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Bladder Cancer

Molecular Lymph Node Staging in Bladder Urothelial Carcinoma: Impact on Survival

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Abstract

Background: Routine histologic analysis of lymph nodes (LN) for detecting disseminated bladder urothelial carcinoma (BUC) lacks sensitivity.
Objective: To identify and test potential mRNA markers of BUC dissemination in LN that has been missed by histological analysis, and to compare the performance of selected markers with patients' clinical outcome.
Design, setting, and participants: Microarray data and a literature search were used to identify potential markers expressed in BUC but absent in LN. Five genes were finally selected to be studied by quantitative real-time RT-PCR (qRT-PCR) in 181 and 29 LN from 102 BUC patients and 29 controls, respectively, collected from 2002 to 2004 (median follow-up of 35 mo).
Measurements: The three most expressed genes plus two additional markers selected from the literature were finally evaluated by qRT-PCR. Gene expression values were statistically compared with histologic results and clinical outcome.
Results and limitations: A discriminant analysis showed that the combination of *FXYD3* and *KRT20* genes yielded a 100% sensitivity and specificity differentiating LN with BUC dissemination from controls. Combined, the expression of both genes allowed the identification of urothelial cells in LN in 20.5% of patients with previous histologically negative LN. These patients did not have a significantly worse survival than those who were negative by qRT-PCR.
Conclusions: Using molecular markers it was possible to improve the sensitivity of LN histologic analysis. However, since 20.5% of patients that reclassified as positive by qRT-PCR did not have a significantly worse survival, we assume lymphadenectomy was important to remove residual disease.
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1. Introduction

The presence of tumour cells in lymph nodes (LN) at the time of cystectomy is a major prognostic factor in patients with muscle-invasive bladder urothelial carcinoma (BUC) [1]. Lymphadenectomy complementing this radical surgery is an essential tool that not only provides staging of the tumour but also therapeutic benefits. Currently, this staging is usually limited to routine pathological evaluation of hematoxylin-eosin (HE)-stained sections of regional LN. Unfortunately, this technique occasionally misses small cancer foci which are thought to be histologically undetectable micrometastasis in the regional LN. Whatever it may be, around 50% of patients with pT3-4 tumours but histologically node negative disease die within 5 yr of radical cystectomy [2].

A molecular technique such as reverse-transcription PCR (RT-PCR) has been applied in various solid tumours to determine the presence of missed tumour cells in LN during routine pathologic examination [3–5]. However, quantitative real-time RT-PCR (qRT-PCR) has been proven to be more efficient than conventional RT-PCR in the detection of rare events [6].

To develop an efficient approach for detecting disseminated tumour cells, not only is a highly sensitive technique such as qRT-PCR needed but also suitable markers. In this respect, a high-throughput technique such as DNA microarrays allows the study of gene expression profiles in different tissues providing a rich source of information.

In this study, DNA microarrays were used for identifying genes specifically expressed in bladder which could indicate BUC dissemination in LN. Detection of selected markers in LN by qRT-PCR was correlated with histologic findings and patients' clinical outcome.

2. Material and methods

2.1. *Molecular markers selection*

To determine a panel of highly specific mRNA markers of BUC dissemination in LN, gene expression from bladder tissue (normal and tumour) and blood were compared. Specifically, public data from U133A Affymetrix GeneChip (Affymetrix, Santa Clara, CA, USA), hybridized with RNA from 16 blood samples (http://www.ncbi.nlm.nih.gov/geo/; GEO accession code GSE1343) were compared with data obtained from our group (GSE7476) using U133 plus 2.0 Affymetrix GeneChip, hybridized with 3 normal and 9 tumoral urothelium pools from 55 RNA samples. In order to select genes that were overexpressed in the urothelium but minimally or unexpressed in blood, a set of maximum expression cutoffs for intensity values (arbitrary units) in blood samples (50, 100, 150) was empirically established, as well as a set of minimum cutoffs in urothelial tissues (3000, 2000, 1000). Those four genes with the highest mean expression value in both normal and tumour urothelial cells but with low expression in blood were selected for evaluation by qRT-PCR. In addition, the expressions of two conventional markers for epithelial cells that have been widely tested as BUC dissemination markers in the bibliography [7–10] were also studied. The selected candidate genes (see Results) were tested in a population of 102 patients described below (section 2.2.1).

