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Research Paper

# A promoter variant of lncRNA GAS5 is functionally associated with the development of osteosarcoma

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ABSTRACT

*Background:* Previous studies showed that genetic variant rs145204276 in the promoter region of GAS5 was associated with the development of human cancer including colorectal cancer and hepatocellular cancer. This study aimed to investigate the role of rs145204276 in the development of osteosarcoma (OS).

*Methods:* 132 OS patients and 1270 healthy controls were recruited for the genotyping analysis of rs145204276. Promoter methylation level of GAS5 was determined for all patients. The tumor tissues and the adjacent normal tissue were collected from 42 patients during surgery and the relative expression of GAS5 was then quantified by Real-time PCR. The Chi-square test was used to determine the difference of genotype and allele frequency between the patients and the controls. The gene expression and the percentage of methylation alleles were compared among different genotypes of rs145204276 with One-way ANOVA test.

*Results:* Compared with the controls, patients were found to have significantly lower rate of genotype del/del (7.6% vs. 8.7%, $p = 0.024$). The frequency of allele del was significantly lower in the patients than in the controls (23.5% vs. 30.1%, $p = 0.021$). Compared with than patients with genotype ins/ins, those with genotype del/del had remarkably higher expression of GAS5 ($0.0033 \pm 0.0019$ vs. $0.0018 \pm 0.0006$, $p < 0.001$). Patients with genotype del/del were found to have obviously hypermethylation at the 7th CpG site as compared with those with genotype ins/ins (38.7% ± 21.1% vs. 20.5% ± 8.2%, $p < 0.001$).

*Conclusions:* The genetic variant rs145204276 is functionally associated with the susceptibility of OS, which can function as a protective factor in the incidence of OS possibly through the regulation of GAS5.

## 1. Introduction

Osteosarcoma (OS) is the most common pediatric bone cancer with the 5-year survival rate estimated to range from 40% to 75% [1]. Over 30% of OS patients may finally experience distant metastases and a poor prognosis [2]. Although the genetic background of OS has been extensively studied, the molecular mechanisms underlying the initiation and progression of OS remains poorly understood. Therefore, investigations on the molecular etiology of OS are essential to improve the treatment of this devastating disease.

The functional role of long non-coding RNA (lncRNAs) in the biological activity of malignant tumor has been widely investigated [3,4]. By regulating the expression of target gene through different mechanisms, lncRNAs have been reported to be associated the growth and metastasis of a variety of cancer [5-7]. Aberrant upregulation of oncogenic lncRNA TUG1 was observed in B-cell malignancies, bladder cancer, hepatocellular carcinoma and OS [8]. The role of lncRNA CCAT1 has been well documented in OS, gastric cancer (GC), colorectal cancer and hepatocellular carcinoma [9]. Recently, the growth arrest specific transcript 5 (GAS5) was identified as a tumor-suppressor, including breast cancer, gastric cancer, colorectal cancer and bladder cancer [10-13]. Ye et al reported that overexpression of GAS5 may suppress cell growth and EMT of OS through the miR-221/ARHI pathway [14]. However, the regulatory mechanism underlying down-expressed GAS5 in OS tissues remains poorly understood.

The polymorphism rs145204276 in the promoter region of GAS5 may regulate its expression level by influencing the methylation status [15]. Tao et al. [15] reported that the allele del of rs145204276 could regulate the expression of GAS5 and thus increased the risk of hepatocellular carcinoma. Zheng et al.[10] observed that the allele del of rs145204276 was associated with a decreased risk of colorectal cancer. To our knowledge, however, there is a paucity of knowledge concerning the association between rs145204276 and the expression of GAS5 in OS. The objective of the current study was to investigate the role of rs145204276 in the development of OS.

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## 2. Methods

### 2.1. Subjects

The current study was approved by the local institutional review board. A total of 132 OS patients who came to our clinic center for treatment between 2006 January and 2017 March were included. The diagnosis of OS was validated by a multiple disciplinary team composed of a senior orthopedic surgeon, a radiological specialist and a pathologist. Patients received no previous medical treatment before the surgery. 1270 healthy controls were recruited during physical exanimations. Demographic data of the patients were collected from the medical record, including age, gender, tumor size, histological differentiation, Anneking Stage, and presence of distant metastasis. Written informed consent was obtained from all the subjects for the collection of blood or tissue samples.

### 2.2. Genotyping assay

The genomic DNA was extracted from peripheral blood of each subject using genomic DNA purification kit (Qiagen, Tokyo). DNA fragments containing rs145204276 were amplified using the following primers: F-TCCCGACTGAGGAGGAAGAGCA; R-AACACCGTCCCGGAA GTGAAA as previously reported [15]. The PCR products were then analyzed by 7% non-denaturing polyacrylamide gel electrophoresis, and the results were visualized by silver staining. The genotype was classified as del/del, del/ins or ins/ins for each patient. 10% samples were randomly sequenced for duplication analysis, which yielded a concordance rate of 100%.

