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ORIGINAL ARTICLE

Polymorphisms of survivin -31 G/C gene are associated with risk of urothelial carcinoma in Serbian population

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Summary

Purpose: *Survivin is thought to play an important role in carcinogenesis and is found to be associated with poor clinical outcome in various malignancies. Gene -31 G/C polymorphism has been identified as a risk factor for the development of several types of tumors. The purpose of this study was to investigate the association between survivin gene promoter -31C/G polymorphism and urothelial carcinoma (UC) risk in Serbian population and to compare the different expressions of survivin in UC of different disease stages, histological grades and tumor location in the upper or lower urinary tract.*

Methods: *DNA from 94 patients with primary UC and from 82 healthy subjects was subjected to PCR restriction fragment length polymorphism analysis (PCR-RFLP) to identify individual genotypes. UC samples were subjected to immunohistochemical analysis to assess survivin expression in these lesions.*

Results: *It was observed that the frequency of G/G genotype was greater in patients with UC (58.7%) than in controls (32%). Compared with study subjects carrying the C/G or C/C genotypes, significantly increased UC risk was found for individuals carrying the G/G genotype. Those carrying the G/G genotype had a significantly increased UC risk compared with those with C/G or C/C genotypes. Patients with UC carrying the G/G genotype had a greater prevalence of muscle-invading (stage T2-T4), high-grade (G2) tumor and immunohistochemicaly overexpressed survivin compared with those carrying the C/G or C/C genotypes.*

Conclusions: *G/G genotype of the -31C/G polymorphism might be a risk factor for UC development.*

Key words: *immunohistochemistry, PCR-RFLP, survivin, urothelial carcinoma*

Introduction

Defects in apoptosis, an important mechanism to control cell growth and division, are known to be involved in carcinogenesis through prolonging cell survival, promoting accumulation of transforming mutations, and enhancing resistance to therapy [1]. Survivin, as a member of the inhibitor of apoptosis protein family, is abundantly expressed in embryonic tissues as well as in various human malignancies. It is almost undetectable in normal tissue [2]. Survivin is thought to play an important role in carcinogenesis and is found to be associated with poor clinical outcome in various malignancies [3]. Mechanisms of survivin upregulation are still poorly understood, but a common functional polymorphism in the survivin gene promoter has been shown to affect

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its expression and consequently the risk for some types of cancer.

UC is the second most common cancer and the second leading cause of death among malignancies of the genitourinary tract [4]. UC usually arises from the urothelium with transitional cell differentiation, including renal pelvis, ureter, urinary bladder and very rarely urethra [5-7]. Increased survivin expression has been found in various malignancies, including bladder, colorectal, lung, and oral cancer [8-11]. One study reported that survivin was detected in the urine samples from 46 patients with new or recurrent bladder cancer but was not found in 16 healthy volunteers [12]. Another study also found that greater level of survivin in urine was associated with increased bladder cancer risk and higher tumor grade [13]. Survivin gene variants have been shown to modulate the risk of urothelial carcinoma [14], sporadic colorectal carcinoma [15], gastric cancer [16], lung cancer [17] etc.

The gene coding survivin is located on chromosome 17q25, and it is composed of 142 amino acids [18]. A feature of the human survivin gene promoter is the existence of a cell cycle dependent element and a cell cycle homology region [2]. Deletion of this promoter region results in lack of cell cycle-dependent expression in HeLa cells [19]. The -31G/C polymorphism located in the promoter region of survivin gene apparently may influence survivin expression. Recent studies [8-17] have shown that there exists an association between -31G/C polymorphism of the survivin gene promoter and cancer susceptibility.

The objectives of the present study were to examine the significance of immunohistochemical expression of survivin and to detrmine variations of its expression in different tumor stages, grades, and the intensity of survivin expression in single and multicentric tumors. Another objective was to find out possible differences of survivin expression in the upper and lower urinary tract. A third objective was to determine the frequency of genotypes C-31G polymorphism using PCR-RFLP in the survivin gene in a group of patients with urothelial carcinoma in comparison to healthy controls, and to determine whether there is a connection between the C-31G polymorphism of survivin and tumor stage, grade, appearance of single or multiple tumors, tumor localization in the upper or lower urinary tract, as well as to compare the results obtained by PCR-RFLP with the results obtained with immunohistochemistry.