2.2. *Candidate marker genes validation*

2.2.1. *Subjects and samples of study*

A total of 181 right and left lymph node specimens, from 102 BUC patients (10 women and 92 men; average 66 yr; range 42–85) who underwent radical cystectomy and pelvic lymphadenectomy between August 2002 and July 2004 were included in this study. Lymphadenectomy in our institution consists of removing the obturator, internal, external, and common iliac nodes. As controls, 29 lymph node samples from 29 patients who were recipients for kidney transplantation (5 women and 24 men; average 41 yr; range 18–61), without any evidence of having malignant diseases were analyzed. The time to recurrence was the interval from cystectomy to the confirmation of the metastases. In patients who did not have metastases, follow-up was recorded as the number of months from the cystectomy to the last patient observation. In patients with metastases or death, follow-up was recorded until the date of the event. The hospital ethics committee approved this study and the patients and controls provided their informed consent before participating in the study.

Tissue sections of right and left nodes (if available) from each patient were immediately frozen after collection in liquid nitrogen and were subsequently stored at −80 °C until RNA extraction. The remnants were stained with HE for routine pathological examinations [11]. According to the pathological results (Table 1), LN were classified into three groups: histologically positive [N(+)], histologically negative [N(−)], and controls from patients with nonneoplasic disease. To test the ability of selected genes as markers of tumour dissemina-

Table 1 – Pathological stage and node status of BUC patients at time of cystectomy

Patholgical stage*	All patients		pN0*		pN1–3*	
	n	%	*n*	%	*n*	%
pT0	23	22.5	21	20.6	2	2
pTis	6	5.9	6	5.9	–	–
pTa	5	4.9	4	3.9	1	1
pT1	12	11.8	11	10.8	1	1
pT2	17	16.7	12	11.8	5	4.9
pT3	23	22.5	18	17.6	5	4.9
pT4	16	15.7	11	10.8	5	4.9
Total	102	100	83	81.4	19	18.6

BUC = bladder urothelial carcinoma.
* According to International Union Against Cancer 2002 [30].

tion, these samples were divided in two sets: the training set, which included N(+) and controls samples, and the validation set, which comprised N(−) samples.

2.2.2. Quantitative real-time PCR analysis

Total RNA was extracted using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Two μg of total RNA were reverse-transcribed with a random hexamer primer mix in a 20 μl reaction mix using SuperScriptII Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Gene expression quantification of the selected genes was performed in the 181 LN biopsies using TaqMan Gene Expression Assays and an ABI PRISM 7000 SDS (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations, except that the final volume of the reaction was 20 μl. Beta-glucuronidase gene (*GUSB*) was used as endogenous control. All samples were analyzed in duplicate and the Cycle threshold (Ct) mean was obtained for further calculations. Each experiment included a negative nontemplate control and an interexperiment control. The relative expression level of the marker genes for each sample was described as the difference between the average Ct from the target gene and the average Ct from *GUSB*.

2.2.3. Statistical analysis

The Mann-Whitney test was performed to compare gene expression values between control and N(+) samples (samples from the training set).

In order to evaluate the ability of individual genes to distinguish between N(+) and control LN in the training set, a receiver operating characteristic (ROC) curve for each selected gene was constructed using the markers' relative expression values.

Finally, to obtain the combination of genes that provided the best discrimination between both sample types, a discriminant analysis was also performed using the aforementioned training set of samples. A ROC curve for the discriminant function was also constructed. The cutoffs obtained by both ROC analyses, for independent and for combined genes, were evaluated in the validation set of samples [N(−)]. According to these cutoffs, samples were classified as qRT-PCR(+) and qRT-PCR(−). Recurrence-free survival and cancer-specific survival curves were calculated using the Kaplan-Meier method.

SPSS v13.0 and MedCalc v8.1 softwares were used for statistical analyses.

