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LKB1/ AMPK / TSC2 signaling pathway alterations in Non-Small-Cell-Lung-Carcinoma

Present by:

Itziar de Aguirre Egaña

Thesis Advisors:

Director: Dr. Paula Vertino

Director: Dr. Rafael Rosell i Costa

Itziar de Aguirre Egaña   Dr. PM Vertino   Dr. Rafael Rosell Costa

Badalona, 2014

Paula M. Vertino , Professor at the Department of Radiation Oncology at Emory University School of Medicine and leader of ¨Cancer Genetics and Epigenetics Program¨, Winship Cancer Institute of Emory University (Atlanta, Georgia).

CERTIFICA

Que la Tesi Doctoral titulada: ¨ **LKB1/ AMPK / TSC2 signaling pathway alterations in Non-Small-Cell-Lung-Carcinoma** ¨ , ha estat realitzada per la llicenciada Itziar de Aguirre Egaña sota la seva direcció, en codirecció amb el Dr. Rafael Rosell i Costa, i considera que és apta per a la seva defensa pública davant d'un Tribunal per optar al grau de Doctora per la Universitat Autònoma de Barcelona.

I per tal que quedi constància , signa aquest document .

Dra. Paula M. Vertino

Badalona, Juny 2014

En Rafael Rosell i Costa, Professor Associat del Departament de Medicina de la Universitat Autònoma de Barcelona i director del ¨Cancer Biology & Precision Medicine Program¨ de l´Institut Català d'Oncologia, Hospital Universitari Germans Trias i Pujol,

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Dr. Rafael Rosell i Costa

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Abbreviations

1x TE	*1 mM Tris-HCl (pH 7.5), 1 mM EDTA, sterile solution.*
4EBP1	*Eukaryote initiation factor 4E Binding Protein 1*
5aza-Dc	*5-azadeoxycytidine*
AC	*Adenocarcinoma*
ADP	*Adenosine diphosphate*
AJCC	*American Joint Committee on Cancer*
AKT	*Serine/threonine protein kinase B*
AMPK	*AMP-activated protein kinase*
APC	*Adenomatous Polyposis Coli*
ASC	*Adenosquamous*
ATCC	*American Type Culture Collection*
ATM	*Ataxia-Telangiectasia-Mutated kinase*
ATP	*Adenosine-5'-triphosphate*
BAC	*Bronchioloalveolar carcinoma*
bp	*Base pairs*
BRSK	*Brain Specific protein Kinase*
C	*Cytosine*
CBS domain	*Cystathionine β-Synthase domain*
CH_3	*Methyl group*
CO_2	*Carbon dioxide*
COPD	*Chronic Obstructive Pulmonary Disease*
Cys	*Cysteine*
DECP	*Diethylpyrocarbonate*
DNA	*Deoxyribonucleic acid*
DNMT	*DNA-5-methyltransferase*
EDTA	*Ethylenediaminetetraacetic acid*
FBS	*Fetal bovine serum*
GADPH	*glycerradehyde 3-phophate dehydrogenase*
GAP	*GTPase- activating protein*
GBD	*Glycogen-Binding Domain*

GTP *Guanosine triphosphate*

GTPase *Family of hydrolase enzymes that can bind and hydrolyze GTP*

HDAC *Histone Deacetylase*

IGF1 *Insulin-like growth factor1*

IRS1 *Insulin Receptor Substrate-1*

LCC *Large cell carcinoma*

LKB1$_{l}$ *LKB1 splice variant, generate a protein of 50-kDa*

LKB1 *Serine-Threonine Protein Kinase,*

LKB1$_{s}$ *LKB1 splice variant, generate a protein of 48-kDa*

LOH *Loss of Heterozygosity*

MAPK *Mitogen Activated Protein Kinase*

MARK *MAP/ microtubule affinity regulating kinase*

MBD *Methyl Binding Domain*

MDR1 *Multidrug resistance gene 1*

MECP2 *5-Methyl-cytosine binding protein*

MgCl2 *Magnesium chloride*

MGMT *O6-methylguanine-DNA-methyltransferase*

M-MLV retrotranscriptase *Moloney Murine Leukemia Virus retrotranscriptase*

MO25 *Mouse protein 25*

mRNA *Messenger Ribonucleic Acid*

MSP *Methylation Specific PCR*

mTOR *Mammalian Target of Rapamycin*

NaOH *Sodium hydroxide*

NH4SO4 *Ammmonical Nitrogen*

NSCLC *Non Small Cell Lung Cancer*

PAR *Partitioning defective gene family*

PCR *Polymerase Chain Reaction*

PI3K *phosphoinositide 3-kinase*

PIP3 *Phosphatidylinositol 3,4 ,5-triphosphatase*

PJS *Peutz-Jeghers Syndrome*

PKA *cAMP-dependent protein kinase*

PTEN	*Phosphatase and tensin homolog*
PTT	*Protein Truncation Test*
PVDF	*Polyvinylidene fluoride membrane*
qRT-PCR	*Quantitative PCR real-time*
RARβ	*Retinoic Acid Receptor β*
RB	*Retinoblastoma tumor-suppressor*
Rheb	*Ras-homolog enriched in brain*
RNA	*Ribonucleic acid*
RNase	*Ribonuclease*
RPMI	*Roswell Park Memorial Institute medium*
RSK	*p90 ribosomal S6 protein kinase*
RT-PCR	*Reverse Transcription Polymerase Chain Reaction*
S6K	*p70 ribosomal S6 kinase 1*
SAD	*Synapses of amphids defective*
SAM	*S-adenosylmethionine*
SCC	*Squamous cell carcinoma*
SCLC	*Small Cell Lung Cancer*
SDS	*Sodium dodecyl sulfate*
SDS-PAGE	*sodium dodecyl sulfate polyacrylamide gel electrophoresis*
siRNA	*Small interfering RNA*
STK11	*Serine-Threonine Protein Kinase,*
STRAD	*STe20 Relater ADaptor,*
T	*Thymine*
TNM	*Tumor Node Metastasis*
TSA	*Trichostatin A*
TSC	*Tuberous Sclerosis Complex*
TSG	*Tumor Supressor Gene*
UBA	*Ubiquitin Associated*
UICC	*Union for Cancer Control*
WHO	*World Health Organization*
WPWS	*Wolff-Parkinsin-White syndrome*

I. Introduction

1. Lung Cancer

1.1 Epidemiology of Lung Cancer

1.1.1 Incidence

Cancer is one of the leading causes of morbidity and mortality worldwide (Peto 2001). According to GLOBOCAN 2012 (Ferlay J 2013), an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. Prevalence estimates for 2012 show that there were 32.6 million people (over the age of 15 years) alive who had had a cancer diagnosed in the previous five years. The most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%) Figure 1.

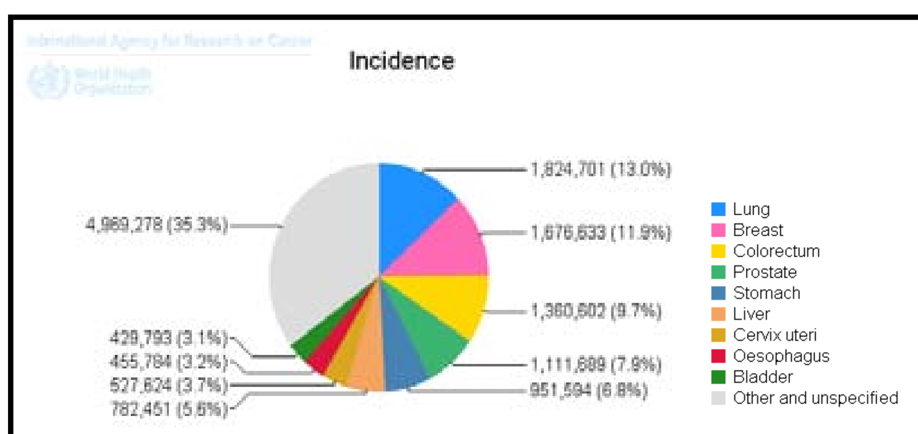


Figure 1. The most commonly diagnosted cancers worldwide. Excluding non melanoma skin cancer, 2012 estimates. The data are derived from the IARC GLOBOCAN 2012 database (Ferlay J 2013).

Projections based on the GLOBOCAN 2012 (Ferlay J 2013) estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and ageing of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025.

Lung cancer has been estimated as the most common cancer in the world for several decades (Figure 2) (Ferlay J 2010), (Parkin, Stjernsward et al. 1984), (Parkin, Laara et al. 1988; Parkin, Pisani et al. 1993), (Parkin, Pisani et al. 1999), (Parkin, Bray et al. 2001). An estimated 1.61 million people across the world were diagnosed with lung cancer in 2008, accounting for 13% of the total.

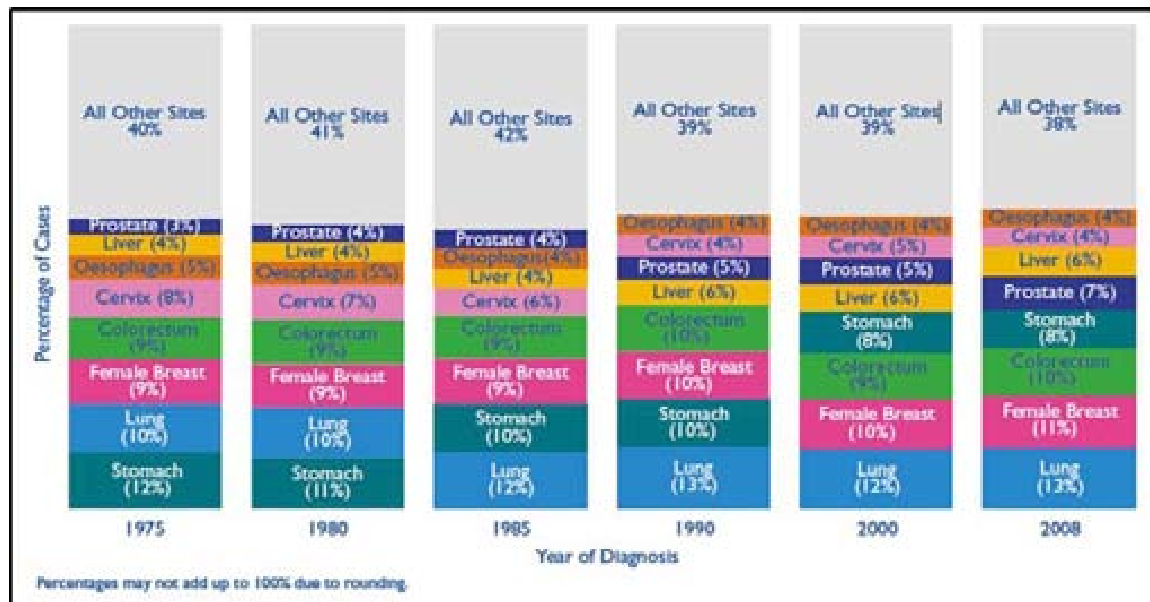


Figure 2. Trends in the Ranking of New Cases of Cancer Worldwide, 1975-2008. The data are derived from the IARC GLOBOCAN 2008 database.GLOBOCAN 2008 (Ferlay J 2010).

Lung cancer has a high incidence in both developing countries and areas undergoing economic development such as China (Parkin 2002). Although these regional differences might be explained by genetic differences among populations, variations in lifestyles, environmental exposures and medical practices such as screening are also likely to be important determinants of cancer risk. This assumption is reinforced by migration patterns that show that incidence of cancer among migrants more closely reflects the rates in the adoptive country. Lung cancer incidence rates are highest in Europe and Northern America and lowest in parts of Africa (Figure 3). More than half (55%) of the cases occurred in the developing world (Ferlay J 2010).

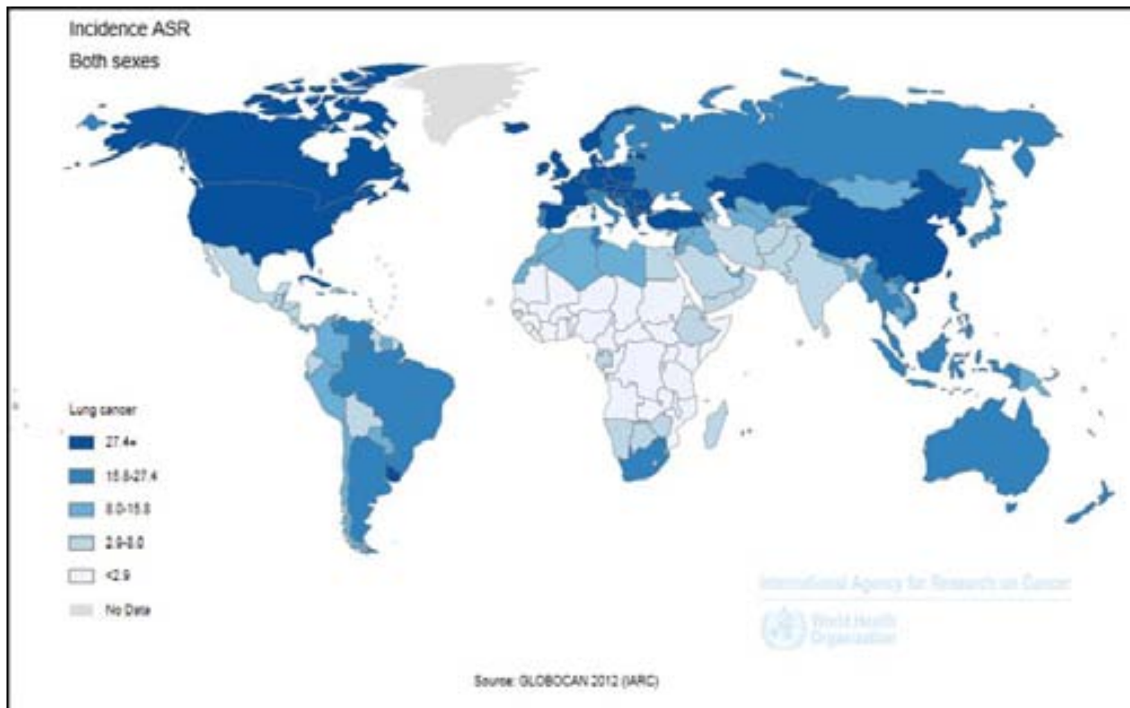


Figure 3. Incidence of lung cancer. There is substantial global variability in lung cancer incidence (measures as age-standardized rates) occurring in people living in development countries. Lung cancer incidence is currently high in development countries as well as those countries undergoing economic transition us China. (Ferlay J 2013).

The disease remains as the most common cancer in men worldwide (1.2 million, 16.7% of the total) with the highest estimated age-standardised incidence rates in Central and Eastern Europe (53.5 per 100,000) and Eastern Asia (50.4 per 100,000). Notably low incidence rates are observed in Middle and Western Africa (2.0 and 1.7 per 100,000 respectively) (Figure 4).

In women, the incidence rates are generally lower and the geographical pattern is a little different, mainly reflecting different historical exposure to tobacco smoking. Thus the highest estimated rates are in Northern America (33.8) and Northern Europe (23.7) with a relatively high rate in Eastern Asia (19.2) and the lowest rates again in Western and Middle Africa (1.1 and 0.8 respectively) (Figure 5).

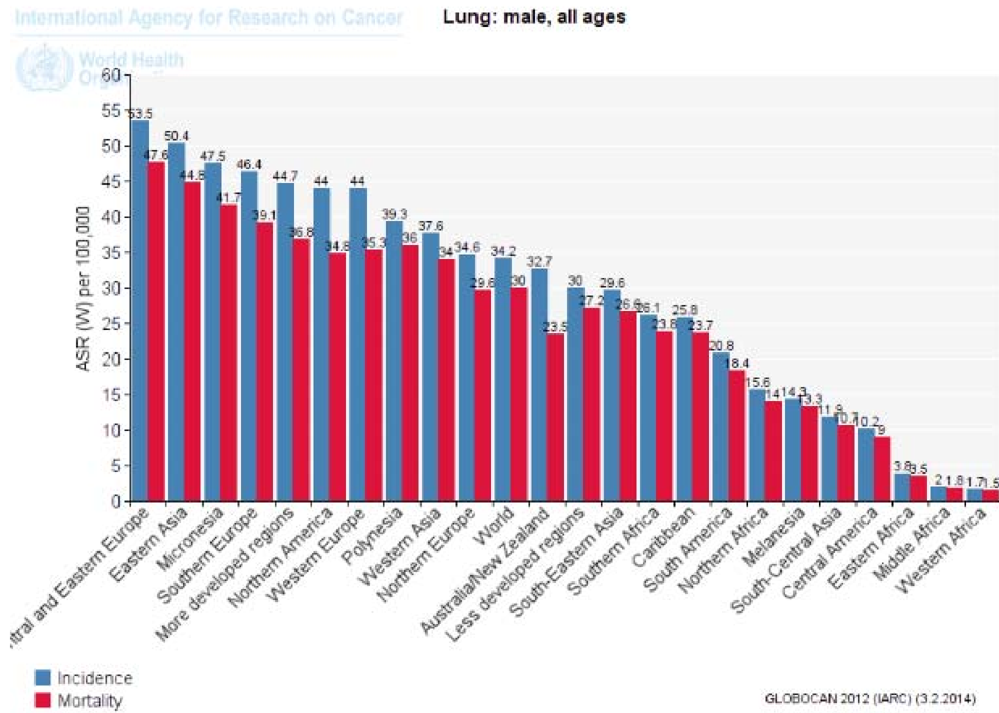


Figure 4. Incidence / mortality of Lung Cancer in Males by World Regions. (Ferlay J 2013).

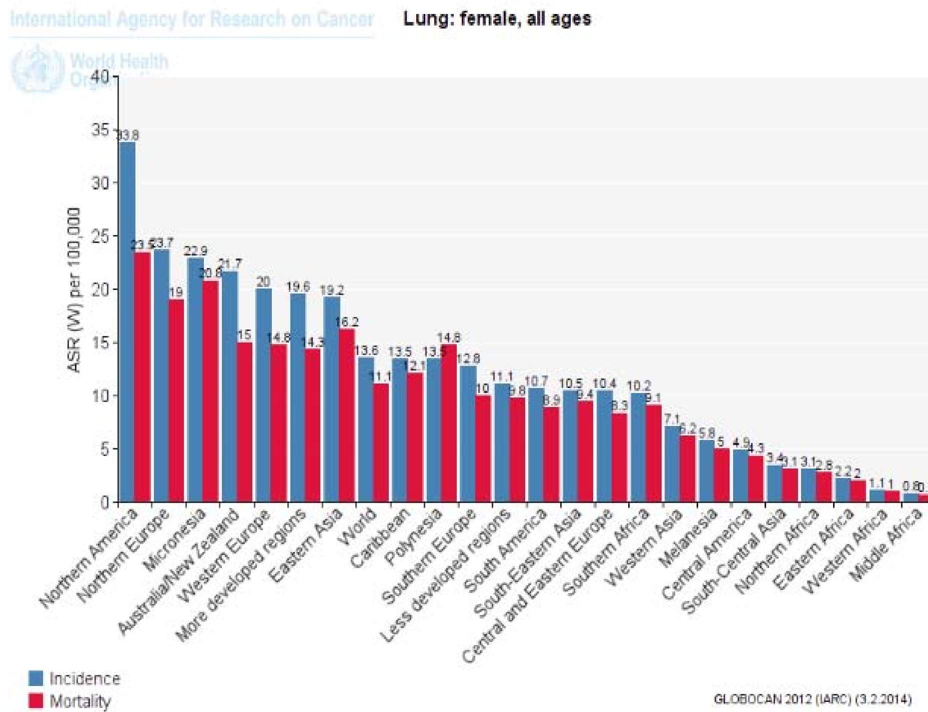


Figure 5 . Incidence / Mortality of Lung Cancer in Females by World Region. (Ferlay J 2013).

1.1.2 Mortality

Deaths from cancer in the world are projected to continue rising, influenced in part by an increasing and aging global population.

Lung cancer is the most common cause of death from cancer worldwide, estimated to be responsible for nearly one in five (1.59 million deaths, 19.4% of the total) (Figure 6). Because of its high fatality (the overall ratio of mortality to incidence is 0.87) and the relative lack of variability in survival in different world regions, the geographical patterns in mortality closely follow those in incidence.

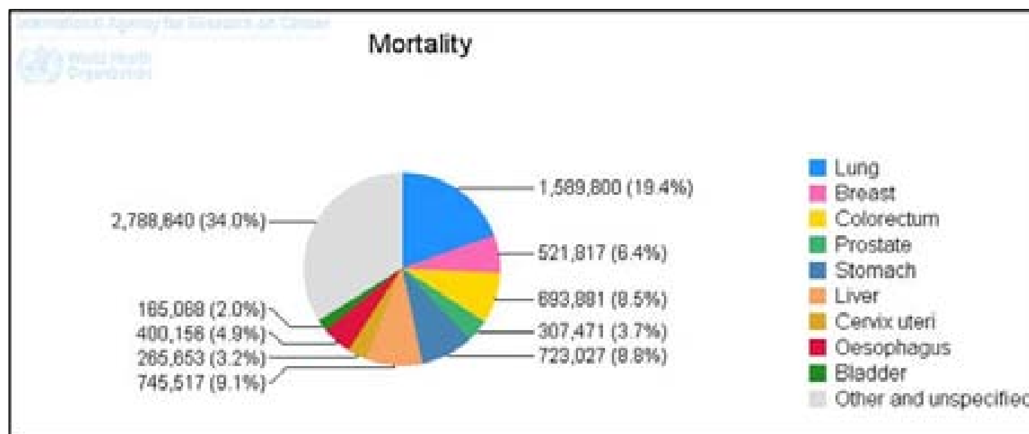


Figure 6. The most commonly causes of cancer death worldwide. Excluding non melanoma skin cancer, 2012 estimates. The data are derived from the IARC GLOBOCAN 2012 database (Ferlay J 2013).

Due to the long time-lag between exposure to lung cancer risk factors, such as smoking, and the onset of the disease itself, lung cancer incidence and mortality for women and men tends to reflect prior and long-term exposures to risk. Broadly speaking, patterns of lung cancer incidence and mortality show higher rates of the disease among men than women (Figure 4-Figure 5). In the United States of America (USA), for example, in 2000 the age-adjusted lung cancer incidence rate was 79.7 per 100 000 population for males, compared with a rate of 49.7 per 100 000 for females (SEER 2003). Similarly, in the United Kingdom, the age-standardized lung cancer incidence rate among males is approximately twice that in women (70.4 per 100 000 population in men and 34.9 per 100 000 population in females in 1999) (Cancer.Research.UK

2003). But in 2012, lung cancer was expected to account for 26% of all female cancer deaths and 29% of all male cancer deaths (American Cancer Society 2012).
Lung cancer accounts for more deaths than any other cancer in both men and women. An estimated 160,340 deaths, accounting for about 28% of all cancer deaths, are expected to occur in 2012 (American Cancer Society 2012). Death rates began declining in men in 1991; from 2004 to 2008, rates decreased 2.6% per year. Lung cancer death rates did not begin declining in women until 2003; from 2004 to 2008, rates decreased by 0.9% per year. Gender differences in lung cancer mortality patterns reflect historical differences between men and women in the uptake and reduction of cigarette smoking over the past 50 years (American Cancer Society 2012).

1.2 Lung cancer causes

- **Smoking**

Lung cancer is unique among human solid cancers in that a single environmental factor-tobacco smoke- is believed to promote sequential changes in target cells that lead to carcinogenesis.

The first references on the carcinogenic effect of the tobacco are of more than 200 years ago in the book *Cautions* against the inmoderate uses of snuff and the book: *Chirurgical Observations* published on 1775 by the Dr. Percivall Pott. It was not until 1914 that the carcinogenic nature of the contained hydrocarbons was demonstrated in the solid and tarred particles of the tobacco smoke (Yamagawa and Ichikawa 1915) .

Manufactured cigarettes were introduced at the beginning of the twentieth century. Since then the global consumption of cigarettes has been rising progressively. While consumption is leveling off, and even decreasing in some countries, worldwide more people are smoking and they are smoking more cigarettes. The numbers of smokers will increase mainly due to expansion of the world's population.

Pipe and cigar smoking can also cause lung cancer, although the risk is not as high as with cigarette smoking (Wald and Watt 1997).While someone who smokes one pack of cigarettes per

day has a risk for the development of lung cancer that is 25 times higher than a nonsmoker, pipe and cigar smokers have a risk of lung cancer that is about five times that of a nonsmoker.
In former smokers, the risk of developing lung cancer begins to approach that of a nonsmoker about 15 years after cessation of smoking.
In Europe the percentage of smokers is about 30%. Overall, ~33% of the adult world population smokes; this equates to 1.1 billion people (of which 200 million are women). The percent of the male population that smokes is 47% while the rate among females is 12%. In developing countries, the percentages are 48% in men and 7% in women, while in the developed countries 42% of the men are smoking and 24% of women (Fuster, O'Rourke et al. 2005).
At the moment it is believed that 90% of all the deaths for lung cancer are caused by tobacco. Worldwide, tobacco use causes more than 5 million deaths per year. In total, tobacco use is responsible for the death of about 1 in 10 adults worldwide (WHO 2011).
Based on the current trends, the World Health Organization (WHO) predicts that by the year 2020 , tobacco will cause in the world more than 10 million deaths a year, (Warren, Jones et al. 2008), causing more deaths than AIDS, tuberculosis, traffic accidents, deaths at birth, suicide and homicides together.

- **Passive smoking**

The National Cancer Institute’s 10th *Smoking and Tobacco Control Monograph* reviewed studies published between 1991 and 1997 in the United States, Europe, and Asia (Wu 1999). It included studies on environmental tobacco smoke exposure from spouses and the workplace and exposure in other social settings. They concluded that environmental tobacco smoke exposure resulted in an excess risk of 20% for developing lung cancer in the never smokers.

- **Asbestos fibers**

Asbestos fibers are silicate fibers that can persist for a lifetime in lung tissue following exposure to asbestos. The workplace is a common source of exposure to asbestos fibers, as asbestos was widely used in the past for both thermal and acoustic insulation materials.
Lung cancer can occur in nonsmokers exposed to asbestos; however, the risk is magnified several-fold by smoking (Boffetta 2004) .

Cigarette smoking drastically increases the chance of developing an asbestos-related lung cancer in exposed workers. Workers exposed to asbestos who do not smoke have a fivefold greater risk of developing lung cancer than nonsmokers not exposed to asbestos, and those asbestos workers who smoke have a risk that is 50 to 90 times greater than nonsmokers.

- **Environmental and occupational exposures**

People in developing countries are exposed to broader ranges of occupational and environmental risks, as more people are involved in manufacturing, farming, mining or other industrial occupations than developed countries. Research in China , for instance, has shown a positive association between lung cancer and radon gas exposure (Lubin, Wang et al. 2004), which is high in some homes and among underground miners. Radon gas is a natural, chemically inert gas that is a natural decay product of uranium. An estimated 12% of lung cancer deaths are attributable to radon gas. As with asbestos exposure, concomitant smoking greatly increases the risk of lung cancer with radon exposure.

In both China and India, indoor air pollution due to burning of coal and biomass for cooking and heating in homes has also been associated with lung cancer (Kleinerman, Wang et al. 2000; Smith 2000).

- **Air Pollution**

Air pollution from vehicles, industry, and power plants can raise the likelihood of developing lung cancer in exposed individuals. Up to 1% of lung cancer deaths are attributable to breathing polluted air, and experts believe that prolonged exposure to highly polluted air can carry a risk similar to that of passive smoking for the development of lung cancer.

- **Genetic predisposition**

While the majority of lung cancers are associated with tobacco smoking, the fact that not all smokers eventually develop lung cancer suggests that other factors, such as individual genetic susceptibility, may play a role in the causation of lung cancer.

Genetic variation causes many metabolic differences between individuals, and there is much interest in understanding the potential impact of this variation on susceptibility to cancer and

cancer pathogenesis. Specific mutations in single genes have been reported to greatly increase the risk of some types of cancer, although the prevalence of these mutations is rare at a population level. By contrast, common genetic polymorphisms that contribute only a modest variation in risk can have a greater impact on public health, especially in conjunction with environmental exposures.

- **Lung diseases**

The presence of certain diseases of the lung, notably chronic obstructive pulmonary disease (COPD), is associated with a slightly increased risk (four to six times the risk of a nonsmoker). Survivors of lung cancer have a greater risk than the general population of developing a second lung cancer. Survivors of non-small cell lung cancers, have an additive risk of 1% -2% per year for developing a second lung cancer. In survivors of small cell lung cancers (SCLCs), the risk for development of second cancers approaches 6% per year.

- **Diet**

So far, much of the diet and cancer research conducted in developing nations has focused on specific diet components.

There are many dietary variations, especially among populations in Asia, Africa or Latin America, that might be associated with disease risk (Rastogi, Hildesheim et al. 2004). Turmeric, a yellow-colour spice and flavor commonly consumed by millions of people, particularly in South Asia, has traditionally been used as a remedy for liver ailment. Curcumin, a constituent of turmeric, is a phytochemical that is currently being researched for its anti-tumour properties, such as inducing cell-growth inhibition and apoptosis.

- **Age**

The population of the world is ageing; this is important because cancer predominantly affects older people. Almost 70% of people diagnosed with the condition are over 65 years of age, while less than 3% of cases occur in people under age 45.

The median age increased from 23.5 years in 1950 to 26.4 years in 1999. By 2050, the median age is projected to reach 37.8 years. The proportion of people in the world aged 60 or older will rise from the current 10% to 22% in 2050 (UnitedNations 1999) .

There are big variations in the age structures of populations of more developed compared with less developed countries (UnitedNations 1999) (Ferlay J.B.F 2004). Currently 20% of the populations in the more developed regions are aged over 60 compared with 8% in the less developed regions. By 2050 these proportions are expected to rise to 33% and 19% respectively. The countries with the oldest populations in the world include Italy, Japan and Germany and the countries with the youngest include Uganda, Niger and Yemen (UnitedNations 1999). World life expectancy at birth is now at 65 years, having increased by a remarkable 20 years since 1950. By 2050, life expectancy is expected to exceed 76 years (UnitedNations 1999).

1.3 Types of Lung Cancer

Lung cancers are broken down into two major types, small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). This classification is based upon the microscopic appearance of the tumor cells themselves. These two types of cancers grow and spread in different ways, so a distinction between these two types is important.

1.3.1 SCLC

Small cell lung cancers comprise approximately 20-25% of all lung cancer cases. SCLC is strongly related to cigarette smoking, with only 1% of these tumors occurring in nonsmokers. This type of lung cancer originates in an inner layer of the walls of the bronchi called the bronchial submucosa, and grows aggressively (in comparison with non small cell lung cancers), quickly spreading into surrounding tissues, and ultimately, through the body. Symptoms are generally not noticeable until the cancer has spread into other parts of the body. Because of their rapid growth pace and tendency to metastasize, small cell cancers are described with only two stages, limited– when spread is contained to the localized area of the lung and immediate surrounding tissues, and extensive– when the cancer has spread throughout the body.

Referring to a specific cell type often seen in SCLC, these cancers are sometimes called oat cell carcinomas.

