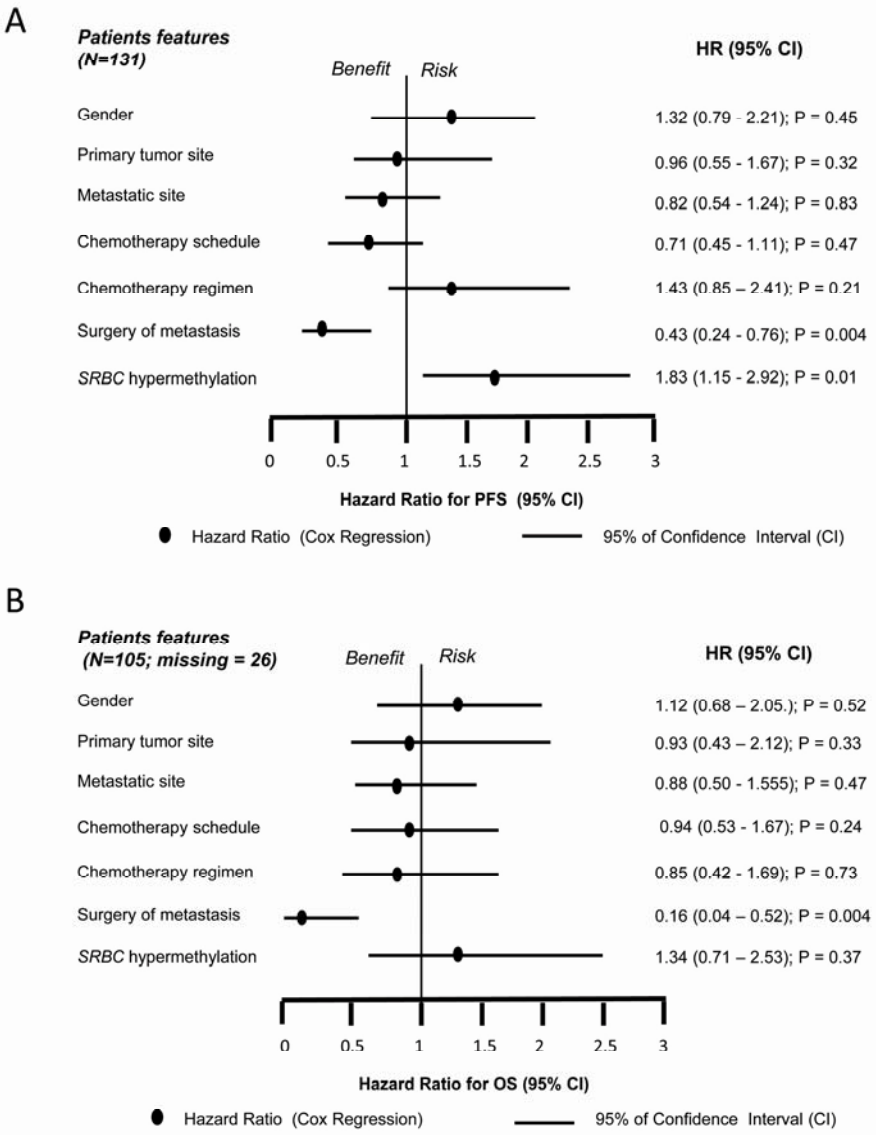
Supplementary Figure 2: A) Western blot analysis of SRBC expression in a panel of human colorectal cancer lines and upon silencing and expression enhancement in our *in vitro* models. **B)** Oxaliplatin and 5-Fluorouracil IC50 for HCT-15, HCT-116 and LoVo stable clones. Upon *SRBC* stable transfection hypermethylated HCT15 cell line, proved to be 1.8-fold more sensitive to oxaliplatin, when compared with HCT15-mock cell line. Stable silencing of *SRBC* in non-methylated HCT116 showed a 2-fold enhancement of drug resistance. Depletion or enhancement of SRBC activity in all cell lines, did not change the sensitivity to 5-fluorouracil. IC50- inhibitory concentration 50. Drug dose that inhibit 50% of cell viability. P-values were obtained from 10000 permutations one sided test, where we tested the difference between two groups of growth curves (data not shown), at each concentration point. P-values were calculated assuming one of the conditions as referent. n.s- not statistically significant.

Supplementary Figure 3



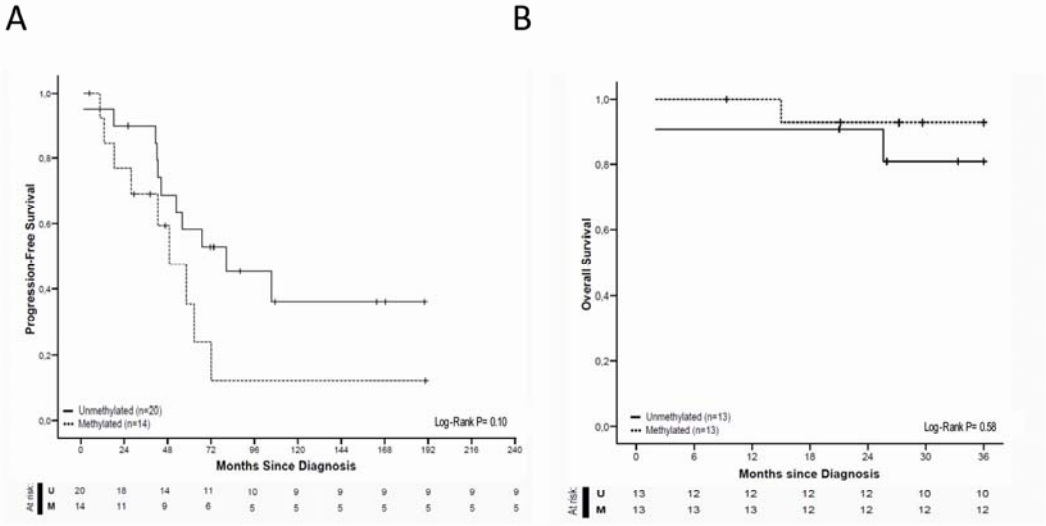
Supplementary Figure 3. SRBC methylation status analyzed by pyrosequencing. Tumor 1 is methylated and Tumor 2 unmethylated. Blue square marks the analyzed CGs and columns represent the analyzed CpGs, where black color is the methylation percentage. TSS- Transcription Start Site; CG- CpG analyzed in the Illumina Methylation microarray. Black arrows represent pyrosequencing primers localization.

Supplementary Figure 4



Supplementary Figure 4: Forest plot of multivariate Cox proportional hazards regression model, taking into account different clinical features of the discovery cohort. Parameters with two-sided test associated p-value (P) under 0.05 were considered as independent predictive factors. A) HR associated with Progression free survival (PFS). Surgery of metastasis is a good prognosis factor, in contrast to *SRBC* hypermethylation that represents a significant risk of progression. B) HR associated with 3-year overall survival (OS). Surgery of metastasis is a good prognosis factor.

Supplementary Figure 5



Supplementary Figure *5.* *SRBC* hypermethylation is not associated with PFS neither with OS in mCRC patients with successful resection of metastasis. A) Kaplan–Meier analysis for progression free survival (PFS) among cases that received oxaliplatin as neoadjuvant therapy, followed by successful resection of liver metastases (n = 34) according to the *SRBC* methylation status. The p-value corresponds to the Log-rank two-sided test. Numbers of events (progression) are shown from 24 to 240 months in unmethylated (U) and methylated (M) groups. B) Kaplan–Meier analysis for overall survival (OS) among cases that received oxaliplatin as neoadjuvant therapy, followed by successful resection of liver metastases (n =26) according to the *SRBC* methylation status. The p-value corresponds to Log-rank two-sided test. Numbers of events (exitus) are shown from 6 to 36 months in unmethylated (U) and methylated (M) groups.

STUDY III

"Orthoxenografts of Testicular Germ Cell Tumors Enable Identification of Glucosylceramide Synthase as a Cisplatin Resensitizing Target"

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RESUMEN

Para investigar la base genética de la resistencia al cisplatino, hemos desarrollado un modelo de ratón representativo de tumores testiculares germinales del tipo no seminoma, sensibles/resistentes al cisplatino. Los tumores humanos han sido ortotópicamente trasplantados al ratón, *orthoxenografts*. Aplicando un enfoque genético global, se identificaron cambios recurrentes en todos los tumores refractarios (ganancias: 9q22.11 - q33.3, 9q32 - q33.1, 15q23 - q24.1 y 15q26.3, pérdida: Xp22.3). Clínicamente, la presencia de las ganancias de 9q32 - q33.1 se asoció con una peor supervivencia global en una serie de tumores de células germinales metastásicos, que incluían pacientes con resistencia al cisplatino. Perfiles de expresión génica de la región 9q32 - q33.1 y estudios funcionales *in vitro* e *in vivo* destacan la relevancia de *GCS*, *ATP6V1G1*, *POLE3*, *PAPPA* y *CTR1/2* como genes de resistencia relacionados al cisplatino y candidatos prometedores para las terapias de resensibilización-dirigida. Como prueba de concepto, se presentan fuertes evidencias preclínicas que la inhibición de *GCS* con DL-treo-PDMP en modelos *orthoxenograft* resistentes al cisplatino resensibiliza los tumores al cisplatino, proporcionando de este modo una justificación para el reposicionamiento de este inhibidor en el desarrollo de futuros ensayos clínicos.

SUMMARY

To investigate genetic basis of cisplatin resistance, we have developed in nude mice a representative panel of matched orthotopically transplantable subject-derived nonseminomatous (NSE) cisplatin-sensitive/resistant tumors, named orthoxenografts. Using genome-wide approach, recurrent changes were identified across refractory tumors (gains: 9q22.11-q33.3, 9q32-q33.1, 15q23-q24.1 and 15q26.3; loss: Xp22.3). Clinically, the presence of 9q32-q33.1 gains was associated with poorer overall survival in a series of metastatic germ cell tumors, which included cisplatin-refractory patients. Gene expression profiling of 9q32-q33.1 region and functional *in vitro,* and *in vivo* RNAi knockdown assays of their *Caenorhabditis elegans* ortholog genes highlights the relevance of *GCS, ATP6V1G1*, *POLE3*, *PAPPA* and *CTR1/2* as cisplatin resistance-related genes, making them promising candidates for targeted cisplatin-sensitizing therapies. As a proof-of-concept, we present strong preclinical evidences that drug inhibition of glucosylceramide synthase (GCS) with DL-threo-PDMP in cisplatin-resistant orthoxenograft models newly sensitizes tumors to cisplatin, thereby providing a drug-repositioning rationale for development of future clinical trials.

INTRODUCTION

Testicular germ cell tumors of adolescent and young adults (TGCTs) are the most common malignancy in young men [1-3]. They can be classified as seminomas [123], which represent around 40% of cases, or nonseminomas (NSEs) (60%). SEs are radio- and chemo-sensitive tumors, and are highly curable at all stages. With the exception of teratomas, NSEs are highly sensitive to cisplatin-based chemotherapy and, when combined with surgery, patients achieve high cure rates [4]. In contrast with most advanced solid tumors, approximately 80-90% of metastatic GCTs will achieve complete cures after standard doses of cisplatin chemotherapy [5,6]. Nevertheless, 10-15% of patients die from cisplatin refractoriness and from the absence of alternative effective resensitizing therapies.

Cisplatin resistance has been attributed in GCTs to various mechanisms [2,3], although the molecular basis underlying treatment failure in refractory patients is understood [7]. Perhaps it is this success in treating advanced testicular cancer that has meant that few studies of underlying treatment failure in refractory patients have been conducted [8]. Additionally, the absence of relevant preclinical animal models reproducing human testicular GCT properties has made it difficult to identify any underlying resistant mechanism and to develop novel therapeutic approaches. Knowing why a curable cisplatin-treated tumor becomes resistant could help in the search for improved treatments for other tumors that are less successfully treated with cisplatin [8]. Here we report the perpetuation of serially cisplatin-refractory orthotopic transplantable patient-derived nonseminomatous tumor grafts in mice, named orthoxenografts, as a system to investigate cisplatin refractoriness from a genetic perspective and for the preclinical development of novel targeted therapies based on overcoming cisplatin-resistance.

RESULTS

Establishment and characterization of engrafted NSE tumors

Fourteen of 40 primary human NSE tumors (35%) were grown as orthotopic implants, named orthoxenografts, in nude mice (**Supplementary Table S1**). Tumors included three choriocarcinomas (CHs) (**Fig. 1a**), four embryonal carcinomas (ECs), three yolk sac tumor (YS) and four mixed tumors. Five orthoxenografts were derived from several extragonadal tumor locations, and in four cases from patients treated with cisplatin-based chemotherapy (**Supplementary Table S1 and Fig. S1a, b and c**). None of the 22 implanted pure gonadal seminomas (SEs) grew in nude mice. Of the mixed tumors, comprising both SE and NSE components, only the NSEs grew in mice.

A very close correlation was found between the primary tumor and its paired orthoxenograft with respect to histological appearance (**Supplementary Table S1**), and their genetic and epigenetic characteristics. They were kept stable throughout serial passages. Immunohistochemical expression of conventional clinical diagnostic markers for GCTs was evident, enabling the successful classification of the tumors (**Fig. 1a; Supplementary Fig. S1a, b and c**). The early embryonic markers OCT4 and NANOG (**Fig. 1b and Supplementary Fig. S1d**) were exclusively detected in pure EC, and EC of mixed tumors. As described for primary tumors [2,9], we did not detect the presence of any gene point mutation and any tumor exhibited a microsatellite instability phenotype (MSI) (**Supplementary Table S2**). Likewise [10,11], there was a good correlation respect to the methylation status of 5' CpG promoter islands in a group of selected genes and this remained stable throughout mouse-to-mouse passages (**Supplementary Table S3**). Orthoxenografts also reproduce in mice the dissemination patterns observed in humans (**Supplementary Fig. S1e and Supplementary Table S1**). Finally, as occurred in patients [2,12], the secreted β-hCG and/or AFP could be readily detected in mouse serum as follow-up markers (**Supplementary Table S1 and Fig. 1C**).

Orthoxenografts of NSE recapitulate the responses to cisplatin treatment in humans

We studied the pattern of responses to chemotherapy for nine orthoxenografts. Mice were treated with low (2 mg/kg) and high (5 mg/kg) doses of cisplatin, and their short- and long-term responses were evaluated. All tumors had a good short-term response to low doses of cisplatin (**Fig. 1c, left panel and Supplementary Fig. S2 and S3**), as indicated by a significant reduction in tumor weight in eight cases and complete response in the tumor TGT21BX (**Supplementary Fig. S2a**). A good correlation between tumor weight and reduction or absence of serum β-hCG and/or AFP levels was found, supporting its use as a dynamic surrogate marker of treatment efficacy (**Fig. 1C, right panel and Supplementary Fig. S2 and S3**). Differences among tumor weight and serum markers observed in TGT21AX after treatment can be explained by the predominance of a teratoma with a few microscopic islands of viable cells (**Supplementary Fig. S2b and S2c**), while those in TGT34X (**Supplementary Fig. S3c**) were due to a high percentage of necrosis. Administration of higher doses of cisplatin (5 mg/kg) was associated with a better response in all cases (**Fig. 1c; Supplementary Fig. S2 and S3**). Additionally, there was a complete response in tumors TGT21AX (**Supplementary Fig. S2b**) and TGT34X (**Supplementary Fig. S3c**).

To investigate long-term cisplatin responses, a subgroup of the treated mice was kept alive post-chemotherapy until tumor regrowth was observed. Tumors regrew in seven out nine cases, over a period of 15 to 135 days, independently of the cisplatin dose in most instances. However, high doses of cisplatin prevented tumor relapse in TGT34X (**Supplementary Fig. S3c**), whereas in TGT39X both treatments yielded a long and sustained response, as was confirmed by constant levels of AFP over a latency period of 90 days (**Supplementary Fig. S3d**). Histological and immunohistological analysis of relapsed masses demonstrated the presence of a viable tumor in most cases, and the maintenance of cell heterogeneity, as indicated in mixed tumors by the presence of different components. As observed in patients [12], cisplatin induced increasing teratoma differentiation in TGT21AX (**Supplementary Fig. S2c**).

In vivo development of representative NSE orthoxenograft models of cisplatin refractoriness

To investigate cisplatin resistance against the same genetic background (sensitive *vs.* resistant) we developed several cisplatin refractory tumor models. Thus, five engrafted tumors, which had not been exposed to cisplatin before implantation (TGT1X, TGT12X, TGT21BX, TGT34X and TGT38X), were treated with cisplatin. After five iterative cycles of treatment in different mice, and applying increasing doses of cisplatin, refractory tumors were obtained (**Fig. 1d**). During the process, a progressive shortened time-lag between tumor treatment and tumor regrowth was noted, and the mice to mice passage time stabilized after five cycles of treatment in all cases (**Fig. 1f**). To demonstrate cisplatin resistance, we performed paired short-term response assays between untreated (TGTX) and resistant (TGTXR) tumors at cycle #5 (**Fig. 1d**). High levels of resistance were observed in all tumors at both cisplatin doses, these being of particular note in the case of TGT21BXR and TGT34XR, which were derived from highly sensitive tumors. No major differences in the histological (**Supplementary Fig. S4**) and immunohistochemical patterns were observed between original and cisplatin-resistant tumors.

Recurrent chromosomal imbalances are associated with acquired cisplatin resistance

We investigated whether the acquisition of cisplatin resistance was associated with the selection of specific genomic imbalances and genetic alterations against the relatively stable genetic background of GCTs. No additional mutations or changes in the MSI status in resistant engrafted tumors were detected (**Supplementary Table S2**). Fine-scale comparative whole-genome mapping using array-based comparative genomic

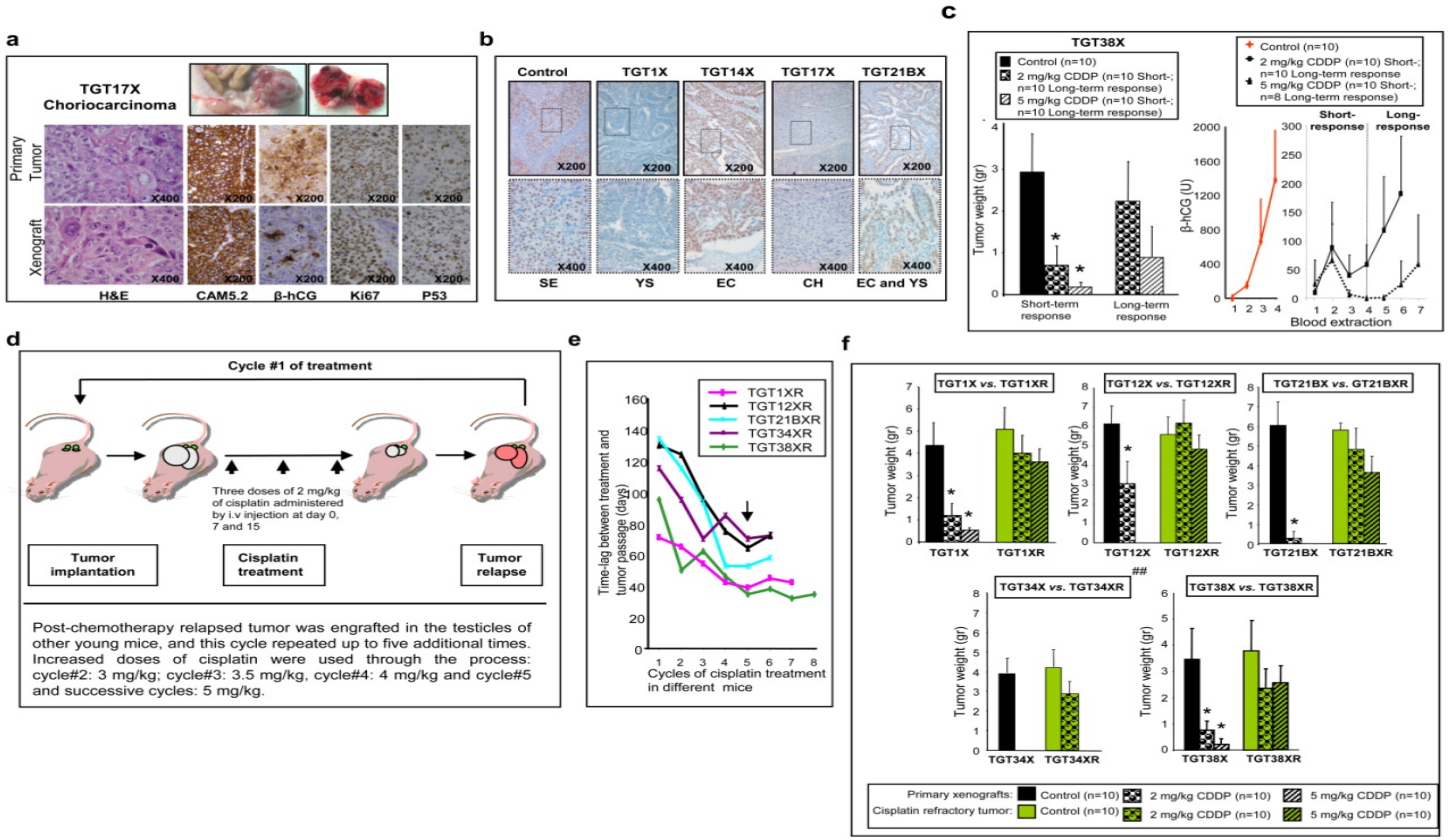


Fig. 1 Representative studies of the characterization of engrafted tumors and development of cisplatin refractory tumors. a) Hematoxylin and eosin (H&E) staining and immunohistochemical analysis reveals a close correlation between the primary tumor and its corresponding engrafted tumor. b) OCT4 protein immunostaining. High levels of nuclear staining were only identified in the embryonal component (EC) of pure TGT14 or TGT21B mixed tumors. Absence of protein expression is noted in TGT1, pure yolk sac (YS) and TGT17, pure choriocarcinoma (CH). Seminoma (SE) was used as a positive control. c) Mice were treated with low and high doses (2 and 5 mg/kg, respectively) of cisplatin and short- and long-term responses to both treatments were analyzed (left panel). Dynamic CDDP response curves were generated by serial -hCG determinations in the serum of controls (right panel). d) Generation of engrafted tumors refractory to cisplatin treatment combines: (i) repetitive cisplatin treatments (one cycle: three doses of cisplatin administered by i.v. injection for three consecutive weeks, on days 0, 7 and 14) 21 for five separate and independent cycles; and (ii) increased doses ranged from 2 to 5 mg/kg applied during the process. e) A short time-lag between tumor treatment and tumor regrowth occurs for the different cycles. Tumors at cycle #5 of treatment (arrow) were used to assess the response to chemotherapy. f) Comparative short-term cisplatin response assays for paired non-treated vs. cisplatin-resistant tumor. Mice were treated with low (2 mg/kg) and high (5 mg/kg) doses of cisplatin. TGT21B and TGT34 showed a complete response at high doses. **, for paired TGT34 vs. TGT34R, only the response to the high dose was assessed. When mice were treated at doses higher than 3.5 mg/kg, the signs of cisplatin-induced toxicity were ameliorated by administration of saline containing 5% glucose for 2 days.

hybridization (CGH) was performed in four paired untreated parental engrafted tumors and their resistant counterparts. Genomic stability of engrafted tumors was confirmed by the low number of chromosome changes identified in untreated tumors. As shown in **Fig. 2a,** few additional recurrent genomic changes were consistently detected in resistant tumors. Gains at 9q were found in three of four cases, and in two at 9q21.11-q33.3. There was also a small gain (5.1 Mbp) overlapping the 9q32-q33.1 sub-region in another case (**Fig. 2b**). Gains at 15q23-q24.1 and 15q26.3 were identified in two tumors. All gains were confirmed by FISH analysis (data not shown). The loss of the Xp22.33 region was identified in three of four tumors (**Supplementary Fig. S5**). We subsequently focused our attention on studying the 9q short (5.1 Mbp) overlapping 9q32-q33.1 region, since several genes within this region had been previously found to be associated with drug response (**Supplementary Table S4**).

Amplification at 9q32-q33.1 is associated with an increased risk of death in advanced GCT patients

To evaluate the clinical relevance of the mouse results, we investigated the gains at 9q32-q33.1 by FISH in a tissue microarray (TMA), comprising a series of tumors from 75 patients with metastatic GCTs (63 NSEs and 12 SEs) homogeneously treated with cisplatin-based chemotherapy in our research center. The series included 24 patients (22 NSEs and 2 SEs) refractory to first-line cisplatin-based chemotherapy. Amplification at 9q32-q33.1 was identified in 18 of 75 (24%) cases, including 16 NSEs (5 CEs, 2 CHs, 1 YS, 2 TEs and 6 mixed tumors) and two pure SEs (**Fig. 2c**). Those results were consistent for tumor sample replicates, as in the different histological tumor components in the majority of tumors. Nevertheless, amplified and non-amplified areas coexisted in five cases (2 pure CEs and 3 mixed tumors) (**Fig. 2c and Supplementary Table S5**).

Analysis of overall survival (OS) adjusted for histology (SE and NSE) showed that amplification at the 9q32-q33.1 region was associated with a 2.79-fold greater risk of death in patients with metastatic GCTs (p = 0.036; hazard ratio (HR) = 2.79; 95% confidence interval (CI) = 1.11–7.0) (**Table 1 and Fig. 2d, left panel**). A higher risk of death was revealed when considering only patients with NSE (n = 63) (p = 0.026; HR = 3.03; 95% CI = 1.18–7.76), but there was no difference in those with SE (p=0.54). OS subgroup analyses in NSE patients showed a trend towards good and intermediate prognosis; the relationship was statistically significant when we analyzed the two groups together (p = 0.014; HR = 5.16; 95% CI = 1.47-18.12). Amplification was also associated with shorter progression-free survival (PFS) (p = 0.043; HR = 2.46; 95% CI

= 1.07–5.63) (**Table 1 and Fig. 2d, right panel**); this relationship was significant even when the NSE group alone was analyzed (p = 0.024, HR = 2.8, 95% CI = 1.19–6.57). There was a trend for tumors harboring the 9q32-q33.1 amplification to have a worse cisplatin response. Fifty percent of tumors with the amplification were considered resistant to first-line chemotherapy compared with 26.3% of tumors without it (p = 0.060). Up to 27.8% of tumors with the 9q32-q33.1 amplification did not achieve a tumor marker complete response or progressed during first-line treatment (p = 0.007) (**Supplementary Table S6**).

Table 1 Analysis of 9q32-q33.1 amplification in metastatic germ cell tumors

			Overall Survival			Progression-Free Survival		
	n	%	HR	95% CI	*P*	HR	95% CI	*P*
Chromosome copy number at 9q31-q32.1 (n=75)								
WT	57	76	1		0.036 *	1		0.043 *
Amplification	18	24	2.79	(1.11 - 7.0)		2.46	(1.07 - 5.63)	
Stratified analysis								
Pathological classification								
Nonseminoma (n=63)								
WT	47	74.6	1		0.026	1		0.024
Amplification	16	25.4	3.03	(1.18 - 7.76)		2.8	(1.19 - 6.57)	
Seminoma (n=12)								
WT	10	83.3	1	(0 - Inf)	0.54	1	(0 - Inf)	0.38
Amplification	2	16.7	0			0		
IGCCCG classification								
NSE with good prognosis (n=33)								
WT	27	81.8	1		0.096	1		0.22
Amplification	6	18.2	5.89	(0.82 - 42.52)		3.29	(0.55 - 19.71)	
NSE with intermediate prognosis (n=14)								
WT	10	71.4	1		0.15	1		0.28
Amplification	4	28.6	3.41	(0.68 - 17.02)		2.33	(0.52 - 10.44)	
NSE with bad prognosis (n=16)								
WT	10	62.5	1		0.88	1		0.30
Amplification	6	37.5	0.9	(0.21 - 3.79)		2	(0.55 - 7.21)	
Grouping NSE according to good and intermediate prognosis (n=47)								
WT	37	78.7	1		0.014	1		
Amplification	10	21.3	5.16	(1.47 - 18.12)		3.28	(1.03 - 10.37)	0.056

Abbreviations: WT, No amplification at 9q32-33.1; HR, hazard ratio; CI, confidence interval.
* *P* values are from multivariate Cox models adjusted for pathological diagnostic classification.

Identification of a group of cisplatin resistance-related genes at 9q32-q33.1

Next, to find cisplatin resistance-related genes in 9q32-q33.1 region, the profiling expression patterns of the 60 genes and two miRNAs annoted were generated by quantitative PCR (qPCR) (**Fig. 3 and Supplementary Fig. S6**) in the five paired (sensitive *vs.* resistant) engrafted tumors. Thirty-seven genes were expressed in GCTs and recurrent changes occurred in eleven of them: eight genes (*ATP6V1G1*, *POLE3*, *EDG2*, *FLJ31713*, *GCS*, *PAPPA*, *TNC*, and ZNF883) were overexpressed in cisplatin-refractory tumors, while three genes (*CTR1*, *CTR2*, and *AKNA*) were underexpressed. These changes were mainly correlated with tumor resistance rather than specifically with the presence of amplification. Nevertheless, despite the small number of samples analyzed, overexpression and 9q32-q33.1 amplification were correlated with the presence of *PAPPA,* an IGF-binding protein protease (IGFBP) involved in the IGF

release process, and *POLE3,* also known as CHRAC17, a histone-fold protein, which forms part of the CHRAC chromatic-remodeling complex. Although changes in the expression levels of miRNA4688 and miRNA455 were observed among the different resistant tumors (**Supplementary Fig. S7a)**, we did not found a clear association with resistance or with amplification.

a

Tumor	Histology of resistant tumor [b]	Chromosome region [c]	Status	Scores [a]	Region	Size (Mbp)	Number of genes [d]
TGT12R	EC	9q21.11-q33.3	gain	+ 0.31	67.950 K - 123.690 K	55.7	502
		15q23-q24.1	gain	+ 0.36	69.690 K - 71.430 K	1.74	26
		15q26.3	gain	+ 0.37	97.770 K - 98.310 K	0.54	6
		Xp22.33	loss	- 0.29	30 K - 2.730 K	2.70	25
TGT21BR	YS, EC and CH	5p15.33-p15.2	gain	- 0.30	90 K - 15.090 K	15.0	94
		9q21.11-q33.3	gain	+ 0.39	70.170 K - 123.690 K	53.5	461
		15q23-q24.1	gain	+ 0.39	69.690 K - 71.370 K	1.68	25
		15q24.3	gain	+ 0.30	74.970 K - 75.750 K	0.78	9
		15q26.3	gain	+ 0.41	97.830 K - 98.310 K	0.48	6
		Chromosome 21	gain	+ 0.28	0 K - 47.000 K	47	386
		Xp22.33	loss	- 0.45	30 K - 2.730 K	2.70	25
		Xp22.2	loss	- 0.42	9.510 K - 9.870 K	0.36	4
TGT34R	EC	No changes					
TGT38R	CH	9q32-q33.1	gain	+ 0.35	112.950 K - 118.050 K	5.10	60
		16p11.2-p11.1	gain	+ 0.34	34.350 K - 34.590 K	0.24	11
		20q13.13-q13.2	gain	+ 0.25	49.050 K- 49.770 K	0.72	5
		Xp22.23	loss	- 0.41	30 K - 2.730 K	2.70	25

[a] NimbleGen microarray data processed at 60 kbp average window. All the chromosomal gains or losses had a $\log_2$ score ≥0.30 or ≤0.30. No microarray data were available for TGT1R, a pure YS.
[b] Tumor histology: YS, yolk sac tumor, EC, embryonal carcinoma, CH, choriocarcinoma.
[c] Genomic changes were consistently detected in chemoresistant tumors at cycles #3 and #5 of chemotherapy.
[d] Location of genes in each region was based on build 36.3 from NCI.

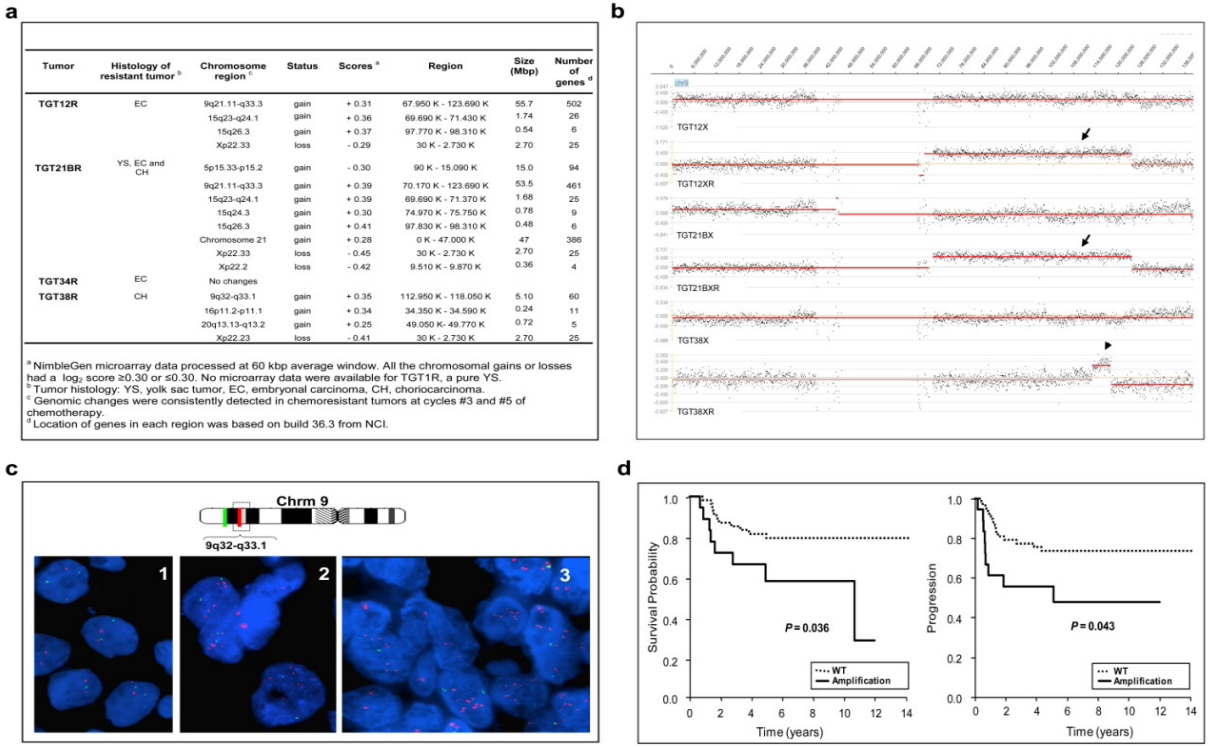
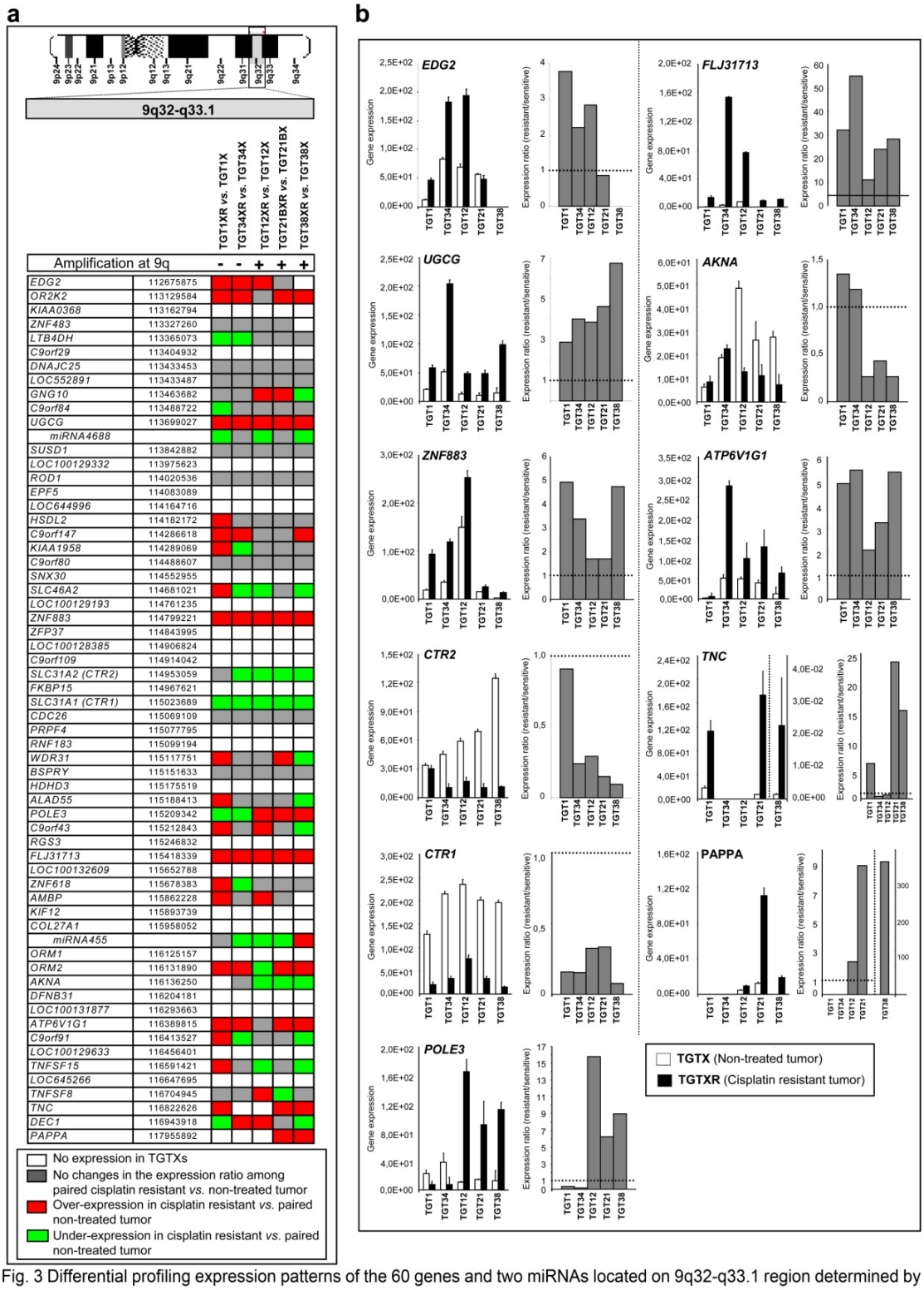


Fig. 2 Recurrent gains at 9q in different refractory tumors. a) The long 9q21.11-q33.3 gain region (arrow) was identified in TGT12R and TGT21BR, while in tumor TGT38R there was a small overlapping region of 5.1 Mbp at 9q32-q33.1 (arrow). Whole-genome mapping was performed by oligonucleotide array CGH analysis (60 kbp window averaging) and visually depicted with the SignalMap graphical interface tool from Nimblegen Systems. b) FISH analyses of the copy number of 9q32-q33.1 in metastatic CGT samples contained in the TMA. Interphase FISH with RP11-582I20 (red) and RP11- 616C16 (green) probes. Panel 1 shows the absence of amplification characterized by two red and two green signals in all interphase nuclei; and panels 2 and 3 show amplification of the region. c) Kaplan–Meier plots by status of 9q32-q33.1 gains. Left panel, overall survival (OS), and right panel, progression-free survival (PFS). P-values are those from multivariate Cox proportional hazards regression models, controlling for the pathological diagnostic classification.

