BRIEF REPORT

Association of 758 G/A polymorphism of 3'untranslated region of prohibitin with risk of gastric cancer

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Background: A polymorphic variant allele (T-allele) in the 3'-UTR of prohibitin (C-to-T at nucleotide 729) was reported to be associated with an increased risk of breast cancer. However, the association between the 3'-UTR polymorphism of prohibitin and the susceptibility to gastric cancer remains unknown. Thus, we investigated the distribution of prohibitin genotypes in Chinese patients with gastric cancer and subsequently analyzed the association between the 3'-UTR polymorphism of prohibitin and the risk of gastric cancer in that population.

Methods: The distribution of 3'-UTR polymorphism of prohibitin in 82 gastric cancer patients was determined by sequencing and compared with that of 171 healthy controls. Luciferase reporter assay was used to investigate the effect of 3'-UTRs variant on PHB expression.

Results: Our study discovered two major polymorphic sites in the 3'-UTR of prohibitin (C-to-T at nucleotide 729 and G to A at nucleotide 758). The C/T polymorphism at 729 site was not associated with the increased risk of gastric cancer ($P=.961$, $OR=1.044$, $95\%CI: 0.187-5.818$); however, G/A polymorphism at nucleotide 758 increased the risk of gastric cancer ($P=.017$, $OR=1.923$, $95\%CI: 1.119-3.305$). Luciferase reporter constructs containing the 758A allele showed higher luciferase activity compared with the wild-type allele, which indicated that 758 G>A in 3'-UTR increased PHB expression.

Conclusions: The G to A transition but not the C-to-T transition in the 3'-UTR of prohibitin was associated with an increased risk of gastric cancer in Chinese population.

KEYWORDS

3'-UTR, cancer risk, gastric cancer, polymorphism, prohibitin

Gastric cancer, the fourth most common cancer, is prevalent in East Asia, Eastern Europe, and particularly in China. The prognosis of gastric cancer is generally rather poor with 5-years relative survival below 30% in most countries.¹ The prohibitin (PHB) gene, located in chromosome region 17q21, is ubiquitously expressed in a wide range of organisms, such as bacteria, plant, yeast, protozoans, and mammals.² Prohibitins are a highly conserved protein family that plays specific roles in cell cycle control, differentiation, senescence, and

anti-proliferative activity.³ Prohibitin was originally thought to be a negative regulator of cell proliferation and a tumor suppressor because its RNA, encoded by the 3'- untranslated region (UTR), arrested cell proliferation between the G1 and S cell cycle phases in tumor cells.⁴ Further studies have shown that a C-to-T transition within the 3'-UTR (at nucleotide 729) of the PHB gene increased the risk of breast cancer in North American and Polish women.^{5,6} However, subsequent studies have suggested that a polymorphism in the PHB 3'-UTR was not associated with a risk of ovarian cancer or breast cancer.⁷⁻⁹

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Previously, we reported that both the protein and mRNA levels of PHB were increased in cancerous gastric tissues compared with adjacent normal gastric tissues. The elevated expression of PHB was correlated with the degree of differentiation in gastric cancer.¹⁰ The specific role of PHB overexpression in gastric cancer cell differentiation remains uncertain; however, PHB might play an important role in the development or progression of gastric cancer. Thus, we studied the potential relationship between PHB polymorphisms and the risk of gastric cancer.

Given the abnormal expression of PHB in gastric cancer, we investigated the distribution of the PHB 3'-UTR polymorphism in Chinese gastric cancer patients and healthy individuals and analyzed the association between PHB 3'-UTRs and the susceptibility to gastric cancer.

Eighty-two tumor tissue samples were collected from gastric cancer patients treated at the Affiliated Putuo Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai, China. The study subjects were gastric cancer patients who lived in the urban area of Shanghai, China. Among these individuals, 52 cases were men, and 30 cases were women; 37 cases were under the age of 56 years, and 45 cases were above 56 years of age; and 31 cases were poorly differentiated cancer, and 51 cases were moderately differentiated cancer. Blood samples obtained from 171 healthy individuals were used as controls. Among these individuals, 85 cases were men, and 86 cases were women; and 79 cases were under the age of 56 years, and 92 cases were above 56 years of age. The protocol was approved by the institutional ethics committee.

Genomic DNA was isolated from the tumor and control tissue samples using a Genomic DNA Extraction Kit (Takara, Japan). Total DNA was used as a template for subsequent PCRs. The following primers were designed based on the PHB sequence obtained from GenBank (no. U49725): 5'-GAGGAAGTGGTCTGTCTGTAC-3' (Forward primer) and 5'-TTCCTCCCAGTGCAGGCATAG-3' (Reverse primer). These primers were used to amplify the reported mutation region. The PCR was performed for 35 cycles (each cycle was conducted at 98°C for 10 seconds, 60°C for 15 seconds, and 68°C for 60 seconds), followed by a final extension at 72°C for 10 minutes. The PCR products were separated on agarose gels, and the corresponding bands were excised and purified for the sequencing analysis. Sequencing was conducted using reverse primers on the ABI 3730xl DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA) using ABI BigDye Terminator V3.1 Kit (Applied Biosystems). Genotypes were determined using Chromas software.