1.3.2 NSCLC

NSCLC are the most common lung cancers, accounting for about 80% of all lung cancers. NSCLC can be divided into three main types that are named based upon the type of cells found in the tumor:

- **Adenocarcinomas** are the most commonly seen type of NSCLC. While adenocarcinomas are associated with smoking like other lung cancers, this type is especially observed as well in nonsmokers who develop lung cancer. Most adenocarcinomas arise in the outer, or peripheral, areas of the lungs. The most frequents subtypes are: acinar adenocarcinoma, papillary adenocarcinoma, micropapillary and solid (Travis, Brambilla et al. 2011).
- **Squamous cell carcinomas were** formerly more common than adenocarcinomas; at present, they account for about 30% of NSCLC. Cancer that begins in squamous cells, which are thin, flat cells that look like fish scales. Also known as epidermoid carcinomas, squamous cell cancers arise most frequently in the central chest area in the bronchi.
- **Large cell lung cancer** sometimes referred to as undifferentiated carcinomas, are the least common type of NSCLC.

1.4 Stage of lung cancer

1.4.1 Stages of Small Cell Lung Cancer

The objectives of staging in SCLC are to identify localized disease, for which radiation therapy may be suitable, and to quantify the extent of the disease before therapy. Small cell lung cancer is typically classified according to the 2-stage system development by the Veterans Administration Lung Cancer Study Group:

Limited stage: when spread is contained to the localized area of the lung and immediate surrounding tissues.

Extensive stage: **c**ancer is found in tissues of the chest outside of the lung in which it began or cancer is found in distant organs, therefore when the cancer has spread throughout the body

1.4.2 Stages of Non-Small Cell Lung Cancer

The International Staging System for Lung Cancer has provided a common language for communication about patients with this disease, and the scientific community has been served well by its application. This system classifies the extent of disease based mostly on anatomic information on the extent of the primary tumor, regional lymph nodes, and distant metastases. This classification was developed in the 1940s by Pierre Denoix PF of France and formalized by the Union for Cancer Control (UICC) in the 1950s with the formation of the Committee on Clinical Stage Classification and Applied Statistics. The American Joint Committee on Cancer (AJCC) was founded in 1959 to complete this work.

The classification of malignant tumors is according to tumor-node-metastasis (TNM) that describes the extent of a person´s cancer. But the concept of stage grouping came later ((UICC) 1988) .

The TNM system is based on 3 key pieces of information

- **T** describes the size of the original (primary) tumor and whether it has grown into nearby areas.
- **N** describes the spread of cancer to nearby (regional) lymph nodes that are involved.
- **M** describes distant metastasis (spread of cancer from one part of the body to another).

Revisions in stage grouping of the TNM subsets in the schema of the International System for Staging Lung Cancer were made to provide greater specificity for identified patient with similar prognoses and treatment options (Table1-Table2). The rules of classification and staging correspond to those appearing in the seventh edition of the AJCC Cancer Staging Manual 2009 and have approval of all national TNM committees.

Table 1 . TNM Staging system

Primary tumor (T)	
TX	Primary tumor cannot be assessed, or tumor proven by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor 3cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus (*ie*, not in the main bronchus) *
T1a	Tumor 2cm or less in greatest dimension
T1b	Tumor more than 2cm but 3cm or less in greatest dimension
T2	Tumor more than 3cm but 7cm or less or tumor with any of the following features (T2 tumors with these features are classified T2a if 5cm or less); Involves main bronchus, 2cm or more distal to the carina; Invades visceral pleura (PL1 or PL2); Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but not involve the entire lung.
T2a	Tumor more than 3cm but 5cm or less in greatest dimension
T2b	Tumor more than 5cm but 7cm or less in greatest dimension
T3	Tumor more than 7cm or one that directly invades any of the following: parietal pleural (PL3) chest wall (including superior sulcus tumors), diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium; or tumor in the main bronchus (less than 2 cm distal to the carina∗ but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung or separate tumor nodule(s) in the same lobe.
T4	Tumor of any size that invades any of the following; mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, separate tumor nodule(s) in a different ipsilateral lobe.
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph nodes metastasis
N1	Metastasis to ipsilateral peribronchial and/or ipsilateral hilar lymph nodes, and intrapulmonary nodes involved by direct extension
N2	Metastasis to ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis to contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node (s)
Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis
M1a	Separate tumor nodule(s) in a contralateral lobe tumor with pleural nodules or malignant pleural (or pericardial) effusion∗
M1b	Distal metastasis

*The uncommon superficial spreading tumor of any size with its invasive component limited to the bronchial wall, which may extend proximal to the main bronchus, is also classified T1a.

∗Most pleural (and pericardial) effusions with lung cancer are due to tumor. In a few patients, however, multiple cytopathologic examinations of pleural (pericardial) fluid are negative for the tumor, and the fluid is nonbloody and

is not exudate. Where these elements and clinical judgment dictate that the effusion is not related to the tumor, the effusion should be excluded as a staging element and the patient's disease should be classified as M0.

Table 2. Anatomic Stage/ Prognostic Groups

Occult carcinoma	**TX**	**N0**	**M0**
Stage 0	Tis	N0	M0
Stage IA	T1a	N0	M0
	T1b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
	T1a	N1	M0
	T1b	N1	M0
	T2a	N1	M0
Stage IIB	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1a	N2	M0
	T1b	N2	M0
	T2a	N2	M0
	T2b	N2	M0
	T3	N1	M0
	T3	N2	M0
	T4	N0	M0
	T4	N1	M0
Stage IIIB	T1a	N3	M0
	T1b	N3	M0
	T2a	N3	M0
	T2b	N3	M0
	T3	N3	M0
	T4	N2	M0
	T4	N3	M0
Stage IV	Any T	Any N	M1a
	Any T	Any N	M1b

2. Epigenetic alterations in DNA

The term "epigenetic" is the study of heritable changes in the pattern of gene expression mediated by mechanisms other than alterations in the primary nucleotide sequence, i.e. heritable changes in gene expression that do not change the DNA sequence.

Mediators of epigenetic regulation include: DNA methylation, histone modifications, nucleosome positioning and other factors that control chromatin structure or gene expression (eg. miRNAs, long non-codings RNA).

2.1 Epigenetic modifications

Epigenetics is involved in many normal cellular processes. Consider the fact that our cells all have the same DNA, but our bodies contain many different types of cells: neurons, liver cells, pancreatic cells, inflammatory cells, and others. How can this be? In short, cells, tissues, and organs differ because they have certain sets of genes that are "turned on" or expressed, as well as other sets that are "turned off" or inhibited. In other words, epigenetic changes can switch genes on or off and determine which proteins are transcribed. Several phenomena employ epigenetic systems in humans:

2.1.1 X chromosome inactivation

X chromosome inactivation is the epigenetic system where ¨stamping¨ of the genetic information enables men and women to have equal expression of the genes carried on the X chromosome; despite the fact that women have two X chromosome copies and men have only one-in addition to a Y chromosome.

Epigenetics is important for X chromosome inactivation in female mammals, which is necessary so that females do not have twice the number of X chromosome gene products as males (Egger, Liang et al. 2004). Thus, the significance of turning genes off via epigenetic changes is readily apparent.

2.1.2 Genomic imprinting

Genomic imprinting is parent of origin specific allele silencing, or relative silencing of one parental allele compared with the other parental allele. It is maintained, in part, by differentially methylated regions within or near imprinted genes, and it is normally reprogrammed in the germline (Feinberg and Tycko 2004).

Thus, there are two copies of every autosomal gene, one copy from our mother and one from our father. Both copies are functional for the majority of these genes; however, in a small subset one copy is turned off in a parent-of-origin dependent manner. These genes are called “imprinted” because one copy of the gene was epigenetically marked or imprinted in either egg or the sperm. Thus, the allelic expression of an imprinted gene depends upon whether it resided in a male or

female the previous generation. Imprinted expression can also vary between tissues, developmental stages, and species (Reik and Walter 2001).

Imprinted genes are susceptibility targets for numerous human pathologies because their functional haploid state enables a single genomic or epigenomic change to dysregulate their function causing potentially disastrous health effects. Imprinting anomalies are often manifested as developmental and neurological disorders when they occur during early development, and as cancer when altered later in life (Falls, Pulford et al. 1999; Jirtle 1999).

2.2 DNA methylation

2.2.1 What is methylation?

One epigenetic modification in humans is methylation of cytosine located within the dinucleotide CpG.

DNA methylation plays an important role in determining whether some genes are expressed or not. By turning genes off that are not needed, DNA methylation is an essential control mechanism for the normal development and functioning of organisms. Alternatively, aberrant DNA methylation of the promoter region is a key mechanism for inactivation of genes that suppress tumorigenesis; this occurs early in tumor development and has been implicated in neoplastic transformation.

2.2.2 Distribution of methylated cytosines and CpG islands

Cytosine DNA methylation is a covalent modification of DNA, in which a methyl group is transferred from S-adenosylmethionine (SAM) to the C-5 position by a family of cytosine (5)-methyltransferases (DNMT) (Figure 7) (Feinberg and Tycko 2004).

DNA methylation occurs almost exclusively at CpG nucleotides in differentiated cells and has an important contributing role in the regulation of gene expression and the silencing of repeat elements in the genome (Feinberg and Tycko 2004).

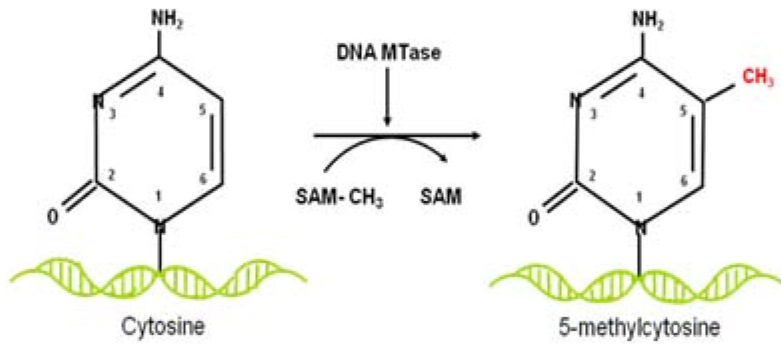


Figure 7. Pathway for the Methylation of Cytosine in the Mammalian Genome. 5-methylcytosine is produced by the action of DNA methyltransferases which catalyse the transfer of a methyl group (CH_3) from SAM to the carbon-5 position of cytosine.

The distribution of CpG dinucleotides in human genome is not uniform, for example there is short stretch of DNA in which the frequency of the CG sequence is higher than other regions of the genome known as ¨CpG islands¨, where "p" simply indicates that "C" and "G" are connected by a phosphodiester bond.

CpG islands are ~ 200-1,000 bp in length and often coincide with the 5´ ends of genes. There are approximately 29,000 CpG islands in the human genome, although estimates vary widely, depending on the stringency of the definition (Antequera and Bird 1993). About 3-4% of all cytosines are methylated in normal human DNA.

CpG islands are often associated with sites where transcription of DNA into RNA begins, the promoter regions (untranslated region and exon1) of housekeeping genes (which are essential for general cell functions) or other genes frequently expressed in a cell. At these locations, the CG sequence is not methylated. By contrast, the CG sequences throughout most of the rest of the genome are methylated; approximately 80% of all CpGs are methylated. Methylation of CpG islands in the promoter region silences gene expression and is a normal event that occurs in cells to regulate gene expression at imprinted genes and on the inactive X chromosome. Most CpG islands remain unmethylated in normal tissues regardless of gene expression (Figure 8). It is becoming increasingly apparent that aberrant methylation of the promoter regions of genes is the major mechanism of gene silencing in tumors (Baylin, Esteller et al. 2001).

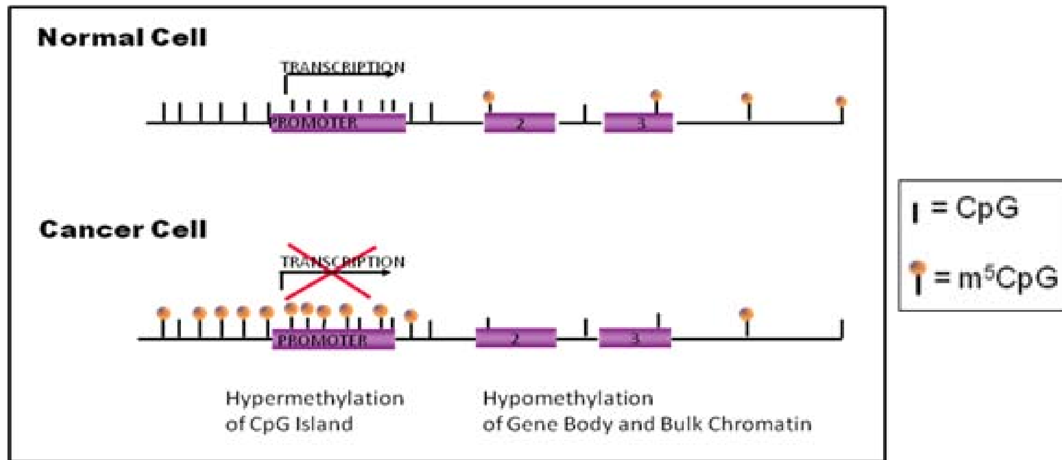


Figure 8. DNA Methylation change in cancer. There are differences in the Methylation patterns and DNA distribution of CpG dinucleotide in the Human Genome between normal cells and tumor cells.

Over the life span of an organism, DNA methylation serves as a number of functions in the body. In normal cells, the pattern of DNA methylation is conserved after DNA replication and cell division by the methylation of cytosine by a maintenance DNA methylase (DNMT1). Various disease states are associated with changes in CpG methylation, including Fragile X syndrome (i.e., gene, as well as promoter methylation), and Prader-Willi and Angelman syndromes (i.e., aberrations in methylation –dependent transcriptional silencing of imprinted genes in maternal or paternal chromosomes).

DNA methylation patterns fluctuate in response to changes in diet, inherited genetic polymorphisms and exposures to environmental chemicals (Sutherland and Costa 2003). Methyl groups are acquired through the diet and are donated to DNA through the folate and methinonine pathways (Ulrey, Liu et al. 2005). Changes in DNA methylation may occur as a result of low dietary levels of folate, methionine or selenium, which can have profound clinical consequences such as neural tube defects, cancer and atherosclerosis. Such imbalances in dietary nutrients can lead to hypomethylation and genetic instability (Friso and Choi 2002). Enviromental agents such as metals (e.g. arsenic) and aromatic hydrocarbons (e.g benzopyrene) can also destabilize the genome or modify cellular metabolism or both (Rossman 2003). These environmental contaminants are found in occupational chemicals, fossil fuel emissions, contaminated drinking water and cigarette smoke.

2.2.3 DNA methyltransferases

This family of three active enzymes (DNMT1, DNMT3a, DNMT3b), catalyzes the methylation of 5 position of the cytosine ring, using S-adenosyl-methionine as the donor molecule for methyl group (CH_3). This modification is imposed only on cytosines that precede a guanosine in the CpG dinucleotide. This reaction can be blocked by the drug 5-azacytidine (Figure 9). When this compound is integrated into DNA, replacing the natural base cytosine, it acts as a direct and irreversible inhibitor of the DNA methyltransferases (DNMTs), since it contains nitrogen in place of carbon at the 5 position of the cytidine ring. This process reactivates the affected genes and restores production of the corresponding protein in cultured cancer cells (Herman and Baylin 2003).

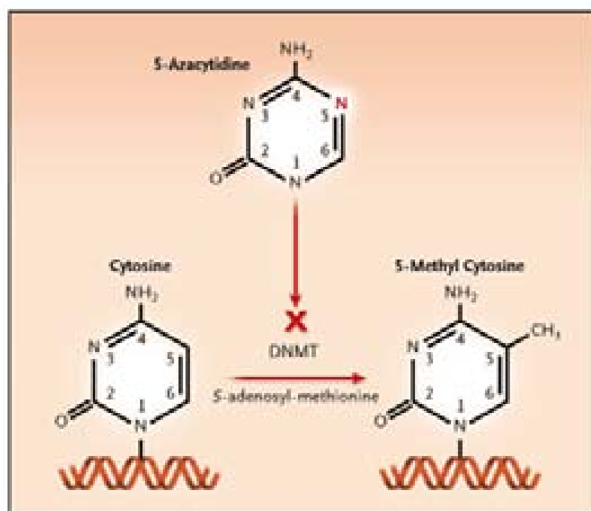


Figure 9. Pathway for the Methylation of Cytosine in the Mammalian Genome and Effects of Inhibiting Methylation with 5-Azacytidine (Herman and Baylin 2003).

The first DNMT to be identified was DNMT1 (Bestor, Laudano et al. 1988). This enzyme is believed to function primarily to maintain the DNA methylation pattern after the synthesis of the new DNA during cell division, because it exhibits much higher activity on hemimethylated DNA than on unmethylated DNA (Bestor 1992). The two de novo methylating enzymes DNMT3a and DNMT3b use unmethylated DNA as their template and play an important role in embryonic development (Okano, Bell et al. 1999).

2.2.4 DNA Methyation in Cancer

In primary human tumors, cancer cells exhibit two apparently opposing changes in their pattern of DNA methylation. An overall decrease in DNA methylation (*hypomethylation)* is observed throughout the genome and increased methylation of CpG islands (*hypermethylation*), is observed in the promoter regions of some tumor suppressor genes which in frequently related with gene silencing. These two processes might play important roles in the tumourigenic process.

- **Hypomethylation and gene activation:**

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells in 1982. Southern blotting was used to analyze DNA that had been digested with methylation-sensitive restriction enzymes and found that a substantial proportion of CpGs that were methylated in normal tissues were unmethylated in cancer cells (Feinberg and Vogelstein 1983)**.**

Hypomethylation of DNA has mechanistic implications (Feinberg and Tycko 2004):

❖ It can lead to **gene activation**. It has been found that around 10% of CpG islands are methylated in somatic tissues (Strichman-Almashanu, Lee et al. 2002). These methylated islands can become hypomethylated in cancer and nearby genes become activated, for example, oncogenes as HRAS (Feinberg and Vogelstein 1983).

❖ Tumour hypomethylation in cancer has been linked to **chromosomal instability**. Hypomethylation is particularly severe in periocentromeric satellite sequences, and several cancers (Wilms tumour, ovarian and breast carcinomas) frequently contain unbalanced chromosomal translocations with breakpoints in the pericentromeric DNA of chromosomes 1 and 16 (Qu, Grundy et al. 1999).

❖ Hypomethylation is a mechanism of drug, toxin and viral effects in cancer. In addition to gene amplification, hypomethylation of multidrug-resistance gene MDR1 correlates with increased expression and drug resistance in acute myelogenous leukaemia (Nakayama, Wada et al. 1998). Toxic carcinogens might also act through methylation alterations. For example, cadmium inhibits DNA methyltransferase activity and leads to acute

hypomethylation, which is followed by hypermethylation of DNA after chronic exposure to this "epigenetic carcinogen" (Takiguchi, Achanzar et al. 2003).

- **Hypermethylation and gene silencing:**

DNA methylation in the promoter region is important to the control of gene expression during the development of an organism. In some cases, methylation controls normal gene expression in adults (e.g., inactivation of the X chromosome in females). However, when aberrant DNA methylation in the promoters of tumor suppressor genes, it is implicated in neoplastic transformation .The aberrant methylation of genes that suppress tumorigenesis appears to occur early in tumor development and increases progressively, eventually leading to the malignant phenotype. Genes involved in every step of tumorigenesis can be silenced by this epigenetic mechanism.

Direct confirmation of epigenetic silencing of a tumor suppressor gene was provided by Sakai's group in 1993, who showed a 92% reduction of RB expression in tumours with promoter hypermethylation (Ohtani-Fujita, Fujita et al. 1993) and by Horsthemke's group in 1994 (Greger, Debus et al. 1994). In 1995, several groups, confirmed promoter hypermethylation at numerous other loci in cancer cells, supporting the principle of epigenetic gene inactivation in cancer.

A cellular **"methylator phenotype"** has been linked to mismatch repair. In 1997 it was shown that the cancer cells that are deficient in DNA mismatch repair silenced retroviral construct promoters by DNA methylation (Lengauer, Kinzler et al. 1997).

While hypomethylation may permit expression of oncogenes, cause chromosome instability, and activation of retrotransposons (transposable elements that perform a retrovirus-like process of reverse transcription); hypermethylation may lead to decrease expression of tumor-suppressor and DNA-repair genes (Figure 10).

To recapitulate, the epigenetic aberrations observed can be summarized as follows:

- Transcriptional silencing of tumour suppressor genes by CpG island promoter hypermethylation and histone deacetylation.
- Global genomic hypomethylation.

- Loss of imprinting events.
- Epigenetic lack of the repression of intragenomic parasites.
- The appearance of genetic defects in chromatin-related genes.

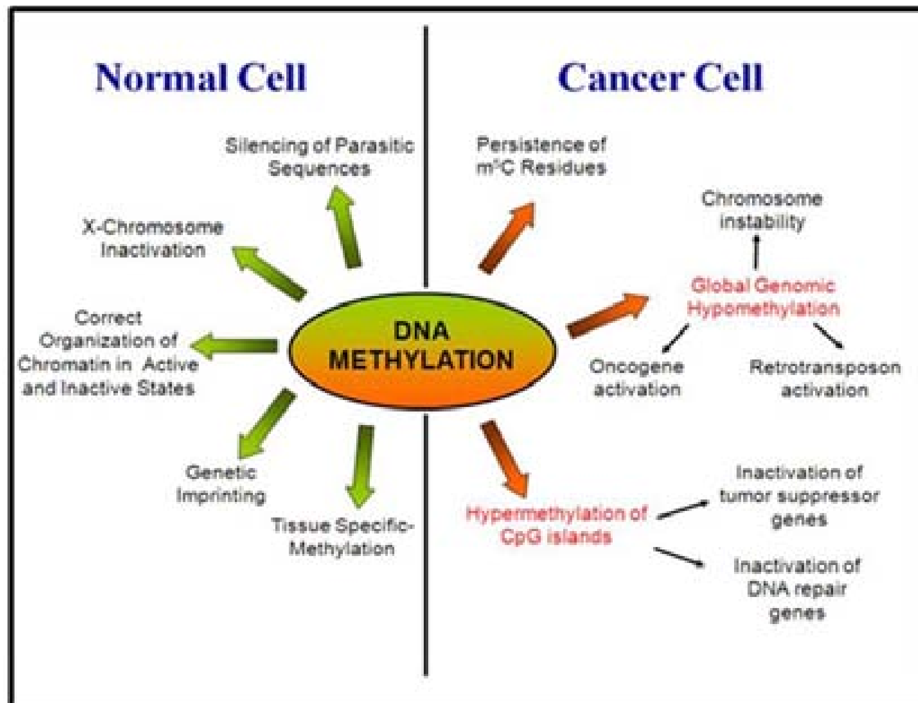


Figure 10 . Possible roles of increased CpG island and decrease global DNA methylation in tumour development.

DNA methylation markers have obvious applications in diagnostics, but can also contribute indirectly to therapeutics as predictors of response to therapy. Methylation level determinations offer a variety of oncology-related clinical applications:

• Changes in the regulation of DNA methylation are an early signal in tumor development.
• By characterizing methylation processes, practitioner will be able to classify tumors.
• DNMT inhibitors are being tested as anticancer agents, with the associated monitoring of surrogate tissues or, in the case of leukemia, repeat samples. There are, however, concerns that long-term exposure to such agents may lead to chromosomal instability.

2.2.5 Techniques to study DNA methylation

Changes in methylation patterns particularly in CpG islands, can be indicative of changes in gene expression. Conventionally, changes in gene expression are studied via expression arrays or proteomics. However, DNA methylation studies offer a number of advantages for studying phenotypic changes. The methylation pattern in a DNA molecule is relatively stable, in contrast to RNA transcripts and changes in methylation patterns may be both qualitative and quantitative, leading to assays with high specificity and sensitivity. In addition, such assays are more general than those for individual mutations and are localized to promoter regions in contrast to mutations that can be spread out in the gene (Widschwendter and Jones 2002; Laird 2003).

The methods for analysis of DNA methylation are divided into two major categories:
methods that utilize **chemical methods or restriction enzymes** to differentially cleave at cytosine versus 5-methylcytosine sites, and **the methods that utilize sodium bisulfite**, this type of analysis permits the identification of the specific positions of 5-methyl-cytosines in genomic DNA. This latter method appears to be more sensitive.

The bisulfite method has been one of the most significant developments in methylation analysis. The bisulfite treatment of DNA results in a deamination of cytosine to form uracil; 5-methyl-cytosine is resistant to this chemical treatment. If PCR is performed on the bisulfite-treated DNA, sequence analysis reveals that all the cytosines are replaced by thymine (C to T conversion) whereas 5-methyl-cytosine is not modified (C remains C) (Figure 11). Thus, the retention of cytosine in a specific position indicates methylation, whereas the appearance of thymine in a position that normally contains cytosine indicates the presence of unmethylated cytosine in the original DNA sample.

Due to differences in the DNA sequence of methylated and unmethylated CpGs after bisulfite treatment, it is possible to design specific primers for methylation analysis by PCR (Polymerase Chain Reaction) (Herman, Graff et al. 1996). This method is called MSP (Methylation –Specific-PCR) and is a rapid technique that requires a minimal amount tissue and can be used to analyze several cancer-related genes in tumors.

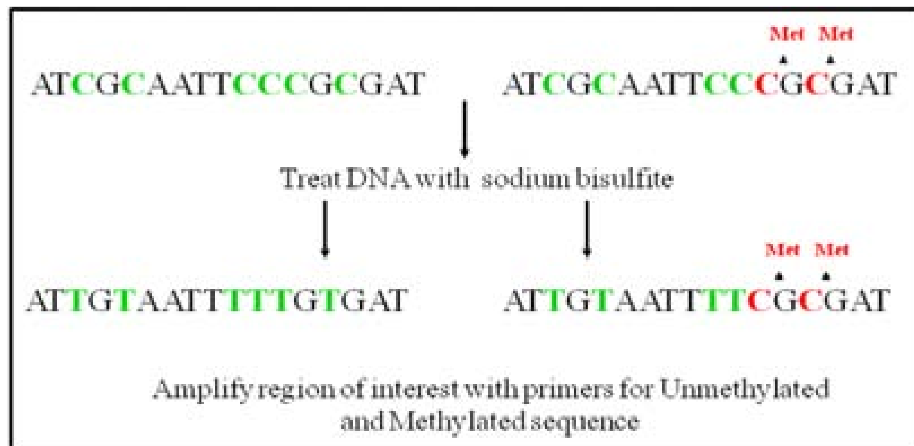


Figure 11. Bisulfite conversion of sample sequence of genomic DNA. Nucleotides in green are unmethylated cytosines converted to uracils by bisulfite, while red nucleotides are 5-methylcytosines resistant to conversion.

2.2.6 Clinical implications of DNA methylation

Determining the methylation levels in DNA of cells in bodily fluids offers the possibility of obtaining information on gene expression through noninvasive sampling. Current methods for the analysis of methylation in bodily fluid samples have fairly low sensitivity but excellent specificity, making them valuable in population screening where the clinical follow-up of false-positives can be costly and invasive. In population screening, methylation markers can be used as a supplemental tool in risk assessment or disease detection. Such markers can enhance the specificity of existing screening methods with low specificity (e.g., prostate-specific antigen screening for prostate cancer) (Laird 2003).

2.2.7 Methylation &Tumor suppressor genes

Tumor suppressor genes involved in cancer pathogenesis require inactivation of both alleles. One allele is frequently inactivated by allelic loss, while another one is inactivated by multiple mechanisms, including point mutation and allelic homozygous deletions, or by aberrant methylation (Zochbauer-Muller, Minna et al. 2002) (Figure 12).

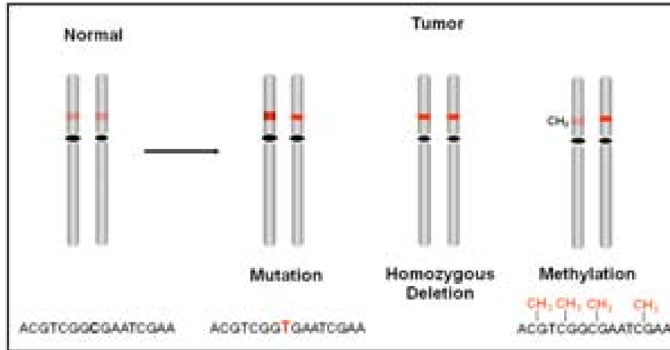


Figure 12. Tumor Suppressor Gene inactivation in human cancer. One of the allele can be inactivated by point mutation, homozygous deletion or by aberrant Methylation.

In the case of primary lung cancer, several genes have been shown to be frequently inactivated by DNA methylation. Some genes associated with tumor cell invasion or tumor architecture (E-cadherin, APC), growth factor response (RAR β), altered ell cycle control (p16), repair or DNA damage (MGMT). In the majority of these cases, gene expression was reactivated by treatment of lung cancer cells with the demethylating agent 5-aza-2'-deoxycytidine, causing re-expression of silencing silence genes in cancer cells.

2.3 Chromatin and Methylation

The human genome contains 23000 genes that must be expressed in specific cells at precise times. The compaction of DNA together with histones into a highly organized structure, termed chromatin, not only overcomes the space limitations within the nucleus but also serves as an important means to regulate gene activity. The basic unit of chromatin is the nucleosome, which consists of 146 base pairs (bp) of DNA wrapped around an octomer of the core histone proteins: H2A, H2B, H3 and H4.

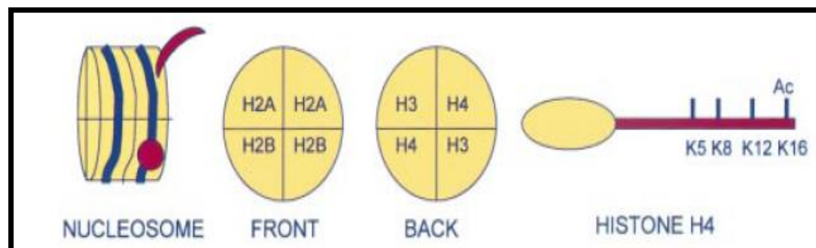


Figure 13. Schematic representation of a nucleosome. Yellow represents the histones. Dark red depicts the histone tail that can be modified to loosen DNA (purple) winding. The dark red circle represents a tail without an acetyl (Ac) group. The dark red ¨banana shape¨ represents a histone tail with an acetyl group, relieving the tight packaging of the DNA (de Ruijter, van Gennip et al. 2003).

2.3.1 Histone Acetylation/ Deacetylation

The modification of the δ amino group of lysine in histones by acetylation or deacetylation changes the configuration of nucleosomes .The positive charge on unacetylated lysines in the histones is attracted to the negatively charged DNA producing a compact chromatin state that is repressive for transcription. Therefore, genes are inactivated (switched off) when the chromatin is condensed (silent). Conversely, acetylation of the lysines by histone acetylase removes their positive charge and results in an open chromatin structure, which facilitates gene transcription. As a consequence, the genes are expressed (switched on) when the chromatin is open (active) (Jones and Baylin 2002).

A link between chromatin and DNA methylation dates back to the 1980s. Cedar, H et al. showed that naked DNA templates, pre-methylated in vitro and then transfected into cells, only became transcriptionally silenced after packaging into a repressive form of chromatin (Keshet, Lieman-Hurwitz et al. 1986). Thus dynamic chromatin states are controlled by epigenetic patterns of DNA methylation and histones modifications. Enzymes implicated in this process include DNMTs, histone deacetylase (HDACs) histone acetylases, histone methyltransferases and the methyl-binding domain protein (MECP2 and MBD2), which bind to methylated CpGs (Figure 14).

