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Association between survivin gene promoter -31G/C and -644C/T polymorphisms and non-small cell lung cancer

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ABSTRACT. Lung cancer is the most common cancer worldwide. Survivin is one of the first reported inhibitors of apoptosis proteins, which is an important family of proteins that regulate apoptosis. The survivin gene is located on human chromosome 17q25, which is composed of 142 amino acids. A common polymorphism of the survivin gene promoter -31G/C has been shown to influence cancer risk. This genetic variant has been associated with overexpression of survivin at both protein and mRNA levels in cancer cells. We examined promoter (-31G/C) genotype frequency in a patient group (N = 146), 77.4% GG, 18.5% GC, 4.1% CC, and in a control group (N = 98), 57.1% GG, 34.7% GC, 8.2% CC. These distributions were significantly different. Promoter (-644C/T) genotype frequency in the patient group was 40.4% TT, 48.6% TC, 11% CC, and in

the control group it was 55.1% TT, 40.8% TC, 4.1% CC; these distributions were also significantly different. Individuals carrying the survivin 31 GC genotype and those carrying the survivin 644 CC genotype had a significantly decreased risk of having non-small cell lung cancer.

Key words: Non-small cell lung cancer; Survivin; Gene polymorphism; Genetics; Biomarker

INTRODUCTION

Lung cancer is the second most common cancer, and it causes the highest number of cancer-related deaths among all cancers worldwide (Park et al., 2010). Survivin, a member of the inhibitor of apoptosis (IAP) protein family, is involved in both regulation of cell division and inhibition of apoptosis. Survivin was one of the first reported IAPs (Deveraux et al., 1997; Marusawa et al., 2003). The human survivin gene maps to chromosome 17q25 and encodes a protein made up 142 amino acids (Chiou et al., 2003). A survivin gene polymorphism may affect survivin production and activity, thus resulting in susceptibility to the development of lung cancer. A common polymorphism on the survivin gene promoter, -31G/C, has been shown to influence survivin expression and the risk of cancer development. It has been associated with the overexpression of survivin at both the protein and the mRNA levels in cancer cells.

The overexpression of survivin has been associated with disease development, recurrence, and prognosis in various malignancies, including cancers (Gazouli et al., 2009). Loss of survivin function results in disorganized mitosis and embryonal death (Altieri, 2003). The presence of the polymorphism G/C at position -31 seems to be correlated with increased expression of survivin (Xu et al., 2004). Previous research has shown that promoter elements such as cell-cycle-dependent elements and cell cycle homology regions regulate the cell-cycle-dependent expression of survivin during G2/M phase (Li and Altieri, 1999). The -31G/C polymorphism is located in the cell-cycle-dependent element/cell cycle homology region repressor-binding site and may change the cell-cycle-dependent transcription of survivin through functional disruption of this motif (Xu et al., 2004). The aim of this study was to demonstrate the prognosis-related associations between the -644C/T and -31G/C gene polymorphisms in the survivin gene promoter region and non-small cell lung cancer (NSCLC) in a Turkish population.

MATERIAL AND METHODS

Study population and collection of specimens

Samples were collected from the Istanbul Yedikule Chest Diseases and Thoracic Surgery Training Hospital and the Istanbul University Cerrahpaşa Medicine Faculty Department of Thoracic Surgery Clinic. The study examined 146 diagnosed NSCLC patients and 98 healthy controls. The mean ages and percentage of the patients and controls are shown in Table 1. Ninety-eight healthy subjects without any malignancy were selected for the control group, which comprised only individuals with a negative family history of cancer. The patient and control groups were matched for age. NSCLC patients were all newly diagnosed with histopathologically confirmed

primary lung cancer and had been treated surgically but had not yet received radiotherapy and chemotherapy. All participants signed an informed consent form before enrollment, and institutional Ethics Committee approval was obtained for the study. The study protocol was approved by both the Ethics Committee of the Istanbul Faculty of Medicine (November 10, 2010; No. 849) and the Scientific Research Projects Coordination Unit of Istanbul University (Project No. 11276).

