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Association between indel polymorphism in the promoter region of lncRNA GAS5 and the risk of hepatocellular carcinoma

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Abstract

The growth arrest special 5 (GAS5) is known to be involved in various cancers. However, its expression regulation remains unclear. Polymorphisms in the promoter region of GAS5 may affect its expression and be associated with cancer susceptibility. In this research, we first evaluated the association of a 5-base pair indel polymorphism (rs145204276) in the promoter region of GAS5 with hepatocellular carcinoma (HCC) susceptibility in Chinese populations. Logistic regression analysis showed that the deletion allele of rs145204276 significantly increased the risk of HCC in two independent case control sets (1034 HCC and 1054 controls). Further genotype-phenotype association analysis revealed that the deletion allele was markedly correlated with higher expression of GAS5 in HCC tissues. The luciferase activity analysis in an *in vitro* reporter gene system suggested that the deletion allele improved an increased expression of GAS5 in three hepatoma cell lines. Intriguingly, overexpression of GAS5 displayed an anti-apoptosis effect in HCC cell lines, GAS5 knockdown could partially revert this anti-apoptosis effect, suggesting that GAS5 may act as a proto-oncogene in HCC, in contrast with its inhibitory role in other cancers. Further pyrosequencing revealed that the genotypes of rs145204276 were associated with methylation status of GAS5 promoter region. Taken together, our findings provided evidence that rs145204276 may contribute to hepatocarcinogenesis by affecting methylation status of the GAS5 promoter and subsequently its transcriptional activity thus serving as a potential therapy target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver with a high mortality rate (1). More than 80% of the HCC cases are from Asia and Africa among which

over half are from mainland China (2). HCC has a complicated carcinogenesis involving multiple risk factors for its initiation, promotion and progression. In addition to hepatitis B virus

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Abbreviations

bp	base pair
GAS5	growth arrest special 5
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
indels	insertions and deletions
lncRNA	long non-coding RNA
SNP	single-nucleotide polymorphism

infection, a well-known risk factor, genetic factors also play an important role in HCC carcinogenesis (3). In spite of great effort for the research of HCC carcinogenesis, the molecular mechanisms of HCC remain unclear (4).

Recently, high throughput transcriptome analysis has revealed that up to 98% of human genome would transcript into non-coding RNA (5,6). Among them, the long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides, cannot be translated (7). The growth arrest special 5 (GAS5), one lncRNA encoded at 1q25, was identified in a functional screen for suppressing apoptosis in a mouse thymoma cell line (8). A large number of studies have shown that GAS5 is downregulated in many kinds of cancer cells (9–11) and functions as a tumor-suppressor lncRNA (12–14). More importantly, the expression of GAS5 is downregulated in majority of HCC patients thus can serve as an independent prognostic predictor (15). It is known that polymorphisms in protein-coding genes may affect their expression levels or their functions through different mechanisms depending on their locations. For example, single-nucleotide polymorphisms (SNPs) in the promoter region of matrix metalloproteinases (MMP9) affect the serum level of MMP9, and as a result correlate with the susceptibility to metabolic syndrome (16). Moreover, one SNP located in the promoter region of telomerase reverse transcriptase is associated with the progress of malignant glioma by disturbing the binding of transcription factor Ets2 and in turn inhibiting the transcriptional activity of telomerase reverse transcriptase (17). There is also one report that one SNP in the promoter region of lncRNA IRAIN can regulate its expression level through affecting the methylation status of CpGs island in breast cancer (18). However, it is little known about the association between polymorphism in the promoter region of lncRNA GAS5 and susceptibility to HCC, as well as the regulation mechanisms of GAS5 expression in HCC.

As one of the most abundant genetic variations in human genome, small insertions and deletions (indels) polymorphisms are likely to have a major impact on human biology and diseases (19). By using a candidate-gene-based approach, we have analyzed hundreds of indels within putative regulatory regions of cancer-related genes, and identified several indels significantly associated with HCC susceptibility (20–23). Considering the important roles of GAS5 in cancer development, we analyzed the association between a 5-base pair (bp) indel polymorphism (rs145204276) in the promoter region of GAS5 and HCC susceptibility in Chinese populations, and investigated the possible mechanisms by which the SNP influenced GAS5 expression.

Materials and methods

Study populations

This study was composed of two independent case-control sets, Suzhou case-control set and Shanghai case-control set. A total of 1034 newly diagnosed patients with HCC and 1054 non-cancer controls were enrolled. Six hundred four HCC patients were diagnosed, hospitalized and treated in the affiliated hospitals of Soochow University from 2010 to 2013; the

remaining 430 HCC patients were recruited from Qidong Liver Cancer Research Institute of Jiangsu Province from 2010 to 2013. All patients were newly diagnosed without treatment of any medications. The inclusion and exclusion criteria for both cases and controls were the same as described previously (20–22). A total of 1054 age- and gender-matched controls (634 from Suzhou and 420 from Shanghai) were recruited from the community nutritional survey conducted in the same regions during the same period as the HCC patients. Peripheral blood was collected from both cases and controls after informed consent was obtained from each participant. All participants were negative for antibodies to hepatitis C virus, hepatitis D virus or HIV. The demographic data and related risk factors including smoking and drinking status were obtained through a structured questionnaire. The definitions of current, former or non-smokers and heavy, light or non-drinkers were described as before (23). The tumor tissues from 72 patients with HCC were collected after surgical resection and immediately stored at -80°C till use. All the 72 HCC cases were newly diagnosed and confirmed by pathologic diagnosis without preoperative chemotherapy or radiotherapy. A modified American Joint Committee on Cancer and International Union Against Cancer standard was used to determine tumor stages. The research design was approved by the Ethical Committee of Soochow University.