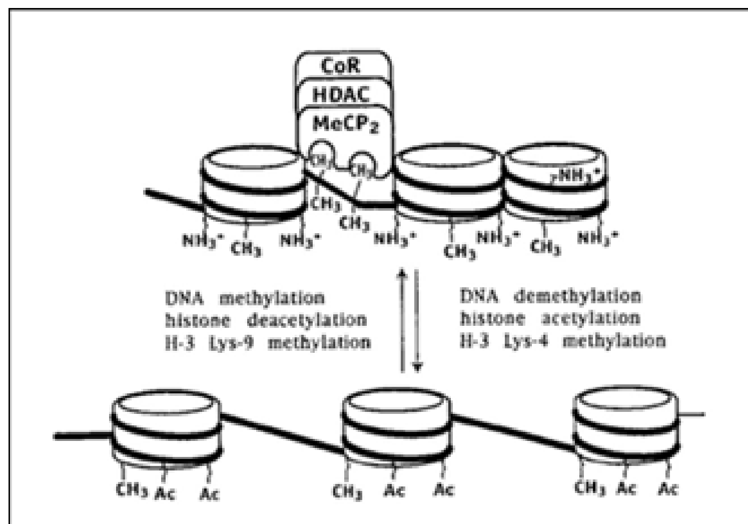


Figure 14. Silencing of gene expression by aberrant DNA methylation and histone modification. (Momparler 2003).

2.3.2 Histone deacetylase and Methyl-binding domain

DNA methylation leads to the binding of proteins known as methyl-binding domain (MBD) proteins. The members of this protein family all share a common MBD, which allows them to bind specifically to DNA containing methylated CpG sites (Hendrich and Bird 1998). At least three of the five known members of this family (MeCP2, MBD2 and MBD3) have been shown to be associated with large protein complexes (Zhang, Ng et al. 1999) containing histone deacetylase (HDAC1 and HDAC2) and chromatin-remodelling (Sin3a and Mi-2) activities. The action of these histone deacetylase and chromatin-remodelling activities is thought to result in the production of compacted chromatin that is refractory to transcription (Tyler and Kadonaga 1999).

3. Tumor suppressor genes & familial cancer

Several familial cancers have been shown to be associated with the loss of function of a tumor suppressor gene. A few of these tumor suppressor genes are described in more detail below (Table 3). They include the retinoblastoma susceptibility gene (RB), Wilms´tumors (WT1), neurofibromatosis type-1 (NF1), tuberous sclerosis complex (TSC) and Peutz-Jeghers Syndrome (LKB1). Many of these genes function to inhibit cell division and cell proliferation, stimulate cell death, and repair damaged DNA.

The products of tumor suppressor genes may act at the cell membrane, in the cytoplasm, or in the nucleus. Mutations in these genes result in a loss of function so they are usually recessive. This means that the trait is not expressed unless both copies of the normal gene are mutated. In some cases, the first mutation is already present in a germ line cell; thus, all the cells in the individual inherit it. Later a mutation occurs in the second copy of the gene in a somatic cell. In that cell both copies of the gene are mutated and the cell develops uncontrolled growth.

Table 3. Familial cancers syndromes caused by loss of function of a TSG

Familial Cancer Syndrome	Affected gene	Protein function	Chromosomal Location	Tumor Spectrum in affected patients
Li-Fraumeni Syndrome	P53	cell cycle regulation, apoptosis	17p13.1	brain tumors, sarcomas, leukemia, breast cancer
Familial Retinoblastoma	RB1	cell cycle regulation	13q14.1-q14.2	retinoblastoma, osteogenic sarcoma
Wilms Tumor	WT1	transcriptional regulation	11p13	pediatric kidney cancer
Neurofibromatosis Type 1	NF1	catalysis of RAS inactivation	17q11.2	neurofibromas, sarcomas, gliomas
Neurofibromatosis Type 2	NF2	linkage of cell membrane to actin cytoskeleton	22q12.2	Schwann cell tumors, astrocytomas, meningiomas, ependymonas
Familial Adenomatous Polyposis	APC	signaling through adhesion molecules to nucleus	5q21-q22	colon cancer
Tuberous sclerosis 1	**TSC1**	interacts with tuberin, exact function unknown	9q34	facial angiofibromas
Tuberous sclerosis 2	**TSC2**	GTPase activation of RAP1 and RAB5	16p13.3	benign growths (hamartomas) in many tissues, astrocytomas, rhabdomyosarcomas
Deleted in Colorectal Carcinoma	DCC	transmembrane receptor involved in axonal guidance via netrins	18q21.3	colorectal cancer
Familial Breast Cancer	BRCA1	cell cycle control, controlling protein degradation, DNA damage repair, and transcriptional regulation; interacts with Rad51 in DNA repair	17q21	breast and ovarian cancer
Familial Breast Cancer	BRCA2	transcriptional regulation of genes involved in DNA repair and homologous recombination	13q12.3	breast and ovarian cancer
Cowden syndrome	PTEN	phosphoinositide 3-phosphatase protein tyrosine phosphatase	10q23.3	gliomas, breast cancer, thyroid cancer, head & neck squamous carcinoma
Peutz-Jeghers Syndrome	**LKB1**	phosphorylates and activates AMP-activated kinase (AMPK), AMPK involved in stress responses, lipid and glucose metabolism	19p13.3	hyperpigmentation, multiple hamartomatous polyps, colorectal, breast and ovarian cancers
Hereditary Nonpolyposis Colon Cancer type 1	MSH2	DNA mismatch repair	2p22-p21	colon cancer
Hereditary Nonpolyposis Colon Cancer type 2	MLH1	DNA mismatch repair	3p21.3	colon cancer

4. Peutz –Jeghers Syndrome (PJS)

Peutz-Jeghers Syndrome (PJS), also known as Hereditary Intestinal Polyposis Syndrome, was first identified by a Dutch physician Peutz in 1921 (Peutz 1952), and later by an American physician Jeghers in 1949 (Jeghers, Mc et al. 1949).

PJS is a rare, autosomal dominantly inherited condition characterized by micocutaneous pigmentation, as well as predisposition to gastrointestinal hamartomatous polyposis (Tomlinson and Houlston 1997). The relative incidence of PJS is approximately 1/120 000 births (Lindor and Greene 1998). Patients with PJS almost always develop malignancies of the epithelial tissues, particularly of the gastrointestinal tract. For example, they have an 84- fold increased risk of developing colon cancer, a 213 fold increased risk of gastric cancers, and a 520- fold increased risk of developing small intestinal cancers (Giardiello, Welsh et al. 1987; Karuman, Gozani et al. 2001). It is estimated that 93% of PJS patients have a lifetime risk of cancer development at an average of 43 years old (Giardiello, Brensinger et al. 2000).

Some rare tumor types can be found relatively frequently in PJS patients, including cervical adenoma malignun, ovarian sex cord tumors with annular tubes and testicular Sertoli cell tumors (Giardiello, Welsh et al. 1987; Tomlinson and Houlston 1997). Additional PJS related malignancies include cancers of the breast, lung, uterus and cervix. Peutz-Jeghers syndrome patients show an increased risk of lung cancer but this is not the most frequent tumor type in these patients.

4.1 PJS & LKB1

Although the majority of PJS patients have a family history, 10%-20% of the cases are apparently caused by de novo LKB1 mutations (Boardman, Couch et al. 2000).

In 1997, linkage analysis of multiple hamartomas derived from PJS patients suggested that the causative locus for this disorder was located at chromosome 19p13.3 (Hemminki, Tomlinson et al. 1997), which is frequently lost in several types of cancer. In 1998, two groups reported that the gene mutated in PJS families was a previously uncharacterized serine-threonine protein kinase, termed LKB1 (Hemminki, Markie et al. 1998) or STK11 (Jenne, Reimann et al. 1998).

Most (80%) patients with autosomal dominant PJS show germline mutations in the LKB1 gene (Hemminki, Tomlinson et al. 1997; Ylikorkala, Avizienyte et al. 1999; Olschwang, Boisson et al. 2001; Hearle, Rudd et al. 2006; Volikos, Robinson et al. 2006). In these patients, the most important associated health-related concern is the increased risk of cancer (Giardiello, Brensinger et al. 2000). The loss of LKB1 leads to the formation of benign hamartomatous polyps composed primarily of epithelial cells. Nevertheless , a small but significant number of inherited forms of PJS found in certain families do not exhibit mutations in the LKB1 gene (Resta, Stella et al. 2002), indicating that there could be other causative loci for PJS.

LKB1 germline alterations detected in patients with PJS generate premature truncated proteins, either by nonsense or frameshit mutations in the coding sequence or by partial or complete deletions of the gene (Hemminki, Markie et al. 1998). Those mutations likely abolish some or all of the kinase activity of the protein.

Inactivating mutations in LKB1 have also been found in patients without PJS, for instance those with sporadic lung adenocarcinoma , where as many as 33% of the lesions analyzed displayed somatic mutations in the LKB1 gene (Sanchez-Cespedes, Parrella et al. 2002) (Carretero, Medina et al. 2004) (Fernandez, Carretero et al. 2004). LKB1 mutations have also been observed in ovarian carcinomas (Papageorgiou and Stratakis 2002), breast cancers (Shen, Wen et al. 2002) and pancreatic and biliary adenocarcinomas (Sahin, Maitra et al. 2003).

5. LKB1

LKB1 (Gen-Bank accession number U63333, MIM# 602216) has been identified by linkage analysis on chromosome 19p13.3 and encodes a novel serine/threonine kinase LKB1, also known as, STK11. The LKB1 gene spans 23 kb and is composed of 10 exons, nine coding exons and a final noncoding exon. LKB1 encodes an mRNA of 2.4kb transcribed in the telomere to centromere direction and for a protein of 433 amino acids and approximately 48kDa (Hemminki, Markie et al. 1998) . The protein possesses a nuclear localization signal in the N-terminal non-catalytic region (residues 38-43) and a kinase domain (residues 49-309) (Alessi, Sakamoto et al.

2006). The N-terminal and C-terminal noncatalytic regions of LKB1 are not related to any other proteins and possess no identifiable functional domains.

The carboxy terminus of LKB1 contains a CAAX- box a consensus sequence for prenylation (Figure 15). Transfection experiments have shown that LKB1 is prenylated in cultured cells at cysteine (Cys) 433 (Collins, Reoma et al. 2000), (Sapkota, Kieloch et al. 2001). One of the naturally occurring PJS mutations is a stop mutation that would prevent translation of the last 20 amino acids (Wang, Churchman et al. 1999). This indicates that the extreme carboxy terminus of LKB1 is important for its function.

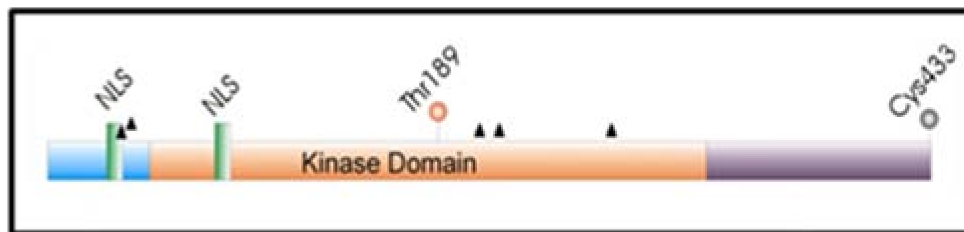


Figure 15. LKB1 gene structure. LKB1 has two nuclear leading sequences (NLS), are indicated in green, a central kinase domain (residues 50-319), and N- and C-terminal regulatory domain. Cys433 at C-terminal is the site for farnesylation.

To date, more than 250 different mutations in LKB1 have been identified in PJS patients and sporadic cancers according to the Sanger Institute Catalogue of Somatic mutations in Cancer website:http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=STK11&start=1&end=434&coords= AA% 3AAA (Zhao and Xu 2014). These mutations at LKB1 are nonsense (Lim, Olschwang et al. 2004), frameshift (Amos, Keitheri-Cheteri et al. 2004) or large intragenic deletions (Wei, Amos et al. 2003), which predict the generation of truncated protein. In case of Lim et al report, they suggested some evidence that the mutations in exon 3 of LKB1 could be associated with a higher cancer risk than the mutations within other regions of the gene (Lim, Olschwang et al. 2004).

Half of these mutations are missense or nonsense mutations, which mostly lead to truncations of the catalytic domain and impair LKB1 catalytic activity. However, there are also a significant number of point mutations, which are located in the kinase domain and in the C-terminal noncatalytic region (Alessi, Sakamoto et al. 2006).

Interestingly, two studies one in PJS samples (Launonen 2005) and the other one in lung cancer samples (Koivunen, Kim et al. 2008), either found the *LKB1* mutation spectrum very similar (deletions, insertions, splice site mutations, missense mutations, and nonsense mutations).

LKB1 is ubiquitously expressed in fetal and adult tissues with high expression in the pancreas, liver and skeletal muscle (Collins, Reoma et al. 2000). LKB1 protein expression is mainly localized in the nucleus, although small fraction is present in the cytoplasm (Nezu, Oku et al. 1999; Smith, Radzio-Andzelm et al. 1999; Tiainen, Vaahtomeri et al. 2002). LKB1 possesses a nuclear localization signal at its N-terminal non-catalytic region and mutation of this motif results in LKB1 being located throughout the cell (Smith, Radzio-Andzelm et al. 1999; Sapkota, Boudeau et al. 2002). A mutant of LKB1 lacking the nuclear localization signal still retains the ability to suppress cell growth (Tiainen, Vaahtomeri et al. 2002), suggesting that the cytosolic pool of LKB1 plays an important role in mediating its tumour suppressor properties.
LKB1 has a catalytic core that is common to both serine/threonine and tyrosine protein kinase family members. This domain contains 12 conserved subdomains that fold into common catalytic core structure (Hanks and Hunter 1995). The kinase domain of LKB1 is reasonably similar to the kinase domains of other serine/threonine protein kinases [SNF1 kinases and AMP-activated protein kinases (AMPKs)] (Hardie, Carling et al. 1998); however, several LKB1 subdomain sequences differ significantly from these and other kinases (Hanks and Quinn 1991).

A role for LKB1 in cell polarity has been described, and its ortholog in *C. elegans* (Par4) is one of six polarity regulators governing embryonic development. (Alessi, Sakamoto et al. 2006).
A *C.elegans* homologue, termed PAR-4, was identified as a member of the maternally expressed PAR (partitioning defective of LKB1) gene family. It has 42% amino acid identity to human LKB1 within the kinase domain but only 26% overall identity to the human LKB1 protein, as the non-catalytic regions of these proteins differs. PAR-4 is required for establishing cell polarity during the first cycle of *C.elegans* embryogenesis. The *Drosophila* homologue of human LKB1, which possesses 44% overall identity to human LKB1 and 66% identity within the kinase domain, also regulates cell polarity, as it is required for establishing the polarity of the anterior-posterior embryonic axis (Martin and St Johnston 2003).

Recent findings show that variants of LKB1 exist because of alternate splicing at the 3´ end of the mRNA. The newly described short variant (LKB1s) contains an unique 38-residue sequence at the C terminus and lacks the Ser-431 site (Towler, Fogarty et al. 2008). The two LKB1 proteins have different C-terminal sequences generating a 50-kDa form (termed $LKB1_L$) and a 48-kDa form ($LKB1_S$). $LKB1_L$ is widely expressed in mouse tissues, whereas $LKB1_S$ has a restricted tissue distribution with predominant expression in the testis. $LKB1_S$, like $LKB1_L$, forms a complex with MO25 and STRAD, and phosphorylates and activates AMPK both *in vitro* and in intact cells. A phosphorylation site (serine 431 in mouse) and a farnesylation site (cysteine 433 in mouse) within $LKB1_L$ are not conserved in $LKB1_S$ raising the possibility that these sites might be involved in differential regulation and/or localization of the two forms of LKB1 (Denison, Hiscock et al. 2009).

5.1 Posttranslational modifications of LKB1

There are at least eight residues on LKB1 that are phosphorylated. Ser31, Ser325, Thr366 and Ser431 are phosphorylated by distinct upstream kinases whereas Thr185, Thr189, Thr336 and Thr402 comprise autophosphorylation sites (Figure 16). Thr185 and Thr402 sites were mapped in human LKB1 (Baas, Boudeau et al. 2003), whereas the other residues were mapped in mouse LKB1 (Sapkota, Kieloch et al. 2001). In addition, there has also been a report that claims that phosphorylation of serine 428 at the C- terminus of human LKB1 (equivalent to Ser-431 in the mouse sequence) is necessary for LKB1 export from the nucleus (Xie, Dong et al. 2008).
In a recently study, Xie et al. group have identified S 307 of LKB1 as a putative novel phosphorylation site which is essential for its nuclecytoplasmatic transport (Xie, Dong et al. 2009).

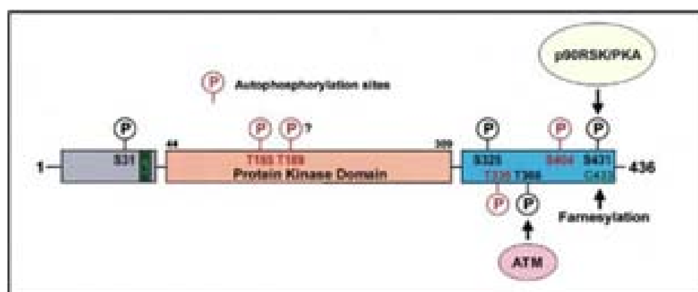


Figure 16. Location of the phosphorylation and prenylation site on mouse LKB1. The Ser 31, Thr 185 and Thr189, Ser325 and Ser336 are identical in number in human and mouse LKB1. In human Thr366 is Thr363, Ser 404 is Thr402, Ser 431 is Ser428 and Cys 433 is Cys430. NLS is nuclear localization signal (Boudeau, Sapkota et al. 2003).

LKB1 is phosphorylated at Ser431 by the p90 ribosomal S6 protein kinase (RSK) and cAMP-dependent protein kinase (PKA) in response to agonists, which trigger the activation of these kinases (Collins, Reoma et al. 2000; Sapkota, Kieloch et al. 2001). Consequently, RSK and PKA may regulate cell growth through phosphorylation of LKB1. Genetic analysis in *Drosophila* indicated that both phosphorylation of the Ser431 residue by PKA and prenylation of Cys433 are essential for LKB1 to regulate cell polarity (Martin and St Johnston 2003). However, the mechanism by which prenylation regulates LKB1 is unknown.
Therefore although some studies suggested that the phosphorylation of Ser-431 on LKB1 appears to be required for axon specification in the developing nervous system in response to agents such as brain-derived neurotrophic factor, by promoting the ability of LKB1 to activate BRSK1/BRSK2 (SAD-B/SAD-A) (Barnes, Lilley et al. 2007; Shelly, Cancedda et al. 2007), others have suggest that LKB1 is constitutively active and is not rate-limiting for activation of AMPK, BRSK1 or BRSK2 (Fogarty and Hardie 2009).

Phosphorylation of LKB1 at Thr366 is triggered only following exposure of cells to ionizing radiation, and it is likely that the DNA-damage activated ataxia-telangiectasia-mutated (ATM) kinase mediates this phosphorylation in vivo (Sapkota, Deak et al. 2002). This evidence that LKB1 may be controlled by ATM and could thus play a role in mediating DNA damage responses in cells. Ser31 lies in a consensus sequence for phosphorylation by AMP-activated protein kinase (AMPK) (Sapkota, Boudeau et al. 2002; Scott, Norman et al. 2002). As Ser325 is followed by a proline, it is likely to be phosphorylated by one of the proline-directed kinases (Sapkota, Boudeau et al. 2002).

Many of the sites of phosphorylation and prenylation are located in the C-terminal non-catalytic region of LKB1, and numerous mutations that affect only this region of LKB1 have been identified in tumours. Thus, the C-terminal region of LKB1 is likely to play important roles.

Collins et al.(Collins, Reoma et al. 2000) observed that LKB1 terminated in the amino acid sequence Cys-Lys-Gln-GLn, lying in an optimal consensus sequence for protein prenylation. This motif is conserved in LKB1 homologues in Xenopus (Su, Rempel et al. 1995), *Drosophila* and *C.elegants* (Kemphues, Priess et al. 1988).

Mutation of any of these sites of phosphorylation to either Ala to abolish phosphorylation or to Glu to mimic phosphorylation, had no effect on LKB1 in vitro catalytic activity or its in vivo cellular localisation (Sapkota, Kieloch et al. 2001; Sapkota, Boudeau et al. 2002).

Mutation of Ser431 to either Ala or Glu prevented LKB1 from suppressing the growth of G361 cells (cell lines G361 do not express endogenous LKB1) in a colony formation assay, suggesting that phosphorylation of this residue is essential for LKB1 to inhibit cell growth (Sapkota, Kieloch et al. 2001).

Cys433 is located two residues away from Ser431, the site of RSK and PKA modification, but mutation of Ser431 to either Ala or Glu did not affect farnesylation of Cys433. Mutation of Cys433 to Ala also had no effect on phosphorylation of LKB1 at Ser431 in response to agonists that activate RSK and PKA (Sapkota, Kieloch et al. 2001).
Mutation of Thr336, the major autophosphorylation site on LKB1, to Glu but not to Ala prevented LKB1 from inhibiting G361 cell growth, suggesting that phosphorylation of this residue may inhibit LKB1 tumor suppressor function (Sapkota, Boudeau et al. 2002). In contrast, mutation of Ser31, Ser325 or Thr366 had no major effect on the ability of LKB1 to suppress G361 cell growth (Sapkota, Boudeau et al. 2002).
The overexpression of wild type LKB1 in G361 cells, which do not express endogenous LKB1, suppressed the growth of these cells by inducing a G1 cell cycle arrest (Tiainen, Ylikorkala et al. 1999). Catalytically inactive LKB1 mutants including some of those isolated from PJS patients failed to suppress cell growth. This was the first indication that LKB1 functioned as a tumor suppressor and the serine-threonine protein kinase catalytic activity was required for this function.

5.2 LKB1 functions

The LKB1 protein has been shown to be involved in two biologically critical pathways (Figure 17) (Spicer and Ashworth 2004); dysfunction of both of these pathways may be important in the pathogenesis of Peutz-Jeghers syndrome:

- LKB1 has been shown to play a **fundamental role in controlling** the spatial orientation of structures required to maintain an ordered, **polarized epithelium** (Baas, Kuipers et al. 2004).
- LKB1 activity has been suggested to be the elusive **master regulator of AMP-dependent kinase** (AMPK), which controls the balance of cellular energy consumption and generation (Hawley, Boudeau et al. 2003; Woods, Johnstone et al. 2003; Shaw, Kosmatka et al. 2004).

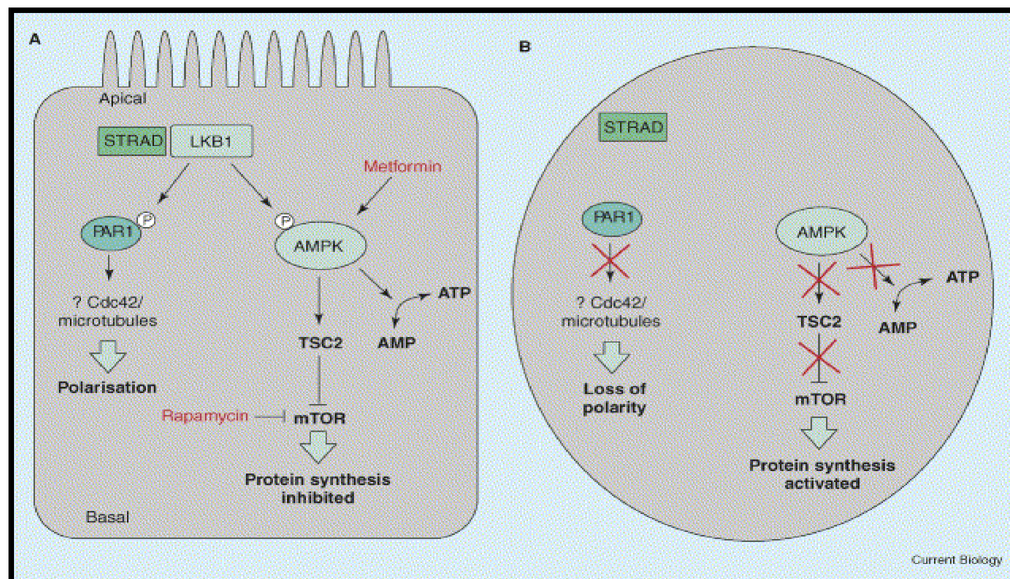


Figure 17. LKB1 kinase activity coordinates diverse cellular processes. (A) Both AMPK and PAR1 are phosphorylated and activated by LKB1. LKB1-directed PAR1 activation promotes correct polarisation, including brush border formation in an intestinal epithelial cell. In parallel, AMPK redirects metabolic priorities towards the generation of ATP, and downregulates anabolic processes, including protein synthesis. Metformin is an activator of AMPK used in the treatment of diabetes. Rapamycin is an inhibitor of mTOR. (B) Absence of LKB1 results in failure of PAR1 activation and loss of cell polarity. In addition, failure of AMPK activation releases inhibition of mTOR, switching on anabolic pathways required for cell division.(Spicer and Ashworth 2004)

The mechanism by which **LKB1 induces polarization** may involve another group of mammalian serine/threonine kinases, collectively termed the PAR1 family. LKB1 associates with PAR1 (Brajenovic, Joberty et al. 2004) and causes its phosphorylation and activation (Spicer, Rayter et al. 2003; Lizcano, Goransson et al. 2004). The connection between the LKB1

and PAR1 kinases in the regularity of cell polarization requires asymmetry in the localization of structural components including microtubules, and it may be that these structures are targeted by LKB1, via PAR1. In support of this, two members of the mammalian PAR1 family were initially characterized in the screen for kinases able to phosphorylate microtube-associated proteins (Drewes, Ebneth et al. 1997), an event which destabilizes microtubules and leads to their dissociation into tubulin subunits. The coordinated dissociation and reassociation of tubulin is essential to asymmetric microtubular function in a number of processes including mitotic spindle formation. As well as effects on cellular polarity, downregulation of proliferative signaling may be a consequence of PAR1 acting downstream from LKB1.
PAR1 is known to phosphorylate and activate Dishevelled (Sun, Lu et al. 2001), a key component of the Wnt pathway that is upregulated in most colorectal cancers.

The finding that LKB1 regulates cell polarity is important as it suggests that hamartoma formation in patients with Peutz-Jeghers syndrome may arise from inappropriate overgrowths of differentiated cells, which have lost their ability to regulate their polarity as a consequence of LKB1 inactivation (Boudeau, Sapkota et al. 2003).

5.3 Regulation of LKB1: LKB1-STRAD-MO25 complex

In vivo, LKB1 forms a heterotrimeric complex with two proteins termed STE20-related adaptor (STRAD) and mouse protein 25 (MO25) (Baas, Boudeau et al. 2003; Boudeau, Baas et al. 2003). Bass et al. (Baas, Boudeau et al. 2003) identified a novel STE20-related kinase, which was termed STRAD (STe20 Relater ADaptor), as an LKB1-specific adaptor and substrate. Members of the STE20-like kinase family are implicated in the stimulation of mitogen-activated protein kinase (MAPK) pathways, by direct activation of MAPKKK (Dan, Watanabe et al. 2001).
The STRAD (with α and β isoforms in mammals) is a polypeptide of 45-48 kDa that is localizes on human chromosome 17, and its 13 exons produce a 2.1Kb cDNA. STRAD possesses a domain with high sequence homology to protein kinases but is non-functional because it lacks two key motifs in subdomains VIb and VII of the catalytic domain that are essential for its catalytic activity. This explains why STRADα is catalytically inactive and has therefore been classified as a pseudokinase.

The interaction of LKB1 with STRADα stimulates LKB1 autophosphorylation and is absolutely necessary for downstream phosphorylation of AMPK (Hawley, Boudeau et al. 2003).
A key function of MO25α is to stabilize the binding of STRADα to LKB1, which interacts only weakly in the absence of MO25α (Boudeau, Baas et al. 2003). LKB1 expressed on its own is localized mainly in nuclei, but becomes re-localised in the cytoplasm following its interaction with STRADα and MO25α (Baas, Boudeau et al. 2003; Boudeau, Baas et al. 2003; Brajenovic, Joberty et al. 2004).

5.4 LKB1 & Lung Cancer

Somatic mutations in LKB1 have been identified in many tumor types (Hearle, Schumacher et al. 2006) (Sanchez-Cespedes 2007), including colon (Avizienyte, Roth et al. 1998), breast (Bignell, Barfoot et al. 1998), ovarian (Wang, Churchman et al. 1999) and brain (Sobottka, Haase et al. 2000), but their frequency is extremely low. Mutation of LKB1 does occur in a small fraction of malignant melanomas (Guldberg, thor Straten et al. 1999; Rowan, Bataille et al. 1999). Recent reports showed that 20% primary cervical cancers posses somatically-acquired mutations of LKB1 (Wingo, Gallardo et al. 2009).

In case of NSCLC, LKB1 inactivation is a common event (Sanchez-Cespedes, Parrella et al. 2002; Carretero, Medina et al. 2004; Matsumoto, Iwakawa et al. 2007). Thus far, somatic mutations in LKB1 have been observed in 30% of NSCLC cell lines (Matsumoto, Iwakawa et al. 2007). Interestingly, mutations in the LKB1 tumor suppressor gene were found to widely vary in frequency across different racial groups. LKB1 mutational frequency as identified in multiple studies was reported in approximately 17% to 35% of NSCLC in the white population (Koivunen, Kim et al. 2008) compared to only 3% to 14.4% in the Asian NSCLC population (Gao, Sun et al. 2010; Suzuki, Oonishi et al. 2012) (El-Telbany and Ma 2012) Figure 18.

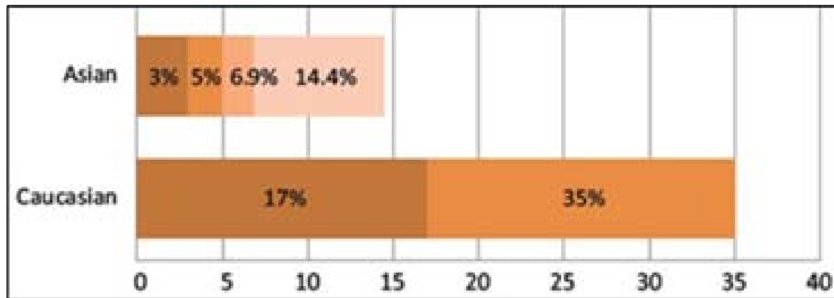


Figure 18. Spectrum of LKB1 oncogenic mutations among different racial groups with NSCLC. The different color shades represent LKB1 mutational rates reported by different studies.

Similar to mutations in LKB1, methylation of LKB1 is relatively rare in many common tumor types, but has been observed in a small number of colorectal tumors, testicular carcinomas and cervical carcinomas (Esteller, Fraga et al. 2001). In contrast, methylation of LKB1 is frequently observed in papillary breast carcinomas (45%) and colonic hamartomas (22%). Two tumors associated with PJS (Esteller, Fraga et al. 2001). The epigenetic status of LKB1 in human NSCLC has not been extensively studied. In one study Sanchez-Cespedes et al. showed alterations in LKB1 in 6 of 20 sporadic lung cancer adenocarcinomas 5 were mutated and one was methylated (Sanchez-Cespedes, Parrella et al. 2002).