Knockdown of *Caenorhabditis elegans* ortholog genes, *GCS*, *ATP6V1G1*, *AMBP* and *CTR1/CTR2, by* RNAi impaired worm cisplatin response

We investigated the individual contribution of the 9q32-q33.1 genes to cisplatin resistance/sensitivity in the nematode *Caenorhabditis elegans* by RNAi their corresponding ortholog genes. We identified *C. elegans* ortholog genes in 15 of the 37 genes expressed in engrafted tumors; 12 genes were selected on the basis of our previous qPCR results to be knocked down by feeding RNAi (**Fig. 4a**). Cisplatin-induced toxicity was subsequently evaluated in these worms by measuring the track locomotor activity over 24 hours of cisplatin exposure (**Fig. 4b, 4c and Supplementary Fig. S8**). We observed a clear correlation with sensitivity/resistance to cisplatin with *ctg-1* and *ctg-3* (*GCS* orthologs) and *F27C1.2* (*CTR1/CTR2* ortholog). In the case of *ctg* genes, the functional redundancy was overcome by doing *ctg-3* RNAi in the *ctg-1*(ok1045) mutant background. *mig-6* (*AMBP* ortholog) and *vha-10* (*ATP6V1G1* ortholog) also contribute to cisplatin resistance, but not other genes as *gpc-1* (*GNG10* ortholog) or *ten-1* (*TNC* ortholog)(**Supplementary Fig S8**). Although, the role of *mig-6* and *vha-10* genes under cisplatin exposure may need further exploration since their RNAi inactivation by itself produces an effect on locomotor activity in the absence of cisplatin. Functional redundancy probably explains the absence of association with cisplatin response for *nfyb-1* (*POLE3/CHARC17* ortholog) [13] in *C. elegans*, while we did not identity a specific worm ortholog for *PAPPA* gene.

Next, by the absence of an ortholog gene or by the difficult to study in worms its role in cisplatin response, the expression changes of candidate genes *POLE3*, *PAPPA*, *ATP6V1G1*, AKNA and *AMBP* were determined by qPCR in a set of three paired sensitive *vs*. cisplatin-derived resistant testicular germ cell tumor cell lines (SuSaS *vs* SuSaR, 833KS *vs* 833KR and GC27S *vs* GC27R), confirming their association with cisplatin resistance for the majority of them (**Supplementary Fig S7b**).



Amplification at 9q		-	-	+	+	+
EDG2	112675875					
OR2K2	113129584					
KIAA0368	113162794					
ZNF483	113327260					
LTB4DH	113365073					
C9orf29	113404932					
DNAJC25	113433453					
LOC552891	113433487					
GNG10	113463682					
C9orf84	113488722					
UGCG	113699027					
miRNA4688						
SUSD1	113842882					
LOC100129332	113975623					
ROD1	114020536					
EPF5	114083089					
LOC644996	114164716					
HSDL2	114182172					
C9orf147	114286618					
KIAA1958	114289069					
C9orf80	114488607					
SNX30	114552955					
SLC46A2	114681021					
LOC100129193	114761235					
ZNF883	114799221					
ZFP37	114843995					
LOC100128385	114906824					
C9orf109	114914042					
SLC31A2 (CTR2)	114953059					
FKBP15	114967621					
SLC31A1 (CTR1)	115023689					
CDC26	115069109					
PRPF4	115077795					
RNF183	115099194					
WDR31	115117751					
BSPRY	115151633					
HDHD3	115175519					
ALAD55	115188413					
POLE3	115209342					
C9orf43	115212843					
RGS3	115246832					
FLJ31713	115418339					
LOC100132609	115652788					
ZNF618	115678383					
AMBP	115862228					
KIF12	115893739					
COL27A1	115958052					
miRNA455						
ORM1	116125157					
ORM2	116131890					
AKNA	116136250					
DFNB31	116204181					
LOC100131877	116293663					
ATP6V1G1	116389815					
C9orf91	116413527					
LOC100129633	116456401					
TNFSF15	116591421					
LOC645266	116647695					
TNFSF8	116704945					
TNC	116822626					
DEC1	116943918					
PAPPA	117955892					

Fig. 3 Differential profiling expression patterns of the 60 genes and two miRNAs located on 9q32-q33.1 region determined by quantitative PCR. a) Results are presented as changes in the expression levels in cisplatin refractory tumors relative to the untreated tumors, grouped by 9q32-q33.1 gain status. No expression changes (in grey), underexpression in resistant tumors (in green), overexpression in resistant tumors (in red) and no expression in engrafted tumors (in white). b) Graphs showing qPCR expression results for relevant genes. For each gene, normalized gene expression (left graph), and the expression ratio among refractory vs. sensitive tumors (right graph) are shown. Reactions were performed in triplicate and all data were normalized with two endogenous control genes.

DL-threo-PDMP, a competitive inhibitor of GCS, re-sensitizes refractory NSE orthoxenografts to cisplatin

Our study enabled six candidate (*GCS*, *POLE3*, *PAPPA*, *ATP6V1G1*, *AMBP* and *CTR1/2*) targets to be identified that are of use for developing novel therapeutic approaches for overcoming cisplatin resistance. As a proof-of-concept we decide to deep into the therapeutic value of one of these genes/proteins at the preclinical level. GCS was chosen on the grounds that: (i) it increased mRNA expression in all cisplatin refractory orthoxenografts; (ii) it increased GCS enzymatic activity (2.5-3-fold induction) occurs in 4 out 5 cisplatin-refractory orthoxenografts (**Fig. 5a**); (iii) its knockdown by RNAi in worms confers cisplatin sensitivity; (iv) its central role in the pathway of sphyngolipid synthesis; and (v) specific inhibitors of it are available, some of which are currently in clinical use for other pathologies.

NSE testicular germ cell line SuSAS and its paired cisplatin resistant SuSaR were used as cellular models to deep into the functional relationship among GCS expression/activity and cisplatin resistance. Significant differences among protein expression and activity levels measuring glycosilceramide formation were observed for both cell lines (**Fig. 4d**). At low doses of cisplatin (5 μM) SuSaR cells exhibit increased GCS activity through the time (**Fig. 4d**). Transfected SuSaS cells overexpressing GCS has a significant cisplatin-resistance increase (5-fold) (**Fig. 4e)**; while shRNAi knockdown of the endogenously overexpressed *GSC* gene (70% of inhibition) in SuSaR cells correlates with a partially (57.6%) cisplatin resensitization (**Fig. 4e**). Likewise, the treatment of SuSaR cells with the specific GCS inhibitor DL-thhreo-PDMP (PDMP) (**Fig. 4f**) mimics this cisplatin sensitization (44.8%). Effect mediated by a significant increase in the intracellular levels of ceramide for combined cisplatin+PDMP treatment (**Fig. 4f**). Thus, we demonstrate that impaired GCS expression/activity *in vitro* resensitizes cisplatin-resistant NSE cell line newly to cisplatin treatment.

Engrafted animals with cisplatin-refractory TGT1XR and TGT38XR orthoxenografts, which exhibit increase GCS expression and enzyme activity (**Fig. 5a**), were treated daily with PDMP, a competitive inhibitor of GCS for 21 days (**Fig. 5b**). As a single agent PDMP did not produce a significant response with respect to the vehicle-treated animals, and no significant differences were observed among individual PDMP and cisplatin treatments. Nevertheless, both tumors experienced significant tumor weight reductions (TGT38XR, 73.5% and TGT1XR, 42.8%) for combined PDMP+cisplatin treatment (**Fig. 5b**).

a

Gene expressed in TGCTs	Ortholog *C.elegans* gene	*RNAi*	Cisplatin treatment
EDG2	*AC7.1*	yes	NA
OR2K2	-	-	-
ZNF483	-	-	-
LTB4DH	*M106.3*	-	-
DNAJC25	*dnj-2*	yes	NA
LOC552891	n.i	-	-
GNG10	*gpc-1*	yes	NA
C9orf84	-	-	-
GCS	*cgt-1,-2, -3*	yes	Sensitive
SUSD1	*B0393.5*	-	-
ROD1	*ptb-1*	yes	NA
HSDL2	*dhs-6*	-	-
C9orf147	-	-	-
KIAA1958	-	-	-
C9orf80	-	-	-
SLC46A2	*Y4C6B.5*	yes	NA
ZNF883	n.i	-	-
CTR2	*F27C1.2*		Resistant
CTR1			
CDC26	-	-	
WDR31	*T05A8.5*	yes	NA
BSPRY	-	-	-
ALAD55	-	-	-
POLE3	*nfyb-1*	yes	NA
C9orf43	-	-	-
FLJ31713	-	-	-
ZNF618	-	-	-
AMBP	*mig-6*	yes	Sensitive
ORM2	-	-	-
AKNA	-	-	-
ATP6V1G1	*vha-10*	yes	Sensitive
C9orf91	-	-	-
TNFSF15	-	-	-
TNFSF8	-	-	-
TNC	*ten-1*	yes	NA
DEC1	-	-	-
PAPPA	-	-	-

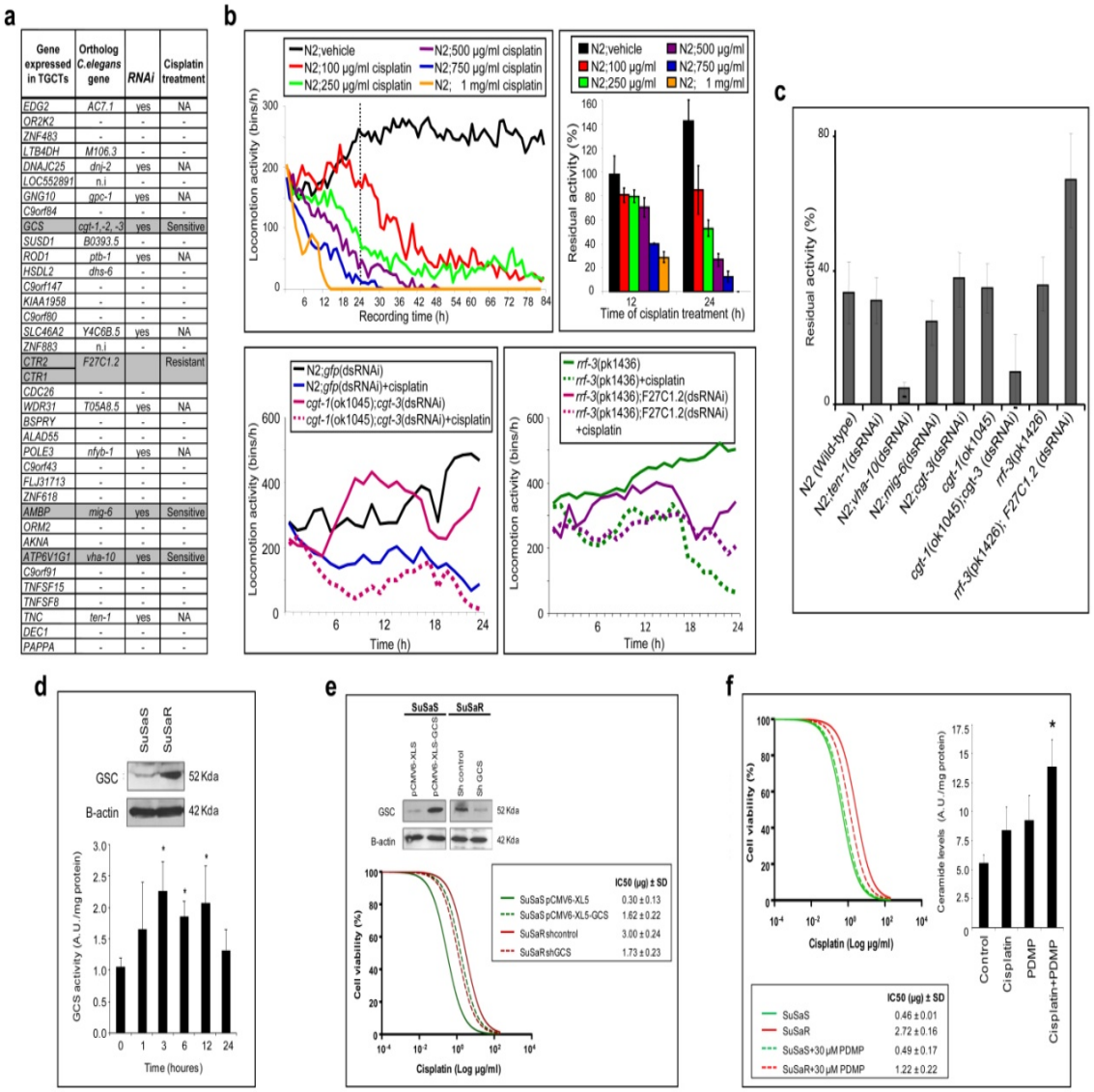


Fig 4 Knockdown of C. elegans ortholog genes by RNAi and correlation with cisplastin response. a) Correspondence between expressed 9q32-q33.1 human genes in xenografts and their corresponding C. elegans orthologs. Twelve selected genes were independently knocked down by RNA interference in worms, and the cisplatin response was evaluated measuring worm track locomotion activity over 24 hours of exposure to the drug (500 µM cisplatin). b) Representative cisplatin worm assays. Knockdown of ctg- 1/ctg-3, mig-6 or vha-10 genes sensitizes worms to cisplatin treatment, while knockdown of the F27C1.2 gene is associated with increased cisplatin resistance. c) Percentage of conserved locomotor activity after 24 h of exposure to 500 µM cisplatin doses for different genes. N2 control worms maintained 35-40% of their locomotor activity after 24 hours of treatment. All the assays were performed in triplicate (20 worms/well and five replicates). d) Glycosilceramide synthase (GCS) activity in engrafted tumors was determined as described (ref) in the five paired sensitive vs. refractory engrafted tumors. The levels of NBD-labeled glucosylceramide formation were determined by HPTLC (90:20:0.5; chloroform:methanol:ammonia) after running on silica gel 60A plates 23 (Whatman), and quantified using Biorad Quantity One software. e) Graphs illustrate responses of cisplatin-refractory engrafted TGT1R and TGT38R tumors to treatment with the GCS inhibitor DL-threo-PMDP. Animals were treated with (i) vehicle, (ii) cisplatin (3.5 mg/kg), (iii) DL-threo-PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1- propanol hydrochloride) (Santa Cruz), 50 mg/kg dissolved in 5% of Tween 80 in saline; and (iv) DL-threo-PDMP+cisplatin (50 mg/kg+3.5 mg/kg) (see Material and Methods). Cisplatin was i.v. administered once a week for three consecutive weeks (days 0, 7 and 14), while DL-threo-PDMP was administered daily by intraperitoneal injection over the 21-day| period and mice were sacrificed on day 22 of treatment. In combined treatments, PDMP was administered one hour before cisplatin treatment. Cer, ceramide; GluCer, glycosilceramide. TGT, untreated tumor; TGTR, cisplatin-refractory tumor. *, P<0.05.

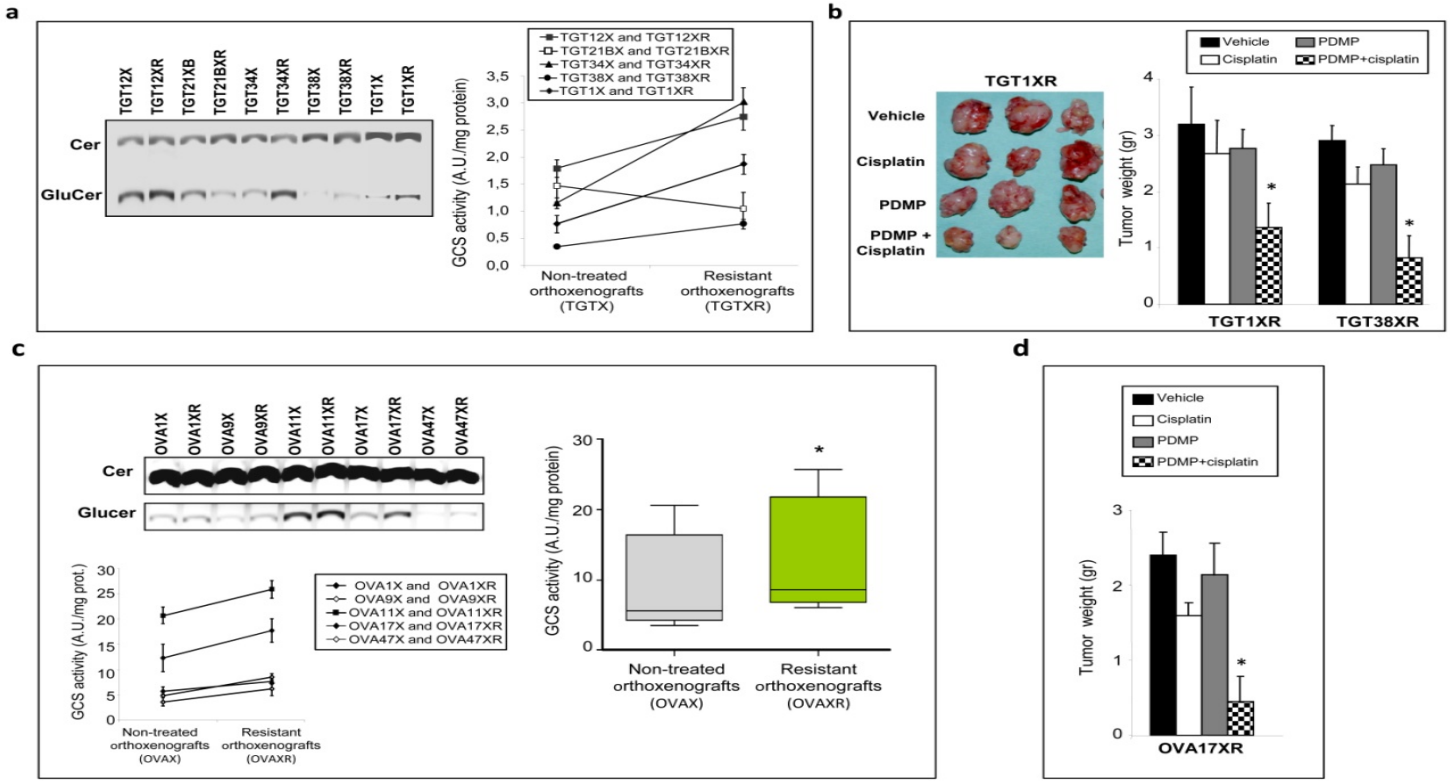


Fig 5. Glucosylceramide synthase (GCS) activity in orthoxenografts of NSE and the epithelial ovarian cancer (EOC) was determined. a) Activity in five paired sensitive vs. refractory engrafted testicular germ cell tumors. The levels of NBD-labeled glucosylceramide formation were determined by HPTLC (90:20:0.5; chloroform:methanol:ammonia) after running on silica gel 60A plates (Whatman), and quantified using Biorad Quantity One software. b) Graphs illustrate responses of cis-platin-refractory engrafted TGT1XR and TGT38XR tumors to treatment with the GCS inhibitor DL-threo-PMDP. Tumors were implanted in mouse testicle and when homo-geneous tumor sizes were detected they were randomized to three treatment groups (n = 6-8 mice/group): (i) vehicle, (ii) cisplatin (3.5 mg/kg), (iii) DL-threo-PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride) (Santa Cruz), 50 mg/kg dissolved in 5% of Tween 80 - 0.85% NaC l36 and (iv) DL-threo-PDMP+cisplatin (50 mg/kg+3.5 mg/kg). Cisplatin was i.v. administered once a week for three consecutive weeks (days 0, 7 and 14), while DL-threo-PDMP was administe-red daily by intraperitoneal injection over the 21-day period and mice were sacrificed on day 22 of treatment. In combined treatments, PDMP was administered one hour before cisplatin treatment. c) GCS activity was also determined in six paired sensitive vs. refractory orthoxenografts of epithelial ovarian cancer (EOC). Increase levels of glucosylceramide were detected in 5 out 6 resistant cases. d) Graph illustrates response of cisplatin-refractory engrafted OVA17XR orthoxenograft (serours tumor) treated with the GCS inhibitor DL-threo-PMDP. Tumor was implanted in the mouse ovary and when homogeneous tumor sizes were detected they were randomized to three treatment groups (n = 6 mice/group) and treated as described. Cer, ceramide; GluCer, glycosilceramide. TGTX, untreated testicular germ cell tumor; TGTXR, cisplatin-refractory testicular germ cell tumor; OVAX, epithelial ovarian tumor; OVAXR, cisplatin-refractory epithelial ovarian tumor; *, P<0.05.

Glucosylceramide synthase activity also associated with acquired cisplatin resistance in orthoxenografts of epithelial ovarian cancer

Finally, we investigate whether the identified association among GCS and cisplatin resistance was specific of germ cell tumors, or it happens in other cisplatin treated tumors. Thus, GCS expression/ activity were determined in six paired cases of sensitive and cisplatin-resistant orthoxenografts of epithelial ovarian cancer (EOC). In 5 out 6 (83.3%) serous tumors a median increase of 52.5% ± 9.4 GCS activity and RNA/protein levels (data not shown) were also observed in the resistant orthoxenografts respect to its paired sensitive tumors (**Fig. 5c**). Furthermore, PDMP treatment of OVA17XR, having high levels of GCS activity, has a cisplatin resensitizing effect (**Fig. 5d**) (tumor weight reduction of 76,5% in combined cisplatin+PDMP treatment). Together, the GCS inhibitor PDMP resensitizes cisplatin-refractory orthoxenografts to cisplatin treatment, providing a promising therapeutic opportunity for GCT refractory cases, and a strong preclinical rationale for further clinical trials.

DISCUSSION

In this paper we report the generation of a unique series of orthotopically engrafted nonseminomatous tumor, named orthoxenografts, including several paired cisplatin-sensitive/resistant tumors and endogenous refractory cases. Preclinical models enabled several cisplatin resistance-related chromosomal regions to be identified, as the development of novel therapeutic approaches to overcome cisplatin refractoriness. We then focused on the recurrent gained 9q32-q33.1 region, demonstrating that its presence in metastatic GCTs was associated with poorer overall survival. Combined differential expression profiling studies, and functional RNAi gene knockdown in *C. elegans* and *in vitro* cell lines we identified and validate a group of cisplatin resistance-related genes. Finally, as proof-of-concept we preclinically validated the PDMP as a therapeutic strategy that resensitizes to cisplatin treatment producing a change that overcomes cisplatin refractoriness.

Rao et al [14], analyzing human refractory GCTs samples, provided the first evidence of chromosomal amplification associated with cisplatin resistance by comparing unpaired tumors obtained from relapse-free patients with chemotherapy-resistant tumors. Our approach of comparing against the same genetic background (paired sensitive vs. cisplatin-refractory tumor cases) identified fewer recurrent changes across the different refractory tumors. The genetic concurrence observed among pure primary refractory tumors analyzed Rao et al [14] and our refractory-derived orthoxenografts by the

identification of 9q and 15q amplified regions strongly reinforced their relevance as models for studying the genetic basis of cisplatin refractoriness.

Until now, the presence of the b-raf (V600E) mutation in some refractory NSE has been the only molecular marker associated with cisplatin resistance [15] in GCT. Here we have demonstrated that the presence of the 9q32-q33.1 amplification was associated with increased risk of progression and death in one of the largest cohort of patients with metastatic GCTs, of whom, 32% are truly refractory to cisplatin treatment. Thus, their presence as early high-risk patients marker may be especially important in the good prognostic group and may allow clinicians to include them under more aggressive protocols, or to offer alternative drug treatments. Although it is a single retrospective analysis it is important to note its relevance, given how difficult is to obtain representative GCT series that include patients with a poor prognosis, and refractory tumors.

Differential expression profiling of the 9q32-q33.1 region among orthoxenografts highlights the complexity of the mechanism for developing cisplatin tumor resistance. We found two groups of genes coexisting in refractory tumors: (i) those whose expression changes correlate with 9q32-q33.1 gain status (PAPPA, POLE3, and AKNA), and mainly (ii) those that do not, and are associated solely with cisplatin refractoriness (GCS, EDG2, ZNF883, FLJ31713, TNC, ATP6V1G1, CTR1, and CTR2). Together, functional results of RNAi experiments in C. elegans and in paired sensitive/resistant cell lines strongly suggest that targeting GCS, ATP6V1G, CTR1/2, PAPPA or POLE3 should be useful strategies for treating unresponsiveness in cisplatin tumors. Targeting GCS, due is central role in the glycosphingolipid synthesis pathway, has emerged as a novel approach for treating metabolic diseases such as Gaucher, Niemam-Pick and diabetes. In this context, several GCS inhibitors are in clinical use or under development, including Miglustat, PDMP and EXEL-0346 among others [16-19]. In the present work we deep into the relevance of targeting GCS as a novel approach to resensitize tumors to cisplatin. Here we demonstrate the relevance of the over-expression/increased GCS activity as a biological mechanism that mediate tumor cell protection against cisplatin exposure, and they denoted the significance of sphingolipid metabolism through cisplatin-induced tumor cell death. Thus, we hypothesize that PDMP or other GCS inhibitors blocking the conversion of ceramide to glucosylceramide should open an important therapeutic window in patients with refractory tumors by fuel ceramide pools after cisplatin treatment strengthen the cell-death pathways. In this way, our preclinical results in advanced refractory cisplatin

orthoxenografts of GCTs and EOC tumor models demonstrate that PDMP resensitizes to cisplatin treatment, providing a firm preclinical rationale of drug repositioning and for developing further clinical trials in the field.

In summary, we report the generation of cisplatin-refractory orthoxenografts of germ cell tumors as preclinical models and demonstrate their proficiency identifying cisplatin-resistance genes. As a proof-of-concept, we present strong preclinical evidence that they are outstanding tools to provide the rationale for the design of future trials for the treatment of patients with cisplatin resistant/refractory tumors.

MATERIAL AND METHODS

Material Methods are in Supplementary Material

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SUPPLEMENTARY INFORMATION

MATERIAL AND METHODS

Human primary testicular germ cell tumors implantation and perpetuation in nude mice as orthoxenografts

Primary tumor samples were obtained after surgical resection (Hospital Universitari de Bellvitge and Fundació Puigvert, Barcelona (Spain and placed at room temperature in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Fresh surgical specimens of 62 human GCTs were implanted in nude mice. Twenty-two tumors were classified as pure SEs, 21 as pure NSEs, and 19 as mixed tumors containing different proportions of SE and NSE components. NSE includes pure histologies (yolk sac tumor, YS; choriocarcinoma, CH; embryonal carcinoma, EC), and mixed tumors containing one or more histological subtypes. Animals were housed in a sterile environment, cages and water were autoclaved and bedding and food was γ-ray sterilized. Tumors were implanted in the testis of five-week old male *nu/nu Swiss mice* (Charles River, France) weighting 18-22 g. After anesthesia by isofluorane inhalation, a median laparatomy was performed and the testes were mobilized. Tumor pieces were anchored to the testis surface with prolene 7.0 sutures. After implantation, mice were inspected twice a week, and if no tumor growth was apparent, mice were sacrificed six months after implantation. Serial tumor passaging was performed in two to five animals. Time lags varied for each tumor, depending upon their growth kinetics (**Supplementary Table S1**). Four orthoxenografts were derived from patients previously treated with cisplatin-based chemotherapy. All patients gave written consent to participate in the study. The Ethics Committee of the hospitals approved the study protocol, and the animal experimental design was approved by the IDIBELL animal facility committee.

Immunohistochemistry tumor characterization

Tissues taken for histological studies were fixed in 10% buffered formalin and 3-μm slices of paraffin-embedded tissues were used for immunohistochemistry (IHQ) studies. Primary antibodies were monoclonal antibodies for TP53 (clone BP53-12-1, dilution 1:1000, Biogenex); PLAP (clone PL8-F8, dilution 1:30, Biogenex); EMA (clone E29, dilution 1:100, Dako); Vimentin (clone V9, dilution 1:2000, Dako); CD-30 (clone Ber-H2, dilution 1:20, Dako); EGFR (clone 31G7, dilution 1:50, Zymed); Ki-67 (clone BGX-297, dilution 1:40, Biogenex); Cam 5.2 (dilution 1:100, Becton Dickinson) and polyclonal antibodies for β-hCG (dilution 1:5000, Dako); CD117 (dilution 1:50, Dako); AFP (dilution 1:3, Dako); OCT3/4 (dilution 1:300, Santa Cruz) and NANOG (dilution

1:50, RD). Reactions were visualized using the EnVision anti-mouse antibody system, and developed using the DAB-Plus Kit (Dako, Copenhagen, Denmark). Slides were counterstained with Harry's modified hematoxylin.

Determination of mouse serum levels of tumor markers

Serum concentrations of alpha-fetoprotein (AFP) and the β-subunit of human chorionic gonadotropin (β-hCG) concentrations were measured as subrogate tumor growth markers in the serum of nude mice using commercially available two-site enzyme chemiluminometric assays automated on the Immulite® 2000 analyzer [1,2].

Genetic characterization of engrafted NSE tumors

DNA was extracted following standard phenol-chloroform protocols, while total RNA was extracted using TRIZOL reagent following the manufacturers' instructions (Invitrogen). Nude mouse tissues were included in all PCR experiments to avoid mouse DNA and RNA contamination.

Presence of point mutations: Mutations in *TP53* (exons 4-10); K-*ras* (codon 12 and 13), *b-raf* (exons 11 and 15), *EGFR* (exons 18, 19, 20 and 21), c-*Kit* (exons 9, 11, 13 and 17), *PDGFRα* (exons 12 and 14), *PDGFRβ* (exon 12) and *PI3KCA* (exons 9 and 20) were analyzed. All exons were amplified in independent PCR reactions using human intronic primers to avoid amplification of mouse DNA. PCR reactions were carried out using 100-200 ng of genomic DNA in a mixture containing PCR buffer, 100 mM deoxynucleotide triphosphates, 0.5 μM of each primer and 1 unit of Taq DNA polymerase (Invitrogen). RNA was reverse-transcribed to cDNA using $pd(N)_6$ and the M-MLV retrotranscriptase kit (Invitrogen) and the entire coding *Smad*4 region was analyzed in five overlapping reactions. Primer sequences and PCR conditions are available on request. The presence of gene mutations was detected by direct sequence and/or single-strand chain polymorphism (SSCP). Homozygous deletions or microdeletions in *p15*, *p16* and *Smad4* were evaluated in agarose gels and were defined by the absence of PCR product in three independent experiments.

5' CpG promoter methylation studies: The DNA bisulfite reaction was carried out on 2 μg of restriction-digested DNA for 16 h at 55ºC. 5' CpG promoter islands of *APC*, *MGMT*, *DAPK*, *CDH1* and *RASSF1* were analyzed by the methylation-specific polymerase chain reaction (MSP) [3-5]. Primers and PCR conditions are available on request. MSP results were confirmed after sequencing individual clones using the TOPO system (Invitrogen).

Microsatellite instability (MSI) analysis: Genetic instability was analyzed using Bethesda's set of five microsatellite markers (D2S123, BAT25, BAT26, D5S346 and BAT40).

Primary response of engrafted NSEs to cisplatin treatments

Small fragments of engrafted tumors were reimplanted in the testicles of 30 nude mice, as described above. When palpable intra-abdominal masses and increased levels of serum tumor marks had both been detected, usually 7–30 days after implantation, mice were randomized into three groups: (i) control group (n = 10), treated with vehicle; (ii) low-dose treatment group (n = 20) (2 mg/kg of cisplatin); and (iii) high-dose treatment group (n = 20) (5 mg/kg of cisplatin). Each treatment group was randomly divided into a *short-term response group* (n = 10), defined by tumor weight at the time of sacrifice of the control group, and a *long-term response group* (n = 10), defined by recurrent tumor mass regrowth post-chemotherapy. Cisplatin was intravenously administered (i.v.) once a week for three consecutive weeks (days 0, 7 and 14). Animals were sacrificed seven days after the final dose (day 21) to examine their short-term response.

Generation in mice of refractory engrafted NSE to cisplatin treatment

Five engrafted tumors, TGT1, TGT12, TGT21B, TGT34 and TGT38, from patients without prior exposure to cisplatin, were allowed to grow until intra-abdominal palpable masses were noted. Animals were administered with cisplatin i.v. at a dose of 2 mg/kg for 3 consecutive weeks (days 0, 7 and 14) (cycle#1 of treatment). Post-cisplatin relapse tumors were harvested, prepared as previously described, and engrafted in new animals. This process was repeated up to five times by treating tumor-bearing mice with stepwise increasing doses of cisplatin: cycle#2, 3 mg/kg; cycle#3, 3.5 mg/kg; cycle#4, 4 mg/kg; and cycle#5, 5 mg/kg (**Fig 2d**), as recently we described for ovarian tumors [6]. Dynamic CDDP responses were evaluated after assessing β-hCG and/or AFP serum levels, as described above.

Whole genome analysis by NimbleGen CGH arrays

The CGH oligonucleotide array was carried out by NimbleGen Systems, Inc., at their facility in Wisconsin [13]. Array design descriptions were: 2006-07-27_HG18_WG_CGH, single array CGH design for whole human genome (hg18; NCBI Build 36). Methods of DNA labeling array construction, hybridization, array normalization and data analysis have been described in detail by Seltzer et al. [7].

FISH analysis

FISH was done by standard methods. We used the UCSC genome browser to select three bacterial artificial chromosomes (BACs) from the K32 BAC library (kindly provided by Dr L. Pérez-Jurado). BAC RP11-582I20 is contained in the amplified 9q32-9q33.1 region while RP11-616C16 flanks it at its distal end. FISH results were analyzed under an Olympus BX60 microscope and images were captured with a Cytovision (Applied Imaging) workstation. One hundred non-overlapping nuclei were scored for each sample.

Quantification of gene and miRNA expression

Total RNA was extracted using Trizol (Invitrogen, San Diego, CA), following the manufacturer's instructions, and reverse-transcribed to cDNA. Quantitative RNA and miRNA analyses was performed as described. Quantitative real-time RT-PCR analyses were performed using the Light-Cycler 2.0 Roche System and LightCycler FastStart DNA Master SyBR Green I kit (Roche). All the primers were designed specifically to amplify human RNA. Primer sequences and PCR conditions are available on request. Experiments were performed in triplicate using three independent RT reactions. Gene expression was normalized with respect to β-actin.

For miRNA, RNA samples were DNase-treated with Turbo DNA-free (Ambion, Austin, TX), and determined as described [8]. Reactions were performed in triplicate and incubated in an Applied Biosystems 7900HT Fast Real-Time PCR system in 384-well plates. All data were normalized with endogenous controls: PPIA, HPRT1 and RPLPO. The relative miRNA levels were calculated using the formula $2^{-\Delta\Delta Ct}$ [9].