Methods

The study was performed after approval of the Institutional Ethics Committee. Informed consent has been granted by all relevant parties at the Clinical Center "Dr Dragisa Misovic", Belgrade, as well as at the Medical School of Belgrade. All clinical parameters were analyzed from the hospital's documentation. We analyzed the following clinical data: gender, age, multiple tumor appearance, localization in the upper or lower segment of the urinary tract. Pathologic confirmation was performed by regular urologic practice, including endoscopic biopsy and surgical resection of urinary tract tumors. The tumor stage and grade were determined using the TNM classification and the 2004 World Health Organization (WHO) classification system. The pathologic stage was classified in 5 groups (Stage 0a, T1-T4). Tumor grade was divided into 3 groups (G1, G2, G3). The control group which consisted of 82 healthy individuals was age and sex matched with UC patients. The study included 94 patients with primary cancers of the urinary tract, 71 (75.5%) males and 23 (24.4%) females, who had been diagnosed between 2007 and 2011. The average age was 66.94±10.1 years (range 33-92). Samples were obtained from 60 patients from the urinary bladder, in 15 patients from the renal pelvis, in 9 cases from the ureter, and in 3 cases from the urethra. In 7 patients multicentric urothelial cancer was found, with 5 patients having carcinoma of the ureter and renal pelvis, and 2 with tumor located along the entire urinary tract (renal pelvis, ureter, bladder).

Immunohistochemistry

The bioptic material was stained by immunohistochemistry. To unmasking the antigens specimens were processed in a microwave oven in citrate buffer (pH 6.0) during three cycles of 5 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 min. In order to reduce nonspecific staining, we used the normal, nonimmune swine serum dilution 1:10 for 30 min. After that, primary survivin antibody (DAKO, Ghostrup, Denmark), dilution 1:100 for 60 min was applied. Staining was performed by streptavidin-biotin technique using DAKO LSAB + kit. As a chromogen 3.3 diaminobenzidine (DAB substrate) was used, and for contrast staining Mayer's hematoxylin was used. Survivin positivity was expressed as brown coloration of the nucleus. Survivin immunoreactivity was assessed in 10 consecutive fields, under optical microscope at 400 x magnification. The total number of epithelial cells was counted, and the percentage of positive cells was calculated. Survivin expression was classified as normal (less than 10% cells expressing survivin) or overexpressed (10% or more cells expressing survivin).

Survivin -31 C/G genotyping

DNA was extracted from paraffin blocks using QIAamp DNA Mini Kit (Qiagen, GmbH, Germany), as

Table 1. Distribution of genotypes in patients with urothelial cancer and in healthy controls

Patients and healthy controls	*Number of patients with C/C genotype (%)*	*Number of patients with C/G genotype (%)*	*Number of patients with G/G genotype (%)*	*Total*
Patients with UC	7 (7.6)	31 (33.7)	54 (58.7)	92 (100)
Healthy controls	11 (13)	45 (55)	26 (32)	82 (100)

UC: urothelial cancer

recommended by the manufacturer. Survivin promoter polymorphism was determined by PCR-RFLP. A 151 base pair (bp) fragment, surrounding the -31 position, was amplified using the following primers: 5´-AAGAGGGCGTGCGCTCCCGACA-3´ and 5¢- GAGATGCGGTGGTCCTTGAGAAA-3¢. The PCR was performed in a total volume of 20 µl containing 2 µl of 10X PCR buffer (MBI Fermentas, Lithuania), 1.5 µl of $MgCl_2$, 0.2 mM dNTPs, 0.375 µM of each primer, 200 ng of genomic DNA and 1 unit of Taq DNA polymerase (MBI Fermentas, Lithuania). The amplification conditions were as follows: initial denaturation at 95^{0}C for 5 min, followed by 35 cycles consisting of denaturation at 94^{0}C for 45 s, annealing at 60^{0}C for 45 s, elongation at 72^{0}C for 1 min, and a final elongation at 72^{0}C for 10 min. The amplified fragment was digested with 5 units of Msp I (MBI Fermentas, Lithuania), resulting in products of 151 bp for the GG genotype, two fragments of 90 and 61 bp for the CC genotype and three fragments of 151, 90 and 61 bp for the CG genotype. Genotypes were confirmed by randomly re-genotyping 10% of the samples. There were no discrepancies between genotypes determined in duplicate.