5.5 LKB1/ AMPK/ TSC pathway

There are different studies which demonstrate a biochemical and genetic link from LKB1 to mTOR signaling via the sequential activation of AMPK and the TSC2 tumor suppressor. This interrelationship, particularly hyperactive mTOR signaling, provides a rational explanation for the shared features of three human disorders characterized by the development of hamartomas (benign tumors consisting of normal cellular differentiation but disorganized tissue architecture) including PJS, Cowden's disease, and the tuberous sclerosis complex, which possess autosomal dominantly inactivating mutations in the tumor suppressor LKB1, PTEN and either TSC1 and TSC2 respectively.

The PTEN tumor suppressor encodes a lipid phosphatase that serves to dephosphorylate the D3 position of phosphatidylinositol 3,4,5-triphosphatase (PIP3) second messenger which controls proliferation and survival of cells (Cantley 2002) (Figure 19). Thus biochemically directly opposing the activity of phosphoinositidine 3-kinase (PI3-kinase) and its dowmnstream effectors. Key among downstream PI3-kinase effectors is the Akt serine/threonine kinase, which mediates

cell proliferation survival, and growth. Akt activates mTOR signaling by directly phophorylating and inactivating the TSC2 tumor suppressor, tuberin (Inoki, Li et al. 2002; Manning, Tee et al. 2002).

In another study, microarray analysis revealed that overexpression of LKB1 in A549 lung adenocarcinoma cells resulted in a significant increase in the tumor suppressor PTEN mRNA expression. Therefore, lack of LKB1 could result in reduced expression of PTEN, thereby increasing PIP3 levels and enhancing proliferation and survival of these cells (Jimenez, Fernandez et al. 2003). The PI3K pathway is implicated in human diseases including diabetes and cancer, and understanding the intricacies of this pathway may provide new avenues for therapeutic intervention.

The mechanisms acting upstream to couple stress stimuli to the activation of LKB1 are at this point largely unknown. As previously stated, LKB1 kinase activity is stimulated by interaction with STRADα and MO25. The interaction of LKB1 with STRADα stimulates LKB1 autophosphorylation and is absolutely necessary for the downstream phosphorylation of AMPK at (Thr-172) on the α subunit of AMP-activated protein kinase (AMPK) (Hawley, Boudeau et al. 2003). This in turn activates AMPK kinase activity and the downstream phosphorylation of the TSC2 tumor suppressor protein (Tuberin) at two sites (S1270, S1388) distinct from the Akt sites. TSC1-TSC2 functions as a heterodimer, through Rheb to inhibit cell growth by negatively regulating the mammalian target of rapamycin (mTOR). mTOR controls cell growth by phosphorylating key substrates such as p70 ribosomal S6 kinase 1 (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1). So alterations in the LKB1 pathway can allow cancers cells to escape cell growth arrest (Figure 19). LKB1 is therefore a suppressor of cell-proliferation and tumor formation, but its downstream target(s) were until recently largely unknown.

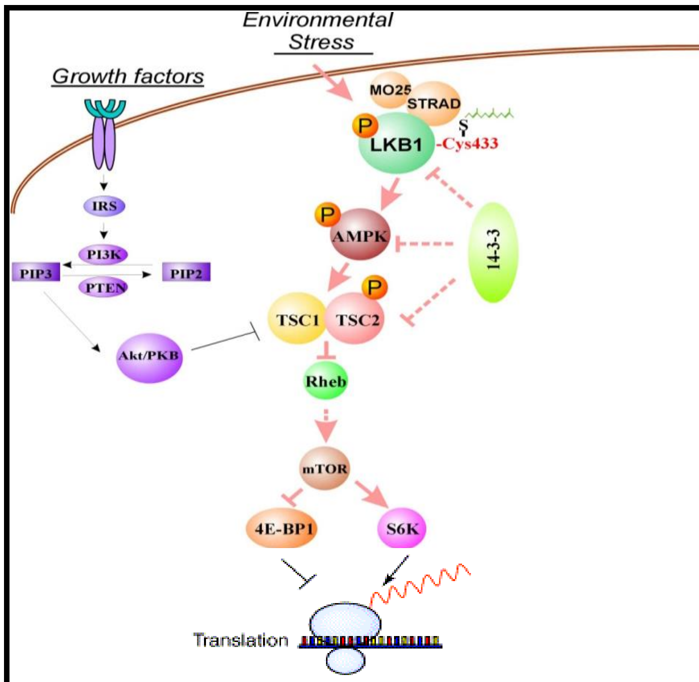


Figure 19. LKB1 signaling pathway in NSCLC. Environmental stress leads to LKB1 activation, which activates AMPK and TSC1-TSC2. The activation of this pathway down regulates mTOR activity that leads to cell growth arrest. mTOR activity can also be regulated by growth factors through PI3K/PTEN/ATK pathways.

5.5.1 AMP-activated protein kinase (AMPK)

AMP-activated protein kinases (AMPKs) are a class of serine/threonine protein kinases that are conserved throughout eukaryotes and function as an intracellular energy sensor maintaining the energy balance within the cell. AMPK is a key regulator of cellular pathways that consume and generate cellular energy.

Living cells normally maintain a high ratio of ATP to ADP (typically around 10:1). This represents a store of energy that can be used to drive energy-requiring processes. Catabolism (and photosynthesis when available) “charges up the battery” by converting ADP to ATP, whereas most other cellular processes require ATP hydrolysis and tend to discharge the battery (Figure 20). Most cells manage to maintain their ATP:ADP ratio within fairly narrow limits, which indicates that the rate of ATP synthesis exactly matches the rate of ATP consumption.

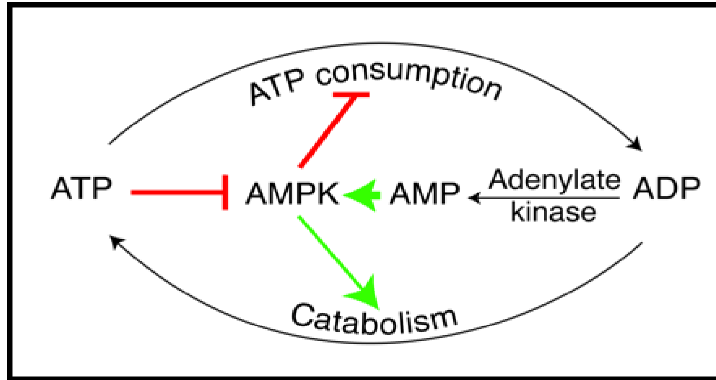


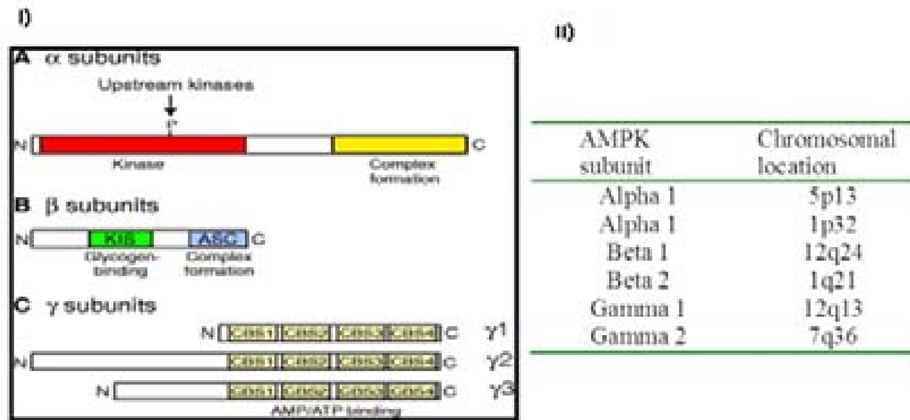
Figure 20. Physiological role of AMPK in the cell. Catabolism ¨charges up the battery¨ by converting ADP to ATP (bottom curved arrow) whereas ATP- consuming process convert ATP to ADP (top curved arrow). If a cellular stress causes the rate of catabolism to fail to keep pace with the rate of ATP consumption, ADP levels will rise and ATP levels will fall. ADP is converted into AMP by adenylate kinase and this, combined with the fall in ATP, will activate AMPK. AMPK then promotes the restoration of energy balance by stimulating catabolism and inhibiting ATP-consuming processes. (Hardie 2004)

AMPK is only active after phosphorylation of a critical threonine residue (Thr-172) within the kinase domain by upstream kinases (Hawley, Davison et al. 1996; Stein, Woods et al. 2000); the major upstream kinase being a complex between the tumour suppressor LKB1 and its two accessory subunits, STRAD and MO25 (Stein, Woods et al. 2000; Woods, Johnstone et al. 2003).

- **AMPK structure**

In mammals AMPKs are heterotrimetic complexes consisting of a catalytic α subunit and two regulatory subunits β and γ (Hardie 2003), all three subunits have multiple isoforms (α1, α2; β1, β2; γ1, γ2, γ3) encoded by distinct genes , and all twelve possible heterometric combinations are able to form when these are coexpressed in cell (Figure 21).

❖ The **α1** and **α2 subunits** (Figure 21) contain a conventional protein kinase catalytic domain at the N-terminus, containing features conserved throughout the protein kinase superfamily (Hanks, Quinn et al. 1988). The C-terminal regions of the α subunits contains a region of ~ 150 amino acid residues at the extreme C- terminus that is required for association with the β and γ subunits (Crute, Seefeld et al. 1998), whereas



AMPK subunit	Chromosomal location
Alpha 1	5p13
Alpha 1	1p32
Beta 1	12q24
Beta 2	1q21
Gamma 1	12q13
Gamma 2	7q36

Figure 21. I) Conserved domains in AMPK subunits: (A) αsubunits, (B) ß subunits and (C) γ subunits. The proposed function of each domain is indicated. The two isoforms of the α(α1, α2) and ß (ß1, ß2) subunits have very similar structures, but the three isoforms of the γ subunit (γ1, γ2, γ3) contain variable N-terminal regions of unknown function, and are drawn separately. (Hardie 2004). **II) AMPK subunits are encoded by distinct genes located at different chromosomal region** (Li, Liu et al. 2012).

a region immediately downstream of the catalytic domain (residues 312-392 in the α1 isoform) appears to have an inhibitory function. The α subunit also contains several residues that can be phosphorylated, one of these is Thr 172 and its phosphorylation is essential for AMPK activity; the mutagenesis of Thr172 to alanine completely abolishes kinase activity.

- Comparison of **β subunit (**Figure 21**)** sequences from different species revealed that they contained two conserved regions originally termed the KIS and ASC domain (Jiang and Carlson 1997). The ASC domain is sufficient for the formation of a complex with the α and γ subunits (Witczak, Sharoff et al. 2008). Whereas the KIS domain is not involved in subunit interactions is in fact a glycogen-binding domain (GBD) (Hudson, Pan et al. 2003). The exact function(s) of the GBD remains unclear, although there are several experimental findings suggesting the concept that the GBD is a regulatory domain that allows AMPK to act as a glycogen sensor in vivo (McBride, Ghilagaber et al. 2009)

- The **γ subunit** (Figure 21) contains four tandem repeats of a sequence motif first identified by Bateman as a ¨CBS domain¨ (cystathionine β-synthase) (Bateman 1997).

Bateman domains in the γ subunits represent the regulatory AMP- and ATP-binding sites of the AMPK complex (Scott, Hawley et al. 2004). The N-terminal and C-terminal Bateman domains from γ2 can each bind one molecule of AMP or ATP with strong positive cooperativity.

- **How do the mutations affect AMPK activity?**

The CBS domains importance was emphasized by findings that point mutations within them cause several hereditary diseases in humans. These include: homocystinuria (Kluijtmans, Boers et al. 1996), retinitis pigmentosa (Bowne, Sullivan et al. 2002), congenital myotonia, idiopathic generalized epilepsy, hypercalciuric nephrolithiasis, and classic Bartter syndrome (Konrad, Vollmer et al. 2000), and Wolff-Parkinsin-White syndrome (WPWS) (Blair, Redwood et al. 2001).

There are 5 naturally occuring missense mutations (R302Q, H383R, R531G ,T400N and N 4881) identified within γ2 that cause cardiac abnormalities (Daniel and Carling 2002). Three of these mutations within the CBS domains (R302Q, H383R and R531G) decrease AMPK activity by dramatically reducing the AMP activation of the kinase, providing strong support for the hypothesis that the CBS domains are involved in nucleotide-binding (Daniel and Carling 2002). Two other mutations (T400N and N 4881) are identified in individuals with Wolff-Parkinsin-White syndrome and associated cardiac hypertrophy, and these mutations caused activation of AMPK (Arad, Benson et al. 2002).

All these discoveries could show the possibility that distinct mutations leading to the same disease phenotype could have different effects on AMPK activity. It would suggest that alternative mechanisms can contribute to similar pathogenesis.

5.5.2 LKB1 activates 12 kinases of AMPK family

Examination of the human kinome indicates that there are a total of 12 human protein kinases, NUAK1, NUAK2, BRSK1, BRSK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4 and MELK, which are related to AMPK α1 and AMPK α2.

LKB1 has been shown to regulate 11 of the 12 AMPK family members in vitro (Lizcano, Goransson et al. 2004), including MARK/PAR-1, suggesting that one of the tumour suppressor functions of LKB1 may be regulation of AMPK signaling (Figure 22) .

Therefore, if LKB1 is an upstream activator of AMPK and 11 other related kinases, LKB1 could be a potential target for treatment of both cancer and metabolic disorders, especially diabetes.

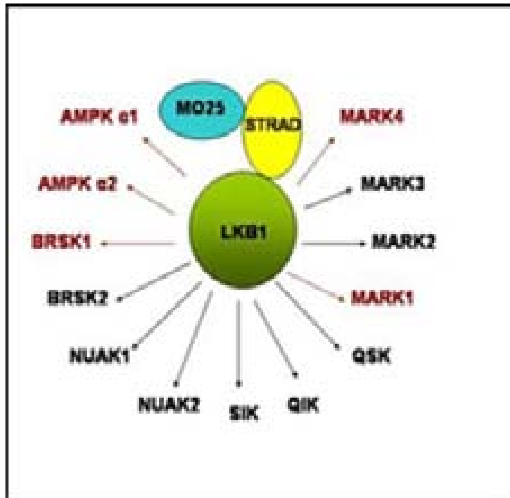


Figure 22 .5'-AMP-activated protein kinase (AMPK) subfamily of protein kinases, which are activated by LKB1. Abbreviations: BRSK, brain-selective kinase; MARK, microtubule-affinity-regulating kinase; NUAK1, AMPK-related protein kinase 5; NUAK2, SNF/ AMPK-related kinase; QIK, SIK 2;SIK,salt-inducible kinase.

As previously stated, the binding of LKB1 to STRAD and MO25 activates LKB1, resultating in phosphorylation of a critical threonine residue (Thr172) in the AMPKα subunit.

The Thr-172 residue of AMPKα1 and AMPKα2 that is phosphorylated by LKB1, and many of the surrounding residues, is conserved in the family of kinases whose catalytic domains are closely related to those of AMPK. This indicates that LKB1 might also phosphorylate the T-loop Thr residue and hence may also regulate other AMPK-related kinases (Lizcano, Goransson et al. 2004) in the same way that PDK1 regulates the activity of a group of related AGC kinases (Alessi 2001) (Figure 23).

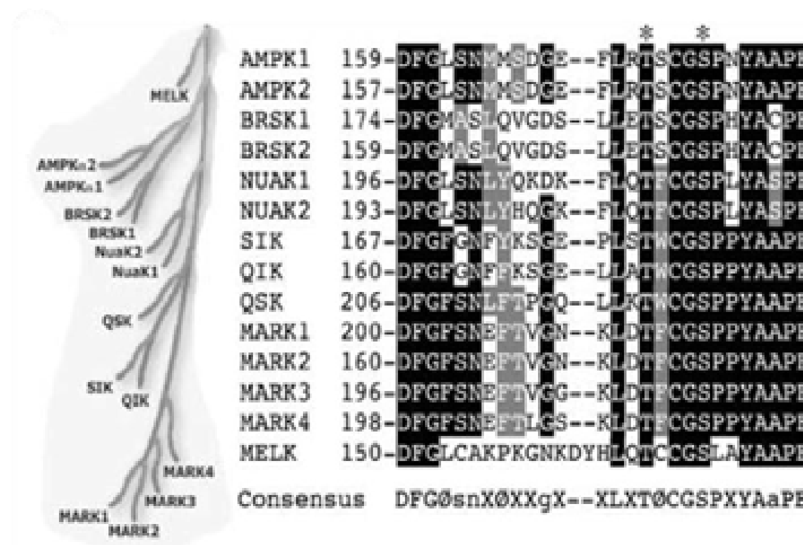


Figure 23. Activation of AMPK-related kinases by LKB1. Dendrogram and T-loop sequences of AMPK subfamily of protein kinases. The identical residues are shaded black and the conserved residues in grey. The T-loop Thr and Ser are indicated with an asterisk. (Lizcano, Goransson et al. 2004; Jaleel, Villa et al. 2006)

Sequence analysis indicates that many of the AMPK –related kinases that are activated by LKB1 posses a UBA (ubiquitin-associated) domain that is located immediately after the kinase catalytic domain. The AMP K-related kinase s are the only protein kinase s in the human g enome that possess a UB A domain, which is located immediately C -terminal to their catalytic domain. Although the mammalian AMPKα1 and AMPKα2 catalytic subunits do not possess an obvious UBA domain (Jaleel, Villa et al. 2006) (Figure 24).

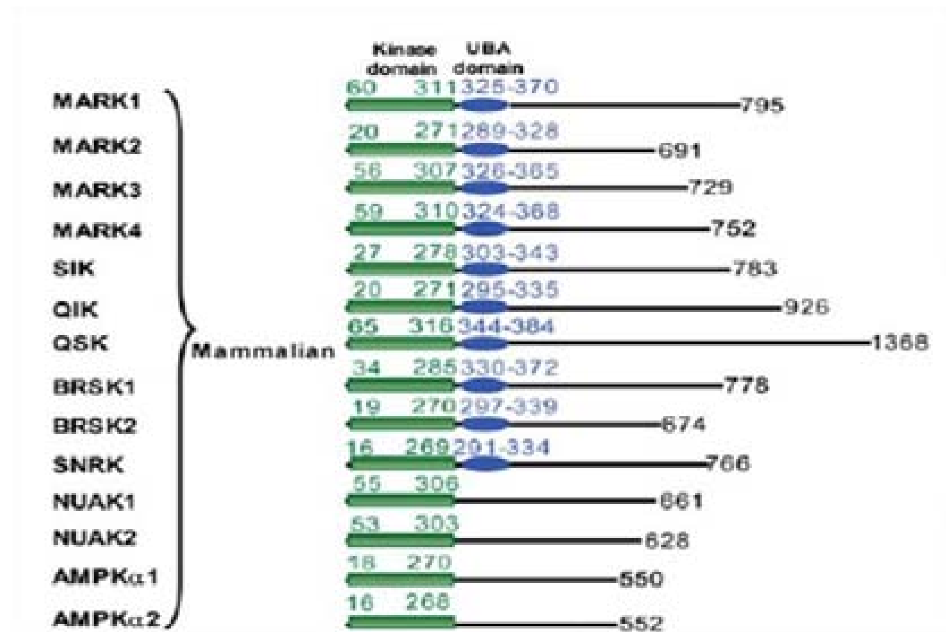


Figure 24. The mammalian AMPK subfamily kinases that are activated by LKB1. The presence or absence of the UBA domain. (Jaleel, Villa et al. 2006)

UBA domains comprise ~ 45 amino acid residues, are found in diverse proteins and can function as ubiqui tin- or po lyubiquitin-interacting mot ifs (Hicke, Schubert et a l. 2005). However, the function of these UBA domains within the AMP K-related kinase s are unc lear. Recent biochemical analysis suggests that none of the UBA domains in the AMPK-related kinases bind polyubiquitin or other ubiquitin –related molecules.(Jaleel, Villa et al. 2006).

Structural studies of the AMPK-related kinases, MARK2 and MARK3, have indicated that the function of the UBA domain may be to regulate phosphorylation , and therefore activity, of the kinase, throu gh c onformational c hanges (Jaleel, Villa e t al. 2006 ; Murphy, Kor zhnev e t al. 2007). B ased on struc tural analysis of the catalytic and U BA domains of MARK3 (Murphy, Korzhnev et al. 2007), it has been proposed that the ubiquitin-binding function of UBA domain may have evolved to interact with the kinase domain, at the expense of binding ubiquitin. This

interaction can stabilize the open conformation of the kinase domain, allowing phosphorylation by LKB1.

Within the UBA domain of BRSK1 the mutation of a key residue (Gly-343) almost completely abolished its activation by LKB1. However, mutation of the equivalent residue in BRSK2 (Gly-310) reduced, but did not prevent, activation by LKB1. It is possible that the kinase domain of BRSK2 can exist in an open conformation, even in the absence of a functional UBA domain (Bright, Carling et al. 2008).

There is evidence that the presence of the ubiquitin-associated (UBA) domains is required for phosphorylation and activation of AMPK-related kinases by LKB1 (Jaleel, Villa et al. 2006). The functional UBA domain is not as essential for BRSK2 activity, or phosphorylation by LKB1, as it is for BRSK1 activation (Bright, Carling et al. 2008)

5.5.2.1 MARK (MAP/ microtubule affinity regulating kinase)

The MARK kinases are the mammalian homolog of PAR1, a serine/threonine kinase that plays a central role in directing polarity in *C.elegans* and *D.melanogaster* (Guo and Kemphues 1995; Shulman, Benton et al. 2000; Tomancak, Piano et al. 2000).

The MARK kinases were originally identified as microtubule affinity-regulating kinases, after their role in regulating microtubule dynamics by phosphorylating microtubule associated proteins (Drewes, Ebneth et al. 1997). Subsequently they were shown to be required for neurite outgrowth and establishment of neuronal polarity, indicating a conserved role for the PAR-1 homologs in controlling polarity (Biernat, Wu et al. 2002).

The ability of LKB1 to phosphorylate and active MARK isoforms was described in two additional reports (Spicer, Rayter et al. 2003; Brajenovic, Joberty et al. 2004) and suggest that LKB1 and MARK kinases act in a common pathway.

5.5.2.2 BRSK (Brain Specific protein Kinase)

Synapses of amphids defective (SAD) kinases are the only vertebrate proteins shown to date to be required for neuronal polarization in vivo (Kishi, Pan et al. 2005) and to modulate presynaptic vesicle clustering (Crump, Zhen et al. 2001) but also phosphorylate MAPs (Kishi, Pan et al. 2005).

BRSK1(Kishi, Pan et al. 2005) (also referred to as SAD-B) and BRSK2 (SAD-A) are mammalian serine/threonine kinases that along with 10 other kinases form the AMPK-related family of protein kinases (Lizcano, Goransson et al. 2004). BRSK1 and BRSK2 (NCBI Acc.AF533878) are evolutionarily conserved, and orthilogs exist in mice, *Caenorhabditis elegans* (SAD-1), *Drosophila* (CG6114), and ascidians (HrPOPK-1).

BRSK1 has been shown to localize and associate with synaptic vesicles in mouse hippocampus and cerebellum as well as in cultured rat primary hippocampal neurons (Inoue, Mochida et al. 2006). Knock-out mice that lack both BRSK1 and BRSK2 have defects in neuronal polarity and die at ~2 H after birth (Kishi, Pan et al. 2005). Some studies have demonstrated that BRSK1 and BRSK2 are expressed in the brain (Kishi, Pan et al. 2005; Inoue, Mochida et al. 2006) and also in pancreas (Hezel, Gurumurthy et al. 2008). The Bright group detected expression of BRSK1 and BRSK2 in pancreas, although at a greatly reduced level when compared with brain. In both tissues, BRSK2 activity is substantially greater than BRSK1 (Bright, Carling et al. 2008). Recently Chen X et al. found that BRSK2 is also expressed in some tumor cell lines, such as HeLa and Panc-1 (Chen, Gu et al. 2012). Although the function of BRSK2 is not well known, there are some signs that implicate BRSK2 in the regulation of the cell cycle. A recent study has demonstrated that the phosphorylation of Ser-431 on LKB1 does not affect progress through the cell cycle. However, cells expressing the wild type $LKB1_L$ and mutants of Ser-431 of $LKB1_L$ showed clear evidence for a G_1-S phase arrest, with a much higher proportion of cells in G_1 (60%) and a lower proportion in S (10%) and G_2 (30%). Cells expressing $LKB1_S$ gave similar results (Fogarty and Hardie 2009). It is interesting to note that BRSK1 was recently shown to be involved in the G2/M checkpoint (Lu, Niida et al. 2004). Recently, BRSK2 was identified as a novel centrosome co-localized protein, implying that BRSK2 regulates the cell cycle through interaction with γ-tubulin or other mitosis regulators such as polo-like kinase (Alvarado-Kristensson, Rodriguez et al. 2009) (Vazquez-Martin, Oliveras-Ferraros et al. 2011).

On the other hand, in the nervous system SAD1 kinase is involved in presynaptic differentiation in *C.elegans*. Either deletion or overexpression of SAD1 is associated with synaptic abnormalities (Crump, Zhen et al. 2001). Recent works suggest that mammalian SAD kinases are also required for neuronal polarization, a critical step in neuronal differentiation, in mouse brain (Kishi, Pan et al. 2005).

Information regarding the potential downstream targets for BRSK1/2 is very limited ; however, a number of AMPK-related kinases, including BRSK1 and BRSK2, phosphorylate tau, a microtubule-associated protein that regulates stability of microtubule network (Kishi, Pan et al. 2005). The phosphorylation of tau may contribute to the polarity phenotype observed in BRSK1/2 knock-out mice.

Lizcano et.al demonstrated, that phosphorylation of threonine 174 is essential for activation of BRSK2. The sequence surrounding the equivalent threonine residue in BRSK1 (Thr-189) is identical to that in BRSK2, and phosphorylation of Thr-189 is essential for BRSK1 activity. (Lizcano, Goransson et al. 2004). In that same study, it was reported that mutation of the T-loop residue to glutamic acid led to a constitutively active form of the kinase. In contrast, another group observed the glutamic acid substitution is not sufficient for maximal activity of BRSK2 in mammalian cells (Bright, Carling et al. 2008). Similar findings have been reported recently for other AMPK-related kinases, SIK (Katoh, Takemori et al. 2006), Par 1 in *Drosophila* (Wang, Imai et al. 2007), and QIK (Hemminki, Markie et al. 1998).

5.5.3 Tuberous sclerosis complex (TSC)

Tuberous sclerosis complex (TSC) is an autosomal dominant syndrome characterized by the development of benign tumors termed hamartomas present in many organs, including the brain, skin, kidneys, lung, retina, teeth and others.
The prevalence of tuberous sclerosis complex syndrome is estimated at 1/10,000, and about two-thirds of the cases are sporadic. Familial cases were first reported in 1910, and the autosomal dominant pattern of inheritance was recognized in 1935.
The majority of TSC cases are caused by mutation in either the tsc1 or tsc2 tumor suppressor genes (Young and Povey 1998). The tumour suppressor genes TSC1 and TSC2 encode, respectively, proteins called hamartin and tuberin, which form a functional complex that seems to be important for the stability of the both proteins (van Slegtenhorst, Nellist et al. 1998).

- **TSC1-TSC2 structure**

❖ The tumour suppressor **TSC1**, maps to chromosome 9 (9q34) (Fryer, Chalmers et al. 1987), and encodes a 130kDa protein, hamartin, containing 1164 amino acid (van

Slegtenhorst, de Hoogt et al. 1997). The gene is composed of 23 exons with coding sequence from exon 3 to exon 23, and exon 2 is alternatively spliced. TSC1 is transcribed into an 8.6-kb messenger ribonucleic acid that is widely expressed. Hamartin has a single putative transmembrane domain.

- The **TSC2** gene maps to chromosome 16 (16p13.3) and contains 41 exons distributed over 44 kbp of genomic DNA. It encodes a 198-kDa protein, tuberin, containing 1807 amino acids. It contains several potential serine-threonine and tyrosine phosphorylation sites (Figure 25). Phosphorylation of TSC2 by AMPK is involved in the mechanism by which the TSC complex inhibits cell growth and protects against apoptosis during glucose starvation (Shaw, Kosmatka et al. 2004), (Inoki, Zhu et al. 2003).
  Tuberin is phosphorylated at serine and tyrosine residues in response to growth factors, and tuberin phosphorylation affects the interaction between hamartin and tuberin (Aicher, Campbell et al. 2001).

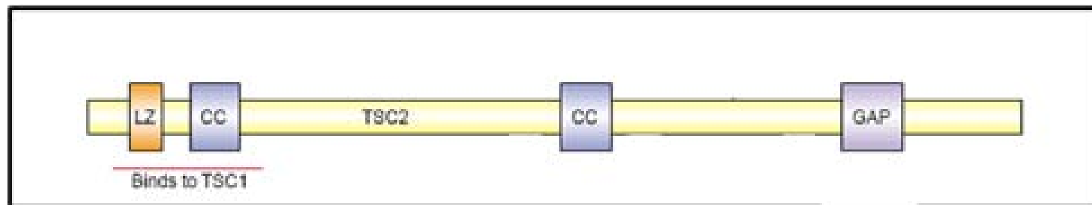


Figure 25. Simplified view of the TSC2 domain structure. Among other putative domains, TSC2 consists of two coiled-coil domains (CC), one leucine zipper domain (LZ) and a GTPase-activating protein (GAP) domain. (Li, Corradetti et al. 2004)

A C-terminal domain within TSC2 protein contains a GTPase activating activity (GAP), and appears to inhibit mTOR by promoting the GTPase activity of the small G protein Rheb, which activates mTOR in its GTP-bound state (Garami, Zwartkruis et al. 2003) (Figure 26). Phosphorylation of TSC2 by PKB/Akt is thought to inhibit the GAP activity, thus switching on the TOR pathway. Loss of TSC1–TSC2 results in the mTOR pathway becoming active in the absence of growth factor stimulation.

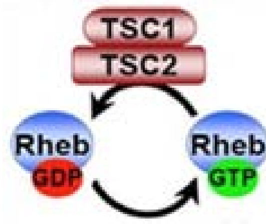


Figure 26 .TSC1-TSC2 complex acts as GTPase activating protein (GAP) for Rheb, thereby turning Rheb off by stimulating its intrinsic GTPase activity.

A study suggests that in the absence of growth factor or insulin stimulation, TSC1 localizes TSC2 at the intracellular membrane, which is required for the stabilization of TSC2 and the efficiency of TSC2 function as a RhebGAP (Cai, Tee et al. 2006).

- **TSC1 or TSC2 mutations**

TSC1 or TSC 2 mutations include nonsense, missense, insertion and deletion mutations, involving nearly all of the exons of TSC1 and TSC2. However both missense mutations and large genomic deletions are rare in TSC1 (Cheadle, Reeve et al. 2000).
Mutations in the TSC1 or TSC2 genes that interfere with the assembly of functional tuberin-hamartin complexes cause unregulated activation of mTOR, and lead to abnormal cell growth. This discovery of the importance of mTOR in the pathogenesis of tuberous sclerosis raises the possibility of using drugs (like rapamacyn) that inhibit mTOR or other downstream elements for treating tuberous sclerosis complex syndrome.

