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Long Stress-induced Non-coding Transcript 5 rs2935641 Polymorphism and the risk of Bladder Cancer in Patients from Fars Province

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ABSTRACT

Background: Bladder cancer is one of the most common cancers in the world with a considerably higher frequency among men than women. In case of early diagnosis, it is possible to treat the disease; therefore, it is essential to identify useful markers. LSINCT5 (long stress-induced non-coding transcript 5) is a stress-regulated lncRNA involved in response to oxidative stress, also in cell proliferation and cancer development, especially in the presence of tobacco carcinogens. In this study, we aimed to evaluate the influence of LSINCT5 gene polymorphism with the susceptibility of bladder cancer among a group of patients from the South of Iran.

Methods: One hundred patients with bladder cancer and 100 healthy participants as the control group were investigated in this case-control study. Genotyping of the rs2935641 polymorphism was carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach.

Results: It was found that the single base substitution C to T at the coding region of the LSINCT5 was not significantly associated with bladder cancer. Genotype and allele frequencies were distributed evenly among cases and controls, but no TT genotype was observed. The history of smoking was significantly higher in bladder cancer patients compared to controls ($p<0.001$).

Conclusion: Results showed that the LSINCT5 polymorphism is not a key factor in the pathogenesis of bladder cancer. However, tobacco use is one of the strongest risk factors for bladder cancer.

Keywords: Bladder cancer, LSINCT5, LncRNA, Polymorphism

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Introduction

Bladder cancer (BC) is one of the most prevalent genitourinary carcinomas with a worldwide distribution[1]. Based on the epidemiological studies in Iran, BC is the most common cancer of the genitourinary system and the third most common cancer among men[2]. Painless hematuria, the most prevalent symptom, is found in only 4-10% of the diagnosed BC cases[3]. From the histological point of view, bladder cancer is divided into several subtypes: transitional cell carcinoma (TCC), the most common types of BC, is originated from the urothelial cells lining the inside of the bladder; rare subtypes such as adenocarcinomas which form specialized structures in glands that produce and release fluids such as mucus; and squamous cell carcinomas that originate from the thin and flat cells and result in inflammation or irritation lasting for many months or years[3]. The etiology of BC is still incompletely characterized, although recent studies have suggested that it is the result of multiple environmental, ethnic, dietary, and genetic factors[4].Tobacco use and being male are the two well-established risk factors for BC[5,6].

One of the great surprises of modern biology was the discovery that the human genome encodes only ~20,000 protein-coding genes, representing <2% of the total genome sequence. However, the high-throughput RNA sequencing technologies revealed that more than 90% of the genome is actively transcribed[7,8]. The RNA pool of the human cells is more complex than a collection of protein-coding transcripts and their splice variants, but is composed of a large number of non-coding RNAs (ncRNAs), including short (less than 200 nucleotides (nt) long) and long (200 nt up to 100 kb long) non-coding RNAs. Long non-coding RNAs (lncRNAs) share many features of the mRNAs and are mainly transcribed by RNA polymerase II, polyadenylated, spliced and mostly localized in the nucleus[9,10]. The function of the lncRNAs is not completely clear, but growing evidence provided by recent investigations points to the involvement of the aberrant lncRNA expression in human cancers, their efficient role as a biomarker for the diagnosis and prognosis of malignancies, and their oncogenic and tumor suppressive function in cancer pathobiology[11,12]. LSINCT5 (long stress-induced non-coding transcripts 5) is an intergenic 2.6 kb LncRNA located in Chromosome 5p, somewhere between IRX2 and IRX4 genes. LSINCT5 is potentially transcribed by RNA polymerase III and polyadenylated from the negative strand[13]. LSINCT5 with proto-oncogenic function is a stress-regulated lncRNA involved in response to oxidative stress, and also in cell proliferation and cancer development[14,15].

The aim of the present study is to investigate the association between the LSINCT5 rs2935641 C/T polymorphism and the risk of BC in patients from Fars province.

