UROLOGY – ORIGINAL PAPER

Lack of evidence for increased level of circulating urothelial cells in the peripheral blood after transurethral resection of bladder tumors

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Abstract

Purpose Aggressive intervention against the bladder wall during transurethral resection of bladder tumors (TURBT) causes damage and leakage from blood vessels to the bladder lumen. The aim of this study was to determine whether TURBT could increase the level of circulating urothelial cells.

Methods Expression of tumor markers, discriminative for nucleated blood cells and urothelium, was evaluated by quantitative (q) RT-PCR on RNA isolated from peripheral blood samples of 51 patients who underwent TURBT for $\geq$cT1c bladder tumors.

Results Four of 14 studied genes, epidermal growth factor receptor (EGFR), Collagen α-1(I) chain, Mast/stem cell growth factor receptor (KIT) and CD47, exhibited significant differences in gene expression between controls and cancer patients. While TURBT did not significantly increase the number of PCR-positive results of any transcripts, positive RT-PCR detection for EGFR was significantly less frequent on day 30 compared to results obtained before surgery.

Conclusions Although the results of our study do not provide evidence for increased tumor cell release into the peripheral blood after TURBT, they seem to indicate that EGFR mRNA measurement in the blood may provide useful information for urologists.

Keywords Bladder tumor · Circulating tumor cells · Gene expression · mRNA · RT-PCR

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Introduction

Most bladder cancers (BCa) are derived from the uroepithelium. Tumors confined to the bladder lining are regarded as non-invasive, while those that invade the bladder muscle are invasive. Transurethral resection of the bladder tumor via a wire loop (TURBT) remains a standard procedure for the diagnosis of BCa, as well as for the treatment of non-muscle invasive tumors [1].

While circulating tumor cells (CTCs) in the peripheral blood have been reported in patients with several cancer types, including BCa [2–9], it is well

documented that diagnostic and therapeutic procedures [6, 9] can additionally release dysplastic cells from the tumor and may affect the level of CTCs in the peripheral blood. In turn, dissemination of tumor cells from locally advanced tumors may significantly increase risk for metastatic disease, although only limited number of CTCs may colonize distant sites propagating the metastases.

An optimal method for detecting CTCs should be sufficiently specific and sensitive with the most popular methods utilizing polymerase chain reaction (PCR)-based analyses. They allow detection of expression of so-called epithelium-specific genes, which are expressed in cancer cells but not in nucleated cells of the blood. While the detection of mRNA markers specific for CTCs by reverse transcription (RT)-PCR is sensitive, its specificity may significantly differ depending on the marker. Therefore, marker selection is considered vital for a proper analysis of CTCs.

Here, we asked the question whether the electroresection of a bladder tumor, which uses high pressure of washing fluid, may induce a spread of cells from resected tissues into blood stream leading to the increase level of circulating urothelial cells in the peripheral blood. To answer it, we used quantitative RT-PCR to amplify the transcripts of selected genes, which might be specific and sensitive indicators of circulating urothelial cells.

Methods

Patients

A total number of 51 patients (43 men and 8 women; mean age 67.5 years, range 34–89) with primary or recurrent urothelial BCa submitted to TURBT between April 2009 and May 2010 were prospectively selected for the study. Pathological staging was as follows: pTa—2 pts (2 patients; 4%), pT1—26 pts (26 patients; 51%), pT2—11 pts (11 patients; 21%), while in 12 cases (24%) primary tumor stage could not have been definitely assessed (pT $\geq$1; lack of muscularis propria in the specimen). The grade of the tumor was defined as G1, G2 and G3 according to 1973 WHO classification in 8 (16%), 25 (49%) and 18 (35%) cases, respectively. Details on patient's age, sex and pathological diagnosis are given in the Online Resource 1. The control group consisted of 32 healthy

volunteers, 17 among them were recruited from the laboratory staff and 15 were selected among individuals who underwent a screening colonoscopy with no known history of any malignancy. The study protocol was approved by the Ethics Committee, and all patients signed informed consent before inclusion.

