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Original Article

Functional indel polymorphism within lncRNA GAS5 and colorectal carcinoma risk

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Abstract: LncGAS5 was formerly identified as a tumor suppressor gene, involved in multiple cancers. GAS5 was recently reported to be an indicator for CRC progression and prognosis, but its role in colorectal carcinogenesis was still unclear. The current case-control study was to investigate the association of the indel polymorphism rs145204276 within promoter of GAS5 and CRC susceptibility. Statistical analysis showed that when compared with genotype ins/ins, ins/del or del/del genotype upregulated CRC susceptibility (OR=1.33, 95% CI=1.08-1.62, *P*=0.006 and OR=2.09, 95% CI=1.50-2.91, *P*=0.006) in the codominant model, and other genetic models betrayed similar tendency. The positive genotype and phenotype correlation was identified by *in vivo* analysis, and gain-of-function assay *in vitro* found the modulation was modulated through influencing GAS5 transcription activity. In addition, stratified analysis found the association was more prominent in cases with higher tumor stage. Taken together, we firstly reported that a new functional indel polymorphism could modulate CRC risk by affecting GAS5 transcription activity. Further support from other similar investigations and functional validations are required for our current study.

Keywords: GAS5, CRC, risk, indel polymorphism, association

Introduction

Colorectal carcinoma (CRC) still claimed over 600,000 deaths globally in the recent decade whereas great progress was made on investigation of CRC mechanism [1]. Recent reports on CRC demonstrated that genetic variations were involved in colorectal carcinogenesis [2, 3], and genetic variations within protein-coding genes were continuously identified based on candidate genes analysis method. However, non-coding genes and non-coding RNAs attracted little attention until the genome-wide association study (GWAS) method was applied in the last decade [4].

Non-coding RNAs especially long non-coding RNAs (lncRNAs) were reported to play a crucial role in cancer pathogenesis and metastasis [5], and more and more aberrantly expressed lncRNAs were identified in human CRC tissues [6]. Several functional polymorphisms within lncRNAs have been reported, including rs944289 for papillary thyroid carcinoma [7], rs10680577 for hepatocellular carcinoma (HCC) [8], and rs145204276 for HCC [9]. A latest report of functional polymorphism identified in CRC strengthens the conception that genetic polymorphisms within lncRNAs involved in CRC pathogenesis [10].

LncRNA GAS5 was known to be a tumor repressor in multiple cancers including CRC [11-14], its effect mainly was on cell proliferation repression and poor diagnosis prediction. The newly identified functional polymorphism (rs145204276) within lncRNA GAS5 was found to have association with HCC [9], but its association with CRC was not elucidated. Thus, we performed a hospital-based case-control study to analyze the correlation between rs145204276 and CRC risk in Chinese populations and validated the possible modulating manner by Gain-of-function assay.

Table 1. The clinicopathological characteristics of the subjects enrolled in the study

Characteristics	Cases N (%)	Controls N (%)	*P*-value
Total	813	926	
Age (Mean ± SD)	58.9±10.1	59.3±9.2	0.34
Gender			
Male	591 (72.7)	693 (74.8)	0.32
Female	222 (27.3)	233 (25.2)	
Tumor site			
Colon	380 (46.7)	-	
Rectum	433 (53.3)	-	
Tumor stage			
I	79 (9.7)	-	
II	240 (29.5)	-	
III	311 (38.3)	-	
IV	183 (22.5)	-	

Materials and methods

Study populations

The enrolled CRC group consisted of 813 histopathologically confirmed cases diagnosed, hospitalized and treated in the affiliated hospital of Xuzhou medical University from January 2012 to December 2015. All cases underwent no medical treatment before sampling. Clinical tumor stages were judged based on a modified American Joint Committee on Cancer (AJCC) and international union against cancer (UICC) standard. A total of 926 controls were cancer-free individuals selected from a routine physical survey in the same areas during the investigation as CRC counterparts. Tumor tissues from a total of 84 patients with a diagnosis of CRC were collected according to the availability of frozen stored tissues of CRC resections from January 2012 to December 2015. The 84 CRC cases were confirmed by pathologic diagnosis and none of these patients had ever received preoperative chemotherapy or radiotherapy, these 84 patients was a part of 813 cases recruited. Each CRC case signed informed consent for the research. All subjects recruited were Han Chinese with non-kinship. Ethical approval for current investigation was obtained from the Ethical Committee of Xuzhou Medical University.