Methods

Study participants

The present study consisted of 200 individuals, divided into the two patient and control groups. The control group included 100 healthy individuals without a history of any type of cancer (aged 30-81 years). The age- and gender-matched patient group was composed of 100 cases with BC (aged 26-89 years), diagnosed by a urologist, whose samples were kept in Shiraz Institute for Cancer Research (ICR) in the Shiraz University of Medical Sciences, School of Medicine. Patients were diagnosed based on symptoms such as gross hematuria. Each case underwent careful physical examination, complete urine analysis, and culture and urine cytology, which were followed by ultrasonography and a CT scan of the urinary system. Their diagnosis of BC

was later confirmed by cystoscopy.

The research protocol was approved by the Ethics Committee of the Islamic Azad University, Arsanjan Branch, and written informed consent forms were obtained from controls and BC patients.

Genotyping

The extracted DNA was obtained from the DNA bank of Shiraz Institute for Cancer Research. LSINCT5 rs2935641 C/T genotyping was done based on restriction fragment length polymorphism (RFLP) analysis after PCR amplification. Details including primer sequences, Tm of the reaction, and the restriction site for NsiI enzyme are presented in **Table 1**. A single C to T nucleotide substitution in the exon of LSINCT5 creates a recognition site for the NsiI restriction enzyme. PCR analysis of this SNP was carried out in a total volume of 15 µL, containing 1 µL of genomic DNA, 7.5 µL of PCR master mix, and 0.7 µL of each primer. The thermocycling procedure consists of initial denaturation at 95 ºC for 6 min, followed by 35 cycles of denaturation (at 94 ºC for 30 sec), annealing (at 60 ºC for 30 sec) and extension (at 72 ºC for 30 sec), and a final extension (at 72 ºC for 7 min) resulting in a product of 602 bp. Amplification was achieved with a gradient BioRad t100 thermocycler (USA). Amplicons were resolved as a single band by 1.5% agarose gel electrophoresis prior to RFLP analysis to ensure that a specific single product was amplified. PCR products were therefore digested for 15 min at 37 °C using NsiI and separated by electrophoresis in 1.5% agarose gel electrophoresis. Digestion of the amplified 602-bp product with this enzyme resulted in 235-bp and 367-bp fragments. The schematic representation of PCR-RFLP and the electrophoresis resolution are shown in **Figure 1**.

Statistical analyses

All statistical analyses were performed in SPSS 16. Chi-squared test was applied to determine whether or not the control sample demonstrated Hardy–Weinberg equilibrium. The association between genotypes and BC was assessed by computing the odds ratio (OR) and 95% confidence intervals (CI) from logistic regression analyses. A p-value <0.05 was considered statistically significant.

Results

The main characteristics of the study participants (cases, n=100; controls, n=100) are demonstrated in Table 2. The sex ratio was calculated to be more than 7-fold higher in men than women. About half of the participants in the studied population were beyond the 7th decade of their life. As the data in **Table 2** shows, more than 90% of the patients were diagnosed with transitional cell carcinoma (TCC). About 89% of the patients with BC had a history of cigarette smoking, and the difference in this variable between BC cases and controls was statistically significant (OR: 7.5, 95% CI: 4.3-13.3, p<0.001).

LSINCT5 rs2935641 T/C polymorphism and BC risk

The distribution of LSINCT5 rs2935641 genotypes and the ORs associated with BC are presented in **Table 3.** Neither the wild-type allele C (0.88 vs.