The pmirGLO Dual-Luciferase vector containing both the firefly luciferase gene and the Renilla luciferase gene was purchased from Promega (Promega, Madison, WI, USA). The PHB 3'-UTR sequence (758A allele or wild-type allele) was amplified from control samples. The amplified DNA fragments were subcloned into the 3'-UTR region downstream of the firefly luciferase gene of the pmirGLO vector. Prior to transfection, BGC823 cells were plated onto 24-well plates at a density of 50 000 cells/well and grown overnight. The cells were subsequently transfected with 1 µg/well of the pmirGLO-PHB construct using Lipofectamine 3000 (Invitrogen, Shanghai, China). After 24 hours, the luciferase activity was assayed using a dual-luciferase

reporter assay system (Promega) on a luminometer (GloMax 20/20; Promega). Renilla luciferase activity was normalized to the corresponding firefly luciferase activity and presented as fold-expression to the control.

The frequency distribution of genotypes was examined for the cases and controls. The deviation of genotype frequencies from Hardy-Weinberg equilibrium was assessed in the controls using a chi-square test, and the associations are expressed as odds ratios (ORs) with 95% confidence intervals (95% CIs). We used a chi-square test for multivariate regression analysis to assess the primary effects of the genetic polymorphism on gastric cancer risk, assuming a recessive model of inheritance. All statistical calculations were performed using SPSS 16.0. We analyzed the Linkage Disequilibrium for 82 cases and 171 controls, showing $r^2=.131$ and $D'=0.818$, indicating that there was no linkage disequilibrium.

In the present study, tissues and control were used in the present study. There was no significant difference in the distribution of patients and the controls of age and gender ($P>.05$). Direct PCR sequencing analysis revealed two major polymorphic sites in the 3'-UTR of prohibitin (C-to-T at nucleotide 729 and G to A at nucleotide 758). The frequencies of C/C, C/T, and T/T genotypes at nucleotide 729 were 62 (75.6%), 18 (22.0%) and 2 (2.4%), respectively, in gastric cancer patients whereas the frequencies were 129 (75.4%), 38 (22.2%), and 4 (2.3%), respectively, in the healthy controls. The frequencies of G/G, G/A, and A/A genotypes at nucleotide 758 were 19 (23.2%), 25 (30.5%), and 38 (46.3%), respectively, in gastric cancer patients, whereas the frequencies were 37 (21.6%), 81 (47.3%), and 53 (31.0%), respectively, in the healthy controls. The C/T polymorphism at nucleotide 729 was not associated with the increased risk of gastric cancer ($P=.961$, OR=1.044, 95%CI: 0.187-5.818, Table 1) but the G/A polymorphism at nucleotide 758 increased the risk of gastric cancer ($P=.017$, OR=1.923, 95%CI: 1.119-3.305, Table 1). Furthermore, these data showed that patients carrying the A/A genotype at nucleotide 758 were more likely to carry the C/C genotype at nucleotide 729 than the controls, indicating that the C/C+ A/A genotype increased the risk of developing gastric cancer ($P=.017$, OR=1.935, 95%CI: 1.122-3.335).

Previous studies have indicated that PHB expression is increased in gastric cancer tissues.¹⁰ Thus, we further investigated the impact of this variant on PHB expression. The wild-type (containing the G-allele) and mutant (containing the A-allele) PHB 3'-UTRs were cloned into the dual-luciferase pmirGLO reporter vector. Luciferase assays showed higher luciferase activity of mutant construct compared with the wild-type plasmid (1.62 vs 1.33, $P=.028$), indicating that the 758 G>A mutation in the 3'-UTR increased the transcription of PHB mRNA.

Research have indicated that PHB expression is increased in cancerous tissues, including both gastric cancer¹⁰ and colorectal cancer,¹¹ but the mechanism underlying PHB overexpression remains uncertain. Jupe and colleagues proposed that the increased PHB expression might reflect a mutation in the 3'-UTR in an effort to produce additional 3'-UTR suppressors.⁴ Because microRNAs (miRs) play an important role in gene regulation, we analyzed the conjugation of microRNAs at nucleotide 758 in the 3'-UTR of prohibitin using bioinformatics. We observed that nucleotides 745-765 were potential targets

TABLE 1 The 3'-UTR polymorphism of PHB and the association with the risks of gastric cancer

Genotype	Controls (N=171) (%)	Cancers (N=82) (%)	P-value	OR, 95%CI
Nucleotide 729				
C/C+C/T ^a	167 (97.7%)	80 (97.6%)	.961	1.044 (0.187-5.818)
T/T	4 (2.3%)	2 (2.4%)		
Nucleotide 758				
G/G+G/A ^a	118 (69.0%)	44 (53.7%)	.017	1.923 (1.119-3.305)
A/A	53 (31.0%)	38 (46.3%)		

^aReference genotype.

of miR-345-3p. Tang and colleagues reported that colorectal cancer tissues with significantly downregulated miR-345 had increased rates of lymph node metastasis and poorer histological types. In vitro, decreased miR-345 function causes a significant increase in the proliferation of HT29 and SW1116 cells.¹² In addition, miR-345 in whole blood has been reported as a potential biomarker for clinical outcomes, and miR-345 was used as a single prognostic biomarker for both the overall survival and PFS in all patients and those in the non-KRAS mutant population.¹³ We speculated that a mutation at nucleotide 758 in the 3'-UTR of prohibitin inhibited the conjugation of miR-345-3p with this region, causing PHB mRNA overexpression in gastric cancer tissues. Further research is warranted to confirm these hypotheses.

In summary, we identified a novel polymorphism (A/A) at nucleotide 758 in the 3'-UTR of prohibitin, and the elevated frequency of this mutation increased the risk of gastric cancer in the Chinese population. In addition, luciferase assays demonstrated that the 758 G to A mutation in the 3'-UTR increased the transcription of PHB mRNA, suggesting that overexpression of PHB mRNA in gastric cancer might reflect the G to A transition. Therefore, further studies are needed to determine the association between G to A genetic variants and the expression level of PHB in cancerous tissues.

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