Different Gene Expression and Activity Pattern of Antioxidant Enzymes in Bladder Cancer

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Abstract. *The aim of this study was to evaluate the possible role in and contribution of antioxidant enzymes to bladder cancer (BC) etiology and recurrence after transurethral resection (TUR). We enrolled 40 patients with BC who underwent TUR and 100 sex- and age-matched healthy controls. The analysis was performed at diagnosis and recurrence, taking into account the time of recurrence. Gene expression of catalase (CAT), glutathione peroxidase 1 (GPX1) and manganese superoxide dismutase (SOD2) was determined in peripheral blood leukocytes. The activity of glutathione peroxidase 3 (GPX3) was examined in plasma, and GPX1 and copper-zinc containing superoxide dismutase 1 (SOD1) in erythrocytes. SOD2 and GPX1 expression and GPX1 and SOD1 activity were significantly higher in patients at diagnosis of BC in comparison to controls. In patients who had recurrence earlier than 1 year from TUR, CAT and SOD2 expression was lower (at diagnosis $p=0.024$ and $p=0.434$, at recurrence $p=0.022$ and $p=0.010$), while the GPX1 and GPX3 activity was higher (at diagnosis $p=0.242$ and $p=0.394$, at recurrence $p=0.019$ and $p=0.025$) compared to patients with recurrence after 1 year from TUR. This study revealed that the gene expression and activity of the antioxidant enzymes are elevated in blood of patients with BC, although a low expression of CAT might contribute to the recurrence of BC, in early prognosis.*

Bladder cancer (BC) is the most frequent malignancy of the

urinary system in males (1). The highest incidence rates worldwide have been reported in Europe, with highest male mortality rates in Spain and Poland (2). BC is characterized by frequent recurrence (3). There are two major types of BC which usually arise from urothelium: non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC). MIBC is usually of a higher tumor stage and grading, with poorer prognosis than NMIBC. Recurrence of NMIBC has been found as rapidly as in a few weeks after treatment/surgery (4). The most commonly applied therapy is transurethral resection (TUR) or partial cystectomy. Generally, in management of MIBC, adjuvant treatment is recommended. Unfortunately, the surgical management of BC is complicated by the development of recurrence (5). The recurrence of BC has a crucial effect on survival time because recurrent BC is more aggressive in its growth pattern than the original lesion. In recent years, great efforts have been made to elucidate the factors contributing to recurrence preventing it after treatment (6, 7). In 2013, the criteria of the European Association of Urology (EAU) guidelines for individual patients defined tumors with low, intermediate, and high risk of both recurrence and progression. Thus the European Organization for Research and Treatment of Cancer (EORTC) scoring system and risk tables are commonly used for BC estimation (8).

Formation of free radicals and reactive oxygen species (ROS) can lead to changes in the redox state of cells and may further contribute to oxidative stress (OS). ROS, by regulating gene expression and enzyme activation, serve a role as signaling molecules which regulate genes important in tumor pathogenesis (9). Moreover, generation of ROS through OS can potentially induce cancer (10, 11).

Manganese superoxide dismutase (SOD2), copper-zinc containing superoxide dismutase (SOD1), catalase (CAT), and glutathione peroxidase 1 (GPX1) are most important in endogenous antioxidant-scavenging. The enzymatic protection

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Key Words: Urinary bladder, bladder cancer recurrence, gene expression, activity, antioxidant enzymes, CAT.

of cells from oxidative injury is present in the mitochondrial matrix as SOD2 and cytosolic SOD1 and GPX1. The first step of the antioxidant defense system is SOD2, which converts ROS to hydrogen peroxide (H_2O_2), which GPX1 and CAT reduce H_2O_2 into water and oxygen. Glutathione peroxidase 3 (GPX3) is plasma selenoprotein involved in the metabolism of H_2O_2 , fatty acid hydroperoxides and phospholipid hydroperoxides. It is, therefore, understandable that different antioxidant enzymes contribute to a number of disease processes, including cancer, neurodegeneration, and cardiovascular and kidney diseases (11). The role of endogenous antioxidant enzymes such as GPX1, GPX3, SOD1, SOD2 and CAT as potential markers of tumor progression and metastasis was shown in recent studies (12-15).

