Abstract. Protein tyrosine phosphatase non-receptor type 11 (PTPN11) encodes the tyrosine phosphatase SHP-2 that is overexpressed in gastric cancer (GC). In the present study, the association of PTPN11 methylation levels with the incidence of GC and its correlation with SHP-2 overexpression were investigated. The methylation levels of PTPN11 in tumor and adjacent normal tissues of 112 GC patients were assessed by quantitative methylation specific PCR (qMSP). The Cancer Genome Atlas (TCGA) public database was used to analyze the association between PTPN11 methylation and PTPN11 expression. Survival analyses were conducted in order to evaluate the prognostic value of PTPN11 methylation for GC. The results of the qMSP analysis indicated that the methylation levels of PTPN11 in GC tumor tissues were significantly decreased compared with those noted in the normal adjacent tissues (mean with standard deviation: 40.91±26.33 vs. 51.99±37.37, P=0.007). An inverse correlation between PTPN11 methylation levels and PTPN11 mRNA expression levels (P=4x10^{-6}, r=-0.237) was noted. Subgroup analyses indicated that the association of PTPN11 hypomethylation with the incidence of GC was specific to male subjects (P=0.015), heavy drinking patients (P=0.019), patients with poor tumor differentiation (P=0.010) and patients with tumor node and metastasis (TNM) stage III+IV (P=0.008). Kaplan-Meier analyses and log-rank test suggested that PTPN11 hypomethylation was not associated with GC patient overall survival (P=0.605) and recurrence (P=0.485), although it could predict the recurrence of GC patients up to and including 60 years (≥60, P=0.049). The results indicated that PTPN11 hypomethylation is associated with gastric cancer progression. TCGA data analysis suggested that PTPN11 hypomethylation could cause an upregulation in the transcription levels of PTPN11. Although, this may explain the pattern of SHP-2 overexpression in GC, additional studies are required to verify this hypothesis. The association of PTPN11 hypomethylation with GC incidence may be specific to male patients, heavy drinking patients, patients with poor tumor differentiation and patients with TNM stage of III+IV. PTPN11 hypomethylation can be considered a biomarker for the recurrence of GC patients with an age of 60 years or lower.

Introduction

In developing countries, stomach cancer is the third most frequently diagnosed cancer and is one of the leading causes of cancer-related death (1). The annual incidence and mortality of gastric cancer (GC) in China is estimated to ~679,100 and 498,000 cases, respectively (2). Despite recent advances in chemotherapy for GC, the outcomes of anticancer therapy remain unsatisfactory.

Tyrosine-protein phosphatase non-receptor type 11 (PTPN11) encodes Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2), which participates in multiple intracellular signaling pathways and plays an important role in tumor cell proliferation, apoptosis, invasion, metastasis and drug resistance (3, 4).

H. pylori (Hp) infection is the primary risk factor for GC (5). Previously, evidence suggests that the well-known carcinogenic protein Cag-A of Hp is associated with SHP-2 expression in gastric mucosal epithelial cells. The Cag-A protein is released by Hp, enters epithelial cells via the type IV secretion system and is activated by tyrosine phosphorylation, which enables this protein to acquire the ability to interact with the tumor promoting enzyme tyrosine phosphatase SHP-2. This process is regulated by host cell phosphatase and

PTPN11 hypomethylation is associated with gastric cancer progression

LELE XU1*, CONG ZHOU2*, RANRAN PAN2, JUNJIAN TANG3, JINZHI WANG4, BIN LI2, TIANYI HUANG2, SHIWEI DUAN2 and CHUNFANG XU1

1Department of Gastroenterology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215200; 2Medical Genetics Center, School of Medicine, Ningbo University, Ningbo, Zhejiang 315211; 3Department of Vascular Surgery, Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu 214000; 4Department of Cell Biology, School of Medicine, Soochow University, Suzhou, Jiangsu 215007, P.R. China

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Correspondence to: Dr Chunfang Xu, Department of Gastroenterology, The First Affiliated Hospital of Soochow University, 889 Pinghai Road, Suzhou, Jiangsu 215200, P.R. China

Dr Shiwei Duan, Medical Genetics Center, School of Medicine, Ningbo University, 818 Fenghua Road, Ningbo, Zhejiang 315211, P.R. China

E-mail: duanshiwei@nbu.edu.cn

*Contributed equally

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affects a large number of downstream pathways ultimately leading to decreased adhesion and polarity of epithelial cells (3,6,7). Therefore, SHP-2 is considered one of the key proteins that links Cag-A with gastric cancer. However, only a limited number of patients with Hp-positive chronic gastritis or a peptic ulcer eventually develop into GC (8). This suggests that specific differences may appear between Hp-infected hosts, such as genetic or epigenetic changes associated with the PTPN11 gene, or the differences noted in SHP-2 protein expression.