Genotyping

The genomic DNA was isolated from peripheral vein blood with Genomic DNA purification kit (Qiagen). Amplification of target DNA fragment with the indel polymorphism and allele discrimination was performed as previously reported protocol [9].

RT-qPCR analysis in vivo

Total RNA was isolated from fresh tissue samples with RNA isolation kit (Qiagen). cDNA was generated using random primers and Superscript II reverse transcriptase (Invitrogen). A SYBR Green qPCR was performed using Roche Light Cycler 480 to quantify relative PTPN11 expression in these samples. GAPDH was chosen as the internal control. Primer sequences for GAS5 and GAPDH were as follows: GAS5-F: 5'-AGCTTACTGCTTGAAAGGGTC-3', GAS5-R: 5'-TCTTCTTGTGCCATGAGACTC-3', GAPDH-F: 5'-CTCTCTGCTCCTCCTGTTAC-3', GAPDH-R: 5'-TGAGCGATGTGGCTCGGCT-3'. The amplification protocol and reaction system was performed as previously described [8]. The expression levels of target genes were normalized with GAPDH using a $2^{-\Delta\Delta CT}$ method [15].

Constructs, cell culture and Luciferase assay

Given that the indel polymorphism resided in the promoter region of *lncGAS5*, amplification fragment that had the polymorphism with length at 300 bp was sent to direct synthesis by Sangon Company (Shanghai). Then the wild-type and mutant amplicons were cloned into pGL3-basic (Promega) at the common H*ind* III and B*gl* II restriction enzyme cutting sites respectively and finally named pGL3-WT and pGL3-MT. The sequence orientation was confirmed by direct sequencing.

Human CRC cell lines including HT29, SW480 and HCT116 were cultured in Roswell Park Memorial Institute media 1640 added with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO_2 in cell culture box (Thermofisher).

Cells were seeded at a density of 2.5×10^6 cells/well in 6-well plates (Corning). After 16 hours, cells were transfected by Lipofectamine 2000 (Invitrogen) conformed to manufacturer's protocol. In each well, 500 ng pGL3-WT or pGL3-MT and 50 ng pRL-TK vector (Promega) were cotransfected. pGL3-basic vector with none load was seen as the current negative control. 24 hours after transient transfection, cells were harvested and treated with

Table 2. Association between rs145204276 and CRC risk

Comparison	Cases N (%)	Controls N (%)	OR (95% CI)[a]	*P*-value	*P*-trend
Codominant model					
ins/ins	317 (39.0)	444 (47.9)	1.00 (Reference)		
ins/del	387 (47.6)	409 (44.2)	1.33 (1.08-1.62)	0.006	0.00001
del/del	109 (13.4)	73 (7.9)	2.09 (1.50-2.91)	0.00001	
P_{HWE}		0.11			
Dominant model					
ins/ins	317 (39.0)	444 (47.9)	1.00 (Reference)		
ins/del+del/del	496 (61.0)	482 (51.1)	1.44 (1.19-1.74)	0.0002	
Recessive model					
ins/ins+ins/del	704 (86.6)	853 (92.1)	1.00 (Reference)		
del/del	109 (13.4)	73 (7.9)	1.81 (1.32-2.73)	0.0002	
Additive model					
ins allele	1021 (62.8)	1297 (70.0)	1.00 (Reference)		
del allele	605 (37.2)	555 (30.0)	1.38 (1.20-1.59)	<0.00001	

[a]Adjusted for sex, age, tumor site and tumor stage.