Patients and Samples

Eighty-eight consecutive patients diagnosed with metastatic germ cell tumors and treated at the Institut Català d'Oncologia between 1989 and 2004 were initially included in this study [10]. Thirteen cases were not evaluated because of the lack of paraffin-embedded tissue blocks. Patient demographics and clinical characteristics of the 75 patients finally evaluated are listed in **Supplementary Table S7**. Sixty-three patients (84%) had NSE tumors and 12 (16%) had SE tumors. Four patients presented with mediastinal extragonadal disease. Sixty per cent of the patients were classed as having a good prognosis, 19% as having an intermediate prognosis and 21% as being of poor prognosis according to the IGCCCG categorization. Twenty-four patients were considered resistant, defined by progression or relapse despite adequate first-line chemotherapy treatment. Cases with mature teratoma only in the resected post-

chemotherapy mass and without posterior tumor relapse were considered sensitive. Tumor samples from primary tumors and/or resected metastases obtained before chemotherapy were included in a newly generated TMA, as described [10].

Cell culture, transfection and *in vitro* shRNAi knockdown experiments

The human NSE cell lines SuSaS (from teratocarcinoma origen), GC27S (from embryonic carcinoma origen) and 833KS ("S" for sensitive to CDDP) and their matched SuSaR, GC27R and 833KR ("R" for CDDP-resistant derived cell line) were growth for different experiments as described [11, 12]. For overexpression experiments, SuSaS cells were transfected with plasmid pCMV6-XL5-*GCS* containing the whole GCS human cDNA from Origene (SC118052; Rockville, USA). Knockdown expereriments were realized in SuSaR with four pre-designed small hairpin RNAs (shRNA) for the human GCS gene from Qiagen (KH02376P; Manchester, UK) that were transfected with the jetPrime transfection kit (Polyplus, Strasbourg), following manufacturer instructions. GCS expression levels was analyzed by Western blot at 24, 48, 72 and 96 hours post-transfection by anti-GCS (1/1000)(ProteinTech, Chicago, USA) using as a control the anti-β-actin-HRP antibody (1/20000)(Sigma, St. Louis, USA). The chosen time to perform the experiments was 48 hours.

***In vitro* determination of drug resistance assays**

Cisplatin (1mg/ml) dissolved in NaCl (TEVA, North Wales, USA), and DL-threo-PDMP (Sigma, St. Louis, USA) in dimethyl sulfoxide (DMSO) at a final concentration of 59 mM were assessed. Cell viability was determined by MTT assay. Briefly, 1 x 10^3 cells were plated onto 96-well plates, after 4 hours of transfection, fresh medium was added and cells were treated for 48 hours with different drugs concentration ranged from 0 to 20 µg/ml doses. MTT was added at a final concentration of 0.1% and after 2.5 hours of incubation (37ºC, 5% CO_2), metabolic product formazan was dissolved in DMSO and the absorbance measured at 570 nm. Prism Software (La Jolla, USA) was used to calculate drugs half maximal inhibitory concentration (IC50).

***C. elegans* gene knockdown by RNAi and cisplatin-response assay**

The *C. elegans* N2 strain (wild type), and the *rrf-3* (pk1436) and *cgt-1* (ok1045) mutant strains were provided by the Caenorhabditis Genetic Centre (CGC). The bacterial RNAi clones used were obtained from the ORFeome-based RNAi library [13] and the JA library [14]. RNAi feeding was done as described [15, 16]. Synchronized worm populations in the L1 state were cultured in NGM plates containing 50 µg/ml ampicillin and 3 mM IPTG and seeded with thick bacterial lawns of each selected bacterial RNAi clone. Then, at the

young-adult stage, they were transferred to a 96-well plate with S-medium containing 50 µg/ml ampicillin, 3 mM IPTG and 750 µg/ml cisplatin fresh made (Sigma). Cisplatin-induced toxicity was evaluated by measuring worm locomotor activity over a 24-h period by an automated tracking system (wmicrotracker) [17, 18]. All assays were performed at 20ºC in triplicate, tracking 20 worms per well each time in each of five replicates. For the genes tested, the efficiency of worm RNAi was confirmed by qPCR (data not shown).

Determination of GCS activity and determination of ceramide levels

Tumor samples were homogenized in lysis buffer (Tris-HCl 10 mM, EDTA 1mM, 0.1% Triton X-100 at ph 7.4) and centrifuged at 600g for 5 minutes. GCS activity was determined from NBD-C6-ceramide and UDP-glucose, the conversion product separated by TLC with chloroform/methanol/32% ammonia (70:30:5, v/v), and quantified by densitometry (Préférence/DVS, Sebia) as described before previously 19. Briefly, for each assay 200 µg of protein extract was suspended in reaction buffer (5 mM MgCl2, 5 mM MnCl2, and 1 mM EDTA in 50 mM HEPES, pH 7.2) and the substrate mixture containing 10 µM NBD-C6-ceramide and 250 µM UDP-glucose. After a 30 min incubation at 37°C, reactions were terminated by adding 2.5 ml of chloroform/methanol (2:1, v/v), the samples were centrifugated (1000 x g, 5 min), the lower phases dried under nitrogen and subjected to TLC by using chloroform/methanol/32% ammonia (70:30:5, v/v) as the mobile phase.

Ceramide was quantified after labeling of cells to isotopic equilibrium with [^{14}C]palmitic acid (2 µCi/ml) (Amersham, Piscataway, NJ) for 24h. Ceramide was resolved in LK6D gel 60A TLC plates using chloroform/acetic acid (90:10).

Statistical analysis

For the clinicopathological features, *P* values were calculated using the X^2 test. Survival curves were estimated using the Kaplan-Meier method, and differences between individual curves were evaluated by multivariate Cox proportional hazards regression modeling. Analyses were adjusted for pathological diagnostic classification. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated. Likelihood ratio tests were used to assess the prognostic value of genomic amplification of 9q32-q33.1 by FISH in the TMA of metastatic GCTs. Values of $P<0.05$ were considered significant.

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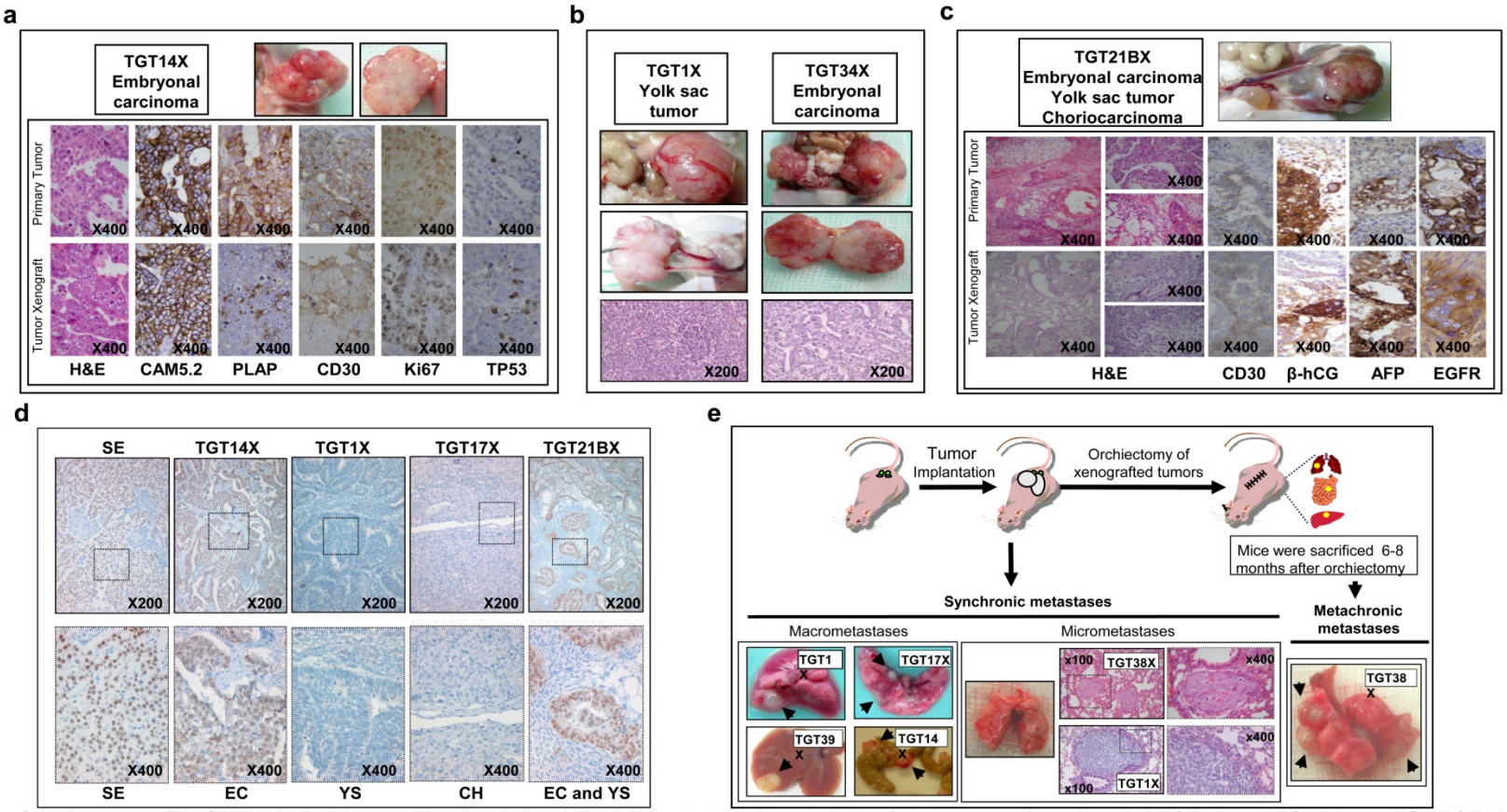
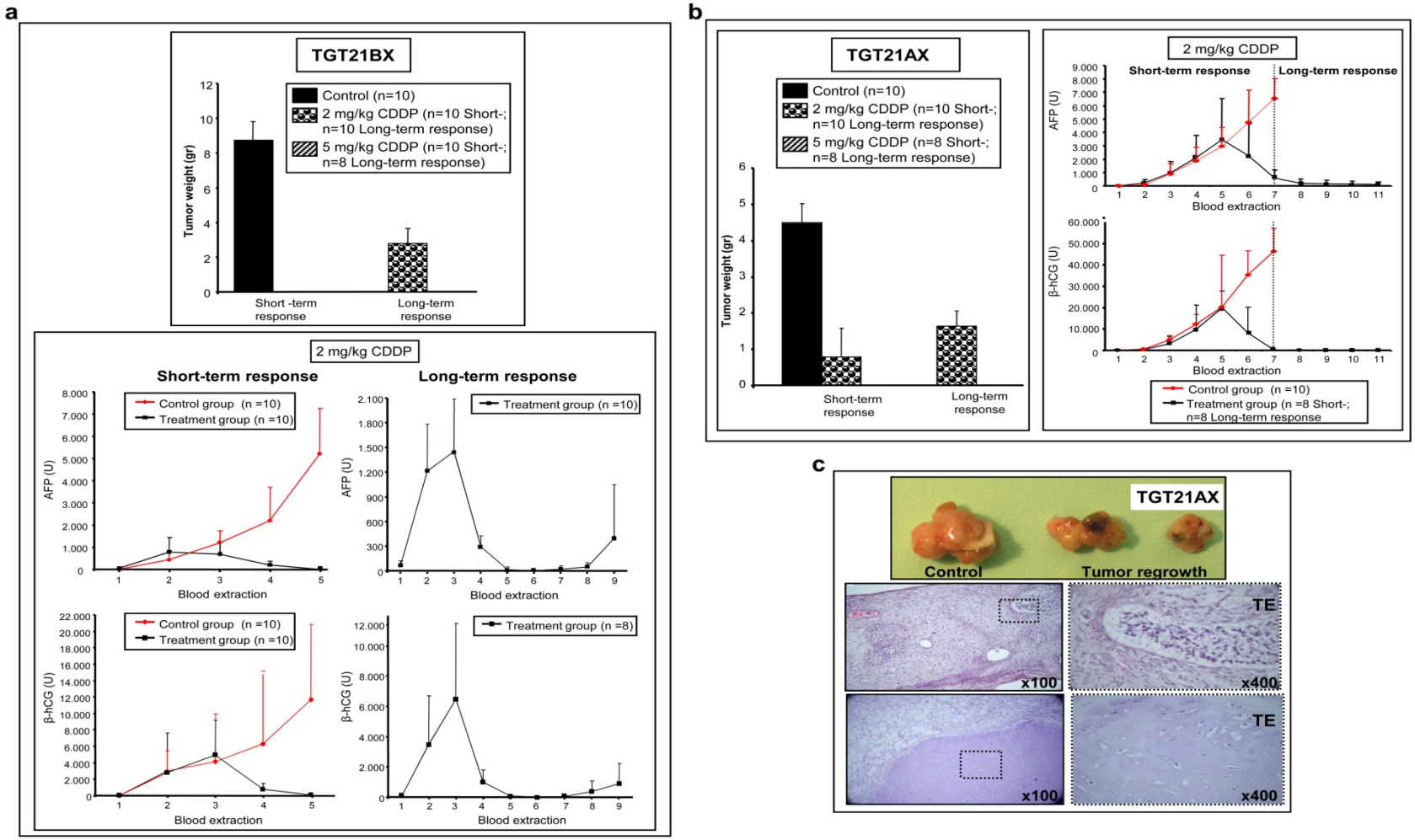
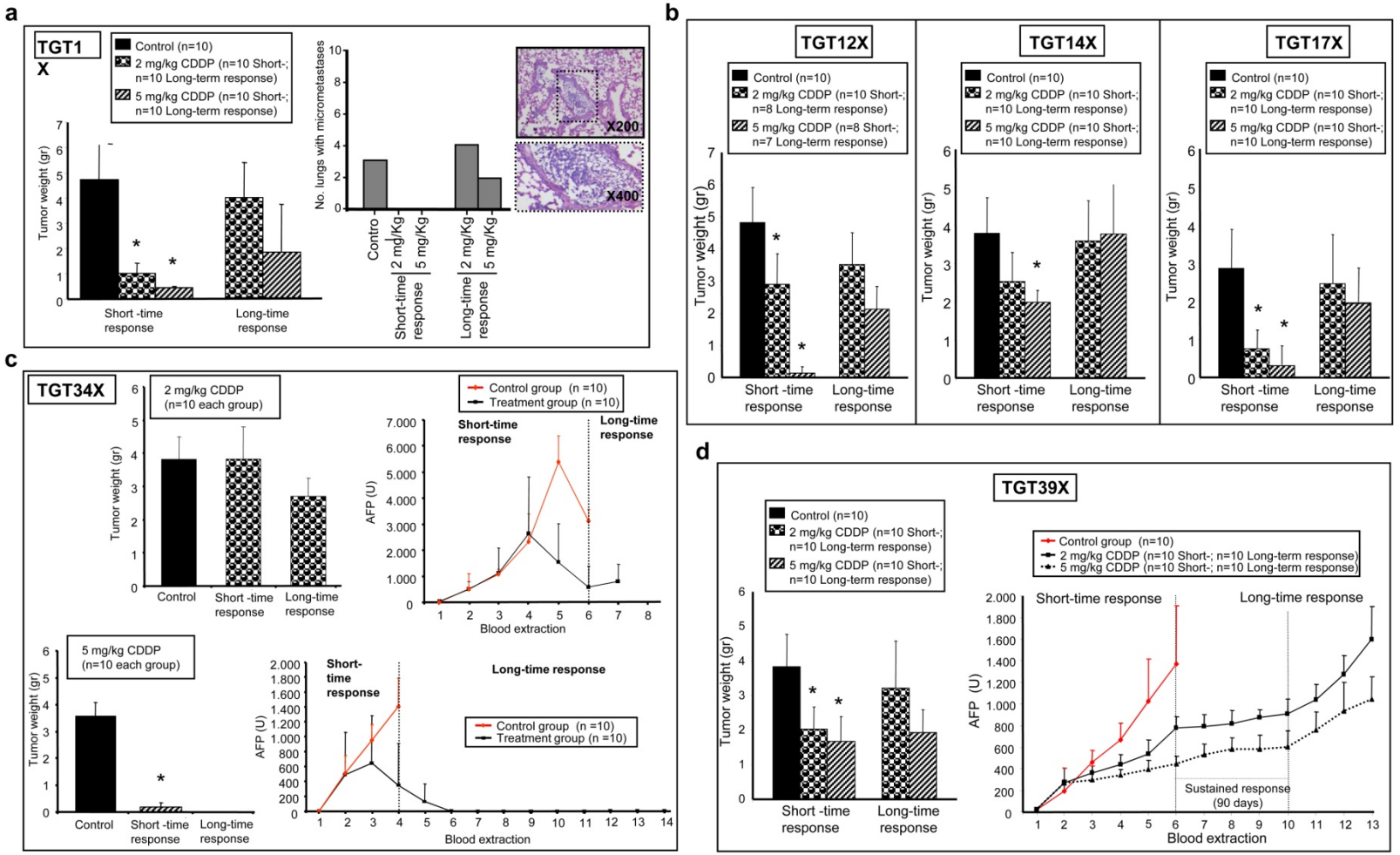


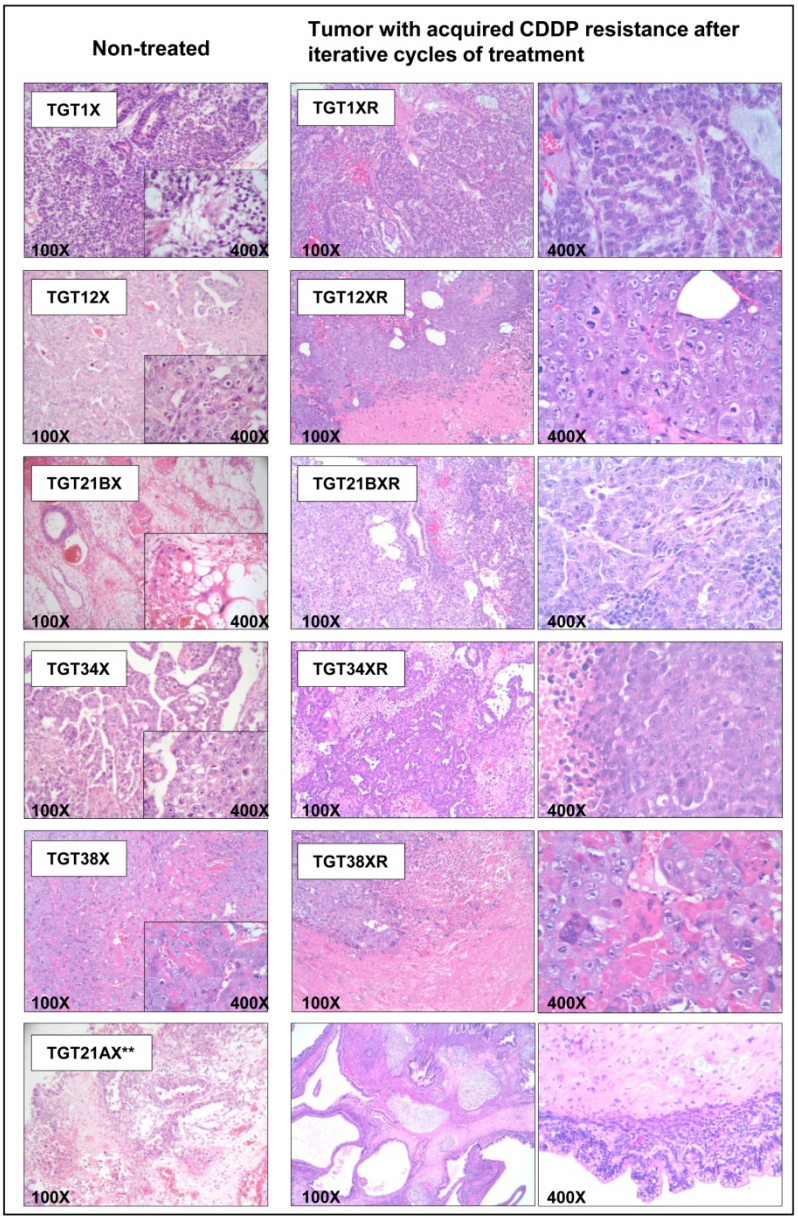
Supplementary Fig. S1 Histological, immunohistochemical and distal dissemination characteristics of tumor xenografts. **a, b and c)** TGT14X and TG34X, pure ECs, TGT1X, a pure YS, and TGT21BX, a mixed tumor composed of EC, YS and CH, all grew on mouse testicles as a big solid mass. H&E staining and immunohistochemical analysis showed a close correlation between primary and corresponding xenografted tumors. **d)** NANOG protein immunostaining. High levels of nuclear staining were only identified in TGT14X, a pure EC, and TGT21BX, a mixed tumor, only in the EC component, (i.e., negative for the YS and CH areas). There was no protein expression in TGT1X (pure YS) and TGT17X (pure CH). Seminoma was used as a positive control. **e)** Study of patterns of distal tumor dissemination. Synchronous metastases were identified when mice were sacrificed by histological evaluation of lung, liver, brain, retroperitoneal lymph nodes and mesentereum tissues. (i) Macrometastases: lung metastases (LMs) were identified in TGT1X and TGT17X. Hepatic metastases and multiple peritoneal implants were identified in TGT39X and TGT14X, respectively. Arrow indicates presence of different macroscopic metastases. (ii) Micrometastases: LMs were identified in TGT1X, TGT17X and TGT38X. All dissemination patterns were confirmed by the presence of large macrometastases after orchiectomy tumor resection; mice were kept alive for an additional 6–8 months. Large LMs were identified in TGT38X at sacrifice. SE, seminoma; YS, yolk sac tumor; EC, embryonal carcinoma; and CH, choriocarcinoma.



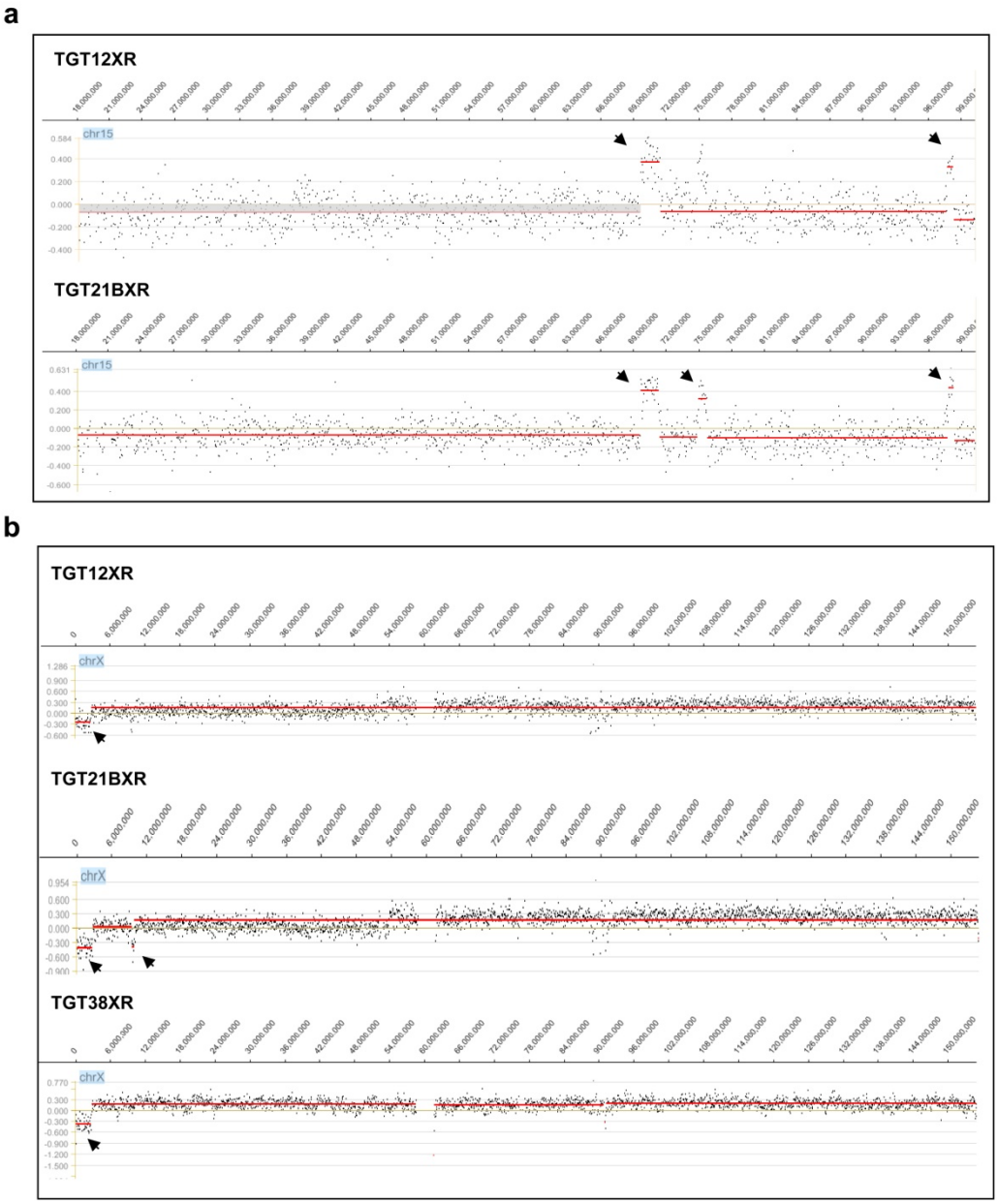
Supplementary Fig. S2 Characterization of differential CDDP responses of TGT21AX and TGT21BX, two tumors obtained from the same patient. **a and b)** Short- and long-term responses were analyzed in mice treated with low (2 mg/kg) and high (5 mg/kg) doses of CDDP. Dynamic CDDP response curves were generated by serial AFP and β-hCG determinations in the serum of control (saline-treated) and drug-treated mice in both types of responses and for 2 and 5 mg/kg doses (not shown) of CDDP. A complete short- and long-term response was obtained at high doses for both tumors. **c)** H&E analyses of relapsed masses in long-term response (2 mg/kg) of TGT21AX, showed the presence of a teratoma (TE), characterized by the absence of tumor serum markers.



Supplementary Fig. S3 Short- and long-term CDDP responses at low (2 mg/kg) and high (5 mg/kg) doses of CDDP were characterized in other xenografted tumors. **a)** TGT1X, a pure YS. Absence of lung micrometastases was revealed by H&E analyses in short-term responses at low (n=10) and high (n=10) CDDP doses. Nevertheless, micrometastases were identified upon sacrifice of the long-term response groups: 4/10 (40%) of lungs of mice treated with a 2 mg/kg dose and 2/10 (20%) of those receiving a 5 mg/kg dose of CDDP. **b)** Responses of TGT12X and TGT14X, two pure ECs, and TGT17X, a pure CH. **c)** TGT34X, a pure EC. Dynamic CDDP response curves were generated by serial determinations of AFP in the serum of control (saline-treated) and drug-treated mice. There was a complete long-term tumor response at 5 mg/kg of CDDP characterized by the absence of detectable serum markers over a follow-up period of 8 months. **d)** TG39X, a mixed tumor with EC and YS components. Dynamic curves of CDDP treatments show a sustained response, characterized by levels of AFP that were maintained for 90 days.

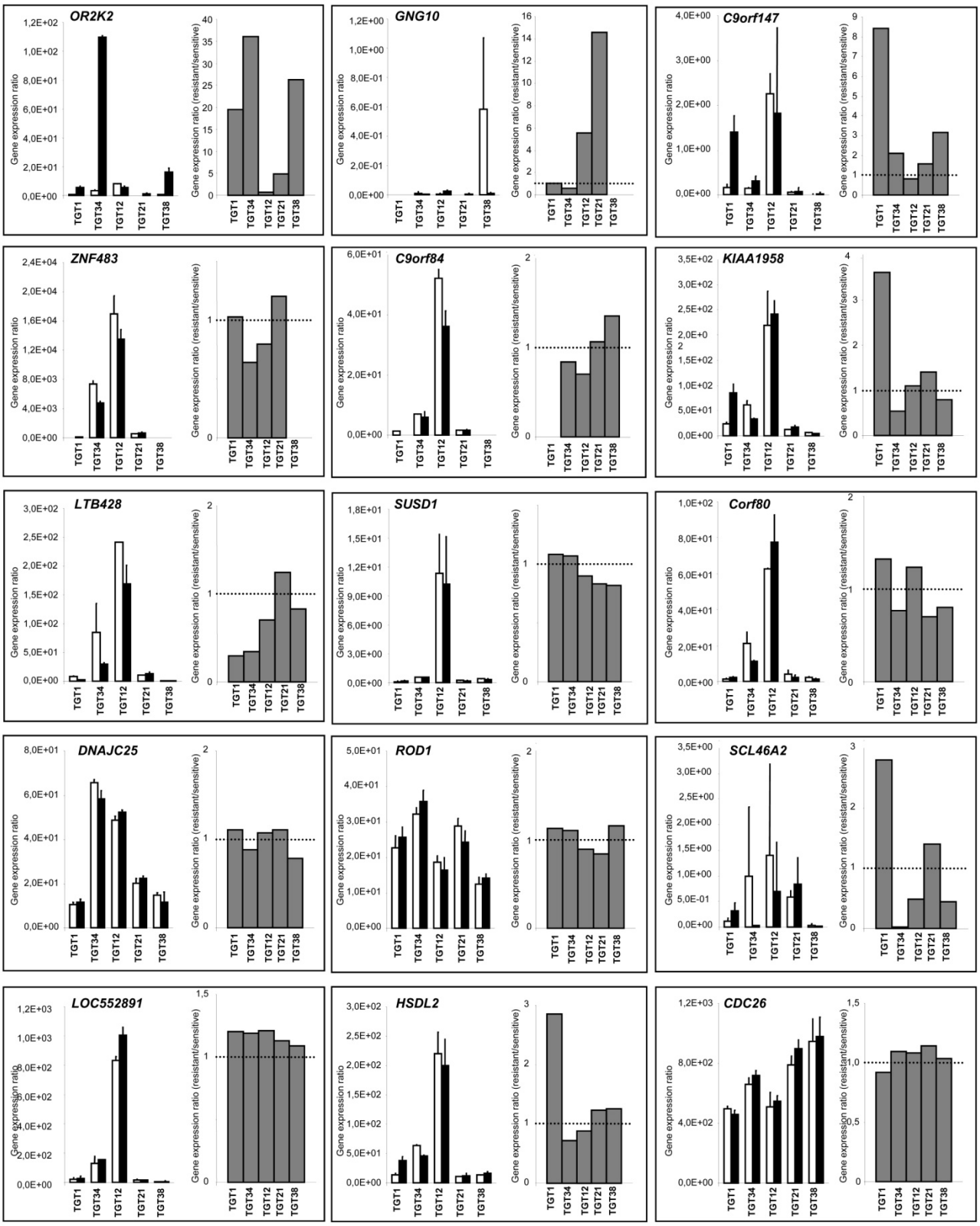


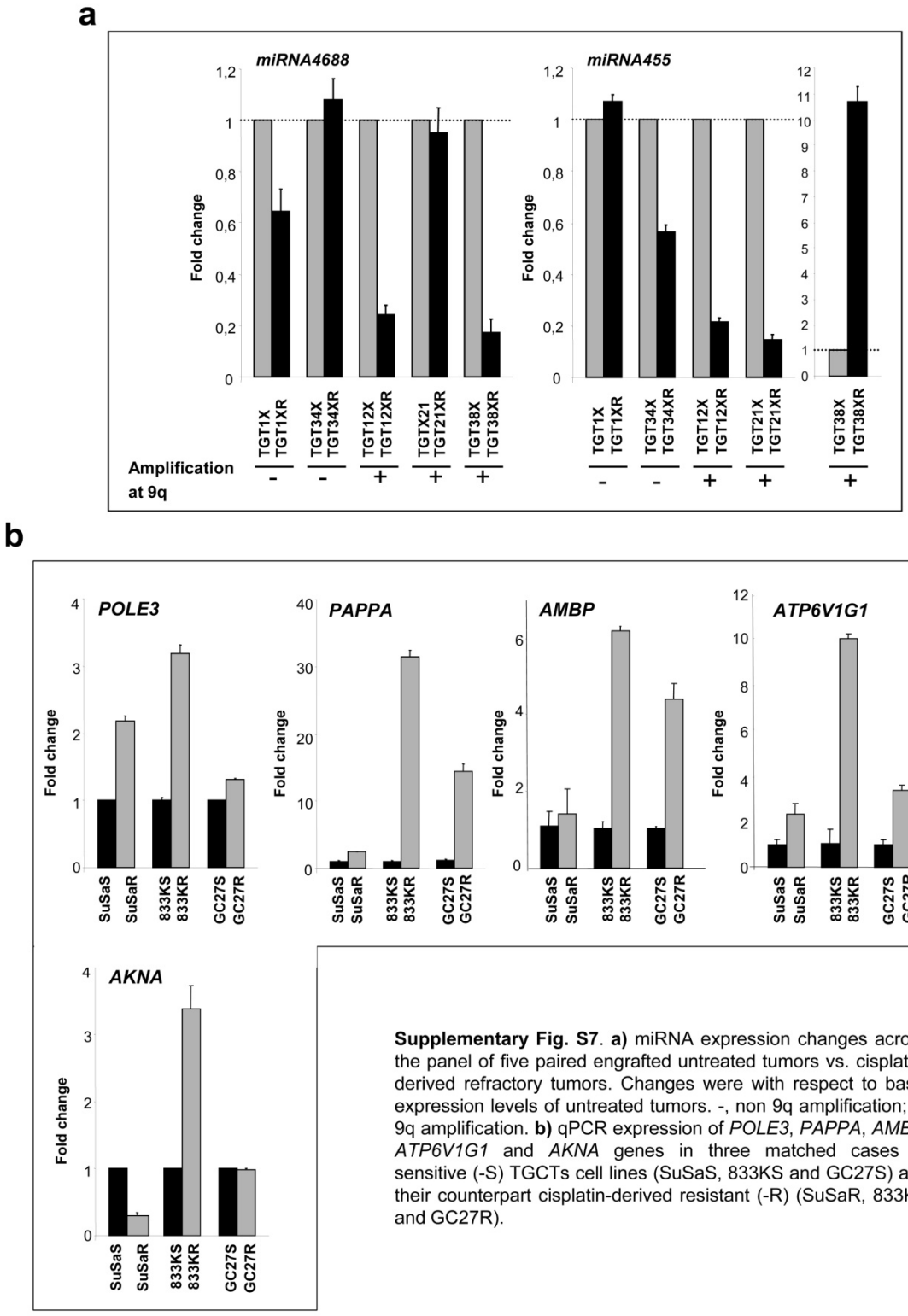
Supplementary Fig. S4 Comparative histological analysis of original non-treated orthoxenografts and their pairs with acquired resistance to CDDP after five cycles of treatment in mice. All tumors maintained the same histological appearance, and unlike untreated tumors, higher levels of fibrosis and necrosis were observed. **TGT21AX differentiated to a growing teratoma after the first cycle of CDDP treatment, and did not regrow after mouse reimplantation.



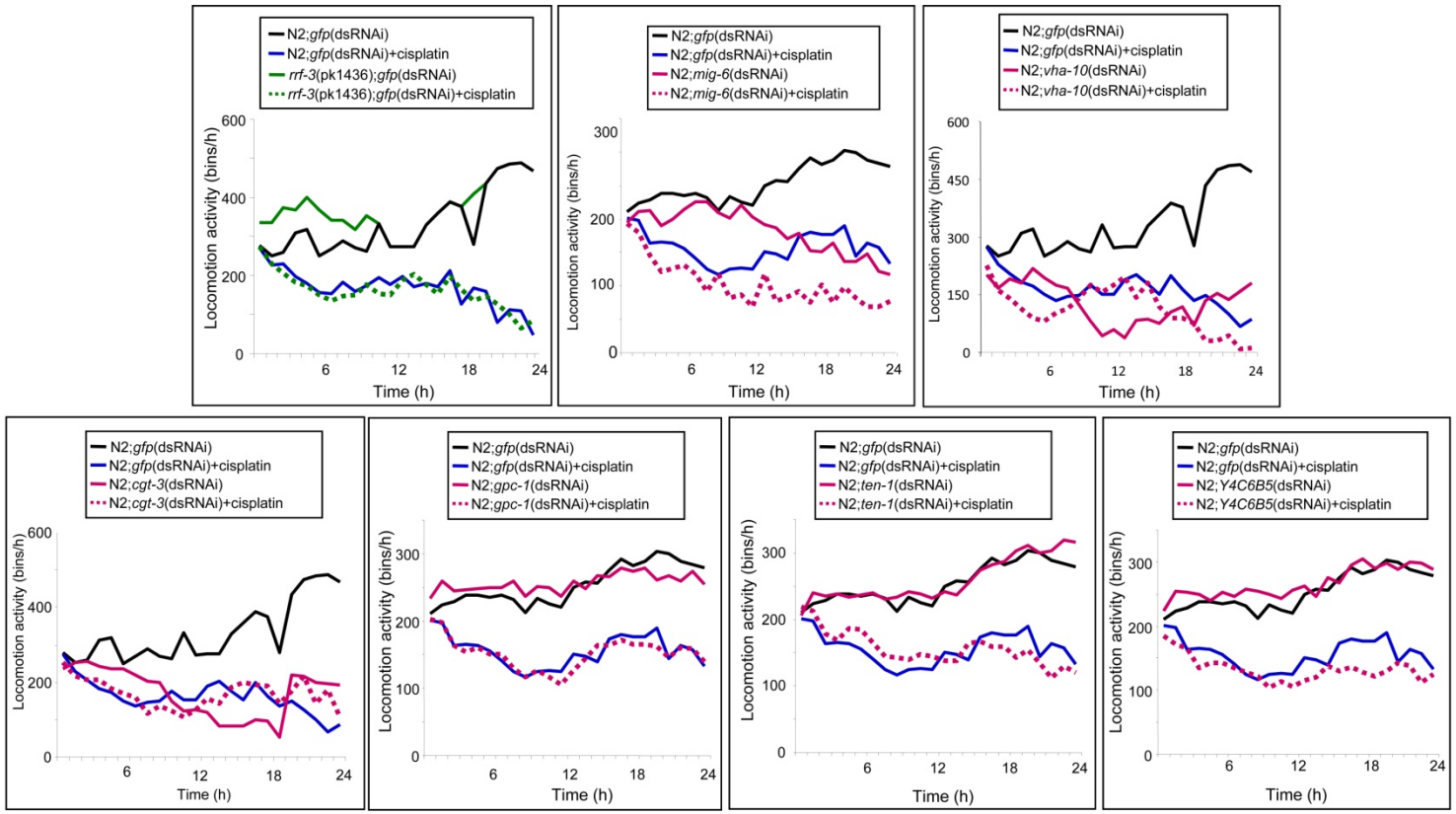
Supplementary Fig. S5 Five representative recurrent imbalanced regions in different xenografted tumors with acquired CDDP resistance. **a)** Gains at 15q23-q24.1 and 15q26.3 were identified in TGT12XR and TGT21BXR. **b)** Loss of the Xp22.33 region happens in TGT12XR, TGT21XR and TGT38XR. Whole-genome mapping was performed by oligonucleotide array CGH analysis (60 kbp window averaging) visually depicted with the SignalMap graphical interface tool from Nimblegen Systems. Arrows indicate regions of new gain/loss in resistant tumors.