PTPN11 mutations have been extensively investigated in the past years. Germline mutations in PTPN11 cause Noonan syndrome (9-11) and its clinically related Leopard syndrome (12), whereas somatic mutations of PTPN11 contribute to leukemogenesis (13-17), as well as in the development of specific solid tumors, including neuroblastoma (18,19), metachondromatosis (20,21), brain tumors (22-24), neurofibromatosis (25), optic nerve pilomyxoid astrocytoma (26), breast carcinoma (27,28), colorectal cancer (29,30) and Ewing sarcoma (31). However, oncogenic mutations of PTPN11 are rare in the majority of solid tumors including GC (32,33).

Previous studies have detected the presence of PTPN11 polymorphisms in GC (34-36). However, these PTPN11 polymorphisms were shown to be associated with gastric atrophy instead of GC in Chinese and Japanese subjects (37,38). These findings indicated that with the exception of mutations and polymorphisms, additional abnormal expression changes in the PTPN11 gene were involved in the development of GC.

Previously, the role of DNA methylation in the study of GC has received increasing attention in the identification of GC has received increasing attention in the identification of GC formation (39). However, the association between PTPN11 methylation and GC has not been reported to date. Therefore, the current study aimed to investigate the contribution of PTPN11 methylation in GC.

Materials and methods

Study subjects. A total of 112 GC patients (mean, 56.56; range, 21-83 years), including 76 male and 36 female patients, were recruited in the First Affiliated Hospital of Soochow University between December 2010 and April 2014. Gastric mucosa tissues of the primary tumor site and the corresponding adjacent normal tissues (5 cm away from the tumor) were collected from the patients. During this period, the GC patients were followed up and their survival data was collected. The patients were diagnosed by pathological examination and none of them received radiotherapy or chemotherapy prior to surgical resection. All patients who participated in the present study had signed an informed consent form. The study was approved by the Ethics Committee of Ningbo University.

DNA extraction, bisulphite conversion and sequencing. Total DNA was extracted from the tissue samples by the EZNA™ Tissue DNA kit (Omega Bio-Tek, Inc.) and its concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Bisulphite treatment was achieved using the EZ DNA Methylation-Gold kit™ (Zymo Research Corp.). Typically, 500 ng of the original DNA sample was denatured by NaOH and bisulphite was used to convert the unmethylated cytosine to uracil, while the methylated cytosine remained unchanged (40). The total volume of the reaction was 30 µl. In addition, a part of the bisulphite-converted products were randomly selected for Sanger sequencing to verify the specificity of the quantitative methylation specific PCR (qMSP) assay.

SYBR green-based qMSP. The SYBR green-based qMSP used β-actin (ACTB) as an internal control. The qMSP reaction consisted of 10 µl SYBR Green I Master mix (Roche Diagnostics), 1 µl primers and 1.0 µl bisulphite-modified DNA template (10 ng/µl). The reaction volume was made up to 20 µl by addition of ddH2O. The primer sequences used in the qMSP assays were the following: 5'-GAGGTTCCGG AGATAGTAGGTAAT-3' for the PTPN11 forward primer, 5'-GATTTCATTCTTCTGGTCCCAAA-3' for the PTPN11 reverse primer, 5'-TGTTGATGGAGGGTTTAGTAAG T-3' for the ACTB forward primer and 5'-AACCAATAAAC CTACTCTCTTTAATA-3' for the ACTB reverse primer. The primers used in the present study were designed by the Primer Premier 5.0 software (PREMIER Biosoft Inc.). The designed primers were evaluated using Oligo 6.0 software (DBA Oligo Inc.) and NCBI primer-blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The PCR reactions were initiated at 95°C for 10 min, followed by 45 cycles at 95°C for 30 sec and 58°C and 72°C for 30 sec. A melting curve analysis was used at 95°C for 15 sec and at 58°C for 1 min. Subsequently, the temperature was increased every sec for 0.11°C until it reached 95°C. The percentage of methylated reference (PMR) was calculated so as to represent the PTPN11 methylation levels. The equation used was as follows: PMR=2−ΔΔCq ×100%, ΔΔCq=sample DNA (CqPTPN11- CqACTB)-fully methylated DNA (CqPTPN11- CqACTB)] (41).