5.6 Rheb (*R*as-*h*omolog *e*nriched in *b*rain) /mTOR (target of rapamacyn), downstream effectors of LKB1/AMPK/TSC pathway.

Rheb is a small GTPase initially isolated as a Ras homolog enriched in brain (Yamagata, Sanders et al. 1994). Nevertheless, it is widely expressed. Rheb shares higher sequence identity with Ras than with Rho family members. There is direct biochemical data demonstrating that TSC2 has GAP activity toward Rheb in vitro (Inoki, Li et al. 2003). One of the roles of Rheb is to stimulate phosphorylation of p70S6k1 or S6K (ribosomal protein S6 kinsae 1) and 4E-BP1 (eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1), two of the best characterized cellular downstream targets of mTOR. Both the effector domain and the GTP binding domain are essential for Rheb function. The ability of Rheb to stimulate S6K

phosphorylation requires the function of mTOR, indicating that mTOR acts downstream of Rheb, which stimulates the autophosphorylation of mTOR on serine residue 2448.

The **TOR** genes were first identified in yeast by mutations that conferred resistance to Rapamycin (Schmelzle and Hall 2000). The TOR pathway is activated by amino acids and by growth factors like insulin-like growth factor1 (IGF1), and stimulates protein synthesis and cell growth via multiple mechanisms including phosphorylation of the protein S6K1 and the translation factor 4E-BP1 (Fingar and Blenis 2004; Hardie 2005). IGF1 stimulates the TOR pathway by activating the insulin receptor substrate-1 (IRS1) this to phosphatidylinosotol -3-kinase and finally to PKB pathway (also known as Akt) then phosphorylates the protein TSC2 (Inoki, Li et al. 2002; Manning, Tee et al. 2002).

II. Rationale

Screening for regions with loss of heterozygosity (LOH) in tumors is widely used to search for novel tumor suppressor genes in cancer. In the particular case of NSCLC, LOH in chromosome 19 is an extremely frequent event occuring in around 80% compared with <30% in SCLC (Virmani, Fong et al. 1998; Sanchez-Cespedes, Ahrendt et al. 2001). These observations strongly suggested that chromosome 19p, specifically the 19p13 region, contained one or more tumor suppressor genes for NSCLC.

Peutz-Jegher Syndrome is a disorder characterized by benign hamartomas of the gastrointestinal tract and mucocutaneous melanin deposits and predisposition to increased risk of certain cancers, including lung. The main PJS locus has been mapped to chromosome 19p13.3, and the gene responsible was identified as LKB1. Therefore, STK11b was originally identified through its involvement in the inherited cancer syndrome, PJS. Emerging evidence has implicated LKB1 as a novel tumor suppressor involved in the development of sporadic cancers.

Previous studies suggested that approximately 30% of somatic lung adenocarcinomas harbour an inactivating mutation in LKB1 (Sanchez-Cespedes, Parrella et al. 2002), and a recent study lists LKB1 as one of the four most frequently mutated genes in lung adenocarcinomas (Ding, Getz et al. 2008), implicating LKB1 defects in the pathogenesis of the lung cancer. However, these latter studies are based on analysis of adenocarcinomas. In case of squamous cell carcinoma there is a large study -The Cancer Genome Atlas project- (Network 2012) where it is identified as a potential therapeutic target in most tumours, offering new avenues of investigation for the treatment of squamous cell lung cancers.

The epigenetic status of LKB1 represents an alternative mechanism of tumor suppressor gene inactivation in human cancer. Although methylation of LKB1 is relatively rare in many common tumor types, previous methylation studies have shown, in colorectal and cervical carcinoma cell lines, a direct correlation between LKB1 promoter hypermethylation and absence of gene transcription (Esteller, Avizienyte et al. 2000). There is at least one study indicating that LKB1 may be inactivated in NSCLC primary tumor by promoter methylation (Sanchez-Cespedes, Parrella et al. 2002).

Recent evidence suggests that LKB1 signaling plays a role in protecting cells from apoptosis in response to energy or nutrient deprivation, which is achieved in part through the suppression of mTOR activity. Evidence from the literature supports a protein kinase cascade model in which LKB1 phosphorylates AMPK. AMP regulated kinase (AMPK) is a heterotrimeric complex comprising a catalytic subunit (α) and two regulatory subunits (β and γ) that act as an intracellular energy sensor maintaining the energy balance within the cell. AMPK subsequently phosphorylates TSC2. Together the TSC1-TSC2 heterodimer acts to suppress the activity of mTOR, which is essential for the control of cell growth and proliferation. Thus, LKB1 functions as a negative regulator of mTOR. Thinking about the essential role of mTOR as an integrator of cell growth and cell stress signals, it stands to reason that acquired mutations that lead to activation of mTOR survival signaling would provide a selective advantage during tumor progression.

As mentioned above, LKB1 has been shown to regulate 11 of the 12 AMPK family members in vitro (Lizcano, Goransson et al. 2004) including MARK/PAR-1, suggesting that one of the tumour suppressor functions of LKB1 may be regulation of AMPK signaling.

Therefore, if LKB1 is an upstream activator of AMPK and 11 other related kinases, LKB1 could be a potential target for treatment of both cancer and metabolic disorders, especially diabetes.

Consequently our hypothesis has two points, the first goal is to examine the frequency of genetic and epigenetic alterations in the signal transduction LKB1/AMPK/TSC pathway that contribute to the pathogenesis NSCLC. We hypothesized that mutations and/or methylation of the different members of the pathway may not be confined to adenocarcinomas, and the possibility of detecting new alterations could be expanded by increasing the spectrum of cell lines to include adenocarcinomas, large cell carcinomas, squamous cell carcinomas and immortalized bronchial epithelial cells. In addition, we will analyze a total of 58 paraffin embedded tumor tissues from NSCLC patients

The second goal is to test the hypothesis that mTOR signaling may be dysregulated by other signals acting in parallel to the LKB1/AMPK pathway in NSCLC. There is evidence to suggest that LKB1 exerts its effects by phosphorylating and activating other members of the AMPK-related kinase family. We hypothesized that other components of AMPK-related kinases that are

activated by LKB1 could be affected by epigenetic alterations and therefore could lose their function. Functional loss will lead to the inactivation of LKB1/AMPK-related kinases pathway and as a result relieve the negative regulation of mTOR.

III. Objectives

The goal of the thesis is to exploit the altered signal transduction pathway that contributes to the pathogenesis NSCLC to derive novel treatment strategies, and perhaps provide a molecular basis for future ¨individualized¨ therapies.

Aim I. The first objective is to determine the frequency of LKB1/AMPK/TSC2 signaling pathway alterations in NSCLC.

I-A. Determine the frequency of genetic/epigenetic alterations of LKB1/AMPK/TSC2 signaling pathway in a panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines). Mutations and promoter methylation analyses will be used to evaluate the status of genes involved in the LKB1/AMPK/TSC2 pathway in a panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines).

Aim II. The second objective is to study LKB1/AMPK-related kinases alterations as an additional molecular mechanisms for the development of lung cancer.

II-A. Analyze the methylation status of 4 from these 12 kinases: BRSK1, BRSK2, MARK1, MARK4 in a 23 cell lines lines (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines).

Aim III. The third objective is to examine LKB1/BRSK2 signaling.

III-A. Evaluate the expression of BRSK2 gene in a total of 23 cell lines (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines). Expression levels of this gene are analyzed by reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR.

III-B. Check if 5-azadeoxycytidine (5 aza-Dc), inhibitor of DNA methylation, and Trichostatin A (TSA) inhibitor of HDAC class I and class II, can reverse this epigenetic event in BRSK2 gene.

Aim IV. The fourth objective is to make a clinical validation of BRSK2 methylation status, in paraffin embedded tumor tissues of patients with lung cancer.

IV-A. Examine the methylation status of BRSK2 in a total of 58 NSCLC paraffin embedded tissues of patients.

Aim V. The fifth objective was to determine the impact of LKB1 depletion on NSCLC cell lines.

V-A. Evaluate the effect of downregulation of LKB1. A small interfering RNA approach was used to knockdown LKB1 in the Calu-1 cell line. The decrease in LKB1 protein expression by si RNA was confirmed by western blot.

IV. Material & Methods

6. Cell Lines

23 cell lines were examined, a group of 4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) (Table 4). All the cell lines were cultured in RPMI 1640 (Life Technologies, cat# 31870025) supplemented with 10% FBS and antibiotics. Cells were cultured at 37ºC in a humidified atmosphere and 5% CO_2 in air.

Table 4. Cell lines. Histology [a] -LCC, large cell carcinoma; AC, adenocarcinomas; SCC, squamous cell carcinoma; ASC, adenosquamous; BAC,bronchioloalveolar carcinoma; N/A: normal bronchial epithelial cells immortalized with SV40.

	Cell lines	Histology [a]
1	H1155	**LCC**
2	H1299	**LCC**
3	H522	**AC**
4	H157	**LCC**
5	H460	**LCC**
6	H1792	**AC**
7	H1648	**AC**
8	H226	**SCC**
9	A549	**AC**
10	H596	**ASC**
11	Calu-1	**SCC**
12	SK-MES1	**SCC**
13	H1944	**AC**
14	1198	**N/A**
15	1170-I	**N/A**
16	1799	**N/A**
17	BEAS-2B	**N/A**
18	EKVX	**AC**
19	HOP 62	**AC**
20	H-23	**AC**
21	H322M	**BAC**
22	H358	**BAC**
23	H1703	**AC**

7. NSCLC Paraffin Embedded Tissues

Lung cancer tissues (n=58), obtained from surgically removed tissues, were collected immediately after surgery and stored in embedded paraffin. Patients were routinely followed clinically after surgery. The presence of tumour cells in the collected tissues was verified by a consultant pathologist, who examined H&E stained tumour tissues. Patients were routinely followed on a regular basis and details stored in a database.

Paraffin-embedded tissues of NSCLC histology were retrieved from the pathology archives of Hospital Germans Trias i Pujol (n=58).

The non small cell lung paraffin embedded tissues samples were classified according to the 6th TNM classification and staging system. The guideline of the World Health Organization (WHO) of 2004 was used too, but without the last modifications of Experts internationally (Travis, Brambilla et al. 2011). Clinicopathologic characteristics of all patients are described in Table 5.

All the specimens had more than 80% tumor infiltration in an area similar or greater than 2.2 mm^2, so that they were macrodissected. This refers to a gross manual dissection of the tissue samples embedded in paraffin guided by a histologic section. A diagnostic pathologist analyzed the histologic section in order to identify areas of the specimen containing the tissue of interest and marks the guide slide appropriately. This marked guide slide was used to isolate those portions of the sample.

Table 5. Clinicopathologic characteristics of patients with NSCLC (N=58).

	Patients (N=58)	
Characteristic	**No.**	**(%)**
Age , years		
Mean	64	
Range	37-79	
Sex		
Male	55	(94, 8 %)
Female	3	(5, 2 %)
Histology		
Adenocarcinoma-bronchioloalveolar carcinoma	18	(31%)
Squamous	29	(50%)
Large-cell carcinoma	9	(15,5%)
Others	2	(3,4 %)

Characteristic	Patients (N=58) No.	(%)
Clinical stage		
IA	13	(22, 4%)
IB	11	(19%)
IIA	0	(0%)
IIB	12	(20, 7%)
IIIA	14	(24, 1%)
IIIB	7	(12, 1%)
IV	1	(1, 7%)
Patological stage		
IA	12	(20, 7%)
IB	22	(37, 9%)
IIA	2	(3, 4%)
IIB	5	(8, 6%)
IIIA	14	(24, 1%)
IIIB	3	(5, 2%)
IV	0	(0%)
Performe Status		
0	40	(69%)
1	15	(25, 9%)
2	3	(5, 2%)
Tumor location		
Righ & left upper lobe	36	(62, 1%)
Righ & left lober lobe	22	(37, 9%)
Tumor diameter (cm)		
4≥	31	(53, 4%)
4<	27	(46, 6%)
Nodules affected (patological)		
0	37	(63, 8 %)
1	5	(8, 6 %)
2	16	(27, 6 %)
Types of lung surgery		
Lobectomy & bilobectomy	33	(56, 9%)
Pneumonectomy	18	(31%)
Others	7	(12, 1%
Treatment		
Neoadyuvant	10	(17, 3%)
Adyuvant	34	(58, 6%)
Surgery	14	(24, 1%

8. Acid Nucleic isolation from cell line

8.1 Genomic DNA isolation

Genomic DNA was obtained from cell lines by digestion with proteinase K (Quiagen, cat #50919131), followed by phenol: chloroform (1:1) extraction (Table 6).

Table 6. Isolation from DNA from cell lines. DNA Lysis Solution: 10mM Tris (pH 8.0), 2mM EDTA, 1% SDS. 1X TE [b]: 1 mM Tris-HCl (pH 7.5), 1 mM EDTA, sterile solution.

Trypsinization	aspirate medium from top of the cell
	wash with 2 ml of PBS
	add 1ml Trypsin to the T75 flask
	after trysinization is complete add 9ml rich medium
	transfer to a 15ml conical tube
Obtain the pellet	centrifuge 6 min 800 r.p.m 4ºC
	wash pellet of 250g-300g with PBS 1X(3ml) 800r.p.m. 3-5min at 4ºC
Lysed	add 3ml [a]DNA Lysis Solution and 150µl proteinasa K (10mgr/ml)
	mix by inversion
	incubate 50ºC o.n
DNA extraction	add 0,3 ml 10M NH_4OAc
	1- add 3ml Phenol:chloroform:isoamilalcohol (25:24:1, AppliChem)
	2-mix by inversion
	3- spin at 3.000r.p.m 15 min to 4ºC
	4- wait 5 min. To separate the phases
	5-transfer aqueous phase (top) to new 15ml conical tube
	repeat the steps 1-2-3-4-5
	add 3ml chloroform:isoamilalcohol (24:1, AppliChem)
	repeat the steps 2-3-4-5
	add 0.3ml 10M NH4OAc and 7.2 ml 100% ethanol
	Mix by inversion
	-20ºC o.n
	Centrifuge 8000r.p.m 30 min at 4ºC
	Remove the ethanol
	Resuspend pellet in 1 ml 70%ethanol
	Centrifuge 10 min 14000g at 4ºC
	Remove the ethanol
	Air dry
	Resuspend the pellet in 400µl of 1X TE [b] and 4µl RNAse A (100µg/ml)
	Incubate 2 hours to 37ºC
	Add 20µl 10% SDS, 20µl proteinase K (10mgr/ml)
	Incubate to 37ºC o.n.

8.2 RNA isolation

Briefly, RNA was isolated from cell lines. Total RNA was extracted with RNeasy kit (Qiagen, Valencia, CA, cat # 74104), according to the manufacturer´s protocol.

Six ng of total RNA was pretreated with amplification grade DNase I (Invitrogen-Life Technologies, cat # 18068-015) to avoid DNA contamination, according to the manufacturer's

instructions. First, we added the following components to an RNase-free, 0.5-ml microcentrifuge tube(s) on ice:

Components
1 µl 10X DNAse I Reaction Buffer 1µl DNAse I, Amp Grade, 1 U/µl DECP-treatment water to 10µl

After, incubating tube(s) for 15 min at room temperature, we inactivated the DNAse I for 10 min at 65ºC. In that moment the RNA sample was ready to use in reverse transcription, prior to amplification.

9. Genomic DNA isolation from Paraffin Embedded Tissue

Total of 58 NSCLC samples of paraffin embedded tissues were analyzed. Paraffin-embedded samples and slides were obtained by standard procedures.

9.1 DNA isolation

For isolation of DNA from deparaffinated, macrodissected tissue, the material was incubated with proteinase K (Qiagen, cat #50919131), and DNA was extracted with phenol: chloroform and ethanol precipitation (Table 7).

Table 7. DNA isolation from Paraffin Embedded Tissue.

Deparafinizaton	55-57ºC from 45 min to 1 hour (in the oven)
	xilol , 10 min
	Dry
Macrodissection	
Incubation	at 60ºC for 16 hours (o/n)
DNA Extraction	add 10µl of Proteinase k 20mg/ml (Qiagen) wait 15 min at 60ºC
	add 600 µl of Phenol:chloroform:isoamilalcohol (25:24:1, AppliChem)
	shake vigorously, wait 5 min. at room temperature
	centrifuge for 10 min, (max.speed 12 to 15.000rpm) at room temperature separating the aqueous and organic phases
	transfer the upper aqueous layer to a new tube

add a 0,1 volume of sodium acetate 3M (AppliChem) and 2,5 µl glycogen (Roche) and 1 volume of isopropanol (Panreac).
mix by inversion
precipitate nucleid acids for 30 to 40 min or o/n at -20º
Centrifuge for 10 min at 12-15,000 rpm at room temperature
remove the supernatant without disrupting the pellet
wash with 75% ethanol (150ml)
centrifuge at 12-15,000 rpm for 5 min
remove the supernatant
let the pellet dry at 65ºC
dilute with DEPC water (20µl)
incubate 5 min at 65ºC

10. Mutations of LKB1/AMPK/TSC2

We have established collaboration with Dr. Wei Zhou and Dr. Diansheng Zhong, MD (Hematology and Oncology Genetics Winship Cancer Institute, Emory University School of Medicine Atlanta, Georgia).

Dr. Diansheng Zhong has analyzed the mutations of LKB1/AMPK/TSC2 pathway. (Zhong, Guo et al. 2006)

10.1 Analysis of LKB1 and AMPK mutations by DNA sequencing

Point mutations of LKB1 and AMPK were determined in cell lines by direct sequencing of amplified full-length cDNA. The primers for amplification of coding region of LKB1 and AMPK-α2 are present in Table 8.

Table 8. Primer Pairs used for the amplified full length cDNA of the LKB1/STK11 and AMPK-α2 genes.

Primers for amplification	Primer Sequence	PCR product size
LKB1-F2	GAAGGGAAGTCGGAACACAA	1506 bp (F2/R1)
LKB1-R1	GTGGTCGGCACAGAAGCAT	
AMPK-α2- FS	GCCGAA GATGGCTGAG AAG	1677 bp (FS/RS)
AMPK-α2- RS	CTAGAGACAGATCAACGGGCTAA	

PCR reaction was carried out with the following cycle condition:
initial denaturation step at 94 ◦C for 2 min, then 45 cycles at 94 ◦C for 30 s, 55 ◦C for 30 s and extension at 68 ◦C for 60 s.
After PCR, every sample was run on 1% agarose gel to confirm the size of amplified products. PCR products were purified using Qiagen PCR purification kit (cat# 28104).
Samples were sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S. cat # 4337455) according to the manufacturer's manual, and analyzed on a ABI Prism 3130 DNA analyzer (Applied Biosystems, Foster City, CA).

Length Analysis of Fluorescently Labeled PCR

All PCR products were sequenced in both directions forward and reverse (Table 9). The sequences were aligned with the published genomic sequence using the DNA Sequencing Analysis Software version 5.1.1 (Applied Biosystem).

Table 9. Primer Pairs used for direct sequencing LKB1/ STK11 and AMPK-α2 genes.

Primers for Sequence analysis	Primer Sequence
LKB1- FS1	CTG GTGGATGTGT TATACAACGA
LKB1-FS2	CCAAGA GGTTCTCCAT CCG
LKB1-RS1	TCGTTGTATAACACATCCACCAG
LKB1-RS2	CGGATGGAGAACCTCTTGG
AMPK-α2-FS1	TGCGGATCTC CAAATTATGC
AMPK-α2-FS2	CAGAAAGGAT GCCACCTCTT AT
AMPK-α2RS1	TTCAGGACCTGCATACAATCTG
AMPK-α2RS2	CATCCAATGGACATCTTGCTT

10.2 Protein truncation analysis of TSC2

TSC2 is a large protein and direct DNA sequencing of this gene would be expensive and time consuming. Most known TSC2 mutations are nonsense mutations that lead to protein truncation. Protein truncation tests have been developed for TSC2, where genomic DNA from cell lines is used as a template for PCR using gene-specific primers, that incorporate a T7 promoter in-frame and upstream of the target sequence. The PCR product is then subjected to in vitro transcription and translation in the presence of 35-S-methionine. Full length versus truncated protein products

are visualized by SDS-polyacrylamide gel electrophoresis and autoradiography. This method was used to analyze TSC2 mutations in a panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines). DNA primers for TSC2 have been described previously (Mayer 2003) (Table 10-Table 11), and we used TNT T7 Quick kit for PCR DNA (Promega, cat# L5540).

Table 10. Primer Pairs Used for amplification of RT-PCR fragments applied to Protein Truncation Test (PTT). a:Each TSC specific forward primer was supplemented with a T7 promoter and translation initation sequence (the partially overlapping region between T7 and gene- specific sequences is underlined). b:Each TSC specific reverse primer was supplemented with a M13 reverse sequence for sequencing with fluorescence labeled primers.

PCR fragment	Forward primer [a]	Reverse primer [b]	T_M (^oC)
Exon 1-11	CCACCATGGCCAAACCAACAAGC	CCACCAGTTCAAAGTATCTCTCCTG	64/63
Exon 9-20	TGCTGAGAGGAGCCGTGTTTTTG	CCGCTCCAGTGTCTTTGGGCC	63/65
Exon 17-26	GCCTTTGACTTCCTGTTGCTGCTG	CCGTGAAGTTGGAGAAGACGTATC	64/63
Exon 23-34	AATCAGTACATCGTGTGTCTGGCC	CATTGGGCAGCAGGATTGGCTTG	63
Exon 28-37	TGCATGTGAGACAGACCAAGGAG	CCTTGATGGTGCCAAGCTTGAAG	64/63
Exon 33-41	GTCGTCCTCAGTCTCCAGCCAG	TCACTGACAGGCAATACCGTCCAA	66/63
T7 promoter sequence	GGATCCTAATACGACTCACTATAGGAACAGACCACCATG		
M13 reverse sequence	CAGGAAACAGCTATGACC		

Table 11. Continuation of Table 10.

PCR fragment	TSC specific fragment length (bp)	Overlapping region (bp)	Polypeptide size (kD)
Exon 1-11	1239	362	45.3
Exon 9-20	1376	408	50.5
Exon 17-26	1279	475	46.8
Exon 23-34	1924	1269	67.3
Exon 28-37	1692		64.5
Exon 33-41	1510	413	54.0

11. Bisulfite modification-MSP

2µg of DNA diluted in 50 µl water, and denatured by 2M NaOH for 10 minutes at 37°C. Thirty microliters of 10mM hydroquinone (Sigma, cat#H9003) and 520 µl of 3M sodium bisulfite

(Sigma, cat#243973) at pH 5, both freshly prepared were added. Samples were incubated under mineral oil at 50°C for 16 hours.

Modified DNA was purified using Wizard PCR Clean-Ups DNA purification resin (Promega, cat#A7280) according to the manufacturer's instruction, and eluted into 50 µl of water. The eluted DNA was treated with 3M NaOH for 5 minutes at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at -80°C for future used.

Methylation Specific PCR (MSP) was performed using bisulfite-modified DNA (~50 ng) as a template in parallel PCR reactions with the following conditions:
67mM Tris-HCl, pH 8.8, 16.6mM NH_4SO_4, 10mM β-mercaptoethanol, 6.7mM $MgCl_2$, 1µM each primer in a 25µl reaction.
A hot start was performed (5 min, 95 ºC) followed by the addition of 0.5 u Taq polymerase (Invitrogen-Life Technologies, cat#10342) and 35 cycles of PCR (95 ºC for 30 s, T° annealing for 30 s and 72 ºC for 30 s).
To check the methylation status of BRSK2 gene in the embedded paraffin tissues, PCR cycling conditions were are follow 95°C for 15 min then 50 cycles of 95°C for 30 s, 50º for 30 s, and 72°C for 30s; and a final extension of 4 min at 72°C. Hot start PCR was used (Qiagen, cat#203203). Hot star DNA polymerase is activated by a 15 minute, 95°C incubation step, which can easily be incorporated into existing thermal cycling programs.
Negative and positive controls for the PCR reaction were carried out in parallel with samples using water only without DNA and commercially universal methylated DNA (Chemicon, cat# 57821) and universal unmethylated DNA (Chemicon, cat#57822), respectively.
Reaction products were separated by electrophoresis on a 6% polyacrylamide/Tris-borate-EDTA gel, stained with ethidium bromide and visualized under UV illumination.
Primers were designed from the interpolated sequence following bisulfite conversion assuming the DNA was either methylated or unmethylated at CpG sites. To ensure maximal discrimination of unmethylated and methylated DNA, primers were designed to overlap three potential methylation sites (ie, CpG). Table 12 and Table 13 contain the sequence, melting temperature and products size of PCR of primers specific for methylated (M) and unmethylated (U) regions of the 11 gene promoters studied**.**

Table 12. PCR primer sequences. M: methylated-specific primers; U: unmethylated-specific primers.

GENE	Forward primer (5'-3')	Reverse primer (5'-3')
LKB1 (M)	ACGAAGTTGATTTTGATCGGGTC	CGATACAAAATCTACGAACCGACG
LKB1 (U)	GGATGAAGTTGATTTTGATTGGGTT	ACCCAATACAAAATCTACAAACCAACA
AMPKα1 (M)	GTCGAAGGTGTCGATTTTTAGC	CCGACGCGACCCTACCCG
AMPKα1 (U)	TGTTGAAGGTGTTGATTTTTAGTG	CCCCAACACAACCCTACCCA
AMPKα2 (M)	AGAAGTAGAAGTACGACGGGC	CGAAACCGCTCCACCTACCG
AMPKα2 (U)	TGAGAAGTAGAAGTATGATGGGT	CCAAAACCACTCCACCTACCA
AMPKβ1 (M)	CGAGGTTTGTAGAGACGGTTC	CTCAACGACTTCCGCTTCCG
AMPKβ1 (U)	TTTTGAGGTTTGTAGAGATGGTTT	CCTCAACAACTTCCACTTCCA
AMPKβ2 (M)	GGTTATAAGGTCGGCGGCGC	CGACTACCCACCATCGAAACG
AMPKβ2 (U)	AAATGGTTATAAGGTTGGTGGTGT	ATACAACTACCCACCATCAAAACA
TSC1 (M)	AAGGAAGGATAGACGGTCGC	CCAATCACGTAACCGCCCG
TSC1 (U)	GAAAGGAAGGATAGATGGTTGT	CACCAATCACATAACCACCCA
TSC2 (M)	GAGGCGGATTTCGTAGTGTTC	ACACGAATTAAAACTACCGCCG
TSC2 (U)	GAGAGGTGGATTTTGTAGTGTTT	AATACACAAATTAAAACTACCACCA
BRSK2 (M)	ATTGTCGCGTCGGAGTGGAC	CCAACATACTACGCGTACTACG
BRSK2 (U)	GGATTGTTGTGTTGGAGTGGAT	ACCCAACATACTACACATACTACA
BRSK1 (M)	AGCGTGTCGGAGAGAAAGGAC	CGACCCCCCGCTTCCTACG
BRSK1 (U)	GTTAGTGTGTTGGAGAGAAAGGAT	ACCAACCCCCCACTTCCTACA
MARK1 (M)	TTATAGTTCGGTCGGCGAGATTTC	CGTATCCCGCTCGTTCACCG
MARK1 (U)	TTATAGTTTGGTTGGTGAGATTTTG	TTCCATATCCCACTCATTCACCA
MARK4 (M)	CGGGGTTATTAGGATTTTCGGC	CCGAATTCCGATCGTT ACCCG
MARK4 (U)	TATGGGGTTATTAGGATTTTTGGT	ATATCCAAATTCCAATCATTACCCA

Table 13 PCR primer annealing temperatures and PCR product length. M: methylated-specific primers; U: unmethylated-specific primers.

GENE	Melting Temperature(° C)	Product size (bp)
LKB1 (M)	57.3	117
LKB1 (U)	57.3	122
AMPKα1 (M)	57.3	161
AMPKα1(U)	57.3	164
AMPKα2(M)	58.2	207
AMPKα2 (U)	58.2	210
AMPKβ1 (M)	58.2	160
AMPKβ1 (U)	58.2	164
AMPKβ2 (M)	59.2	122
AMPKβ2 (U)	59.2	129
TSC1 (M)	57.3	256
TSC1 (U)	57.3	260
TSC2 (M)	57.3	216
TSC2 (U)	57.3	221
BRSK2 (M)	58.2	239
BRSK2 (U)	58.2	243
BRSK1 (M)	59.2	164
BRSK1 (U)	59.2	169
MARK1 (M)	59.2	94
MARK1 (U)	59.2	97
MARK4 (M)	59.2	242
MARK4 (U)	59.2	248

12. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was converted to cDNA by reverse transcriptase. As a negative control a separate reaction was be carried out without reverse transcriptase.

The first-strand cDNA was synthetized using random hexamer primers (Invitrogen, Life Technologies,cat#4819001) and M-MLV retrotranscriptase (Moloney Murine Leukemia Virus retrotranscriptase)(Invitrogen, Life Technologies, cat#28025013), according to the manufacture's protocol, using 6 μg of total RNA.

The cDNA was amplified using primers specific for BRSK2. The reaction was performed with PCR program:

Hot start 95ºC 5 mins
Hold 80ºC
Add 2ul of 0.25u/μl of Taq polymerase (1:20 dilution of 5U/μl)

Continue program- 95ºC 30 secs
57, 2ºC 1 min
72ºC 1 min
} x 35 cycles

Final extension 72ºC 4 min
Hold 4ºC

Table 14. Primer Pairs used for the amplified full length cDNA of the BRSK2.

Primers for amplification	Primer Sequence	PCR product size
BRSK2-F	ACGATGACAACTTGCGACAG	189pb
BRSK2-R	CTGGTTCGGGCTCATTCTT	

Reaction products were separated by electrophoresis on a 6% polyacrylamide/ Tris-borate-EDTA gel, for 1 hour at 180 V, stained with ethidium bromide, viewed on UV transilluminator and photographed.

13. Quantitative PCR real-time (qRT-PCR)

For real-time analysis, 1 µl of a 50-fold dilution of the reverse transcriptase reaction was amplified using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, cat#170-8880) and 0.25µl of each 20µM primer. Primers for real-time PCR analysis of BRSK2 were 5´-CAGCACGCGCAGTATGTTG -3´ and 5´-CATCAGCACCGACTCGCTG-3´ (152 bp product), primers for 18S were 5´-GAGGGAGCCTGAGAAACGG-3´ and 5´-GTCGGGAGTGGGTAATTTGC-3´ (68 bp product).
Real-time quantitation was performed using the MyIQ real-time detection system (Bio-Rad, Hercules, CA) under the following cycling conditions: (step1) 95ºC (3 min); (step2) 50 cycles of the 95ºC (10s), 55ºC (60s). Melt curve analysis was performed to ensure a single product species. Reactions were carry out in triplicate in 96-well plates to control for experimental variability. Parallel reactions were performed using primers to 18S rRNA as an internal control to normalize the amount of input cDNA. To control for variation between experiments, we used H1155 cDNA as a standard curve for all experiments.

