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Differential Expression of IGF-I Transcripts in Bladder Cancer

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Abstract. *Background/Aim: A growing body of evidence shows that the differential expression of E domain-related insulin-like growth factor-I (IGF-I) transcripts (IFG-IEa, IGF-IEb and IGF-IEc) in normal and cancerous tissues, implicating specific biological roles for the putative Ea, Eb, and Ec peptides, beyond IGF-I. Herein, we investigated the expression profile of IGF-IEa, IGF-IEb and IGF-IEc transcripts in bladder cancer and compared them with samples from the normal adjacent bladder tissue. Materials and Methods: Biopsies from 46 patients (39 men and 7 women), aged 73.3±10.9 years, were analyzed for the expression of IGF-I transcripts using semi-quantitative real time-PCR (qRT-PCR). Results: The presence of all three IGF-I transcripts was detected in both normal urothelium and bladder carcinomas. The relative expression of the IGF-IEa and IFG-IEb was marginally increased in bladder cancer tissues compared to normal tissue ($p>0.05$). In contrast, the expression of the IGF-IEc was significantly decreased in bladder cancer as compared to normal adjacent urothelium ($p<0.05$). This specific suppression of IGF-IEc expression was evident and positively correlated with the histological and/or clinical characteristics of an advanced disease, referring to clinical stage, tumor grade and disease recurrence ($p<0.05$); however, in situ carcinomas exhibited an increased expression of all IGF-I transcripts. Conclusion: Our data confirm the differential expression of IGF-I transcripts in bladder cancer, revealing a distinct suppression of IGF-IEc. These findings suggest that IGF-IEc expression and putative Ec product may possess discrete biological role in disease progression beyond IGF-I.*

Insulin-like growth factor-I (IGF-I) regulates various aspects of cellular function such as cell proliferation, differentiation, survival and migration, including cancer biology (1-6), thus IGF-I is implicated in the pathophysiology and disease progression of several human cancers (7-10). Using alternative splicing of exons 5 and 6 (E domain), the *Igf1* gene encodes three IGF-I transcripts, namely IGF-IEa, IGF-IEb, and IGF-IEc (9). Several studies have investigated the differential expression of these IGF-I transcripts in various pathologies vis-à-vis the potential role of their putative products, namely the Ea, Eb and Ec peptides, beyond IGF-I (11-21). The biological significance of the IGF-I splice variants and their products is not fully understood, so far, despite the fact that an increasing body of evidence is pointing out for a complex and distinct IGF-I isoform-related expression pattern in the pathophysiology of various cancer models, both *in vitro* and *in vivo* (9, 14, 22-24).

Although the role of IGF-I bioregulation system has been extensively investigated in several cancers, including prostate, breast and colon cancer (2, 14, 24-28), the available data in bladder cancer is rather limited, while much less is known about the expression pattern of the IGF-I transcripts. High serum IGF-I levels have been associated with the risk of developing bladder cancer, however, no relationship was established between circulating IGF-I levels and disease stage (29). In addition, no significant correlation was documented either between preoperative plasma IGF-I levels and tumor characteristics in bladder cancer and disease outcome (30), or between serum IGF-I levels and tumor grade in patients with superficial bladder cancer (31). Nevertheless, there is a report suggesting that IGF-I may participate in the development and disease progression in bladder cancer, since the local expression of IGF-I was found

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to be increased in biopsies of bladder cancer as compared to normal urothelial tissues (32). It was further reported that a close relationship exists between IGF-I expression levels and disease recurrence, as well as, between IGF-I receptor (IGF-IR) expression levels and tumor grade, tumor differentiation, and disease recurrence (32).

These data suggested that IGF-I may play an important role in bladder cancer, although no report exists on the expression pattern of E domain-related IGF-I transcripts in bladder cancer. Therefore, we characterized the expression profile of the IGF-I mRNA splice variants in bladder cancer biopsies in comparison with normal adjacent urothelium and in correlation to disease stage, tumor grade, disease recurrence and patient characteristics. Our data suggest that the IGF-IEc transcript is differentially regulated *vis-à-vis* the IGF-IEa and IGF-Eb in bladder cancer, being significantly suppressed. Such significant decrease of the IGF-IEc expression was correlated with advanced disease (*i.e.*, high stage, grade, and recurrence). Interestingly, all the E domain-related IGF-I transcripts, including IGF-IEc, were up-regulated in biopsies from *in situ* carcinomas of urothelium as compared with normal adjacent urothelium.