Bioinformatic analysis. The methylation levels of PTPN11 and the expression profiles of this gene [Stomach Adenocarcinoma, The Cancer Genome Atlas (TCGA), Provisional] were available from cBioPortal (http://www. cbioportal.org/). The data comprised 478 GC samples from which DNA was extracted. The samples were used to evaluate the correlation between PTPN11 methylation and mRNA expression levels. A total of 177 samples were derived from subjects with Hp infection. The PTPN11 methylation levels were obtained from the TCGA gastric cancer database, including three tumor Hp(+), 17 tumor Hp(-), 58 non-tumor Hp(+) and 99 non-tumor Hp(-) samples.

Statistical analysis. SPSS 16.0 software (SPSS Inc.) was used for statistical analysis. The normal distributed data were described as mean ± standard deviation, and the non-normal distributed data were described as median with interquartile ranges. The paired sample t test was used to assess the differences in the methylation levels between tumor and adjacent normal tissues. The data were analyzed following subgroup analysis. The spearman rank test was used to assess the association between PTPN11 methylation levels and PTPN11 expression levels. Kaplan-Meier and log-rank test analyses were applied to assess patient survival. A non-parametric Mann-Whitney U test and two independent sample t-tests were used to calculate differences in PTPN11 methylation in Hp-infected samples and non-Hp-infected samples. A
two-sided P<0.05 was considered to indicate a statistically significant difference.

**Results**

Verification of experimental reliability. The specificity of primers in this experiment was verified by Oligo 6.0 software and NCBI primer-blast tool. The evaluation indicated that the primers were methylation-specific. Each of the upstream and downstream primers used in the present study contained one CpG cytosine site and multiple non-CpG cytosine sites (Fig. 1A). The SYBR-green qMSP product formed a single dissolution curve at ~76°C, suggesting that the qMSP product exhibited a uniform melting temperature (Fig. 1B). In addition, the qMSP product was further analyzed by automated capillary electrophoresis and the results indicated a single band of 85 bp, confirming that the amplified qMSP products were homogenous (Fig. 1C). To further verify the specificity of the primers, random qMSP products were selected for Sanger sequencing and the results confirmed that the bisulfite conversion of the DNA template was complete (Fig. 1D). Therefore, all the aforementioned quality control results indicated that the qMSP process was unlikely to amplify fragments that were incompletely converted.

PTPN11 hypomethylation exists in GC and upregulates PTPN11 expression. The results indicated that the PTPN11 promoter was significantly hypomethylated in GC tissues compared with its corresponding methylation levels in the adjacent normal tissues [mean with standard deviation (SD): 40.91±26.33 vs. 51.99±37.37, P=0.007, Fig. 2A]. In addition, PTPN11 expression data was extracted from 478 GC samples present in the TCGA database of Stomach Adenocarcinoma. The results indicated an inverse correlation between PTPN11 methylation and PTPN11 expression (P=4x10^-6, r=-0.237, Fig. 2B).

Results of the subgroup analyses. Subgroup analyses by different clinical phenotypes were performed to compare...
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The data demonstrated that PTPN11 hypomethylation was specific to male subjects (39.44±25.93 vs. 52.06±37.10, P=0.015) and the patients with history of heavy drinking (36.01±21.16 vs. 60.07±46.66, P=0.019, Fig. 3A). In addition, the association of PTPN11 hypomethylation with GC was specific to patients with low/no tumor differentiation (39.97±26.01 vs. 50.96±35.90, P=0.010), positive lymphatic metastasis (39.45±26.20 vs. 53.34±38.49, P=0.002), and TNM stage III+IV (38.62±25.72 vs. 49.91±36.00, P=0.008). TNM, tumor, node, metastasis; LN, lymphatic metastasis; T, tumor tissue; N, adjacent normal tissue.

Figure 2. PTPN11 hypomethylation in the current study and the inverse correlation between PTPN11 methylation and mRNA expression in the TCGA data mining study. (A) Comparisons of PTPN11 methylation between tumor and adjacent normal tissues revealed that PTPN11 was hypomethylated in gastric cancer (40.91±26.33 vs. 51.99±37.37, P=0.007), the plot described presents the mean with standard deviation. (B) The expression data comprising 478 GC samples were extracted from the TCGA database of the Stomach Adenocarcinoma (TCGA, Provisional). An inverse correlation between PTPN11 methylation and mRNA expression levels was found (P=3.8x10^-6, r=-0.237). PTPN11, tyrosine-protein phosphatase non-receptor type 11; TCGA, The Cancer Genome Atlas.