Supplementary Figure 6





Supplementary Fig. S7. **a)** miRNA expression changes across the panel of five paired engrafted untreated tumors vs. cisplatin-derived refractory tumors. Changes were with respect to basal expression levels of untreated tumors. -, non 9q amplification; +, 9q amplification. **b)** qPCR expression of *POLE3*, *PAPPA*, *AMBP, ATP6V1G1* and *AKNA* genes in three matched cases of sensitive (-S) TGCTs cell lines (SuSaS, 833KS and GC27S) and their counterpart cisplatin-derived resistant (-R) (SuSaR, 833KR and GC27R).



Supplementary Fig. S8. Comparative cisplatin response assays. For each gene (*mig-6*, *vha-10*, *cgt-3*, *gpc-1*, *ten-1* and *Y4C6B5*) assays were performed in triplicate and all data were normalized with two control genes. All assays were performed in triplicate (20 worms/well, five replicates) treating worms at 500 µM of cisplatin.

Supplementary Table S1 Summary of characteristics of primary nonseminoma (NSE) tumors growing as xenografts in nude mice.

Human Primary Tumor					Orthoxenografts					
Primary tumor location	Tumor	Histology [a]	Stage [b]	CDDP-treated	Histology	Xenograft perpetuated [c]		Time-lag between passages (days) [d]	Mouse serum markers [e]	Pattern of distal dissemination
						Orthotopic growth	Subcutaneous growth			
Testicle	**TGT1X**	YS	Stage I	No	YS	Yes	No	69 ± 17	AFP	Lung [f, h1]
	TGT11X	YS, EC, CH, TE	Stage I	No	YS, EC, CH	No	No	ND	ND	ND
	TGT12X	EC	Good prognosis	No	EC	Yes	No	97 ± 25	β-hCG, AFP	None
	TGT14X	EC SE	Stage I	No	EC	Yes	No	56 ± 17	β-hCG	Peritoneal implants [g, h2] Lymph node affection [g, h4]
	TGT21AX	YS, EC, CH, TE SE	Stage I	No	YS, EC, CH	Yes	Yes	49 ± 11	β-hCG, AFP	None
	TGT21BX	YS, EC, CH, TE SE	Stage I	No	YS, EC, CH	Yes	Yes	64 ± 14	β-hCG, AFP	None
	TGT34X	EC	Bad prognosis	No	EC	Yes	No	51 ± 8	β-hCG, AFP	Lymph node affection [g,h4]
	TGT38X [J]	CH	Bad prognosis	No	CH	Yes	No	20 ± 6	β-hCG	Lung [f, h1]
	TGT40X	YS, TE	Stage I	No	YS	Yes	No	42 ± 8	AFP	None
Lymph node	**TGT39X**	YS, EC, TE	Bad prognosis	Yes	YS, EC	Yes	No	59 ± 13	β-hCG, AFP	Liver [g, h3] Peritoneal implants [g,h2]
	TGT41X	CH	Refractory	No	CH	Yes	No	18 ± 4	β-hCG	ND
	TGT44X [J]	YS, TE	Refractory	Yes	YS	Yes	No	50 ± 8	AFP	ND
Lung metastasis	**TGT17X**	CH	Refractory	Yes	CH	Yes	No	24 ± 5	β-hCG	Lung [f ,h1]
Brain metastasis	**TGT42X**	EC	Refractory	Yes	EC	Yes	No	62 ± 10	β-hCG, AFP	ND

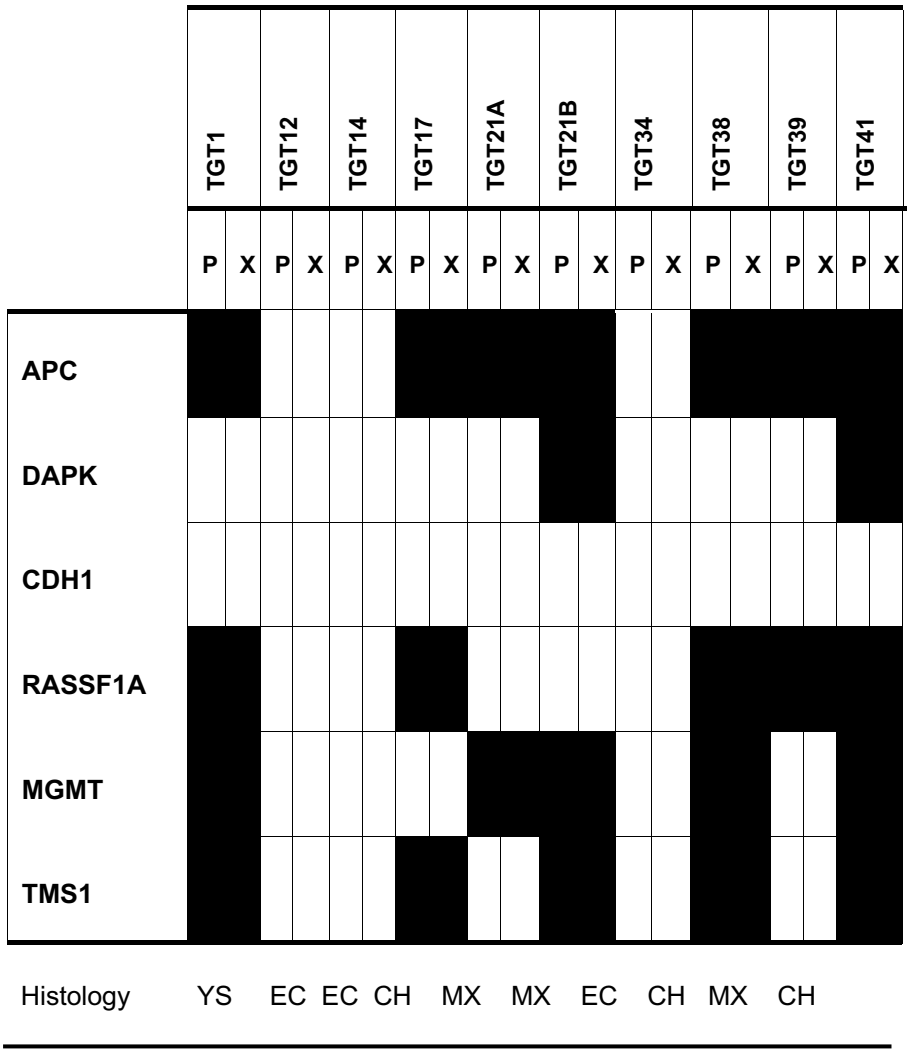
[a] Tumor histology: YS, yolk sac; EC, embryonal carcinoma; CH, choriocarcinoma; TE, teratoma; SE, seminoma.
[b] Stage at first diagnosis (Stage II to IV based on International Germ Cell Cancer Collaborative Group (IGCCCG)).
[c] Primary tumor was simultaneously implanted in the testicles and subcutaneous tissues of nude mice. The tumor was considered perpetuated after at least six consecutive passages in nude mice.
[d] Time-lag between passages was calculated on the basis of the first six passages, for a median of 15 mice implanted with each tumor.
[e] Levels of alpha-fetoprotein (AFP) and/or β-subunit of human chorionic gonadotropin (β-hCG) were analyzed as tumor growth markers in the nude mouse serum.
[f] Synchronous lung micrometastases were detected when nude mice were sacrificed.
[g] Liver macrometastases, peritoneal implants and lymph node affection were observed when nude mice were sacrificed.
[h] Orchiectomy was performed to confirm the dissemination patterns when palpable intra-abdominal masses were detected in 5 to 10 mice for each tumor. Animals were sacrificed 6-8 months after surgery, or when they lost weight: [h1], metachronic lung metastasis; [h2], metachronic peritoneal implants; [h3], metachronic liver metastases; [h4], metachronic lymph node.
[J] Xenografted tumors TGT38X and TGT44X characteristics were previously described [20,21].
None, absence of metastasis; ND, not determined.

Supplementary Table S2 Genetic analyses of paired primary tumors, xenografted NSE, and xenografted tumors with acquired resistance to CDDP.

												Tumors with acquired resistance [c]				
	TGT1X	**TGT11X**	**TGT12X**	**TGT14X**	**TGT17X**	**TGT21AX**	**TGT21BX**	**TGT34X**	**TGT38X**	**TGT39X**	**TGT41X**	**TGT1XR**	**TGT12XR**	**TGT21BXR**	**TGT34XR**	**TGT38XR**
Point mutations [a]																
K-ras	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
b-raf	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
EGFR	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
PI3KCA	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
TP53	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
p15	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
p16	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
Smad4	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
c-Kit	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
PDGFR-α	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
PDGFR-β	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
MSI [b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] We analyzed: codons 12 and 13 of *K-ras*; exons 11 and 15 of *b-raf;* exons 18, 19, 20 and 21 of *EGFR*; exons 9 and 20 of *PI3KCA;* exons 4 to 10 of *TP53*; exons 9, 11, 13 and 17 of *c-Kit* ; exons 12 and 14 of *PDGFRα*; exon 12 of *PDGFRβ;* and whole *p15, p16* and *Smad4*.
[b] Microsatellite instability (MSI) status was determined by the Bethesda panel including D2S123, BAT25, BAT26, D5S346 and BAT40 markers.
[c] Xenografted tumors with acquired CDDP resistance were analyzed at cycles #3 and #5 of chemotherapy treatment.
wt, presence of wild-type sequence; -, absence of microsatellite instability.

Supplementary Table S3 Epigenetic analyses of paired primary and xenografted NSE tumors.



Methylation status of 5' CpG promoter islands determined by MSP analysis (■ methylated; □ unmethylated). *APC*, *adenomatous polyposis coli, RASSF1A, TMS,* PYCARD (PYD and CARD domain-containing), *MGMT*, O-6-methylguanine-DNA methyltransferase, *DAPK, death-associated protein kinase and CDH1,* type 1, E-cadherin. P, primary human tumor; X, xenografted tumor at third and fifth mouse passages; YS, yolk sac; EC, embryonal carcinoma; CH, choriocarcinoma; MX, mixed tumor.

Supplementary Table S4

Sixty genes located at 9q32-q33.1, a region of 5.1 M bp. Build 36.3 from NCI.

112675875	112840186	*EDG2*	9q31.3	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
113129584	113130534	*OR2K2*	9q31.3	olfactory receptor, family 2, subfamily K, member 2
113162794	113286846	*KIAA0368*	9q31.3	KIAA0368
113327260	113379945	*ZNF483*	9q31.3	zinc finger protein 483
113365073	113401532	*LTB4DH*	9q31.3	leukotriene B4 12-hydroxydehydrogenase
113404932	113415654	*C9orf29*	9q31.3	chromosome 9 open reading frame 29
113433453	113456452	*bA16L21.2.1*	9q31.3	DNAJ-like protein
113433487	113472330	*LOC552891*	9q31.3	hypothetical protein LOC552891
113463682	113472347	*GNG10*	9q31.3	guanine nucleotide binding protein (G protein), gamma 10
113488722	113585600	*C9orf84*	9q31.3	chromosome 9 open reading frame 84
113699027	113735260	*GCS*	9q31	UDP-glucose ceramide glucosyltransferase
113842882	113977377	*SUSD1*	9q31.3-q33.1	sushi domain containing 1
113975623	113976072	*LOC100129332*	9q32	hypothetical LOC100129332
114020536	114135733	*ROD1*	9q32	ROD1 regulator of differentiation 1 (S. pombe)
114083089	114083509	*EPF5*	9q32	EPF5 pseudogene
114164716	114165217	*LOC644996*	9q32	similar to 60S ribosomal protein L32
114182172	114274505	*HSDL2*	9q32	hydroxysteroid dehydrogenase like 2
114286618	114294681	*LOC100133204*	9q32	similar to chromosome 9 open reading frame 147
114289069	114462527	*KIAA1958*	9q32	KIAA1958
114488607	114520208	*C9orf80*	9q32	chromosome 9 open reading frame 80
114552955	114677088	*SNX30*	9q32	sorting nexin family member 30
114681021	114692866	*SLC46A2*	9q32	solute carrier family 46, member 2
114761235	114764362	*LOC100129193*	9q32	similar to hCG1795014
114799221	114814293	*LOC169834*	9q32	hypothetical protein LOC169834
114843995	114858817	*ZFP37*	9q32	zinc finger protein 37 homolog (mouse)
114906824	114913864	*LOC100128385*	9q32	hypothetical protein LOC100128385
114914042	114921947	*C9orf109*	9q32	chromosome 9 open reading frame 109
114953059	114966243	*SLC31A2 (CTR2)*	9q31-q32	solute carrier family 31 (copper transporters), member 2
114967621	115023462	*FKBP15*	9q32	FK506 binding protein 15, 133kDa
115023689	115066593	*SLC31A1 (CTR1)*	9q31-q32	solute carrier family 31 (copper transporters), member 1
115069109	115077690	*CDC26*	9q32	cell division cycle 26 homolog (S. cerevisiae)
115077795	115094877	*PRPF4*	9q31-q33	PRP4 pre-mRNA processing factor 4 homolog (yeast)
115099194	115101141	*RNF183*	9q32	ring finger protein 183
115117751	115142388	*WDR31*	9q32	WD repeat domain 31
115151633	115173334	*BSPRY*	9q32	B-box and SPRY domain containing
115175519	115178162	*HDHD3*	9q32	haloacid dehalogenase-like hydrolase domain containing 3
115188413	115203391	*ALAD*	9q33.1	aminolevulinate, delta-, dehydratase
115209342	115212773	*POLE3 (CHRAC17)*	9q33	polymerase (DNA directed), epsilon 3 (p17 subunit)
115212843	115231703	*C9orf43*	9q32	chromosome 9 open reading frame 43
115246832	115399839	*RGS3*	9q32	regulator of G-protein signaling 3
115418339	115418830	*FLJ31713*	9q32	hypothetical protein FLJ31713
115652788	115672935	*LOC100132609*	9q32	hypothetical LOC100132609
115678383	115858696	*ZNF618*	9q32	zinc finger protein 618
115862228	115880536	*AMBP*	9q32-q33	alpha-1-microglobulin/bikunin precursor
115893739	115901158	*KIF12*	9q32	kinesin family member 12
115958052	116112796	*COL27A1*	9q32	collagen, type XXVII, alpha 1
116011534	116011629	*MIRN455*	9q32	microRNA 455
116125157	116128578	*ORM1*	9q31-q32	orosomucoid 1
116131890	116135357	*ORM2*	9q32	orosomucoid 2
116136250	116196506	*AKNA*	9q32	AT-hook transcription factor
116204181	116307551	*DFNB31*	9q32-q34	deafness, autosomal recessive 31
116293663	116293843	*LOC100131877*	9q32	hypothetical LOC100131877
116389815	116400973	*ATP6V1G1*	9q32	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G1
116413527	116448524	*C9orf91*	9q32	chromosome 9 open reading frame 91
116456401	116548297	*LOC100129633*	9q32	similar to hCG1651427
116591421	116608229	*TNFSF15*	9q32	tumor necrosis factor (ligand) superfamily, member 15
116647695	116650692	*LOC645266*	9q32	similar to PRP4 pre-mRNA processing factor 4 homolog B
116704945	116732591	*TNFSF8*	9q33	tumor necrosis factor (ligand) superfamily, member 8
116822626	116920307	*TNC*	9q33	tenascin C (hexabrachion)
116943918	117204744	*DEC1*	9q32	deleted in esophageal cancer 1
117955892	118204421	*PAPPA*	9q33.2	pregnancy-associated plasma protein A, pappalysin 1

Twenty-six genes located at 15q23-q24.1, a region of 1.7 M bp. Build 36.3 from NCI

69220842	69862776	THSD4	15q23	thrombospondin, type I, domain containing 4
69889948	69897654	NR2E3	15q22.32	nuclear receptor subfamily 2, group E, member 3
69898125	69907176	LOC100132473	15q23	hypothetical protein LOC100132473
69905405	70197476	MYO9A	15q22-q23	myosin IXA
70197808	70220358	SENP8	15q23	SUMO/sentrin specific peptidase family member 8
70239202	70277180	GRAMD2	15q23	GRAM domain containing 2
70278424	70310738	PKM2	15q22	pyruvate kinase, muscle
70320576	70350682	PARP6	15q23	poly (ADP-ribose) polymerase family, member 6
70364123	70399579	BRUNOL6	15q24	bruno-like 6, RNA binding protein (Drosophila)
70422832	70455457	HEXA	15q23-q24	hexosaminidase A (alpha polypeptide)
70455567	70456379	C15orf34	15q24.1	chromosome 15 open reading frame 34
70458553	70464260	LOC400389	15q24.1	hypothetical gene supported by AK026491; NM_000976
70477722	70487762	TMEM202	15q24.1	transmembrane protein 202
70530158	70530833	LOC100130579	15q24.1	hypothetical protein LOC100130579
70553721	70662877	ARIH1	15q24	ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosophila)
70666611	70666707	MIRN630	15q24.1	microRNA 630
70684573	70691719	LOC646665	15q24.1	golgi autoantigen, golgin subfamily a, 6 pseudogene
70687876	70716282	LOC100129119	15q24.1	hypothetical protein LOC100129119
70716473	70730171	LOC646670	15q24.1	similar to COMM domain containing 4
70734092	70746791	GOLGA	15q24.1	golgin-like protein
70755176	70765543	HIGD2BP	15q24.1	HIG1 domain family, member 2B pseudogene
70765588	70817869	BBS4	15q22.3-q23	Bardet-Biedl syndrome 4
70830763	70863114	ADPGK	15q24.1	ADP-dependent glucokinase
70978467	70979746	LOC729686	15q24.1	similar to nucleophosmin 1 isoform 1
71131928	71384599	NEO1	15q22.3-q23	neogenin homolog 1 (chicken)
71400988	71448230	HCN4	15q24-q25	hyperpolarization activated cyclic nucleotide-gated potassium channel 4

Six genes located at 15q26.3, a small region of 0.5 M bp. Build 36.3 from NCI

97956185	98071524	MEF2Ax	15q26	myocyte enhancer factor 2A
98070287	98074525	LOC100129079	15q26.3	hypothetical protein LOC100129079
98085133	98091149	LYSMD4	15q26.3	LysM, putative peptidoglycan-binding, domain containing 4
98116151	98151146	LOC644800	15q26.3	similar to Golgi autoantigen, golgin subfamily a, 2
98147884	98164655	C15orf51	15q26.3	chromosome 15 open reading frame 51
98164259	98165704	LOC400464	15q26.3	similar to FLJ43276 protein

Twenty five genes are located at Xp22.33, a region of 2.7 M bp. Build 36.3 from NCI.

110310	112812	CXYorf11	Xp22.33; Yp11.32	chromosome X and Y open reading frame 11
132991	160020	PLCXD1	Xp22.33; Yp11.32	phosphatidylinositol-specific phospholipase C, X domain containing 1
161426	170887	GTPBP6	Xp22.33; Yp11.32	GTP binding protein 6 (putative)
214970	267627	PPP2R3B	Xp22.33; Yp11.3	protein phosphatase 2 (formerly 2A), regulatory subunit B", beta
505079	540146	SHOX	Xpter-p22.32;Yp11.3	short stature homeobox
834110	840111	LOC100132775	Xp22.33	hypothetical LOC100132775
877093	889906	LOC100132256	Xp22.33	hypothetical LOC100132256
889945	890836	LOC442442	Xp22.33;Yp11.32	60S ribosomal protein L14-like
1274894	1291529	CRLF2	Xp22.3; Yp11.3	cytokine receptor-like factor 2
1305011	1306462	LOC100132270	Xp22.33	hypothetical LOC100132270
1347701	1388827	CSF2RA	Xp22.32 and Yp11.3	colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)
1415509	1461582	IL3RA	Xp22.3 or Yp11.3	interleukin 3 receptor, alpha (low affinity)
1465045	1470998	SLC25A6	Xp22.32 and Yp11.3	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
1472923	1473639	LOC729629	Xp22.33	hypothetical protein LOC729629
1480380	1492584	CXYorf2	Xp22.33; Yp11.3	chromosome X and Y open reading frame 2
1482032	1531844	ASMTL	Xp22.3; Yp11.3	acetylserotonin O-methyltransferase-like
1541465	1616000	P2RY8	Xp22.33; Yp11.3	purinergic receptor P2Y, G-protein coupled, 8
1670486	1681413	SFRS17A	Xp22.32; Ypter-p11.2	splicing factor, arginine/serine-rich 17A
1674348	1721974	ASMT	Xp22.3 or Yp11.3	acetylserotonin O-methyltransferase
2147547	2428975	DHRSX	Xp22.33; Yp11.2	dehydrogenase/reductase (SDR family) X-linked
2414455	2429008	ZBED1	Xp22.33;Yp11	zinc finger, BED-type containing 1

2521398	2544212	LOC100130595	Xp22.33	similar to hCG1653094
2546623	2566764	LOC401577	Xp22.33:Yp11.31	hypothetical protein LOC401577
2619228	2669350	CD99	Xp22.32; Yp11.3	CD99 molecule
2680115	2743968	XG	Xp22.33	Xg blood group

Supplementary Table S5 Tumors with amplification at 9q32-q33.1 in metastatic GCTs.

Patient	Histology [a]	Status of 9q32-q33.1 [b]	Cisplatin response
# 1	CE	High amplification	Resistant
#2	CE	High amplification	Sensitive
	CE	Low amplification	
	CE	NA	
#3	SE	NA	Resistant
	CE	High amplification	
#4	YS	High amplification	Resistant
	CE	High amplification	
	CH	High amplification	
	SE	High amplification	
#5	SE	High amplification	Sensitive
	CH	High amplification	
	TE	Low amplification	
#6	CH	Low amplification	Resistant
#7	CH	High amplification	Resistant
	CH	Low amplification	
#8	CE	Low amplification	Resistant
	YS	NA	
	TE	High amplification	
#9	SE	High amplification	Sensitive
#10	CE	High amplification	Sensitive
#11	YS	Low amplification	Resistant
#12	SE	Low amplification	
#13	TE	Low amplification	Resistant
#14	TE	Low amplification	Sensitive
#15	CE	High amplification	Sensitive
	CE	NA	
#16	CE	Low amplification	Sensitive
#17	CE	High amplification	Sensitive
	CE	NA	
	TE/CE	NA	
	CH	NA	
#18	CE	Low amplification	Resistant
	TE	Low amplification	

Supplementary Table S6 Patients classified with respect to 9q32-q33.1 amplification status.

	9q32-q33.1 status [e]				
	Non-amplification (N = 57)		Amplification (N = 18)		
	Number	%	Number	%	*P*
Age, years					0.25
Median	27.6		29.1		
Range	(15 - 56)		(16 - 53)		
Histology					0.52
Seminoma	10	17.5	2	11.1	
Nonseminoma	47	82.5	16	88.9	
Localization					0.96
Testis	54	94.7	17	94.4	
Mediastinum	3	5.3	1	5.6	
IGCCCG stage at diagnosis of metastasis [a]					0.26
Good	37	64.9	8	44.4	
Intermediate	10	17.5	4	22.2	
Bad	10	17.5	6	33.3	
First line of chemotherapy treatment [b]					0.83
EP	9	15.8	2	11.1	
BEP	35	61.4	10	55.6	
Taxol-BEP	2	3.5	1	5.6	
BOMP/EPI	11	19.3	5	27.8	
Response to first line of chemotherapy treatment [c]					0.007
Good response (CR, PR-)	54	94.7	13	72.2	
Poor response (PR+, SD, PD)	3	5.3	5	27.8	
Sensitivity to cisplatin [d]					0.060
Sensitive	42	73.7	9	50.0	
Resistant	15	26.3	9	50.0	

[a] IGCCCG International Germ Cell Cancer Collaborative Group.
[b] EP, etoposide/cisplatin; BEP, bleomycin/etoposide/cisplatin; BOMP/EPI, bleomycin/vincristine/methotrexate/ cisplatin-etoposide/cisplatin/ifosfamide
[c] CR, complete remission characterized by tumor mass reduction by CT scan and negative value of serum tumor marks; PR-, partial remission characterized by normalization of CT scan and negative value of serum tumor markers; PR+, partial remission characterized by reduction of tumor mass by CT scan and positive value of serum tumor markers; SD, stable disease; PD, progressive disease.
[d] Patients who achieved durable complete response with first-line cisplatin-based chemotherapy were considered sensitive. Patients who had either a poor response or relapsed after first-line chemotherapy were considered resistant to cisplatin.
[e] Amplification at 9q determined by FISH using two different probes (see *Material and Methods*).

Supplementary Table S7 Clinicopathological characteristics of patients, by response to cisplatin.

[a] IGCCCG International Germ Cell Cancer Collaborative Group.
[b] EP, etoposide/cisplatin; BEP, bleomycin/etoposide/cisplatin; BOMP/EPI, bleomycin/vincristine/methotrexate/ cisplatin-etoposide/cisplatin/ifosfamide.
[c] CR, complete remission characterized by tumor mass reduction by CT scan and negative valor of serum tumor marks; PR-, partial remission characterized by normalization of CT scan and negative valor of serum tumor markers; PR+, partial remission characterized by reduction of tumor mass by CT scan and positive valor of serum tumor markers; SD, stable disease; PD, progressive disease).
[d] Relapse >24 months after first diagnosis.

Characteristic	Sensitive (N = 51)		Resistant (N = 24)	
	No.	%	No.	%
Age, years				
Median	27.9		28.1	
Range	(16 – 56)		(15 - 53)	
Histology				
Seminoma	10	19.6	2	8.3
Nonseminoma	41	80.4	22	91.7
Localization				
Testis	51	100	20	83.3
Mediastinum	0	0	4	16.7
IGCCCG stage at diagnosis of metastasis [a]				
Good	38	74.5	7	29.2
Intermediate	7	13.7	7	29.2
Bad	6	11.8	10	41.7
First line of chemotherapy treatment [b]				
EP	9	17.6	2	8.3
BEP	33	64.7	12	50.0
Taxol-BEP	2	3.9	1	4.2
BOMP/EPI	7	13.7	9	37.5
Response to first line of chemotherapy treatment [c]				
Good response (CR, PR-)	51	100	16	66.7
Poor response (PR+, SD, PD)	0	0	8	33.3
Late relapse[d]				
Non	0		22	91.7
Yes	0		2	8.3

STUDY IV

"Loss of MGMT Promoter Methylation and Resistance to Cisplatin in Non-Seminoma Testicular Germ Cell Tumors"

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In Preparation

RESUMEN

Para explorar si los cambios de metilación en el promotor del gen *MGMT* tienen un papel en la resistencia al cisplatino, primero estudiamos el estado de metilación en células humanas de cáncer testicular de células germinales del tipo no seminoma, resistentes y sensibles al cisplatino. En segundo lugar estudiamos su estado de metilación en *orthoxenografts* emparejados y finalmente en tumores primarios humanos, de pacientes metastasicos tratados con quimioterapia basada en cisplatino. En general, se encontró que la hipermetilación del promotor de *MGMT* se relaciona con la sensibilidad al cisplatino. La resistencia está presente cuando el promotor *MGMT* está hipermetilado y consecuentemente el gen no es expresado. Clínicamente, la presencia de *MGMT* hipermetilado se relaciona con una mejor supervivencia global (p=0,025) en los pacientes con cáncer testicular de células germinales metastásico. La inhibición de la enzima MGMT con O^6-benzilguanina *in vitro* e *in vivo* aumenta la sensibilidad al cisplatino y a la temozolomida, siendo este un posible enfoque quimioterapéutico para resensibilizar tumores humanos resistentes del tipo no seminoma.

SUMMARY

To explore if *MGMT* promoter methylation changes have a role in cisplatin chemoresistance, first we study it methylation status in cisplatin sensitive and paired resistant human non-seminoma cancer cell lines. Secondly in xenograft paired tumors and after in human non-seminoma primary tumors, from metastasic patients treated with cisplatin-based chemotherapy. In general we found that cisplatin sensitive samples are related with *MGMT* promoter hypermethylation associated with its loss of expression. Resistance is present when *MGMT* promoter is not methylated and expressed. Clinically, the presence of *MGMT* promoter methylation is related with better overall survival (p=0.025) in metastasic patients with testicular germ cell cancer. Inhibition of MGMT with O^6-benzylguanine *in vitro* or *in vivo* increases the sensitivity to cisplatin and temozolomide, being this a possible chemotherapeutic approach to re-sensibilize human non-seminoma refractory tumors.

INTRODUCTION

Testicular cancer is the most commonly diagnosed malignancy among young men aged 15 to 40 years, and its incidence has doubled in the past 40 years. An annual increase of 3–6% is reported for Caucasian populations. Testicular germ cell tumors represent over 95% of the testicular cancers and histopathologically are classified into two major groups of seminomas or non-seminomas [1]. Patients with testicular germ cell tumors, even those with advanced metastatic disease, are often successfully treated with cisplatin-based chemotherapeutic regimens [2,3]. However, 15–20% of patients is refractory to this treatment and succumbs to progressive disease [4]. Some non-seminoma patients, who initially respond to treatment can exhibit a late relapse and have a poor prognosis [3, 4].

MGMT is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA [5, 6]. Alkylation of DNA at the O^6 position of guanine is an important step in the formation of mutations in cancer, primarily due to the tendency of the O^6-methylguanine to pair with thymine during replication, resulting in the conversion of G>C to A>T pairs in DNA [7]. Furthermore, the O^6-alkylguanine-DNA adduct may crosslink with the opposite cytosine residues, blocking DNA replication [8]. The MGMT protein rapidly reverses the formation of adducts at the O^6 position of guanine via transfer of the alkyl adduct to a cysteine residue within the protein, in a reaction that inactivates one MGMT molecule for each lesion repaired [5]. This averts the formation of lethal cross-links and other mutagenic effects. Loss of MGMT function is most frequently due to epigenetic changes, specifically gene promoter region methylation [9]. MGMT has important implications in cancer treatment since its expression correlates inversely with sensitivity to alkylating drugs, being MGMT activity a major mechanism of chemotherapy resistance [10].

Taking the previous into account, we aim to discover if changes in *MGMT* promoter methylation status can be responsible for cisplatin intrinsic or acquired resistance in testicular germ cell tumors, especially in non-seminomas.

MATERIAL and METHODS

Cell Lines and Drugs Treatment

Human non-seminoma cancer cell lines (SUSA-S and SUSA-R) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% (w/v) fetal bovine serum, 100U/penicillin and 100µg/L streptomycin (Invitrogen, Carlsbad, CA), at 37ºC in an atmosphere of 5% (v/v) dioxide carbon in air. Cell lines were

authenticated by short tandem repeat profiling. Cisplatin (50mg/L) was obtained from TEVA (North Wales, PA), temozolomide and O^6-benzylguanine were obtained from Sigma (St. Lois, MO). Temozolomide was diluted in dimethyl sulfoxide (20mg/ml) and O^6-benzylguanine diluted in methanol (80μM).

Xenograft Tumors and Drugs Treatment

All analysed non-seminoma xenograft tumors were generated in a previous work [11]. Two acquired and two intrinsic xenograft cisplatin resistant tumors were unfrozed in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin, at room temperature. Animals were housed in a sterile environment, cages and water were autoclaved and bedding and food was X-ray sterilized. Tumors were implanted in the testis of five-week old male nu/nu Swiss mice (Charles River, France) weighting 18-22 g. After anesthesia by isofluorane inhalation, a median laparatomy was performed and the testes were mobilized. Tumor pieces were anchored to the testis surface with prolene 7.0 sutures. After implantation, mice were inspected twice a week, and if no tumor growth was apparent, mice were sacrificed six months after implantation. Then for each tumor, mice were randomized into eight groups: control group, cisplatin, O^6-benzylganine, temozolomide, cisplatin+O^6-benzylguanine, cisplatin temozolomide, temozolomide+O^6-benzylguanine, cisplatin+O^6-benzylguanine+temozolomide. O^6-benzylguanine and cisplatin were intravenously administered (i.v.). Temozolomide was given by oral administration. The different drugs were given once a week for three consecutive weeks (days 0, 7and 14). O^6-benzylguanine was administrated always one hour before cisplatin and temozolomide. Animals were sacrificed seven days after the final dose (day 21).

All patients gave written consent to participate in the study. The Ethics Committee of the hospitals approved the study protocol, and the animal experimental design was approved by the IDIBELL animal facility committee.

Patients and Clinical Samples

We analyzed 72 testicular germ cell tumors from metastasic patients treated with cisplatin based therapy. Formalin-fixed paraffin-embedded tumors obtained by surgical resection came from ICO-Hospitalet hospital. Clinical features of the patients are showed in **Table I**. Signed informed consent was obtained from each patient, and the Clinical Research Ethical Committee from ICO-Hospitalet provided approval for the study. DNA extraction was performed using a commercial kit (Qiagen) following the manufacturer's instructions.

Table1. Clinicalpathological features of patients, by *MGMT* DNA promoter methylation status

			MGMT methylation status					
	N	%	Unmethylated (U)		Methylated (M)		OR (95% CI)	P****
			N	**%**	**N**	**%**		
Histology								0.413
Seminoma	15	20.8	5	27.8	10	18.5	1.00	
Non-seminoma	57	79.2	13	72.2	44	81.5	1.69	
Localisation								
Testis	72	100%	18	25%	54	75%		
IGCCCG[a] stage at diagnosis of the metastasis								0.923
Good	49	68.1	12	66.7	37	68.5	1.00	
Intermediate	13	18.1	3	16.7	10	18.5	1.08	
Bad	10	13.9	3	16.7	7	13	0.76	
First Line of Chemotherapy								0.430
Ep[b]	14	19.4	5	27.8	9	16.7	1.00	
BEP[c]	44	61.1	8	44.4	36	66.7	2.50	
Taxol-BEP	3	4.2	1	5.6	2	3.7	1.11	
BOMP/EPI[d]	11	15.3	4	22.2	7	13	0.97	
Response to First Line of Chemotherapy *								1.00
Good (CR[e], PR[f]-)	60	93.8	15	93.8	45	93.75	1.00	
Poor (PR[g]+, SD[h], PD[i])	4	6.25	1	6.25	3	6.25	1.00	
Sensitivity to Cisplatin**								
Sensitive	56	77.8	12	66.7	44	81.5	1.00	0.204
Resistant	16	22.2	6	33.3	10	18.5	0.45	
Late relapse***								0.368
Yes	1	6.7	0	0.0	1	10.0	1	
No	14	93.3	5	100.0	9	90.0	0	

[a]IGCCCG International Germ Cell Cancer Collaborative Group; [b]EP-etoposide/cisplatin; [c]BEP-bleomycin/etoposide/cisplatin; [d]BOMP/EPI- bleomycin/vincristine/methotrexate/ cisplatin-etoposide/cisplatin/ifosfamide; [e]CR, complete remission characterized by tumor mass reduction by CT scan and negative valor of serum tumor marks; [f]PR- partial remission characterized by normalization of CT scan and negative valor of serum tumor markers; [g]-PR+, partial remission characterized by reduction of tumor mass by CT scan and positive valor of serum tumor markers; [h]SD, stable disease;[i]PD, progressive disease.
*Information available for only 64 patients. **Patients who achieved durable complete response with first-line cisplatin-based chemotherapy. Patients who had either a poor response or relapsed after first-line chemotherapy; *** Relapse >24 months after first diagnosis; **** P-value was assessed according Chi-Square test; p<0.05 as statistical significant.