Materials and Methods

Patients. Forty-six consecutive patients, 39 men and 7 women, (aged 73.3±10.9 years) were included in the study. These patients were diagnosed with bladder cancer after clinical investigation of a history of macroscopic haematuria. They had undergone a full clinical, laboratory and imaging examination, while all patients had undergone cystoscopic examination, confirming exophytic lesions of their bladder and biopsies have histologically proven the diagnosis. The recommendation for surgery was according to the guidelines of the European Urology Association (EUA). No modification of the chronic medication of the patients was made except for the discontinuation of anticoagulant drugs before operation, while there was no restriction on their diet, physical activity, or caffeine and alcohol consumption. Because of the positive oncologic history of the patients, there were not exclusion criteria for the operation or participation in the study. A written informed consent was obtained by all patients before their inclusion to this study. The research protocol has been approved by the National and Kapodistrian University Ethics Committee and all the experimental procedures conformed to the Declaration of Helsinki.

Bladder biopsies and tissue processing. Bladder tissue biopsies were obtained from both the pathologic bladder lesion and the adjacent normal urothelial tissue, under spinal anaesthesia at surgery. The bladder tissue sample (~50 mg) obtained from each biopsy was snap-frozen in liquid nitrogen and then stored at –80˚C until analyzed for RNA and protein content.

RNA extraction and semi-quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis. In order to identify differences in gene expression at the mRNA level between normal and pathological condition, expression profile of the IGF-I splice variants was considered on total RNA isolated from bladder biopsies of normal and cancerous tissue of the same patient, as previously described (11, 14).

Table I. *The sequence of the primer sets used in IGF-I mRNA variant RT-PCR analyses*.

Target mRNA	PCR primer sequence	Product size (bp)
IGF-IEa	5'-GTGGAGACAGGGGCTTTTATTTC-3'	251
	5'-CTTGTTTCCTGCACTCCCTCTACT-3'	
IGF-IEb	5'-ATGTCCTCCTCGCATCTCT-3'	411
	5'-CCTCCTTCTGTTCCCCTC-3'	
IGF-IEc	5'-CGAAGTCTCAGAGAAGGAAAGG-3'	150
	5'-ACAGGTAACTCGTGCAGAGC-3'	

Specifically, each tissue sample was homogenized and total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The extracted total RNA was dissolved in RNAases free water (Invitrogen) and the concentration and purity were determined spectrophotometrically (Nanodrop, ThermoFiser Scientific, Waltham, MA, USA) by absorption at 260 and 280 nm. The quality and integrity of the RNA were further assessed by visual inspection of the electrophoretic pattern of 18S and 28S ribosomal RNA in ethidium bromide-stained 1% agarose gels under ultraviolet (UV) light and electrophoresis of the RNA confirmed that it was intact.

Total RNA (1 μg) from each sample was used for the production of single-stranded cDNA by reverse transcription (SuperScript™ IV First-Strand Synthesis System, Invitrogen) and the resultant cDNA samples were utilized in real-time PCR. Real-time PCR analyses were performed using the Bio-Rad 96-well iCyclerThermal Cycler (iQ5 Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA) and iTaq™ Universal SYBR® Green Supermix (Bio-Rad). The primer set sequences used for the specific detection of IGF-I transcripts are shown in Table I. To prevent detection of genomic DNA, the primer sets were designed to lie within different exons while, particularly, each set of primers was specific to detect only one specific IGF-I splice variants.

