

Gas6 expression and Tyrosine kinase Axl Sky receptors: Their relation with tumor stage and grade in patients with bladder cancer

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Summary Objectives: It has been shown that the dysregulation of tyrosine kinase Axl receptor and its ligand growth arrest-specific gene (Gas6) are associated with poor prognosis in various types of tumors but there is not enough study about their importance in bladder cancer (BC). We evaluated the relation of Gas6 gene expression and tyrosine-kinase Axl and Sky (Tyro 3) receptors with tumor stage and grade in patients with BC.

Material and Methods: The study group consists of 55 patients whose transurethral resection of bladder (TUR-B) has been performed due to BC and the control group consists of 12 patients with normal bladder mucosa. In tissues mRNAs of Gas6, Axl, and Sky receptors were examined by quantitative (Real-Time) PCR (qPCR). Protein expression was measured by immunohistochemistry. Plasma Gas6 protein levels were compared with control group by ELISA method.

Results: Patients with BC were grouped as Ta low (n=17), Ta high (n=5), T1 low (n=9), T1 high (n=8) and T2 (n=16) according to their TUR-B pathologies. The qPCR analysis showed that the expression of Gas6 gene and Axl receptor is higher in the tumor-positive group and the immune-histochemical showed that the bladder samples of the tumor-positive group stained significantly positive. When the patients are grouped according to the TUR-B pathologies, a statistical significant difference was observed among groups in the qPCR analysis ratios of Gas6 gene and Axl receptor by ($p < 0.05$) but no significance was found for Sky receptor ($p > 0.05$). When Gas6 protein levels in plasma samples were compared by ELISA method, a statistical significance was determined among groups ($p = 0.001$).

Conclusions: Our findings indicate that mRNAs of Gas6 and Axl receptor are closely related to tumor stage and grade in patients with BC. Further studies are needed for understanding the role of Gas6 and its receptors on the neoplastic transformation in terms of novel biomarkers and potential therapeutic targets.

KEY WORDS: Bladder cancer; Gas6; Axl; Sky; Tyro3.

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INTRODUCTION

According to the GLOBOCAN data, bladder cancer (BC) is the 7th most commonly diagnosed cancer in men and it declines to 11th worldwide, when both sexes are considered (1). Worldwide age-standardized mortality rate has been reported as 3.2 for men vs. 0.9 for women per 100.000 persons, however incidence and mortality rates vary depending on the healthcare systems, management protocols and development level of the countries.

The most common subtype of BC is urothelial carcinoma (UC) and the molecular mechanism of UC is not completely understood as in other cancers (2).

Receptor tyrosine kinases (RTKs) play key roles in cellular signal transduction and they are one of the most common types of molecules investigated for this purpose. In humans, 20 distinct subfamilies of RTKs exist that are categorized according to their aminoacid sequence identities and structural similarities in their extracellular regions (3). One of these is the subfamily of TAM receptors comprising Sky (Tyro3), Axl, and Mer. They participate in a signaling axis where growth arrest-specific 6 (Gas6) protein is a ligand (4). The oncogenic nature of Axl, Sky and Mer is demonstrated through activation of signaling pathways involved in proliferation, migration, invasion, angiogenesis, inhibition of apoptosis, and therapeutic resistance (5). It has been shown that overexpression of Axl, Sky, Mer RTKs, and their ligand Gas6 is associated with poor prognosis in various types of tumors (6). The close relationship with the pathogenesis of many cancers suggests that Gas6 and its TAM receptors could be potential biomarkers and targets for treatment (7).

The molecular biology of BC is complex and not fully understood. There is a very limited number of studies investigating the relationship between BC and Gas6/TAM receptors. Yeh *et al.* investigated the role of Axl in the pathogenesis of locally advanced and metastatic BC patients (8). They indicated that c-Met and its crosstalk with Axl could contribute to the progression of

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human BC. *Rui et al.* investigated the relationship between long noncoding RNA and BC (9). They showed that GAS6-AS2, a long noncoding RNA, was significantly up-regulated in BC tissues and positively correlated with tumour stages and poor prognosis. Identification of new prognostic markers and therapeutic targets for BC is urgently required. In the present study, we aimed to elucidate the relation between Axl/Sky/Mer RTKs and its ligand Gas6 in patients with UC of bladder.