DNA extraction and genotyping

Genomic DNA purification kit (Qiagen) was used for extraction of the genomic DNA from blood samples and HCC tumor tissues. DNA fragments containing the indel polymorphism were amplified using genotyping primers (genotyping-F and genotyping-R) listed in [Supplementary Table S1](#), available at [Carcinogenesis Online](#). Genotyping was conducted as described previously in a double-blinded way (23). The quality control was done as follow: 50 randomly selected DNA samples were sequenced following genotyping in order to validate the genotyping method; approximately 10% of the total DNA samples were randomly selected for genotyping in duplicate by two independent technicians to confirm a 100% consistency.

Real-time PCR analysis

Total RNA was extracted from HCC tissue samples with different genotypes according to the manufacturer's protocol (Cat #74106, Qiagen), and reversely transcribed using the Superscript II reverse transcriptase (Cat #18064-014, Invitrogen). Next, SYBR Green real-time PCR (reverse transcriptase-PCR) was performed on Roche Light Cycler 480 system to quantify relative GAS5 expression in these samples. Glyceraldehyde 3-phosphate dehydrogenase was chosen as the internal control. Primer sequences used for GAS5 (GAS5-F and GAS5-R) and glyceraldehydes 3-phosphate dehydrogenase were shown in [Supplementary Table S1](#), available at [Carcinogenesis Online](#). The amplification system was described previously (23). The $2^{-\Delta\Delta C_T}$ algorithm was applied to calculate the expression levels for different groups.

Cell culture

Three human HCC cell lines, Sk-Hep-1, BEL-7404 and Huh7, were obtained from Shanghai Cell Bank of Chinese Academy of Sciences on 9 December 2013. All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified chamber supplemented with 5% CO_2 . Upon resuscitation, the Sk-Hep-1 and Huh7 cell lines used were re-authenticated by DNA fingerprinting analysis using short tandem repeat markers.

GAS5 promoter-reporter and GAS5 expression vector constructs

The fragment about 300bp including the insert allele of rs145204276 in the upstream of GAS5 transcript start point was directly synthesized by Genewiz Company (Suzhou, China) and cloned into Hind III and Bgl II sites of pGL3-basic (Cat # E1751, Promega) yielding the wild-type vector (pGL3-GAS5-WT). The mutant-type vector (pGL3-GAS5-MT) including the delete allele of rs145204276 was generated using QuikChange Lightning Site-Directed Mutagenesis Kit (Cat # 210518, Stratagene). The resulting constructs were verified by direct sequencing. The mature lncRNA GAS5 sequence (651-bp, NR_002578.2) was synthesized and subcloned into

pCDH-CMV-MCS-EF1-copGFP vector to construct the GAS5 expression vector pCDH-CMV-MCS-EF1-copGFP-GAS5.

Knockdown of GAS5

For depletion of GAS5, three small interfering RNAs targeting different regions of human GAS5 were designed and commercially synthesized (RiboBio, Guangzhou, China). The sequences of three small interfering RNAs were shown in [Supplementary Table S1](#), available at *Carcinogenesis* Online. Scrambled sequence was used as a negative control. Cells were transiently transfected with 100 nmol of these small interfering RNA sequences using Lipofectamine 2000.

Flow cytometry

Cell apoptosis was detected by using the PE Annexin V apoptosis kit (BD Biosciences). Briefly, cells were plated in 6-well plates (2×10^5 cells/well), after 24 h treatment with pCDH-CMV-MCS-EF1-copGFP-GAS5, cells were double stained with Annexin V-FITC and 7-aminoactinomycin D and incubated at room temperature for 15 min in dark. Flow cytometer (FACS Calibur, Becton-Dickinson) was used to detect apoptosis of the transfected HCC cell lines by determining the relative amount of Annexin V-FITC-positive-7-aminoactinomycin D-positive cells. Each experiment was repeated at least three times with three replicates for each group.

Western blot

Total protein was obtained from HCC cell lines and approximately 50 μ g was separated on a 12% polyacrylamide gel followed by transferring to a polyvinylidene difluoride membrane (GE Healthcare) and probed with primary antibodies against caspase3 (Cat # sc-65496, 1:1000, Santa Cruz Biotechnology), Bcl-2 (Cat # sc-7382, 1:1000, Santa Cruz Biotechnology) and glyceraldehydes 3-phosphate dehydrogenase (Cat # sc-48167, 1:2000, Santa Cruz Biotechnology) and secondary antibodies (1:2500, Santa Cruz Biotechnology). Enhanced chemiluminescence system was used to visualize the bands and the intensity of the bands (Cell Signaling Technologies).