Urothelial-specific genes

Literature searching [10–19] allowed selection of the following genes: uroplakin 2 (UPK2), EGFR, α-type platelet-derived growth factor receptor (PDGFRA), Insulin-like growth factor-binding protein 7 (IGFBP7), Sorting nexin-16 (SNX16), TP53, KIT, Vascular endothelial growth factor A (VEGFA), CD47 and telomerase reverse transcriptase (TERT), which expression was reported previously to be highly specific and sensitive markers for the detection of CTCs, including circulating urothelial cells. In addition, computation of the fold change of transcript levels between nucleated blood cells and urothelium was performed in searching for new candidate biomarkers of CTCs. Appropriate data were imported from the GSE4251 [20] dataset, which contained gene expression measurements for blood cells analyzed in three healthy subjects, and from the GSE3167 [21] and GSE5287 [22] datasets, which contained measurements of normal bladder and various dysplastic lesions. Datasets were deposited in the Gene Expression Omnibus [23] database and represented measurements performed on Affymetrix® Human Genome U133A and U133A 2.0 platforms, respectively. All imported measurements were processed with the GCRMA algorithm [24] and then averaged and sorted according to minimal fold change between any given blood cell population (white blood cells, peripheral blood monocytes and T lymphocytes) and urothelia. Calculation basing on the highest minima transcript levels has selected several urothelial-specific genes (not shown). Of them, four genes, COL1A1, COL3A1, KRT7 and AGR2, were considered as potential markers of circulating urothelial cells.

RNA studies

Blood samples were collected a day before, immediately after (1 day) and 3, 7 and 30 days after surgery by venipuncture into PAXgene Blood RNA Tubes (QIAGEN) and were kept at −80°C until further use.

Total RNA from blood samples was isolated using the PAXgene Blood RNA Kit following the manufacturer's instructions. One μg of RNA was used in reverse transcription (RT) reactions performed with the SuperScript II Reverse Transcriptase (Invitrogen Co., Carlsbad, CA, USA) as per the manufacturer's protocol. Quantitative evaluation of cDNA was performed on the ABI 7900HT Real Time PCR system (Applied Biosystems) using either Sensimix SYBR or Probe Kit (Bioline, UK) in a 5 μl reaction mixture containing 2.5 μl 2 × Sensimix, 2 μl cDNA (1:5 dilution) and 200 nM primers or preformulated TaqMan Gene Expression Assays (Applied Biosystems). Q-PCR was carried out at 40 cycles, consisting of 15 s of denaturation at 95°C and hybridization for 1 min at 60°C in a 384-well reaction plate. Five serial dilutions of cDNA from normal colon tissue samples (pool of cDNAs) were used to construct the standard curves. The gene expression was regarded as continuous when amplification signal was detected across all the samples, and relative transcripts levels were calculated using the $\Delta\Delta CT$ method. For the transcripts with dichotomous expression (positive/negative signal of expression), we calculated the percent of positive results for a group of patients. Oligonucleotide primers for the analyzed gene transcripts, used for Sybr Green I Dye Assays, were either designed using Primer Express Software (Applied Biosystems) or taken from qPrimer Depot database [25] and are listed in Table 1.

The results of gene expression levels were analyzed with the Mann–Whitney U test, and differences between numbers of samples with detectable signal were analyzed using the Fisher's exact test. P values of less than 0.01 were considered significant.

Results

Selection of potential biomarkers

Gene expression was analyzed using qRT-PCR with 40 amplification cycles using either pairs of designed primers with SYBR Green chemistry or TaqMan Gene Expression Assay. To determine the potential specificity of both literature- and microarray-selected genes (listed in the 'Methods' section), analyses of expression were firstly conducted on RNA isolated from blood samples collected from two groups of control subjects: 17 controls recruited from the laboratory staff and 15 controls from whom blood samples were taken immediately before colonoscopy.

As shown in Table 1, while the expression of 8 of 14 studied genes was found for most control blood samples, detectable signals of EGFR, COL1A1 and AGR2 could be determined for only a few control samples. Expression of TERT, TP53, SNX16 and VEGFA significantly varied between series of PCR assays due to very low signal level (detectable above 37 cycles of amplification), which led to low reproducibility of results. In addition, qRT-PCR analyses revealed significant differences in expression of two genes between the two control groups. The detectable signal of UPK2 mRNA was found in one of 17 control blood samples obtained from laboratory staff and in 14 of 15 blood samples collected immediately before colonoscopy ($P = 8,551E{-}07$). Also, the relative levels of KIT expression were significantly higher ($P = 4,55E{-}06$) in blood samples collected in healthy controls before colonoscopy compared to laboratory staff controls.