Statistics

Chi square test and Mann-Whitney U test were used to determine a significant difference between immunohistochemical expression of survivin and tumor stage, grade of UC, appearance of single or multiple tumors or tumor localization in the upper or lower urinary tract. x^2 test was used to test the Hardy-Weinberg equilibrium by comparing the observed genotype frequencies with the expected frequencies among the controls. The correlation between the -31C/G polymorphism in survivin gene promoter and the tumor stage, grade of UC, appearance of single or multiple tumors or tumor localization in the upper or lower urinary tract was also examined using the x^2 test. The differences between the compared groups were considered statistically significant if p values <0.05. Calculations were performed with the statistical package SPSS 17.0.

Results

Based on the analysis of PCR-RFLP digestion products for the genes in the PAA gel and upon determination of individual genotypes for the survivin gene -31 G/C polymorphism in patients with urothelial cancer, we obtained results on the distribution of genotypes (Table 1). In the group that contained the healthy population the most common genotype was C/G (55%), and in the group that contained UC patients the most common genotype was G/G with 58.7% (54 out of 92 cases; x^2=30.382, df =2, p<0.01).

Single urinary tract tumors were most frequent in patients with G/G genotypes (30 out of 31 cases; 96.8%). Single urinary tract tumors were also most frequent in patients with C/G genotype (51 out of 54 cases; 94.4%), and in patients with C/C genotype (6 out of 7 cases; 85.7%).

Analyzing the genotypes of tumors obtained from different locations (upper or lower urinary tract) no statistically significant differences were observed (x^2=5.216, df=2, p>0.05). However, when we compared the most common genotype groups (G/G and C/G) in patients with UC in the upper or lower urinary tract we documented a statistically significant difference between the compared groups (x^2=4.078, df=1, p<0.05). A much greater number of G/G genotype (25 out of 31 cases; 80.6%) and C/G genotype (32 out of 54 cases; 59.3%) were observed among the individuals in whom the tumor was localized in the lower urinary tract (Table 2).

Analysis of -31 G/C polymorphism of survivin in different tumor grades showed that the most common genotype was G/G (in G1 59.1% had G/G genotype, in G2 57.5%, and in G3 60.9%) without statistically significant difference (x^2=0.0372, df=2, p>0.05).

Analysis of C-31G polymorphism of survivin in different tumor stages revealed that the most

Table 2. Distribution of patients according to the most common genotype in relation to tumor localization

Survivin PCR-RFLP	*Tumor localization*		*Total*
	Upper urothel N (%)	*Lower urothel N (%)*	
C/G genotype	6 (19.4)	25 (80.6)	31 (100.0)
G/G genotype	22 (40.7)	32 (59.3)	54 (100.0)
Total	28 (32.9)	57 (67.1)	85 (100.0)

Table 3. Relationship between tumor grade and stage of urothelial cancer and survivin immunoexpression

	Survivin expression		*p value*
	Normal expression (N=41) N (%)	*Overexpression (N=51) N (%)*	
Grade			
1	16 (39.0)	6 (11.8)	0.000
2	23 (56.1)	24 (47.1)	
3	2 (4.9)	21 (41.2)	
Stage			
0a	19 (46.3)	6 (11.8)	0.000
T1	11 (26.8)	8 (15.7)	
T2	6 (14.6)	11 (21.6)	
T3	4 (9.8)	19 (37.3)	
T4	1 (2.4)	7 (13.7)	

common genotype was G/G (in 0a 56% had G/G genotype, in T1 57.9%, in T2 47.1%, in T3 56.5%, and in T4 100%) without statistically significant difference (x^2=1.258, df=2, p>0.05).

In normal urothelial tissue, survivin was not expressed (Figure 1A). However, UC showed elevated expression of survivin in the nucleus and negative cytoplasmic expression (Figure 1 B,C).