Genomic DNA Extraction and Bisulfite Conversion

Genomic DNA was extracted from human cancer cell lines using DNAsol method, according the manufactures protocol. For frozen and paraffin tissue samples, were used commercial DNA extraction kits, following manufacturer's instructions (QIAamp DNA Mini Kit and QIAamp DNA FFPE Tissue Kit). DNA was subjected to bisulfite using EZ DNA methylation kit (Zymo Research, Orange, CA) as described previously [12]. Briefly 1ug of genomic DNA was denaturated by incubating with 0.2 M NaOH. Aliquots of 10 mM hydroquinone and 3M sodium bisulfate (pH 5.0) were added, and the solution was incubated at 50ºC for 16h. Treated DNA was purified on a Zymo-Spin I column, desulfonated with 0.3 M NaOH, repurified on a Zymo-Spin column and resuspended in 25 µl water. Following bisulfate treatment, all DNA were stored at -20ºC.

Genomic DNA Bisulfite Sequencing and MSP

The DNA methylation status of *MGMT* promoter was analyzed by bisulfite sequencing and by MSP. Both were performed in a 25µl volume containing 1µl of the sodium bisulfite modified DNA. For bisulfite sequencing both strands were sequenced and at least 20 clones were analyzed per sequence. The characteristics of the MSP reactions and the primer sequence have been described previously [10]. SW620 human cancer cell line DNA was used as a positive control for methylated alleles of *MGMT*, and DNA from normal lymphocytes used as a negative control, as previously described [13].

Cell viability and Proliferation Assays

Cell viability and proliferation was determined by the MTT assay. Briefly, 1 x 10^3 cells were plated onto 96-well plates. At different time points (24, 48, 72, 96 and 120 hours), MTT (5mg/ml in phosphate-buffered saline-PBS) was added at 0.1% final concentration. After 3 hours incubation (37ºC, 5% dioxide carbon), the MTT metabolic product, formazan, was dissolved in dimethyl sulfoxide and absorbance at 570 nm was measured. Prism Software was used to calculate drugs IC_{50}. Cell viability and proliferation assays were made for 120 hours. All the other treatments were made for 48 hours.

Cell Cycle Analysis

After 48 hours of the respective treatment, floating and attached cells were collected, and washed with phosphate-buffered saline solution. The cell cycle was assessed with propidium iodide-stained cells (distribution of cells in G0/G1, S and G2/M phases) by flow cytometry. Approximately 2x10^6 cells were fixed in ice-cold 70% ethanol overnight

at -20ºC. Cells were subsequently washed and resuspended in phosphate-buffered saline solution. After 30 minutes, DNA was stained with 25 mg/mL propidium iodide (Sigma, St Louis, MO) in a reaction solution containing 50 mg/mL RNAse A (Sigma, St Louis, MO) for 30 minutes at 37ºC in the dark. Fluorescence emitted from the propidium iodide–DNA was measured for individual cells using a FACS flow cytometer (FACSCalibur; BD Bioscience).

Apoptosis and Necrosis Determination

First we perform a western blot for PARP protein (explained in the next point), we determine caspase-3 and -7 activities using the Caspase-Glo® 3/7 Assay (Promega, Madison, WI) and we determine the possibility of chromatin cleavage using DNA ladder kit as manufacturer's instructions. For determine necrosis we used the Apoptotic/Necrotic/Healthy Cells Detection Kit (Pomokine, Heidelberg, Germany). Cells were grew directly on a coverslip, washed twice with 1X Binding Buffer and stained with a mix of 3 fluorocromes (FITC-Annexin V+ Ethidium Homodimer III+ Hoechst 33342) over 15 minutes. Cells were then fixed with 2% formaldehyde, washed 2 times and mounted each coverslip onto a slide with mowiol (Sigma, St Louis, MO). Finally we observed cells under a fluorescence microscope (DMI6000, acquisition software LEICA application suite advanced fluorescence (LAS AF). Data were analyzed using FlowJo software.

Western Blot

A standard protocol was used to extract cells total protein. Anti-MGMT (1/1000) was acquired from Cell Signaling (Boston, MA), Anti-PARP (1/2000) from BD Pharmigen (San Diego,CA) and H2AX.P (1/1000) from Abcam (Cambridge, UK). An anti-β-actin-HRP antibody (1/20000) was purchased from Sigma (Sigma, St Louis, MO).

Immunoflurescence

Cells were cultured directly on coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline solution for 20 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline solution for 5 minutes and blocked with 1% bovine serum albumin for 1 hour. Double immunostaining with primary antibodies for α tubulin (1/1000, Abcam; Cambridge, UK) and pericentrin (1/1000, Abcam; Cambridge, UK) were performed by simultaneous incubation for 1 hour. We also used H2AX.P in an independent experiment (1/1000; Abcam; Cambridge, UK). Finally, 1/1000 dilutions of appropriate fluorescent-labeled secondary antibodies from Invitrogen (Carlsbad, CA) (anti-rabbit IgG, A11011; anti-mouse IgG,

A21235) were used. The coverslips were mounted on glass slides using mowiol (Sigma) with DAPI. Multi-color immunofluorescence imaging was then performed on a (DMI6000, acquisition software LEICA application suite advanced fluorescence (LAS AF; Leica Microsystems, Germany).Data were analysed by Fiji program. For determine the amount of multipolar mitotic spindles, 100 mitotic spindles were counted for each condition. The counting was repeated 3 independent times.

Statistical Analysis

Survival curves were estimated using the Kaplan-Meier method, and differences between individual curves were evaluated by multivariate Cox proportional hazards regression modeling. Hazard Ratio and 95% confidence intervals were calculated. Values of $P<0.05$ were considered significant.

RESULTS

MGMT Epigenetic Silencing and Cisplatin Sensitivity

In order to analyze if changes in *MGMT* promoter methylation status can modulate cisplatin sensitivity in non-seminoma testicular germ cell tumors, we studied one pair of human cancer cells lines composed by a sensitive (SUSA-S; IC_{50}=75.3 ng/mL) and a resistant derivate (SUSA-R; IC_{50}=596.2 ng/mL) to cisplatin. Bisulfite sequencing methylation profiles revealed a loss of CpG methylation in the resistant cell line, in comparison with sensitive counterpart (28% vs. 3%) (**Fig. 1A**).This fact was associated with a significant increase in MGMT protein level in the resistant cell line (**Fig. 1B**).

Inhibition of MGMT Changes Sensitivity to Cisplatin and to Temozolomide

Considering the increased level of MGMT in resistant cells, we decided to evaluate the effect of its inactivation in the modulation of cisplatin sensitivity. Inhibition of MGMT upon O^6-benzylguanine treatment lead to a decrease in cisplatin IC_{50} in SUSA cancer cells (2 fold), becoming them more sensitive when compared with the vehicle treatment **(Table II and Fig. 2A)**. Based on previously publications, where cells lacking MGMT were sensitive to temozolomide, we add this chemotherapeutic drug to our cells treatments. Furthermore, addition of O^6-benzylguanine in cell culture significantly sensitizes cells to cisplatin and/or temozolomide drugs (**Fig. 2B**).

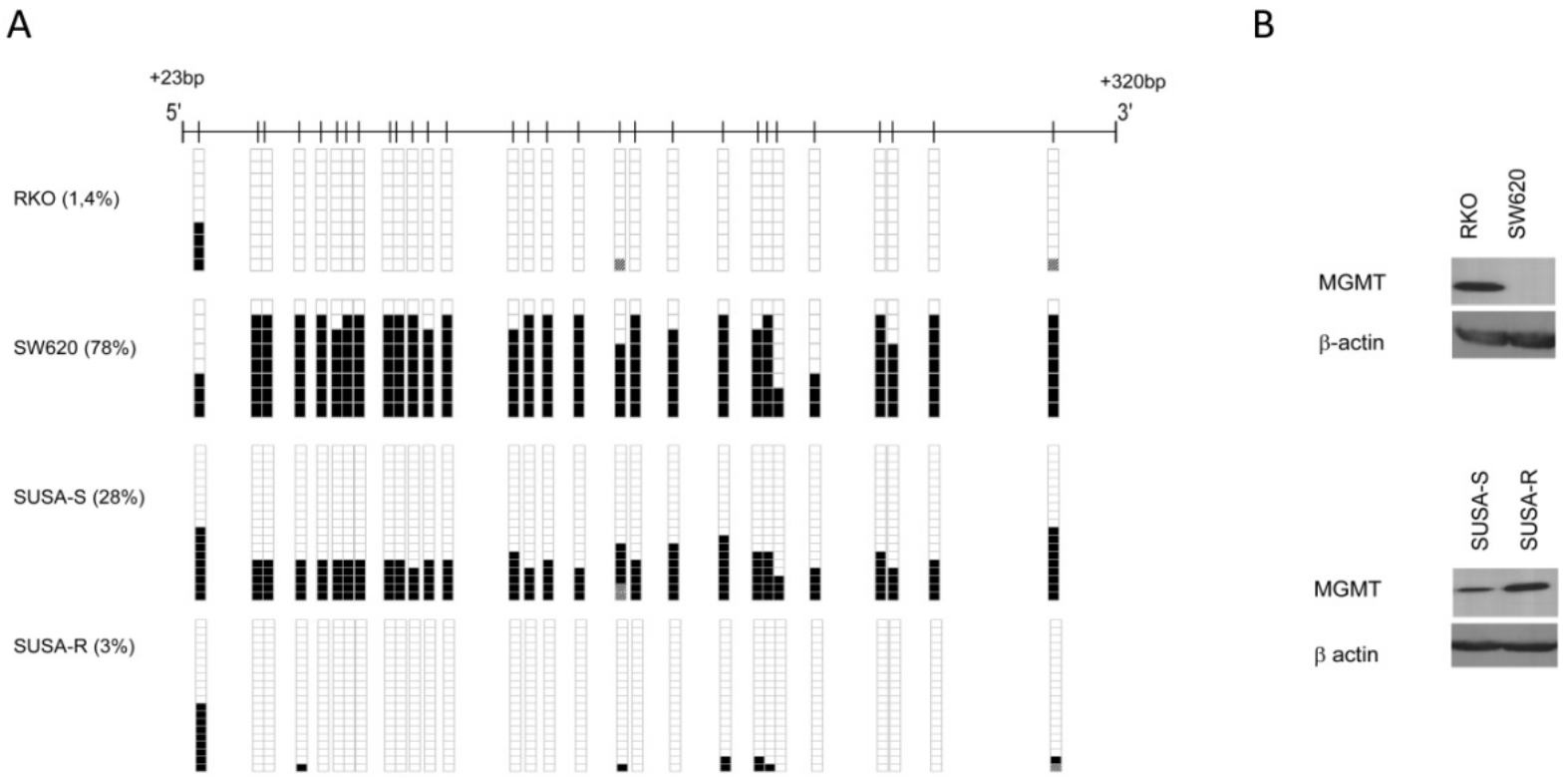

Figure 1 : *MGMT* promoter methylation and expression in SUSA cell lines: **A. *MGMT* promoter bisulfite DNA genomic sequencing.** SUSA-R present a loss of methylation when compared with SUSA-S. RKO and SW620 human colorectal cancer cell lines were used as negative and positive control for *MGMT* promoter methylation, as previously described. Vertical bars represent the different CpGs analysed and the horizontal ones, the different clones. Black squares represent CGs methylated and white ones GCs unmethylated. **B. MGMT protein expression.** SUSA-R cells express more MGMT than the sensitive counterpart. β-actin was used as endogenos control.

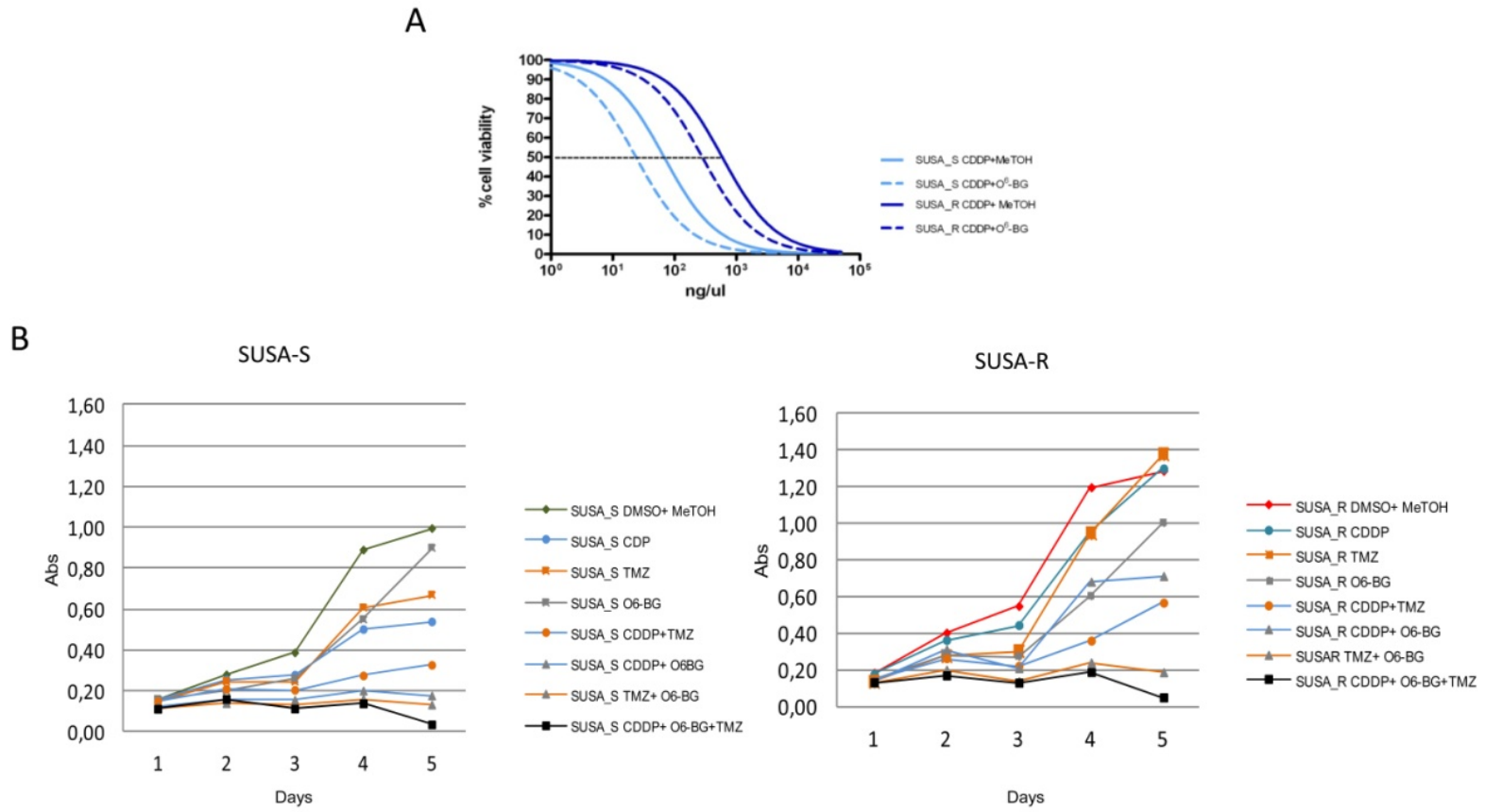

Figure 2: Effects of MGMT inhibition in SUSA cells viability and proliferation. A: Cell lines IC50 plots for CDDP+MeTOH and for CDDP+ O^6 –BG treatments. Cell lines treated with CDDP+ O^6-BG present a significant IC50 decrease when compared with the ones treated with the vehicle (MeTOH). **B: Proliferation plots for SUSA different drug treatments.** MGMT inhibition by O^6-BG treatment sensibilizes cells to CDDP and TMZ treatments. CDDP: Cisplaitin; O^6-BG: O6-benzylganine; TMZ: Temozolomide; MeTOH- metanol; Abs-absorvance at 560 nm.

Table II: IC_{50} for SUSA cell lines

	Cell Lines IC50	
Drugs	SUSA_S	SUSA_R
CDDP (ng/ml)	75,3	598,2
TMZ (µg/ml)	55.9	204.1
CDDP (ng/ml)+ MeTOH	68,3	595,7
CDDP(ng/ml)+ O^6-BG (µM)	23,4	282,7

MGMT Inhibition and Co-treatment with Cisplatin and/or Temozolomide Leads to Cell Death, Necrosis and Mitotic Cell Arrest

In order to elucidate the causes of the decrease in proliferation rates, we performed flow cytometry analyses in SUSA treated cells, to detect possible alterations in cell cycle. A significant increase in the sub-diploid population was detected upon temozolomide+O^6-benzylguanine (5% *vs*. 52% and 30%) and cisplatin+O^6-benzylganine+temozolomide (5% *vs*. 44% and 49%) treatments (**Fig. 3A**). To evaluate the possible mechanism responsible for this cellular death, we analyzed apoptotic markers including PARP cleavage, caspase 3/7 activity, DNA degradation and Anexin V; however, none of them revealed the presence of a apoptotic process (**Supp. Fig. 1A, 1B, 1C and 2**). Therefore, we proceeded to study necrosis. We performed a double cellular immunostaining using Anexin V and ethidium homodimer III. Co-treatment of cisplatin with O^6-benzylguanine increased the percentage of necrotic cells, more evident with the addition of temozolomide (**Supp. Fig. 2**).

In addition to the increase of the sub-diploid peak, we also observed an arrest in G2-M in all cellular treatments, with exception for cells treated only with O^6-benzylguanine (**Fig. 3 B**). Considering that a mechanism that leads to G2-M cell cycle arrest is the mitotic catastrophe, we decided to analyze this phenomenon by immunocytochemistry. A significant increase in prometaphase and metaphase upon cisplatin or/and temozolomide treatment was observed. Moreover, these cells presented a high amount of mitotic multipolar spindles (**Fig. 3C and 3D**). The presence of double strand breaks was also evaluated and the highest values were found in the triple treatment cisplatin+O^6-benzylguanine+temozolomide (**Sup. Fig. 3A and 3B**).

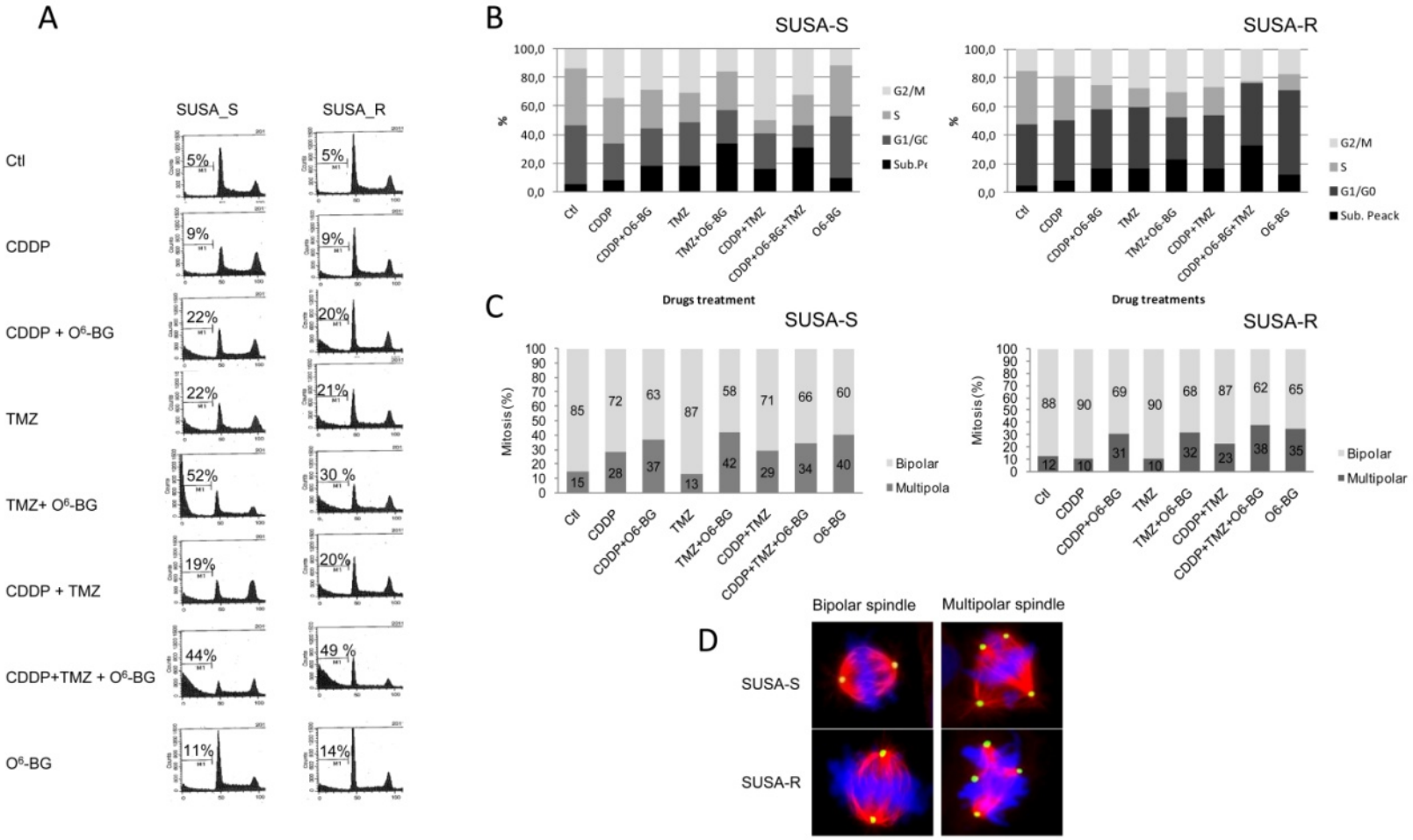

Figure 3: Effects of different drugs treatment in SUSA cell cycle. A. Cell cycle for treated (48h) and untreated cells. Addition of MGMT inhibitor O^6-BG increase cell dead in CDDP and/or TMZ treated cells. **B. Percentage of cells gated in the different cell cycle phases.** Treatments that include CDDP and/ or TMZ lead cells to G2/M cell cycle arrest. **C. Percentage of different mitotic spindles.** CDDP and/or TMZ cell treatments leads to appearance of mitotic mutipolar spindles. **D. Examples of bipolar and multipolar cells mitotic spindles.** Microtubules in red (α tubulin), green for centrosomes (pericentrin) and blue for DNA (DAPI). CDDP-cisplatin; O^6-BG- O^6 benzylmethylaguanine; TMZ-temozolomide; Sub. Peak- subdiploid peak.

Changes of *MGMT* Promoter Methylation Status in Generated Cisplatin Resistant Non-seminoma Xenograft Tumors

In view of the previous *in vitro* results we evaluated the role of the epigenetic regulation of *MGMT* in cisplatin resistant non-seminoma orthoxenograft tumors. Analogous to the profiles observed in the cell lines, we detected a loss of methylation in 4 out of 6 of the cisplatin resistant generated non-seminoma xenografts, when compared with the original tumors (**Fig.4A and Table III**). We also studied a set of human intrinsic non-seminoma cisplatin resistant tumors, where *MGMT* promoter was mainly unmethylated (4 out of 6) (**Fig.4A and Table III).**The loss of CpG methylation in resistant tumors resulted in the re-expression of MGMT protein (**Fig. 4B**). Because previously it was described an association between *MGMT* and *MLH1* promoters hypermethylation in temozolomide resistant cases [14], we also studied this second DNA repair gene in our cases. We only detected 2 out of 6 methylated tumors in the human non-seminoma cisplatin intrinsic resistant tumors, those tumors that were also methylated for *MGMT* (**Supp.Table I**).

Table III: Methylation status of *MGMT* promoters

		Sensitivity to cisplatin	*MGMT* methylation status
Cispaltin generated refractory xenograft tumors	*P1 **	*Sensitive*	***methylated***
		Resistant	*unmethylated*
	P2	*Sensitive*	*unmethylated*
		Resistant	*unmethylated*
	P3	*A -Sensitive*	***methylated***
		B-Sensitive	***methylated***
		B-Resistant	*unmethylated*
	P4	*Sensitive*	*unmethylated*
		Resistant	*unmethylated*
	*P5**	*Sensitive*	***methylated***
		Resistant	*unmethylated*
	P6	*Sensitive*	***methylated***
		Resistant	*unmethylated*
Primary human endogenously cisplatin resistant tumors	*R1**	***Resistant***	***methylated***
	R2	***Resistant***	*unmethylated*
	R3	***Resistant***	*unmethylated*
	*R4**	***A- Resistant***	***methylated***
		B- Resistant	***methylated***
	R5	***Resistant***	*unmethylated*
	R6	***Resistant***	*unmethylated*

MGMT Promoter Methylation Status as a Prognostic Biomarker in Human Testicular Germ Cell Tumors Patients

We analyzed *MGMT* promoter methylation in 72 testicular germ cell tumor patients and we observed a positive correlation between *MGMT* promoter hypermethylation and high overall survival (p=0.025) (**Fig. 4D**). These results suggest *MGMT* promoter methylation status as a prognosis marker in this type of tumors.

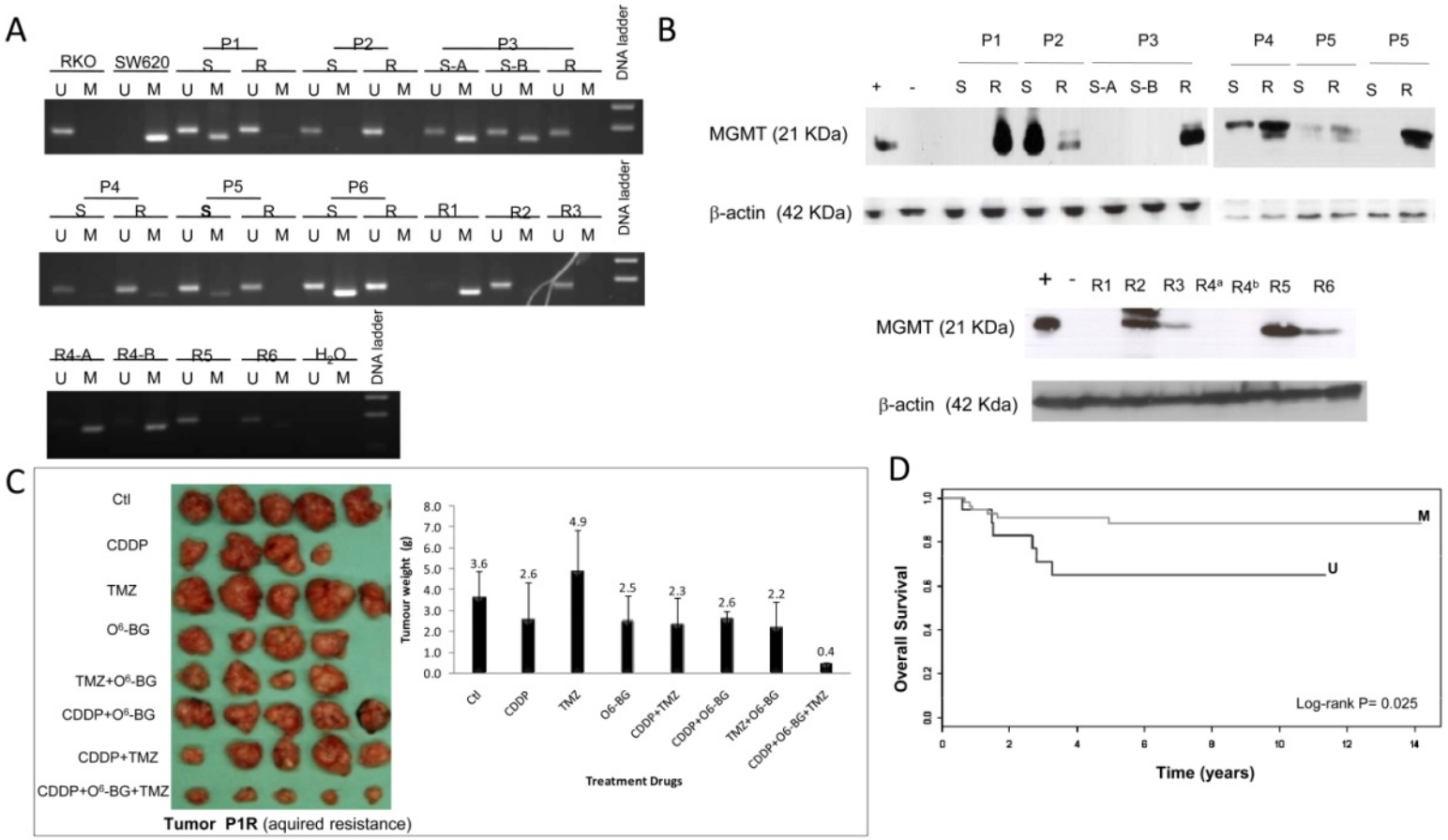

Figure 4: **MGMT study in TGCT. A. Methyl Specific PCR for *MGMT* promoter.** P1-P6 CDDP generated refractory TGCT xenograph; R1-R-6 Primary human intrinsic CDDP refractory TGCT. P1, P5 and P6 present a change in *MGMT* methylation between sensitive and CDDP refratory tumors. Primary TGCT tumors R1 and R4 present *MGMT* methylation. **B. MGMT protein expression.** *MGMT* unmethylated tumors present higher protein expression. **C: Xenograph tumours upon drugs treatment.** P1 R xenograph tumours, that respond to CDDP+ O^6-BG+TMZ treatment, decreasing 9 times the size when compared with the tumor controls.**D: Overall Survival.** Patients that present *MGMT* methylated tumours, present high survival rate. U for umethylated and M for methylated. SW480 was the human cancer cell line used as negative control for methylation and SW620 the human cancer cell line used as positive one. H_2O is the experiment negative control.

New Chemotherapy Proposal for Human Non-seminoma Refractory Cisplatin Tumors

Once confirmed the clinical relevance of *MGMT* DNA promoter methylation in sensitivity to cisplatin agent, we proceeded to test the use of O^6-bezylguanine to inhibit MGMT expression in 3 cisplatin refractory non-seminoma xenograft models: 2 generated cisplatin resistant xenógrafts and 1 non-seminoma cisplatin intrinsically resistant. The inhibition of MGMT was confirmed by western blot (**Supp. Fig. 5A**).The triple combination cisplatin+O^6-benzylguanine+temozolomide resulted in a high decrease of tumor growth (**Fig. 4C and Supp. Fig. 5B**). Additionally, the tumor with *MGMT* hypermethylation (tumor R4) showed a high sensitivity also to temozolomide alone **(Supp. Fig. 5B)**. However, the tumors treated only with temozolomide re-growth faster than those tripled treated (cisplatin+O^6-benzylguanine+temozolomide).

DISCUSSION

Chemoresistance to conventional chemical drugs is a well recognized issue that hampers many of the clinical expectations to improve the survival of oncology patients. Intrinsic and/or acquired resistance appears due to cellular mechanisms that permit cells to escape the chemical antitumoral effects. This involves a wide “repertoire” of genetic and epigenetic events. What concerns to the epigenetic events, little is known about its influence in non-seminoma tumors resistance to cisplatin. In testicular germ cell tumors MGMT was described to be frequently inactivated by promoter hypermethylation [15-17], especially in the cisplatin sensitive ones [18]. In non-seminoma tumors we found the same that in the previous study. *MGMT* promoter methylation was present in sensitive tumors.

MGMT is a tumor suppressor gene that encodes O^6-methylguanine-DNA methyltransferase and plays an important role in DNA repair, removing DNA adducts formed by alkylating agents. So, cells that express this protein present a defense system against alkylating agents, being more difficult to kill them, in comparison with MGMT knock-out cells. This was initially described in human gliomas in relation to temozolomide. Although in this type of tumor there is an exception. If besides *MGMT* methylation, *MLH1* methylation is also present, the patients are resistant to temozolomide [14]. We also found the same in two non-seminoma tumors.

A possible way of revert resistance to temozolomide is the use of a MGMT inhibitor that competes for the same target (O^6-methyl adducts). *O*^{6}-benzylganine is a potent, specific and irreversible blocker of MGMT that enhances the cytotoxic action of

alkylating agents (e.g. cisplatin, temozolomide) [19]. However the clinical trials are not very clear in the benefit for the use of this compound [20, 21], with our study we afford scientific data that indicate the possibility O6-benzylguanine be applied as a treatment option to revert non-seminoma resistant cisplatin tumors. Besides, the combined used of temozolomide can also increase the cure rate in non-seminoma refractory tumors.

In preclinical studies temozolomide has demonstrated antitumor activity against human non-seminoma cell lines [22], being one reason for testing this in the clinics. However three clinical phase II studies were already performed without positive outcome [23-26]. Based on our results we think that the major limitation to the successful treatment of these patients was the absence of information about *MGMT* promoter methylation, as a major issue to include patients in those studies. Therefore, a further rationale for testing this new agent again is first study of *MGMT* promoter methylation and then decide if the chemotherapy schedule should include an MGMT inhibitor, like O^6-benzylganine drug. Other reason for testing temozolomide and when needed a MGMT inhibitor, is that these chemical agents have clinical activity against brain tumors [27]. Brain metastases occur in 8-15% of patients with testicular tumors, almost always associated with relapse at other sites or as a terminal event [23, 28]. Temozolomide may provide in this way a more effective treatment for testis tumors which have metastasized to the brain.

Dolan et al, described that cisplatin, temozolomide and O^6-benzylguanine as chemotherapeutic drug, cause cellular dead [19]. Although they do not refer which dead mechanism is behind that. Our results indicate that the use of these three agents lead to mitosis catastrophe and necrosis. Mitotic catastrophe occurs when cells cannot carry on mitosis. One of many problems that can lead to this process is the accumulation of DNA damage. We believe that inhibiting MGMT action in these cells, make them more sensitive to these alkylating agents since cells are not able of repair their DNA efficiently. If the repair mechanism fails, O^6-methylguanine results in the formation of toxic double strand breaks, due to faulty MMR during proliferation, fact that we also found. About the other dead mechanism, necrosis normally results from a severe cellular insult. It was described that happens as a consequence of extreme stress, such as heat, osmotic shock, and mechanical stress, freeze thawing and high concentration of hydrogen peroxide. In these conditions, cell death occurs quickly due to the direct effect of the stress on the cell, and therefore this cell death process has been described as accidental and uncontrolled [29]. We think that this happens in non-seminoma cell lines, because treating them with DNA damage agents and at the same

time block a pathway of DNA repair should be a shock for the cells, not having them time to enter in a controlled cell death program, apoptosis.

Despite the high cure rate obtained with combination chemotherapy of testicular germ cell tumors, the management of patients with an adverse prognosis at presentation, or of those who fail to respond to first-line chemotherapy, remains a therapeutic challenge [28]. *MGMT* methylation is already used as a predictive temozolomide response biomarker in glioblastomas [10]. It is worth to mention the possibility to extend this to non-seminoma patients. If methylated a possible chemotherapeutic schedule could be O^6-benzylguanine plus cisplatin and temozolomide. However further studies should be performed.

In conclusion, we have demonstrated that the loss of *MGMT* promoter DNA methylation is associated with the acquisition of chemoresistance to the DNA damaging agent cisplatin in non-seminoma tumours both *in vitro* and *in vivo*. The validation of *MGMT* methylation as a predictive marker will require further prospective studies. If successful, clinical trials would also be necessary to develop strategies to overcome or prevent the development of MGMT-mediated epigenetic resistance.