Considering the scavenging role of CAT, GPX1 and SOD2 in the antioxidant pathway, we hypothesized that the gene expression of these enzymes in peripheral blood leukocytes (PBLs) as a 'surrogate' tissue might be related to BC invasiveness and recurrence. We also speculated that the genes encoding *CAT*, *GPX1* and *SOD2* may also influence their activity. Moreover, as far as we are aware, the role of gene expression of these important players has not been examined in PBLs of patients with recurrent BC.

Patients and Methods

Study design. Patients with BC, both female and male (n=40), treated at the first Department of Urology, Medical University, Lodz, Poland, were recruited and blood specimens were collected between 2005-2011, at two time points: at diagnosis and at recurrence. The median interval from time of diagnosis to recurrence was 12 months (range=2-48 months). Therefore patients with first recurrence were stratified into two groups: those with recurrence earlier than 1 year after TUR (n=20) and those with recurrence after 1 year or more from TUR (n=20) (Figure 1). All BC were diagnosed histologically according to 1973 World Health Organization criteria as low (G1), intermediate (G2) and high (G3) grade using specimens obtained before TUR (8, 16). Tumor stage (T) was evaluated according to the tumor-node-metastasis (TNM) classification (17).

Age-matched healthy controls (n=100) were recruited between 2011-2012, from the Nofer Institute of Occupational Medicine (NIOM), Lodz, Poland. The study was approved by the Ethics Committee of the NIOM (approval number 1/2011). Written informed consent was obtained from all participants of the study.

For the smoking-stratified analyses, patients and controls were divided into two subgroups as those who currently smoked (smokers) and those who did not and had not smoked within the previous 5 years (non-smokers).

Blood sampling. Blood samples were collected into EDTA-containing tubes. Cells were lysed in RLT lysis buffer with 0.1% β -mercaptoethanol. Leukocyte lysate samples were preserved in sterile microtubes immediately after delivery to the laboratory and maintained at -70°C until RNA isolation. Whole blood samples were collected into heparinized tubes. This material was further fractionated by centrifugation into plasma and erythrocytes and stored at -20°C , until biochemical analyses.

Figure 1. Study design.

Total RNA isolation and cDNA synthesis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was eluted in 30 μl RNase-free water and stored at -70°C until use. cDNA was synthesized from 250 ng of total RNA, using QuantiTect cDNA synthesis kit (Qiagen) according to the instructions of the manufacturer. The cDNA was stored at -20°C for further gene expression analysis.

mRNA expression of antioxidant enzymes (GPX1, CAT, and SOD2). Gene expression analyses were performed using PBLs of all controls and patients with BC. Synthesized oligonucleotide primers were used in the assay (Metabion International AG, Martinsried, Germany). The mRNA expression levels of target genes were normalized to that of the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The nucleotide sequences of primers used were as follows: *GPX1*, 5'-CAA CCA GTT TGG GCA TCA G-3' (forward) and 5'-GTT CAC CTC GCA CTT CTC G-3' (reverse); *SOD2*, 5'-AAG TAC CAG GAG GCG TTG G-3' (forward) and 5'-TGA ACT TCA GTG CAG GCT GA-3' (reverse); *CAT*, 5'-CCA TCG CAG TTC GGT TCT-3' (forward) and 5'-GGG TCC CGA ACT GTG TCA-3' (reverse); *GAPDH*, 5'-AGC CAC ATC GCT CAG ACA C-3' (forward) and 5'-GCC CAA TAC GAC CAA ATC C-3' (reverse). cDNAs were mixed with primers and 2xFastStart SYBR Green Master (Roche Applied Science, Mannheim, Germany). The final volume of the reaction was 10 μl . The conditions for the amplification reactions were 95°C for 15 s, annealing at 60°C for 45 s and extension at 72°C for 45 s (LightCycler 96; Roche Diagnostics, Mannheim, Germany). Each reaction was run in duplicate. The mean normalized expression (MNE) was calculated using the software package Q-Gene (18).

