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• BRIEF REPORTS •

Hypermethylation of Syk gene in promoter region associated with oncogenesis and metastasis of gastric carcinoma

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Abstract

AIM: To investigate the rrelationship between methylation of Syk (spleen tyrosine kinase) gene in promoter region and oncogenesis, metastasis of gastric carcinoma. The relation between silencing of the Syk gene and methylation of Syk promoter region was also studied.

METHODS: By using methylation-specific PCR (MSP) technique, the methylation of Syk promoter region in specimens from 61 gastric cancer patients (tumor tissues and adjacent normal tissues) was detected. Meanwhile, RT-PCR was used to analyse syk expression exclusively.

RESULTS: The expression of the Syk gene was detected in all normal gastric tissues. Syk expression in gastric carcinoma was lower in 14 out of 61 gastric cancer samples than in adjacent normal tissues (χ^2=72.3, P<0.05). No methylation of Syk promoter was found in adjacent normal tissues. hypermethylation of Syk gene in promoter was detected 21 cases in 61 gastric carcinoma patients. The rate of methylation of Syk promoter in gastric carcinoma was higher than that in adjacent normal tissues (χ^2=25.1, P<0.05). In 31 patients with lymph node metastasis, 17 were found with Syk promoter methylation. A significant difference was noted between two groups (χ^2=11.4,P<0.05).

CONCLUSION: Hypermethylation leads to silencing of the Syk gene in human gastric carcinoma. Methylation of Syk promoter is correlated to oncogenesis and metastasis of gastric carcinoma. Syk is considered to be a potential tumor suppressor and anti-metastasis gene in human gastric cancer.

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INTRODUCTION

Gastric cancer development and progression are thought to occur through a complex, multistep process, including oncogene activation and mutation or loss of tumor suppressor genes. Determining the function of genetic alterations in gastric carcinoma tumorigenesis and metastasis has been the focus of intensive research efforts for several decades. One group of proteins that play a critical role in gastric cancer cell signaling pathways is tyrosine kinases. The decrease or loss of spleen tyrosine kinase (Syk) expression seems to be associated with increased motility and invasion of malignant phenotype[1-5].

Syk, a non-receptor type of protein-tyrosine kinase that is widely expressed in hematopoietic cells, is one of the two members of the Syk family (Syk and ZAP-70). Syk is activated upon the binding to its tandem Src homology 2 (SH2) domains to immunoreceptor tyrosine-based activation motif (ITAM) and plays an essential role in lymphocyte development and activation of immune cells. Emerging evidence indicates that Syk may be a potent modulator of epithelial cell growth and a potential tumor suppressor in human breast carcinoma[6,7]. But the role of Syk in gastric carcinoma remains unclear, so we examined Syk mRNA expression in human gastric cancer and the adjacent non-cancerous tissues, and explored whether methylation of Syk promoter region was associated with the loss of Syk expression in gastric cancer and the relationship between Syk mRNA expression in gastric cancer tissue and clinicopathological factors in the present study.

MATERIALS AND METHODS

Tissues

Gastric cancer and matched adjacent non-cancerous tissues were obtained during surgical excision from 61 patients with gastric cancer in our department between March 2001 and October 2002. The age of 38 male and 23 female patients was rang from 26 to 78 (mean, 58.1) years. All samples were placed in liquid nitrogen immediately after resection and stored at -70 ℃ until RNA extraction. No patient had received chemotherapy or radiation therapy prior to surgery. All patients were confirmed to have gastric carcinoma by pathologic test.

RNA extraction and semi-quantitative reverse transcription (RT)–polymerase chain reaction (PCR)

Total RNA was isolated from each specimen by Trizol reagent according to the manufacturer's recommendations. Samples were ground into a fine powder using a mortar and pestle, incubated in Trizol solution (100 g/L) for 15 min, and then 1/5 volume of choloform was added. After vigorous agitation for 5 min, the inorganic phase was separated by centrifugation at 12 000 g for 20 min at 4 ℃, RNA was then precipitated in the presence of 1 volume of isopropanol and centrifuged at 10 000 g for 15 min at 4 ℃. RNA pellets were washed with 700 ml/L ice-cold ethanol and then dissolved in diethyl pyrocarbonate (DEPC) - treated H_2O. Total RNA concentration and quantity were assessed by absorbency at 260 nm by using a nucleic acid and protein. Semi-quantitative RT-PCR of 40 pairs of gastric cancer tissues and 15 pair of benign fibroadenoma tissues was performed for intergrin beta1 displaying expression alterations. Five micrograms of total RNA in each hybridization sample was used to synthesize the first strand cDNA with SuperScript preamplification system for first strand cDNA synthesis kit. Then 1 mL product was used as the template to amplify specific fragments in 25 mL reaction mixture under the following conditions:

denaturation at 94 ℃ (3 min); 40 cycles at 94 ℃ (45 s), at 60 ℃ (45 s) and at 72 ℃ (60 s), then extensions at 72 ℃ (3 min). The PCR primers of Syk were as follow: Syk forward, 5' -CATGTCAAGGATAAGAACATCATAGA-3' ; reverse: 5' -AGTTCACCACGTCATAGTAGTAATT-3' . A 514 bp nucleotide fragment of the human Syk cDNA was amplified. In each PCR reaction, primers for the human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene were used as an internal control. GAPDH primer sequences were as forward: primer 5' -AGAAGGCTGGGGCTCATTTGCAGGG-3' reverse primer 5' -GTCACTGGCGTCTTCACCACCATG-3' . Ten mL RT-PCR reaction product was analyzed by electrophoresis on a 15 g/L agarose gel. The electrophoresis images were scanned by Fluor-S MultiImager and the original intensity of each specific band was quantitated with the software Multi-Analyst. The data were compared after normalized by the intensity of GAPDH. After normalization, the adjusted intensities were calculated for the amplified gene products, and the ratios were calculated. The sequences of PCR primer pairs of intergrin beta1 and GAPDH were designed using Primer3 Internet software program. Their specificity was confirmed by a BLAST Internet software assisted search for a nonredundant necleotide sequence database.

DNA extraction, purification, bisulfite modification and Sequencing

Genomic DNA from cell lines or frozen gastric tissues was extracted by using a Dneasy kit. Genomic DNA was treated with sodium bisulfite. DNA (20 mg/L) was denatured by NaOH (concentration 0.2 mol/L) for 10 min at 37 ℃. Thirty μL of 10 nmol/L hydroquinone and 520 μL of 3 mol/L sodium bisulfate were added, followed by incubation at 50 ℃ for 16 h. The modified DNA was purified using Wizard DNA purification columns. The purified DNA was treated again with NaOH and precipitated. DNA was resuspended in 30 μL of TE buffer (3 mmol/L Tris (P8.0/0.2 mmol/L EDTA) and subjected to PCR amplification using a primer set (forward 5' -GATTAAGATATATTTTAGGGAATATG-3; reverse 5' -CACCTATATTTTATTCACATAATTTC-3) that spanned the Syk CpG island. Fifteen μL reaction containing 30 ng of bisulfite-treated DNA and 1xRDA buffer (67 mmol/L Tris (pH8.8)/16 mmol/L $(NH_4)_2SO_4$, 100 mmol/L 2-mercaptoethanol, 1 g/L BSA) was processed in 30 thermal cycles at 94 ℃ for 45 s, at 58 ℃ for 45 s, and at 72 ℃ for 45 s. One aliquot (2 μL) of diluted PCR Product (40-fold) was subjected to PCR amplification in a 15-μL volume.

Methylation-specificPCR (MSP)

Methylation-specific primers were designed to cover 9 CPG dinucleotides numbered 17-21 (forward) 47-50 (reverse). Similarly, unmethylation-specific primers were designed cover 8 CpG dinucleotides numbered 18-22 b, (forward) and 35-37 (reverse). Primers specific for methylation DNA (forward) 5' -CGATTTCGCGGGTTTCGTTC-3; (reverse) 5' -AAAACGAACGCAACGCGAAAC-3; and unmethylation DNA (forward 5' -ATTTTGTGGGTTTTGTTTGGTG-3, reverse 5' -ACTTCCTTAACACACCCAAAC-3 were added to the same reaction and PCR products were subjected to electrophoresis on a 10 g/L agarose gel. The m-specific primer set yielded a band at 243 bp and the u primer set yielded a band at 140 bp. PCR conditions were 24 cycles at 94 ℃ for 30 s, at 67 ℃ for 30 s, and at 72 ℃ for 30 s.

Statistical analysis

t- test was used to compare the Syk mRNA expression levels with primary gastric cancer, the adjacent non-cancerous tissues. χ^2 test was also used to estimate the relationship between the silencing of Syk mRNA and methylation of Syk promoter region. Values of $P<0.05$ were considered statistically significant.

RESULTS

Expression of Syk in gastric cancer and adjacent non-cancerous tissues

The expression of Syk was found in 14 out of 61 gastric cancer tissues, Syk mRNA expression was detected in all adjacent normal gastric tissue (Figure 1). The rate of expression of Syk in gastric caner tissue was significantly lower than that in adjacent non-cancerous normal gastric tissues ($\chi^2=72.3$, $P<0.05$).