3. Results

3.1. Molecular markers selection

A list of 49 candidate marker genes specifically expressed in bladder was obtained from microarray data analysis (Table 2). The four genes with the highest expression values in urothelial tissue, together with an expression value in blood of lower than 50 in all the samples analyzed, were initially selected (*C10orf116*, *KRT19*, *FXYD3* and *AGR2*) for evaluation in the training set of samples [N(+) and controls]. Subsequently, *C10orf116* was discarded as it presented similar expression levels in control and in N(+) samples.

According to previous results from our group and data published by other groups [8,12], *KRT20* and *UPK2* were also included for testing as molecular markers for BUC dissemination. Moreover, *KRT20* and *UPK2* presented average expression values of 31.9 and 18.69 in blood, and 4669 and 954 in bladder, respectively, according to microarray analysis.

3.2. Candidate marker genes validation

3.2.1. Pathological analysis

Paraffin-embedded slices from all 181 LN biopsies were evaluated after HE-staining. In terms of individual biopsy specimens, this pathological examination detected tumour cells in 21 samples (11.6%) [representing 19 of the 102 patients (18.6%)]. In contrast, 160 samples (88.4%) showed no sign of tumour dissemination by this technique [83 patients, (81.4%)] (Table 1).

3.2.2. Quantitative real-time PCR analysis

Table 3 shows the expression values for each marker according to samples' pathological classification. Differences between control and N(+) samples (training set) were statistically significant for the gene expression values of each marker ($p < 0.0001$) (Fig. 1; Table 3).

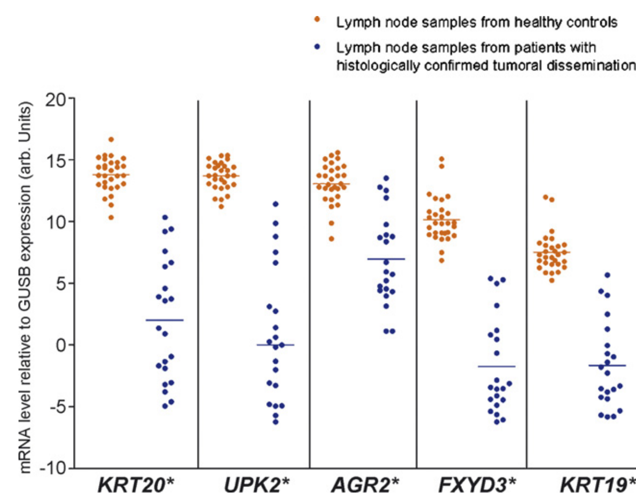

Fig. 1 – Gene expression values of the five selected mRNA markers.
Relative gene expression values of five markers genes *KRT20, UPK2, AGR2, FXYD3,* and *KRT19* in the training set [control and N(+)samples]. The median expression level for each marker gene within a group is indicated by a horizontal line. * Significant, $p < 0.05$.

Table 2 – Average expression values of candidate genes resulting from the comparison of microarray expression data between bladder (normal and tumour) and blood tissues