Biomarkers differentiating between healthy controls and bladder cancer patients

Then, we compared gene expression estimated in blood samples collected from BCa patients a day before TURBT and from control individuals. The expression level of CD47 was significantly higher in blood samples collected from BCa patients compared to both control groups ($P = 1.19E{-}12$ and $P = 1.83E{-}12$, respectively), and the expression of KIT was significantly higher compared to controls selected only from laboratory staff ($P = 3.68E{-}03$). Among genes with expression detected in a limited number of blood samples, mRNAs of EGFR and COL1A1 were found significantly more frequently in cancer patient samples than in controls ($P = 0.000605$ and $P = 0.006737$, respectively). Expression of AGR2 was not detectable in blood samples from cancer patients. Expression of all other genes selected for this study did not differentiate the bladder cancer patient group from healthy controls.

Histological grading is an important prognostic factor for bladder tumors, especially for prediction of progression [1, 26, 27]. Superficial low-grade cancers frequently recur, but they exhibit a minimal risk for progression, while high-grade, locally advanced tumors often metastasize and therefore, result in poor

Table 1 Number of positive qRT-PCR results for mRNAs selected from published reports (genes 1–10) or from analysis of microarray data (genes 11–14) in blood samples collected from two groups of controls: group A—healthy subjects recruited from the laboratory staff; group B—healthy subjects with no illnesses or known history of malignancy selected among individuals who underwent a screening colonoscopy

Candidate markers	No.	Gene name	Control subjects		qRT-PCR primers sequences $5' \geq 3'$ (Sybr Green chemistry) or Taqman Gene Expression assay ID
			Group A ($n = 17$)	Group B ($n = 15$)	
Literature-based	1	EGFR	0	0	Hs01076092_m1
	2	UPK2	1	14	Hs00171854_m1
	3	CD47	17	15	Hs00179953_m1
	4	IGFBP7	17	15	*F*:TGCGAGCAAGGTCCTTCCATA†
					R:ATGAGGACAGGTGTCGGGATTC‡
	5	SNX16	17	15	*F*:AGTCCTCTGCACTTGAGGTTGA
					R:CAGCATCATATGCCACTTCTGCT
	6	TP53	17	15	*F*:TTGCAATAGGTGTGCGTCAGA
					R:AGTGCAGGCCAACTTGTTCAG
	7	KIT	17	15	*F*:GGCGACGAGATTAGGCTGTT
					R:CATTCGTTTCATCCAGGATCTCA
	8	PDGFRA	17	15	*F*:GGCATTCTTTGCAATACTGCTTAA
					R:CATCTGCCGATAGCACAGTGA
	9	TERT	16	14	Hs99999022_m1
	10	VEGF	17	15	*F*:AGGAGGAGGGCAGAATCATCA
					R:CTCGATTGGATGGCAGTAGCT
Microarray-based	11	COL1A1	1	0	Hs00164004_m1
	12	COL3A1	2	7	Hs00943809_m1
	13	KRT7	5	8	Hs00559840_m1
	14	AGR2	1	0	Hs00180702_m1

† Forward primer sequence; ‡ Reverse primer sequence

clinical outcome [28]. However, we could not find any correlation between the level of expression or the PCR-positive-signal frequency of any of the studied markers for CTCs with the grading of bladder tumor (not shown).

Blood biomarkers levels after TURBT

To detect whether TURBT may influence the level of the circulating urothelial cells, in the peripheral blood, expression of all studied marker genes was analyzed in blood samples collected before and 1, 3, 7 and 30 days after surgery. Four genes (Table 2) exhibited significant differences in gene expression between controls and cancer patients found at least at one time point after TURBT. While TURBT did not significantly increase the number of PCR-positive results of any transcripts, a positive EGFR expression was significantly less frequent ($P = 0.0021$) at 30th day compared to results obtained on day 0.

Discussion

The detection of CTCs has been demonstrated in the blood of patients with colon, breast, prostate and other cancers [4–8]. Some of these markers may specifically detect circulating urothelial cells. Most reported studies employed RT-PCR-based analyses, and while they generally exhibited well-documented sensitivity, their specificity might be questionable.