Of 92 patients, 51 (55.4%) had survivin overexpression, 38 of 51 (74.5%) were male, and 13 of 51 (25.5%) female. No statistically significant difference in survivin expression was noticed between male and female patients. Patients with multiple tumors along the entire urinary tract were excluded from analysis due to their extremely low numbers. Out of 92 cases, 41 didn´t show survivin expression. Thirty-nine of these 41 cases had solitary tumors (95.1%), and 2 cases had multicentric tumors. Out of 51 cases with survivin overexpression 48 had solitary tumors (94.1%), and 3 had multicentric tumors (5.9%). There was no statistically significant difference between the overexpression of survivin in solitary tumors and multicentric urothelial carcinoma (x^2=0.000, df=1, p>0.05).

Analyzing the groups with normal and overexpression of survivin and localization of the tumor, i.e. whether they occurred in the upper or lower urinary tract, we didn´t find statistically significant differences between the two groups (x^2=0.000, df=1, p >0.05). In 65.9% of the cases (62 of 94) the tumor was localized in the urinary bladder, in 23.4% (22 of 94 cases) in the renal pelvis, in 17.1% (16 of 94 cases) in the ureter, and very rarely in the urethra 3.2% (3 of 94 cases).

The relationship between pathologic stage and grade of UC and survivin immunoexpression is listed in Table 3. Increased expression of survivin was also more frequent in the higher stages of tumors, especially in cases of bladder cancer (z=- 4.646, p <0.05, Table 3).

No statistical difference was revealed when analyzing whether a certain genotype was predominant in patients showing overexpression of survivin obtained by immunohistochemistry. (x^2=1.776, df =2, p>0.05, Table 4).

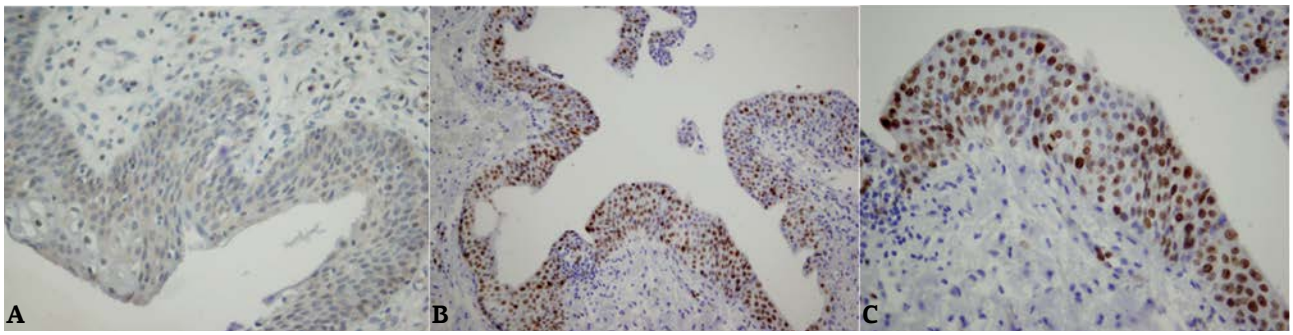

Figure 1. Immunoreactivity patterns of survivin expression in normal urothelial tissue and malignant tissue. Immunostaining using the swine polyclonal antibody against the survivin protein. **A**: In normal urothelial tissue surviving is not expressed (less than 10% cells expressing survivin, original magnification, x200). **B** and **C** show overexpression of survivin in malignant cells (10% or more cells expressing survivin. Original magnifications x200 **(B)** and x400 **(C)**.