MATERIALS AND METHODS

The sample size was calculated based on the formula according to the previously published studies (6, 8). Our study group includes 55 patients with transurethral resection of bladder tumor, where the histopathological diagnosis of the tumors was UC. The control group consists of the 12 patients whom bladder mucosa has been biopsied during radical prostatectomy operation. Tissues from bladder tumor or mucosa were stored at -80°C .

In this study, mRNA expression of Axl, Sky, and Gas6 in the tissues was analyzed by *quantitative (real-time) polymerase chain reaction* (qPCR). Protein expression of Gas6, Axl, and Sky was analyzed by immunohistochemistry. Plasma and urine samples of patients were collected before transurethral resection and *Enzyme linked Immunosorbent Assay* (ELISA) was used to measure Gas6 protein level. Plasma and urine samples of a control group, who are older than 40 years of age and without any history of malignancy, has been used for comparison. The urine and venous plasma aliquots were stored at -80°C before analysis after centrifugation at 2300 g for 15 min at room temperature.

The study has been approved by the local institutional ethics committee (Approval number: 09.2012.0087) and conducted in conformity with the Declaration of Helsinki in 1995. Written informed consent were obtained from all patients included in the study.

Real-time PCR (qPCR)

Total RNA was isolated using TriPure isolation reagent solution (*Roche, Mannheim, Germany*) according to the manufacturer's instructions. The mRNA expression of the Gas6, Axl and Sky were determined using specific primer sequences.

Specific primer sequences used for the Gas6 gene:

- 5'-TGCTGTCATGAAAATCGCGG-3' (gas6-5'; 1328-1347)
- 5' CATGTAGTCCAGGCTGTAGA-3' (gas6-3'; 1594-1613)

Specific primer sequences used for the Axl receptor gene:

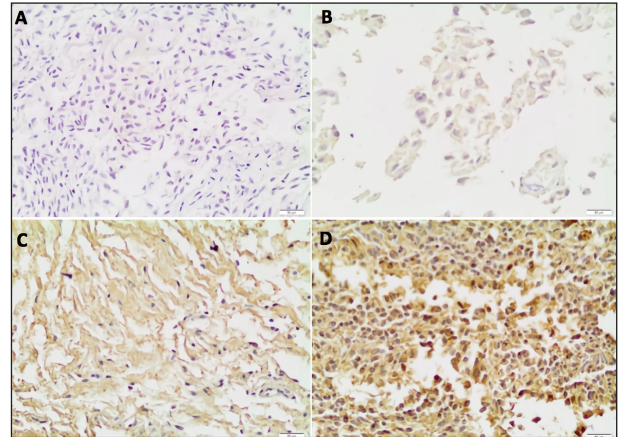
- 5'-GGTGGCTGTGAAGACGATGA-3' (Axl-5'; 1820-1839)
- 5' CTCAGATACTCCATGCCACT-3' (Axl-3'; 2103-2122)

Specific primer sequences used for the Sky receptor gene:

- 5'-CACTGAGCTGGCTGACTAAGCCCC-3'(Sky-5'; 2719-2742)
- 5'-AATGCATGCACTTAAGCAGCAGGG-3'(Sky-3'; 3039-3062)

Figure 1.

The cytoplasmic staining of immune expression for Gas6 protein and Axl, Sky receptors proteins positivity in BC tissue. According to the intensity of staining, it was scored as 1A: 0, 1B: +1, 1C: +2, 1D: +3.



The qPCR analysis was performed using SYBR Green dye (*Light Cycler-RNA Amplification Kit SYBR Green I; Roche, Mannheim, Germany*). Target gene expression was determined by comparing the amount of threshold loop with the *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) gene.