Each PCR reaction contained 50 ng of cDNA, 12.5 μl SYBR green master mix, 0.4 μM of each primer, and nuclease free water to a total volume of 25 μl. The real-time PCR parameters were the following: initial denaturation at 95˚C for 4 min followed by 40 cycles of 15 sec at 95˚C, and 30 sec at 61˚C for annealing, and 30 sec at 72˚C for extension. To normalize the amount of total RNA present in each reaction, the geometric mean of two housekeeping genes, β-actin and cyclophilin, was used as internal standards. Transcript levels of the mRNA isoforms of interest were assessed by automatically calculating the threshold cycle (C_t), as the number of cycles at which the measured fluorescence exceeds the threshold for detection, which is set slightly above background. Each sample was analyzed in duplicate, and the resulting data were averaged.

A melting curve (T_m) was also generated by the Bio-Rad iQ5 Real-Time PCR Detection System software following the final cycle for each experimental sample, by continuous monitoring the SYBR green fluorescence throughout the temperature ramp from 65˚C to 95˚C, in order to confirm the specificity of the primers. Moreover,

Table II. *Clinical and epidemiological characteristics of the patients.*

n	Gender	Recurrence	Cis	Deaths	Smokers	Upper urinary tract urothelial cell carcinoma
39	Male	6	4	6	21	4
7	Female	-	-	-	-	-

Cis: Carcinoma *in situ*.

all target IGF-I mRNA sequences were identified by sequencing analysis to ensure specificity of the primers and to further verify each target, while electrophoretic analysis of the real-time PCR products further verified the specificity of each target IGF-I mRNA. Controls for specificity included cDNA-free (*i.e.*, RNA not reverse transcribed), and template-free (water) reactions.

Protein extraction and Western blot analysis. Total proteins were extracted from the same biopsy sample used for total RNA isolation using the Trizol Reagent protocol. The extracts were analyzed for total protein concentration using the Bradford procedure (Bio-Rad Protein Assay). Samples were stored in aliquots at –80˚C until analyzed by western blot, as previously described (11, 14). The following IGF-I isoform-specific primary antibodies were used for the immunodetection of the IGF-I isoforms at the protein level: anti-IGF-IEa antibody (provided by Dr. Elisabeth R. Barton Lab), anti-IGF-IEb antibody (provided by Dr. Julia Durzynska) and anti-IGF-IEc antibody [raised in our laboratory (33)].

Statistical analysis. Wilcoxon signed-rank test was employed to evaluate potential differences in the expression level of each IGF-I splice variant between the normal and pathological samples (SPPS v. 21 statistical package; SPSS Inc., Chicago, IL, USA). All data are presented as mean±standard error of the mean (SEM). Statistically significant changes were considered at $p<0.05$.

Results

The demographics and disease characteristics of patients with bladder cancer participating in the study are presented in Table II. In addition, the tumor characteristics of the analyzed biopsies are given in Table III.

All the E domain-related IGF-I splice variants were documented at the mRNA level in both normal adjacent urothelium and bladder cancer biopsies. The expression of IGF-IEa and IGF-Eb were marginally ($p>0.05$) increased compared to normal urothelium, while the expression of IGF-IEc was significantly ($p<0.05$) decreased in bladder cancer biopsies (Figure 1A). These data suggest that IGF-I transcripts are differentially expressed in bladder cancer. Western blot analysis did not detect the IGF-IEa and IGF-IEb isoform in either bladder cancer biopsies or normal urothelial tissues, probably due to their low protein expression levels in these tissues. The IGF-IEc was detected in normal urothelial tissues (Figure1B), while it was undetectable in bladder cancer biopsies, possibly due to the down-regulation of this transcript in bladder cancer, as documented by qRT-PCR analysis.

Table III. Pathologoanatomic features of the patient bladder lesions.

Stage	Ta	T1	T2	T3	T4	Total
Low grade	3	4	-	-	-	7
High grade	1	15	17	2	1	36
Total	4	19	17	2	1	43*

*In one sample pathologoanatomical examination was not performed and in two samples no grade was reported.