Table 4. Statistical analysis of tumor stage

Test	*Tumor stage*			
	Renal pelvis	*Urinary bladder*	*Ureter*	*Urethra*
Mann-Whitney	33.000	134.000	18.500	0.000
p value	0.381	0.000	0.463	0.221

Table 5. Survivin expression in urothelial carcinoma samples according to -31C/G genotypes

Genotype	*Survivin expression*		
	Normal expression N (%)	*Overexpression N (%)*	*Total N (%)*
C/G	16 (51.61)	15 (48.39)	31 (100.00)
C/C	4 (57.14)	3 (42.86)	7 (100.00)
G/G	21 (38.89)	33 (61.11)	54 (100.00)

Discussion

CDE/CHR (Eng. cell-dependent Cell/Cell cycle - homology region) is part of the survivin gene promoter that is important for the regulation of its expression. Numerous literature data indicate that polymorphism in this region at position -31 significantly contributes to increased risk of getting different types of tumors, as it leads to increased expression of survivin [16,17,20,21]. This polymorphism is associated with increased expression of survivin mRNA and protein level, a consequence dependent on aberrant transcription of cell cycle. Mutation that leads to depression of transcription of cell cycle and overexpression of survivin mRNA and protein levels was described in their study by Xu et al. [21]. Expression of survivin is 10-fold higher in G2/M phase than in G2 or S phase [22]. Survivin expression is very high in different types of tumors, particularly colon, lung, breast, brain and melanoma [23]. Mechanisms of overexpression of survivin in cancer are only partially understood. Several mechanisms involved in the expression of survivin have been clarified. Demethylation has been demonstrated in ovarian cancer. Recent studies have investigated transcription factors, such as p53, which can regulate the expression of survivin in various cancers. So, in many types of cancer, such as gastric, pancreatic, prostatic, and lung cancer, correlation between the accumulation of p53 and survivin expression was demonstrated [2].

In several studies this polymorphism was identified as a risk factor for cancer development. Most of the clinical studies related the C/C genotype with increased risk of malignant disease. Cheng et al. [16] examined the association of survivin gene polymorphism and risk of gastric cancer. In their study they showed that the incidence of C/C was 39.6% and of C/G 39.6% in patients with gastric cancer, while their frequency in a healthy population was 11.9% and 41.8%. Borbely et al. [20] examined the association of this polymorphism with the occurrence of cervical cancer and showed that the most common genotypes that lead to its development are C/C and C/G. They reported that the frequency of C/C and C/G genotype in patients with cervical cancer was 8.0% and 36.0%, while in the group of healthy individuals was 14.0 and 39.0%. The main finding of the study conducted by Wang et al. [24] was a proven link between promoter polymorphism of survivin gene and the risk of UC. They pointed out that the incidence of C/C and C/G genotypes was significantly higher in affected individuals (34.7 and 47.9%) than in those from the control group (20.9 and 41.0%). On the other hand, several studies related the G/G genotype with increased risk of malignant disease. For instance, Jang et al. [17] found out that the frequency of C/C genotypes was 31.6% in their patients and C/G was 44.5% in patients with lung cancer. In the control group C/C genotype was found in 25.3% and C/G genotype in 50.3% of samples, thus showing that these polymorphisms are not related to the appearance of lung cancer. They also showed that patients with G allele had significantly reduced risk of developing lung cancer. In our study, G/G genotype was recorded in 58.7% of the cases, followed by C/G genotype in 33.7% and C/C genotype in 7.6% of patients with UC. In our control group, however, the most common and statistically significant genotype was C/G. Our research revealed that the G allele was associated with an increased risk of UC. Divergences in reported data may also be attributed to geographic or ethnic differences between the study populations. For instance, Srivastava et al. [25] reported that an increased risk of cancer caused by the presence of the -31C allele was significant only in an Asian population.

We found that 34.5% of our patients with solitary UC had C/G genotype, 58.6% had G/G genotype and 6.9% C/C had genotype. In our cases with multicentric UC 60% of the patients had G/G genotype, 20% had C/C genotype, and 20% had C/G genotype.

Our research showed that C/G and G/G genotypes are by far more common in patients with UC of the lower urinary tract. In the literature, unfortunately, there are no reports to confirm or

contradict these findings. We could conclude that these two genotypes are associated with the frequent occurrence of cancer in bladder and urethra, but due to the small sample size this data should be backed-up by studies with larger samples, especially of the urethra.