Activity of antioxidant enzymes (GPX1, SOD1, and GPX3). Measurement of GPX1 and SOD1 activity was conducted in erythrocytes, while GPX3 activity was determined in plasma. The activity of GPX1 and GPX3 were measured spectrophotometrically by a method published by Paglia and Valentine (19) using *t*-butyl hydroperoxide as a substrate. The decrease in absorbance at 340 nm is proportional to the activity of GPX. The activity of SOD1 was quantified by measuring its ability to inhibit the reduction of nitro-blue tetrazolium using the method of Beauchamp and Fridovich (20). The oxidation product was determined spectrophotometrically at 540 nm. All absorption spectra were read using a spectrophotometer (Unicam UV4 UV/Vis, Cambridge, UK).

Statistics. Nominal variables are given as numbers with corresponding percentage, whereas continuous variables are presented as means with standard deviation. A log2 transformation was applied to normalize

Table I. Demographic, and clinical and pathological characteristics of groups.

Characteristic	Controls, n (%)	Patients with BC, n (%) [‡]	p-Value
Age mean±SD (years)	68.7±8.1	66.2±9.0	0.151 ^b
BMI mean±SD (kg/m ²)	27.7±3.7	25.6±3.0	0.001 ^b
Gender			
Female	19 (19.0)	9 (22.5)	0.815 ^a
Male	81 (81.0)	31 (77.5)	
Smoking status*			
Non-smoker	79 (79.0)	9 (23.7)	<0.001 ^a
Smoker	21 (21.0)	29 (76.3)	
Tumor stage			
T1	n/a	30 (75.0)	
T2	n/a	5 (12.5)	
T3	n/a	4 (10.0)	
unknown	n/a	1 (2.5)	
Tumor grade			
G1	n/a	20 (50.0)	
G2	n/a	13 (32.5)	
G3	n/a	7 (17.5)	

BC: Bladder cancer; BMI: body mass index; n/a: not applicable. [‡]At diagnosis, *Two patients with BC had unknown smoking status; ^achi-square test; ^bt-test.

the distribution of MNE. Chi-square tests, with appropriate corrections, were used to test associations between categorical variables. For pairwise comparisons of continuous variables, Student's *t*-test was used. For multi-group comparisons, one-way analysis of variance (ANOVA) was used. *Post-hoc* comparisons were performed with Tukey's test. *p*-Values lower <0.05 were considered as being statistically significant. Statistical analyses were carried out in Statistica 12.5 software (StatSoft, Tulsa, OK, USA) and GraphPad Prism 7 (GraphPad Software, Inc, San Diego, CA, USA).

Results

Group characteristics. No statistically significant differences were noted for sex ($p=0.815$) and age ($p=0.151$) between controls and patients at diagnosis. The control group was more frequently shown to be obese ($p=0.001$) and reported smoking less frequently ($p<0.001$) compared to patients with BC (Table I).

Higher gene expression of GPX1 in patients with BC. We examined differences in gene expression of *CAT*, *GPX1*, *SOD2* between controls and patients at two time points (at diagnosis and recurrence) (Figure 2). Comparison of the three groups demonstrated a significant difference between controls and patients at two time points in *GPX1* gene expression ($p=0.014$). *Post-hoc* analysis demonstrated that gene expression of *GPX1* was significantly higher in patients at diagnosis compared to controls ($p=0.029$). Moreover, *GPX1* gene expression was higher in patients at recurrence

Figure 2. Relative mRNA expression levels of catalase (*CAT*), glutathione peroxidase 1 (*GPX1*) and manganese superoxide dismutase (*SOD2*) in controls and patients with bladder cancer at diagnosis and recurrence. * $p<0.05$ Post-hoc test. Horizontal lines represent mean values.

compared to controls ($p=0.097$), but the difference was not significant. Comparison of the three groups demonstrated that gene expression of *SOD2* was non-significantly increased in patients at diagnosis compared to control ($p=0.067$). Gene expression of *CAT* was at the same levels ($p=0.782$).

The analysis shows that smoking status did not affect the differences in gene expression of *CAT*, *GPX1*, *SOD2* in BC patients at diagnosis ($p=0.501$, $p=0.059$, and $p=0.979$, respectively).