Luciferase reporter assay

Cells were seeded at a density of 1×10^5 cells/well in 24-well plates (Cat # 3524, Corning). Sixteen hours later, cells were transfected by Lipofectamine 2000 (Cat #11668-019, Invitrogen) according to manufacturer's protocol. In each well, 500 ng reconstructed vector pGL3-GAS5-WT or pGL3-GAS5-MT and 50 ng pRL-CMV vector (Promega) were cotransfected. The empty pGL3-basic vector cotransfected group was regarded as a negative control. Twenty-four hours after transfection, cells were harvested immediately after addition of 100 ml passive lysis buffer. Firefly luciferase activity in cell lysate was measured with the Dual Luciferase assay system (Cat #E1910, Promega) in FilterMax F5 and normalized with the Renilla luciferase activity. Each experiment was repeated at least three times with six replicates for each group.

DNA methylation by pyrosequencing

The bisulfite conversion was carried out with 50 ng extracted DNA using the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's protocol. The methylation status of the GAS5 promoter region was assayed by quantitative bisulfite pyrosequencing. The bisulfite converted DNA was amplified using PyroMark PCR Kit (QIAGEN) with primers GAS5-F and GAS5-R. A total of 25 μ l final reaction mixture consisted of 12.5 μ l PyroMark PCR Master Mix (QIAGEN), 2.5 μ l CoraLoad Concentrate, 5 μ l Q-solution, 0.2 μ mol/l of each primer and 50 ng DNA. The reaction began with 95°C for 15 min followed by 45 cycles of denature at 94°C for 30 s, annealed at 56°C for 30 s, extended at 72°C for 30 s and finally extended at 72°C for 10 min. Three independent PCR replicates were performed for each sample and the biotin labeled products were sequenced using the PyroMark Q96 ID system. Three primers (GAS5-S1, GAS5-S3 and GAS5-S4) were used in pyrosequencing and PyroMark CpG software 1.0.11.14 was used to exclude samples that did not meet the bisulfite conversion criteria for quantification of methylation.

Statistical analysis

The genotype distribution was analyzed by Hardy-Weinberg equilibrium using chi-square test. Logistic regression was used to analyze the association

between rs145204276 and HCC risk, adjusted by gender, age, smoking, drinking, tumor stage and hepatitis B virus (HBV) infection. The relative expression levels of GAS5 in tissue samples among different genotypic groups were compared using non-parametric Mann-Whitney *U*-test. Student's *t* test was used to examine the difference of the luciferase activity. The methylation status of HCC tissues with variant genotypes were analyzed by one-way analysis of variance. When necessary, Bonferroni correction for multiple comparisons was performed. These statistical analyses were implemented with Statistic Analysis System software (version 8.0, SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significance and all statistical tests were two-sided.

Results

The associations of rs145204276 with HCC susceptibility

The demographic characteristics of HCC patients and controls recruited in this study were shown in [Table 1](#). There were no differences in the frequency based on age, gender, smoking and drinking status between HCC patients and controls. As expected, the HBsAg positive rate was 69% in the patients, whereas 11% in the controls, suggesting that HBV infection was an obvious risk factor for HCC. Data from sequencing and genotyping assays for rs145204276 were presented in [Figure 1](#). The observed genotypic frequencies of rs145204276 in the controls were in Hardy-Weinberg equilibrium ($P > 0.05$). As shown in [Table 2](#), in either a single case-control set or pooled analysis, the ins/del or del/del genotype had a significantly increased risk of HCC as compared with the ins/ins genotype under a codominant model (adjusted odds ratio: 1.24 and 2.09, respectively). Similar results were also observed in other genetic models ([Table 2](#)). Moreover, the 5-bp deletion allele was associated with a 36% increased risk in pooled analysis (odds ratio: 1.36, 95% confidence interval: 1.19-1.55). Collectively, these results suggest an association of rs145204276 with susceptibility to HCC.

The genotype-phenotype correlation between rs145204276 and GAS5 expression

Next, we further examined the expression of GAS5 in HCC tissue samples with different genotypes. As illustrated in [Figure 2](#), the relative GAS5 expression level in samples with del/del genotype was significantly higher than that in samples with ins/del and ins/ins genotype (fold change, 2.55).

The influence of rs145204276 on GAS5 transcription activity

Due to the location of rs145204276 (at 268-bp upstream of GAS5 transcription start site) and variability of the expression level in HCC tissues with different genotypes, we wanted to know whether the differential promoter polymorphism as a regulatory mechanism may contribute to genotype-phenotype correlation. We utilized luciferase transfection system to examine the effects of the polymorphism on the promoter activity. As predicated, we found that the luciferase activity of the cells transfected with pGL3-GAS5-WT or pGL3-GAS5-MT was higher than that of cells transfected with control constructs. Furthermore, the luciferase activity of the cells transfected with pGL3-GAS5-MT was higher than that of the cells transfected with pGL3-GAS5-WT in all of the three cell lines tested ([Figure 3](#)). Taken together, these data indicate an effect of the promoter polymorphism on GAS5 transcription activity.