Figure 3. Subgroup analysis by clinical characteristics. (A) Subgroup tests by gender and drinking history. (B) Subgroup tests by gastric cancer differentiation, LN and TNM stage. The plots are presented as mean with standard deviation. The P-value was calculated by the paired-samples t test. A significant P-value was indicated in the following subgroups: Male subjects (39.44±25.93 vs. 52.06±37.10, P=0.015), heavy drinking subjects (36.01±21.16 vs. 60.07±46.66, P=0.019), low/no differentiation (39.97±26.01 vs. 50.96±35.90, P=0.010), positive LN (39.45±26.20 vs. 53.34±38.49, P=0.002) and TNM stage III+IV (38.62±25.72 vs. 49.91±36.00, P=0.008); TNM, tumor, node, metastasis; LN, lymphatic metastasis; T, tumor tissue; N, adjacent normal tissue.
PTPN11 vs. normal tissues were defined as the hypomethylation cohort. Compared with those noted in the adjacent normal tissues were defined as the hypermethylation cohort, whereas those with lower methylation levels in tumor tissues were defined as the hypomethylation cohort. PTPN11, tyrosine-protein phosphatase non-receptor type 11.

Hypomethylation cohort aged ≤60 tends to have a higher recurrence rate. During a seven-year follow-up of 112 GC patients, five patients were lost to follow-up and 34 patients did not survive. The groups that exhibited higher tumor methylation levels compared with those noted in the adjacent normal tumors were defined as the hypermethylation cohort. The subjects that exhibited lower methylation levels in tumor vs. normal tissues were defined as the hypomethylation cohort. Kaplan-Meier analysis indicated no statistical significance between PTPN11 methylation levels and overall survival (P=0.484) or tumor recurrence (P=0.485). However, when stratified by age, the hypomethylation cohort aged ≤60 years demonstrated a higher recurrence rate of GC (mean recurrence: 25.03 vs. 22.25 months, P=0.049; Fig. 4A). In addition, no significant association was noted between PTPN11 methylation levels and GC recurrence in different methylation cohorts aged >60 years (mean recurrence: 22.19 months vs. 22.76 months, P=0.289, Fig. 4B).

Association between Hp infection and PTPN11 hypomethylation. To further investigate the association between Hp infection and PTPN11 hypomethylation, the data from the samples and those from the TCGA database were analyzed (eight CpGs, Fig. 5A). In China, the detection of Hp is not a routine assay used in the screening of gastric cancer. In the present study, the samples were isolated from 9 patients with Hp, including one Hp(+) patient and eight Hp(-) patients. The data indicated that the tumor Hp(+) tissues exhibited decreased PTPN11 methylation levels compared with those noted in the non-tumor Hp(+) tissues (PMR: 46.88 vs. 53.62). Similarly, the tumor Hp(-) tissues exhibited lower PTPN11 methylation levels than the non-tumor Hp(-) tissues although the difference was not significant (PMR: 43.59±27.90 vs. 50.76±31.21, P=0.664, Fig. 5B). In addition, the methylation levels of eight CpG sites from the TCGA database with Hp infection were compared (Fig. 5A). The analysis demonstrated that the Hp infection status in the tumor samples was not associated with the levels of PTPN11 methylation. However, Hp infection was associated with hypermethylation of 2 PTPN11 CG sites in non-tumor tissues (cg09337511: P=0.027, cg24032304: P=0.016). A total of four PTPN11 CG sites were noted that exhibited significant hypomethylation in the tumor Hp(-) compared with the corresponding non-tumor Hp(+) tissues, (cg10069827: P=0.003, cg08573574: P=0.011, cg09337511: P=0.023, cg27541540: P=0.0003).

Discussion

Deregulation of SHP-2 (a protein encoded by PTPN11) by the Hp-related protein Cag-A can lead to GC risk (3). Although ~70% of patients with GC are Hp-positive, only 1-2% of patients with chronic gastritis or Hp-positive peptic ulcer eventually develop GC (42). The expression levels of the SHP-2 protein were increased in Hp(+) GC patients, although the differences noted were not significant (43). In the current study, it was demonstrated that PTPN11 was hypomethylated in GC, which has not been previously reported. Since abnormal gene methylation always interferes with expression (44), it was hypothesized that hypomethylation of PTPN11 may be one of the mechanisms involved in the development of GC. PTPN11 is a specific gene, which exhibits a two-sided effect in cancer progression (45). PTPN11 has been characterized as a tumor suppressor gene (46,47) in liver cancer and as a proto-oncogene in leukemia (45). PTPN11 expression was increased in leukemia (48), breast cancer (49) and in thyroid tumors (50), whereas it was decreased in colon (51) and liver cancer (46,52). Previous studies have shown an increase in PTPN11 mRNA and protein levels in GC (43,53,54), suggesting
that PTPN11 may be a proto-oncogene in GC. In the present study, the results indicated that PTPN11 was hypomethylated in GC. In addition, TCGA data analysis revealed an inverse correlation between PTPN11 methylation and PTPN11 expression. These findings may explain the decreased methylation pattern and the high expression profile of PTPN11 in GC.