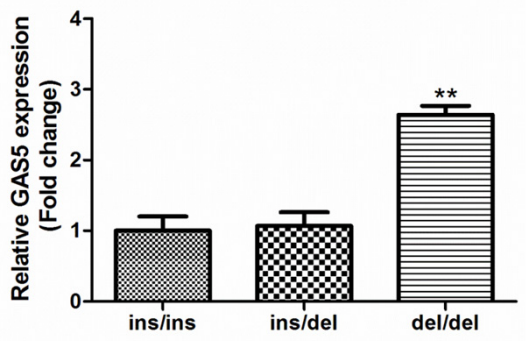

Figure 1. Expression of GAS5 in CRC tissues. $^{**}P<0.01$ compared with ins/ins genotyped tissues.

the protocol from Dual-Luciferase® Reporter Assay System kit (Promega).

Statistics analysis

Genotype distribution in control group was assessed by Hardy-Weinberg equilibrium with χ^2 test. Method of genotypic and allelic frequency comparison between CRC cases and controls was χ^2 test. Unconditional Logistic regression was performed to assess the correlation of the indel polymorphism and CRC risk after adjusted for gender, age, tumor site and tumor stage. Tumor stage was a main indicator for therapy, thus further stratification analysis by tumor stage (I+II, III+IV) for enrolled CRC cases and controls was carried out using binary logistic regression. One-way ANNOVA was used to analyze the difference in Luciferase reporter gene expression. The normalized expression difference of GAS5 in tissues was compared using Student's *t* test. Stratified analysis was applied under four genetic models. The statistical analyses were performed using the statistical software package SPSS 18.0 (SPSS Inc.). $P<0.05$ were considered statistically significant.

Results

Clinicopathological characters of enrolled subjects

Summary of clinicopathological characters was listed in **Table 1**. Distribution of age and gender in both groups demonstrated none obvious deviation ($P=0.34$ and $P=0.32$ respectively), indicating that the case-control study was in appropriate matching status in age and gender. Tumor sites and tumor stage of CRC cases were also collected in the current study. Tumors located at rectum were more popular than at colon, and more than half CRC cases were diagnosed at higher stage (III+IV).

Positive correlation between the indel polymorphism and CRC risk

Genotypic and allelic frequency distribution of the indel polymorphism in both groups in four genetic models was shown in **Table 2**. Genotype distribution in controls was in Hardy-Weinberg equilibrium. As was revealed in codominant model, compared with genotype ins/ins, ins/del or del/del genotype upregulated CRC risk (OR=1.33, 95% CI=1.08-1.62, $P=0.006$ and OR=2.09, 95% CI=1.50-2.91, $P=0.00001$). This tendency was also found in dominant

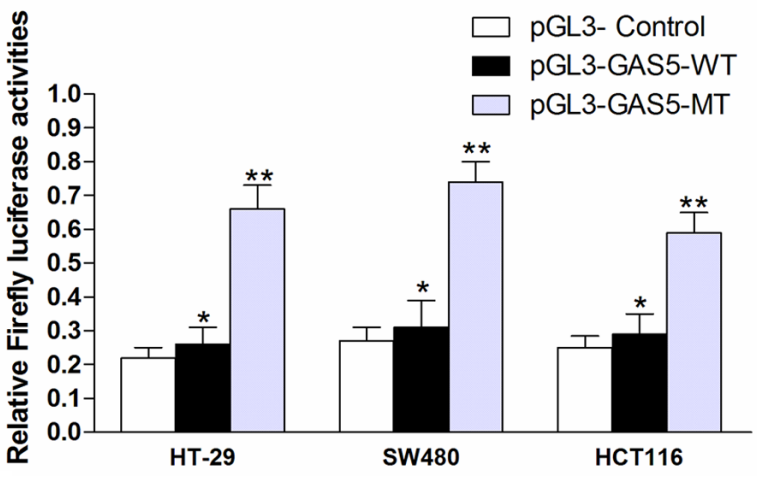

Figure 2. Influence of rs145204276 on transcription activity by Luciferase reporter assay. *P<0.05, **P<0.01 compared with empty pGL3-Basic control.

model and recessive model. Data from additive model proved that the del allele showed 1.38 fold increased CRC risk for ins del (95% CI=1.20-1.59, P<0.00001), furthermore, data of linear-by-linear association analysis exhibited the obvious dose-dependent effect (P_{trend}= 0.00001). In summary, the indel polymorphism rs145204276 was positively correlated with CRC risk.