Table 1. Characteristics and laboratory parameters of non-small cell lung cancer (NSCLC) patients and controls.

Characteristic	NSCLC patients (N = 146)	Controls (N = 98)	P*
Male [N (%)]	132 (90.4)	56 (57.1)	
Female [N (%)]	14 (9.6)	42 (42.9)	<0.001
Age (years, means ± SD)	58.74 ± 8.24	55.39 ± 7.92	0.760
Smoking history (pack/years)	47.56 ± 30.18	28.88 ± 20.89	0.094
FEV1	65.85 ± 17.53	97.16 ± 10.24	<0.001
FVC	68.77 ± 17.61	95.48 ± 9.47	<0.001
FEV1/FVC	78.95 ± 15.85	93.75 ± 16.05	0.203
DLCO	77.63 ± 34.33	90.62 ± 19.52	0.280

*P obtained by the Student *t*-test. Data are reported as number (percentage in parentheses) or as means ± standard deviation. FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity; DLCO = diffusion lung capacity for carbon monoxide.

DNA extraction

DNA was isolated from blood leukocytes in 10 mL ethylenediaminetetraacetic acid using the method reported by Miller et al. (1988) based on sodium dodecyl sulfate lysis, ammonium acetate extraction, and ethanol precipitation (Drábek and Petrek, 2002). The concentration and purity of DNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) at A_{260} and A_{280}. Template DNA (0.5-1.0 µg) was used in a polymerase chain reaction (PCR) under sterile conditions.

Genotyping

A PCR-restriction fragment length polymorphism method was used for genotyping studies. We investigated the -31G/C and -644C/T polymorphisms in the promoter region of the survivin gene using 0.25 µM of each primer shown in Table 2 for the reaction in a volume of 25 µL containing (MBI Fermentas) 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 0.16 mM of each deoxyribonucleotide triphosphate, and 1 U Taq polymerase. Amplification was performed using the following protocol: initial denaturation at 94°C for 5 min; followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s; and a final extension cycle at 72°C for 10 min (Miller et al., 1988). The appropriate primers (see Table 2) were used to amplify the corresponding gene of the subjects via PCR, and the reaction products were digested using the appropriate enzyme at 37°C. The PCR product exhibited a 329-bp fragment for the -31G/C region polymorphism. PCR product (10 µL) was digested with *Bcn*I (MBI Fermentas). The resulting PCR product exhibited a 300-bp fragment for the -644C/T region polymorphism. The PCR product (10 µL) was then digested with *Sty*I (MBI Fermentas), subjected to electrophoresis on 3% agarose containing 0.5 mg/mL ethidium bromide, and examined under transillumination. Samples were repeated if conflict occurred. The expected results after restriction for each gene are given in Table 2.

Table 2. PCR-RFLP procedures and products of survivin -31G/C and -644 C/T.

	Primers	PCR product	Restriction enzyme	Restriction products
-31G/C (rs9904341)	F: 5'-CGTTCTTTGAAAGCAGTCGAG-3' R: 5'-TGTAGAGATGCGGTGGTCCT-3'	329 bp	*Bcn*I	CC: 329 bp CG: 329/234/92 bp GG: 234/92 bp
-644C/T (rs8073903)	F: 5'-AGGTCGTGCAGTCAACGATGT-3' R: 5'-CAGACGGGCATGAAGGACCCATG-3'	89 bp	*Sty*I	CC: 89 bp CT: 89/66/23 bp TT: 66/23 bp

F = forward; R = reverse.