In addition, subgroup analysis indicated that PTPN11 hypomethylation was specific for male subjects and GC patients with a history of heavy drinking. Chinese men were more likely to suffer from GC and the male mortality rate in China was ~twice that noted in Chinese women (2). Heavy drinking is a risk factor for GC (55). Therefore, whether PTPN11 hypomethylation occurs only in male subjects and heavy drinkers requires further studies. In addition, it was found that PTPN11 hypomethylation was specific for poorly differentiated GC patients and TNM III+IV GC patients. Patients with advanced TNM staging exhibited a poor prognosis (56). Poorly differentiated cancer cells are also a feature of advanced GC. Therefore, the present study hypothesized that PTPN11 hypomethylation may be associated with the progression of GC, which can be further studied in the future.

Previous studies have shown that PTPN11 overexpression indicates poor prognosis in liver cancer patients (57). The PTPN11 rs2301756 polymorphism has been shown to be associated with decreased risk of GC and with an improved response to chemotherapy (34). In addition, the gene panel containing PTPN11 in colorectal cancer and oral squamous cell carcinoma has a high prognostic value (58,59). The 3-year survival rate of GC patients with high SHP-2 expression was significantly decreased compared with patients with low SHP-2 expression and the postoperative recurrence mortality of high SHP-2 expression was also significantly increased compared with patients with low SHP-2 expression (53). The correlation between the methylation status of the PTPN11 gene and the prognosis of GC has not been reported previously. Therefore, a 7-year follow-up of GC patients was performed in the current study and the parameters survival time and postoperative recurrence time were assessed. Although the current analysis indicated that PTPN11 methylation exhibited no prognostic value on the survival and recurrence of patients with GC, following stratification by age, it was shown that the hypomethylation cohort with an average age ≤60 years exhibited a higher recurrence rate of GC. SHP-2 abnormalities were associated with tumor cell proliferation, invasion and metastasis (3). Young cancer patients may be more prone to tumor progression due to increased body metabolism compared with elderly cancer patients. Therefore, PTPN11 hypomethylation may be a prognostic indicator for postoperative recurrence of GC patients under 60 years of age.

Several studies have indicated that the SHP-2 protein, which is encoded by PTPN11 is an intracellular target of Cag-A (3,6,8). This protein is a virulence factor of Hp (3,6,8). Recently, Jiang et al (43) demonstrated that although the expression levels of SHP-2 in the gastric cancer Hp(+) group were increased compared with those noted in the gastric cancer Hp(-) group, the differences noted were not statistically
significant. The present study revealed that both Hp infection and tumour status may change the methylation levels of specific \textit{PTPN11} CpG sites. However, only one and three tumor Hp (+) samples were found in the samples used in the present study and in those derived from the TCGA database. Therefore, the present findings require verification in the future with larger sample sizes of subjects with Hp infection.

The present study exhibits certain limitations. Firstly, the samples used and those derived from the TCGA database were not sufficient to ensure a plausible association of GC incidence and \textit{PTPN11} methylation with the status of Hp (positive or negative). Therefore, the correlation between Hp infection and \textit{PTPN11} methylation should be further tested in larger datasets with known Hp infection status. Secondly, although an inverse association between \textit{PTPN11} methylation and mRNA expression was noted by TCGA data analysis, future work is required to evaluate whether \textit{PTPN11} hypomethylation can lead to elevated SHP-2 expression in GC patients.

In summary, the present study indicated that \textit{PTPN11} hypomethylation can be associated with SHP-2 overexpression in GC. Future study is required to verify this hypothesis. Hypomethylation of \textit{PTPN11} may be specific for men, patients with a history of heavy drinking, patients with poor tumor differentiation and patients with TNM III+IV stage GC. In addition, the present study further demonstrated that \textit{PTPN11} hypomethylation could predict recurrence of GC in patients aged ≤60 years.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SD, CX and LX conceived and designed the study and gave the final approval of the submitted version. CZ, RP, JT, JW, BL and TH performed the data analyses and conducted the experiments. CZ contributed to figure preparation. LX collected samples and wrote the paper. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

All patients who participated in the present study had signed an informed consent form. The study was approved by the Ethics Committee of Ningbo University.

Patient consent for publication

Written informed consent was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

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