Table 1: The sequence and other characteristics of the primer pairs used in the amplification process

Gene	Polymorphism	Sequence 5' to 3'	Tm	Restriction enzyme
LSINCT5	rs2935641 C/T	F: 5'- GTGTCTGGTCTTGAGGCTCTTG3' R: 5'-GTGGAGAAGGAGGCTCTGGAA3'	60°C	NsiI 5'… ATGCA↓T…3' 3'… T↑ACGTA…5'

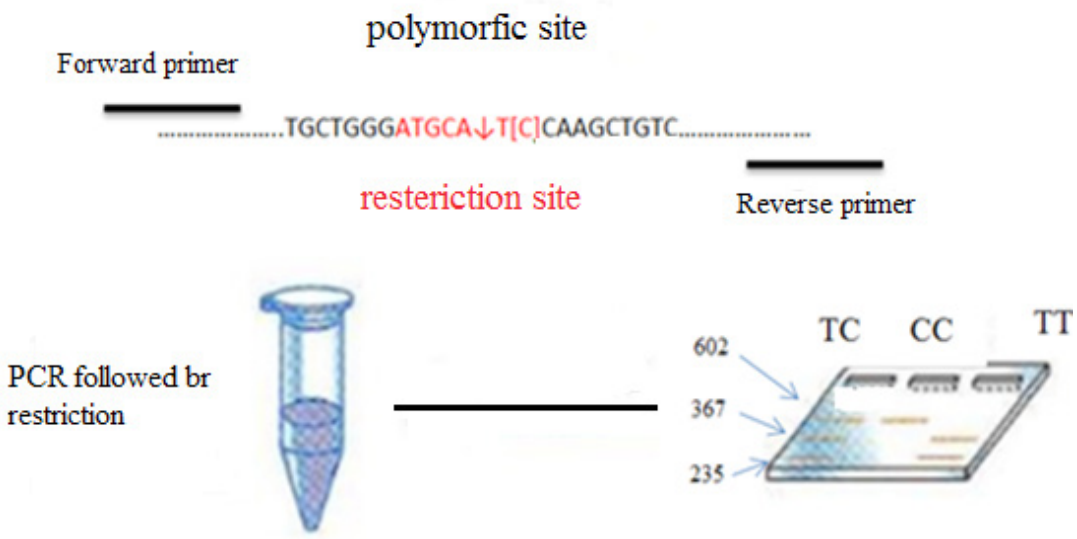

Figure 1: Schematic representation of the PCR-RFLP for LSINCT5 rs2935641 polymorphism. The polymorphic region was amplified by a pair of F and R primers resulting in a 602bp fragment (CC wild type homozygote). Digestible fragments 367 & 235 bp with 602bp band represents the TC heterozygote. The presence of 367bp and 235bp fragments shows the TT genotype.

Table 2: Demographic characteristics of bladder cancer

Variables	Cases n= 100	Controls n= 100	P* value
Age (years)			
Mean (±SD)	64.3±12.5	63.5±11.4	
Median Range	26-89	30-81	
Classified			
<50	14	10	
50-59	25	23	
60- 69	20	17	
>70	41	50	
Sex			
Male	88	88	
Female	12	12	
History of smoking	89	17	< 0.001
Tumor type			
TCC	92	-	
Squamous	6	-	
Adenocarcinoma	1	-	
Unknown	1	-	

*Logistic regression

Table 3: Distribution of LSINCT5 rs2935641 genotypes and allelic frequencies among cases and controls

rs2935641 Polymorphism	Controls (100) n, %	BC (100) in, %	P value	*OR	95% CI
Genotypes					
CC	74	75	-	1	Reference
CT	26	25	0.87	0.95	0.5- 1.8
Alleles					
C	174	175	-	1	Reference
T	26	25	0.88	0.96	0.5- 1.7

OR: Odds ratio; CI: Confidence interval; *Logistic regression; BC: Bladder cancer

0.87; case: control) nor polymorphic allele T (0.12 vs. 0.13) significantly affected BC risk in the studied population.

No significant association was found between the genotype frequencies, 75% (CC) and 25% (CT) of the cases, compared with their respective frequencies (74% and 26%, respectively) in the control group. No one was found to have the homozygote TT genotype.