The effect of overexpression/knockdown of GAS5 on apoptosis in HCC lines

To next determine the role of overexpressed GAS5 in apoptosis of HCC cells, flow cytometry analysis and western blot were performed. The results from flow cytometry analysis revealed that

Table 1. Demographic characteristics of HCC cases and controls recruited from Suzhou and Shanghai during 2010–13

Characteristics	Suzhou		Shanghai		Overall	
	Case (n = 604)	Control (n = 634)	Case (n = 430)	Control (n = 420)	Case (n = 1034)	Control (n = 1054)
Age (mean ± SD)	54.5±9.7	53.9±10.1	52.8±10.5	53.1±9.9	53.7±9.9	53.5±10.0
Gender						
Male	399 (0.66)	418 (0.66)	267 (0.62)	260 (0.62)	666 (0.64)	678 (0.64)
Female	205 (0.34)	216 (0.34)	163 (0.38)	160 (0.38)	368 (0.36)	376 (0.36)
Smoking status						
Non-smokers	441 (0.73)	476 (0.75)	297 (0.69)	307 (0.73)	738 (0.71)	783 (0.74)
Former smokers	97 (0.16)	76 (0.12)	65 (0.15)	59 (0.14)	162 (0.16)	135 (0.13)
Current smokers	66 (0.11)	82 (0.13)	68 (0.16)	54 (0.13)	134 (0.13)	136 (0.13)
Drinking status						
Non-drinkers	381 (0.63)	431 (0.68)	249 (0.58)	273 (0.65)	630 (0.61)	704 (0.67)
Light drinkers	151 (0.25)	165 (0.26)	116 (0.27)	84 (0.20)	267 (0.26)	249 (0.24)
Heavy drinkers	72 (0.12)	38 (0.06)	65 (0.15)	63 (0.15)	137 (0.13)	101 (0.10)
Tumor stages						
Ia+Ib	428 (0.71)		298 (0.69)		726 (0.70)	
IIa+IIb	133 (0.22)		82 (0.19)		215 (0.21)	
IIIa+IIIb	43 (0.07)		50 (0.12)		93 (0.09)	
HBsAg						
Positive	417 (0.69)	51 (0.08)	292 (0.68)	38 (0.09)	709 (0.69)	89 (0.08)
Negative	187 (0.31)	583 (0.92)	138 (0.32)	382 (0.91)	325 (0.31)	965 (0.92)

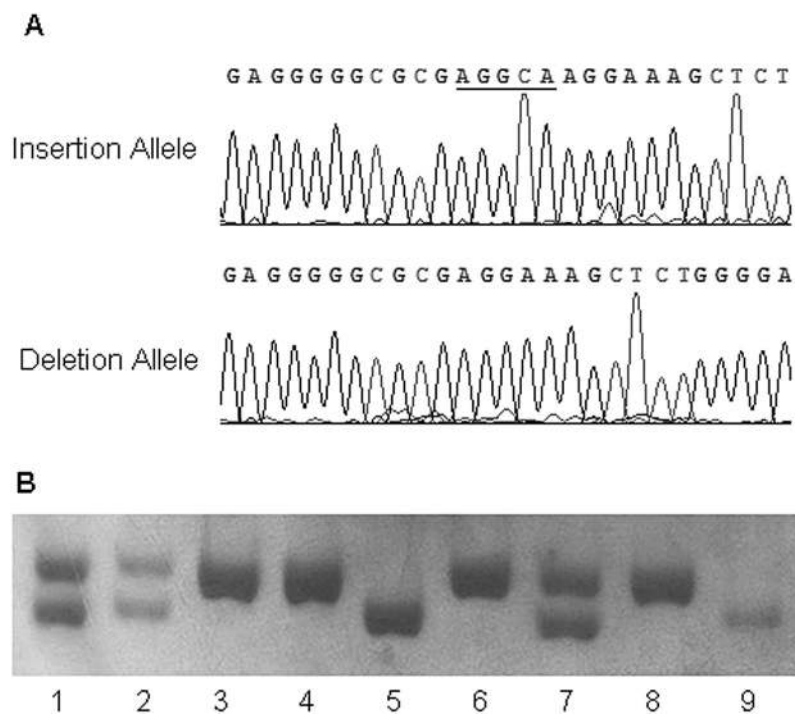


Figure 1. Example output from sequencing and genotyping assays of rs145204276. The upper (A) panel shows the sequence of insertion and deletion allele. The underlined base-pairs indicate the “AGGCA” insertion. The lower panel (B) displays the genotyping results by using 7% non-denaturing polyacrylamide gel electrophoresis (PAGE) and silver staining (lane 5 and 9, del/del genotype; lane 1, 2 and 7, ins/del genotype; remaining lanes, ins/ins genotype).

the rate of apoptosis in GAS5-overexpressed groups was significantly lower than that in control group ($36.52 \pm 0.97\%$ versus $47.95 \pm 1.26\%$, $P < 0.001$; **Figure 4A**). Moreover, western blot results showed the expression level of caspase 3 (an apoptosis-associated key player) was lower in GAS5-transfected group than in control group (**Figure 4B**). In contrast, the expression level of Bcl-2 (a survival-associated crucial mediator) was higher in GAS5-transfected group than that in control group (**Figure 4C**). Therefore, these results indicated that overexpression of GAS5 inhibits apoptosis

of HCC. To further evaluate the specificity of GAS5 on HCC cell apoptosis, a GAS5 gene silence group was also included. As shown in **Figure 4D** and **Figure 4E**, with the knockdown of GAS5, the anti-apoptosis effect of GAS5 could be at least partially reverted.