Immunohistochemical analysis

Streptavidin biotin peroxidase immunohistochemical staining method was applied to show the immune expression of Gas6 protein and Axl, Sky receptors proteins in paraffin-embedded tissues. The immune expression positivity in BC tissue was observed as cytoplasmic staining. According to the intensity of staining, it was scored as 0 (Figure 1A), +1 (Figure 1b), +2 (Figure 1c), +3 (Figure 1d). Immunohistochemistry expression index was obtained by multiplying the cytoplasmic staining density and the ratio of stained cells. Two authors, who were blinded to the clinical course of the patients, independently evaluated immunoreactivity, and the average counted by these two authors was used for statistical analyses.

ELISA method

The human GAS6 sandwich ELISA development kit (*R&D Systems, Inc., Minneapolis, MN, USA*) and a *Substrate Reagent Pack (Color reagent A&B)* (*R&D Systems, Inc.*) were used to measure plasma GAS6 levels. Our group has been optimized the development kit to measure human plasma GAS6 levels and established reference intervals (10). Briefly, the following parameters were tested for optimization: type of antibody; capture antibody concentration; dilution solution; dilution ratio of samples and calibrators; blocking agent (BSA or non-fat dry milk), and incubation time and temperature. Dilution solution for samples and calibrators was PBST containing 1 mM EDTA and 1% BSA. Dilution ratio of samples and calibrators was 1/40. Incubation time during antigen antibody interaction (both capture and detection antibodies) was 1h at 37°C . After analytical validation studies of the method, samples were analyzed.

Statistical analysis

In descriptive statistics of the data, frequency, ratio, mean and standard deviation values were used. The distribution of data was tested with Kolmogorov-Smirnov. Student t test and ANOVA were used to analyze the parametric section data. Mann-Whitney U and Kruskal-Wallis tests were used for the analysis of non-parametric data. SPSS 15.0 software (SPSS, USA) was used in the analysis.

RESULTS

Patient demographics

The BC patients were grouped according to their pathological degrees and stages: Ta low grade (n=16), Ta high grade (n=5), T1 low grade (n=10), T1 high grade (n=10), T2 high grade (n=14) and normal bladder mucosa control group (n=12). Mean ages of the patients with BC and the control group were 62.8 ± 10.7 and 57.3 ± 7.0 years, respectively (p > 0.05). Patients with BC, were male in 81.8% and female in 18.2%, while the control group consisted of male patients.

Tissue analyses

The qPCR results showed that the mRNA expression of Gas6 and Axl receptor were higher in the tumor-positive patient group, where Sky receptor gene were higher in control group, these differences were not significant (p > 0.05) (Table 1). When the patients are grouped according to the grade and stage, significant differences were observed for Gas6 and Axl receptor genes (p < 0.05). But no significant difference was found for Sky receptor (p > 0.05) by qPCR (Figure 2).

Table 1.

Gas6, Axl, Sky receptor gene expression copy level assessed by the qPCR method.

	Control group			Tumor-positive patient group				p
	n	Median	Min. Max.	n	Median	Min.	Max.	
Gas6	11	60.6	7 294	50	73.1	10	1810	0.223*
Axl	12	288.7	145 578	55	421.5	87	1997	0.288*
Sky	11	10000	2888 15497	54	8048	1591	31333	0.588*

*Mann Whitney U test.

Figure 2.

Expression of Gas6 and Axl receptor mRNAs in BC against tumor stage and grade by qPCR.