Gene expression of CAT, GPX1 and SOD2 and tumor characteristics. The gene expression of *CAT*, *GPX1* and *SOD2* was compared by tumor characteristics such as tumor stage and tumor grade in patients at diagnosis of BC. There were no significant differences between gene expression according to tumor stage ($p=0.692$, $p=0.222$ and $p=0.352$, respectively). Elevated gene expression of *GPX1* was observed in poorly differentiated tumors (G2 and G3), but was not significant ($p=0.317$). *CAT* and *SOD2* gene expressions were not associated with tumor grade ($p=0.664$ and $p=0.457$) (Figure 3).

Lower gene expression of CAT and SOD2 among patients with BC with recurrence at <1 year. The analysis as performed at diagnosis and recurrence in patients with BC in relation to the time of recurrence (<1 year and ≥ 1 year) (Figure 4). We show that gene expression of *CAT* was significantly higher in patients with recurrence ≥ 1 year from TUR compared to that at <1 year, and this was shown at two time points, both diagnosis and recurrence ($p=0.024$ and $p=0.022$, respectively). The gene expression of *GPX1* at both time points did not differ significantly in patients with

Figure 3. Association between relative mRNA expression of catalase (CAT), glutathione peroxidase 1 (GPX1) and manganese superoxide dismutase (SOD2) and tumor grade at diagnosis. Horizontal lines represent means.

recurrence at ≥ 1 year compared to those with recurrence at < 1 year (diagnosis: $p=0.489$; and recurrence: $p=0.786$). We observed at diagnosis, *SOD2* gene expression did not differ significantly in patients with recurrence at ≥ 1 year compared to those with recurrence at < 1 year ($p=0.434$) (Figure 4A). At recurrence, however, gene expression of *SOD2* was higher in patients with recurrence at ≥ 1 year compared to those with earlier recurrence ($p=0.010$) (Figure 4B).

Higher activity of GPX1 and SOD1 in patients with BC. Comparison of patients at diagnosis and recurrence with controls demonstrated an increase of GPX1 ($p<0.001$) and SOD1 activity ($p<0.001$) (Figure 5). Activity of GPX1 and SOD1 was significantly higher in patients at diagnosis ($p=0.021$ and $p=0.021$, respectively), and at recurrence of BC ($p=0.011$ and $p=0.017$, respectively) compared to controls. Activity of GPX3 in controls was at the same levels as those observed at diagnosis and at recurrence ($p=0.126$).

Activity of GPX1, SOD1 and GPX3 and tumor characteristics. The correlation between activity and pathological features of the tumors at diagnosis was also examined. Activity of GPX1, SOD1 and GPX3 was not significantly associated with tumor stage ($p=0.381$, $p=0.797$ and $p=0.340$, respectively), as shown in Table II. Activity of GPX1, SOD1 and GPX3 in T1 tumors was at the same levels as those observed in T2 and T3 tumors. Moreover, activity of GPX1, SOD1 and GPX3 was not associated with tumor grade ($p=0.174$, $p=0.367$ and $p=0.900$, respectively). Activities of GPX1, SOD1 and GPX3 in G1 tumors were at similar levels as those observed in G2 and G3 tumors.

Table II. Association between activity of glutathione peroxidase 1 (GPX1), copper-zinc containing superoxide dismutase 1 (SOD1) and glutathione peroxidase 3 (GPX3) and tumor characteristics.

Tumor stage/grade	GPX1 (U/gHb)	SOD1 (U/mgHb)	GPX3 (U/ml)
T1	21.91 $\pm$ 5.55	7.72 $\pm$ 1.97	0.16 $\pm$ 0.03
T2	17.85 $\pm$ 6.80	7.30 $\pm$ 2.63	0.18 $\pm$ 0.02
T3	21.17 $\pm$ 8.27	7.08 $\pm$ 2.02	0.17 $\pm$ 0.02
<i>p</i> -Value	0.381	0.797	0.340
G1	22.53 $\pm$ 6.06	7.45 $\pm$ 2.10	0.18 $\pm$ 0.04
G2	18.99 $\pm$ 5.18	7.26 $\pm$ 1.65	0.17 $\pm$ 0.03
G3	23.44 $\pm$ 6.83	8.54 $\pm$ 2.27	0.17 $\pm$ 0.02
<i>p</i> -Value	0.174	0.367	0.900