Furthermore, by classifying bladder cancer samples in different subgroups based on the clinical stage, degree of tumor differentiation and clinical recurrence, or based on a combinational analysis of those characteristics, our study revealed that in T1 stage as well as in all high-grade tumors (Table III) the expression of IGF-IEc was found to be significantly decreased ($p<0.05$). This was coming along with a steady differential, albeit non-significant ($p>0.05$) increase in the expression of the transcripts IGF-IEa and IGF-IEb compared to the adjacent normal urothelium (Figure 2A, 2B). A similar expression pattern was detected in the subgroup of patients who were exhibited both the above pathological characteristics (*i.e.*, stage T1 plus high-grade tumors) (Figure 2C).

Interestingly, the analysis of *in situ* carcinomas (Cis) revealed increasing expression levels of all three IGF-I transcripts vis-à-vis normal urothelium (data not shown), although Cis are superficial bladder cancers, as the T1 cancers.

When the expression of the IGF-I transcripts in papillary urothelial carcinoma was analyzed, either as an independent factor (Figure 3A), or in combination with the stage and degree of differentiation (stage T1, High-grade, or stage T1-High grade tumors; Table III), or with the coexistence of solid urothelial cancer, IGF-IEc transcript exhibited significantly decreased expression levels ($p<0.05$) while, again, the other splice variants (IGF-IEa and IGF-IEb) showed marginally ($p>0.05$) elevated expression as compared with their relative expression in normal tissues (Figure 3A).

Moreover, in the group of the recurrent disease, a significant down-regulation of the IGF-IEc expression

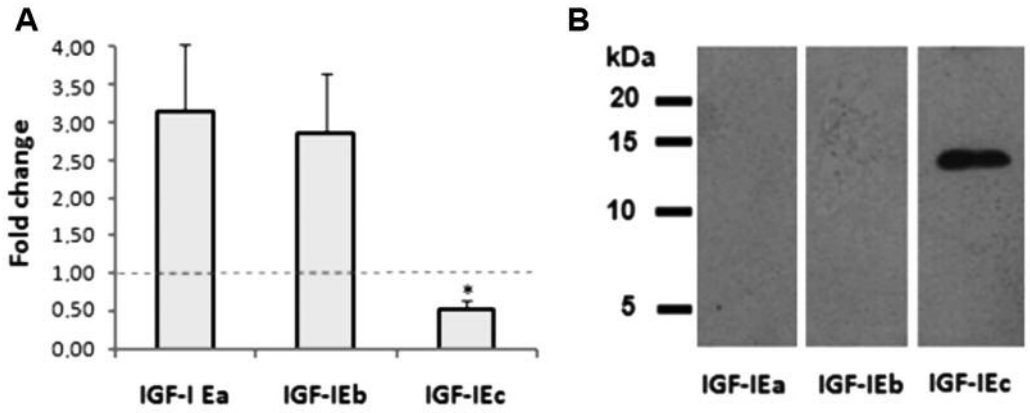

Figure 1. *A: Expression of the different IGF-I isoforms at the transcriptional level (mRNA), in cancerous bladder tissue compared to normal adjacent urothelial tissue of the same patient (n=43). Values (means±SE) were normalized to the geometric mean of two housekeeping genes (β-actin and cyclophilin) and expressed as fold changes compared to the values of the corresponding normal tissue for each patient. *p<0.05. B: Evidence for the expression of IGF-IEc transcript at the protein level in normal urothelial tissue detected by the anti-IGF-IEc specific antibody. IGF-IEa and IGF-IEb protein isoforms were not detected by the anti-IGF-IEa and anti-IGF-IEb antibodies.*