Statistical analysis

All statistical analyses were carried out using the SPSS version 17.0 statistical package for Windows. Numeric values were analyzed with the Student *t*-test. The chi-square test was used to assess both the prevalence of the genotype of the survivin promoter region and allele differences between groups. The relative associations between NSCLC patients and controls were assessed by calculating crude Gart's odds ratios (ORs) and 95% confidence intervals (95%CIs). The threshold for significance was $P < 0.05$. Linkage disequilibrium between the survivin -31G/C and -644C/T polymorphisms was assessed using D^0 and r^2 values obtained with the Haploview program (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). A multivariate logistic regression model was used to investigate the effects of genotypes and alleles after adjustment for age. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Genotype and allele frequencies for survivin -31G/C and -644C/T in NSCLC patients and controls are listed in Table 3. The distribution of the survivin -31G/C genotypes in these groups was significantly different (P = 0.004). The prevalence of -31G/C heterozygosity was 18.5% (27/146) in patients and 34.7% (34/98) in the control group. Individuals with the -31G/C genotype had a 1.24-fold decreased risk for NSCLC (P = 0.004; OR = 0.801; 95%CI = 0.680-0.944). The distribution of the survivin -644C/T genotypes in controls and NSCLC patients was significantly different (P = 0.032). Although the frequencies of the variant -644T/T genotype in patients were higher than those of the controls, the difference did not reach statistical significance (P = 0.055; OR = 1.077; 95%CI = 1.004-1.155). However, homozygous carriers of the -644C/C genotype had a 1.32-fold decreased risk for NSCLC (P = 0.024; OR = 0.753; 95%CI = 0.583-0.974). Distributions of the survivin -31G/C and -644C/T genotypes according to characteristic features in NSCLC patients are summarized in Table 4. No association was found between the -31G/C and -644C/T frequencies and tumor stage, lymph node, or metastasis status in NSCLC patients. Haplotypes were evaluated for association with NSCLC (Table 5). No linkage disequilibrium was found between survivin -31G/C and -644C/T polymorphisms (D': 0.11; r^2: 0.001) (Figures 1 and 2).

The results of the logistic regression are presented in Table 6. A multivariate logistic regression model was performed to investigate possible independent effects of smoking (pack/year), gender, and survivin -31G/C and -644C/C genotypes on risk of NSCLC. Logistic

regression analysis revealed that the survivin -644C/C genotype is associated with decreased risk, but gender is a risk factor for NSCLC. The relationship between the survivin -31G/C genotype and smoking (pack/years) did not reach significance.

Table 3. Risk of non-small cell lung cancer (NSCLC) associated with survivin -31G/C and -644 C/T genotypes.

Genotypes/alleles	Patients		Controls		OR (95%CI)	P
	N	%	N	%		
Survivin -31G/C						
GG	113	77.40	56	57.10	1.896 (1.300-2.765)	0.001
GC	27	18.50	34	34.70	0.801 (0.680-0.944)	0.004
CC	6	4.10	8	8.20	0.958 (0.895-1.025)	0.182
G allele	253	86.64	146	74.48		0.0006
C allele	39	13.35	50	25.51		
Survivin -644C/T						
CC	59	40.40	54	55.10	0.753 (0.583-0.974)	0.024
CT	71	48.60	40	40.80	1.152 (0.917-1.447)	0.230
TT	16	11.00	4	4.10	1.077 (1.004-1.155)	0.055
C allele	189	64.72	148	75.51		0.011
T allele	103	35.27	48	24.48		

OR = odds ratio; 95%CI = 95% confidence interval.

Table 4. Distribution of survivin -31G/C and -644C/T genotypes with characteristic features of non-small cell lung cancer patients.