14. Reagents and 5-aza-dC and TSA treatment

Calu-1 cells were seeded at a density of $3x10^5$ per 100-mm tissue culture dish. After 24 hours of incubation, the culture media were changed to media containing 0.5µM 5-aza-2-deoxycytidine (5-aza-dC; Sigma, cat#A3656).
For the combined treatments, after 48 hours cells were treated with 0.5µM of 5-aza-2-deoxycytidine again and 100ng/ml Trichostatin (TSA; Sigma cat# T8552) was added for the last 12 h and 24h.
DNA from Calu-1 cell line was extracted as explained previously. RNA from Calu-1 cell line was extracted using the RNeasy kits (Qiagen, Valencia, CA, cat # 74104) according to the manufactures protocol.
TSA stock solutions were made in DMSO and 5-aza-2dC stock solutions were made in water, and both stored in aliquots at -80°C. The compound was diluted in incubation media immediately prior to each experiment. Thawed stock solutions were used once and discarded.

15. LKB1 small interfering RNA treatment

An RNA interference approach was used to generate Calu-1 cell line that is stably knocked down for LKB1.

Cells were cultured up to 70% confluence on 6-well plates. Usually 2 wells per transfection were needed to get enough protein for an experiment.

Example: Sample 6 well Plate Set-up for 3 different transfections.

Mock	Lamin A/C	LKB1
Mock	Lamin A/C	LKB1

- Mock = negative control. (No siRNA, just Optimen).
- Lamin A/C= internal control. (lamin siRNA+ Optimen).Lamin A/C is recommended as a positive silencing control in human, mouse and rat cell lines. Lamin A/C was used to control for any nonspecific off-target effects of siRNA transfection Lamins are intermediate filament-type proteins, which form major components of the nuclear lamina. Lamins A and C have close sequence homology and are derived by differential splicing and alternative polyadenylation from one gene. This gene is abundantly expressed in most cells and because it is non-essential, knockdown of the corresponding mRNA does not affect cell viability.
- LKB1= the one to be knocked down (LKB1 siRNA + Optimen).

LKB1 small interfering RNA (siRNA) duplexes were purchased from Dharmacon. The sequence of LKB1 siRNA was 5′-GGACUGACGUGUAGAACAATT-3′.

Oligofectamine reagent (Invitrogen, cat#12252011) was incubated with Opti-MEM1 (Life Technologies, cat#31985062) reduced serum medium for 10 min, and a mixture of siRNA was then added. After incubation for 15 min at room temperature, the mixture was diluted with medium and added to each well. siRNA (200nM) was used per well in 2 mL medium and incubated at 37º for 5 hours. Transfection media was then replaced with normal growth media and growth allowed to continue for 48 hours before proceeding with protein collection for Western blot analysis. Total cell lysates were used for Western blot analysis.

15.1 Cell Lysis & Protein extraction

Aspirate medium from the well
Wash the well with 2ml of DPBS ⇒ aspirate the DPBS
Add 300µl Trypsin/EDTA (0.05%) } per well to count the cells Quench with complete media
Add 2ml per well of DPBS and wash them (repeat it 3 times) Transfer cells to 15 ml conical tube
Centrifuge pellets cells of the conical tube at 200g during 5 minutes to 4ºC Remove the supernatant
Wash the cell pellet with 500µl cold DBPS Centrifuge at 200g during 5 minutes to 4ºC } repeat it 3 times Remove the supenatant
Lysed cell pellet in a volume of 3 times cell pellet with: RIPA buffer conting complete mini protease tablet (1 tablet/10ml RIPA*) Phosphatase inhibitors { 2.5 mM Sodium pyrophosphatase 1 mM β-Glycerolphosphatase 1 mM $NaVO_4$
Incubated in ice 10 minutes
Spin 13.000 g
Protein in supernatant is removed and placed into a new 1.5 ml eppendorf
Store in -80ºC

*RIPA(Roche cat#11836153001)

15.2 Assessment of protein

Protein concentrations were measured by the method of Bradford according to the manufactures instructions (Bio-Rad, Hercules, USA). The Bradford assay is the classic colorimetric assay for measuring total protein concentration.

15.3 Western Blotting

Proteins (50-75ug/lane) from whole cell lysates were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride membrane (PVDF) (Bio-Rad, cat# 162-0177). The membranes were blocked with 5% nonfat milk and incubated with mouse monoclonal antibody against human Anti-LKB1 protein from Abcam (Cambridge, USA), followed by horseradish peroxidase-conjugated secondary antiserum. Immunoreactivity was visualized by enhanced chemiluminescence reagent (Thermo Scientific, cat # 34077). Actin and GADPH was used as loading controls.

V. Results

16. Genetic and Epigenetic alterations in LKB1/AMPK/TSC

Aim I. The first objective is to determine the frequency of LKB1/AMPK/TSC2 signaling pathway alterations in NSCLC:

Approximately one-third of primary sporadic lung adenocarcinomas and half of lung cancer cells lines of adenocarcinoma histological subtype, harbor mutations in the LKB1 gene. This indicates that LKB1 plays a significant role in an extremely frequent cancer type. LKB1/AMPK/TSC signaling pathway is frequently altered in NSCLC. We were investigating whether components of this pathway undergo epigenetic alteration. In order to do that, we analyzed the promoter methylation status and mutations of LKB1, different components of the AMPK heterotrimeric complex AMPKα1, AMPK α2, AMPK β1, AMPK β2, and the TSC1-TSC2 complex.

CpG island promoter methylation was analyzed by methylation specific PCR (MSP) and mutations analysis were performed by direct sequencing of amplified full-length cDNA except for TSC2 which was analyzed by the protein truncation test (Table 15) . Twenty three cell lines were examined, nineteen were NSCLC cell lines (9 adenocarcinomas, 3 squamous cell carcinoma, 4 large cell carcinoma, 1 adenosquamous carcinoma, 2 bronchioloalveolar carcinoma) and 4 cell lines were from normal bronchial epithelia transformed with SV40.

Table 15. Summary of methylation patterns and mutations in LKB1, and two of three different subunits of heterotrimeric AMPK complex. U = unmethylated; LCC=large cell carcinoma; AC=adenocarcinoma; SCC= squamous cell carcinoma; ASC= adenosquamous; BAC=bronchioalveolar carcinoma; N/A = normal bronchial epithelial cells immortalized with SV40.

Cells lines		Histology	LKB1		AMPKα1	AMPKα2		AMPKβ1	AMPKβ2
			Mut.	Methyl.	Methyl.	Mut.	Methyl.	Methyl.	Methyl.
1	H1155	LCC	NO	U	U	NO	U	U	U
2	H1299	LCC	NO	U	U	NO	U	U	U
3	H522	AC	NO	U	U	NO	U	U	U
4	H157	LCC	YES	U	U	NO	U	U	U
5	H460	LCC	YES	U	U	NO	U	U	U
6	H1792	AC	NO	U	U	NO	U	U	U
7	H1648	AC	NO	U	U	NO	U	U	U
8	H226	SCC	NO	U	U	NO	U	U	U
9	A549	AC	YES	U	U	NO	U	U	U
10	H596	ASC	NO	U	U	NO	U	U	U
11	Calu-1	SCC	NO	U	U	NO	U	U	U
12	SK-MES1	SCC	NO	U	U	NO	U	U	U
13	H1944	AC	YES	U	U	NO	U	U	U
14	1198	N/A	NO	U	U	NO	U	U	U
15	1170-I	N/A	NO	U	U	NO	U	U	U
16	1799	N/A	NO	U	U	NO	U	U	U
17	BEAS-2B	N/A	NO	U	U	NO	U	U	U
18	EKVX	AC	YES	U	U	NO	U	U	U
19	HOP 62	AC	NO	U	U	NO	U	U	U
20	H-23	AC	YES	U	U	NO	U	U	U
21	H322M	BAC	NO	U	U	NO	U	U	U
22	H358	BAC	NO	U	U	NO	U	U	U
23	H1703	AC	NO	U	U	NO	U	U	U

As detailed in Table 15, in the genetic analysis we identified LKB1 point mutations in 6 of 19 NSCLC cell lines, 31, 5 % of the cell lines analyzed. These data were consistent with previous reports of ~30% mutation frequenty of LKB1 in NSCLC. Our results demonstrated the presence of mutations in LKB1 gene in H460 and A549 human lung cancer cells lines. Both of them were identical nonsense mutation, involving a C to T mutation at codon 37, leading to a change from Gln to a stop codon. The H1944 cell line contained two missense mutations; both Lys to Asn at codon 62 and 78 in the kinase domain. We also found that the cell line EKVX contained a Ser to Phe mutation at codon 216. The H-23 cell line also contained a nonsense mutation at codon 332.

Interestingly, we found a novel deletion of LKB1 in a large cell carcinoma cell line of the lung, H157. Direct sequencing of cDNA from H157 cells indicated that it lacked exons 2 and 3. Dr. Wei Zhou and Dr. Diansheng Zhong, MD (Winship Cancer Institute, Emory University Atlanta, Georgia) also sequenced genomic DNA isolated from H157 cancer cell line, confirming that the deletion was also present in genomic DNA (data not shown). No other sequence alterations were identified in adjacent genomic regions of exon 3 (Zhong, Guo et al. 2006). Thus, a genomic deletion led to the elimination of both exons 2 and 3 in the mRNA of LKB1, a 174 bp deletion in the coding sequence, and was predicted to result in deletion of 58 amino acids (codons 98-155) in the central kinase domain from the LKB1 protein (Figure 27).

These data indicated that LKB1 mutations are not confined to adenocarcinomas, but also occur in other NSCLC subtypes as large cell carcinomas.

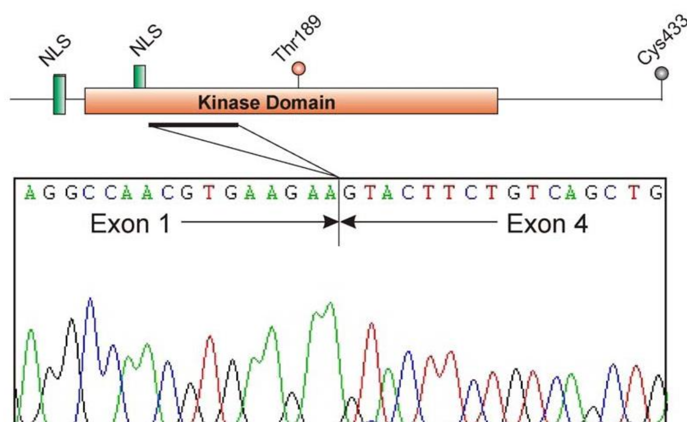


Figure 27. Schematic representation of LKB1 gene and histogram of DNA sequencing reactions of RT-PCR product from H157. The nuclear localization signal (NLS), kinase domain and farnesylation site (Cys 433) of LKB1 are indicated (Zhong, Guo et al. 2006)

In the epigenetic analysis, of the LKB1 (Figure 28) and different isoforms of 2 of the three components of AMPK (α1, α2, β1, and β2) (Figure 29, Figure 30, Figure 31, Figure 32), non promoter methylation was detected in none of the 23 cell lines (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines). On the other hand, no sequence alterations in AMPK α2 were detected in this panel of 23 cell lines.

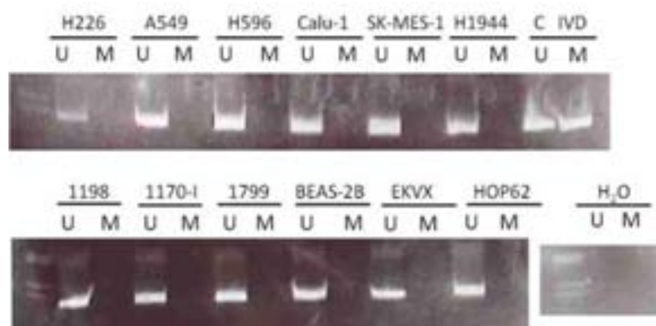


Figure 28. LKB1 Methylation Analysis in some of the 23 cell line panel. CpG island promoter methylation at the LKB1 locus was analyzed in a panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA. Shown are representative examples.

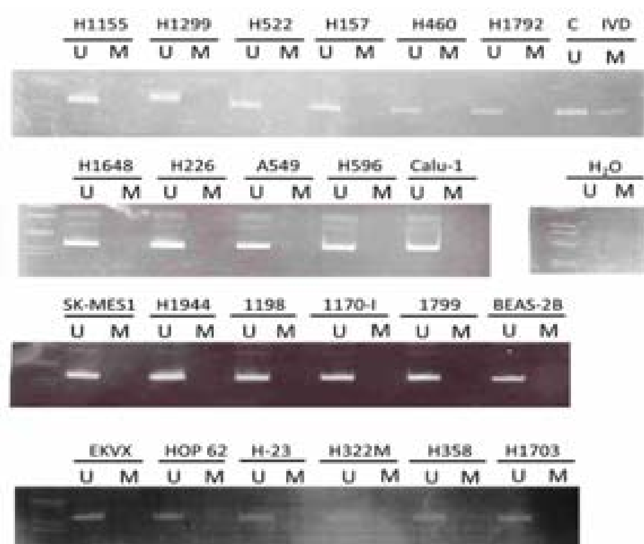


Figure 29. AMPK α1 Methylation Analysis in 23 cell line panel. CpG island promoter methylation at the AMPKα1 locus was analyzed in a panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA.

Figure 30. AMPK α2 Methylation Analysis in 23 cell line panel. CpG island promoter methylation at the AMPK α2 locus was analyzed in a panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA.

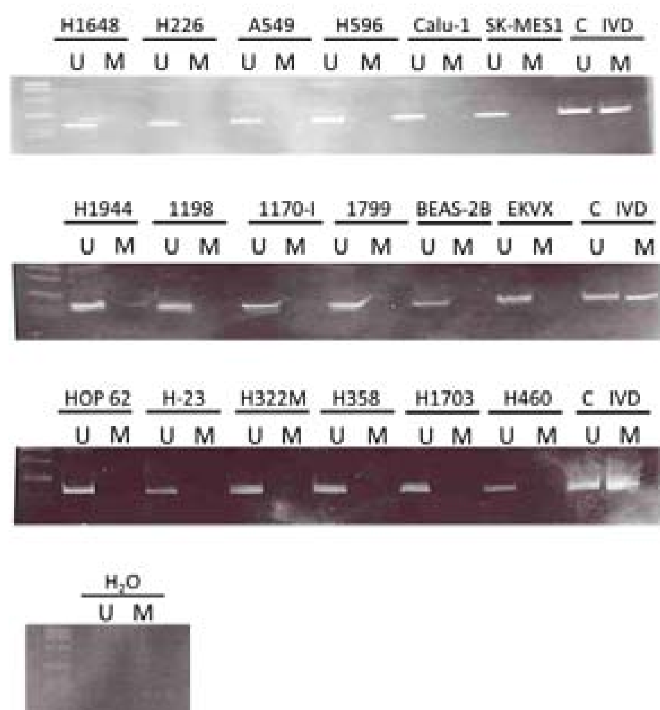


Figure 31. AMPK β1 Methylation Analysis in 23 cell line panel. CpG island promoter methylation at the AMPK β1 locus was analyzed in 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA. Shown are representative examples.

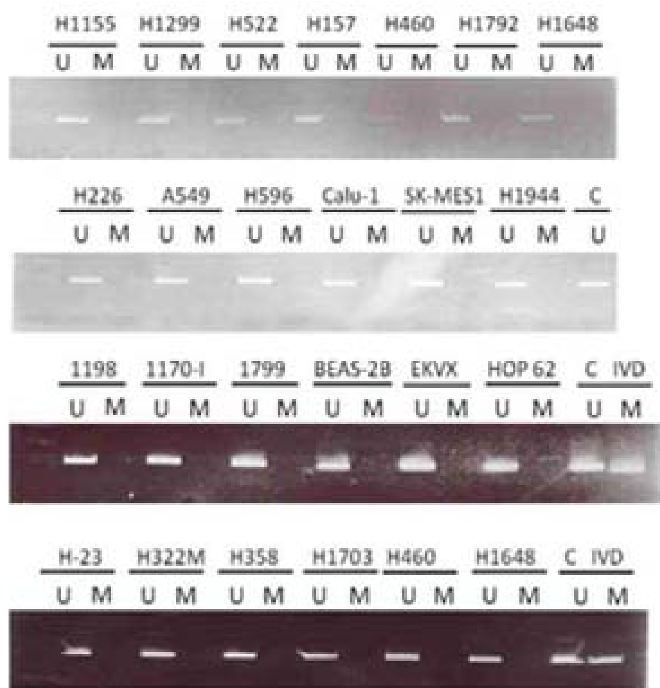


Figure 32. AMPK β2 Methylation Analysis in 23 cell lines. CpG island promoter methylation at the AMPKβ2 locus was analyzed in a panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA.

As mentioned earlier, AMPK protein kinase activates TSC2, which is associated with TSC1. This heterodimer, TSC1-TSC2, regulates negatively mTOR (mammalian target of rapamycin) signaling. Consequently, inactivation of TSC1 or TSC2 is associated with elevated mTOR activity.

Therefore methylation patterns of TSC1 and TSC2 heterodimer were analyzed by MSP and also the mutations of TSC2 tumor suppressor were analyzed by protein truncation test (PTT) (Table 16).

Table 16. Summary of methylation patterns and mutations in TSC1-TSC2 complex. U = unmethylated; LCC=large cell carcinoma; AC=adenocarcinoma; SCC= squamous cell carcinoma; ASC= adenosquamous; BAC=bronchioalveolar carcinoma; N/A = normal bronchial epithelial cells immortalized with SV40.

Cells lines		Histology	TSC1	TSC2	
			Methylation	Mutations	Methylation
1	H1155	LCC	U	NO	U
2	H1299	LCC	U	NO	U
3	H522	AC	U	NO	U
4	H157	LCC	U	NO	U
5	H460	LCC	U	NO	U
6	H1792	AC	U	NO	U
7	H1648	AC	U	NO	U
8	H226	SCC	U	NO	U
9	A549	AC	U	NO	U
10	H596	ASC	U	NO	U
11	Calu-1	SCC	U	NO	U
12	SK-MES1	SCC	U	NO	U
13	H1944	AC	U	NO	U
14	1198	N/A	U	NO	U
15	1170-I	N/A	U	NO	U
16	1799	N/A	U	NO	U
17	BEAS-2B	N/A	U	NO	U
18	EKVX	AC	U	NO	U
19	HOP 62	AC	U	NO	U
20	H-23	AC	U	NO	U
21	H322M	BAC	U	NO	U
22	H358	BAC	U	NO	U
23	H1703	AC	U	NO	U

No sequence alterations in the TSC2 gene were detected in this panel of 23 cell lines. On the other hand, we did not identify any epigenetic alterations in CpG island promoter methylation in TSC1- TSC2 heterodimer in any of this 23 cell lines checked (4 normal bronchial epithelial cell

lines immortalized with SV40 and 19 NSCLC cell lines) (Figure 33, Figure 34). However, the MSP technique is limited to the analysis of the 3-6 CpG sites underlying the PCR primers. Our results thus far reflect the data from a single primer set.

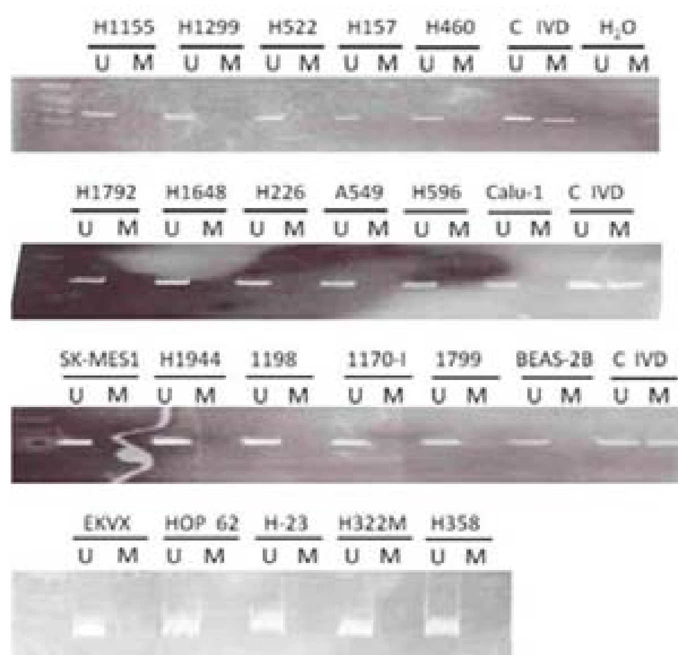


Figure 33. TSC1 Methylation Analysis in some of the 23 cell line panel. CpG island promoter methylation at the TSC1 locus was analyzed in 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA.

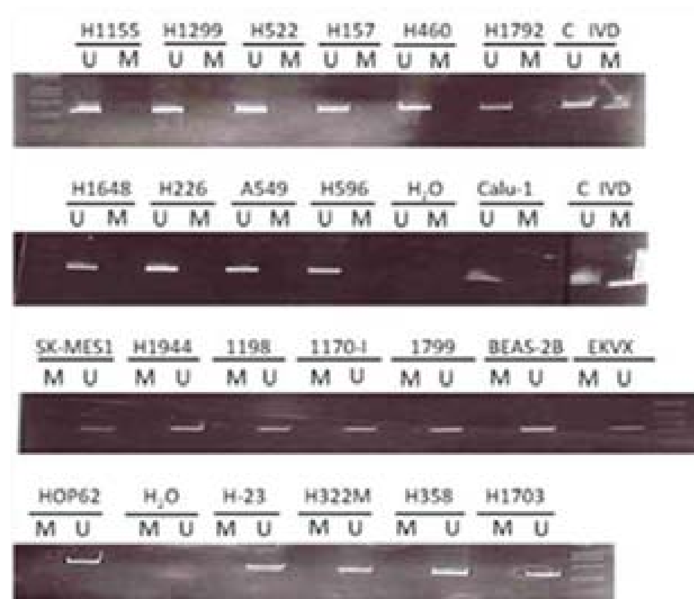


Figure 34. TSC2 Methylation Analysis in 23 cell line panel. CpG island promoter methylation at the TSC2 locus was analyzed in 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA.

17. LKB1 activates 12 kinases of AMPK subfamily.

Aim II. The second objective is to study LKB1/AMPK-related kinases alterations as a additional molecular mechanisms for the development of lung cancer.

LKB1 is a master protein kinase that activates 11 of the 12 AMPK family members in vitro: NUAK1, NUAK2, BRSK1, BRSK2, QIK, QSK, SIK, MARK 1, MARK2, MARK3, and MARK4 (Lizcano, Goransson et al. 2004). So LKB1 is an upstream activator of AMPK and 11 other related kinases.

We considered the possibility of LKB1/AMPK- related kinase alterations as additional molecular mechanisms for the development of lung cancer. Therefore we analyzed the methylation status of four kinases of AMPK subfamily by MSP technique, in 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) (Table 17).

We found a low frequency of methylation status in MARK1 (Figure 35), MARK4 (Figure 36) and BRSK1 (Figure 37).

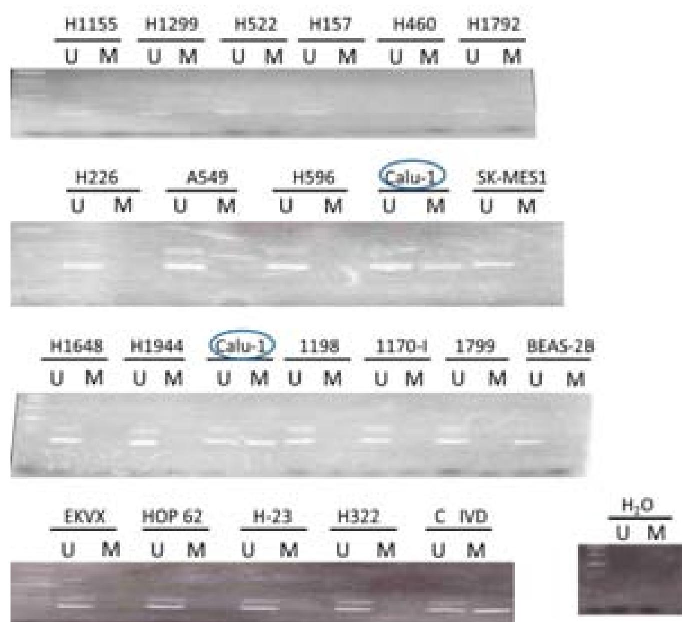


Figure 35. MARK1 Methylation Analysis in some of the 23 cell lines panel. CpG island promoter methylation at the MARK1 locus was analyzed in 23 cell line panel (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA. Shown are representative examples.

Table 17. The methylation status of four kinases was analyzed by MSP.

U-M = both methylated and unmethylated alleles are present.

M*= <10% methylation. LCC=large cell carcinoma; AC=adenocarcinoma; SCC= squamous cell carcinoma; ASC= adenosquamous; BAC=bronchioalveolar carcinoma; N/A = normal bronchial epithelial cells immortalized with SV40.

Cells lines		Histology	LKB1	BRSK1	BRSK2	MARK1	MARK4
			Mutation	Methylation	Methylation	Methylation	Methylation
1	H1155	LCC	NO	U	U	U	U
2	H1299	LCC	NO	U	U	U	U
3	H522	AC	NO	U	U	U	U
4	H157	LCC	YES	U	U-M	U	U
5	H460	LCC	YES	U	U	U	U
6	H1792	AC	NO	U	U	U	U
7	H1648	AC	NO	U	U	U	U
8	H226	SCC	NO	M*	M	U	U
9	A549	AC	YES	U	U	U	U
10	H596	ASC	NO	U	U	U	U
11	Calu-1	SCC	NO	U	M	U-M	U
12	SK-MES1	SCC	NO	U	U	U	U
13	H1944	AC	YES	U	U	U	U
14	1198	N/A	NO	U	U	U	U
15	1170-I	N/A	NO	U	U-M	U	U
16	1799	N/A	NO	M*	U-M	U	U
17	BEAS-2B	N/A	NO	U	U	U	U
18	EKVX	AC	YES	U	U-M	U	U
19	HOP 62	AC	NO	U	U	U	U
20	H-23	AC	YES	U	U	U	U
21	H322M	BAC	NO	U	M	U	U
22	H358	BAC	NO	U	U	U	U
23	H1703	AC	NO	U	U	U	U

When the methylation status of the MARK1 promoter was studied, only one of the NSCLC cell lines was partially methylated. The Calu-1 NSCLC cell line had both methylated and unmethylated alleles present in the promoter region of the gene. In Figure 35, Calu-1 is represented inside a blue circle on a 6% polyacrylamide/Tris-borate-EDTA gel.

In case of MARK4 (Figure 36) we didn't find any methylation in any of the 23 cell lines (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines) that we

analyzed by MSP, however MSP technique is limited to the analysis of the 3-6 CpG sites underlying the PCR primers. Our results thus far reflect the data from a single primer set.

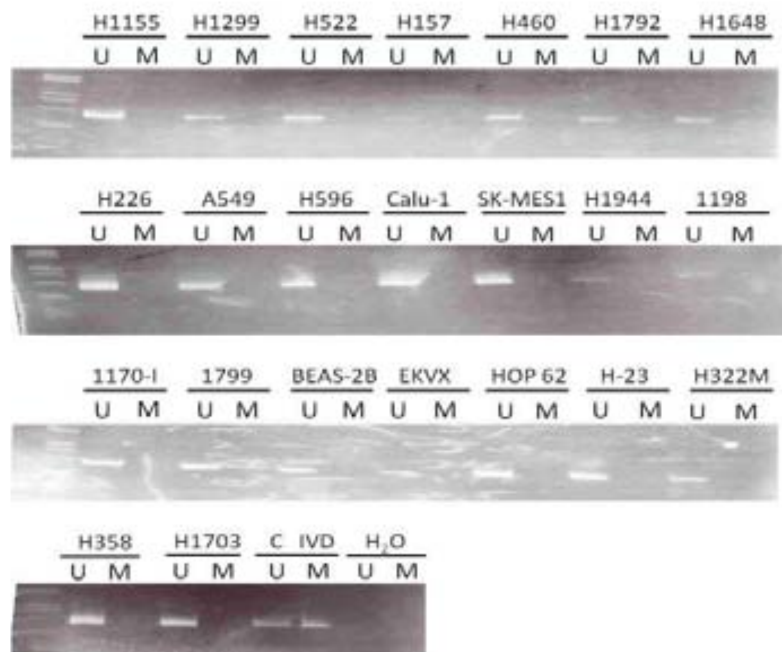


Figure 36. MARK 4 Methylation Analysis in 23 cell lines panel. CpG island promoter methylation at the MARK4 locus was analyzed in 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA.

Also, the methylation status of BRSK1 promoter was analyzed in this panel of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines). Representative examples of unmethylated and the two methylated cell lines are shown in Figure 37. We found two cell lines that were methylated less than 10% (M*) in the BRSK1 promoter. One of them is H226 squamous cell carcinoma the other cell lines was 1799 is from normal bronchus epithelia transformed with SV40. In Figure 37, those cell lines are represented inside a blue circle on a 6% polyacrylamide/Tris-borate-EDTA gel.

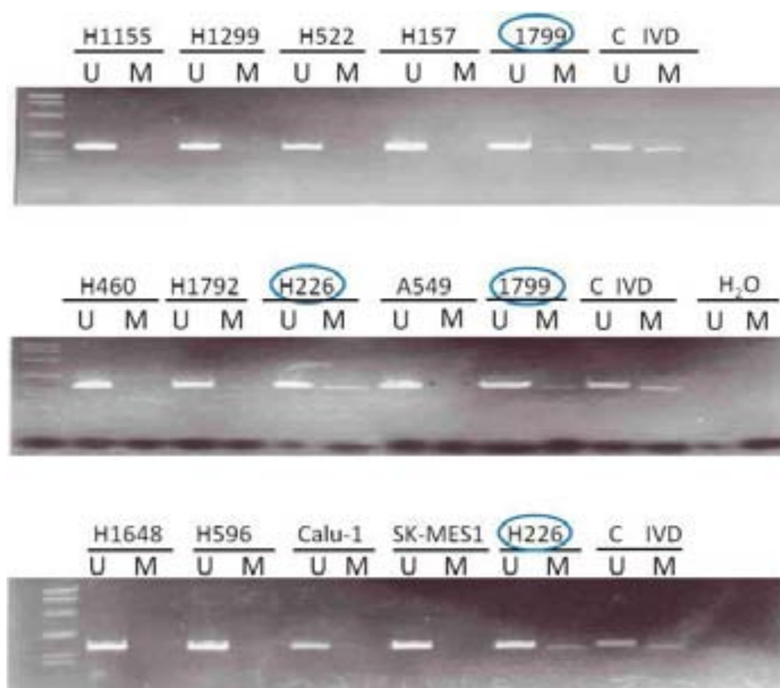


Figure 37. BRSK1 Methylation Analysis in some of the 23 cell lines. CpG island promoter methylation at the BRSK1 locus was analyzed in 23 cell lines (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines) by MSP. Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA. Shown are representative examples.

Next, the CpG island promoter hypermethylation of BRSK2 gene was analyzed in 23 lung cancer cell lines by means of methylation-specific PCR (Table 17).

We found in all, 2 of 19 NSCLC cell lines are "partially methylated", this represents approximately ~10.5% of NSCLC cell lines. But if we include the 4 immortalized bronchial epithelial cell lines with SV40, increase the number of cell lines with methylated and unmethylated alleles present in the promoter of this gene: the H157 cell line is a large cell carcinoma, other one is EKVX a adenocarcinomas cell line, and the other two cell lines are 1170-I and 1799 both of them are normal bronchial epithelial cells immortalized with SV40. This represents a ~17% of our 23 cell line panel (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines). These cell lines with methylated and unmethylated alleles are marked with blue circles on the 6% polyacrylamide/Tris-borate-EDTA gel (Figure 38).