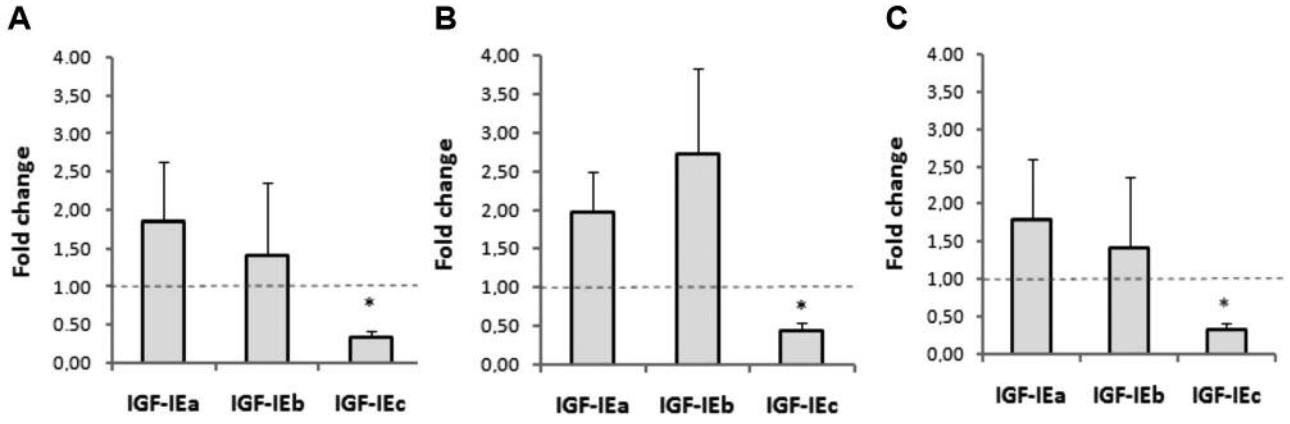

Figure 2. *Expression of different IGF-I isoforms at the transcriptional level (mRNA) in (A) tumors stage T1 (n=19), (B) high-grade tumors (n=36) and (C) in T1 – high-grade tumors (n=15) compared to normal adjacent urothelial tissue of the same patient. Values (means±SE) were normalized to the geometric mean of two housekeeping genes (β-actin and cyclophilin) and expressed as fold changes compared to the values of the corresponding normal tissue for each patient. *p<0.05.*

($p<0.05$) was detected (Figure 3B). However, similar analysis in other classifications based on either tumor (Table III) or clinical characteristics (Table II) revealed no statistically significant differences in the expression profile of all the IGF-I transcripts ($p>0.05$).

Discussion

This study investigated for the first time the expression profile of the different IGF-I splice variants in human bladder cancer with reference to the relative expression of the normal adjacent urothelium, in order to shed light on the possible role of these IGF-I isoforms in the pathophysiology of bladder cancer. Our findings documented that the expression of the IGF-IEc is significantly decreased in bladder cancer and that this regulation is to the opposite direction of the expression detected for the IGF-IEa and IGF-IEb transcripts in bladder cancer. This provide for the first time evidence for a differential regulation of IGF-I transcripts in bladder cancer. Interestingly, this differential regulation was not found in the limited number of samples with *in situ* carcinomas. The down-regulation of the IGF-IEc

