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A functional genetic variant in GAS5 lncRNA (rs145204276) modulates p27^{Kip1} expression and confers risk for gastric cancer

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Gastric cancer is the fifth most common cancer type worldwide [1]. Established risk factors are environmental factors (alcohol, *H pylori*), atrophic gastritis, intestinal metaplasia and genetic factors [2]. Long noncoding RNAs (lncRNAs) are RNA molecules < 200 nucleotides with a coding potential of < 100 amino acids, or non-protein coding transcripts [3]. They have roles in cell regulation through transcription, chromatin remodelling, generation of endo-siRNAs (small interfering RNAs) and modulation of protein activity [4]. Genome-wide approaches have identified many lncRNAs that are transcribed in different manners in normal and malignant tissues [5], and it has been suggested that lncRNAs could be a molecular biomarker for specific cancers. The lncRNA growth arrest-specific transcript 5 (GAS5) is located at 1q25 and contains ~630 nucleotides that is significantly down-regulated in gastric cancer cell lines as well as cancer tissues in comparison with normal tissue specimens [6].

A functional 5-base pair (AGGCA/-) insertion/deletion (indel) polymorphism (rs145204276) exists in the promoter region of GAS5. A deletion (del) allele appears to produce an elevation in gene transcription activity compared to an insertion (ins) allele [7,8]. However, the clinical and metabolic impact of the GAS5 polymorphisms is unclear. GAS5 functions as a tumour suppressor in prostate cancer development and progression via targeting the protein P27^{Kip1}, which mainly inhibits the Cdk2-Cyclin E complex [9]. Several studies have reported links between p27^{Kip1} levels and cancer development [10,11]. Thus, given the critical role played by GAS5 in cancer and its dysregulation in gastric cancer, we hypothesized that a functional polymorphism (rs145204276) in GAS5 lncRNA may modulate the p27^{Kip1} protein level, and may ultimately influence susceptibility to gastric cancer.

To test our hypothesis, we recruited 130 patients with a confirmed diagnosis of gastric cancer by clinical histopathological parameters. They were all newly diagnosed, without a prior history of cancer or previous chemotherapy or radiotherapy. Tumours were excised and staged using the seventh edition of the TNM of the American Joint Committee on Cancer 2010. During the same period, 230 non-cancer healthy unrelated volunteers as controls were included in this study. A 2 ml blood sample was drawn into vacutainer tubes with heparin and stored at −80 °C until DNA extraction. Stomach specimens of gastric cancer were snap frozen in liquid nitrogen and stored at −80 °C until protein analysis. Written informed consent was obtained from all individuals and the approval of the local research ethics committee was obtained. This study was conducted according to the principles expressed in the Declaration of Helsinki.

Purity and quantity of genomic DNA were checked with Nanodrop spectrophotometer (Thermo Scientific, USA). DNA with A260/280 1.75–1.85 was used for the study. Primers for tetra-primer amplification refractory mutation system-PCR were designed with the Oligo primer analysis software (version 7.54, Molecular Biology Insights Inc., Cascade, CO, USA). The primers sequences were ins-F (5′- GCAGAGACATGACCGTCCAC-3′) and ins-R (5′-CCCCATCCCCAGAGCTTTCGTT-3′) for ins allele; del-F (5′-AAAACCCGCAACATTCGCAAA-3′) and del-R (5′-CCCCATCCCCAGAGCTTTCGTC-3′) for del allele. The PCR system contained template DNA (50 ng), buffer solution (10 × PCR), each primer (2 pmol), dNTPs (0.1 M) and *Taq* polymerase 2U plus double-distilled water to 15 µl. PCR was performed as follows: 95 °C for 6 min, 35 cycles of denaturation at 94 °C for 35 s, annealing at 56 °C (ins allele) and 60 °C (del allele) for 40 s, with a final extension step at 72 °C for 3 min. Genotypes were determined as ins/ins (wild type genotype) (109 bp), del/del (homozygous mutant genotype) (348 bp) or ins/del (109 and 348 bp). The yield and specificity of PCR products were evaluated by electrophoresis in 1.5% agarose gel. p27^{Kip1} in homogenised gastric tissue specimens (10 mg

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tissue in 0.2 ml protein lysis buffer, including protease inhibitors (Roche Diagnostics, Sussex, UK) was measured by ELISA (Abcam, Cambridge, UK).