### 2.3. Expression of GAS in tissues of the patients

The tumor tissues and the adjacent normal tissue were collected from 42 patients during surgery. The tissues were stored at −80 °C, and total RNA was extracted with a commercial kit (CWBio. Co. Ltd). The relative expression of GAS5 was quantified by Real-time PCR (RT-PCR) on Roche Light Cycler 480 system with the following primer 5′- CTTC TGGGCTCAAGTGATCCT-3′ and 5′- TTGTGCCATGAGACTCCATCAG-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer for GAPDH designed as 5′-GTCAACGGAT TTGGTCTGTATT-3′ and 5′-AGTCTTCTGGGTGGCAGTGAT-3′. The amplification procedures were composed of an initial denaturation step of 95 °C for 10 min, 40 amplification cycles at 95 °C for 10 s, annealing at 60 °C for 20 s and elongation at 72 °C for 10 s. The relative expression of GAS5 was normalized using the ΔΔCt method as previously reported [15].

### 2.4. DNA methylation analysis

Promoter methylation level of GAS5 was determined using quantitative bisulfite pyrosequencing by the EpigenDx Inc. (Worcester, MA, USA). The target sequence of the 7th CpG site in GAS5 promoter region was listed in Table 1. The bisulfite conversion was carried out with 100 ng extracted DNA using the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's protocol. The converted DNA was then amplified using PyroMark PCR Kit (QIAGEN) with primers mentioned above. The methylation status of each CpG site was analyzed as artificial T/C SNP using QCpG software (Qiagen Pyrosequencing). Each pyrosequencing assay was performed a minimum of three times. DNA methylation analysis was subsequently conducted using the Sequenom EpiTYPER system (Sequenom Inc., USA) as described previously.

### 2.5. Statistical analyses

SPSS version 19.0 (SPSS Inc., Chicago, USA) was used for the data analysis. The Hardy-Weinberg equilibrium (HWE) test was performed for all the subjects. The Student's t test was used to analyze the continuous date and the Chi-square test was used to analyze the categorical data. Odds ratio (OR) was calculated to evaluate the contribution of rs145204276 to the risk of OS. The effect size (ϕ) was defined as the value of the square root of the Chi square value divided by total number of subjects. Specifically, a ϕ value of 0.1 was defined as small effect, 0.3 as medium effect and 0.5 as large effect. The gene expression and the percentage of methylation alleles were compared among different genotypes of rs145204276 with One-way ANOVA test. The relationship between the gene expression level and the percentage of methylation was determined by Pearson correlation analysis. The statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Demographic data

As shown in Table 2, there was no significant difference between the cases and the controls in terms of age and gender. For the subjects included in the genotyping analysis, the mean age was 31.3 ± 18.4 years for the patients and 30.8 ± 12.3 years for the controls, respectively. Clinical features of the patients, including tumor size, histological differentiation, Enneking Stage and presence of distant metastasis, were summarized in Table 2.

### 3.2. Association of rs145204276 with the susceptibility of oS

The frequency of rs145204276 of the cases and the controls were summarized in Table 3. HWE test indicated no selection bias for either group ($p > 0.05$). Compared with the controls, patients were found to have significantly lower rate of genotype del/del (7.6% vs. 8.7%, $p = 0.024$). The frequency of allele del was significantly lower in the patients than in the controls (23.5% vs. 30.1%, $p = 0.021$), with an OR of 0.72 (95% CI = 0.54–0.97). The effect size (ϕ) of rs145204276 was 0.07.

### 3.3. The relationship between rs145204276 and progression and metastasis of oS

The tumor size was compared among different genotypes to determine the relationship between rs145204276 and the progression of OS. As shown in Table 4, the tumor size of patients with genotype del/del were remarkably smaller than that of patients with genotype ins/ins (4.1 cm ± 1.7 cm vs. 5.9 cm ± 2.4 cm, $p = 0.04$).

**Table 1**
Primers sequences and selected CpG sites in the promoter region.

| Primer name | Sequences |
|---|---|
| GAS5-p1 | 5′-AATATAGTTTAGGAAGTGAAATTTT-3′ |
| GAS5-p2 | 5′-GTTTTTTTTTTTTTATTTTTAGA-3′ |
| GAS5-p3 | 5′-TTTTAGTAGGGAGGAG-3′ |
| CpG site | CCACCCCCTCCCACGGAGCGGGCGACGTGCCGGAAGGAAA TCACTCAGCCTTACACCGCCCCCCCTTCCCCCATCCCCA<br>GAGCTTTCCTTGCCTCGCGCCCCCTCCCCTCTCTGCTCTT CCTCCTCAGTCGGGAGGAGGGCGGGAGCACGGCATCACGTGGACGGTCATGTCTCTGCCCACAATGGCG |