Cutoff microarray expression values	Gene symbol	Affymetrix ID	Accession number	Gene expression in bladder			Gene expression in blood		
				Average	Range		Average	Range	
					Max	Min		Max	Min
Bladder_min>3000, Blood_max < 50									
	C10orf116	203571_s_at	NM_006829	14059.2	22677	7360	9.5	26	4
	KRT19	201650_at	NM_002276	12119.2	15774	6097	6.2	23	3
Bladder_min>2000, Blood_max<50									
	FXYD3	202489_s_at	NM_005971	7312.5	12834	2842	20.1	46	5
Bladder_min>1000, Blood_max<50									
	AGR2	209173_at	NM_006408	6051.7	13122	1896	8.4	18	4
	KRT13	207935_s_at	NM_002274	5644.9	11395	1203	16.5	47	4
	KRT7	209016_s_at	NM_005556	5155.6	8386	1184	7.1	24	2
	SEPP1	201427_s_at	NM_005410	3427.7	7187	1236	18.4	46	4
	TM4SF6	209109_s_at	NM_003270	2872.4	7949	1253	15.6	31	6
	NET1	201830_s_at	NM_005863	2426.8	5232	1416	8.9	31	2
		201829_at	AW263232	1559.2	2670	993	44.9	83	4
	EGFR	201983_s_at	AW157070	1970.6	3257	1088	12.7	48	6
	SERPINH1	207714_s_at	NM_004353	1800.8	2628	1182	17.1	35	7
Bladder_min>3000, Blood_max<100									
	DHRS2	214079_at	AK000345	9250.2	14423	6004	38.3	98	3
		206463_s_at	NM_005794	7580.3	11865	4410	24.4	91	5
Bladder_min>2000, Blood_max<100									
	COL3A1	215076_s_at	AU144167	8793.7	13961	2586	28.1	67	5
		201852_x_at	AI813758	7362.4	11351	2235	63.5	151	26
	PSCA	205319_at	NM_005672	6587.1	14025	2128	26.9	73	3
	CDH1	201131_s_at	NM_004360	4251.8	6229	2234	8.9	50	2
Bladder_min>1000, Blood_max<100									
	FN1	210495_x_at	AF130095	5982.0	12388	1016	30.5	80	7
		212464_s_at	X02761	5948.4	12067	893	29.3	55	9
		211719_x_at	BC005858	5940.2	12183	957	8.8	20	4
		216442_x_at	AK026737	5859.7	12084	1001	44.9	138	9
	MAC30	212282_at	BF038366	5878.8	11141	1827	74.0	133	21
		212281_s_at	BF038366	4158.6	8238	1169	37.4	69	8
	RGS5	209071_s_at	AF159570	5020.0	10346	1376	22.0	85	5
		209070_s_at	AI183997	2996.3	7579	796	22.5	41	9
		218353_at	NM_025226	1719.5	4528	379	38.9	95	2
	PTPRF	200636_s_at	NM_002840	3018.6	4527	1505	26.8	74	4
		200635_s_at	AU145351	1440.1	2207	606	29.8	61	10
		200637_s_at	AI762627	1261.5	1868	472	17.5	44	6
	PPAP2A	210946_at	AF014403	2828.2	4039	927	45.3	117	6
		209147_s_at	AB000888	2472.7	3787	1270	31.4	64	5
	COL1A1	202310_s_at	K01228	5433.2	8725	1486	20.9	85	6
	COL1A2	202404_s_at	NM_000089	5432.8	9580	1740	20.0	64	4
	GJA1	201667_at	NM_000165	4817.3	8501	1376	18.6	56	1
	HMGCS2	204607_at	NM_005518	4505.2	11812	1018	28.6	84	4
	SDC1	201286_at	Z48199	3720.8	6683	1525	34.7	67	7
	PERP	217744_s_at	NM_022121	3474.6	6171	1532	40.6	70	7
	LOC51186	217975_at	NM_016303	2690.1	5401	1401	58.1	93	17
	PLS3	201215_at	NM_005032	2529.5	4281	1246	24.9	54	4
	JUP	201015_s_at	NM_021991	2453.3	3919	1206	22.3	68	10
	IGFBP4	201508_at	NM_021991	2355.5	3706	1443	27.8	65	12
	EPS8	202609_at	NM_004447	2075.6	3147	1177	44.0	77	5
	WEE1	212533_at	X62048	1715.9	2921	1051	33.7	99	3
	PTK2	208820_at	AL037339	1600.8	2405	1174	22.6	77	3
Bladder_min>3000, Blood_max<150									
	SPINK1	206239_s_at	NM_003122	17957.2	30345	7522	64.4	150	16
	KRT18	201596_x_at	NM_000224	11845.5	22160	4653	69.1	143	29
	ID1	208937_s_at	D13889	10663.2	15171	5456	52.3	111	4

Table 2 (*Continued*)