Higher activity of GPX1 and GPX3 among patients with BC with recurrence at ≥ 1 year from TUR. The analysis were performed in patients at diagnosis and recurrence in relation to the time of recurrence (Figure 6). There were no differences in GPX1, SOD1 and GPX3 activity levels at diagnosis according to time of recurrence in patients with BC ($p=0.242$, $p=0.775$, and $p=0.394$, respectively) (Figure 6A). When evaluated at recurrence, higher activity of GPX1 and GPX3 was observed in patients with recurrence < 1 year compared to ≥ 1 year recurrence ($p=0.019$ and $p=0.025$, respectively). Activity of SOD1 at recurrence was not associated with time to recurrence in patients ($p=0.664$) (Figure 6B).

Discussion

ROS play an important role in the pathogenesis disease associated with structural remodeling, including carcinogenesis (9, 21). It is generally recognized that anti-stress defense in the cell is very important in carcinogenesis and in the recurrence of cancer (22). Studies show that activity of CAT, SOD2 and GPX1 prevents formation of ROS and hence prevents oxidative damage of proteins, lipids and DNA damage (23, 24). Urine markers for BC progression and surveillance are available and commonly used in clinical settings (25). Non-invasive urine-based tests such as ImmunoCyt, BTA TRAK[®], UroVision using immunoassay, immunohistochemistry, fluorescent *in situ* hybridization and PCR techniques, and these diagnostic tests would also help to reduce the number of unnecessary cystoscopies (26-28). The expression patterns of several genes and proteins as markers still need to be determined (29). Having this in mind, the present study aimed to investigate the expression of selected genes involved in defense against oxidative stress (*CAT*, *GPX1*, *SOD1*) and activity of enzymes GPX1, GPX3 and SOD1 which are primary antioxidant enzymes in cells and are usually up-regulated by severe oxidative stress (21, 30). The role of mRNA expression of antioxidant enzymes in

Figure 4. Association between relative mRNA expression of catalase (CAT), glutathione peroxidase 1 (GPX1) and manganese superoxide dismutase (SOD2) and time to recurrence (earlier than 1 year and 1 year or more) in patients at diagnosis (A), and at recurrence (B) of bladder cancer. * $p < 0.05$. Horizontal lines represent means.

Figure 5. Activity of glutathione peroxidase 1 (GPX1), copper–zinc containing superoxide dismutase 1 (SOD1) and glutathione peroxidase 3 (GPX3) in controls and patients at diagnosis and recurrence of bladder cancer. * $p < 0.05$ Post-hoc test. For the box-and-whiskers plots, the whiskers represent the minimum and maximum values, the box boundaries represent the 25th and 75th percentiles, and the line is the median value.

PBLs in oxidative stress associated with invasiveness and recurrence of BC is unknown. Therefore, the objective of this study was to assess the potential association of activity and gene expression of antioxidant enzymes with invasiveness and recurrence of BC.

Higher gene expression and activity in BC patients at diagnosis. The evidence implicating CAT, GPX1 and SOD2 in the progression of cancer is extensive. Expression of antioxidant enzymes is often up-regulated in patients with BC, because of the protection against cellular and molecular damage caused by ROS. Previous study found alteration of the antioxidant defense system in breast cancer tissue (31).

Alterations caused by tumor in antioxidant enzymes were also observed in our study.

In our study, we noticed the same pattern for antioxidant enzymes in gene expression in PBLs and for activity in the blood. We observed elevated gene expression and activity of antioxidant enzymes in patients with BC patients at both diagnosis and recurrence compared to controls. GPX1 and SOD2 genes were overexpressed at diagnosis and recurrence, and there was significantly higher gene expression of GPX1 at diagnosis compared to controls. In the same way, the activity of antioxidant enzymes was higher in patients at diagnosis and at recurrence than in controls. This observation is consistent with results from other studies (32, 33), which

