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ORIGINAL ARTICLE

# A Single Nucleotide Polymorphism in GAS5 lncRNA is Associated with Risk of Bladder Cancer in Iranian Population

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## Abstract

Down-regulation of the long non-coding RNA (lncRNA) *growth arrest-specific 5* (*GAS5*) has a pathogenic role in bladder cancer. Moreover, genomic variants of this lncRNA have been associated with risk of diverse cancers. In the present project, we genotyped two putative functional SNPs (rs2067079 and rs6790) in 122 bladder cancer patients and 150 age- and sex-matched healthy subjects. The rs2067079 was associated risk of bladder cancer in recessive inheritance model (TT vs.CC + CT: OR (95% Confidence interval (CI)) = 2.67 (1.27–5.62), adjusted *P* value = 0.02). The T G haplotype (rs2067079 and rs6790) increased the risk of bladder cancer in the assessed population (OR (95% CI) = 1.73 (1.18–2.56), adjusted P value = 0.02). Consequently, in the current project we introduced a novel risk locus for bladder cancer in Iranian population.

**Keywords** Bladder cancer · *GAS5* · lncRNA

## Introduction

Bladder cancer as one of the top ten worldwide frequent malignancies has an increasing trend in some regions of the world including Iran [1]. The high rate of morbidity and mortality of this cancer and lack of sensitive and specific biomarkers for early detection of this malignancy have encouraged researchers to identify genetic risk loci for it. Association studies including both candidate gene approaches and genome-wide studies have revealed several loci associated with bladder cancer [2]. Among putative risk loci for bladder cancer are variants located in long non-coding RNAs (lncRNAs). Based on the results of recent studies, several lncRNAs have been dysregulated in bladder cancer tissues [3]. As functional single nucleotide polymorphisms (SNPs) in lncRNA coding genes might alter expression of these transcripts or modulate their function especially their interactions with target genes, these variants are putative targets of association studies [4]. The *growth arrest-specific 5* (*GAS5*) lncRNA is a putative tumor suppressor lncRNA which induces apoptosis and cell cycle arrest in several tissues [5–7]. This lncRNA resides at 1q25, hosts numerous small nucleolar RNAs in its intronic regions and encodes a transcript with riborepressor function [8, 9]. A certain SNP within the promoter region of this lncRNA has been shown to regulate expression of *GAS5* and has been associated with risk of gastric, lung and colorectal cancers [10–12]. This lncRNA has other SNPs among them are rs2067079 and rs6790 which have been proposed as prognostic biomarkers for adverse effects of chemotherapy in nasopharyngeal carcinoma [13]. These two SNPs are located in an active promoter or robust enhancer area of the lncRNA. Moreover, they possess characteristics of expression Quantitative Trait Locus (e-QTL) in numerous cells [13]. Based on the fundamental role of *GAS5* in suppression of bladder cancer [14] and the putative effects of rs2067079 and rs6790 SNPs in modulation of *GAS5* function, we genotyped these SNPs in a population of Iranian patients with bladder cancer as well as healthy individuals.

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**Table 1** Nucleotide sequences of primers used for genotyping of *GAS5* variants

| SNP | Primer sequence | Tm | Annealing temperature | PCR product size (bp) |
|---|---|---|---|---|
| rs2067079 | Forward inner primer (T allele): GTGCTGATTGCATTAAATAAATGTCA | 61 °C | 55 °C | 200 bp (T allele) |
| | Reverse inner primer (C allele): TCATATTAATCATAACAAGACAAGAAGCC | 61 °C | | 276 bp (C allele) |
| | Forward outer primer: CTATCTGTCTGATGTATCTGGGGTAGTT | 61 °C | | 420 bp (two outer primers) |
| | Reverse outer primer: AAATAAGAGTAGTCTTAGAATAGCCACA | 61 °C | | |
| rs6790 | Forward inner primer (G allele): TGTACCTATAATAGGTATGACAGGAAATG | 62 °C | 58 °C | 194 bp (G allele) |
| | Reverse inner primer (A allele): ACTTGCTTGGGTAAGGACATGAATAT | 62 °C | | 129 bp (A allele) |
| | Forward outer primer: AGATTCCCATCTGCTTTAATGTTAAAATC | 62 °C | | 268 bp (two outer primers) |
| | Reverse outer primer: CTGAAATGAGCATGTAGACAAAGGTAAC | 62 °C | | |

## Material and Methods

### Patients and Controls

For the current case-control study, we enrolled 122 Iranian patients with definite diagnosis of bladder cancer and 150 age- and sex-matched healthy individuals. Histopathological studies confirmed the presence of transitional cell carcinoma of bladder in all tissues samples. Persons enlisted in the control group had no former history of cancer and no urinary system disorders. The study protocol was approved by ethical committee of Shahid Beheshti University of Medical Sciences. Written informed consent forms were obtained from all study participants.