Cutoff microarray expression values	Gene symbol	Affymetrix ID	Accession number	Gene expression in bladder			Gene expression in blood		
				Average	Range		Average	Range	
					Max	Min		Max	Min
Bladder_min>2000, Blood_max<150									
	A2M	217757_at	NM_000014	6969.7	16998	2294	49.4	119	6
	CXADR	203917_at	NM_001338	4419.2	6783	2176	54.1	101	13
	AHR	202820_at	NM_001621	3281.8	6446	2016	71.8	147	6
Bladder_min>1000, Blood_max<150									
	UPK1A	214624_at	AA548647	4778.3	7215	1469	69.9	131	22
	TM4SF13	217979_at	NM_014399	2964.9	7654	1343	100.1	128	60
	SEMA3C	203789_s_at	NM_006379	2032.7	3227	1315	64.1	100	15
	FAT	201579_at	NM_005245	1927.8	3525	1049	55.1	113	20
	TJP1	202011_at	NM_003257	1821.2	2476	1185	62.8	131	22
	PON2	201876_at	NM_000305	1716.8	4683	1098	71.1	141	26
	NCKAP1	207738_s_at	NM_013436	1399.3	1972	1149	67.4	144	27
	EIF5B	201024_x_at	BG261322	1331.3	1689	1089	61.6	149	19
	COL4A1	211980_at	AI922605	3674.1	9052	1952	71.8	113	46
		211981_at	NM_001845	1394.8	3892	643	13.1	24	6
	ATP1B1	201243_s_at	NM_001677	3478.8	7633	1618	84.7	150	28
		201242_s_at	BC000006	2725.8	5292	1294	67.9	154	16

Genes are grouped according to nine different expression value cutoffs in both tissues. Expression values are expressed in arbitrary units.

3.2.3. *Determination of expression value cutoffs for independent markers*

ROC curve analysis was applied to each gene to calculate the expression value cutoffs that most efficiently separate both types of samples in the training set (Table 4). Interestingly, considering only *FXYD3* or *KRT20* expression values and by using the cutoffs $\leq$5.395 and $\leq$10.33, respectively, sensitivity and specificity differentiating N(+) from control samples (training set) were 100% for both (Tables 4 and 5). Then, applying the *FXYD3* and *KRT20* cutoffs in the validation set [160 N(–) samples from 83 patients], 24 (15%) and 70 (43.8%) samples, respectively, became positive for the presence of tumour dissemination [equivalent to 15 (18.1%) and 47 (56.6%) patients] (Table 5). Eighteen samples (11 patients) were reclassified by both genes.

3.2.4. *Molecular markers combination*

Despite the 100% sensitivity and specificity obtained individually by both *FXYD3* and *KRT20* genes in the training set, a discordant percentage of reclassified patients by both genes (18.1% and 56.6%, respectively) was found when applying their cutoffs to the validation set. Therefore, a discriminant analysis considering all five genes was performed. This analysis showed that, combined in a function ($Y = 0.140KRT20 + 0.250FXYD3 - 2.532$), *FXYD3* and *KRT20* genes provided the highest statistical power in the discrimination of N(+) and control samples. ROC curve analysis for the discriminant function using the cutoff $\leq$5.68 showed 100% sensitivity and specificity differentiating N(+) from control samples (training set).

Applying this formula in the validation set [N(–)], 24 of the 160 samples (15%) [17 patients (20.5%)]

Table 3 – Average relative expression level by qRT-PCR

Marker gene	Control samples (n = 29)			Histologically positive-lymph nodes samples (n = 21)			p-value*
	Average	St. Dv	Range	Average	St. Dv	Range	
KRT20	13.79	1.37	10.34–16.66	2.03	4.94	–4.91–10.33	<0.0001
UPK2	13.69	1.12	11.22–15.37	0.79	5.38	–6.18–11.41	<0.0001
AGR2	13.13	1.61	8.61–15.59	6.97	3.69	1.14–13.50	<0.0001
FXDY3	10.15	1.81	6.86–15.07	–1.72	3.86	–6.18–5.40	<0.0001
KRT19	7.51	1.54	5.24–11.99	–1.62	3.49	–5.77–5.68	<0.0001

qRT-PCR = quantitative real-time RT-PCR.
Expression values are expressed in arbitrary units.
* Mann-Whitney test. Significant, $p < 0.05$.

Table 4 – Sensitivity and specificity of cross ROC points for individual genes and for the discriminant function for the detection of tumour dissemination by qRT-PCR in the training set [control and N(+) samples]

Marker gene	Cutoff ≤	% Sensitivity	% Specificity
KRT20	10.33	100	100
UPK2	11.41	100	96.6
AGR2	9.76	81	96.6
FXYD3	5.39	100	100
KRT19	5.68	100	96.6
Discriminant Function (KRT20 & FXDY3)	−0.15	100	100

became positive for the presence of BUC dissemination, and 136 of the 160 samples (85%) [66 patients (79.5%)] were classified as negative (Table 5). Of note, from the 24 samples reclassified as positive by the discriminant function, 20 samples (13 patients) were reclassified also by *FXDY3* and 22 samples (15 patients) by *KRT20*, when used as simple markers.