	Survivin -31G/C genotypes [N (%)]			P	Survivin -644C/T genotypes [N (%)]			P
	GG	GC	CC		CC	CT	TT	
T stage								
T1	8 (80)	2 (20)	0 (0)		3 (30)	5 (50)	2 (20)	
T2	50 (82)	8 (13.1)	3 (4.9)		20 (32.8)	35 (57.4)	6 (9.8)	
T3	16 (76.2)	4 (19)	1 (4.8)		8 (38.1)	11 (52.4)	2 (9.5)	
T4	9 (52.9)	6 (35.3)	2 (11.8)		8 (47.1)	9 (52.4)	0 (0)	
				0.330				0.690
Lymph node status								
N0	42 (79.2)	10 (18.9)	1 (1.9)		18 (34)	31 (58.5)	4 (7.5)	
N1	2 (100)	0 (0)	0 (0)		0 (0)	2 (100)	0 (0)	
N2	28 (70)	7 (17.5)	5 (12.5)		15 (37.5)	19 (47.5)	6 (15)	
N3	11 (78.6)	3 (21.4)	0 (0)		6 (42.9)	8 (57.1)	0 (0)	
				0.361				0.498
Metastasis								
M0	54 (73)	15 (20.3)	5 (6.8)		29 (39.2)	38 (51.4)	7 (9.5)	
M1A	12 (100)	0 (0)	0 (0)		6 (50)	6 (50)	0 (0)	
M1B	18 (72)	6 (24)	1 (4)		5 (20)	17 (68)	3 (12)	
				0.316				0.289

Table 5. Haplotype frequencies of polymorphic variants of the survivin gene in patients with non-small cell lung cancer and healthy controls.

No. of haplotype	Haplotype associations	Haplotype frequency		Chi-square	P
		Patient	Control		
1	644C:31G	0.556	0.562	0.015	0.903
2	644T:31G	0.310	0.183	9.868	0.001
3	644C:31C	0.091	0.193	10.694	0.001
4	644T:31C	0.042	0.062	0.913	0.339

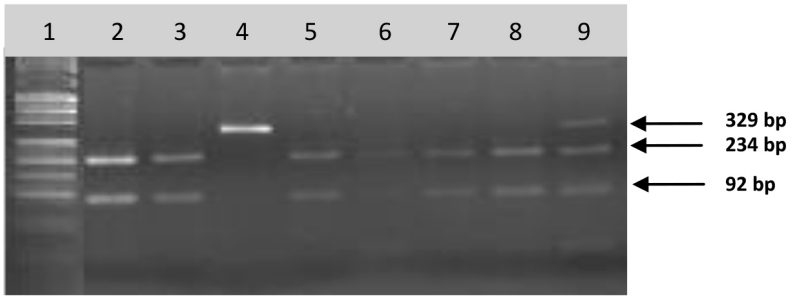

Figure 1. PCR-RFLP to detect -31G/C polymorphism of survivin promoter. Polymerase chain reaction products (329 bp) digested with restriction enzyme *Bcn*I and analyzed by 3% agarose gel. *Lane 1* = Puc8X DNA ladder (MBI Fermentas); *lanes 2*, *3*, *5*, *6*, *7*, and *8* = homozygotic for G allele; *lane 4* = homozygotic for C allele; *lane 9* = G/C heterozygotic.

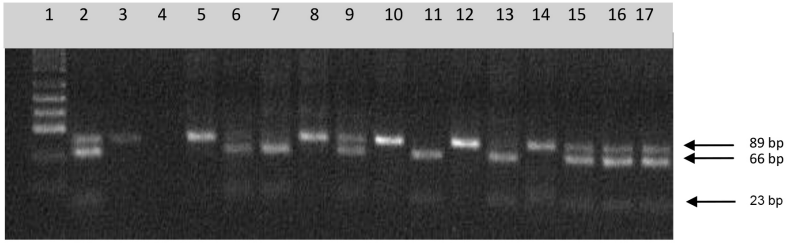

Figure 2. PCR-RFLP to detect -644C/T polymorphism of survivin promoter. Polymerase chain reaction products (89 bp) digested with restriction enzyme *Sty*I (fast digest) and analyzed by 3% agarose gel. *Lane 1* = Puc8X DNA ladder (MBI Fermentas); *lane 4* = left blank; *lanes 2*, *6*, *9*, *15*, *16*, and *17* = C/T heterozygotic; *lanes 7*, *11*, and *13* = homozygotic for C allele; *lanes 3*, *5*, *8*, *10*, *12*, and *14* = homozygotic for T allele.

Table 6. Results of multivariate logistic regression analysis.