### Genotyping

Two non-coding transcript variants namely rs2067079 (Chr 1:17386607, C > T, Minor allele frequency (MAF) =0.19, Minor allele count (MAC) =965) and rs6790 (Chr 1:173865494, G > A, MAF = 0.12, MAC = 602) were genotyped. Genotyping were executed on DNA extracted from peripheral blood samples. Salting out technique was used for DNA extraction. The mentioned SNPs were genotyped by tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) technique. Primer sequences, annealing and melting temperatures and the anticipated product sizes are summarized in Table 1. PCR was performed using the Taq 2x red master mix (Ampliqon, Denmark) and the following program: an initial denaturation phase at 95 °C for 5 min, 35 cycles of 95 °C for 45 s, specific annealing temperatures for 50 s, and 72 °C for 55 s, and a final extension phase at 72 °C for 7 min.

### Statistical Analyses

SNPAnalyzer 2.0 tool [15] was used for statistical analyses. Genotype distributions of the mentioned SNPs were compared with the anticipated frequencies based on the Hardy–Weinberg equilibrium (HWE). The linkage disequilibrium (LD) between rs2067079 and rs6790 were appraised by measurement of D' and r values. Associations between SNPs and bladder cancer risk were appraised in co-dominant, dominant and recessive models. Frequencies of SNP alleles were also compared between study groups. Odds ratios (OR) and 95% confidence interval of OR (95% CI) were calculated. *P* values were corrected for multiple comparisons using the Bonferroni method and adjusted P values were reported. P values less than 0.05 were regarded as significant.

## Results

### Demographic Data of Enrolled People

The general information of enrolled people is shown in Table 2.

**Table 2** General information of enrolled people

| Variable | Patient (%) | Control (%) |
|---|---|---|
| Male/Female [no.(%)] | 90 (73.8)/32 (26.2) | 110 (73.3)/40 (26.7) |
| Age (mean ± SD, Y) | 64 ± 8.1 | 63 ± 0.8 |
| Age range (Y) | 32–79 | 30–76 |
| Age at onset (mean ± SD, Y) | 51 ± 2.3 | – |

**Table 3** Detailed genotype frequencies in cases and controls as well as their compliance with HWE

| SNP | rs2067079 | | | P value | rs6790 | | | P value |
|---|---|---|---|---|---|---|---|---|
| | CC | CT | TT | | GG | AG | AA | |
| Bladder cancer cases | 50 | 49 | 23 | 0.09 | 95 | 23 | 4 | 0.19 |
| Healthy control | 72 | 66 | 12 | 0.56 | 107 | 37 | 6 | 0.35 |

**Table 4** The results of association analysis between rs2067079 and rs6790 SNPs and bladder cancer

| SNP | Model | | Cases (%) | Controls (%) | OR (95% CI) | P value | Adjusted P value |
|---|---|---|---|---|---|---|---|
| rs2067079 | Allele | T vs. C | 95 (39)<br>149 (61) | 90 (30)<br>210 (70) | 1.49 (1.04–2.12) | 0.03 | 0.06 |
| | Co-dominant | TT vs. CC<br>CT vs. CC | 23 (19)<br>49 (40) | 12 (8)<br>66 (44) | 2.78 (1.26–5.88)<br>1.06 (0.64–1.78) | 0.03 | 0.06 |
| | Dominant | TT + CT vs. CC | 72 (59)<br>50 (41) | 78 (52)<br>72 (48) | 1.33 (0.82–2.15) | 0.25 | 0.49 |
| | Recessive | TT vs. CC + CT | 23 (19)<br>99 (81.2) | 12 (8)<br>138 (92) | 2.67 (1.27–5.62) | 0.008 | 0.02 |
| rs6790 | Allele | A vs. G | 31 (13)<br>213 (87) | 49 (16)<br>251 (84) | 0.75 (0.46–1.21) | 0.23 | 0.47 |
| | Co-dominant | AA vs. GG<br>AG vs. GG | 4 (3.3)<br>23 (18.9) | 6 (4)<br>37 (24.7) | 0.66 (0.25–1.75)<br>0.88 (0.64–1.22) | 0.58 | 1.00 |
| | Dominant | AG + AA vs. GG | 27 (22.1)<br>95 (78) | 43 (28.7)<br>107 (71) | 0.71 (0.41–1.23) | 0.22 | 0.44 |
| | Recessive | AA vs. AG + GG | 4 (3.3)<br>118 (96.7) | 6 (4)<br>144 (96) | 0.81 (0.22–2.95) | 0.99 | 1.00 |

### Compliance with HWE

Based on the results of $Chi^2$ test, genotype frequencies of both SNPs were in compliance with HWE in both cases and controls. Table 3 shows the detailed genotype frequencies in cases and controls as well as their compliance with HWE.