Finally, the already evaluated paraffin-embedded sections of the 24 samples reclassified as positive by the discriminant function were reviewed by the pathologist. No evidence of metastasis was found in this second revision.

3.2.5. *Patients' follow-up*

After a median follow-up of 35 mo (range 0.4-61.2), 40 of 102 patients (39.2%) recurred and 35 of them died because of the cancer. Twenty-three patients with pT3-T4 tumours [15 N(−) and 8 N(+)] received adjuvant chemotherapy. This adjuvant treatment was balanced between N(−) qRT-PCR(+) and N(−) qRT-PCR (−) patients.

There is evidence that cancer-specific survival was worse for N(+) patients than for N(−) (p = 0.027).

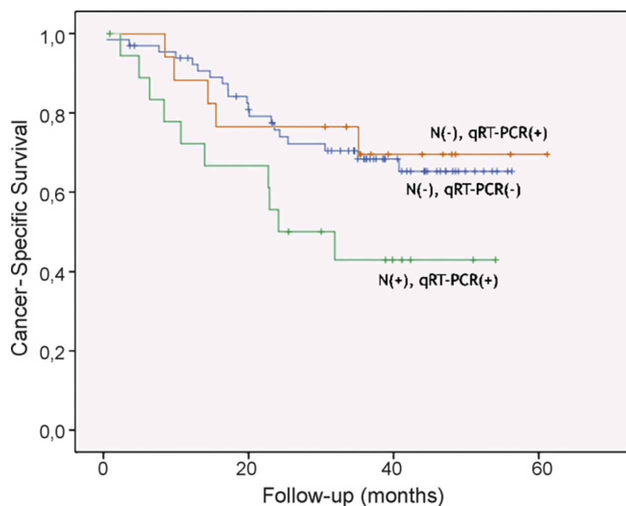

Fig. 2 – Kaplan-Meier curves comparing cancer-specific survival of patients according to the molecular (qRT-PCR) and pathologic (N) detection of the disseminated disease (*p* = ns).

In contrast, neither cancer-specific survival nor recurrence-free survival were significantly worse in patients with N(−) qRT-PCR(+) than in N(−) qRT-PCR(−) patients (*p* = ns) (Fig. 2).

4. Discussion

The presence of tumour dissemination in LN has been shown to be an important risk factor for many neoplasic diseases [13]. In BUC, it seems that the use of the pathological staging alone is not sensitive enough to evaluate tumour dissemination since up to

Table 5 – Percentages of samples in both sets (training and validation) classified according both independent and combined *KRT20* and *FXYD3* gene expression cutoffs

Set of samples	% of samples classified according to *KRT20* and *FXYD3* independent cutoffs		% of samples classified according to discriminant function (combined *KRT20* and *FXYD3* cutoffs)	
	Positive	Negative	Positive	Negative
Training set				
N(+) [*n* = 21]	KRT20: **100** (21/21) *(100% of patients)*	KRT20: **100** (29/29) *(100% of patients)*	**100** (21/21) *(100% of patients)*	**100** (29/29) *(100% of patients)*
Negative controls [*n* = 29]	FXYD3: **100** (21/21) *(100% of patients)*	FXYD3: **100** (29/29) *(100% of patients)*		
Validation set				
N(−) [*n* = 160]	KRT20: **43.8** (70/160) *(56.6% of patients)* FXYD3: **15** (24/160) *(18.1% of patients)*	KRT20: **56.2** (90/160) *(43.4% of patients)* FXYD3: **85** (136/160) *(81.9% of patients)*	**15** (24/160) *(20.5% of patients)*	**85** (136/160) *(79.5% of patients)*

Equivalence to patients is shown in brackets under each percentage.
n = number of samples, *N* = pathological analysis result.