The indel polymorphism could influence GAS5 expression

We then observed the *GAS5* expression between different genotype groups in CRC tissues. The 84 cases were finally identified as 43 cases for ins/ins, 22 cases for ins/del and 19 cases for del/del. Compared with ins/ins cases, del/del counterparts showed more marked increase (2.53 fold change), while the ins/del counterparts displayed no statistically difference (P>0.05) (**Figure 1**).

rs145204276 influence the transcription activity of GAS5

The differential expression of GAS5 in different genotyped CRC tissues and its location in promoter region suggested that the polymorphism rs145204276 contributed to phenotype change possibly through promoter activity. To validate our hypothesis, we did the dual-luciferase reporter assay and found that the luciferase activity of cells co-transfected with pGL3-WT or pGL3-MT was higher than counterparts with empty pGL3-basic vector (**Figure 2**). The cells with pGL3-MT was more pronounced than cells with pGL3-WT in transcription activity (P<0.01). Thus, we found the polymorphism could influence GAS5 expression by modulating promoter activity.

Stratification analysis of rs145204276 and CRC risk based on tumor stage

The above finding confirmed the positive genotype-phenotype association between rs145204276 and CRC susceptibility, and previous report demonstrated that aberrant expression of GAS5 predicts poor prognosis [14], therefore, we performed stratified analysis based on tumor stage under four genetic models to confirm whether tumor stage had association with the indel polymorphism. As was listed in **Table 3**, we found that cases genotyped with ins/del or del/del higher tumor stage (III+IV) conferred increased CRC risk when comparing with those controls counterparts (OR=1.61, 95% CI=1.27-2.04, P=0.0001 and OR=2.91, 95% CI=2.03-4.19, P<0.00001) in codominant model, the propensity was similar in the other three genetic models. However, counterparts of lower tumor stage (I+II) showed no statistical relationship with the polymorphism. Taken together, the correlation of the polymorphism rs145204276 and CRC risk was more prominent in cases with higher tumor stage (III+IV).

Discussion

The conception that cancer was caused by gene-environment interaction prevailed in recent decades, and more and more oncogenes and tumor suppressor genes were identified with the progress of technical innovation in scientific research. To date, profound understanding based on an insight to carcinogenesis still can't explain all types of malignant tumors. The perspective that genomic instability including polymorphisms contributed to tumorigenesis provided a new strategy for completely elucidating tumorigenic mechanism, and appearance of GWAS was a catalyst for the potential causative polymorphisms screening in large scale [16]. However, not all potential polymorphisms were recruited in the genechip, and high cost restricted sample num-

Table 3. Stratified analysis on the association between rs145204276 and CRC risk based on tumor stage

Comparison	Cases N (%)	Controls N (%)	OR (95% CI)[a]	*P*-value	*P*-trend
	Cases (I+II)	Controls			
Codominant model					
ins/ins	150 (47.0)	444 (47.9)	1.00 (Reference)		0.64
ins/del	140 (43.9)	409 (44.2)	1.01 (0.78-1.32)	0.92	
del/del	29 (9.1)	73 (7.9)	1.17 (0.74-1.88)	0.54	
Dominant model					
ins/ins	150 (47.0)	444 (47.9)	1.00 (Reference)		
ins/del+del/del	169 (53.0)	482 (51.1)	1.04 (0.81-1.37)	0.78	
Recessive model					
ins/ins+ins/del	290 (90.9)	853 (92.1)	1.00 (Reference)		
del/del	29 (9.1)	73 (7.9)	1.17 (0.75-1.83)	0.48	
Additive model					
ins allele	440 (69.0)	1297 (70.0)	1.00 (Reference)		
del allele	198 (31.0)	555 (30.0)	1.05 (0.88-1.26)	0.62	
	Cases (III+IV)	Controls			
Codominant model					
ins/ins	167 (33.8)	444 (47.9)	1.00 (Reference)		<0.00001
ins/del	247 (50.0)	409 (44.2)	1.61 (1.27-2.04)	0.0001	
del/del	80 (16.2)	73 (7.9)	2.91 (2.03-4.19)	<0.00001	
Dominant model					
ins/ins	167 (33.8)	444 (47.9)	1.00 (Reference)		
ins/del+del/del	327 (66.2)	482 (51.1)	1.80 (1.44-2.26)	<0.00001	
Recessive model					
ins/ins+ins/del	414 (83.8)	853 (92.1)	1.00 (Reference)		
del/del	80 (16.2)	73 (7.9)	2.26 (1.65-3.19)	<0.00001	
Additive model					
ins allele	581 (58.8)	1297 (70.0)	1.00 (Reference)		
del allele	407 (41.2)	555 (30.0)	1.64 (1.39-1.91)	0.01	