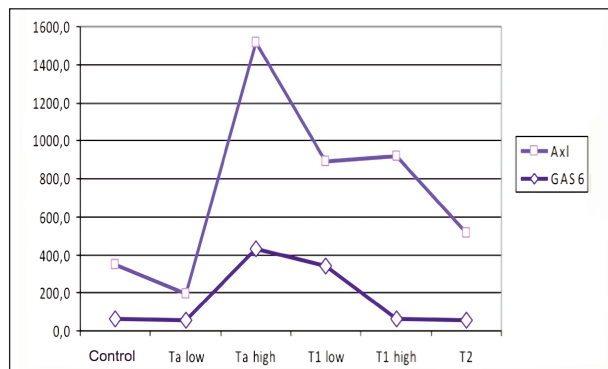


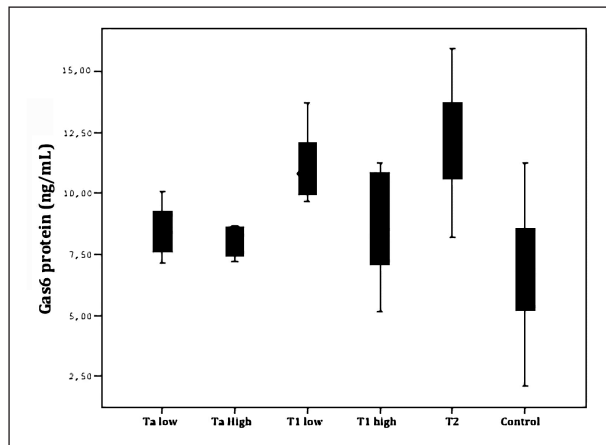
Table 2.

Immunohistochemical expression index of Gas6 protein, Axl, Sky receptors for BC and control group.

	Control group				Tumor-positive patient group				p
	n	Median	Min.	Max.	n	Median	Min.	Max.	
Gas6	6	5	0	90	32	85	0	300	0.006
Axl	6	15	0	70	28	60	10	200	0.037
Sky	6	50	0	200	32	180	0	300	0.261

Figure 3.

Gas6 protein levels in plasma samples according to the stage and the grade of the BC patients with ELISA analysis.



The immunohistochemical expression index for the Gas6 and Axl receptor were statistically higher compared to the control group (p < 0.05). The immunohistochemical expression index of Gas6 protein, Axl, Sky receptors for BC and control group is shown in Table 2.

Plasma and urine analyses

The mean plasma Gas6 protein levels for the control group and the BC patient group were 6.5 ± 2.6 ng/mL and 9.9 ± 2.4 ng/mL, respectively (p < 0.001). The distribution according to the stage and the grade of the patients are shown in Figure 3. Gas6 protein was not detected in urine samples by ELISA.

DISCUSSION

In the present study, an association between BC and expression of Gas6, Axl and Sky was identified using three different methods: qPCR and immunohistochemistry in tissues and Gas6 levels in plasma by ELISA. The qPCR analysis showed that mRNA expression of Gas6 and Axl are higher in the BC group than the control group but not significantly (p > 0.05). mRNAs of Gas6 and Axl was higher in Ta high grade and T1 low grade significantly, but gradually decreasing in T1 high grade and T2 stages. Using the immunohistochemical examination, the BC group showed significantly higher (p < 0.05) Gas6 and Axl receptor expression compared to the control group. ELISA analysis showed higher Gas6 protein levels in plasma of the BC group compared to the control group (p=0.001), where highest levels are detected in patients with T2 tumors.