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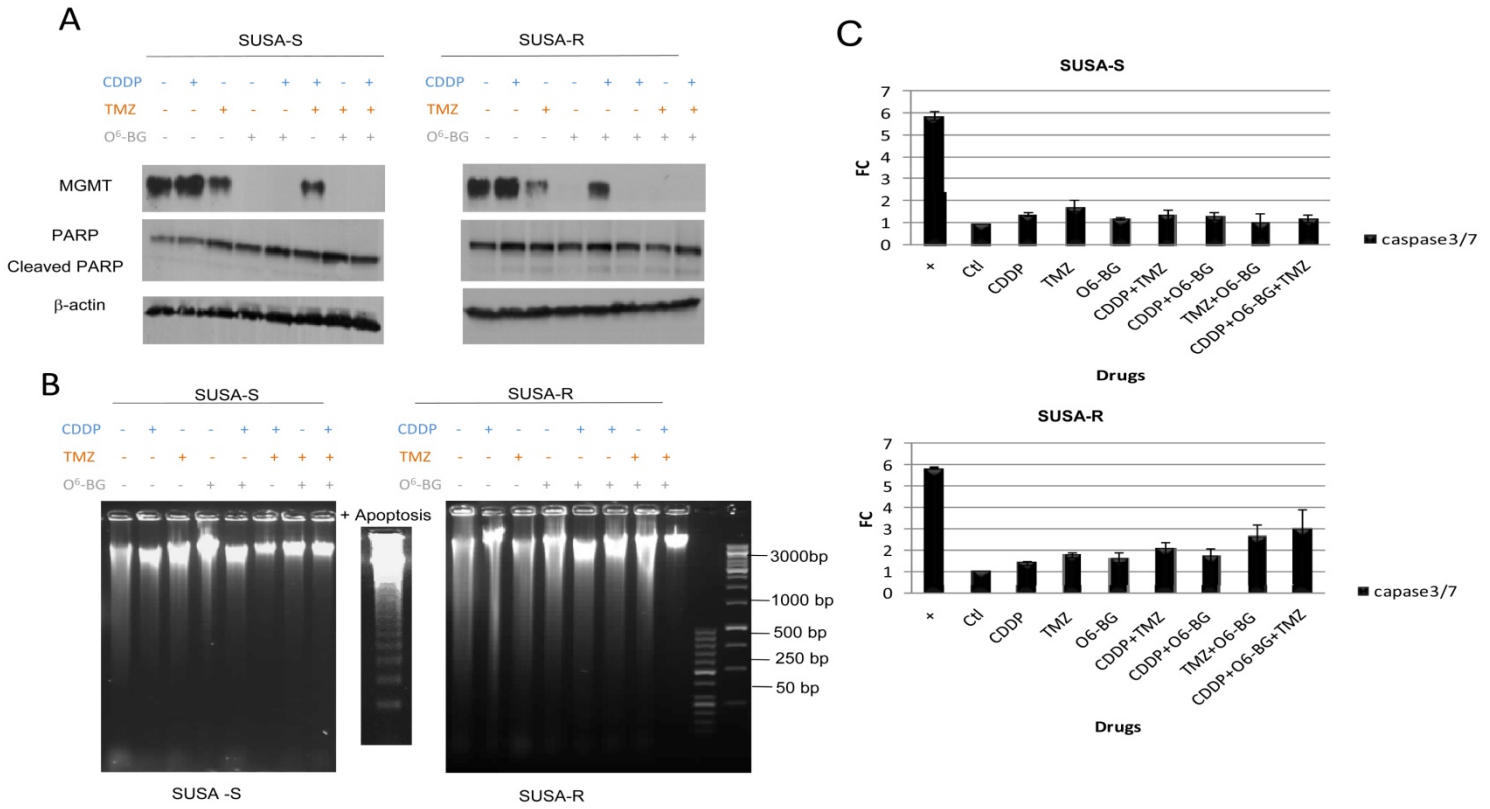
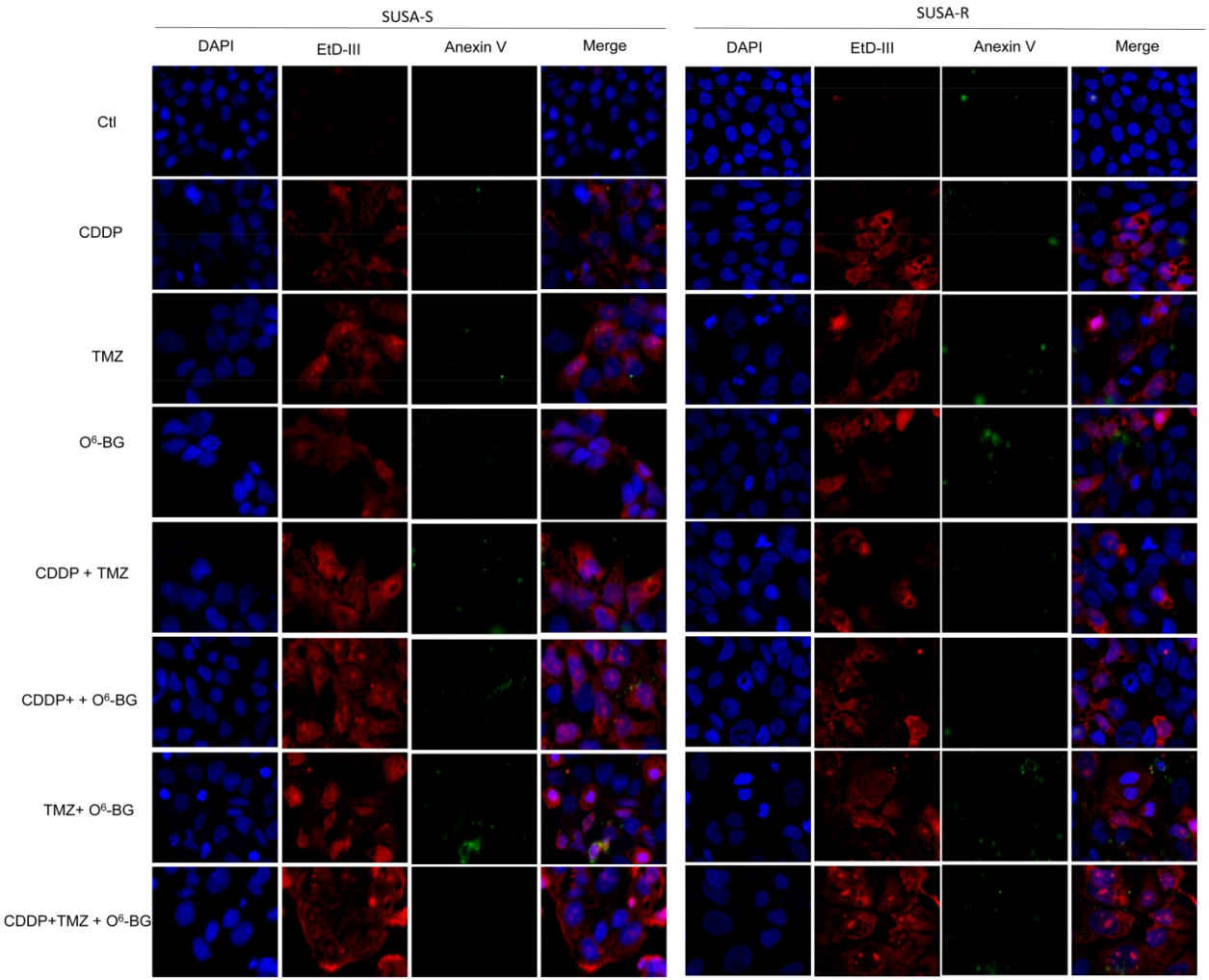
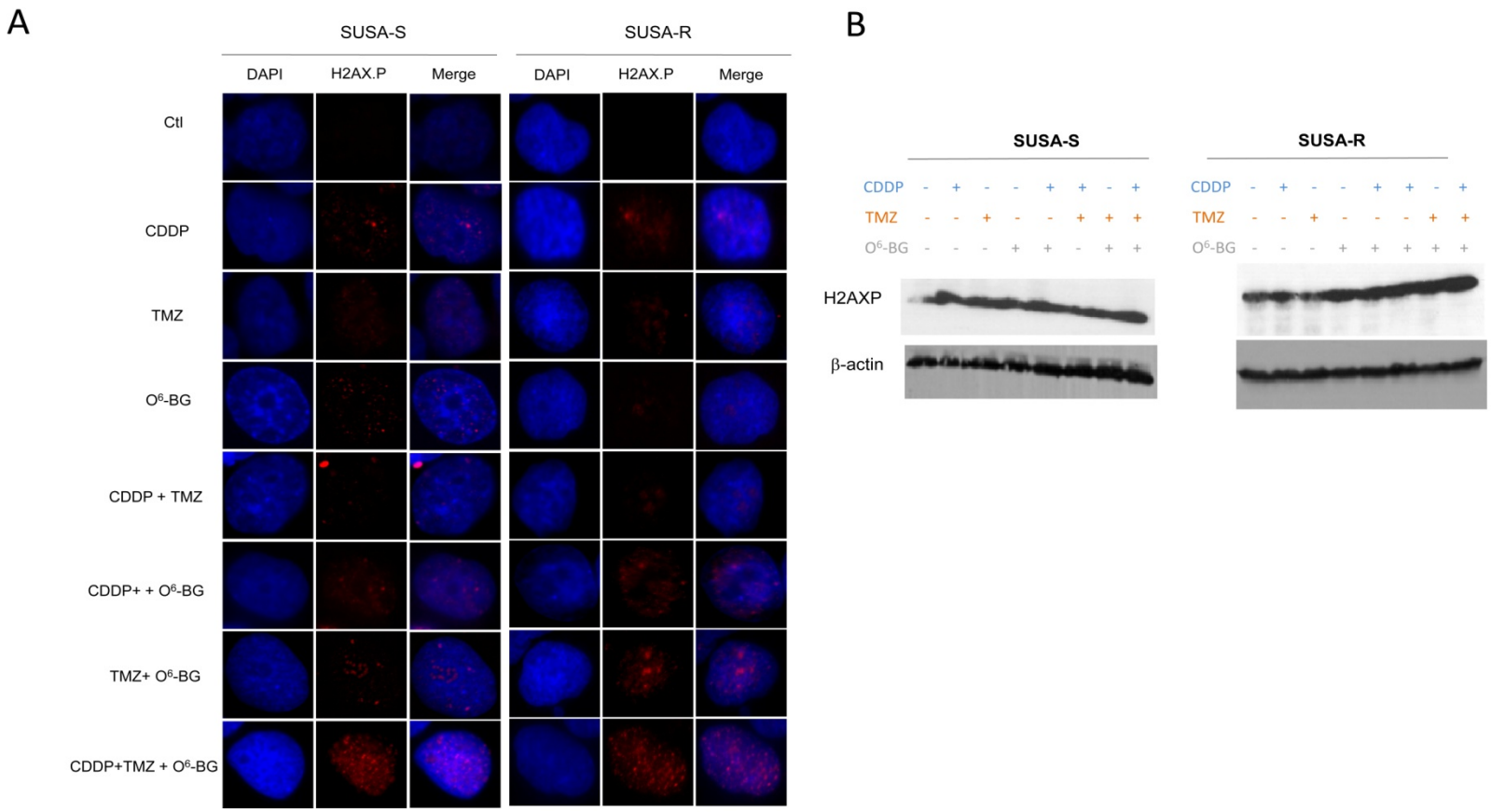
SUPPLEMENTARY INFORMATION



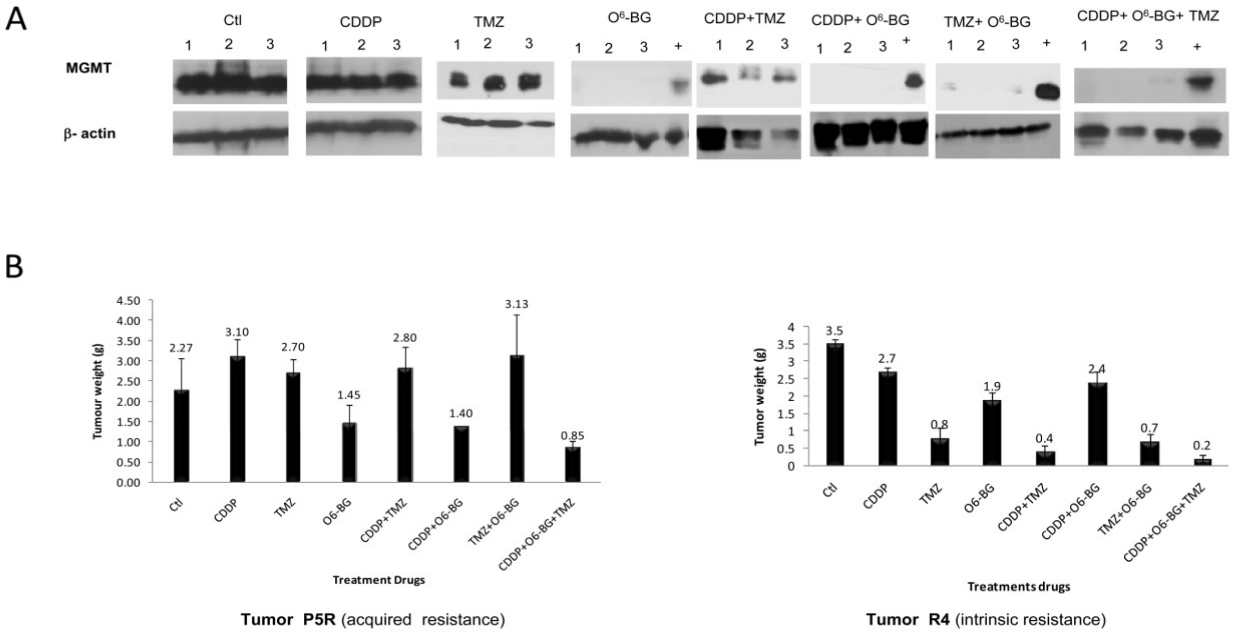
Supplementary Figure 1. Lack of apoptosis upon drug treatments in SUSA cell lines. A. PARP clevadge. There is no PARP cleavadge after drugs treatments. β-actin was used as endogenous control. **B. Apoptotic DNA ladder**. Cells do not present chromatin cleavage upon drugs treatment. **C. Caspase 3/7 activity**. Treated cells present low caspase3/7 activity. + positive control for apoptosis; Ctl-negative control; C-CDDP; T-TMZ; O^6- BG:O^6-benzylmethylguanine.



Supplementary Figure 2. Necrosis. Necrotic cells are detected in red (Ethidium Homodimer III- necrosis), in blue (DAPI-DNA) the nucleus and in green apoptotic cells (Anexin V-apoptosis). CDDP- cisplatin; O^6-BG- O^6 benzylmethylaguanine; TMZ- temozolomide.



Supplementary Figure 3. DNA damage in SUSA cell lines after 48h treatments. A. Immunoflurescence for DNA damage. The higher number of DNA damage focis appear in cells treated with CDDP+ O^6-BG+ TMZ. Blue (DAPI) stain cell nucleos and red (H2AX.P) the DNA damage foci. **B. H2AX.P protein expression.** Cells tripled treated are the ones that present more DNA double strand breakes (DSB). β-actin was used as endogenous control. CDDP-cisplatin; TMZ-temozolomide; O^6- BG-O^6-benzylmethylguanine.



Supplementary Figure 4: **MGMT study of xenograph tumors upon chemotherapeutic drugs treatment. A. MGMT expression control after treatment, for tumors P1R.** Tumors to which was administrated O^6-BG do not express MGMT. **B. Xenograph tumours sizes upon different drugs treatments.** P5R xenograph refractory tumor (left panel) respond to CDDP+ O^6-BG+TMZ treatment, decreasing 3 times the size when compared with the tumor controls. In the right panel is represented R4 tumors, intrinscally CDDP refractory and endogenously MGMT knockout, that respond to all the treatements that include TMZ, decresing the size 17 times, when compared with the control tumors. CDDP-cisplatin; TMZ-temozolomide; O^6- BG-O^6-benzylmethylguanine.

RESULTS and DISCUSSION SYNTHESIS

CHEMORESISTANCE IN COLORECTAL CANCER

The preexistence or the *de novo* development of cellular mechanisms to escape the antitumoral effects mediated by the anticancer compounds, involves different genetic and epigenetic events [220]. From an epigenetics perspective, little is known. Regardless of promising pharmacoepigenetics biomarkers, such as the example of *MGMT* hypermethylation and good response to temozolomide in gliomas have been described [54], for other tumor types like colorectal neoplasms examples are limited, specially if we just center our attention on resistance biomarkers. Herein, in the first part of this thesis we provide two examples that help fill this niche. In one hand we show that hypermethylation of *MGMT* predicts sensitivity for dacarbazine and in the other, that *SRBC* hypermethylation predicts resistance to oxaliplatin, both in metastasic colorectal cancer, a disease stage that represents the second most common cause of death from cancer [221].

STUDY I:

"Promoter CpG Island Hypermethylation of the DNA Repair Enzyme MGMT Predicts Clinical Response to Dacarbazine in a Phase II Study for Metastatic Colorectal Cancer"

Dacarbazine represents an effective chemotherapeutic agent for treatment of Hodgkin's lymphoma [222] and was the mainstay of treatment for metastatic melanoma until the recent approval of ipilimumab and vemurafenib [223].

MGMT promoter methylation status predicts metastasic colorectal cancer tumors response to dacarbazine

MGMT plays an important role in DNA repair, removing DNA adducts formed by alkylating agents. In the present study, *MGMT* promoter methylation was found in 38% of the colorectal tumors analysed, similar to the previously reported for this tumor type (35-40%) [224].

A low response rate to dacarbazine was observed (2 patients met clinical parameters with objective response and 8 with stable diseases) in our study. This could be linked to the fact that the patients included in this study are heavily pre-treated patients (median four lines of chemotherapy). Dacarbazine is activated in liver by CYP450 microsomial N-demethylation with formation of 5-[3-hydroxymethyl-3-methyl-triazen-l-yl]-imidazole-4-carboxaliplatinmide and 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC). It is therefore conceivable that the patients included in this study may have

exhausted the liver function capacity to activate dacarbazine. In this regard temozolomide, other alkylating agent whose activity is also enhanced in tumors with *MGMT* loss and that is hydrolyzed in cells producing the active compound MTIC without requiring liver passage, can be a good option for a new clinical trial [225]. In fact a new Phase II clinical trial (TEMECT EudraCT Number: 2012-003338-17) is already being performed. In this ongoing study, patients in whom standard therapies failed should be treated with temozolomide if present *MGMT* promoter gene methylated. Actually temozolomide is mainly used for the treatment of malignant glioblastomas and melanomas. In the meanwile of our Phase II clinical trial, others described a similar one, with deceptive results. Efficacy of temozolomide for colorectal cancer patients with confirmed *MGMT* promoter DNA methylation was inconclusive. However none achieved a complete response response, almost 45% achieved a stable disease [226].

When looked in more detail, in our study with dacarbazine, the patients that displayed the objective responses were the ones carrying *MGMT* promoter hypermethylated tumors, showing one of them a long-lasting maintenance response (more than six months), which is uncommon in the advanced setting of metastasic colorectal cancer. Important to reinforce that these patients failed standard chemotherapy schemes and any other treatment option is a great achievement. What concerns to progression free survival, a trend toward to a better progression free survival, was shown in the first cases (p=0.098).

The association between *MGMT* promoter hypermethylation and these referred clinical endpoints supports the hypothesis that DNA-repair defective metastasic colorectal cancer tumors are more susceptible to dacarbazine. The same was already described for human gliomas, in relation to temozolomide, an analogue of dacarbazine [54]. Tumors that express MGMT have a specific system for repair O^6-methyl adducts, the principal damage caused by alkylating agents. Thus tumors that present *MGMT* inactivation have high probability of response, since the insuficient DNA repair leads to point mutations or even chromosomal aberrations and subsequent cellular death [227, 228].

MGMT methylation is related with *KRAS* G>A mutations in metastasic colorectal cancer patients

In this trial despite *KRAS* mutational status did not influence patient selection, we check for a possible correlation between this issue and *MGMT* promoter methylation

status. As previously described we found that *MGMT* hypermethylation was more frequent in tumors carrying *KRAS* mutation with guanine-adenine transition (G12D, G12V or G13D) [229]. MGMT transfers methyl groups from the O^6 position of guanine in DNA to a cysteine residue in its active site, thereby inactivating MGMT itself [230]. Inactivated MGMT molecules are ubiquitinated and degraded by the proteasome [231]. If the methyl group is not removed from guanine, this base can pair with thymine during DNA replication which leads to transition of guanine-cytosine to adenine-thymine. However this difference was not statistically significant due to the sample small size for which we had both information (n=26; p=0.238). In contrast to *MGMT* promoter methylation, *KRAS* mutational status was not associated with response rate, progression free survival or overall survival.

STUDY II:

"Epigenetic Inactivation of the BRCA1 Interactor SRBC and Resistance to Oxaliplatin in Colorectal Cancer"

Small methylation differences between oxaliplatin sensitive and resistant paired human colon cancer cell lines

With the use of our first *in vitro* model, where the resistant cell line was derived from the sensitive one, by increasing oxaliplatin concentrations, we try to cut out most part of the genetic variability and focus our study only on genes promoter DNA methylation changes. After our general approach with 27K Methylation Illumina, only three promoter genes were found differentially methylated between oxaliplatin sensitive and resistant cell lines: *SRBC*, *family with sequence similarity 111, member A (FAM111A)* and *family with sequence similarity 84, member A* (*FAM84A)*. The gene with the highest difference in promoter DNA methylation was *SRBC*. This was the only gene later validated. This small methylation difference between sensitive and resistant cell lines could be due to the reduced coverage from the 27K plataform or due the restricted thresholds that we applied. For example we only chose genes that presented differences higher than 75% of methylation between the two samples. With this criteria we thought that the probability of the selected genes be implied in chemoresistance acquisition, would be greater. Nonetheless this does not discard the importance of genes with smaller methylation differences. Other question was that the chosen probes were localized between minus 300 or plus 300 base pairs from the transcription start site. Promoter genes are canonical defined to be localized between minus 1000 base pairs from the transcription start site until the end of the first exon. Based on this, maybe we lost other genes that were differentially methylated. Other fact to have in

account is that chemoresistance process can appear due to different mechanisms beside genes promoter DNA methylation, like genetic alterations or simply due to changes in proteins expression (e.g. alterations in non-coding RNAs or histone modifications).

SRBC expression is regulated by DNA methylation in colorectal cancer

The oxaliplatin resistant human colon cancer cell lines (LoVo-R and HCT15) were the only presenting *SRBC* promoter hypermethylation and diminished protein expression. This data is in agreement with previous reports, where *SRBC* promoter hypermethylation led to a reduction of messenger RNA and protein expression in human colorectal cancer, ovary and gastric cancer cell lines [232]. To be shore that promoter hypermethylation was the cause of *SRBC* inactivation in resistant cells, LoVo-R cell line was treated with 5-AZA, a demethylating agent. 5-AZA is an analog of cytosine that is converted into nucleotide triphosphates *in vivo*. Thus it is able to incorporate into the DNA and influence it structure and stability. Two models have been proposed to explain the mechanisms by which 5-AZA reactivates silenced genes by demethylating CpG islands in the promoters. First, after being incorporated into DNA, 5-AZA forms an irreversible and covalent bond with DNMT1, protein which is mainly responsible for the maintenance of DNA methylation. This therefore leads to demethylation when DNA is replicated [233]. Second, 5-AZA is reported to induce DNMT1 degradation trough an ubiquitin-proteosome-dependent pathway that results in gene re-expression [234]. Upon treatment these resistant cells re-expressed SRBC. The same was reported for other colorectal cancer human cell lines by Xu, *et al* [235]. Before move forward we wondered how was *SRBC* promoter methylation satus and its expression in normal colon mucosa. In normal tissues, *SRBC* was not methylated and was expressed (data not shown). This was in agreement with data published before, where in normal breast, lung, stomach and colorectal tissues, SRBC was expressed and gene promoter was not methylated [232, 235, 236].

***SRBC* promoter methylation plays a role in colorectal cancer resistance to oxaliplatin**

In order to demonstrate that the epigenetic inactivation of *SRBC* gene functionally contributed to oxaliplatin resistance, we restored it expression in colorectal cancer resistant cells and inhibited it in the sensitive ones. Upon SRBC transfection, resistant cells became more sensitive to oxaliplatin, in sharp contrast with sensitive cells that after SRBC downregulation by the short hairpin RNA approach, became considerable more resistant. The observed effects were specific for oxaliplatin because the *in vitro*

depletion or enhancement of SRBC did not change the sensitivity to 5-FU, other drug commonly used in colorectal cancer treatment. Getting or having *SRBC* promoter methylated seems to be an advantage to resistant cells. With HCT15 and HCT-116 cells we studied intrinsic resistance. Important to note that sensitivity changes to oxaliplatin, were higher in LoVo-S and LoVo-R cells than in HCT15 and HCT-116. Endogenously HCT15 and HCT-116, beside *SRBC* methylation differences presented already a different genetic background. This fact for shore led to alterations in different pathways that in HCT15 can be reinforcing resistance to oxaliplatin. Thus, the simple fact of re-introduce or silence *SRBC* in these cells was not enough to produce so great changes in oxaliplatin sensitivity.

A role of SRBC in mediating different sensitivity to oxaliplatin can be clearly justified by its protein interaction with the product of the *BRCA1* gene [235]. The BRCA1 protein exerts an important role in DNA double-strand break repair through homologous recombination 2, so its deficiencies can impair the capacity of cancer cells to repair DNA cross-links caused by chemotherapy drugs such as platinum derivatives [100, 237].Two independent studies reported greater primary chemotherapy sensitivity to platinum based chemotherapy agents in patients with ovarian cancer who were carriers of *BRCA1* germline mutation [237, 238]. These observations have also been extended to *BRCA1* epigenetic silencing in sporadic breast and ovarian tumors, where it also predicts a good response to cisplatin and carboplatin [239-241]. However, the biology of mammary tumors is very different from colorectal malignancies and in all cases of colon cancer, the *BRCA1* promoter has always been found in an unmethylated status [242]. This makes us hypothesise that SRBC could act as a BRCA1 inhibitor, and due to its promoter hypermethylation, BRCA1 stays active and as consequences cells are more resistant to oxaliplatin. Yet a better study about the molecular mechanisms linking SRBC activity and DNA damage repair triggered by oxaliplatin should be investigated. Interestingly, in addition to its BRCA1-related roles, SRBC might have other functions related to the observed chemoresistance phenotype, such as its interaction with caveolin 1, which may putatively affect intracellular vesicule traffic of the drug [243].

Our *in vitro* findings let us think that this gene could be a good candidate as a predictive biomarker for oxaliplatin-based treated colorectal patients. We choose metastasic patients, because they present less therapeutic options.

***SRBC* Hypermethylation predicts short progression free survival in oxaliplatin treated patients with unresectable metastasis**

In the clinical set we started with a discovery cohort (n= 131), composed by stage IV colorectal adenocarcinoma patients, all of whom were treated with oxaliplatin in combination with a fluoropyrimidine. In this cohort we found 29.8% of primary tumors methylated. A similar percentage (30.2%) was found after analyze a dataset from The Cancer Genome Atlas (n=232). However data published before revealed a higher percentage (47.5%) [235]. What concerns to the correlation between *SRBC* methylation and clinical endpoints as response rate, progression free survival and overall survival, in the discovery cohort we only found a significant association with short progression free survival (p=0.01).

According to Cox regression multivariable test, surgery of metastases showed to be an independent progression free survival (p=0.004) and overall survival (p=0.003) prognostic factor. This was expected, since patients that under go metastasis surgery present an advantage when compared with subjects with unresectable metastasis. Taking this into account, our discovery cohort was stratified in relation to this clinical feature and was divided into two groups: patients that underwent metastases resection (n=34) and patients with unresectable metastases (n=97). After subdivide this cohort *SRBC* hypermethylation was only statistically significant associated with shorter progression free survival (p=0.01) in patients that received oxaliplatin as neoadjuvant therapy and were subsequently not eligible for surgery or patients with metastasis that were originally classified as unresectable and were given oxaliplatin as palliative chemotherapy. In this last set of patients, for whom overall survival data was available (n=79), we also observed that *SRBC* hypermethylation was significantly associated with shorter overall survival (p=0.04). To validate this data we study a second independent set of colorectal cancer patients with unresectable metastasis, who also received oxaliplatin based therapy (n=58). With this validation cohort, we only confirmed that the presence of *SRBC* hypermethylation was associated with shorter progression free survival (p=0.045). Based in our *in vitro* and clinical data, we think that *SRBC* promoter methylation is more important for oxaliplatin resistance acquisition that for intrinsic one. Patients with primary tumors in which *SRBC* promoter is methylated, progress faster when receive oxaliplatin-based chemotherapy. Maybe because tumor cells with this alteration present a selective advantage to proliferate under oxaliplatin action.

The validation of *SRBC* promoter DNA methylation as a predictive biomarker requires further prospective studies. If successful, clinical trials would also be necessary to develop strategies to overcome or prevent the development of *SRBC*-mediated

epigenetic resistance. In this regard, research into DNA demethylating agents that might re-sensitize cancer cells to oxaliplatin is reasonable. This can represent a *revival* of the DNA demethylating agents in the therapy of solid tumors. With few therapeutic options against metastasic colorectal cancer once it has become insensitive to oxaliplatin, DNA methylation inhibitors, such as 5-AZA and 5-aza-2′-deoxycytidine, could be used to resensitize these tumors to the oxaliplatin therapy. This idea has been recently explored in non–small cell lung carcinoma patients who had reached the last line of chemotherapy. The subsequent administration of 5-AZA was able to rescue previous chemosensitivity, with low toxicity rates [244]. Other option shows up from a recent publication that links *SRBC* promoter hypermethylation with NF-kB over-expression. Since in the clinical stage, inhibitors of *NF-kB* are already applied in chemotherapy schemes, first it will be interesting to investigate if *NF-kB* expression in our *in vitro* model is related with *SRBC* methylation status. If yes, secondly study the possible reversion of oxaliplatin resistance by the use of NF-kB inhibitors. A good option for test these new drugs combinations will be the establishment of colorectal orthoxenografts. These are mice models where a piece of patient's tumor is implanted, in mice corresponding tumor organ. This allows the perpetuation of the tumor and test different chemotherapeutic schedules in order to study tumors response and pharmacological behavior.

If in the future *SRBC* promoter methylation will be accepted as a predictive marker, in order to simplify its analyses, DNA methylation in circulating tumor cells or stool, approaches less invasive for patients, should be optimized and implemented. These detection techniques will be also important in order to do a prospective study and monitorise *SRBC* promoter methylation status in patients under oxaliplatin-based treatments.

In general, to patients with unresectable metastasic colorectal cancer is typically administered a first-line chemotherapy regimen based in oxaliplatin or in irinotecan. Basically the choice remains on established protocols. The Europeans normally apply FOLFOX scheme. With chemotherapy selection in mind, it will be worth to study *SRBC* promoter methylation status in a cohort composed by patients that received irinotecan-based treatment. If *SRBC* promoter methylation keeps predicting short progression free survival exclusively for oxaliplatin treated patients, in the future we can put patients which present *SRBC* promoter methylation directly under a first line irinotecan-based chemotherapy. Other point that will be interesting is to investigate *SRBC* promoter methylation in paired primary tumors and metastasis and also in posterior treatment

relapse tumors. The extension of our study to stage II, stage III colorectal cancer patients, and to different geographical origins patients are also important points for further investigation.

Other possible avenue of future research derived from our findings is the possibility to extend the observations to other tumor types, beyond colorectal cancer. The described existence of *SRBC* hypermethylation in lung, breast and ovarian carcinomas [235, 236, 245] might prompt the development of translational assays to determine if the epigenetic inactivation of this gene is also associated with lower sensitivity to platinum-derivatives in these neoplasms.

STUDY OF CHEMORESISTANCE IN TESTICULAR GERM CELL TUMORS

Although the elevated sensitivity of testicular germ cell tumors to cisplatin-based therapies (80%), there is still a subset (15-20%) of young men that will not be cured and will die [102]. With our work we afford two potential genes, *GCS* and *MGMT* that can be pharmacologically regulated in order to reverse cisplatin resistance of these last cases, and resensibilize tumors to cisplatin treatment.

STUDY III

"Orthoxenografts of Testicular Germ Cell Tumors Enable Identification of Glucosylceramide Synthase as a Cisplatin Resensitizing Target"

Preclinical inhibition of GCS resensitizes cisplatin refractory germ cell tumors to cisplatin

The genomic high-throughput analyses of our xenograft model, representative from paired non-seminoma cisplatin sensitive and refractory human tumors, prompted us several genes (*GCS, ATP6V1G*, *CTR1/2*, *PAPPA* or *POLE3*) with a possible implication in cisplatin resistance acquisition.

As a proof-of-concept we evaluated the therapeutic value of GCS. This protein transfers a glucose residue from UDP-glucose to ceramide synthesizing glucosylceramide, which allows cellular escape from ceramide-induced programmed cell death. *In vitro* over-expression conferred cellular resistance to doxorubicin and daunorubicin [246, 247]. Its up-regulation was found in multidrug resistance cancer cell lines, in chemoresistant leukemia, and in metastatic breast cancer [247, 248]. We choose this gene since its messenger RNA expression and enzymatic activity was increased in most part of the engrafted refractory tumors; its knockdown by RNA

interference in worms conferred cisplatin sensitivity; and because there are specific inhibitors available, some of which are currently in clinical use.

To investigate if over-expression of GCS contributes to cisplatin resistance in non-seminomas, we decreased its expression in a human non-seminomas cisplatin resistant cell line and over-expressed it in a sensitive one. As in study II, the human non-seminoma resistant cell line was derived from a sensitive parental one, due to cisplatin increasing drug concentrations. Upon GCS inhibition resistant cells became more sensitive to the antiproliferative activity of cisplatin, in contrast with sensitive cells that after GCS expression, became more resistant. The same effect was found when we treated resistant cells with DL-treo-PDMP, an inhibitor of GCS. A possibility explanation is the same that happens in breast, ovary, cervical and colon cancer cells, where GCS activity triggers MDR1 expression, leading that to drug efflux [249], in our case to cisplatin efflux, and like this to appearance of resistant phenotype. Targeting glycosphingolipid synthesis has emerged as a novel approach for treating metabolic diseases such as Gaucher, Niemam-Pick and diabetes. In this context, GCS inhibitors are in clinical use or under development, including Migustat, DL-treo-PDMP, EXEL-0346, etc. Our preclinical results demonstrate that DL-treo-PDMP resensitize cisplatin refractory cells to treatment, providing a firm preclinical rationale for developing further Phase II clinical trials, in order to do drug reposition in the field.

Futures strategies exploring cisplatin resistance are guaranteed in our preclinical models. For example the study of the influence of the subunit of the vacuolar proton-translocating ATP6V1G1 which is responsible for acidifying intracellular compartments, was found overexpressed in cisplatin-resistant cell lines [250]. Yeast genomic screening; have revealed its effect on the sensitivity to DNA-damaging agents such as cisplatin [251]. CTR1 and CTR2, Cu^{2+} influx transporters are also the main cellular cisplatin transporters and its under-expression was associated with cisplatin resistance [252].

STUDY IV:

"Loss of MGMT Promoter Methylation and Resistance to Cisplatin in Non-Seminoma Testicular Germ Cell Tumors"

***MGMT* methylation status influences non-seminoma tumors sensitivity to cisplatin**

MGMT DNA promoter methylation was previously related with non-seminoma tumors resistance to cisplatin [253]. Nonetheless it was never study on *in vitro* or *in vivo* models of cisplatin acquired resistance. This prompted us to examine an *in vitro* model, composed by a human non-seminoma cell line sensitive to cisplatin and it derived resistant counterpart. Once again we found less *MGMT* DNA promoter methylation in the resistant cell line. Although this difference was not too high (20%) it had a biological significance, since it was correlated with protein expression. This is in agreement with data published before where it was described that a cisplatin resistant human non-seminoma cell line presented higher MGMT levels, than the sensitive counterpart [254]. To observe if MGMT higher expression was directly related to cisplatin resistance, first in the human cell lines, we blocked it with O^6-benzylguanine and we found an increase on sensitivity to cisplatin. O^6-benzylguanie is a potent, specific and irreversible blocker of MGMT DNA repair protein and thereby enhances the cytotoxic action of agents that produce lesions at the O^6 position of guanine [255]. Pera *et al*, described before that human non-seminoma cells with highest levels of MGMT, were also more resistant to temozolomide, data that we also found [254].

***MGMT* promoter hypermethylation is correlated with better overall survival in testicular germ cell cancer metastasic patients, treated with cisplatin-based chemoterapy**

We also investigated *MGMT* methylation profile in a clinical set of metastasic patients with testicular germ cell tumors (n=72), where a positive statistical correlation between *MGMT* hypermethylation was related with a higher overall survival (p=0.025). Previously *MGMT* gene was described to be frequently inactivated in testicular germ cell tumors by promoter hypermethylation, especially in the sensitive ones [256, 253]. The same happens in glioblastomas, as mentioned before. Patients that present MGMT promoter hypermethylation are more sensitive to temozolomide, this because their cells cannot repair the DNA damage cause by this chemotherapeutic drug, fading [54]. This result makes us think that *MGMT* DNA methylation status could have in the future a clinical impact, in the same way that it has for gliomas [54]. However further validation studies are required.

Preclinical inhibition of MGMT re-sensitizes cisplatin refractory non-seminoma tumors to cisplatin

Despite the high cure rate obtained with cisplatin-based chemotherapy in testicular germ cell tumors, the management of patients with an adverse prognosis at presentation, or of those who fail to respond to first-line chemotherapy, remains a

therapeutic challenge [257]. Focus on a the possibility of re-sensibilize cisplatin resistant tumors, using the xenograft model established before, we proved that MGMT inactivation by O^6-benzylguanine in resistant tumors over-expressing it, plus additional treatment with cisplatin and temozolomide can rescue these tumors. In preclinical studies temozolomide has demonstrated antitumor activity against human lymphoblastoma, myeloid leukemia, Burkitt's lymphoma, choriocarcinoma, astrocytoma, lung and colorectal human cell lines [258]. The exceptional sensitivity of human non-seminoma cell lines to temozolomide was one reason for testing this new drug in the clinics [254]. Three clinical phase II studies were made before without positive results [259-261]. We think that the major limitation to the successful treatment of these patients was the absence of information about *MGMT* promoter methylation. Therefore, a further rationale for testing this new agent again is first study of *MGMT* promoter methylation and then decides if the chemotherapy schedule should include an MGMT inhibitor, like O^6-benzylguanine drug.

Other reason for testing temozolomide and when needed a MGMT inhibitor, is that this chemical agent has clinical activity against brain tumors [258, 262]. Brain metastases occur in 8-15% of patients with testicular tumors, almost always associated with relapse at other sites or as a terminal event [259]. Temozolomide may provide in this way a more effective treatment for testis tumors which have metastasized to the brain.