[a]Adjusted for age, sex, tumor site.

bers enrolled in GWAS, thus the disadvantages prevented us from full discovery of potential polymorphisms. GWAS reports on CRC were a booster for CRC mechanism investigation [17], but the lack of functional validation was of no vain for further research.

Based on the advantages and disadvantages of GWAS, it was a necessity to find novel functional polymorphisms in traditional way. In the current investigation, we firstly reported the functional indel polymorphism rs145204276 could modulate CRC risk through influencing transcription activity of adjacent gene *GAS5* in a Chinese population. Previous reports identified GAS5 as a tumor repressor lncRNA, and it correlated with multiple cancers including lung cancer [18], breast cancer [19], prostate cancer [12], renal cell carcinoma [20] and HCC [21]. Furthermore, downregulation of GAS5 in CRC tissues was a predictor for cancer progression and poor prognosis [14]. Nevertheless, the role of GAS5 involved in CRC pathogenesis was still unclear; the underlying cause of GAS5 dysregulation was not reported until now. For the first time, we reported the functional polymorphism could modulate CRC risk by change of its adjacent gene promoter activity. We further found the genotype-phenotype correlation between rs145204276 and CRC risk *in vivo*, and confirmed the connection by gain-of-function assay *in vitro*. Stratified analysis of tumor stage betrayed that the association between rs145204276 and CRC susceptibility was apparently in higher tumor stage. Therefore, based on the results of cur-

rent study, we proposed that the functional polymorphism rs145204276 was a promising biomarker for CRC risk prediction and neoplasm staging, and GAS5 upregulation may be involved in colorectal carcinogenesis.

Notably, we noticed the dual role of GAS5 in CRC: its upregulation increased the CRC risk, while its downregulation indicated cancer progression and poor prognosis. Considering of the formerly identified tumor suppressor role, high expression of GAS5 should attenuate the CRC risk, but the current study revealed the reverse trend. The contradiction may be caused by both genetic factor and environmental factor. In high-fat diets condition, intestinal epithelium will be prone to suffering from DNA damage, and constant DNA damage often lead to apoptosis and chronic inflammation [22, 23]. Cellular DNA damage activated *P53*, and *GAS5* expression was consequently enhanced, thus elevated GAS5-derived snoRNAs played a crucial role in DNA damage response with the cooperation of *P53*. Therefore, GAS5 showed enhanced expression in carcinogenesis, and low expression of GAS5 reflected p53 dysregulation and tumors cells escaped from p53-mediated tumor surveillance, thus, downregulated GAS5 was an obvious indicator for cancer progression and prognosis.

Trend of the cancer susceptibility within the current investigation was similar with the previous association investigation on HCC [9], this may due to same ethnicity of enrolled subjects and possible identical manner of pathogenesis. However, stratification analysis in the current investigation found more remarkable correlation between the functional polymorphism rs145204276 and CRC risk, while the report on HCC had no related data.

Present study was carried out only in Xuzhou Chinese Han population, compelling support from multiple centers was required in the future validation. The functional assay in the current study needs further confirmation from other research centers, and more similar investigations in other cancers or in other populations of different ethnicities was warranted.

In brief, the current study firstly reported that the functional indel polymorphism rs145204276 could modulate CRC risk in a Chinese Han population by influencing lncGAS5 transcription activity, the correlation was more pronounced in CRC cases with higher tumor stage. GAS5 was involved in CRC pathogenesis and its dual role in CRC was to be fully elucidated. More similar investigations and further functional validation are needed for the current study.

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Disclosure of conflict of interest

None.

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