The methylation status of GAS5 promoter region in different genotypes

To further investigate the association between the genotype of rs145204276 and methylation rate of GAS5 promoter region,

Table 2. Associations between rs145204276 and HCC susceptibility in Suzhou and Shanghai case control sets recruited during 2010–13

	Genetic model	Genotype	Cases	%	Control	%	OR (95% CI) ^a	P value
Suzhou	Codominant model	ins/ins	242	40.1	299	47.2	1.00 (Reference)	
		ins/del	282	46.7	285	45.0	1.23 (0.96–1.56)	0.089
		del/del	80	13.2	50	7.9	1.95 (1.29–2.95)	7.45 × 10 ⁻³
			<i>P</i> _{trend}					9.91 × 10 ⁻⁴
	Dominant model	ins/ins	242	40.1	299	47.2	1.00 (Reference)	
		ins/del + del/del	362	59.9	335	52.8	1.34 (1.06–1.68)	0.012
	Recessive model	ins/ins + ins/del	524	86.8	584	92.1	1.00 (Reference)	
		del/del	80	13.2	50	7.9	1.76 (1.19–2.60)	2.8 × 10 ⁻³
	Additive model	ins allele	766	63.4	883	69.6	1.00 (Reference)	
		del allele	442	36.6	385	30.3	1.32 (1.12–1.57)	1.02 × 10 ⁻³
Shanghai	Codominant model	ins/ins	172	40.0	205	48.8	1.00 (Reference)	
		ins/del	198	46.0	183	43.6	1.30 (0.97–1.75)	0.068
		del/del	60	14.0	32	7.6	2.25 (1.36–3.72)	6.89 × 10 ⁻⁴
			<i>P</i> _{trend}					7.25 × 10 ⁻⁴
	Dominant model	ins/ins	172	40.0	205	48.8	1.00 (Reference)	
		ins/del + del/del	258	60.0	215	51.2	1.44 (1.09–1.91)	7.96 × 10 ⁻³
	Recessive model	ins/ins + ins/del	370	86.0	388	92.4	1.00 (Reference)	
		del/del	60	14.0	32	7.6	1.97 (1.22–3.17)	2.96 × 10 ⁻³
	Additive model	ins allele	542	63.0	593	70.6	1.00 (Reference)	
		del allele	318	37.0	247	29.4	1.41 (1.14–1.73)	9.21 × 10 ⁻⁴
Overall	Codominant model	ins/ins	414	40.0	504	47.8	1.00 (Reference)	
		ins/del	480	46.4	468	44.4	1.23 (1.02–1.48)	0.024
		del/del	140	13.5	82	7.8	2.10 (1.54–2.87)	1.10 × 10 ⁻⁶
			<i>P</i> _{trend}					2.53 × 10 ⁻⁷
	Dominant model	ins/ins	414	40.0	504	47.8	1.00 (Reference)	
		ins/del + del/del	620	60.0	550	52.2	1.36 (1.14–1.63)	4.82 × 10 ⁻⁴
	Recessive model	ins/ins + ins/del	894	86.4	972	92.2	1.00 (Reference)	
		del/del	140	13.5	82	7.8	1.89(1.40–2.54)	1.11 × 10 ⁻⁵
	Additive model	ins allele	1308	63.2	1476	70.0	1.00 (Reference)	
		del allele	760	36.8	632	30.0	1.36 (1.19–1.55)	3.53 × 10 ⁻⁶

CI, confidence interval; OR, odds ratio.

^aAdjusted for age, sex, smoking status, drinking status, tumor stage and HBV infection.

we analyzed the methylation status of the CpG islands in the promoter region of GAS5 in different genotypes. Based on the predicted results from PyroMark CpG software (version 1.0.11), there were totally 14 potential CpG sites within this region. As seen in Figure 5, there were no differences in the mean methylation index in most of the CpG sites among three genotypes. However, in the seventh site next to the indel polymorphism rs145204276, the mean methylation index of del/del genotype was markedly higher than that of either ins/del genotype or ins/del genotype (the mean methylation index was 41.3% for del/del genotype, 24.6% for ins/del genotype, 21.1% for ins/ins genotype; $P < 0.01$, versus ins/del genotype or ins/ins genotype). This difference was still significant after Bonferroni correction for multiple comparisons (corrected α level = 0.017). Together, these results suggested that the indel polymorphism rs145204276 may influence GAS5 transcription activity by an epigenetic mechanism.