Data was analysed by SPSS Version 20 (SPSS Inc., Chicago, Illinois, USA). Hardy-Weinberg equilibrium (HWE) was assessed by a χ^2 test. The link between single nucleotide polymorphisms (SNPs) and gastric cancer risk was assessed by odds ratio (OR) and 95% confidence intervals (CIs) in heterozygous (ins/del vs ins/ins), homozygous (del/del vs ins/ins), dominant (ins/del + del/del vs ins/ins), recessive (del/del vs ins/ins + ins/del), Over-dominant (ins/del vs ins/ins + del/del) and additive (del allele vs ins allele) models, respectively. Difference in categorical data was sought by χ^2 testing, in continuous data by *t* test. A *P* value < 0.05 was considered statistically significant.

Of the patients, 79 were males and 51 females, whereas in the control subjects 154 were males and 76 females (*P* = 016). Mean [standard deviation] age of the cases was 61.6 [9.4] years versus 64.8 [8.8] in the controls (*P* = 0.23). Histopathologic classification of the tumours and other characteristics are shown in Table 1. The genotypes in the control group were in accordance with the HWE (*P* = 0.27). The difference in the genotype frequencies ins/ins, ins/del and del/del in cases and controls was significant (*P* = 0.045). The most common type of gastric cancer was intestinal (73.1%). Based on TNM staging criteria, patients were classified as stage I-II (*n* = 44) and III-IV (*n* = 86). Based on the dominant model, combination of ins/del + del/del variant showed a lower risk in cases, compared to the ins/ins genotype (OR 0.56; 95%CI 0.34–0.86; *P* = 0.01). The recessive and over-dominant models showed a non-significant association with gastric cancer susceptibility (*P* = 0.15, *P* = 0.08, respectively). Overall, the del allele was more frequent in the controls (*P* = 0.002), and del allele carriage conferred a protective effect on gastric cancer risk. The ins/ins genotype was more frequent in patients with tumours of a higher stage than those of a lower stage (88.6%), compared to ins/del (13.5%) and del/del (50%) (*P* < 0.001). Older patients showed more protective association at del allele. There were no statistical links between sex, type, size, location of tumour and the presence of the del allele.

To evaluate the effect of rs145204276 on the $p27^{Kip1}$, we examined the protein level in alternative genotypes (Figure 1). Compared with ins/ins genotype, with $p27^{Kip1}$ levels of 0.69 [0.1] ng/mL, those with heterozygous ins/del genotype had levels of 0.87 [0.09] ng/ml (*P* = 0.001], while those with del/del genotype had the highest levels of 1.30 [0.31] ng/ml (*P* < 0.001).

Recent studies have investigated the contribution of non-coding RNAs genetic variations and the risk of gastric cancer [8,12]. GAS5 expression is downregulated in gastric cancer tissues and is associated with larger tumour size and advanced stage. In addition, ectopic expression of GAS5 decreased gastric cancer cell proliferation and induced apoptosis *in vitro* and *in vivo*, while downregulation of GAS5 could promote cell proliferation [6]. Several studies have reported a link between GAS5 SNP rs145204276 and increased tumours risk including stomach, osteosarcoma and lung [8,13,14].

Table 1. GAS5 lncRNA (rs145204276) genotype and allele frequencies.