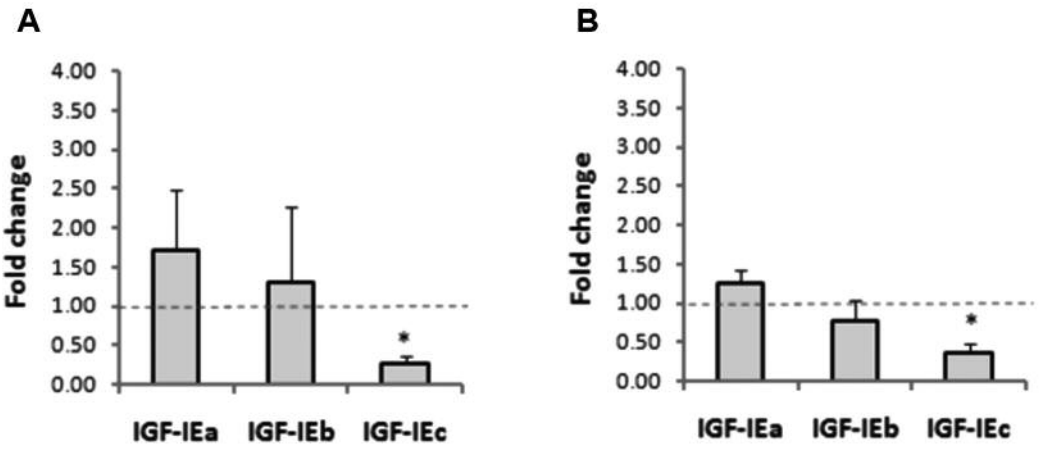

Figure 3. *A: Expression of different IGF-I isoforms at the transcriptional level (mRNA), in papillary urothelial carcinoma compared to normal adjacent urothelial tissue of the same patient (n=21). B: The expression of different IGF-I isoforms at the transcriptional level (mRNA), in recurrent tumors compared to normal adjacent urothelial tissue of the same patient (n=6). Values (means±SE) were normalized to the geometric mean of two housekeeping genes (β-actin and cyclophilin) and expressed as fold changes compared to the values of the corresponding normal tissue for each patient. *p<0.05.*

expression in urothelial cancer compared to normal urothelium, and the modest increases in the expression of the IGF-IEa and IGF-IEb variants in bladder cancer, were independent of the cancer stage or tumor grade.

IGF-I possesses a strong mitogenic and/or anti-apoptotic activity in a wide variety of cancers such as sarcoma, leukemia, prostate, breast, lung, colon, stomach, esophagus, liver, pancreas, kidney, thyroid, brain, ovary and uterus (cervix and endometrium) cancer (27, 34-36). Nevertheless, few studies have addressed the role of IGF-I and/or its receptor (IGF-IR) in bladder cancer. These studies have implicated IGF-I in the development and progression of bladder cancer, reporting that both the IGF-I and IGF-IR expression exhibited a tendency to be increased in bladder cancer (32, 37).

The investigation of the expression pattern of the IGF-I splice variants in various pathologies, including cancer (11-14, 38), is of particular interest as it may indicate a distinct biological role for the putative Ea, Eb and Ec peptides, which are produced by such transcripts and possibly have a discrete role beyond (mature) IGF-I, which is the common product of all splice variants. However, the role of the putative Ea, Eb and Ec peptides remain as yet unclear (39).

Our group focused on the investigation of IGF-I transcripts in cancer biology and for the first time we have reported the differential IGF-IEc expression in human prostate cancer along with strong evidence for IGF-I over-expression in human prostate cancer tissue (14). Moreover, IGF-IEc expression was positively correlated with the prostate cancer stage and Gleason's score (40). In addition, we documented an increased expression of IGF-IEc isoform in secondary compared to primary foci of neuroendocrine neoplasms (18). Other investigators have reported the over-expression of IGF-IEa and IGF-IEb in multiple myeloma patients (41) and of IGF-IEb in uterine cancer (23).

Lower expression of total IGF-I mRNA as well as of each particular IGF-I transcript has been observed in colorectal cancer compared to normal colon tissue (24). More specifically and similarly to our findings, Kasprzak *et al*. (24) found no significant differences between the expression of IGF-IEa and IGF-IEb splice variant in colon cancer, while both these mRNA isoforms exhibited higher expression compared to IGF-IEc. Overall, there is a growing body of evidence indicating a differential regulation of the IGF-I isoforms in the pathophysiology of cancer, which may be tumor type-specific.

Another interesting finding of our study was the increased, though no significant, expression of all three IGF-I isoforms in the *in situ* carcinoma compared to the T1 tumors. Since both carcinoma *in situ* and T1 tumors belong to the superficial disease, similar expression levels might be expected in both cases and this circumstantial evidence may suggest a specific regulation of IGF-I isoforms in surface tumors with possible predictive value.

In conclusion, the differential mRNA expression pattern of the IGF-IEc isoform, compared to the other IGF-I transcripts suggests, an isoform-specific regulation of IGF-I expression in the pathophysiology of urothelial carcinoma and supports the notion that the Ea, Eb, and Ec peptides may possess discrete biological roles, beyond IGF-I. Recently, the synthetic Ec peptide (24 aa) was documented to possess bioactivity which was exerted *via* an IGF-I receptor-

independent manner (20), while its over-expression in PC-3 cells resulted in Ec-PC-3 cells, which were found to possess increased oncogenic potential in SCID mice (26). Further studies shall investigate the mechanism(s) through which IGF-I transcripts may influence bladder cancer biology.

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