Discussion

There is increasing evidence that abnormal expression of GAS5 is involved in many kinds of cancers and its low expression is associated with poor prognosis of gastric cancer, cervical cancer and HCC (12,13,15). Also, SNP in the promoter region of lncRNA IRAIN can regulate the expression level of GAS5 in breast cancer through affecting the methylation status of the CpGs island (18). In this study, we found that the indel polymorphism rs145204276 in the promoter region of GAS5 was associated with susceptibility to HCC and may play its role by influencing one CpG island

methylation status in the GAS5 promoter region, in turn GAS5 transcriptional activity and eventually GAS5 expression level.

GAS5 was identified in a mouse thymoma cell line and further its potential promoter region was suggested (8). Previous studies reported the role of GAS5 in multiple cancers. For example, the low expression level of GAS5 was an indicator of poor prognosis for colorectal cancer, gastric cancer and cervical cancer (12–15). Moreover, the expression level of GAS5 was downregulated in pancreatic cancer, bladder cancer and renal cancer (10,24,25); downregulation of GAS5 attenuates cell responses to apoptotic stimuli including classical chemotherapeutic agents (26,27). In contrast, overexpression of GAS5 inhibits cancer cell proliferation and promotes cancer cell apoptosis (10,24,25). These data suggested a tumor-suppressing role of GAS5. To date, there is no evidence on associations of the polymorphism within GAS5 with the risk for human diseases. In the present study we evaluated the association of the indel polymorphism with the risk of HCC and found that the del allele had a significantly increased risk of HCC in all genetic models. The indel polymorphism (rs145204276) is located within the upstream of GAS5 transcriptional start site where there is a CG rich region. Thus, it is possible that rs145204276 affects the expression level of GAS5 by disturbing its transcript activity and consequently correlates with the risk to HCC. Our results from the expression of GAS5 in different genotypes and luciferase activity supported this possibility. Furthermore, flow cytometry and western blot assay revealed that overexpression of GAS5 leads to an anti-apoptosis effect in HCC cell lines. Taken together, our findings

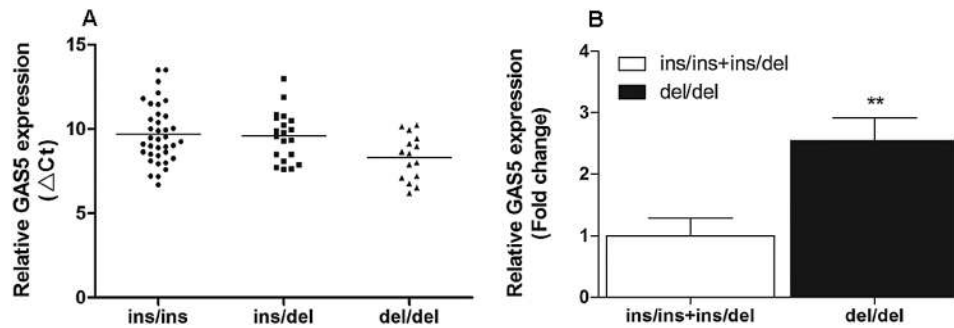


Figure 2. The expression level of GAS5 in HCC tissues with different genotypes. The expression level of GAS5 in HCC tissues with del/del genotype was 2.55-fold higher than that of ins/ins and ins/del genotypes (** $P < 0.01$). ins/ins, $N = 37$, ins/del, $N = 20$, del/del, $N = 15$.

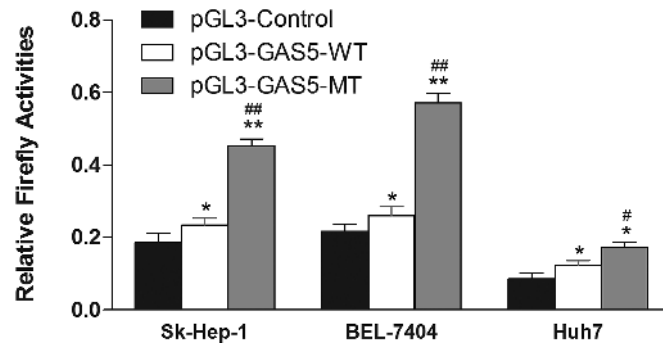


Figure 3. The effect of rs145204276 on GAS5 transcriptional activity as determined by luciferase reporter assay. The relative firefly luciferase activities were compared between insertion construct group and deletion construct group in three cell lines ($\#P < 0.05$, $\#\#P < 0.01$, compared with pGL3-GAS5-WT); In all three cell lines, cells transfected with pGL3-GAS5-WT or pGL3-GAS5-MT exhibited a significantly higher luciferase activity as compared with cells transfected with pGL-3-basic control (* $P < 0.05$, ** $P < 0.01$, compared with pGL-3-basic control).

suggested that GAS5 may act as a proto-oncogene in HCC in contrast with its inhibitory role in other cancer types.