37% of N(−) patients can develop distant metastasis [2]. Tumour deposits located in different areas of the 4–5 μm section of the paraffin-embedded mass examined by the pathologist can be missed on microscopic examination. However, the qRT-PCR based approach allows the detection of a very small number of tumour cells in heterogeneous populations of cells, because there is a previous homogenization of the tissue and a subsequent disseminated tumour cell RNA amplification.

Since there are no well established molecular markers for detecting lymph node dissemination in BUC, DNA microarrays were used to select a group of candidate genes that are selectively expressed in the urothelium, whose expression is preserved in neoplasic urothelial cells. Selected genes were tested in regional LN that are potential sites of early metastasis in BUC patients. We found that all the selected genes were not expressed in control samples except for *c10orf116* that is specifically expressed in adipose tissue which inevitably surrounds LN. Consequently, this marker was discarded from this study, although its usefulness as a BUC dissemination marker in blood should not be ruled out. Finally, the selected genes studied were: *AGR2, KRT20, UPK2, FXYD3, KRT19*. Even though neither *KRT20* nor *UPK2* appears in our restrictive list of 49 highly expressed urothelial candidate markers, they are widely known conventional markers for epithelial cells and have been extensively described as BUC dissemination markers in the literature [7–10]. Furthermore, we found that they are clearly overexpressed in bladder tissue in comparison to blood according to microarray analysis.

To our knowledge, this is the first time that *AGR2* and *FXYD3* have been related with BUC dissemination. However, *AGR2* has been demonstrated to be a potential marker for prostate cancer [14] and *FXYD3* to contribute to the proliferative activity of pancreatic cancer, it is expressed in primary human breast tumours and is upregulated in prostate cancer [15–17]. On the other hand, *KRT20*, *UPK2* and *KRT19* have already been considered as markers for BUC dissemination and other different types of cancer [9,18–23]. It is important to point out that all the candidate genes are expressed in the urothelium but they are not markers of tumour activity.

On the other hand, it has to be taken into account that only the four most differentially expressed marker genes from the microarray analysis were tested. Other 45 genes are candidate to be analyzed in future studies.

In the present work, we found that two of the five genes tested (*FXYD3* and *KRT20*) presented, individually, 100% specificity and sensitivity in differentiating between N(+) and control samples. Thus, theoretically each one could be enough to be used as a marker of BUC dissemination. However, when applying their cutoffs in the validation set [N(−) samples] the number of patients re-classified as positive for BUC dissemination was discordant. Probably the low number of samples included in the training set [control and N(+) samples] accounts for these discrepancies. In any case, in order to give more consistency to the test, all possible combinations of the five candidate genes were considered. *KRT20* and *FXYD3*, combined in a discriminant function, proved to be the best option for detecting disseminated cells in N(−) samples since they maintained the 100% sensitivity and specificity classifying N(+) and control samples. Using this function and its corresponding cutoff, an upstaging of LN containing BUC dissemination in 20.5% of N(−) patients was achieved (17 out of 83).

As expected, N(+) patients in our series had a significantly worse cancer-specific survival than N(−) ($p = 0.027$). However, even though marker genes were meticulously selected and that they seem to be precise enough to discriminate between N(+) LN and controls, no significant worse cancer-specific survival was associated with PCR(+) LN. This result could be explained in two ways. First, the detection of microdisseminated disease in LN from patients with a muscle-invasive cancer is a non relevant finding. Hard to believe since all N(+) patients were also positive by the molecular technique. The second is that a therapeutic procedure such as lymphadenectomy impacts on survival. Since adjuvant chemotherapy has been used sparingly and is balanced within the N(−) group, performing lymphadenectomy in the whole series seems to be the main factor responsible for the potentially curative effect observed. Radical cystectomy and systematic pelvic LN dissection alone can provide a favorable outcome in some patients with regional nodal metastases from BUC [24]. In fact, recurrence-free survival has been significantly associated with N category, with N1 patients having significantly more probabilities of being cured by the lymphadenectomy than N2, and N2 more than N3 patients [25]. From these results, it can be assumed that N(−) patients can still be more susceptible to curation by this surgical process since it eliminates even micrometastases that are not detected during routine histological examination [26–29].

Lastly, since at least 45 other genes from our microarray data remain to be tested, we also consider our findings are a promising basis for developing future studies in order to develop a blood test to diagnose and predict BUC metastasis in this tissue.