Wang et al. [24] showed that the polymorphism of promoter gene of survivin -31C/G was associated with tumor grade and clinical stage of UC. These authors suggest that the prevalence of invasive cancer was significantly higher in patients who have had C/C genotype compared to those with G/G genotype. Many studies showed that survivin expression was increased in patients with high grade carcinomas [26-29]. Such findings led these authors to conclude that polymorphisms of genes for survivin promoter may disrupt the regulation of apoptosis and lead to increased expression of survivin in tumor cells. Kawata et al. [30] found that single nucleotide polymorphism (SNP) located in the region C-31G promoter of survivin disrupts the mRNA level. They examined the relationship between the SNP of survivin and the risk of urinary bladder cancer progression. This study included a total of 346 patients and 235 healthy individuals and the authors reported that patients with the C/C genotype had a higher risk of urinary bladder cancer development than patients with G/G and C/G genotype. The results of our study do not coincide with these results. Our PCR-RFLP investigation showed that there was not a significant association between survivin gene polymorphism at position -31 and the appearance of a higher grade and stage of UC. In our research we found that the majority of patients with UC had G/G genotype. G/G genotype was associated with grade 2 in 50.0% of our patients, and grade 3 in 25.9% of the patients. C/G genotype was more frequently associated with grade 2 (in 51.6% of the patients), then with grade 3 (in 25.8% of the patients). Although statistical significance was not found, we believe that if we included more cases in our study we would have found statistical significance. Our research showed that the C/C genotype occurred in (according to frequency) T3 stage tumors (29.0%), non-invasive papillary carcinoma (29.0%), and in T1 stage (14.8%), while in T4 stage had no patients with this genotype. This finding agrees with findings from the literature, but unlike their results we found no statistically significant association between this genotype and the occurrence of higher stages of UC. In our study, C/C genotype was also commonly detected in T3 stage (24.1%), followed by noninvasive cancers (25.9%), and T2 and T4 stage tumors (14.8%).

Our immunohistochemical investigations showed a statistically significant association between overexpression of survivin and higher histological grade and stage in UC and the results are consistent with literature data. Numerous studies have found association between increased survivin expression and higher stage tumors [8,31]. Other authors [32,33] have also confirmed the overexpression of survivin in non-invasive UC. These authors [32,33] also found a statistically significant correlation between increased expression of survivin and higher stage in UC. They also compared the gender, age, number of tumors (solitary or multicentric), size and shape with overexpression of survivin and concluded that increased expression of survivin may be regarded as unfavorable prognostic factor in non-invasive bladder cancer. Karam et al. [8] and Swana et al. [31] investigated the association between excessive expression of survivin and higher tumor grade and showed that the intensity of surviving expression increased with increasing grade (65-90%). Jin et al. [33] confirmed these findings.

Our investigation showed that elevated expression of survivin does not affect the appearance of single or multicentric UC. Also, we found no statistically significant association between survivin expression and increased occurrence of tumors in the upper or lower urinary tract. Unfortunately, we were unable to find supporting reports in the literature. Nakanishi et al. [34] investigated the prognostic significance of survivin expression in UC of the upper urinary tract. Increased survivin expression was found in only 12.7% of the samples, which was not correlated with clinicopathological findings. They concluded that the expression of survivin does not anticipate the behavior of the upper urinary tract UC.

The immunohistochemical study showed that in our samples increased expression of survivin was recorded only in the nucleus of tumor cells, and was most pronounced in patients who had G/G genotype (61.1% of the patients), while patients with C/G genotype also had increased expression of survivin (48.4% of the patients), and so did the group with the C/C genotype (42.9% of the patients). No statistically significant correlation was found between the results obtained by PCR-RFLP method and immunohistochemistry. Kawata et al. [30] found a correlation between C/C genotype and the expression of survivin in the nuclei of tumor cells and they concluded that patients with C/C genotype and overexpression of survivin in tumors

cell have a higher risk of UC, especially bladder cancer, compared with patients who had a G/G and C/G genotype and increased expression of survivin.

Conclusions

Based on the presented results we can conclude that the increased expression of survivin is associated with higher grade and stage of UC. Survivin may be regarded as unfavorable prognostic factor for progression of transitional cell type of cancer. Promoter gene polymorphism at the position survivin -31C/G is associated with the risk of UC in Serbian population. G/G and C/G genotypes are associated with the appearance of cancer in the urinary bladder and urethra.

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Conflict of interests

The authors declare no confict of interests.

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