### Distribution of Alleles and Genotypes of SNPs in Study Groups

The frequency of T allele of the rs2067079 was higher in cases compared with controls. However, after correction for multiple comparisons, it did not reach the level of significance (adjusted *P* value = 0.06). The rs2067079 was associated risk of bladder cancer in recessive inheritance model (TT vs.CC + CT: OR (95% CI) = 2.67 (1.27–5.62), adjusted *P* value = 0.02). The rs6790 was not associated with bladder cancer susceptibility n any inheritance model. Table 4 shows the results of association analysis between mentioned SNPs and bladder cancer.

### Haplotype Analysis

The rs2067079 and rs6790 SNPs were not in LD in the assessed population (D' = 0.1, r = 0.003). The T G haplotype (rs2067079 and rs6790) increased the risk of bladder cancer in the assessed population (OR (95% CI) = 1.73 (1.18–2.56), adjusted *P* value = 0.02). Table 5 demonstrates the haplotype frequencies in cases and controls.

## Discussion

In the current genotyping project, we genotyped two *GAS5* SNPs in bladder cancer patients and healthy subjects to find putative risk locus for bladder cancer in Iranian population. *GAS5* is a well-known tumor suppressor lncRNA. A recent study has verified the inverse correlation between expression levels of this lncRNA and stage of bladder cancer [14]. Moreover, functional studies have shown the role of *GAS5* in stimulation of apoptosis in bladder cancer cells through suppression of expression of Enhancer of zeste homolog 2 (EZH2) [14]. This lncRNA also suppresses bladder cancer cell proliferation through inhibiting CDK6 transcription [16]. The genotyped SNPs in the current study were located in a genomic region which was predicted to encompass an active promoter or robust enhancer [13]. The role of these SNPs in modifying transcriptional activity of *GAS5* has been verified by bioinformatics tools as they have been shown to possess e-

**Table 5** The haplotype frequencies of rs2067079 and rs6790 SNPs in cases and controls

| rs2067079 | rs6790 | Cases | Controls | Total | OR (95% CI) | P value | Adjusted *P* value |
|---|---|---|---|---|---|---|---|
| C | G | 0.54 | 0.6 | 0.57 | 0.75 (0.53–1.06) | 0.10 | 0.41 |
| T | G | 0.33 | 0.24 | 0.28 | 1.73 (1.18–2.56) | 0.005 | 0.02 |
| C | A | 0.07 | 0.10 | 0.09 | 0.71 (0.35–1.44) | 0.34 | 1.00 |
| T | A | 0.06 | 0.06 | 0.06 | 0.80 (0.43–1.5) | 0.49 | 1.00 |

QTL characteristics [13]. However, we could only demonstrate association between rs2067079 and bladder cancer risk. In addition to the mentioned characteristics of two SNPs, the rs2067079 has been predicted to change the secondary structure of *GAS5*. Characteristic secondary configurations are a principal requirement for the activity of lncRNAs; thus, changes produced by SNPs might lead to malfunction and the susceptibility to certain disorders including cancer [17]. Moreover, miRNA target prediction has shown that rs2067079 changes binding sites for four miRNAs, but the other SNP does not have such effect [13]. Taken together, in silico analyses have provided stronger evidences for functionality of the rs2067079 compared with the other assessed SNP. Consistent with these results, we only confirmed association between the rs2067079 and cancer risk.

The rs2067079 also alters the Yin Yang 1 (YY1) motif as predicted by HaploReg v4.1 [18]. This transcription factor has an acknowledged role in tumor development and progression through modification of expression of several cancer-related proteins and pathways [19]. In bladder cancer, this transcription factor participates in the regulation of expression of differentially expressed genes among distinct tumor categories [20]. This fact further supports participation of the rs2067079 in the pathogenesis of bladder cancer.

We also demonstrated higher frequency of the haplotype that has minor alleles of both SNPs in bladder cancer patients. The presence of minor alleles of these SNPs might have synergic effects in destruction of *GAS5* function. Alternatively, this haplotype might contain another functional SNP.

The current study was performed only in Iranian population. Convincing supports from numerous populations are needed for confirmation of these results. Moreover, increasing the number of assessed patients and genotyped SNPs would increase the study power and enhance haplotype analysis robustness respectively.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare they have no conflict of interest.

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