The tumor-promoting role of GAS5 in HCC may reflect the differential roles of GAS5 in carcinogenesis of different types of cancer. Of note, GAS5 expression was low in liver or spleen but abundant in other adult mouse tissues including brain, heart, thymus, lung, kidney and testis (8). The difference may suggest the differential role of GAS5 in carcinogenesis of different tissues. Importantly, gene-environment interactions in different tissues contribute to its paradoxical role. For example, HBV/hepatitis C virus infection, an independent risk factor for HCC, leads to massive inflammation, fibrosis and eventually cirrhosis, accounting for 80% of HCC (28). Tumor necrosis factor is overexpressed in HBV/hepatitis C virus infection and plays an important role in the immune pathogenesis of HCC (29,30). Recently, it was reported that the pro-inflammatory tumor necrosis factor stimulation can up-regulate GAS5 expression in both epithelial and smooth muscle cells in airway (31). Thus, the higher level of GAS5 in HCC may be associated with overexpression of tumor necrosis factor.

Accumulating evidence suggests that SNPs in the promoter region of lncRNA regulates lncRNA expression by influencing the binding of transcription factor or the methylation status of CpG islands (17,32). It is known that methylation imbalance, including hypermethylation of tumor suppressor genes and hypomethylation of oncogenes (33,34), is one obvious character of cancer. In our study, we found that the methylation of the seventh CpG site (adjacent to rs145204276) located in the promoter region of GAS5 was much higher in del/del genotype as compared with ins/del or ins/ins genotype. Based on our *in vitro* experiments and GAS5 expression in HCC tissues, we propose that hypermethylation in del/del genotype is linked to an

increased expression of GAS5. Hypermethylation of CpG islands usually associates with low transcriptional activity with occasional exceptions. For instance, one report demonstrated that in some conditions hypermethylation activates transcription: new methylation of CpG sites generates active transcription start site; the hypermethylation status disturbs the bind of repressor elements, leading to active transcription; the hypermethylation status results in the transcription start site from one to another, causing active transcription (35). Therefore, the indel polymorphism in the promoter region of GAS5 may play its role by these mechanisms. Further studies are needed to address this observation.

Finally, one limitation in this study should be emphasized. Although we observed a strong correlation between rs145204276 and GAS5 expression, as well as between GAS5 transcriptional activity and CpG methylation status, how genetic variability at this locus can influence methylation status of the GAS5 promoter still need to be further investigated and discussed. In addition, it should be taken into account of the possibility that there are other possible presence of CpG sites in this regulatory region that can be influenced by rs145204276. Genome wide DNA methylation studies on normal and pathological conditions are now warranted to confirm our data.

In summary, we provided evidence that rs145204276 may play a functional role in carcinogenesis of HCC by regulating methylation status of the CpG island in the promoter region of lncRNA GAS5 and subsequently affecting the expression of GAS5. Furthermore, in contrast with the tumor-suppressing role of GAS5 in other cancers, GAS5 may act as a proto-oncogene in HCC. Therefore, GAS5 may be a promising marker for personalized diagnosis and therapy for HCC. The replications of our studies in other populations as well as further systematic