Discussion

Bladder cancer is the fourth most common cancer and the eighth most common cause of cancer death[3]. Gender influences the severity and incidence of BC, which may be due to differences in carcinogenic exposure. Men are at a higher risk than women for developing BC[16]. This is confirmed by the result of the present study which shows a 7-time higher frequency of the disease among men than women. Age is the clearest single risk factor for developing BC. BC can occur at any age, but it is more frequent in middle-aged and elderly people[17]. About half of the BC patients enrolled and evaluated in this study aged more than 70 years. In the reports by Salehi et al.[18], Farahmand et al.[19], Ahmadi et al.[2] and Aminsharifi et al.[20] the mean age of developing BC in Iran is recorded to be 64-68 years old. Paz-y-Mino et al.[21] had also reported the mean age of 64.5±1.2 for BC patients in Ecuador.

It seems the distribution of the different types of the disease does not vary among different ethnic backgrounds; in the United States, more than 90% of the patients are diagnosed with TCC, and the remaining 10% are squamous cell carcinomas and adenocarcinomas[22]. The histological classifications of our studied patients were also compatible with those of the US.

The etiologic factors implicated in bladder carcinogenesis are unclear, but the roles of tobacco use and oxidative stress[23,24] in BC have been established. Cigarette smoke contains a number of carcinogens including acetaldehyde, acrylonitrile, O-anisidine hydrochloride, 4-aminobiphenyl, arsenic, benzene, and 4-(N-nitroso methylamino)-1-(3-pyridyl)-1-butanone (NNK)[25]. Tobacco smoke is also a rich source of reactive oxygen species (ROS) which can induce damage to DNA[26]. Because oxidative damage to DNA can cause mutations, and mutations are known to cause cancer, much effort has been devoted to the study of its role in the carcinogenesis of oxidative DNA damage[27]. Ploeg et al.[28] report the annual frequency of BC new cases worldwide to be

more than 12 million, with 5.4 million occurring in developed countries and 6.7 million in developing countries. The higher frequency of BC among developing countries may be explained by the high prevalence of smoking. A considerable number of patients in our study were cigarette smokers (89%). Aminsharifi et al.[20] also analyzed a group of BC patients in 2010 on samples from Fars province and reported a history of smoking among 69% of them. In an effort to increase our understanding of the genetic factors contributed in the pathogenesis of and predisposition to BC risk, the frequencies and the relative risk (ORs) of LSINCT5 gene polymorphism, as a new player in cancer, were determined for the first time among patients and healthy controls. Our results did not reveal any significant association between the LSINCT5 rs2935641 polymorphism with the minor allele frequency (MAF) of 0.26 and the risk of BC at the genotypic and allelic level. In a comprehensive comparison between the transcriptome of the normal human bronchial epithelial cells (NHBE) and NHBE cells exposed to the tobacco carcinogen NNK, a new group of long noncoding RNAs, named LSINCTs, was characterized[15]. This family of long noncoding transcripts with nearly 12 members showed a highly abundant expression in response to DNA damage induced by NNK[15]. On the other hand, the induction of breast carcinogenesis in the presence of NNK has already been shown[29]. Silva et al.[14,15] showed the overexpression of the LSINCTs in several breast and ovarian cancer cell line panels derived from both primary and metastatic tumors. The greatest change in the expression pattern was recorded for LSINCT5, the overexpression of which was seen in almost all breast cancer cell lines. In the same study, the expression level of the LSINCTs was measured in lung cancer cell lines. Almost all members of this noncoding RNA superfamily were overexpressed[30]. Upregulation of LSINCT5 was also confirmed in gastrointestinal cancer tissues in comparison with normal adjacent tissues, and its role in the promotion of gastric cancer cell growth was determined using loss-of-function and gain-of-function approaches in vitro[31].

The literature did not provide any information on the association between the polymorphism of LSINCT5 with any kind of cancer for a comparison of results.

In conclusion, the results of this study did not show any association between the polymorphism of LSINCT5 and the risk of BC among patients from Fars province, but we cannot ignore the role of this transcript on the pathogenesis of BC. Further investigation in the RNA level is proposed to better understand if the alteration in the expression of LSINCT5 would be involved in the pathogenesis of BC.

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