5. Conclusions

Quantification of *FXYD3* and *KRT20* mRNAs by qRT-PCR in LN at time of cystectomy could achieve an upstaging of LN containing BUC dissemination in 20.5% patients compared to the standard pathological analysis. However, detecting such residual disease in LN by qRT-PCR is not associated with a significantly worse cancer-specific survival. Consequently, lymphadenectomy seems essential as a complement to the radical surgery and its curative effect is specially emphasized in those patients with microdisseminated BUC in LN.

Author contributions: Antonio Alcaraz had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Marín-Aguillera, Mengual, Algaba.
Analysis and interpretation of data: Marín-Aguillera, Mengual, Algaba, Bruset, Ars, Ribal, Alcaraz.
Drafting of the manuscript: Marín-Aguillera, Mengual, Algaba, Burset.
Critical revision of the manuscript for important intellectual content: Ars, Colomer, Mellado, Ribal, Alcaraz.
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Obtaining funding: Alcaraz.
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Supervision: Alcaraz.
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Editorial Comment on: Molecular Lymph Node Staging in Bladder Urothelial Carcinoma: Impact on Survival

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In their study, Marín-Aguilera and colleagues [1] demonstrated that quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) is capable of detecting the presence of small amounts of disseminated urothelial cells in lymph nodes of a subgroup of patients with histopathologically negative lymph nodes at radical cystectomy. The authors suggested that lymphadenectomy improved outcome in those 20.5 % of patients who had positive RT-PCR, but negative conventional histopathologic examination.

The presented data, however, suggest some reservations with drawing this conclusion. Although there is evidence in the literature that extended lymphadenectomy may improve outcome [2], a lack of difference between the survival curves of patients with and without positive RT-PCR test in the current study [1] might also be due to a low statistical power of the study (small sample size, short follow-up) or to a low clinical significance of positive RT-PCR results. With positive lymph nodes as positive controls and lymph nodes retrieved at renal transplantation (ie, without previous transurethral surgery) as negative controls, impressive sensitivity and specifity figures were obtained which are, however, possibly not achievable in the investigated clinical setting. Therefore, at present, we can only speculate on the real performance of the described RT-PCR tests and on the clinical implications of positive results.

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Editorial Comment on: Molecular Lymph Node Staging in Bladder Urothelial Carcinoma: Impact on Survival

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The pathologic stage of the primary bladder tumour and the presence of lymph node metastasis are the most important determinants of survival in patients with bladder cancer undergoing radical cystectomy. The optimal extent of the lymph node dissection for accurate staging, the curative potential of the method, and the prognosis of lymph node-positive disease after such treatment are matters of debate.

In a recent issue of *European Urology* it was shown that there is a relatively common discrepancy between clinical and pathologic stage after extirpative surgery for bladder cancer [1]. But even the histopathologic examination of lymph nodes has its limitations, as indicated by immunohistochemical and reverse transcription polymerase chain reaction (RT-PCR) analysis [2]. As cancer is a disease of cells having abnormal gene expression, different molecular tools are currently being investigated to improve diagnostics and optimize therapy decisions [3].

In the present study, qRT-PCR analysis is used focusing on the sensitivity of routine histologic examination of lymph nodes from bladder cancer patients undergoing radical cystectomy [4]. A whole set of genes was analysed in terms of applicability, ending up with five genes that were evaluated by qRT-PCR. A combination of two of the evaluated genes yielded a 100% sensitivity and specificity differentiating lymph nodes with bladder urothelial carcinoma dissemination from controls. Combined, the expression of both genes allowed the identification of urothelial cells in lymph nodes in 20.5% of patients with previous histopathologically negative classified lymph nodes.

However, the present study showed, as others before [3], no significantly worse survival of patients presenting qRT-PCR positive compared to negative lymph nodes after a median follow-up of 35 mo.

When using RT-PCR analysis, it is important to ask how patient management will be affected when the assay is positive and the histopathologic assessment is negative. If the prognostic value of this method could be determined in prospective series, RT-PCR results could serve as a tool to assess the need for and extent of lymph node dissection, especially if available in a time frame suitable for intraoperative evaluation.

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