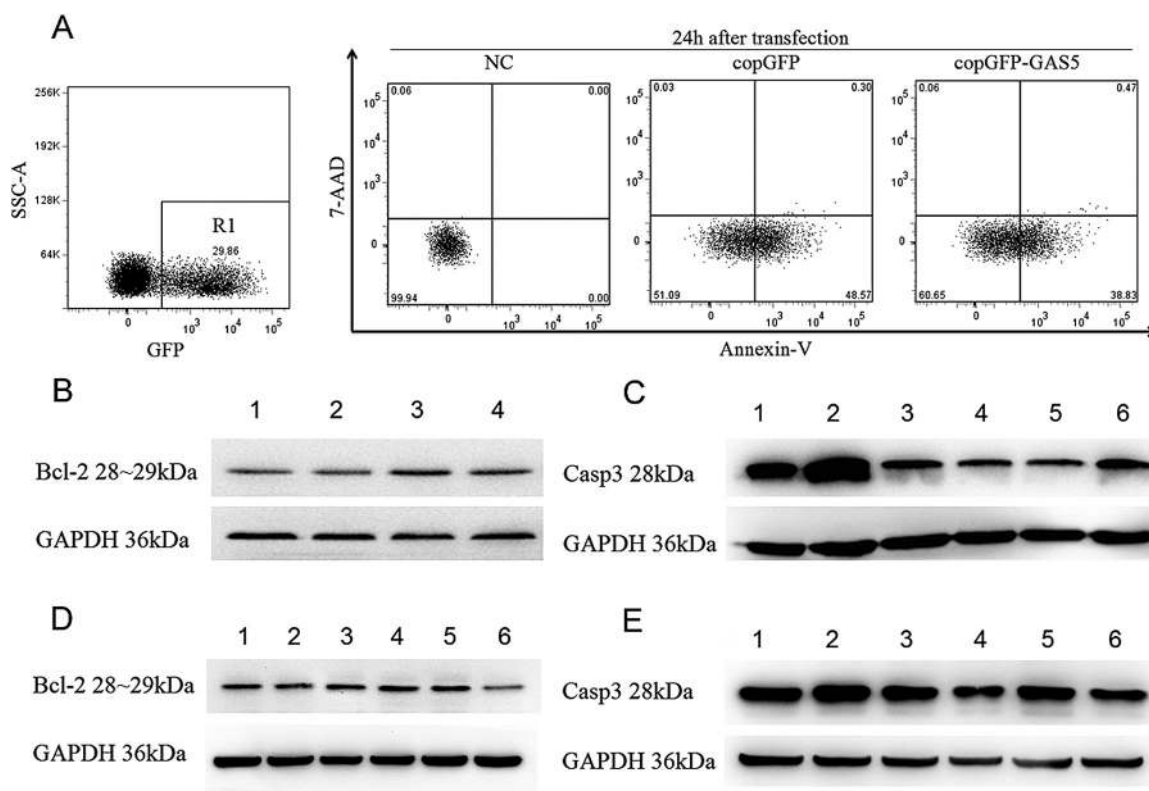


Figure 4. The influence of GAS5 on cell apoptosis as evaluated by flow cytometry and western blot. **A.** Flow cytometry analysis of cell apoptosis. Cells (1×10^5 /well) were transfected with copGFP or copGFP-GAS5 for 24h, and then harvested and double-stained with Annexin V and 7-aminoactinomycin D (7-AAD). Next, apoptotic cell (Annexin V+ and 7-AAD cells) percentage was determined by flow cytometry. The cells analyzed were GFP+, as shown in the R1 region. Data are representative of three independent experiments. **B.** Western blot analysis of Bcl-2 expression level in sk-hep-1 cell line overexpressed GAS5. Line 3 and 4 represent GAS5 transfected group; line 1 and 2 represent the control group. **C.** Western blot analysis for the expression level of caspase3 in sk-hep-1 cell line in the GAS5 transfected group and the empty vector transfected group. Line 1, 2 and 3 represent control group; line 4, 5 and 6 represent GAS5 transfected group. **D.** Bcl-2 expression level in sk-hep-1 cell line with small interfering RNA (siRNA)-GAS5. Line 1, 2 and 3 represent siRNA-GAS5 group; Line 4, 5 and 6 represent negative group. **E.** Caspase3 expression level in sk-hep-1 cell line with siRNA-GAS5. Line 1, 2 and 3 represent siRNA-GAS5 group; Line 4, 5 and 6 represent negative group.

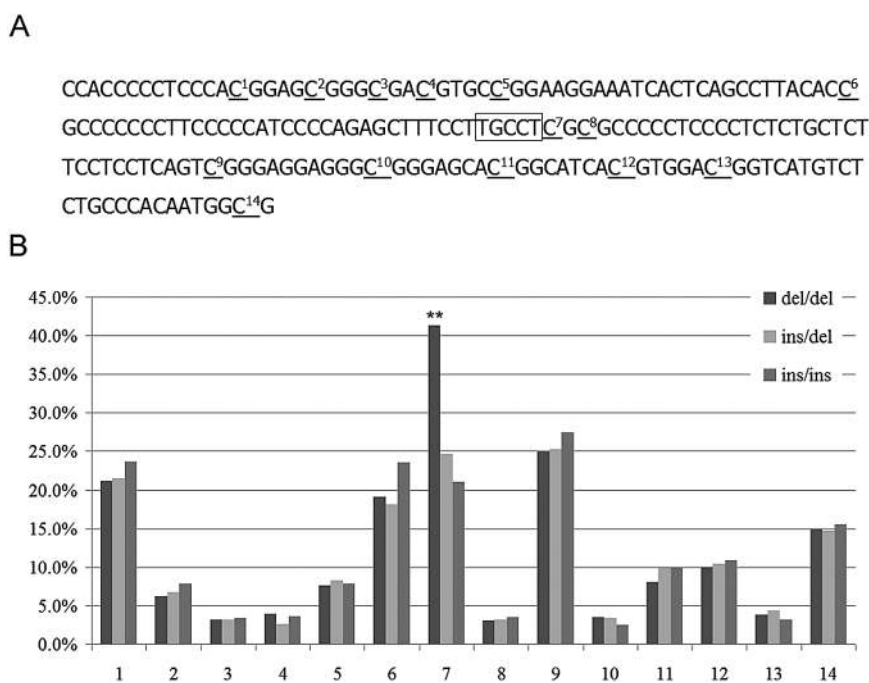


Figure 5. The effect of rs145204276 on methylation status of the GAS5 promoter region. **A.** The potential CpG sites (1-14) within the GAS5 promoter region. The underlined basepairs indicates the exact locations. **B.** The mean methylation index in most of the CpG sites was undifferentiated among three genotypes. However, the mean methylation index on site 7 (adjacent to rs145204276) of del/del genotype is significantly higher than that of both ins/del genotype and ins/ins genotype (**P < 0.01).

investigations are needed to clarify the molecular mechanisms underlying GAS5 regulation.

Supplementary material

Supplementary Table S1 can be found at <http://carcin.oxfordjournals.org>

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