Covariates	P	Odds ratio	95%CI
Smoking (pack/years)	0.051	0.525	0.275-1.002
Survivin -31G/C genotype	0.130	0.598	0.307-1.163
Survivin -644C/C genotype	0.005	0.437	0.244-0.783
Gender	0.000	0.150	0.073-0.310

Variable(s) entered on step 1: smoking (pack/years), survivin -31G/C, survivin -644C/C, and gender. 95%CI = 95% confidence interval.

DISCUSSION

Survivin was one of the first reported IAPs, which is an important family of proteins that regulate apoptosis (Deveraux et al., 1997; Marusawa et al., 2003). A common polymorphism at the survivin gene promoter -31G/C has been shown to influence survivin expression and the risk for cancer. Studies carried out in tissue have suggested that survivin may have a critical role in the diagnosis, prognosis, and prediction of response to therapy (Altieri et al., 2003; Nachmias et al., 2004; Shinohara et al., 2005). The genetic variant -31G/C in the survivin promoter region has been associated with the overexpression of survivin at both the protein and the messenger RNA levels in cancer cells. The overexpression of survivin is associated with disease development,

recurrence, and prognosis in various malignancies, including cancers (Gazouli et al., 2009). A recent meta-analysis has suggested that the survivin -31G/C promoter polymorphism might be associated with an increased risk of cancer, especially in Asian populations (Wang et al., 2012). Single nucleotide polymorphisms in the gene promoter region may affect survivin production and activity, thus providing a sensitivity for the development of lung cancer. To date, several polymorphisms in the promoter region of the survivin gene have been identified.

Defects in the apoptosis mechanism have an important role in cancer development. Apoptosis is an important process controlled by various agents that inhibit or activate it. Apoptosis is inhibited by anti-apoptotic proteins in cancer. Survivin, an anti-apoptotic factor, has a main role in cell cycle regulation. Unlike Bcl-2 and other IAPs, survivin is not expressed in normal tissues with incomplete differentiation but is expressed in various cancers. Clearly, the latter display abnormal survivin gene expression, which causes transcriptional deregulation. Survivin exerts its effect by attaching to caspases and other apoptosis proteins that contain baculoviral IAP repeat BIR regions. Jang et al. (2008) have shown that the -31G allele has significantly decreased transcriptional activity compared to that of the -31C allele, which affects the -31G/C polymorphism, has an important role in the predisposition to lung cancer. Xu et al. (2004) have analyzed cancer cell lines and reported that increased survivin gene expression is related to -31G/C polymorphism. Increased expression occurs at both the protein and the messenger RNA levels. Dai et al. (2010) have reported that polymorphisms at the survivin gene promoter region can cause gene modification in NSCLC.

From a clinical perspective, for predicting disease features with lifestyle habits, survivin gene expression levels in the genetic fingerprint of patients and genetic variants can be early indicators of outcome. In fact, Lan et al. (2010) have shown that high survivin level in pleural effusion is associated with poor prognosis. According to Yang et al. (2009) no relationship exists between susceptibility to esophageal squamous cell carcinoma and polymorphism of both the -644T/C and the -31G/C regions. Interestingly, esophageal squamous cell carcinoma tumors have embryonic origins along with some differences among cancers. The survivin gene has important roles in the control of mitosis, cytokinesis, and apoptosis during the embryonic development of cells (Adida et al., 1998; Murphy et al., 2002; Kawamura et al., 2003; Jiang et al., 2005; Li and Brattain, 2006).

According to previous data, the -31G/C polymorphism might have a role in the predisposition to lung cancer (Jang et al., 2008) and other malignancies. However, whether polymorphism in the -644C/T region causes susceptibility to NSCLC in particular is unknown because studies of this relationship remain scarce. Our data showed that compared to controls, as a result of present study, individuals carrying the survivin -31G/C genotype ($P = 0.004$) and survivin -644C/C genotype ($P = 0.024$) have a decreased risk of NSCLC.

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