The definitive diagnosis of BC ultimately depends on cystoscopic examination of the bladder, which is an invasive method. There are continuous efforts for the development of non-invasive reliable tumor markers to facilitate the diagnosis. However, none of these markers have been accepted for the diagnosis or follow-up in routine practice (11). By the help of further studies, the Gas6/Axl axis may represent an attractive diagnostic tool for BC. Relationship between various physiological events and Gas6, and/or its receptors has been demonstrated, however, its exact mechanism has not been elucidated yet (12, 13). The relationship between Gas6 and/or its receptors with tumor suppressor genes such as *Inositol polyphosphate-4-phosphatase* (INPP4B) remains uncertain (14). The Gas6 gene and particularly the Axl receptor are highly overexpressed in many diseases. Numerous biological dysfunctions, inflammatory diseases and autoimmune disorders are related to the overexpression of Gas6 and its receptors (6). They also participate in the development, progression and metastatization of a range of malignancies (15). Prostate cancer, breast cancer, lung cancer, leukaemia and cancers of gastrointestinal tract including colon are the most studied malignancies (5, 6, 16). Recent studies have also demonstrated an important role of AXL signaling in tumor proliferation, survival, stem cell phenotype, prognosis, metastasis, and resistance to cancer therapy (16). However, there is an opposite situation in renal cell carcinoma, which is a urogenital tumor where the Gas6 and Axl-Sky receptors are associated with good prognosis (17). When we look at the stage and grade of BC, it seems that mRNAs of Gas6 and Axl receptor are very close to each other in the control and Ta low grade group. The malignant potential of low-grade BC, that was formerly regarded as 'low malignant potential papillary urothelial neoplasia', may be related to different molecular pathological and histopathological features. In this respect, it is similar to the new WHO-ISUP classification (18). In the present study, the expression of Gas6 was higher at Ta high grade and T1 low grade, but differentiation was gradually decreasing at T1 high grade and T2 stages. Similarly, in the study of Sun *et al.*, the gene expression of Gas6 and Axl receptor was found to be higher in G1 endometrial cancer, however, it was gradually decreasing in G2 and G3 endometrial cancers where the differentiation is poor (19). Using the immunohistochemical examination, Gas6 and Axl receptor were found to have a significantly higher protein expression at BC compared to the control group. However, similarly to Sky receptor qPCR results, there was no statistically significant difference between the BC and the control group. Using immunohistochemistry examination, Hattori *et al.* investigated the relationship between increased expression of the Axl/Gas6 signal cascade and prognosis of patients with upper tract urothelial carcinoma. They concluded that the protein expression of Axl and its ligand Gas6 is related to worse clinical outcome in upper tract urothelial tumors (20). The plasma levels of Gas6 protein measured by ELISA were significantly higher in the BC group compared to the control group. In addition, mean levels of Gas6 protein in plasma were significantly higher in T2 patients compared to other groups. This may be important for the separation

of the muscle invasive BC from non-muscle invasive BC. Therapeutic potential of AXL inhibition has been explored for cancer therapy (21). A variety of AXL inhibitors have been developed and are efficacious in preclinical studies. These agents offer new opportunities for therapeutic intervention in the prevention and treatment of advanced disease. For the treatment of the breast cancers, BGB324 (R428) and SGI7079, which are the highly selective Axl tyrosine kinase inhibitors, entered in clinical studies (22, 23). Mao *et al.* showed that GAS6 was overexpressed in BC cells. They also found that high levels of GAS6 expression were related to tumor stage, grade, and poor overall survival (24). Unfortunately, there are not enough clinical studies on anti-Axl/Gas6 therapy focusing on BC. However, in light of the future studies, this pathway could be a candidate as novel biomarker and as an approach for treatment for BC.

Limitations of the study

This hypothesis-generating study and our results have some limitations such as small sample size. The control group consists of the normal bladder tissues of the prostate cancer patients is another limitation of the study. Normal bladder tissue is excised as a standard surgical procedure during radical prostatectomy operations. We preferred to use this normal bladder tissue as a control group without an ethical restriction. We could not excise the normal bladder tissue from healthy individuals as a control group because of the ethical problems. Gas6 and its ligands might represent an attractive diagnostic tool in the future. However, expression of these proteins was studied in tissues requiring an invasive biopsy to be obtained. On the other hand, plasma Gas6 ELISA results were also promising for BC diagnosis and could be used as non-invasive diagnostic test. Of course, future studies with larger sample size will provide more reliable information for implementation of plasma Gas6 test as a biomarker. ELISA method was used to detect the urine Gas6 protein levels but Gas6 protein was not detected in urine samples. The sensitivity of this method may not be appropriate for determining the level of Gas6 protein in urine samples. Gas6 protein could make a complex with soluble form of Axl (25). Further studies for optimization of plasma Gas6 ELISA method for measurement of Gas6 in urine samples are necessary.

CONCLUSIONS

We evaluated the relationship between Gas6/RTKs and BC with three different methods by performing qPCR analysis, immunohistochemistry and ELISA analysis. Our findings indicate that mRNAs of Gas6 and Axl receptor are closely related to tumor stage and grade in patients with BC. Further studies are needed for understanding the role of Gas6 and its TAM receptors on the neoplastic transformation in terms of novel biomarkers and potential therapeutic targets.

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