In another hand, 3 of 19 NSCLC cell lines were completely methylated (only the methylated alleles are present). Calu-1 and H226 are squamous cell carcinoma; the third cell line is H322M, bronchioloalveolar carcinoma NSCLC subtype. These cell lines completely methylated in CpG island promoter of BRSK2 are corresponding to red circles on the 6% polyacrylamide/Tris-

borate-EDTA gel (Figure 38). Therefore, BRSK2, which is also a downstream target of LKB1, was completely methylated in ~16% of NSCLC cell lines. Interestingly, whereas there was no correlation between LKB1 mutation status and BRSK2 methylation in cases where the methylation status of BRSK2 promoter was either unmethylated or partially methylated, complete methylation of BRSK2 was only observed in cell lines that were wildtype for LKB1 (Table 17). Therefore, the epigenetic silencing of BRSK2 may be an alternative mechanism for the loss of LKB1 pathway function in these cell lines.

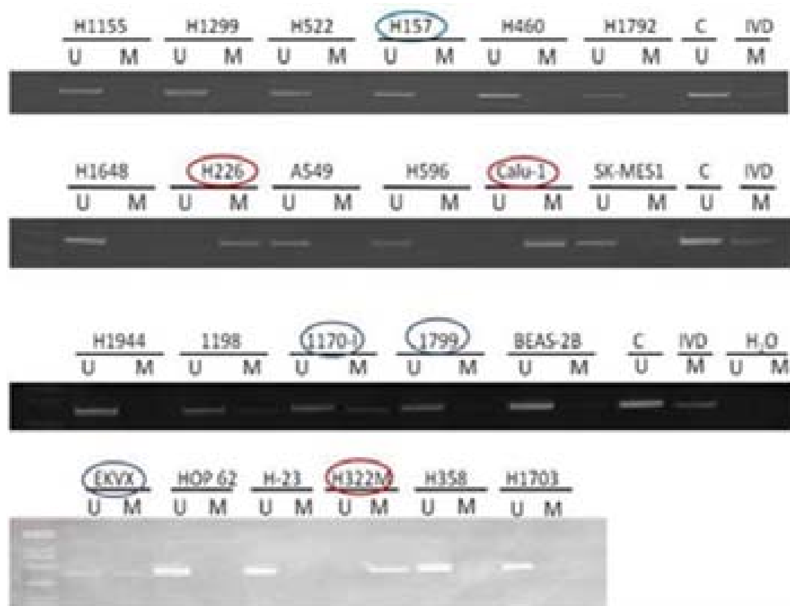


Figure 38 BRSK2 Methylation Analysis in some of the 23 cell lines panel. Methylation of a CpG island surrounding the promoter of the BRSK2 gene was determined by Methylation specific PCR (MSP). DNA from the indicated cell line was modified with bisulfate (which converts C to U and leaves meCintact). Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form; C, Control DNA; IVD, in vitro methylated DNA.

18. Determine the BRSK2 expression in a panel of 23 cell lines.

Aim III. The third objective is to examine LKB1/BRSK2 signaling.

III-A. Evaluate the expression of BRSK2 in a total of 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines).

After determining the methylation status of BRSK2 promoter in the 23 cell line panel, we also evaluated BRSK2 expression in these same cell lines panel by regular RT-PCR analysis. To

check the correlation between the methylation status of BRSK2 promoter, this mean unmethylated or totally methylated or partially methylated of the promoter and the expression of the BRSK2 gene. The results of the reaction products separated by electrophoresis on 6% polyacrylamide/Tris-borate-EDTA gels are shown in Figure 39. These gels show expression of BRSK2 gene in each 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines). Cell lines represented with blue color inside a blue circle (H157-H1170-1799-EKVX) were partially methylated, so they had methylated and unmethylated alleles in the promoter of BRSK2 gene.The other three NSCLC cell lines which were completely methylated (H226-Calu1-H322M), are represented with red color inside a red circle. In the RT-PCR assays, the housekeeping β-actin was used as an internal control. We compared the expression of the BRSK2 gene with the expression of β-actin.

As seen Figure 39, the reaction products that represented the expression of BRSK2 were similar more or less between all the NSCLC cell lines independently of the promoter methylation status. Thus, in the NSCLC cell lines with BRSK2 promoter completely methylated or partially methylated or unmethylated, BRSK2 gene expression was analogous. But one of the problems presented by the regular RT-PCR analysis was that the results are not quantitative at high amplification cycles. For this reason, we also evaluated BRSK2 expression in the 23 cell lines (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines) by another more sensitive technique, quantitative real-time PCR.

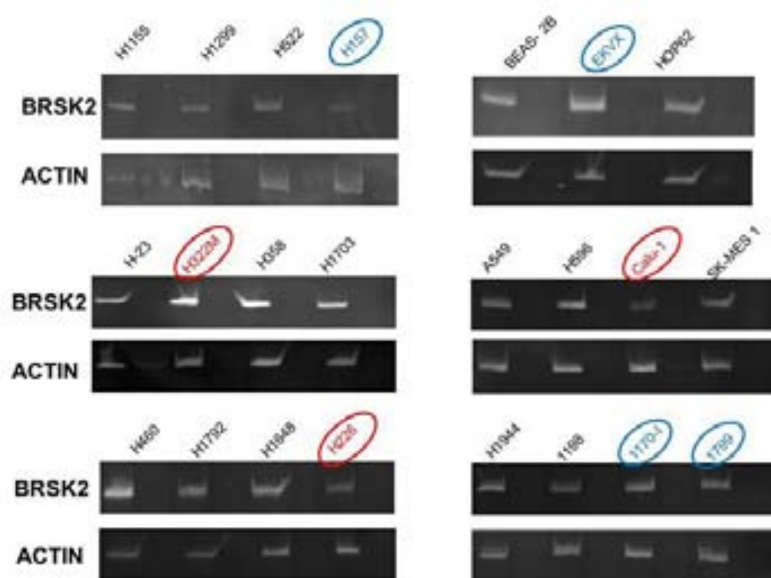
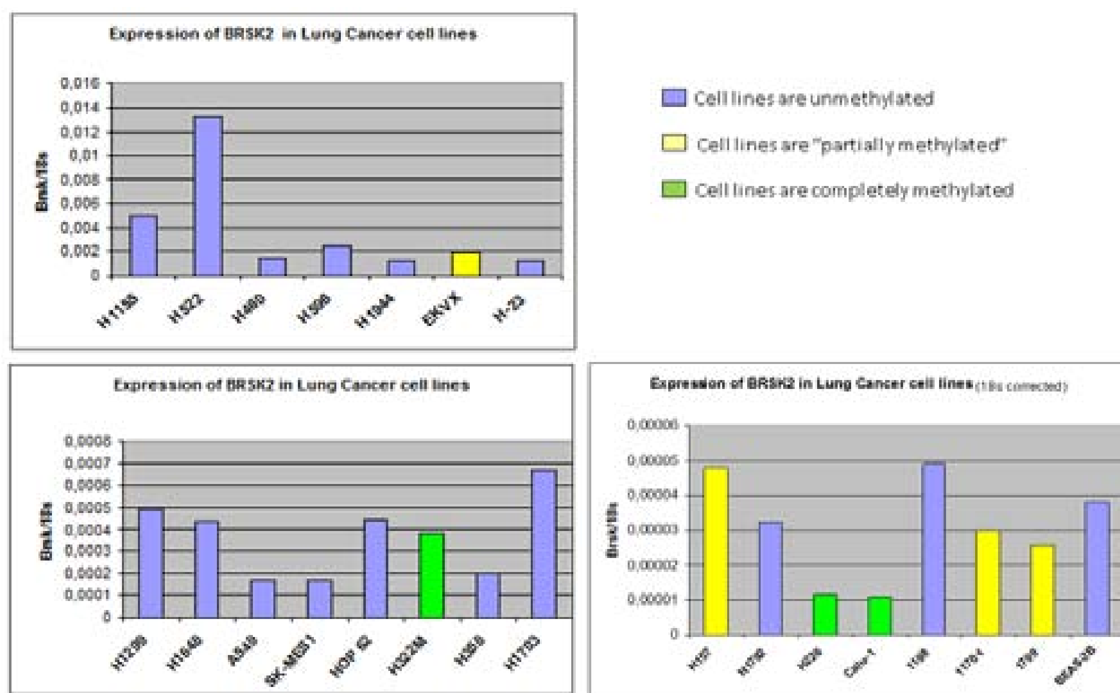


Figure 39. BRSK2 expression of the 23 cell lines (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines) using RT-PCR technique. RT-PCR products were generated from cDNA isolated from cell lines.

The H1155 is a large c ell c arcinoma cell li ne, in which we didn´ t find any mut ation or methylation in any of the genes that we analyzed in LKB1/AMPK/ TSC pathway.

To do the standard c urve we use d cDNA f rom H1155 cell li ne. H1155 c ell li ne was run as undiluted and then as a 1/3 serial dilutions (related as PCR efficiency). Thus the curve goes from undiluted to 1/243 dilution. The H1155 curve gave a correlation coefficient of r^2 =0.99 and the PCR efficiency is 94.1% .We therefore used the H1155 cDNAs curve as a standard for the rest of the experiment to check and analyze the BRSK2 expression in the different cell lines. Samples were run se veral undiluted or with various dil utions 1/10 or 1/50 to ensure that w e obtaine d values that fell on the linear portion of the standard curve. All the cell lines were run in triplicate. Since the cell li nes had a wide ra nge of e xpression of B RSK2 they were plo tted on diff erent graphs with different scales. The diff erent colors in the graphs show methylation status of BRSK2 in each of the cell lines. The level of BRSK2 mR NA expression within each cell li ne was normalized with the c orresponding 18s rRNA. Values w ere c orrected for dil ution. The graphs below show the results obtained from those Real time runs (Figure 40).

Figure 40. BRSK2 expression in 23 cell lines using quantitative real-time RT-PCR. 18s expression was used as an internal standard, and expression levels were reference to BRSK2 expression in H1155 cell line.



In Figure 40, we can observe the lowest expression levels of expression of BRSK2 correspond to H226 and Calu-1 squamous cell carcinoma cell lines, in other words, 2 of 19 NSCLC whose promoter status of BRSK2 were completely methylated. Moreover in this graph the cell lines with lowest expression include 3 of 23 cell lines analyzed (H157, 1170-I and 1799) with both methylated and unmethylated alleles presented in their promoter region of BRSK2. Therefore, 5 of 19 (~26%) NSCLC cell lines with BRSK2 epigenetic alterations, specifically presence of methylation in their promoter region of BRSK2, also exhibited reduced expression of the transcript. In the NSCLC cell lines EKVX (adenocarcinoma) and H322M (bronchioalveolar carcinoma), the expression of the BRSK2 transcript seemed not be alterated despite partially and totally methylated the promoter region of BRSK2 gene, respectivaly.

III-B. Determine whether 5-azadeoxycytidine (5 aza-Dc), inhibitor of DNA methylation, and Trichostatin A (TSA) inhibitor of HDAC class I and class II, can reverse this epigenetic event in BRSK2 gene.

Calu-1 and H226 cell lines were two of the cell lines exhibiting methylated BRSK2 promoter. For this reason, specifically we decided to use Calu-1 as a cell model to test whether both DNMT and HDAC inhibition could reactive BRSK2 expression. To test the hypothesis that pharmacological modulation of DNMT and HDAC by 5-aza-dC and TSA can activate BRSK2 expression, we first performed time-course studies to characterize the effects of 5-aza-dC treatment alone on CpG island methylation in the Calu-1 cell line. DNA was isolated and methylation of the BRSK2 gene was determined by MSP (Figure 41). In this figure we can probe the reversal of methylation after 0.5 μM of 5-aza-dC treatment after 48 hours. This suggests that the maximal BRSK2 gene re-expression was achieved with 0.5 μM of 5-aza-dC during 48 hours; note the reversal of methylation after 5-aza-dC treatment. In these experiments H2O was used as a negative control (data not shown in this figure).

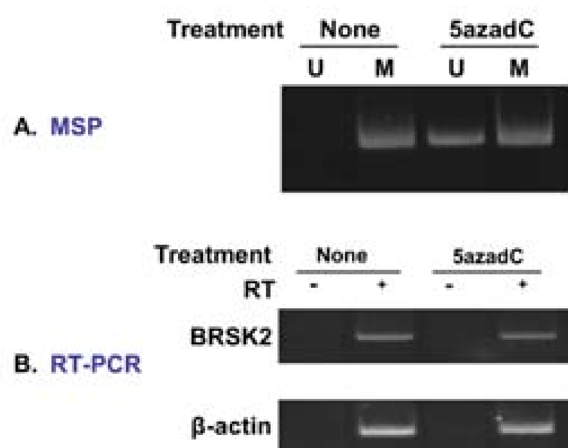


Figure 41. E ffects o f inh ibitors of DNM T o n BRSK2 expression. *A*, MSP an alysis o f B RSK2 CpG i sland methylation p attern was analyzed, u sing a representative primer s et B RSK2 a fter t reatment. A) representative example of three experiments with similar results is shown. Calu-1 without 5 -aza-dC treatment was used a s a methylated co ntrol. Lanes M, methylated p roducts; Lanes U, u nmethylated p roducts. *B*) P redicted 1 89 b p BRSK2 transcript ex pression in Calu-1 ce lls. A r epresentative example of three experiments with similar results is shown. β-actin R T-PCR product provides a control for the amount of i ntact RNA used i n the reaction. Calu-1 w ithout treatment was used as a BRSK2 PCR-positive.

The reactivating effects of the demethylation agents may be potentiated by a short time treatment with the HDAC inhibitor, therefore the next step was use TSA to inhibit histone deacetylases (HDAC) to alter B RSK2 expression b y int erfering with the removal o f acetyl groups from histones. Thus, Calu-1 cell line was treated with 5-aza-dC for 48 h, a nd TSA was added for the last 12 h and the last 24 h (Figure 42). In these experiments H_2O was used as a negative control (data not shown in this figure).

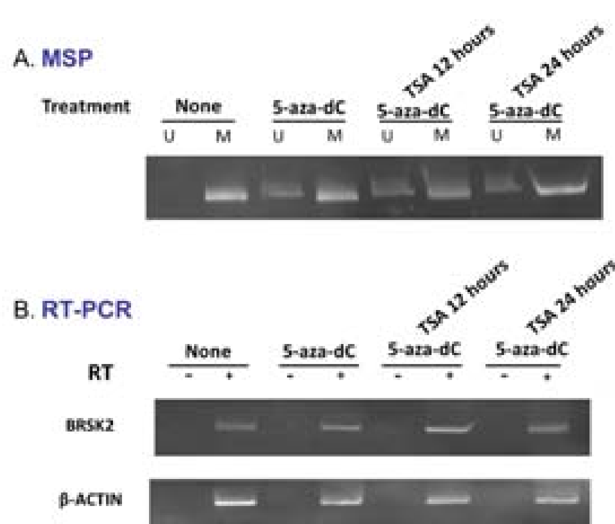


Figure 42. Effect of 5-aza-dC and TSA treatment on BRSK2 gene expression. *A*, MSP as say was u sed to examine the methylation status of BRSK2 CpG island in C alu-1, u sing a representative primer set B RSK2 after tr eatment. A) representative example o f t wo experiment with s imilar r esults is shown. C alu-1 without treatment was used as a BRSK2 PCR-positive control. *B)* Predicted 1 89 b p B RSK2 transcript expression i n C alu-1 N SCLC ce ll line. *Lanes M* , methylated products; *Lanes U*, unmethylated products. A r epresentative example of t wo ex periments with similar results is shown. B-actin R T-PCR p roduct provides a control for the amount of intact RNA used in the reaction. Calu-1 without treatment was used as a BRSK2 PCR-positive control.

We also evaluated BRSK2 expression in the Calu-1 NSCLC cell line after the treatment with 5-aza-dC and TSA, by the quantitative real time RT-PCR (Figure 43). In this figure we can observe how in Calu-1 cells, after 0.5 µM of 5-aza-dC treatment during 48 hours could restore the BRSK2 mRNA expression. But BRSK2 mRNA expression was restored in a time dependent manner when TSA was added for the last 12 h more than when TSA was added the last 24 h, as demonstrated by quantitative real time PCR.

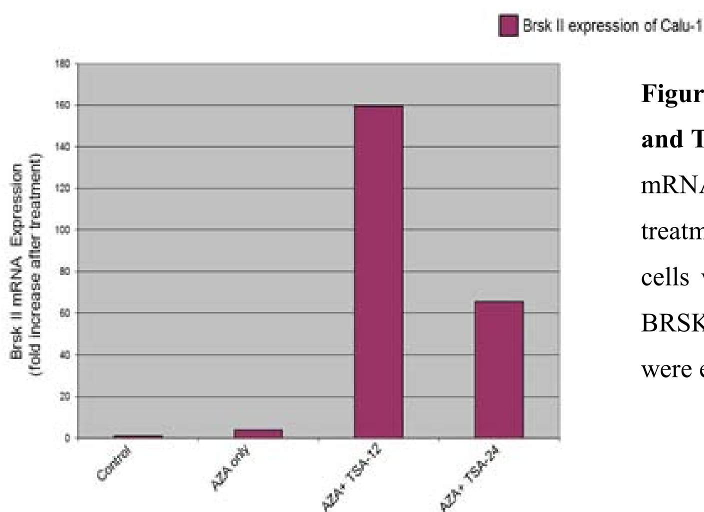


Figure 43 Restoration of BRSK2 expression by 5-aza and TSA in Calu-1 NSCLC cells. Induction of BRSK2 mRNA expression induced by 5-AZA and TSA treatment. The relative expression level of BRSK2 in cells was detected by qRT-PCR, the mRNA levels of BRSK2 were normalized by 18s expression and data were expressed as fold increase after the treatment.

19. BRSK2 methylation status in paraffin embedded tumor tissues

Aim IV. The fourth objective was make a clinical validation of BRSK2 methylation status, in paraffin embedded tumor tissues of patients with lung cancer.

The next step was to examine the methylation status of BRSK2 in a total of 58 NSCLC paraffin embedded tissues of surgical patients.

Table 18. Clinicopathological features of the patients analyzed for BRSK2 methylation by MSP.(*) Fisher´s Exact Test (#) Pearson Chi-Square.

	Patients (N=58)				
Characteristic	**No.**	**(%)**	**BRSK2 promoter Methylation status**		**p**
			Unmethylated	**Partially Methylated**	
Age , years					
Mean		64	63.4	66.13	0,341
Range		37-79			
Sex					
Male	55	(94, 8 %)	41(74.5%)	14(25, 5%)	1(*)
Female	3	(5, 2 %)	2 (66, 7 %)	1 (33,3 %)	
Histology					
Adenoc. -bronchioloalveolar carcin.	18	(31%)	14 (77, 8 %)	4 (22, 2 %)	0, 001(*)
Squamous	29	(50%)	26 (89, 7%)	3 (10, 3%)	
Large-cell carcinoma	9	(15,5%)	3 (33,3 %)	6 (66, 7%)	
Others	2	(3,4 %)	0 (0%)	2 (100%)	
Clinical stage					
IA	13	(22, 4%)	29 (80, 6%)	7 (19,4%)	0, 153(#)
IB	11	(19%)			
IIA	0	(0%)			
IIB	12	(20, 7%)			
IIIA	14	(24, 1%)	14 (63, 6%)	8 (36, 4%)	
IIIB	7	(12, 1%)			
IV	1	(1, 7%)			
Patological stage					
IA	12	(20, 7%)	34 (82, 9%)	7 (17, 1%)	0 ,025(*)
IB	22	(37, 9%)			
IIA	2	(3, 4%)			
IIB	5	(8, 6%)			
IIIA	14	(24, 1%)	9 (52, 9%)	8 (47, 1%)	
IIIB	3	(5, 2%)			
IV	0	(0%)			
Performed Status					
0	40	(69%)	30 (75%)	10 (25%)	1(*)
1	15	(25, 9%)	13 (72, 2%)	5 (27, 8%)	
2	3	(5, 2%)			
Tumor location					
Righ & left upper lobe	36	(62, 1%)			
Righ & left lober lobe	22	(37, 9%)			
Tumor diameter (cm)					
4≥	31	(53, 4%)	20 (64, 5%)	11 (35, 5%)	0, 073(*)
4<	27	(46, 6%)	23 (85, 2%)	4 (14, 8 %)	

Characteristic	Patients (N=58) No.	(%)	BRSK2 promoter Methylation status: Unmethylated	Partially Methylated	p
Nodules affected (patological)					
0	37	(63, 8 %)	31(83, 8%)	6 (16, 2%)	
1	5	(8, 6 %)	4 (80 %)	1(20%)	**0, 04(*)**
2	16	(27, 6 %)	8 (50 %)	8 (50 %)	
Types of lung surgery					
Lobectomy & bilobectomy	33	(56, 9%)	26 (78, 8%)	7 (21, 2%)	
Pneumonectomy	18	(31%)	12 (66, 7%)	6 (33,3 %)	0, 628(*)
Others	7	(12, 1%)	5 (71, 4%)	2 (28, 6%)	
Treatment					
Neoadyuvant	10	(17, 3%)	9 (90 %)	1 (10 %)	0, 427(*)
Adyuvant	34	(58, 6%)	25 (73,5 %)	9 (26, 5 %)	0, 9 (#)
Surgery	14	(24, 1%)	9 (64,3 %)	5 (35, 7%)	0, 484(*)

The patient population consisted of 55 males and 3 females, whose ages ranged from 37 to 79 years (median age: 64). According to histological criteria (6th edition), 16 patients were classified as adenocarcinoma, 2 as bronchioloalveolar carcinoma, 29 as squamous, 9 as large-cell carcinoma, 1 as adenosquamous and 1 as carcinoma poorly differentiated. Both groups of paraffin embedded tissues were checked by a pathologist. The post-surgical pathologic stage was classified according to the 6th TNM classification and staging system. The guideline of the World Health Organization (WHO) of 2004 was used too, but without the last modifications of Experts internationally (Travis, Brambilla et al. 2011).

Complete resection of the tumor was performed in 58 patients. The most frequent surgical procedure was lobectomy and bilobectomy in 56, 9%, while pneumonectomy was performed in the remainder a total of 18 patients (31%). In the rest of 7 the patients, 1 as thoracic biopsy, 3 as mediastinoscopy and 3 as atypical resection. Clinicopathologic features of the patients analyzed for BRSK2 methylation by MSP are described in Table 18 .

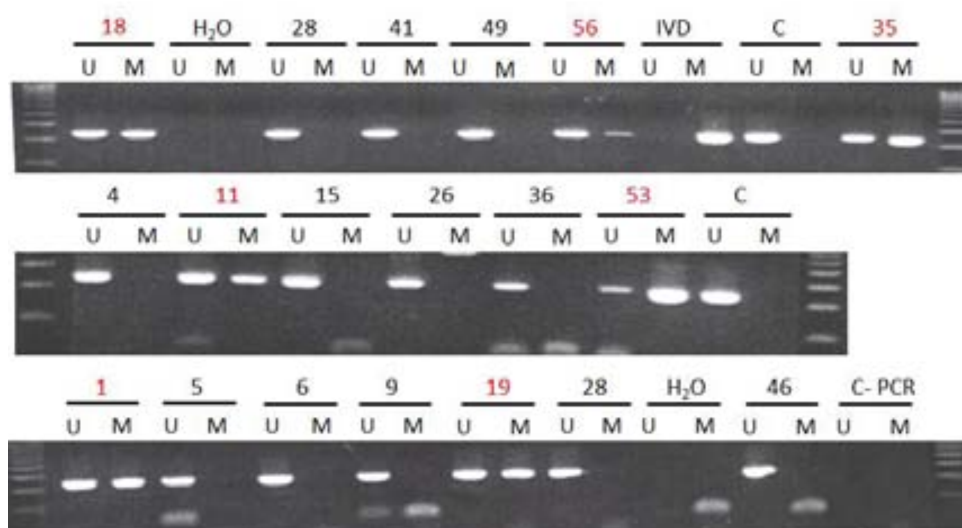
DNA was extracted with phenol: chloroform and ethanol precipitation, and methylation of the BRSK2 locus was determined by MSP as described for the cell lines. In the statistical analysis, Fisher´s Exact or Chi-square test was used to ascertain differences in proportions between groups for the categorical variables and the student t-test for comparison of continuos variables.

Table 18, summarizes the relationship of the methylation status of the BRSK2 promoter and the clinical and pathological characteristics of the patients. Neither gender, age, performance status,

types of lung surgery nor treatments were associated with the promoter methylation status of the BRSK2.

A good correlation was found between methylation and the patients with advanced tumors (stage IIIA-IIIB), when this group of patients are compared with the other group of pacients with early stages (IA-IB-IIA-IIB) (Fisher´s Exact Test p= 0,025). Of the 17 patients with advanced tumors (stage IIIA-IIIB), 8 of them presented prevalence of BRSK2 methylation indicating that 47,1% of patients at advanced stages exhibited BRSK2 methylation. Some examples of BRSK2 methylation in the 58 paraffin embedded tumor tissues from patients are shown in Figure 44.

Figure 44. BRSK2 Methylation Analysis in 58 paraffin embedded tumor tissues. Methylation of a CpG island surrounding the promoter of the BRSK2 gene was determined by Methylation specific PCR (MSP). DNA from the patients (indicated with numbers) was modified with bisulfate (which converts C to U and leaves meCintact). Parallel PCR reactions were performed with primers specific to the unmethylated (U) or Methylated (M) form. C, Control DNA; IVD, in vitro methylated DNA. Shown are representative examples.



A good correlation was found between methylation and Histology (Fisher´s Exact Test P = 0, 001). BRSK2 methylation was more prevaletion in patients with large-cell carcinoma, 6/9 (66,7%) and others histologies that included adenosquamous and carcinoma poorly differentiated were 2/2 (100 %). Thus, the BRSK2 methylation was more prevalent in adenosquamous and carcinoma poorly differentiated than the rest of the histologies described.

Moreover, BRSK2 methylation was associated with a larger tumor size (Pearson Chi-Square P= 0, 073), this means that the tumors with a size greater or equal to 4 cm have a higher percentage of methylation, 11/31 (35, 5%), than the tumor size smaller 4 cm, 4/27 (14, 8%).

Significant differences were observed according to nodules affected (Fisher´s Exact Test P= 0, 04), there is a higher frequency of methylation in patients with lymph node involvement, i.e patients with zero , one or two nodules affected the BRSK2 methylation was 6/37 (16,2 %), 1/5 (20%) and 8/16 (50%), respectively.

In the statistical analysis, overall survival was measured from the date of surgery to the date of death or to the date of loss to follow-up, this mean the last date on which the patient was known to be alive. Survival curves were drawn with the Kaplan-Meier method and compared with the two-sided log-rank test.

Hazard ratios of death with 95% confidence intervals (CI) were estimated with the Cox model. Statistical significance was set at 0,05. Analysis was performed using Statistical Package for the Social Sciences (SPPS) for Windows version 17.0.

We found no correlation between methylation of BRSK2 and survival (P=0.958) (Figure 45). There were no differences in survival according to BRSK2 methylation in tumor.

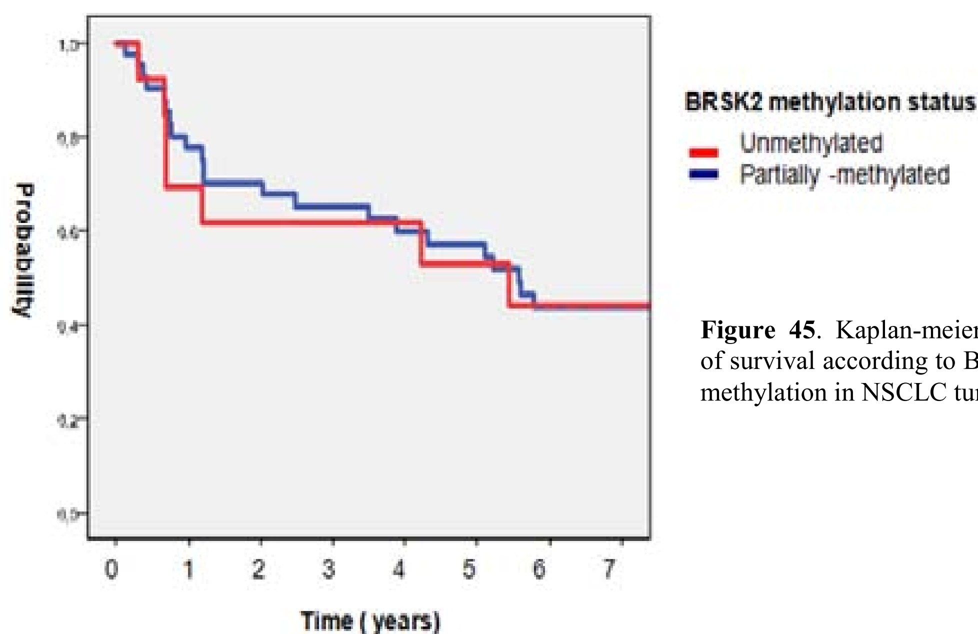


Figure 45. Kaplan-meier plots of survival according to BRSK2 methylation in NSCLC tumors.

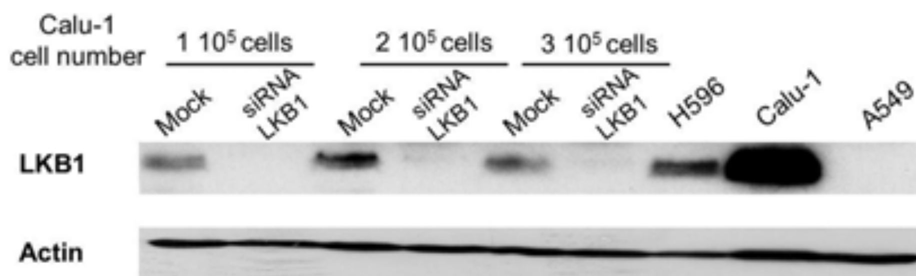
20. Inhibition of LKB1 Protein expression via siRNA.

Aim V. To determine the impact of LKB1 downregulation on NSCLC cell lines.

As had been explained before, LKB1 functions at the apex of pathway that negatively regulates mTOR so LKB1down regulates mTOR activity that leads to cell growth arrest.
So we expected that inactivation in LKB1 could lead to activation of mTOR and make such cells more sensitive to mTOR inhibition and could be a target for future therapeutic drugs.

To do that we evaluated the inhibition of LKB1 protein, using a siRNA approach. We used a cell line that was wildtype for LKB1, Calu-1. In Figure 46 we can observed down-regulation of LKB1 protein expression by LKB1-specific siRNA in NSCLC Calu-1.

Figure 46. Inhibition of LKB1 in Calu-1 NSCLC cell line. Calu-1 cells ($1x10^5$, $2x10^5$and $3x10^5$) were transfected with 200nM of the LKB1-specific siRNA duplex or control siRNA (Mock) using Oligofectamine. Cells were harvested 48h later and analyzed using immunoblots with anti-LKB1 antibody.
Proteins (50-75ug/lane) from whole cell protein were loaded in each lane for Western blot analysis using mouse monoclonal antibody against human Anti-LKB1 protein.
β-actin was used as internal loading control for the experiment. Lysates from LKB1 wt (H596) and mutant cells (A549) were loaded for comparison.
Inhibition of LKB1 protein expression via siRNA gene silencing targeting LKB1 mRNA is obvious in Calu-1 cell line.