	Genotypes			
Characteristics	ins/ins	ins/del	del/del	del allele
Controls (n = 230)	126% (54.8)	84% (36.5)	20% (8.7)	124% (27)
Patients (n = 130)	88% (67.7)	36% (27.7)	6% (4.6)	48% (19)
OR*(95%CI)	Ref	0.63 (0.39–1.02)	0.40 (0.12–0.96)	0.58 (0.41–0.83)
p-value		0.04	0.08	0.01
Age (years)				
≥60/<60	53/35	15/21	1/5	17/31
OR(95%CI)	Ref	0.47 (0.21–10.03)	0.13 (0.01–1.17)	0.41 (0.21–0.76)
P-value		0.061	0.069	0.007
Sex				
Male/Female	50/38	25/11	4/2	33/15
OR(95%CI)	Ref	1.72 (0.75–3.94)	1.52 (0.26–8.73)	1.53 (0.78–2.98)
P-value		0.19	0.63	0.21
Tumour Location				
Lower/Upper	59/29	20/16	5/1	30/18
OR(95%CI)	Ref	0.61 (0.27–1.35)	2.45 (0.27–22.01)	0.89 (0.46–1.71)
P-value		0.22	0.42	0.73
Tumour Size				
>3 cm/≤3 cm	50/38	20/16	3/3	26/22
OR(95%CI)	Ref	0.95 (0.43–2.07)	0.76 (0.14–3.97)	0.90 (0.48–1.70)
P-value		0.89	0.74	0.75
Lauren's classification				
Intestinal/Diffuse	63/25	27/9	5/1	37/11
OR(95%CI)	Ref	1.19 (0.49–2.88)	1.98 (0.22–17.84)	1.72 (0.75–3.94)
P-value		0.69	0.54	0.19
TNM staging				
III-IV/I-II	78/10	5/31	3/3	11/37
OR(95%CI)	Ref	0.02 (0.006–0.06)	0.12 (0.02–0.72)	0.09 (0.04–0.19)
P-value		<0.001	0.02	<0.001

*, Adjusted for age and gender; OR, odds ratio; CI, confidence interval.

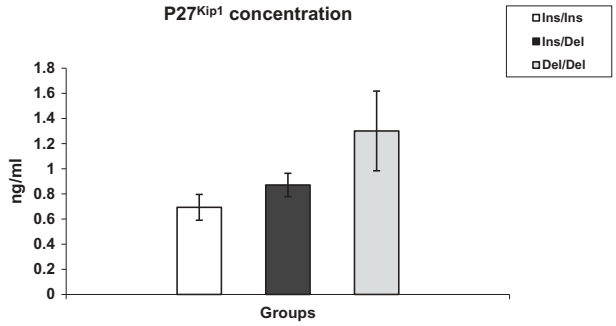

Figure 1. P27^{Kip1} concentration in gastric tissue specimens with ins/ins, ins/del and del/del genotypes.

Our study provides data on a GAS5 lncRNA SNP and is the first that has investigated rs145204276 allele frequency in gastric cancer. We found a significant link with the disease, and in addition, the del allele was also associated with lower risk of cancer with respect to age and higher stages of tumour. Li et al. reported that the del allele of rs145204276 was significantly associated with a decreased risk of gastric cancer in China (OR 0.81; 95%CI 0.70–0.94, P = 0.005), and that this polymorphic site was associated with the tumour size and metastasis [8]. However, our study is contradictory to that of Tao et al., who showed that the deletion allele of rs145204276 significantly increased the risk of hepatocellular carcinoma [7].

Cyclin-dependent kinase inhibitor 1B (p27^{Kip1}) may act as a tumour suppressor, as decreased expression of nuclear p27^{Kip1} is commonly observed in various cancers [10,11], and it linked with lymph node metastasis, depth of invasion, and proliferative activity of gastric cancer [15]. In an *in vitro* and *in vivo* study, Luo and colleagues showed that GAS5 could bind directly to transcription factor E2F1, enhance its binding to P27^{Kip1} promoter, and then activate P27^{Kip1} promoter [9]. We also evaluated the influence of GAS5 (rs145204276) on gastric cancer risk in interaction with gastric tissue p27^{Kip1} protein level, showing that patients with the del allele had higher gastric tissue p27^{Kip1} levels.

Certain limitations need to be taken into consideration upon interpretation of the results. Our population was not large, and so needs confirmation, and the effect of only one polymorphic site of GAS5 lncRNA on p27^{Kip1} expression was evaluated, which may not represent the entire gene. Finally, some potential confounding factors were not considered in this study, such as helicobacter pylori infection and family history.

This work represents an advance in biomedical science because it provides evidence of the protective effect of GAS5 lncRNA (rs145204276) genetic variant on gastric cancer by influencing p27^{Kip1} expression, and so the potential use of this SNP in routine practice.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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