Figure 6. Association between activity of glutathione peroxidase 1 (GPX1), copper-zinc containing superoxide dismutase 1 (SOD1) and glutathione peroxidase 3 (GPX3) and time to recurrence (before 1 year and after 1 year) in patients at diagnosis (A), at recurrence (B) of BC. * $p < 0.05$. For the box-and-whiskers plots, the whiskers represent the minimum and maximum values, the box boundaries represent the 25th and 75th percentiles, and the line is the median value.

also indicate that SOD and GPX are significantly increased in patients with cancer because they may increase protective effects (14). Some studies have shown, in contrast, that CAT activity was lower in patients with breast cancer (14, 34). Previous study confirmed that overexpression of GPX1 alone or in combination with SOD enhanced skin tumor growth in transgenic mice (35). This may suggest the potential impact of the GPX1 and SOD in carcinogenesis. However, we demonstrate that CAT, GPX1 and SOD2 gene expression was not significantly related to tumor characteristics.

Time-to-recurrence associated with different pattern of gene expression and activity of antioxidant enzymes. CAT, GPX1

and SOD2 may prevent carcinogenesis and have a protective effect. Some investigators, in their reports on different cancer types, have concluded that high levels of antioxidant enzymes contribute to cancer progression (13, 36-38). Thus, it could be expected that even early recurrence should be associated with high mRNA expression and activity. But our recent report suggests that different patterns of gene expression and activity are associated with time of recurrence.

All our recurrent cases were divided into early- or late-recurrence groups, using 1 year as the cut-off value. This division was applied in patients at two time points: at diagnosis and recurrence. Analysis at diagnosis offers a chance for early prediction of recurrence. Surprisingly, we observed

changes in gene expression and activity related to time of recurrence. Moreover, the analysis taking into account the time of recurrence showed different patterns of gene expression and activity, with patients with recurrence earlier than 1 year from TUR having higher GPX1 and GPX3 activity compared to patients with recurrence after 1 year. The significantly higher activity of GPX1 and GPX3 was observed at the second time point, recurrence. On the other hand, we observed that gene expression of antioxidant enzymes was lower. *CAT* and *SOD2* gene expression in particular might participate in cancer recurrence. Gene expression of *CAT* and *SOD2* was significantly lower in patients with recurrence at <1 year from TUR. Similar results have also been reported in an in vitro model indicating that lower CAT activity plays a role in tumor progression (39). Reiners *et al.* reported a significant decrease of CAT and SOD activity in mouse skin tumors (40). Nishikawa *et al.* also showed that retention of CAT activity may inhibit pulmonary metastasis (41). We hypothesized that down-regulation of *CAT* and *SOD2* gene expression is accompanied by increased antioxidative potential expressed as activity of GPX1 and GPX3 enzymes in patients with BC with early recurrence.

Urothelial carcinoma of the bladder as a multifocal disease which, among others, depends on different antioxidant enzymes at different levels such as mRNA expression and enzyme activity. General influence of the antioxidant enzymes on cancer invasiveness and recurrence is difficult to predict. In our opinion, high activity of antioxidant enzymes and low gene expression seems to be associated with a particularly unfavorable outcome (early recurrence) after TUR. The prediction of time to recurrence was probably considerably more accurate when mRNA expression and activity were used in conjunction. Therefore, in order to prevent recurrence, we should pay attention to gene expression as well as enzyme activity. This study examined the transcript levels, which may or may not relate to changes in protein levels due to post-transcriptional regulation. Interestingly, we show that the gene expression and activity of antioxidant enzymes are altered in patients with BC, but they possess different pattern of gene expression and activity in regard to recurrence of BC. According to these results, we can conclude that in particular, gene expression of *CAT* is important in BC recurrence and may predict recurrence at any early stage, namely at the time of first diagnosis.

Conflicts of Interest

The Authors declare they have no conflict of interests related to this work.

Funding

This work was supported by the NIOM (internal grant no. 1.22/2013 and 1.39/2016) and the Ministry of Science and Higher Education Grant 1978/B/P01/2009/37.

Ethical Approval

The study was approved by the Ethics Committee of the NIOM. All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Acknowledgements

We would like to thank Stefan Markiewicz for his help in preparing the English text of this article.

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Received November 2, 2016

Revised December 18, 2016

Accepted December 20, 2016