Testicular germ cell cancer as mentioned before is mainly divided in two histological categories: seminoma and non-seminoma. In both studies where we investigate this type of cancer we only were able to use *in vitro* and *in vivo* representative non-seminoma models. Until nowadays only two pure human seminomas cell lines were established. What concerns to mice models, none was established. Our laboratory tried to establish orthoxenographs; nevertheless none of the twenty two implanted pure gonadal seminomas grew in nude mice and from the mixed tumors, comprising both seminoma and non-seminoma components, only the last grew in mice.

CONCLUSIONS

Based on the findings of this PhD thesis we can conclude:

STUDY I

- In metastasic colorectal cancer, *MGMT* inactivation by DNA promoter methylation is associated with susceptibility to dacarbazine.

STUDY II

- Oxaliplatin resistance acquisition in colorectal cancer is related with DNA hypermethylation-associated silencing of *SRBC*.

- *SRBC* promoter hypermethylation is a good candidate as oxaliplatin predictive marker for metastasic colorectal cancer patients that did not underwent metastases surgery, yet further prospective studies are required.

STUDY III

- Over-expression of GCS is related with cisplatin acquired resistance, in human non-seminoma cancer cell lines.

- GCS inhibition decreases non-seminoma cancer cells resistance to cisplatin.

- Inhibition of GCS with DL-treo-PDMP is a possible line of attack to revert cisplatin resistance in refractory non-seminoma tumors.

STUDY IV

- Non-seminoma cell lines and orthoxenografts tumors made resistant to cisplatin, present a re-expression of MGMT.

- Inhibition of MGMT is a possible way to revert non-seminoma tumors cisplatin resistance.

- *MGMT* methylation status is related with better overall survival in metastasic testicular germ cell tumors patients.

- A better phase II study design, based on *MGMT* DNA promoter methylation, may offer the possibility to resensitize refractory patients to cisplatin. Thus we will detect the right patient for the right drug, on the right time.

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SUPPLEMENT I

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Clinical Cancer Research

Promoter CpG Island Hypermethylation of the DNA Repair Enzyme MGMT Predicts Clinical Response to Dacarbazine in a Phase II Study for Metastatic Colorectal Cancer

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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Promoter CpG Island Hypermethylation of the DNA Repair Enzyme MGMT Predicts Clinical Response to Dacarbazine in a Phase II Study for Metastatic Colorectal Cancer

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Abstract

Purpose: O^6-methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein removing mutagenic and cytotoxic adducts from O^6-guanine in DNA. Approximately 40% of colorectal cancers (CRC) display MGMT deficiency due to the promoter hypermethylation leading to silencing of the gene. Alkylating agents, such as dacarbazine, exert their antitumor activity by DNA methylation at the O^6-guanine site, inducing base pair mismatch; therefore, activity of dacarbazine could be enhanced in CRCs lacking *MGMT*. We conducted a phase II study with dacarbazine in CRCs who had failed standard therapies (oxaliplatin, irinotecan, fluoropyrimidines, and cetuximab or panitumumab if *KRAS* wild-type).

Experimental Design: All patients had tumor tissue assessed for *MGMT* as promoter hypermethylation in double-blind for treatment outcome. Patients received dacarbazine 250 mg/m^2 intravenously every day for four consecutive days, every 21 days, until progressive disease or intolerable toxicity. We used a Simon two-stage design to determine whether the overall response rate would be 10% or more. Secondary endpoints included association of response, progression-free survival, and disease control rate with *MGMT* status.

Results: Sixty-eight patients were enrolled from May 2011 to March 2012. Patients received a median of three cycles of dacarbazine (range 1–12). Grades 3 and 4 toxicities included: fatigue (41%), nausea/vomiting (29%), constipation (25%), platelet count decrease (19%), and anemia (18%). Overall, two patients (3%) achieved partial response and eight patients (12%) had stable disease. Disease control rate (partial response + stable disease) was significantly associated with *MGMT* promoter hypermethylation in the corresponding tumors.

Conclusion: Objective clinical responses to dacarbazine in patients with metastatic CRC are confined to those tumors harboring epigenetic inactivation of the DNA repair enzyme MGMT. *Clin Cancer Res; 19(8); 2265–72. ©2013 AACR.*

Introduction

Globally, nearly 1.25 million patients are diagnosed and more than 600,000 patients die from colorectal cancer (CRC) each year (2008 estimates; ref. 1). At least 50% of patients develop metastases (2), and most of these patients have unresectable tumors (2, 3).

In the last 10 years, thanks to a wider clinical use of a multidisciplinary approach, along with the introduction of new cytotoxic drugs and the addition of targeted therapies against the angiogenesis (bevacizumab and aflibercept), the EGF receptor (EGFR) pathway (cetuximab and panitumumab), or multiple receptor tyrosine kinases (regorafenib), the survival of patients with metastatic CRC (mCRC) has considerably been ameliorated (4–6). Nevertheless, prognosis remains poor and patients carrying *KRAS* mutations (35%–40% of CRCs), which preclude responsiveness to cetuximab or panitumumab (6), have limited therapeutic options after failure of 2 lines of standard treatments, although a significant percentage of these patients retain a good performance status potentially allowing further therapies. There is therefore an unmet need of therapeutic

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

O^6-methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein removing mutagenic and cytotoxic adducts from O^6-guanine in DNA. Approximately 40% of colorectal cancers (CRC) display MGMT deficiency due to promoter hypermethylation leading to silencing of the gene. Alkylating agents, such as dacarbazine, exert their antitumor activity by DNA methylation at the O^6-guanine site, inducing base pair mismatch; therefore, activity of dacarbazine could be enhanced in CRCs lacking *MGMT*. Although several reports have shown anecdotal efficacy of dacarbazine in metastatic CRC, there is a lack of translational evidence of CRC sensitivity to this drug based on *MGMT* status. We report here a phase II clinical study showing for the first time that dacarbazine activity is confined to CRC harboring promoter CpG hypermethylation of *MGMT*. These data therefore highlight a previously unidentified subgroup of the patients with CRC who benefit from treatment with alkylating agents based on a specific epigenetic alteration in individual tumors.

options, based on specific molecular alterations that could prove their effectiveness also in the wide *KRAS*-mutated subgroup of CRCs.

O^6-methylguanine-DNA-methyltransferase (MGMT) is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA. MGMT protects cells against these lesions, transferring the alkyl group from the O^6-guanine in DNA to an active cysteine within its own sequence. Such reaction inactivates one MGMT molecule for each lesion repaired (7). The inactivation of tumor suppressor genes by the presence of cytosine methylation encompassing the corresponding transcription start site located in a CpG island is gaining "momentum" in the management of oncology patients (8) and, in this regard, promoter CpG island hypermethylation leads to the transcriptional silencing of *MGMT* (9). The subsequent lack of repair of O^6-methylguanine adducts can result in a higher frequency of G:C > A:T transitions (10, 11). It is known that approximately 40% of CRCs have silencing of *MGMT*. Interestingly, in a retrospective analysis on 244 CRCs samples, it has been found that 71% of tumors with G to A mutation in *KRAS* showed *MGMT* epigenetic inactivation, showing a strong association between the *MGMT* inactivation by promoter hypermethylation and the appearance of G to A mutations at *KRAS* (10). Furthermore, *MGMT* hypermethylation was also found in 35% of wild-type *KRAS* mCRCs. de Vogel and colleagues (12) found that *MGMT* hypermethylation is associated with G:C > A:T mutations in *KRAS*, but not in adenomatous polyposis coli (*APC*), suggesting that *MGMT* hypermethylation may succeed *APC* mutations but it precedes *KRAS* mutations in colorectal carcinogenesis.

In cells, loss of *MGMT* expression leads to compromised DNA repair and may play a significant role in cancer progression and response to chemotherapy as it occurs in glioma (13–16). The mechanism of action of dacarbazine and temozolomide is DNA methylation at the O^6-guanine site, inducing base pair mismatch. The methyl group at O^6-site is removed by MGMT in a one-step methyl transfer reaction. Therefore, we hypothesized that *MGMT* inactivation by hypermethylation may confer sensitivity to these agents (17). However, discrepant data about the clinical activity of these drugs in mCRC are reported in the literature (18–21). A response rate of 19%, including one complete response, was reported in 26 fluoropyrimidine-resistant patients receiving cisplatin and dacarbazine (19). In another study, 48 patients refractory to fluoropyrimidine were treated with dacarbazine, irinotecan, and cisplatin obtaining a 33% of response rate (18). Temozolomide is an imidazotetrazine derivative of dacarbazine. The combination of lomeguatrib and temozolomide did not show activity in unselected mCRC (20). In a pilot study including patients selected by tumor molecular profiling, temozolomide was effective in 2 patients with mCRC exhibiting loss of *MGMT* expression (22). The latter finding was confirmed by a recent report by Shacham-Shmueli and colleagues (23) documenting objective responses to temozolomide in 2 patients with *MGMT*-deficient mCRC.

On the basis of these findings, we designed a phase II trial aimed to assessing the antitumor activity of dacarbazine in patients with mCRC with determined *MGMT* promoter methylation status and refractory to the standard therapies.

Materials and Methods

Trial design

The study was designed as a phase II trial (DETECT-01 trial, EUDRACT number 2011-002080-21). Patients were treated with dacarbazine monotherapy until progression or unacceptable toxicity for 18 weeks (6 cycles). In case of partial response with clinical benefit, treatment was allowed until dose-limiting toxicity. Primary endpoint was to assess response rate to dacarbazine according to Response Evaluation Criteria in Solid Tumors (RECIST 1.1) criteria. Secondary endpoints were to assess: disease control rate (DCR), progression-free survival (PFS), identification of *KRAS*, and O^6-methylguanine-DNA-methyltransferase (*MGMT*) status in individual tumor samples as potential molecular biomarkers of response to dacarbazine. Written informed consent was obtained from each patient. The study followed the Declaration of Helsinki and good clinical practice, being approved by Ethic Committee of Ospedale Niguarda Ca' Granda (Milan, Italy).

Patients

All patients met the following inclusion criteria: age 18 years or more, Eastern Cooperative Oncology Group performance status of ≤ 1, histologically confirmed metastatic colorectal adenocarcinoma. A paraffin-embedded

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block from archival tumor tissue of primary and/or metastases for *MGMT* status analysis was requested. All patients had measurable disease (by RECIST criteria v1.1), and progressed on standard treatment with fluoropyrimidine, oxaliplatin, irinotecan, and cetuximab or panitumumab (the latter 2 drugs if *KRAS* wild-type). An adequate bone marrow, liver, and renal function was required.

Treatment schedules

Dacarbazine 250 mg/m^2 intravenously everyday for 4 consecutive days, every 21 days, was administered until progression, death, unacceptable toxicity, or patient withdrawal of consent. Antiemetic agents and supportive care were provided by treating physician as per standard clinical practice. In case of G3 hematologic toxicity (absolute neutrophil count $< 1.5 \times 10^9$/L and platelet count $< 100 \times 10^9$/L) dacarbazine was delayed by 1-week interval until recovery. Prophylactic use of colony-stimulating factors was allowed as per standard clinical practice.

Evaluation criteria

Patients were evaluated for primary overall response rate (ORR) and secondary endpoint (DCR and PFS) according to RECIST criteria v1.1. Tumors were measured every 8 ± 1 weeks through week 18 and then every 8 ± 1 weeks until the tumor progressed. Complete response was defined as disappearance of all target lesions. Any pathologic lymph nodes (whether target or nontarget) must have reduction in short axis to 10 mm or less. An objective response (partial response) was defined as a reduction of at least 30 percent in the sum of all target lesions on computed tomography or magnetic resonance imaging scanning. Confirmed objective responses were those for which a follow-up scan obtained at least 4 weeks later showed the persistence of the response. Progressive disease was defined as at least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also show an absolute increase of at least 5 mm. Stable disease was defined as shrinkage neither sufficient to qualify for partial response nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum diameters while on study. Clinical investigators and radiologists were blinded as for *MGMT* status of the tumors.

Safety assessment

Safety assessments and blood biochemistry including complete blood counts were carried out at baseline and at the beginning of each treatment cycle. Any toxicity was assessed using the National Cancer Institute (NCI)-CTCAE version 4.0 and recorded at every visit until resolved.

Analysis of MGMT promoter methylation status

Loss of expression of *MGMT* was defined as promoter hypermethylation 25% or more as previously described (9). Tumor samples from patients' primary tumor were obtained from Pathology Department of the Ospedale Niguarda Ca' Granda or others Pathology Departments as referral. Formalin-fixed paraffin-embedded tumor blocks were reviewed for quality and tumor content. A single representative block, from either the primary tumor or metastasis, depending on availability, was selected for each case. White slides (2 cut of 10 μm, if from a tumor tissue paraffin block, or 3 cuts of 10 μm if from a biopsy) were sent to Bellvitge Biomedical Research Institute (IDIBELL; Barcelona, Spain) for DNA extraction and evaluation of *MGMT* promoter methylation status in blind as for clinical outcome. Genomic DNA was extracted from paraffin tissue samples following manufacturer's instructions (QIAamp DNA FFPE Tissue Kit). DNA was then subjected to bisulfate treatment using EZ DNA methylation kit (Zymo Research). Briefly, 1 μg of genomic DNA was denaturated by incubating with 0.2 mol/L NaOH. Aliquots of 10 mmol/L hydroquinone and 3 mol/L sodium bisulfate (pH 5.0) were added, and the solution was incubated at 50°C for 16 hours. Treated DNA was purified, desulfonated with 0.3 mol/L NaOH, repurified on Zymo-Spin columns, and eluted with 25 μL water. *MGMT* promoter methylation status was analyzed by methyl-specific polymerase chain reaction (MSP). It was carried out in a 15 μL volume containing 1 μL of the sodium bisulfite-modified DNA. The characteristics of the MSP reactions and the primer sequence have been previously described (14). SW620 cell line was used as a positive control for hypermethylated alleles of *MGMT* and DNA from RKO cell line used as a negative control (Fig. 1).

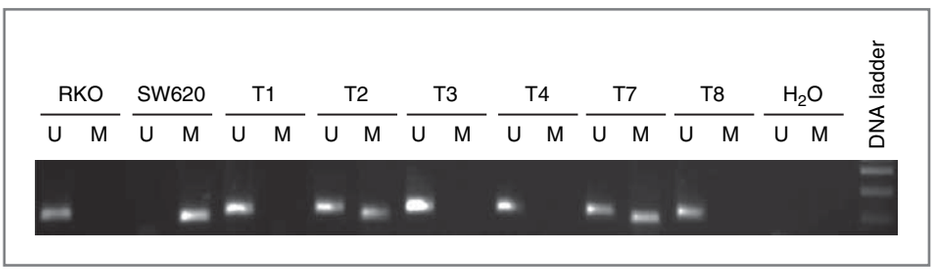

Figure 1. Methyl-specific PCR for *MGMT* promoter. Example of results obtained for 6 metastatic colorectal cancer primary tumors from the study cohort. Tumors T2 and T7 were methylated and all the others unmethylated. U indicates unmethylated tumors and M methylated tumors. RKO was the human colorectal cancer cell line used as negative control for methylation and SW620 the human colorectal cancer cell line used as positive one. H_2O is the experiment negative control.

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Table 1. Patients characteristics

Demographics	Value (%)
Age	
Median	63.5
Range	29–81
Sex	
Male	47 (69)
Female	21 (31)
Clinical characteristics	**No. of patients (%)**
Performance status	
0	37 (54)
1	31 (46)
Tumor grade at diagnosis	
G1	2 (3)
G2	43 (63)
G3	9 (13)
Not available	14 (21)
No. of prior treatments	
2	14 (21)
3	18 (26)
4	23 (35)
5	5 (7)
6	6 (9)
7	2 (3)
Tumor KRAS status	
Wild-type	35 (51)
Mutated	33 (49)
G12V	7
G12C	5
G12S	1
G12D	7
G12A	1
G13D	5
Codon not available	7
Tumor MGMT methylation status	
Hypermethylated	26 (38)
Unmethylated	39 (58)
Not assessable	3 (4)
No. of metastatic sites	
1	2 (3)
2	25 (37)
3	29 (43)
4	11 (16)
5	1 (1)
Patients previously treated with:	
Bevacizumab	59 (87)
Mitomycin	17 (25)
Experimental drugs (clinical trial)	8 (12)

Statistical analysis

According to clinical considerations and on the basis of the available literature, the efficacy of a treatment in this setting of mCRC chemorefractory patients would be considered poor if the ORR is 3% or less, whereas it could be considered of clinical usefulness if the ORR is 10% or more. Assuming $\alpha = 0.05$ and $\beta = 0.20$, a Simon Optimal 2-stage design has been then chosen to test the null hypothesis that $P \leq 0.03$ versus the alternative that $P \geq 0.10$. According to this design, if at least 2 of the first 40 patients would have achieved an objective response, enrollment would have been extended by 28 patients. Overall, objective response rate of dacarbazine monotherapy would have been deemed unacceptable if objective response was 4 or less. The association between *MGMT* promoter methylation status and ORR and DCR was determined by 2-sided Student *t*-tests or Fisher exact test. PFS was estimated by Kaplan–Meier product-limit method followed by log-rank test.

Results

Patients' characteristics

Sixty-eight patients were enrolled in our institution from May 2011 until March 2012. All patients had progressed on fluoropyrimidines, oxaliplatin, irinotecan, and cetuximab or panitumumab (the latter 2 drugs if *KRAS* wild-type). 87% of patients had received prior bevacizumab and 19% patient had received more than 4 lines of treatment. Twenty percent of patients received mitomycin C, 4% raltitrexed, and 12% previous experimental agents within clinical trials. Clinical characteristics of patients in this trial are reported in Table 1. Reasons for discontinuation of dacarbazine treatment included hematologic toxicity (1 patient), progression (61 patients), death (4 patients), and withdrawal of consent (2 patients). Cause of death was recorded as mCRC in all deceased patients.

Toxicity

Adverse events are listed in Supplementary Table S1. Hematologic toxicity was the most frequent adverse event reported and general toxicity was consistent with the known toxicity profile of dacarbazine. We observed 3 hematologic G4 adverse events (2 platelet count decreased and one neutrophil count decreased). Hepatic failure with increased bilirubin due to progression of disease was observed in 3 patients with extensive metastatic liver involvement.

Analysis of MGMT promoter hypermethylation

Sixty-five of 68 patients were tested for *MGMT* promoter CpG island methylation, as showed in Table 1. Overall, *MGMT* hypermethylation was found in 40% (26/65) of the colorectal neoplasms DNAs analyzed, a similar frequency to the previously reported for this tumor type (9). According to the location of the tumor, *MGMT* promoter status was assessed in 69% (45/65) in primary tumor, in 14% (9/65) in metastatic site, and in 17% (11/65) in both primary

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and metastatic site from the same patient. In the latter case, we observed concordance in 10 of 11 pairs, with only one case showing a hypermethylated primary with unmethylated liver metastasis, and the result from liver metastasis was considered for the purpose of analysis. Sites of metastases were: liver 75% (15/20), 5% (1/20) ovary, 10% (2/20) lung, 5% (1/20) spleen, and 5% (1/20) cutaneous. *MGMT* hypermethylation was more frequent (61% and 31%, respectively) in tumors carrying *KRAS* mutation with G > A transition (G12D, G12V, or G13D), as previously described (10, 11), although the difference was not statistically significant due to the small size (only 26 patients were evaluable for both analysis; $P = 0.238$).

Antitumor activity of dacarbazine

ORR was 3%, with 2 partial responses. Stable disease was achieved in 8 of 68 patients (12%), accounting for a DCR (partial response + stable disease) of 15%. Median PFS was 57 days. Preplanned analysis of secondary endpoints based on assessments of MGMT methylation and KRAS mutation status in individual tumors showed that objective responses occurred only in patients displaying MGMT-methylated tumors (Fig. 2A and Fig. 3). In addition, we observed a significantly higher DCR (44.0% vs. 6%, $P = 0.012$) in the MGMT-hypermethylated group (Fig. 2). A trend toward better PFS [HR = 0.66; 95% confidence interval (CI) 0.40–1.10; $P = 0.0982$)] was also found in the MGMT-hypermethylated cases (Fig. 4A). A similar tendency was found between reduction of tumor volume following dacarbazine treatment and MGMT methylation status: tumor shrinkage of any size occurred more frequently in patients displaying *MGMT* hypermethylation (Fisher exact test, $P = 0.093$). In contrast, KRAS status was not associated with PFS, DCR, and ORR (KRAS mutant vs. KRAS wild-type, $P = 0.735$, 0.999, and 0.492, respectively; Fig. 4B).

Discussion

In this study, we document that dacarbazine is active after failure of standard therapies only in those patients with mCRC whose tumor is harboring epigenetic inactivation of the DNA repair enzyme *MGMT*. Overall, we observed 2 objective responses, accounting for 3% of ORR, and 8 stable diseases, accounting for 12% of the cases. The observation of a significant association between *MGMT* promoter hypermethylation and these clinical endpoints supports the

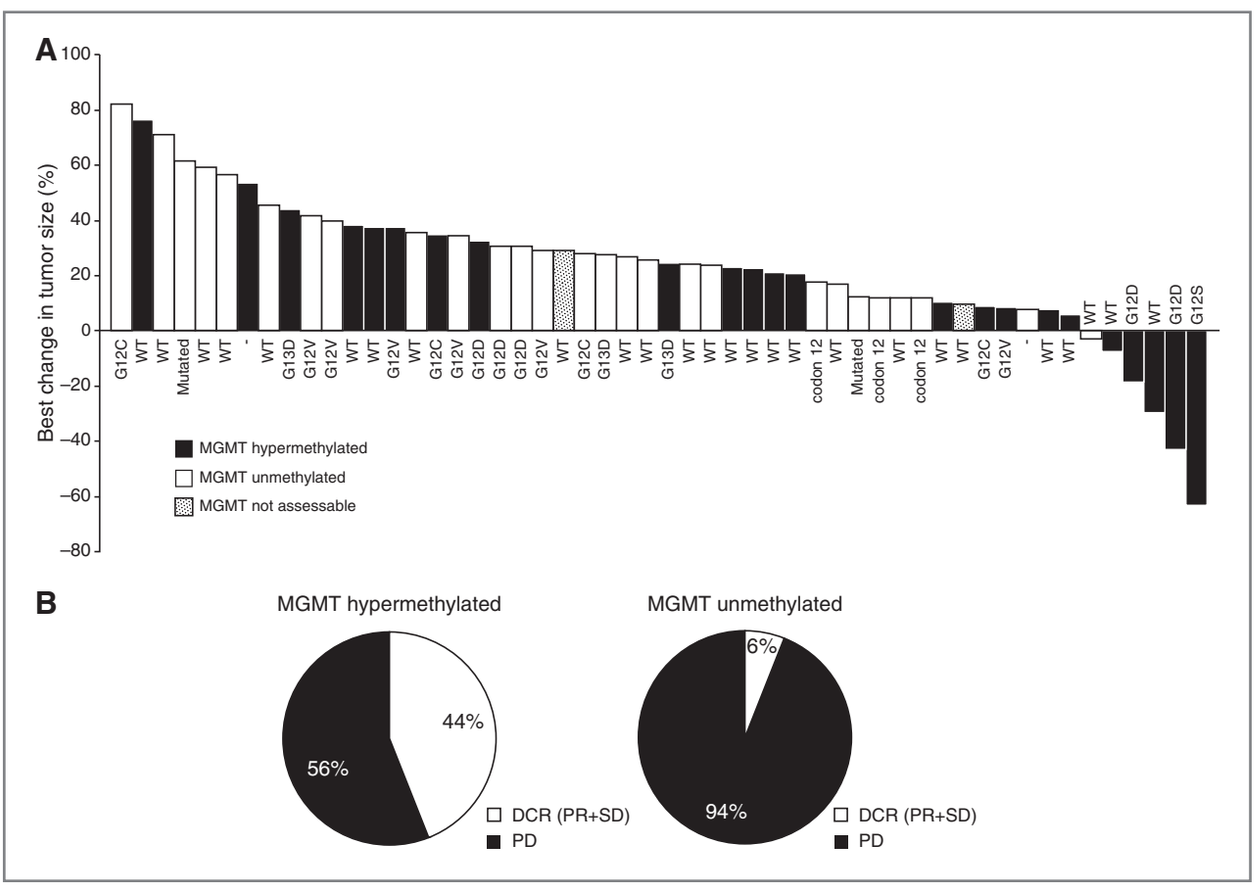

Figure 2. A, waterfall plot showing best change in tumor size (%) along with *MGMT* promoter methylation status (hypermethylated/unmethylated) and *KRAS* mutation status, if available. WT, *KRAS* wild-type; mutated, type of *KRAS* mutation not available. B, pie-charts showing disease control rate [DCR = partial response (PR) + stable disease (SD)] according to *MGMT* promoter methylation in individual CRC tumors.

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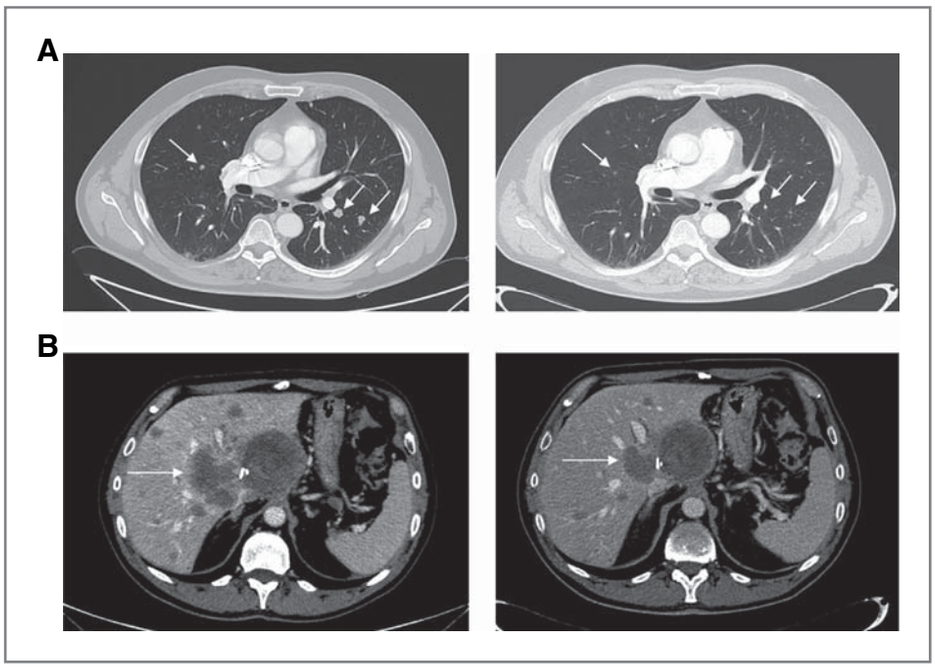

Figure 3. Computed tomography scan showing tumor shrinkage (white arrows) after treatment with dacarbazine in 2 patients, one with lung (A) and another with liver (B) metastases, both displaying *MGMT* promoter hypermethylation in primary tumor.

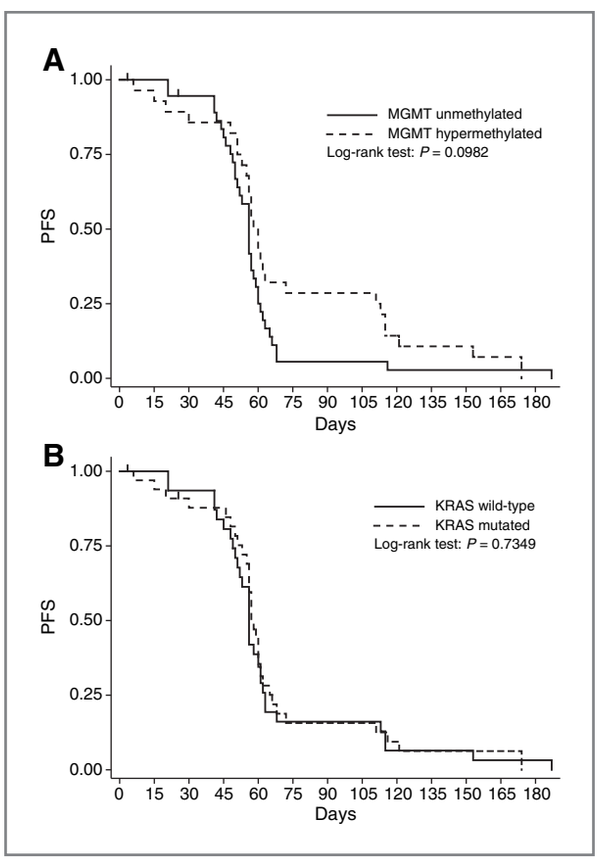

Figure 4. A, Kaplan–Meier PFS survival analysis according to *MGMT* status in individual tumors. B, Kaplan–Meier PFS survival analysis according to *KRAS* status in individual tumors.

hypothesis that DNA repair-defective mCRC tumors are more susceptible to this chemotherapeutic agent. However, even in the case of *MGMT* hypermethylation, we observed that a fraction of 44% of patients achieved control of disease (stable disease + partial response), thus suggesting that a multiparametric signature including the DNA methylation-associated silencing of *MGMT* together with other molecular traits would improve the identification of CRC tumors with defects in DNA repair, susceptible to the action of dacarbazine.

The low response rate observed in the present cohort could be linked to the inclusion of heavily pretreated patients (median 4 lines of previous treatments). To interpret this clinical result in the context of therapy-resistant mCRC, one should consider that second-line treatment with FOLFIRI or FOLFOX combination regimens induces ORR of 10% to 12% (24–26) and dramatically decreases in subsequent lines (6). It is also known that dacarbazine is activated in liver by CYP^{450} microsomial N-demethylation with formation of 5-[3-hydroxymethyl-3-methyl-triazen-l-yl]-imidazole-4-carboxamide and 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC). Rapid decomposition of MTIC produces the major plasma and urine metabolite 5-amino-imidazole-4-carboxamide and the reactive species methane diazohydroxide, which produces molecular nitrogen and a methyl cation supposed to be the methylating species (27). It is therefore conceivable that the multiple (median 4) previous lines of cancer treatment as well as the high (79%) rate of liver involvement in the present study population may have exhausted the liver function capacity to activate dacarbazine.

It was our hypothesis that anticancer activity of dacarbazine could be enhanced by a specific defect in DNA

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repair system as evaluated by *MGMT* promoter hypermethylation in individual tumors. This epigenetic defect occurs in about 35% to 40% of mCRCs (9) and it is detected in more than 70% of *KRAS*-mutated tumors carrying the G > A transitions subtypes of mutation (10, 11), a subgroup of mCRCs with limited therapeutic options. Although the present trial was not designed, and thus, powered to assess a significant difference in PFS between *MGMT*-hypermethylated/unmethylated groups, we observed a trend toward better PFS in the *MGMT*-hypermethylated group, together with a better DCR. The 2 patients displaying objective response were indeed carrying MGMT-hypermethylated tumors (Fig. 2A) and one of them showed a long-lasting maintenance of response of 6 months, which is uncommon in the advanced setting of mCRC.

In conclusion, present data document that specific DNA repair defects can be associated with susceptibility to dacarbazine. The use of an alkylating agent that does not require hepatic activation may be preferable in heavily pretreated patients with metastatic liver disease. In this regard, temozolomide is an alkylating agent whose activity is also enhanced in tumors with *MGMT* loss (17) that is hydrolyzed in cells producing the active compound MTIC without requiring liver passage. A phase II trial with temozolomide has been designed and it is ongoing at our institution to assess the efficacy in patients with *MGMT* hypermethylated mCRCs after failure of standard therapies.

Disclosure of Potential Conflicts of Interest

Andrea Sartore-Bianchi has received honoraria from speakers' bureau from Bayer, Roche, and Amgen and is a consultant/advisory board member of Amgen. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Amatu, A. Sartore-Bianchi, A. Belotti, K. Bencardino, A. Cassingena, F. Rusconi, S. Siena
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Management of data relating the clinical trial in Italian database, drug receipt: A. Esposito

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SUPPLEMENT II

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ARTICLE

Epigenetic Inactivation of the BRCA1 Interactor SRBC and Resistance to Oxaliplatin in Colorectal Cancer

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Background A major problem in cancer chemotherapy is the existence of primary resistance and/or the acquisition of secondary resistance. Many cellular defects contribute to chemoresistance, but epigenetic changes can also be a cause.

Methods A DNA methylation microarray was used to identify epigenetic differences in oxaliplatin-sensitive and -resistant colorectal cancer cells. The candidate gene *SRBC* was validated by single-locus DNA methylation and expression techniques. Transfection and short hairpin experiments were used to assess oxaliplatin sensitivity. Progression-free survival (PFS) and overall survival (OS) in metastasic colorectal cancer patients were explored with Kaplan–Meier and Cox regression analyses. All statistical tests were two-sided.

Results We found that oxaliplatin resistance in colorectal cancer cells depends on the DNA methylation–associated inactivation of the BRCA1 interactor *SRBC* gene. SRBC overexpression or depletion gives rise to sensitivity or resistance to oxaliplatin, respectively. *SRBC* epigenetic inactivation occurred in primary tumors from a discovery cohort of colorectal cancer patients (29.8%; n = 39 of 131), where it predicted shorter PFS (hazard ratio [HR] = 1.83; 95% confidence interval [CI] = 1.15 to 2.92; log-rank $P = .01$), particularly in oxaliplatin-treated case subjects for which metastasis surgery was not indicated (HR = 1.96; 95% CI = 1.13 to 3.40; log-rank $P = .01$). In a validation cohort of unresectable colorectal tumors treated with oxaliplatin (n = 58), *SRBC* hypermethylation was also associated with shorter PFS (HR = 1.90; 95% CI = 1.01 to 3.60; log-rank $P = .045$).

Conclusions These results provide a basis for future clinical studies to validate *SRBC* hypermethylation as a predictive marker for oxaliplatin resistance in colorectal cancer.

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Colorectal cancer (CRC) is the second most common cause of cancer death in the western world (1). In metastatic CRC, polychemotherapy based on fluoropyrimidines plus oxaliplatin or irinotecan, combined with biological agents such as cetuximab and panitumumab, is the gold-standard treatment (2). Oxaliplatin forms intrastrand adducts that disrupt DNA replication and transcription (3,4). DNA damage induced by oxaliplatin is repaired in part by the nucleotide excision repair pathway (5), but the DNA double-strand breaks induced by the drug are also repaired by the BRCA1 complex (6–8). In this regard, epigenetic inactivation of the *BRCA1* gene by promoter CpG island methylation has been associated with increased sensitivity to cisplatin and carboplatin in breast and ovarian cancer (9,10).

Genes critical to colorectal tumor biology are frequently inactivated by hypermethylation of the CpG dinucleotides located in their 5'-CpG island regulatory regions (11–13). We wondered whether this epigenetic alteration was involved in the resistance to oxaliplatin in CRC, where treatment failure due to primary or acquired resistance remains a major obstacle to the management of the disease. Herein, we demonstrate that the epigenetic inactivation of the BRCA1 interactor *SRBC* gene by promoter CpG island hypermethylation is associated with poor outcome upon oxaliplatin treatment.

Methods

Cell Lines

LoVo parental cell line (LoVo-S) and its derived 10-fold oxaliplatin-resistant cells (LoVo-R)(14) were cultured at 37ºC in an atmosphere of 5% (v/v) carbon dioxide in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM-HAM's F12) medium supplemented with 20% (w/v) fetal bovine serum, 100 U penicillin, and 100 µg/L streptomycin (Invitrogen, Carlsbad, CA). The HCT-116, SW48, SW480, SW620, RKO, Co115, and HCT-15 CRC cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were authenticated by short tandem repeat profiling.

Determination of Drug Resistance

Oxaliplatin (5 mg/mL) and 5-fluorouracil (50 mg/mL) were obtained from TEVA (North Wales, PA) and Accord Healthcare SLU (Barcelona, Spain), respectively. Cell viability was determined by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 1×10^3 cells were plated onto 96-well plates. Cells were treated for 120 hours with different drug concentrations (oxaliplatin: 0–250 μM; 5-fluorouracil: 0–35 μM). MTT was added at a final concentration of 0.1%. After 2.5 hours of incubation (37 ºC; 5% carbon dioxide), the MTT metabolic product formazan was dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm. Prism Software (La Jolla, CA) was used to calculate the drugs' half-maximal inhibitory concentration (IC_{50}).

DNA Methylation Analyses

DNA was subjected to bisulfite using EZ DNA methylation kit (Zymo Research, Orange, CA) as previously described (15). To perform the genome-wide DNA methylation profiling we used the Illumina Infinium HumanMethylation27 BeadChip (Illumina, San Diego, CA) microarray following the manufacturer's instructions (15).The Infinium assay quantifies DNA methylation levels at specific cytosine residues adjacent to guanine residues (CpG loci), by calculating the ratio (β value) of intensities between locus-specific methylated and unmethylated bead-bound probes. The β value is a continuous variable, ranging from 0 (unmethylated) to 1 (fully methylated). This microarray assesses the DNA methylation level of 27 578 CpG sites located at the promoter regions of 14 495 protein-coding genes. DNAs were processed on the same microarray to avoid batch effects. The array was scanned by a Bead Array Reader (Illumina), and intensity data were analyzed using Genome Studio software (version 2011.1; Illumina). Further details are described in the Supplementary Methods (available online). The data is freely avalilable at GeneExpressionOmnibus (http://www.ncbi.nlm.nih.gov/geo/) under GEO accession code GSE44446.