Analysis of epithelial-specific mRNAs by a qRT-PCR is a sensitive technique that allows CTCs identification in the peripheral circulation. However, because of the limited specificity of these assays, leading to both false-positive and false-negative results, proper

Table 2 Percent of positive qRT-PCR signal for EGFR, COL1A1 and COL3A1 mRNAs and relative expression levels of KIT and CD47 in blood samples collected from controls and patients treated for bladder tumors

	Controls	Days after TURBT (P values)				
		Before	1	3	7	30
Positive signal of expression (percent)						
EGFR	0%	27%	20%	14%	10%	4%
		(0.0006)	(0.005)	N.S.	N.S.	N.S.
COL1A1	3%	27%	45%	25%	36%	23%
		(0.007)	(0.0004)	(0.007)	(0.0004)	N.S.
COL3A1	28%	40%	38%	30%	36%	22%
		N.S.	N.S.	N.S.	N.S.	N.S.
Relative expression levels						
KIT	0.061†	0.231	0.265	0.224	0.258	0.291
		(8.66E−07)	(0.003)	(1.43E−06)	(1.70E−06)	(1.23E−06)
CD47	0.020	0.279	0.233	0.264	0.219	0.200
		(2.71E−08)	(1.09E−08)	(1.30E−07)	(1.10E−08)	(8.46E−08)

† Control group consisted of 17 healthy volunteers selected from laboratory staff

selection of mRNA markers strongly determines the final results of the study. Therefore, the study started with the identification of urothelium-specific genes. As a result, circulating EGFR mRNA-positive cells were found in 27% of blood samples collected from bladder cancer patients and in none of the control blood samples. Also, positive RT-PCR signals for COL1A1 and CD47 mRNAs were found significantly more frequent and at higher levels, respectively, in blood samples collected from cancer patients compared to controls. EGFR and COL1A1 are not expressed in hemopoietic cells [20], while expression of CD47 seems to be significantly lower in those cells compared to expression in epithelial cells. Thus, from potential molecular markers selected for our analyses, expression of only few genes exhibited the desired specificity.

It should however be noted that the results of our statistical analysis may be underestimated. Comparisons utilizing only qualitative information in datasets of moderate size, such as employed in this study, require large relative frequency differences to conclude significant group bias. Moreover, results are susceptible even to single sample readout alteration. For example, when one group features 20/31 pos/neg ratio, there is no possibility of proving significantly lower pos/neg proportions in other groups using 17 samples (assuming P value < 0.01). Only a 14/3 (or higher) pos/neg ratio is significant in that group (Fisher test, significance level $= 0.01$, Online Resource 2).

Previous studies have reported EGFR expression in 30–50% of bladder tumors [29], and the presence of EGFR transcript in peripheral blood was proposed as a marker for circulating BCa cell detection [13]. A study by De Luca et al. [30], which detected EGFR-expressing cells in the blood of lung, breast and colon cancer patients, suggests that this assay rather may be used in detecting cancer cells of epithelial origin than different carcinoma types. Nevertheless, the combination of several markers could increase specific cancer type detection, and such molecular indicators were also proposed for BCa.

Expression of UPK2 was found to be highly specific to the uroepithelia [31, 32] and was suggested as a highly specific but not sensitive blood marker for CTCs in bladder cancer patients [3]. However, in our study, a standard bowel preparation by polyethylene glycol (Fortrans) followed by a cleansing phosphate enema 30 min before the colonoscopy significantly affected expression of UPK2 and KIT in blood samples from healthy subjects. Therefore, bowel preparation before colonoscopy may release epithelial cells into circulation or activate transcription of epithelia-specific genes in hemopoietic cells or both.

An optimal method for detecting CTCs should be not only sufficiently specific but also sensitive enough to define the relatively low number of tumor cells in blood samples. Recently, the most popular methods employed for analyzing CTCs have used PCR

detection of so-called epithelium-specific genes, which are potentially expressed in CTCs but not in blood nuclear cells. However, most analyzed transcripts exhibited variability in RT-PCR signals that hampered their recognition as specific markers for CTCs. This variability may be caused by many reasons. Although qRT-PCR greatly increases the sensitivity of mRNA detection, its usefulness as a specific method for CTC detection, specifically in blood specimens, is questionable. A low PCR signal significantly decreases the reproducibility of inter- and intra-measurement results. In addition, PCR-based methods may detect both malignant and non-malignant epithelial cells circulating in the blood. Both cell types may be released into the blood due to undefined reasons and with undefined intervals [3], leading to their inconsistent presence in the circulation. For these reasons, the existing PCR-based methods of detecting CTCs have limited usefulness for clinical use.

Despite the above-mentioned reservations, we can draw two conclusions from our study: (1) expression of EGFR in the blood may be considered to be a specific marker for the detection of circulating urothelial cells and its mRNA measurement in the blood may provide useful information for urologists; (2) the results of this study do not provide evidence for increased tumor cell release into the peripheral blood after TURBT.

Conflicts of interest The authors declare that they have no conflict of interest.

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