VI.Discussion

In this study, we have analyzed the alterations in genes that are part of the transduction pathway LKB1/AMPK/TSC. The finality of this aim is that in the future, this will allow the search for new therapies ¨individualized¨ in lung cancer.

To carry this out we set a number of goals, here we will discuss the different results obtained in each of the proposed objectives.

Aim I. Determine the frequency of LKB1/AMPK/TSC2 signaling pathway alterations in NSCLC

We determined the frequency of genetic/epigenetic alterations in the LKB1/AMPK/TSC2 pathway in a panel of human NSCLC cell lines. Using a panel of 4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines, we have systematically evaluated the mutation spectrum and promoter methylation status of LKB1 and downstream effectors of LKB1/AMPK/TSC pathway. We initially focused on genes that reside within regions that undergo a high rate (>30%) of loss of heterozygosity in NSCLs, including LKB1, AMPK α-2, and TSC2. The results of the methylation status and the mutations detected in a panel of 4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell tested in our laboratory , are summarize in Table 15 and Table 16. Using MSP, we observed the presence unmethylated and methylated bands for the LKB1 gene and in the different components of the complex heterotrimeric AMPK and TSC1-TSC2 complex. Of the lung cancer cell lines examined, LKB1 genetic alterations are detected in 6 NSCLC cell lines, this mean 31.5% (6/19) of the lung cancer cell lines.These data are consistent with previous reports of ~30% mutation frequency of LKB1 in NSCLC (Sanchez-Cespedes, Parrella et al. 2002; Carretero, Medina et al. 2004; Matsumoto, Iwakawa et al. 2007). When we confined to adenocarcinomas, the incidence of LKB1 point mutations is 44.4% (4/9). This incidence is comparable to those reported by *Carretero et al* (Carretero, Medina et al. 2004), and *Matsumoto et al* (Matsumoto, Iwakawa et al. 2007). They detected LKB1 mutations in 54% (6/11) and 42% (13/31) of lung adenocarcinomas. Thus, LKB1 mutations at codon 37 in A549, and mutation at codon 332 in H-23 cell line have previously been reported by Sanchez-Cespedes M. et al (Sanchez-Cespedes, Parrella et al. 2002), they reported the presence of LKB1 alterations only in adenocarcinomas among various histological types of lung cancer cell lines. This group have documented that at least one third of

primary lung adenocarcinomas harbor somatic mutations at the LKB1 gene, supporting a role for LKB1 inactivation in the development of lung tumors. A few years later the same group of investigators reported (Carretero, Medina et al. 2004), LKB1 mutation at codon 37 in H460 cell line (although they histologically classified this cell line as a sporadic lung adenocarcinomas, even though ATCC describes H460 cells as derived from a patient with large cell lung cancer), so some of our results are consistent with these other reports. We observed that frequent LKB1 mutations in adenocarcinomas. Importantly, however our data suggest that LKB1 genetic alterations are also present in other NSCLC subtypes.

We demonstrated the presence of an interstitial deletion within the LKB1 gene in large cell carcinomas (H157) (Zhong, Guo et al. 2006), and point mutations in large cell carcinoma (H460) and adenocarcinomas (H-23, A549, H1944, EKVX) cell lines. This mean, LKB1 mutations are not confined to adenocarcinomas but also occur in other types of NSCLC histologies, such as large cell carcinomas.

To determine the epigenetic alterations in LKB1 gene, we investigated the promoter methylation status of LKB1 in NSCLC cancer cells using methylation–specific PCR. DNA samples are treated with bisulfite to convert all unmethylated cytosines to uracils; those that are methylated are resistant to this modification and remain as cytosine. Uracils are then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated CpGs sites. PCR primers are designed to distinguish between methylated and unmethylated DNA. Using this method, we found no evidence of LKB1 methylation in any NSCLC cell lines used in this study (Table 15, Figure 28). Esteller et al. (Esteller, Avizienyte et al. 2000) previously checked the methylation status by MSP technique 51 cell lines, of which were 11 lung cancer cell lines (21.5%). These lung cell lines did not have LKB1 methylation, unlike other cell lines derived from other tumor types, specifically three colorectal and one cervical carcinoma cell lines. All this information suggests that methylation as a mechanism of LKB1/STK11 gene inactivation is not common in sporadic lung carcinomas. It is possible that our analysis is limited however the MSP technique is limited to the analysis of the 3-6 CpG sites underlying of the PCR primers and our results thus far reflect the data from a single primer set. So one option could be to analyze other portions of the LKB1 promoter for evidence of methylation. Phosphorylation of AMPK α subunit in the activation loop at Thr172 by LKB1 is essential for AMPK catalytic activity (Stein, Woods et al. 2000). AMPK exits as a heterotrimer

in the cell and is composed of a catalytic (α) and two regulatory (β and γ) subunits (Steinberg and Kemp 2009). Each subunit has different isoforms, namely, α1, α2, β1, β2, γ1, γ2 and γ3 encoded by distinct genes, which yields 12 possible heterotrimetic combinations. Binding of AMP to the γ subunit allosterically activates the complex, making it a more attractive substrate for its major upstream AMPK kinase, LKB1.

We propose that one of the mechanisms for inactivation of AMPK could be via genetic mutations. We analyzed only the α2 subunit, mainly because there are no mutation found in α1 subunit, these data are available from COSMIC database:
(http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/)
(http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=PRKAA1&ln1=TSC1&start=1&end=1165 &coords=AA%3AAA&sn=&ss=&hn=&sh=&id=6738#). We did not analyze the β1, β2, γ1, γ2 and γ3 subunits, for two reasons, one because they are not the catalytic subunits and the other reason is because no mutations are show in COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Nevertheless the present study analysis did not identify AMPKα2 inactivation in the panel of 4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell tested in our laboratory by genetic analysis (Table 15). Alternatively, given that the hypermethylation of promoters can lead to epigenetic gene silencing, we decided in this study to analyze the promoter hypermethylation of different subunits of AMPK heterotrimer. We did not analyze AMPK γ-1 and AMPKγ-2 methylation status, because these two genes don´t have CpG islands in their promoter regions (UCSC Genome Bioinformatics (http://genome.ucsc.edu/). We also investigated the methylation status of the genes encoding AMPK α1, α2, β1 and β2. No evidence of methylation of the α1, α2, β1 and β2 promoters region were noted (Table 15, Figure 29, Figure 30, Figure 31, Figure 32). It could be the possibility of other gene inactivation located upstream of AMPK with secondary AMPK suppression may be other mechanisms that remain to be explored.

Other groups have demonstrated that activated AMPK phosphorylates and activates the TSC2 tumor suppressor protein to enhance TSC2 function. AMPK activates the TSC1-TSC2 complex by phosphorylating TSC2 on Thr 1227 and Ser1345 in metabolic stress (Inoki, Zhu et al. 2003). Together the TSC1-TSC2 heterodimer acts to suppress the activity of mTOR, which is essential for the control of cell growth and proliferation. So we hypothesized that TSC complex downregulation could be due to inactivating mutations and/or promoter methylations. The TSC

genes are regarded as tumour suppressor genes, primarily in the tuberous sclerosis condition, as mutation of these genes is associated with the occurrence of certain tumours, namely lymphangioleiomyomatosis, the brain tumour giant cell astrocytoma and occasionally renal cell carcinoma. However, there have been few studies on the link between these genes and gene products with sporadic human solid tumours.

We investigated the possibility that mutations of TSC2 gene occur in NSCLC and tested this by analyzing our 23 cell line panel (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines), with the invaluable help and collaboration of of Dr. Wei Zhou and Dr.Dian-Sheng Zhong. For the tumor suppressor TSC1 gene, we only checked the methylation status of the promoter, the genetic analysis was not carried out. The TSC2 gene may be more susceptible to acquiring mutations because the TSC2 gene is larger than the TSC1 gene and spreads over about 44kb of genomic DNA (Consortium 1993). Finally, we did not detect mutations in TSC2 gene in all the exons checked by Protein Truncation Analysis (Table 16). In spite of the exciting link of TSC genes with the mTOR signaling pathway so widely linked with the onset and progression of tumorigenesis, only few studies have investigated the role of TSC genes in sporadic cancers. Hebert et al.(Hebert, Norris et al. 2006) detected a few mutations in both TSC genes in head and neck tumors from the U.S. patients and SCC cell lines. Although reduced expression of tuberin was found in sporadic astrocytomas, no intragenic mutations were detected in either TSC gene (Parry, Maynard et al. 2000). Parry et al (Parry, Maynard et al. 2001) did not detect any intragenic somatic mutation in TSC genes in sporadic renal cell carcinomas. A previous report of mutation screening of the entire coding regions of the TSC genes (Chakraborty, Mohiyuddin et al. 2008), did not detect any mutations in a panel of 25 oral squamous cell carcinoma samples. This was carried out using PCR-SSCP and DNA sequencing techniques.

There are examples of functional modification of TSC gene by methylation in non-hereditary sporadic solid tumor and human breast cancer. The clinical outcome of patients with breast cancer, turns unfavorable, in patients with the TSC promoter is methylated (Jiang, Sampson et al. 2005). They showed TSC1 promoter methylation in most of the breast tumour tissues whereas the methylation of TSC2 promoter appears to be less frequent (Jiang, Sampson et al. 2005). Also the down-regulation of TSC gene function in oral squamous cell carcinoma can be ascribed to an epigenetic alteration by methylation of the TSC promoter (Chakraborty, Mohiyuddin et al.

2008). This group examined the methylation status of the TSC2 gene promoter using combined bisulfite restriction analysis (COBRA) in the tissues samples.

In the present study, we did not detect methylation of TSC2 in our panel of cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) (Table 16, Figure 33, Figure 34). A similar result has been provided by the work of Niida et al. They report no evidence of methylation within TSC2 promoter region of the wild-type allele in hamartomas obtained from TSC patients (Niida, Stemmer-Rachamimov et al. 2001).

While someprevious reports do not preclude the existence of genetic and/or epigenetic alterations our results thus far suggest that genetic/epigenetic alterations in AMPK and TSC1/2 are relatively rare in NSCLC.

To summarize, after the screening AMPKα1, α2, β1, β2, TSC1 and TSC2, there are no sequence alterations or/and promoter methylation of these genes in our 23 cell line panel (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines). More detailed understanding of the involvement of the LKB1/AMPK/TSC cascade will lead to new therapies for cancer /human genetic diseases that are caused by deregulation of this signaling network.

Aim II. Study LKB1/AMPK-related kinases alterations as an additional molecular mechanism for the development of lung cancer.

Although we did not detect mutations or methylation in AMPK or TSC2 in NSCLC, AMPK is only one kinase that can be phosphorylated by LKB1. Genetic and biochemical findings indicate that LKB1 phosphorylates the T loop of at least 14 related protein kinases that belong to the AMPK subfamily, which includes isoforms of AMPK—an important regulator of cellular energy levels—as well as the MARK and BRSK enzymes that control cell polarity (Alessi, Sakamoto et al. 2006).

Previous studies indicated that four members of the AMPK-related kinase subfamily (MARK1,MARK2,MARK3, and MARK4-microtubule affinity-regulating kinases) play roles in regulating cell polarity (Drewes 2004; Tassan and Le Goff 2004). However, little or no previous research had been performed on the remaining members of the AMPK-related protein kinases (BRSK1/SAD-A, BRSK2/SAD-B, NUAK1/ARK5, NUAK2/SNARK, QIK/SIK2, QSK, SIK,

MELK, SNRK, NIM1, TSSK1, TSSK2, TSSK3, TSSK4, SSTK, and HUNK). Evidence to date suggests that 12 of these AMPK-related kinases (BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK,MARK1, MARK2, MARK3, MARK4, and SNRK) are indeed activated over 50-fold by the LKB1-catalyzed phosphorylation of their T loops and that these enzymes are substantially less active when expressed in LKB1-deficient cells (Lizcano, Goransson et al. 2004; Jaleel, McBride et al. 2005). These findings suggest that LKB1 functions as a master kinase, similarly to PDK1 (Mora, Komander et al. 2004), to activate many members of the AMPK-related protein kinase subfamily.In addition, these data suggest that the AMPK-related kinases may mediate someof the physiological effects previously ascribed to LKB1 and that one or more of the AMPK related kinases may themselves function as tumor suppressors.

After reviewing all this information, we focussed on analyzing some of these direct LKB1 targets. We propose the potential mechanism of the inactivation of BRSK1, BRSK2, MARK1 and MARK4 genes in lung cancer is via promoter hypermethylation. To investigate this, we examine the methylation status of the promoters of BRSK1, BRSK2, MARK1 and MARK4 in our 23 cell line panel (4 immortalized bronchial epithelial cell lines with SV40 and 19 NSCLC cell lines) using the MSP assay.

In case of BRSK1, this gene was methylated in H226 (squamous cell carcinoma) and in 1799 a normal bronchial ephitelial cell immortalized with SV40 (Table 17, Figure 37). In both cell lines the methylation status of the promoter is completely methylated. In MARK1 promoter, only the squamous cell carcinoma Calu-1 (squamous cell carcinoma) was ¨partially methylated¨. This means that Calu-1 has both methylated and unmethylated in the promoter (Table 17, Figure 35).

MARK1 presents low frequency of methylation compared to Brain-Specific kinase 1 and 2. The MSP assay of BRSK2 showed that H-157 (large cell carcinoma), 1170-I, 1799 and EKVX (adenocarcinoma) cell lines were partly methylated; H-226 (squamous cell carcinoma), Calu-1 (squamous cell carcinoma) and H322M (bronchioalveolar carcinoma) were fully methylated and the rest of the cell lines were unmethylated (Table 17, Figure 38). Finally the MARK4 gene, whose promoter has not even one of the 23 cell lines of our cell lines panel methylated, regardless of histology to which it belongs (Table 17, Figure 36).

With regards to BRSK2, we found aberrant methylation in ~26% (5/19) of the 19 NSCLC cell lines spanning four different subtypes of NSCLC – large cell carcinoma (H157), squamous cell carcinoma (H226, Calu-1), bronchioloalveolar carcinoma (H322M) and adenocarcinoma (EKVX). We also observed partial methylation of BRSK2 in 2 normal bronchial epithelial cell lines immortalized with SV40 (1170-I and 1799) such that overall the percentage across all 23 cell lines analyzed was ~30% (7/23) (Figure 47).

Figure 47 Frequency of BRSK2 methylation in our panel of 23 cell lines (4 normal bronchial epithelial cells immortalized with SV40 and 19 NSCLC cell lines). (*) In this panel is included the 4 normal bronchial epithelial cells immortalized with SV40.

BRSK2	19 NSCLC cell lines	Percentage in 19 NSCLC cell lines	23 cell lines panel (*)	Percentage in our panel of 23 cell lines
completely methylated	H226 Calu-1 H322M	**~16 %** (3/19)		**~13%** (3/23)
partially methylated	H157 EKVX	**~10,5 %** (2/19)	1170-I 1799	**~17%** (4/23)
Percentage methylation		**~26 %** (5/19)		**~30%** (7/23)

As is show in Table 17 and Figure 38, the degree of promoter methylation of gene BRSK2 ranges from completely methylated (all alleles in the cell population are methylated) to partially methylated (some fraction of alleles in the cell population are methylated), to completely unmethylated (all alleles in the cell population are unmethylated). Another point to consider is that when BRSK2 promoter is totally methylated (only the methylated alleles are present) in H226, H322M and Calu-1 NSCLC cell lines, no LKB1 mutations are detected in those NSCLC cell lines. Therefore, from the group of four AMPK related kinases including BRSK1, BRSK2, MARK1 and MARK4, studied by our group, BRSK2 is a good target to understand better LKB1 in lung cancer. The epigenetic silencing of BRSK2 being an alternative mechanism for disruption of LKB1 pathway in lung cancer, i. e. the finding suggests that the disruption of LKB1/BRSK2 signaling is important in the carcinogenesis of lung.

Aim III. Consequences of BRSK2 methylation.

It is clear that LKB1/AMPK signaling is involved in the regulation of mTOR, but until now the target of LKB1/BRSK2 signaling is not known and nor how BRSK2 exerts its functions.
Considering all this, one of the goals we set is to examine LKB1/BRSK2 signaling in lung cancer. We previously detected tumor-specific promoter hypermethylation of BRSK2 in large cell carcinoma, squamous cell carcinoma, bronchioloalveolar carcinoma and adenocarcinoma cancer cell lines. These findings invite speculation that the disruption of LKB1/BRSK2 signaling is important in the carcinogenesis of lung.
We showed that one of the potential mechanism of inactivation of BRSK2 is via promoter hypermethylation, so the next step was to investigate whether the BRSK2 promoter methylation in NSCLC cell lines cause the low expression levels of BRSK2 mRNA. To research it, we analyzed the expression patterns of the BRSK2 gene in all 23 cell lines by the conventional method of RT-PCR (Figure 39), then re-analyzed by a more sensitive method quantitative real-time PCR (Figure 40). In general, the two cell lines Calu-1 and H226 both squamous cell carcinoma cell lines and completely methylated at the promoter BRSK2, also exhibit a lower expression of BRSK2 mRNA. In case of the third line H322M- a bronchioloalveolar carcinoma cell line- in spite of being heavily methylated at the BRSK2 promoter, BRSK2 mRNA still expressed, but still low relative to the group of NSCLC cell lines. The expression level is similar to other cell line (H358) that doesn´t have any methylation of the promoter in BRSK2 gene (Figure 40). The two NSCLC cell lines are ¨partially methylated¨ in the promoter of BRSK2 gene, H157 and EKVX, have different levels of expression of mRNA BRSK2, with low and high respectively.The explanation could be that, in the partially methylated cells the percentage of methylation is variable causing a different levels of BRSK2 mRNA expression. Probably in EKVX the percentage of methylation of the cell line is too low to affect the expression level, but in the other cell line H157, the methylation percentage is higher causing the low level expression of BRSK2 mRNA. All this information could be interpreted that in cell lines studies which present completely or partially methylation of the promoter in BRSK2 gene there is a trend to decrease the expression level of BRSK2 (Figure 40).
To investigate the possibility that the decreased expression of the BRSK2 gene is due to the methylation of the BRSK2 promoter, we treated the Calu-1 cell line with DNA methyltransferase

inhibitor 5-aza-2'-deoxycytidine at 0.5µM for 48 hours. The reversal of methylation was observed after treatment coinciding with the appearance of unmethylated BRSK2 alleles (Figure 41a) and the restoration of BRSK2 expression (Figure 41b). The Calu-1 cell line, was completely methylated the promoter BRSK2, also represents the lowest expression of BRSK2 mRNA among all 23 cell lines (4 normal bronchial epithelial cell lines immortalized with SV40 and 19 NSCLC cell lines) analyzed.

It is known that the reactivating effects of the demethylation agents may be potentiated by treatment with an HDAC inhibitor (Cameron, Bachman et al. 1999; Yang, Phillips et al. 2001; He, Ji et al. 2012; Guo, Feng et al. 2014). TSA inhibits the eukaryotic cell cycle during the beginning of the growth stage. TSA can be used to alter gene expression by interfering with the removal of acetyl groups from histones (histone deacetylases, HDAC) and therefore altering the ability of DNA transcription factors to access the DNA molecules inside chromatin. Consequently, we treated Calu-1 NSCLC cell line, only with 5-aza-2'-deoxycytidine or in combination with tricostin A (TSA) the last 12 hours and the last 24 hours to alter BRSK2 expression (Figure 42a). The reversal of methylation after treatment can be observed coinciding with the appearance of unmethylated BRSK2 alleles (Figure 42b).

Data in Figure 43 show a significant increase in the expression following the treatment with methyltransferase inhibitor 5-aza-dC in combination with TSA added for the last 12 hours more than TSA added for last 24 hours. We observed a synergistic induction of BRSK2 transcript after combined treatment with 5-aza-dC and TSA in comparison to 5-aza-dC treatment alone, this implicates DNA methylation and histone deacetylation in the silencing of BRSK2. The difference in the ability of TSA to potentiate the effect of 5-aza-dC between 12 h and 24h may be because high dose TSA treatment is toxic to the cells and triggers cell cycle arrest in G1 and G2 (Qiu, Burgess et al. 2000). Alternatively, TSA in the culture medium may have fallen to below effective concentrations within 24 hours (i.e. the initial dramatic effect on histone acetylation has been completely reversed (Travers, Spotswood et al. 2002).

In summary, we can conclude that:

- ❖ The hypermethylation of the BRSK2 promoter was observed in NSCLC cell lines is and is associated with reduced expression.

- ❖ Hypermethylation and downregulation of BRSK2 may represent an alternative mechanism for LKB1 pathway disruption in NSCLC.
- ❖ Downregulation of BRSK2 gene in lung cancer is related to the promoter methylation. But more studies are needed to elucidate the mechanism of the 5-aza-dC induced changes in the BRSK2 gene expression profile.
- ❖ The disruption of LKB1/BRSK2 signaling but not LKB1/AMPK signaling in NSCLC suggested that dys-regulation of mTOR signaling may not be the only events involved in the lung cancer carcinogenesis. It would be interesting in future studies in LKB1/BRSK2 signaling may provide additional molecular mechanisms for the development of lung cancer.

Aim IV. Clinical validation of BRSK2 methylation status, in paraffin embedded tumor tissues of patients with lung cancer.

To investigate if the downregulation of BRSK2 gene in tumors from the patients with NSCLC is due to their promoters being methylated, we proposed to determinate the frequenty of epigenetic alterations in the BRSK2 gene in a panel of 58 NSCLC paraffin embedded tissues of patients. The methylation status in lung cancer tumors are compared with their clinic pathological parameters.

The study population is comprised of 58 patients with NSCLC referred to the Hospital Germans Trias i Pujol (Badalona), the patients are grouped according to age, sex, histology, clinical and pathological state, performed status, tumor location, tumor diameter, nodules affected, types of lung surgery and treatment.

But if we look globally all patients without any kind of grouping the prevalence of BRSK2 hypermethylation in primary NSCLC samples is 15/58 (25,8%)(Table 18). The percentage is similar to that found in the analysis of 19 NSCLC cell lines (Figure 47), in this case ~26% (5/19) of the 19 NSCLC cell lines are completely or partially methylated, i.e. 2 partially methylated and 3 completely methylated.

BRSK2 methylation was significantly correlated with four clinicopatological parameters (Table 18). We have detected a good correlation between methylation and the patients with advanced

tumors (stage IIIA-IIIB) (P=0,025). The prevalence of BRSK2 methylation is 47,1% in advanced stages versus a 17,1% in early stages (IA-IB-IIA-IIB). A good correlation is also found between methylation and histology (P= 0,001). The patients with large-cell carcinoma present 66,7% of methylation versus (1 patient) adenosquamous and (1 patient) carcinoma poorly differentiated which present a 100% of methylation. But it is also important to remember that the number of patients with these last two histologies are only two patients, with one patient each of histology.
Furthermore, statistical analysis of the relationship between BRSK2 and clinicopatological features revealed that larger tumor size is significantly correlated with BRSK2 methylation in patients with NSCLC (P=0,073). The prevalence of BRSK2 methylation was significantly greater among patients with a tumors size greater or equal to 4 cm (35, 5%) than with tumor size smaller than 4cm (14, 8%). Finally, significant differences are observed according to nodules affected (P=0, 04), the higher frequency of methylation is present in patients with lymp node involvement.
However, BRSK2 methylation doesn´t correlate with other, parmeters than have been study: clinical stage, performance status, types of lung surgery (lobectomy-bilobectomy, pneumonectomy and other group that includes thoracic biopsy, medioastinoscopy and atypical resection) and treatment (neoadyuvant, adyuvant and surgery).

The finding that BRSK2 methylation is significantly associated with tumor stage may indicate that BRSK2 methylation accumulates during lung cancer progression. Also, Iliopoulos D et.al have demonstrated the hypermethylation levels of hTERT, MGMT and DAPK are increased during cervival oncogenesis progression (Iliopoulos, Oikonomou et al. 2009).
We also found a significant association with lymph node status. BRSK2 methylation occurs in 20% of patients with one lymp node affected, but rises to 50% of patients with two lymp node affected (Table 18). With all these information we could think about the analysis methylation of BRSK2 as a novel biomarker for disease progression or dissemination in lung cancer so could influence in clinicians decisions as other biomarkers (Ludwig and Weinstein 2005).
Before defining BRSK2 promoter methylation as a biomarker in lung cancer, the five stages of biomarker validation defined by Pepe et al. (Pepe, Etzioni et al. 2001) need to be taken into account. For clinical implications, a biomarker needs to be validated in different institutes and in a large number of samples followed by approval from the FDA. Thus, the collaboration among

investigators in universities and institutes, clinicians, industrial participants and FDA is a must to bring a biomarker from the lab to clinic (Mishra and Verma 2010). To our knowledge epigenetic factors have not yet been used in formal staging, but their application to predict response to treatment is already onging , and can be expected to increase in the future, particularly given the development of DNA-demethylating drugs such as 5-azacytidine (Hasina, Surati et al. 2013).

In our analysis, no differences in survival are observed according to BRSK2 methylation status (P=0.958). This may be due to inclusion of patients, the type of criteria for selection and this do not research statistical significance or actually because BRSK2 methylation status doesn´t affect the survival of the NSCLC patients.

After these results, we can remark:

- The prevalence of BRSK2 methylation in the 58 NSCLC samples show partial methylation in 15/58 (25,8%) and it is similar to that found in the analysis of 19 NSCLC cell lines, in this case ~26% (5/19).
- There is a good correlation between methylation of BRSK2 promoter and pathological stage, histology, tumor diameter and nodules affected of the patients with lung cancer.
- The above data stablish a role for BRSK2 silencing in the pathogenesis of lung cancer , suggesting that BRSK2 might be a potentially molecular biomarker for NSCLC progression, but it should be take into account all the process before: determine the sensitivity, specificity and clinical validation
- The correlation between patients with NSCLC and BRSK2 methylation needs to be further evaluated in future with a group of patients with cohort selection different than it has been analyzed this time.

Aim V. Inhibition of LKB1 protein on NSCLC cell lines.

Finally, the last objective was to evaluate the effect of LKB1 downregulation on NSCLC cell lines, to determine the consequences of LKB1 loss of function on NSCLC and the sensitivity to selected chemotherapeutic agents in a future.

Some reports have analyzed the LKB1 loss of function in NSCLC cell lines using a siRNA. The experience with RNA interference indicates that several target sites must be tested. Our strategy has been to test several target sites using commercially synthesized short interfering (siRNA) duplexes by Dharmacon. The design target sites have <50% C+G content and try to target a region with little secondary structure as predicted using the RNA-fold algorithm. We used a siRNA approach to knockdown LKB1. Using this methodology, we have identified a target site that gives reasonable knock down of LKB1 protein in the transfection.

In Figure 46, we show the siRNA against LKB1 can be used to down-regulate LKB1 expression in Calu-1 NSCLC cells (LKB1 wild-type). Treatment with this LKB1 siRNA resulted in an 80% to 90% reduction in LKB1 protein, whereas, as expected, no reduction of LKB1 is detected with control siRNA untreated with LKB1 siRNA, comparing with Calu-1cell line at different quantitive number cells (from $1x10^5$-$2 x10^5$- $3x10^5$) (Figure 46). Knockdown of LKB1 in the cell line is confirmed by Western blot analysis for LKB1 protein. There are significantly reduced the expression of LKB1 in the Calu-1 treated at different quantities of number of Calu-1 cells $1x10^5$-$2 x10^5$- $3x10^5$.

In summary, we show a reasonable downregulation of LKB1 protein by LKB1 siRNA in NSCLC, specifically in LKB1 wild-type Calu-1 cells. The data suggest that knockdown of LKB1 could be a useful approach for further studies of LKB1 function and relation to drug sensitivity. In the future, it would be interesting to determine the impact of LKB1 loss of function on the regulation of mTOR or on the NSCLC sensitivity to particular anticancer agents. Future studies will be needed to establish a role for LKB1 inhibition as a therapeutic strategy.

VII.Conclusions

In this work we sought to study the signal transduction LKB1/AMPK/TSC pathway alterations that contribute to the pathogenesis NSCLC. Our objetives were to examine the frequency of genetic and epigenetic alterations in the signal traduction LKB1/AMPK/TSC pathway in NSCLC and to study some components of AMPK-related kinase family that could be activated downstream of LKB1.

The major conclusions of this work are:

1. LKB1 point mutations are present in a 31,5 % (6/19) of 19 NSCLC cell lines analyzed. Our findings demonstrate for a novel deletion of LKB1 in a large cell carcinomas cell line of the lung, H157, this deletion led to a 174pb deletion in the coding region, and point mutations in large cell carcinoma (H460). So LKB1 mutations are not confined to adenocarcinomas, they also occur in other NSCLC subtype such as large cell carcinomas. But we found no evidence of LKB1 methylation in any NSCLC cell lines used in this study.

2. There are not sequence alterations or/and promoter methylation of AMPKα1, α2, β1, β2, TSC1 and TSC2 in our 23 panel cell lines (4 normal bronchial epithelial cells immortalized with SV40 and 19 NSCLC cell lines).

3. Of the four AMPK related kinases -BRSK1, BRSK2, MARK1 and MARK4- studied, BRSK2 represents the highest percentage of methylation; ~26 % of the (5/19) NSCLC cell lines are completely or partially methylated at BRSK2. Including four different subtypes of NSCLC – large cell carcinoma (H157), squamous cell carcinoma (H226, Calu-1), bronchioloalveolar carcinoma (H322M) and adenocarcinoma (EKVX). The percentage increase when include the 4 normal bronchial epithelial, ~30% (7/23) because we increase the number of cell lines that have BRSK2 methylated with 1170-I and 1799 (normal bronchial epithelial cell lines immortalized with SV40).

4. The downregulation of BRSK2 gene in lung cancer is related to the promoters being methylated and the hypermethylation of the BRSK2 promoter is associated with the reduced expression.

5. BRSK2 methylation in the 58 NSCLC samples show partial methylation in 15/58 (25,8%) and it is similar to that found in the analysis of 19 NSCLC cell lines ~26% (5/19).

6. BRSK2 methylation is significantly correlated with four clinicopatological parameters. In the group of 58 patients with NSCLC analyzed: tumor stage (P=0,025), histology (P=0,001), tumor size (P=0,073) and nodules affected (P=0,04) were all positively correlated with BRSK2 promoter methylation. This information establishes a role for BRSK2 silencing in the pathogenesis of lung cancer, suggesting that methylation of BRSK2 could provide a novel biomarker for disease progression in lung cancer.

7. In our analysis, no difference in survival are observed according to BRSK2 methylation status (P=0.958). The correlation between patients with NSCLC and BRSK2 methylation needs to be further evaluated in the future in a different group of patients with an independent cohort selection.

8. The disruption of LKB1/BRSK2 signaling could be important in the carcinogenesis of lung, as a molecular mechanism for the development of lung cancer.

9. There is significantly reduced the expression of LKB1 in the Calu-1 treated with siRNA at different quantities of number of Calu-1 cells $1x10^5$-2 $x10^5$- $3x10^5$ cells. Knockdown of LKB1 is useful approach to study the functional significance of LKB1 loss of function in lung carcinogenesis and therapeutic response.

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