We established *SRBC* CpG island methylation status using three different polymerase chain reaction (PCR)–based techniques: bisulfite genomic sequencing of multiple clones, methylation-specific PCR, and pyrosequencing. Further technical details are described in the Supplementary Methods (available online).The used primer sequences are shown in Supplementary Table 1 (available online).

mRNA and Protein Expression Analyses

mRNA extraction, cDNA synthesis, conventional and quantitative real-time PCR (RT-PCR) using Hs00376942_m1Taqman Gene Expression assay (Applied Biosystems. Madrid, Spain) were performed as previously described (16). Primer sequences are shown in Supplementary Table 1 (available online). Anti-SRBC (1/1000) from Cell Signaling and anti-β-actin-HRP antibody (1/20 000) from Sigma (St. Louis, MO) were used to develop the western blot analysis.

SRBC Transfection and Depletion Experiments

Human short hairpin RNAs and cDNA plasmids for SRBC were obtained from Origene (Rockville, MD). After *Escherichia coli* transformation, we proceeded to plasmid DNA purification. Forty-eight hours after electroporation, cells transfected with short hairpin RNAs (TR317747; Origene) were grown in medium containing 0.8 or 0.6 μg/mL of puromycin (LoVo-S and HCT-116). Cells transfected with SRBC cDNA (SC320781; Origene) were grown with DMEM containing 0.8 or 0.6 mg/mL of geneticin (G418, LoVo-R, and HCT-15) to perform clonal selection. Once selected, clones were picked, grown, and tested by Western blot.

Patients

In our study, we analyzed two independent cohorts of white, stage IV CRC patients (17). In the discovery set, 131 metastatic CRC primary tumors that received oxaliplatin plus fluoropirimidines–based therapy were retrospectively included. Formalin-fixed paraffin-embedded tumors obtained by surgical resection came from three different hospitals (ICO-Hospitalet, ICO-Badalona, and Niguarda Ca' Granda). Clinical features of the patients are showed in Table 1. From this cohort, 65 patients could undergo surgery to remove metastases. After neoadjuvant regimen, 34 could be operated, and 31 received palliative regimen. The rest of the patients (n = 66) showed unresectable metastases and directly underwent palliative regimen. The greatest time of follow-up of this group was near 10 years. The validation cohort consisted of 58 stage IV CRC patients from the Hospital Vall d'Hebron with a follow-up of nearly 3 years (Table 1). According to discovery set results, we selected patients with unresectable metastases who received oxaliplatin plus fluoropirimidines–based therapy in a neoadjuvant (n = 20) or palliative regimen (n = 38). The distribution of patients according to the different clinical features was similar in both cohorts. Signed informed consent was obtained from each patient, and the Clinical Research Ethical Committee from ICO-Hospitalet provided approval for the study. DNA from all case patients was obtained from formalin-fixed paraffin-embedded tissue sections (10 μm) by xilol deparafination and digestion by proteinase K (Qiagen, Manchester, UK). Tumor specimens were composed of at least 70% carcinoma cells. DNA extraction was performed using a commercial kit (Qiagen) following the manufacturer's instructions.

Statistical Analysis

In both independent cohorts we analyzed *SRBC* promoter methylation status and its association with response rate, progression-free survival (PFS), and overall survival (OS). The associations between categorical variables were assessed by χ^2 tests or Fisher exact test whenever required. Kaplan–Meier plots and log-rank test were used to estimate PFS and OS. The association between epigenetic variant and clinical parameters with PFS and OS was assessed through univariate and multivariable Cox proportional hazards regression models. The proportional hazards assumption for a Cox regression model was tested under R statistical software (Boston, MA) (cox.zph function). Statistical analysis was performed by using SPSS for Windows, (Armonk, NY) and *P* values less than .05 were considered statistically significant. All statistical tests were two-sided.

Results

Identification of Epigenetics Changes Associated With Oxaliplatin Resistance Using a DNA Methylation Microarray

To address in an unbiased manner whether epigenetic changes can be associated with oxaliplatin resistance, we adopted a whole genomic approach by comparing the DNA methylation status of

Table 1. Clinical features of the discovery and validation cohorts of stage IV colorectal samples included in the study*

	Discovery cohort (n = 131)							Validation cohort (n = 58)						
	SBRC according to methylation status							SBRC according to methylation status						
			Unmethylated		Methylated					Unmethylated		Methylated		
Characteristic	No.	%	No.	%	No.	%	OR (95% CI)	No.	%	No.	%	No.	%	OR (95% CI)
Sex														
Male	85	64.9	61	71.7	24	28.3	1.00 (referent)	35	60.3	29	82.8	6	17.2	1.00 (referent)
Female	46	35.1	31	67.4	15	32.6	1.13 (0.85 to 1.47)	23	39.7	15	65.2	8	34.8	0.60 (0.32 to 1.10)
Primary tumor														
Colon	102	77.8	72	70.6	30	29.4	1 (referent)	41	70.7	32	78.1	9	21.9	1.00 (referent)
Rectum	29	22.2	20	68.9	9	31.1	0.94 (0.47 to 1.25)	17	28.3	12	70.6	5	29.4	0.76 (0.33 to 1.79)
Metastatic site														
Liver	81	61.8	52	64.2	29	35.8	1.00 (referent)	47	81.0	35	74.5	12	25.5	1.00 (referent)
Lung	9	6.9	5	55.5	4	44.5	0.72 (0.21 to 2.51)	3	5.2	2	66.7	1	33.3	0.70 (0.07 to 7.12)
Others	18	13.7	15	83.3	3	16.7	2.39 (0.74 to 7.66)	8	13.8	7	87	1	13	2.10 (0.29 to 16.1)
Unknown	23	17.6	20	86.9	3	13.1	—	0	0	0	0	0	0	—
Chemotherapy schedule														
Oxaliplatin / 5-FU	107	81.7	74	69.2	33	30.8	1.00 (referent)	41	70.7	32	78.1	9	21.9	1.00 (referent)
Oxaliplatin / CAPE	10	7.6	8	80.0	2	20.0	1.71 (0.38 to 7.64)	0	0	0	0	0	0	—
Oxaliplatin / 5-FU / BA	13	9.9	9	69.2	4	30.8	1.01 (0.33 to 3.05)	17	29.3	12	70.6	5	29.4	0.76 (0.33 to 1.79)
Oxaliplatin / CAPE / BA	1	0.8	1	100	0	0	—	0	0	0	0	0	0%	—
Chemotherapy regimen														
Neoadjuvant	65	49.6	41	63.1	24	36.9	1.00 (referent)	20	34.5	15	75.0	5	25.0	1.00 (referent)
Palliative	66	50.4	51	77.3	15	22.7	1.47 (0.95 to 2.27)	38	65.5	29	76.3	9	23.7	1.02 (0.66 to 1.60)
Surgery of metastasis														
No	97	74.1	72	74.3	25	25.7	1.00 (referent)	58	100	44	75.9	14	24.1	—
Yes	34	25.9	20	58.8	14	41.2	0.61 (0.34 to 1.07)	0	0	0	0	0	0	—

* None of the relationships were statistically significant after using the two-sided χ^2 test, considering $P < .05$ as statistical significant threshold. 5-FU = 5-fluorouracil; BA = biological agents; CAPE = capecitabine.

27 000 CpG sites (15) in an oxaliplatin-sensitive CRC cell line (LoVo-S) and an oxaliplatin-resistant clone (LoVo-R) that we derived by exposure to increasing concentrations of the drug (14).

This approach yielded only three differentially methylated target genes: *SRBC* (protein kinase C delta binding protein), *FAM111A* (family with sequence similarity 111, member A) and *FAM84A* (family with sequence similarity 84, member A) (Supplementary Figure 1A, available online). The most noteworthy gene with the highest difference in DNA methylation was *SRBC*; thus, it was the logical option to pursue. However, we also studied initially the other two genes. For *FAM111A*, bisulfite genomic sequencing of multiple clones showed that indeed the CpG site included in the DNA methylation microarray was distinctly methylated in LoVo-S and LoVo-R cells; however, the remaining sites of the CpG island were unchanged (Supplementary Figure 1B, available online). Thus, we excluded this gene from further experiments. For *FAM84A*, bisulfite genomic sequencing confirmed the differential methylation of the CpG island, but both conventional and quantitative RT-PCR did not show any difference in gene expression (Supplementary Figure 1, D and E, available online). Thus, we also excluded this second gene from further analyses. For the main target gene, *SRBC*, the DNA methylation microarray data showed that it had a CpG site located in its 5'-CpG island (–155 base-pair position) that was hypermethylated in LoVo-R but unmethylated in LoVo-S (Supplementary Figure 1A, available online). Interestingly, *SRBC* CpG island methylation-associated silencing has already been found in cancer (18,19), including colorectal tumors (20). From a functional standpoint, it is biologically plausible that *SRBC* is responsible for the different sensitivity to oxaliplatin because its protein interacts with the product of the *BRCA1* gene (18), which is widely accepted as being a mediator of response to DNA damage induced by platinum compounds (21).

To further demonstrate the presence of *SRBC* 5'-CpG island methylation in resistant cells, we undertook bisulfite genomic sequencing analyses. We found CpG island hypermethylation in LoVo-R but mostly an unmethylated CpG island in LoVo-S (Figure 1A). Importantly, SRBC expression was diminished in LoVo-R, showing CpG island methylation, whereas it was expressed in the unmethylated LoVo-S at the mRNA and protein levels (Figure 1B). SRBC re-expression was observed upon treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine in LoVo-R cells (Figure 1B).

SRBC Epigenetic Inactivation and Oxaliplatin Resistance

We next sought to demonstrate that the epigenetic inactivation of this gene functionally contributed to oxaliplatin resistance. We restored the expression of SRBC in LoVo-R by stably transfecting an exogenous expression vector (Figure 1C). Upon SRBC transfection, the cells proved to be statistically significantly more sensitive to the antiproliferative activity of oxaliplatin measured by the MTT

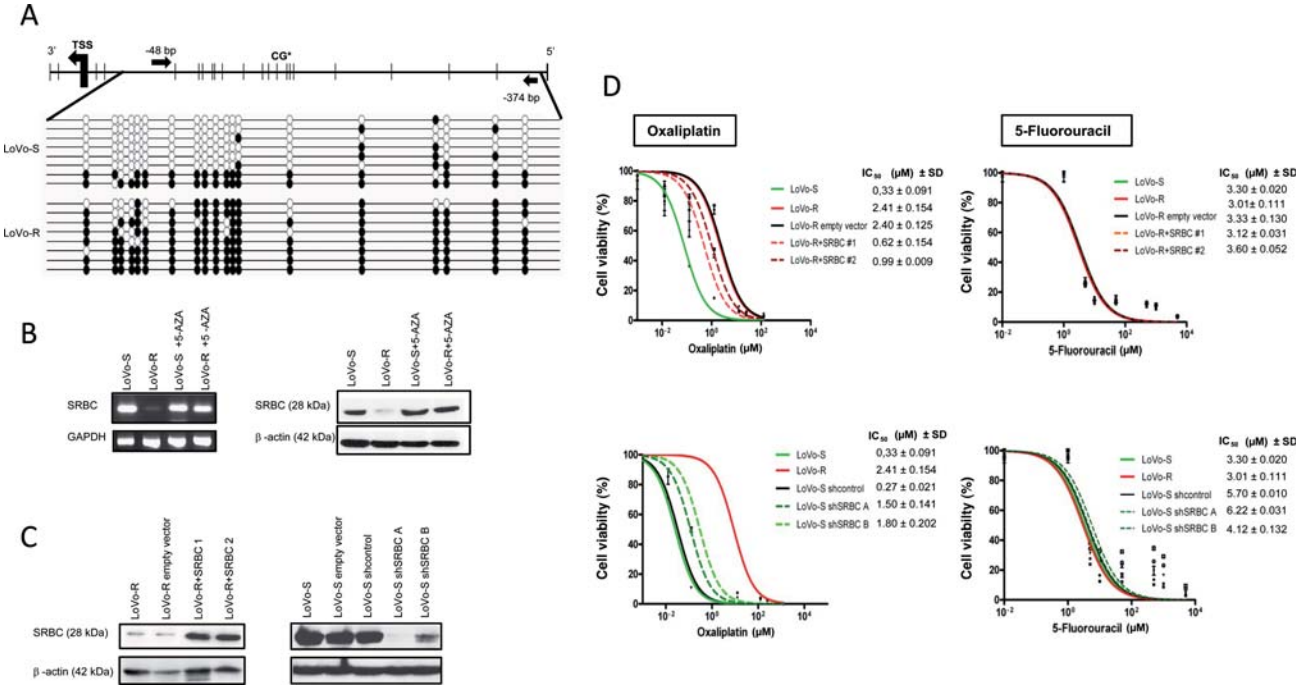
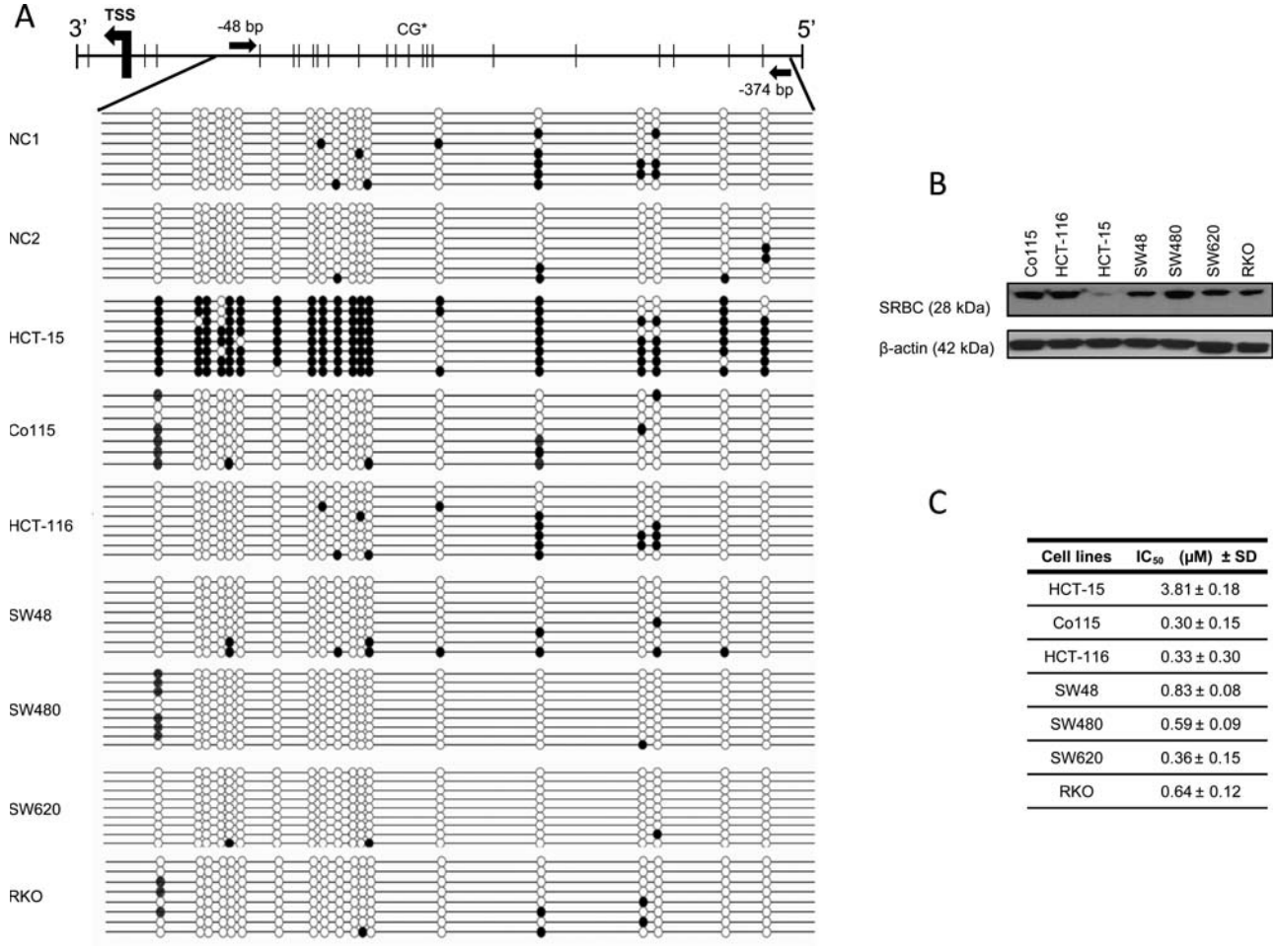


Figure 1. Epigenetic inactivation of *SRBC* is associated with resistance to oxaliplatin in colon cancer cells. **A**) Bisulfite genomic sequencing of eight individual clones in the *SRBC* promoter CpG island was used to determine DNA methylation status. Presence of a methylated or unmethylated cytosine is indicated by a **black** or **white square**, respectively. **Black arrows** indicate the position of the bisulfite genomic sequencing primers. **B**) SRBC expression determined by semiquantitative real-time polymerase chain reaction analyses (**left**) and Western blot (**right**). GAPDH and β-actin were used as controls, respectively. The oxaliplatin-resistant cell line (LoVo-R) features a hypermethylated CpG island that is associated with the downregulation of the SRBC transcript and protein, in comparison with the SRBC-unmethylated and expressing oxaliplatin-sensitive cells (LoVO-S). Pharmacological treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine (5-AZA) restores SRBC expression. **C**) Western blot showing the in vitro enhancement (transfection in LoVo-R, **left**) or depletion (short hairpin [sh] RNA approach in LoVo-S, **right**) of the SRBC protein. **D**) Cell viability determined by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay upon use of oxaliplatin. External intervention by inducing SRBC overexpression (in LoVo-R cells) or depletion (in LoVo-S cells) gives rise to sensitivity or resistance to oxaliplatin, respectively (**left panels**). 5-Fluorouracil sensitivity is not dependent on SRBC activity (**right panels**). The corresponding half-maximal inhibitory concentration (IC_{50}) values are also shown. SD = standard deviation.

assay (Figure 1D) than were the empty vector-transfected cells (LoVo-R + SRBC 1 and 2: $P = .02$ and $P < .001$, respectively). In sharp contrast, we observed that SRBC stable downregulation by the short hairpin RNA approach in SRBC-expressing and unmethylated sensitive cells (LoVo-S) (Figure 1C) had the opposite effect: a considerable enhancement of the resistance to the antiproliferative effect mediated by oxaliplatin (Figure 1D) (LoVo-S short hairpin SRBC A and B: $P = .04$ and $P < .001$, respectively). The observed effects were specific for oxaliplatin because the in vitro depletion or enhancement of SRBC activity did not change the sensitivity to 5-fluorouracil (Figure 1D), other drug commonly used in CRC.

We extended our study to seven additional CRC cell lines (Co115, HCT-15, HCT-116, SW48, SW480, SW620, and RKO), in which we found *SRBC* promoter CpG island hypermethylation (Figure 2A) and the associated loss of expression only in HCT-15 cells (Figure 2B). Interestingly, these cells were the only ones showing resistance to oxaliplatin (IC_{50} ± standard deviation = 3.81 ± 0.18 μM); the remaining cells were sensitive to the drug (Figure 2C) (IC_{50} values ranging from 0.30 to 0.83 μM). As we did with LoVo-S and LoVo-R, we also sought to demonstrate that *SRBC* epigenetic inactivation functionally contributed to oxaliplatin resistance in these cells. We restored the expression of SRBC in the resistant cell line HCT-15 by stably transfecting an exogenous expression vector (Supplementary Figure 2A, available online). Upon SRBC transfection, the cells proved to be statistically significantly more sensitive to the antiproliferative activity of oxaliplatin (HCT15 + SRBC: $P = .02$) (Supplementary Figure 2B, available online). The opposite effect was observed with SRBC stable downregulation using the short hairpin RNA approach in SRBC-expressing and unmethylated sensitive cells (HCT-116): a noteworthy increase in the resistance to the antiproliferative effect mediated by oxaliplatin was found (Supplementary Figure 2B, available online) (HCT-116 short hairpin SRBC A and B: $P < .001$). The described effects were specific for oxaliplatin because the in vitro depletion or enhancement of SRBC activity did not change the sensitivity to 5-fluorouracil (Supplementary Figure 2B, available online). Western blot analyses showed that the level of expression of the SRBC protein in the transfected clones was similar to



Cell lines	IC_{50} (μM) ± SD
HCT-15	3.81 ± 0.18
Co115	0.30 ± 0.15
HCT-116	0.33 ± 0.30
SW48	0.83 ± 0.08
SW480	0.59 ± 0.09
SW620	0.36 ± 0.15
RKO	0.64 ± 0.12

Figure 2. Epigenetic inactivation of *SRBC* is associated with oxaliplatin resistance in colorectal cancer cell lines. **A**) Bisulfite genomic sequencing of eight individual clones in the *SRBC* promoter CpG island was used to determine DNA methylation status. Presence of a methylated or unmethylated cytosine is indicated by a **black** or **white square**, respectively. **Black arrows** indicate the position of the bisulfite genomic sequencing primers. HCT-15 cells are the only cells that present *SRBC* promoter CpG island hypermethylation. Normal colon mucosa samples (NC1 and NC2) are unmethylated. **B**) Western blot analyses for SRBC expression show that the hypermethylated CpG island in HCT-15 cells is associated with loss of protein in comparison with the remaining *SRBC*-unmethylated and -expressing colon cancer cell lines. **C**) Half-maximal inhibitory concentration (IC_{50}) values, determined by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay assay, upon use of oxaliplatin in the panel of colon cancer cell lines. All the studied cells are sensitive to oxaliplatin except the *SRBC*-hypermethylated and -silenced HCT-15 cell line.

that observed in unmethylated CRC cell lines (Supplementary Figure 2A, available online).

SRBC Hypermethylation and PFS in Oxaliplatin-Treated Cases of Unresectable Colorectal Cancer

Given these in vitro findings that colon cancer cells with *SRBC* methylation-associated silencing were resistant to oxaliplatin, we wondered whether the same effect could be observed in clinical samples. The study of a first clinical cohort of 131 stage IV colorectal adenocarcinoma patients (termed "discovery cohort") (Table 1), all of whom were treated with oxaliplatin in combination with a fluoropyrimidine, showed SRBC methylation in 29.8% (n = 39 of 131) of the case patients analyzed by both methylation-specific PCR and pyrosequencing analyses (Figure 3A; Supplementary Figure 3, available online). The described occurrence of *SRBC* hypermethylation in colorectal tumors was identical to the one available in the The Cancer Genome Atlas datasets (30.2%; n = 70 of 232). Considering the whole population of studied advanced CRC case patients (n = 131), we observed that *SRBC* hypermethylation was associated with PFS (HR = 1.83; 95% confidence interval [CI] = 1.15 to 2.92; log-rank P = .01) (Figure 3B). For the 105 case patients for whom OS information was available, SRBC hypermethylation was not associated with this variable (Figure 3C).

According to Cox regression multivariable test, surgery of metastases showed to be an independent PFS (HR = 0.43; 95% CI = 0.24 to 0.76; log-rank P = .004) and OS (HR = 0.16; 95% CI = 0.04 to 0.52; log-rank P = .003) prognostic factor (Supplementary Figure 4, available online). Taking this into account, our cohort was stratified in relation to this clinical feature and was divided into two groups: patients that underwent metastases resection (n = 34) and patients with unresectable metastases (n = 97). Subdividing the discovery cohort into these resectable or unresectable groups, SRBC hypermethylation did not have any predictive effect in PFS and OS for those case patients that received oxaliplatin as neoadjuvant therapy followed by the successful resection of the metastases (Supplementary Figure 5, available online).

However, the scenario was completely different in the context of patients with colorectal adenocarcinomas with unresectable metastases who received oxaliplatin as neoadjuvant therapy and were subsequently not eligible for surgery (n = 31) or patients with tumors that were originally classified as unresectable and were given oxaliplatin as palliative chemotherapy (n = 66). For these 97 oxaliplatin-treated advanced CRC case patients with unresectable metastases, *SRBC* CpG island hypermethylation was statistically significantly associated with shorter PFS (HR = 1.96; 95% CI = 1.13 to 3.40; log-rank P = .01) (Figure 3D). In this set of case patients, for whom OS data were available for 79 patients, we also observed that SRBC hypermethylation was statistically significantly associated with shorter OS (HR = 2.01; 95% CI = 1.13 to 3.40; log-rank P = .04). These interesting results prompted us to study the *SRBC* methylation status in a second independent set of CRC patients with unresectable metastasis who also received oxaliplatin-based therapy (n = 58) (Table 1). In this validation cohort, we confirmed that the presence of *SRBC* hypermethylation was associated with shorter PFS (HR = 1.90; 95% CI = 1.01 to 3.60; log-rank P = .045) (Figure 4). Thus, the clinical data are similar to the results from the aforementioned cell cultures that suggest increased chemoresistance of SRBC hypermethylated colorectal tumors to oxaliplatin treatment.

Discussion

The preexistence (primary resistance) or the de novo development (secondary resistance) of cellular mechanisms to escape the antitumoral effects mediated by the anticancer compounds probably involve a wide repertoire of genetic and epigenetic (22) events. From a genetics perspective in CRC, it has been described that the presence of *KRAS* mutations and gene amplification of the *EGFR* or *MET* genesis is associated with resistance to overall anti-EGFR therapies (23,24,25). However, from an epigenetics perspective, very little is known. In spite of promising pharmacoepigenetics biomarkers, such as the example of *MGMT* hypermethylation and good response to temozolamide in gliomas (26), have been described for other tumor types, the examples in colorectal neoplasms are scarce, even more so if we just focus on resistance biomarkers. Herein, we provide an example to help fill this niche by showing that *SRBC* hypermethylation predicts resistance to the commonly used agent oxaliplatin in metastatic CRC, a disease stage that represents the second most common cause of death from cancer (1).

A role of SRBC in mediating different sensitivity to oxaliplatin can be clearly justified by its protein interaction with the product of the *BRCA1* gene (18). The BRCA1 protein exerts an important role in DNA double-strand break repair through homologous recombination 2, so its deficiencies can impair the capacity of cancer cells to repair DNA cross-links caused by chemotherapy drugs such as platinum derivatives (3–7). Two independent studies reported greater primary chemotherapy sensitivity to platinum-based chemotherapy agents in patients with ovarian cancer who were carriers of *BRCA1* germline mutations (5,6). These observations have also been extended to *BRCA1* epigenetic silencing in sporadic breast and ovarian tumors, where it also predicts a good response to cisplatin and carboplatin (9,10,27). However, the biology of mammary tumors is very different from colorectal malignancies, and in all cases of colon cancer, the *BRCA1* promoter has always been found in an unmethylated status (28–30). Interestingly, in addition to its BRCA1-related roles, SRBC might have other functions related to the observed chemoresistance phenotype, such as its interaction with caveolin 1, which may putatively affect intracellular vesicle traffic of the drug (31).

It is worth mentioning two possible avenues of further research. First, there is the possibility to detect *SRBC* hypermethylation by sensitive user-friendly techniques, such as methylation-specific PCR and pyrosequencing, which could be useful in the clinical setting. Instead of always requiring the use of the surgical tumor sample, stool or serum/plasma DNA could be useful alternative biological materials to predict oxaliplatin resistance in CRC patients. In this regard, DNA methylation changes are also amenable for the development of new powerful molecular techniques, such as those recently referred to as "liquid biopsies" (32). Second, our observation that sensitivity to oxaliplatin can be restored by the re-expression of the *SRBC* gene could represent a revival of the DNA demethylating agents in the therapy of solid tumors. With little therapeutic options against metastatic CRC once it has

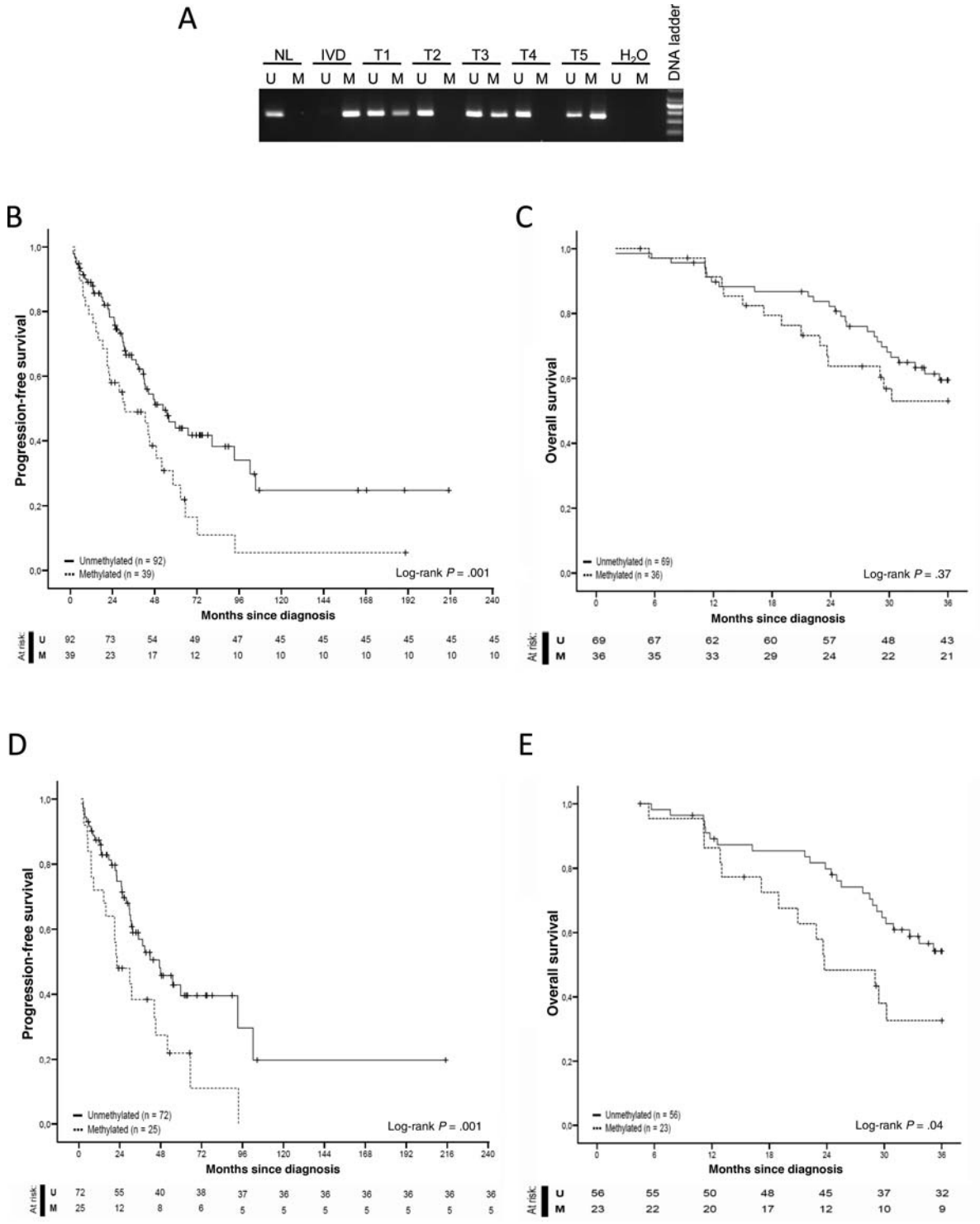


Figure 3. *SRBC* promoter hypermethylation occurs in primary tumors from colorectal cancer patients, where it predicts shorter progression-free survival (PFS) in oxaliplatin-treated case patients. **A**) Analysis by methylation-specific polymerase chain reaction (MSP) of the promoter region of *SRBC* in primary colorectal tumors. The presence of a visible polymerase chain reaction product in lanes marked U indicates unmethylated *SRBC* sequences; the presence of a product in lanes marked M indicates methylated sequences. In vitro methylated DNA (IVD) was used as a positive control for methylated *SRBC* sequences. DNA from normal lymphocytes (NL) was used as a negative control for methylated *SRBC* sequences. MSP of *SRBC* in five colon cancer patients demonstrates *SRBC* promoter hypermethylation in tumors 1, 3, and 5. **B**) Kaplan–Meier analysis of PFS among the whole population of advanced colorectal cancer cases by SRBC methylation status. Numbers of events (progression) are shown from 24 to 240 months in unmethylated (U) and methylated (M) groups. **C**) Kaplan–Meier analysis of overall survival (OS) among the whole population of advanced colorectal cancer cases by SRBC methylation status. Numbers of events (exitus) are shown from 6 to 36 months in unmethylated (U) and methylated (M) groups. **D**) Kaplan–Meier analysis of PFS among the oxaliplatin-treated advanced colorectal cancer case patients with unresectable metastases by *SRBC* methylation status. Numbers of events are shown from 24 to 240 months in unmethylated (U) and methylated (M) groups. **E**) Kaplan–Meier analysis of OS among the oxaliplatin-treated advanced colorectal cancer case patients with unresectable metastases by *SRBC* methylation status. Numbers of events are shown from 6 to 36 months in unmethylated (U) and methylated (M) groups.

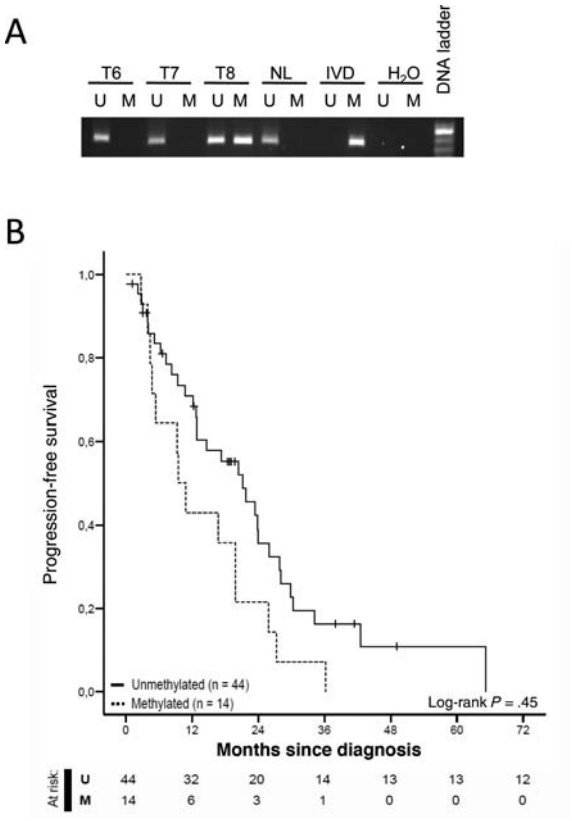


Figure 4. *SRBC* promoter hypermethylation in the validation cohort predicts shorter progression-free survival (PFS) in colon cancer patients with unresectable metastasis treated with oxaliplatin. **A**) Analysis by methylation-specific polymerase chain reaction (MSP) of the promoter region of *SRBC* in primary colorectal tumors. The presence of a visible polymerase chain reaction product in lanes marked U indicates unmethylated SRBC sequences; the presence of a product in lanes marked M indicates methylated sequences. In vitro methylated DNA (IVD) was used as a positive control for methylated SRBC sequences. DNA from normal lymphocytes (NL) was used as a negative control for methylated SRBC sequences. MSP of SRBC in three colon cancer patients demonstrates SRBC promoter hypermethylation in tumor 8. **B**) Kaplan–Meier analysis of PFS among the oxaliplatin-treated advanced colorectal cancer case patients with unresectable metastases (n = 58) by SRBC methylation status. Numbers of events (progression) are shown from 12 to 72 months in unmethylated (U) and methylated (M) groups.

become insensitive to oxaliplatin, DNA methylation inhibitors, such as 5-azacytidine and 5-aza-2′-deoxycytidine, could be used to resensitize these tumors to the oxaliplatin therapy. This idea has been recently explored in non–small cell lung carcinoma patients who had reached the last line of chemotherapy. The subsequent administration of 5-azacytidine was able to rescue previous chemosensitivity (33).

Limitations of our study to be addressed in further research include the lack of knowledge about the molecular mechanisms linking SRBC activity and DNA damage repair triggered by oxaliplatin, the use of nonquantitative DNA methylation assays that will require transformation to quantitative DNA methylation tests to get specific cut offs for a future clinical application, and the extension of our CRC patient data source to stage II and III tumors and samples from other geographical origins.

In conclusion, we have demonstrated that DNA methylation–associated silencing of the BRCA1 interactor gene *SRBC* is associated with the acquisition of chemoresistance to the DNA damaging agent oxaliplatin in CRC both in vitro and in vivo. The validation of *SRBC* hypermethylation as a predictive marker will require further prospective studies. If successful, clinical trials would also be necessary to develop strategies to overcome or prevent the development of SRBC-mediated epigenetic resistance.

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“Never underestimate your ability, to make someone else life better even if you never know it.”

Greg Louganis