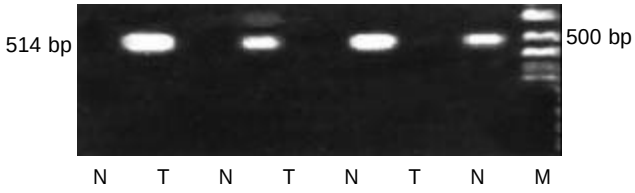

Figure 1 Syk mRNA expression in human gastric cancer and adjacent non-cancerous tissue (RT-PCR detection of Syk mRNA). T: tumor tissue; N: adjacent non-cancerous normal gastric tissue; M: marker.

Syk mRNA expressions were found in 11 out of the 30 gastric cancer patients without lymph node metastasis, but in 3 out of the 31 gastric cancer patients with lymph node metastasis. Expression of Syk mRNA in patients having lymph node metastasis was significantly lower than that in those having no lymph node metastasis ($\chi^2=4.85$, $P<0.05$). Meanwhile no significant difference was found between Syk mRNA expression in the gastric cancer patients and age, tumor size, clinicopathological stage, histological type (data not shown).

Semi-quantitative RT-PCR

A total of 10 μL RT-PCR products were electrophoresed on 15 g/L agarose gel contacting ethidium bromide. The level of GAPDH was used as internal control (Figure 2). The relative expression levels of Syk normalized to the expression level of GAPDH, in the primary gastric cancer tissues and adjacent mammary tissues were, 1.71±1.28 and 3.19±0.59 (mean±SD). A significant difference was found between the level of Syk mRNA expression in the primary gastric cancer tissues and adjacent mammary tissues ($t=2.1$, $P<0.05$). Furthermore, the level of Syk mRNA in patients having lymph node metastasis was significantly lower than that in patients having no lymph node metastasis (1.18±1.13 *vs* 2.14±1.27, $t=3.4$, $P<0.05$).

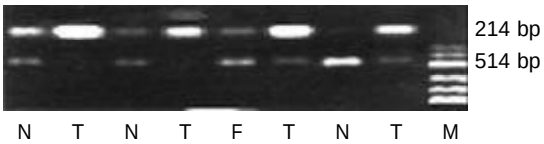

Figure 2 Partial semi-quantitative RT-PCR results in 40 gastric cancer tissues and adjacent non-cancerous tissues. T: tumor tissue; N: adjacent non-cancerous normal gastric tissue; M: marker.

Correlation between methylation of Syk gene in promoter region and oncogenesis, metastasis of gastric carcinoma

MSP was used to analyze the Syk methylation status of gastric carcinoma and its matched normal gastric tissues. Forty nonselective gastric cancers were screened. Among the 61 carcinomas examined, 21 exhibited strong Syk methylation. Representative examples are shown in Figure 3. In contrast to their corresponding carcinomas, the Syk gene of the 61 matched neighboring normal gastric tissues remained unmethylated. The

rate of methylation of Syk promoter in gastric carcinoma was higher than that of adjacent normal tissues (χ^2=25.1, P<0.05).

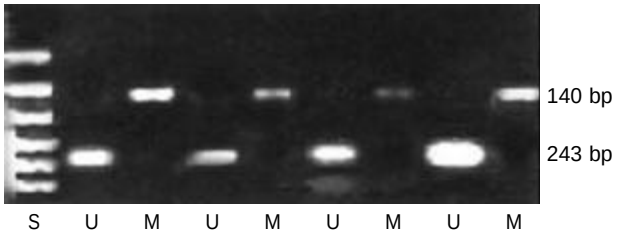

Figure 3 Representative examples of Syk methylation (neighboring normal gastric tissues unmethylated).

As shown in Table 1, of the 31 cases of lymph node metastasis, 17 were found with Syk promoter methylation. Syk promoter methylations in 4 out of 30 without lymph node metastasis were detected. A significant difference was noted between two groups (χ^2=11.4, P<0.05). Correlation between methylation of Syk promoter region and clinicopathologic factors was shown in Table 1.

Table 1 Correlation between methylation of Syk promoter region and clinicopathologic factors (*n*)

Factor	Frequency of Syk promoter region methylation	*P*
Gender		
Male	14 / 38	
Female	7 / 23	0.26
Tumor size		
<3cm	8 / 26	
3	13 / 35	0.61
Depth of invasion		
Mucosa and submucosa	2 / 9	
Muscle and subserosa	7 / 29	
Serosa	12 / 23	0.075
Lymph node metastasis		
Present	17 / 31	
Absent	4 / 30	0.0016
Stage		
I-II	6 / 25	
III-IV	15 / 36	0.24
Tumor location		
Lower third	6 / 15	
Middle third	11 / 35	
Upper third	4 / 11	0.83

Relationship between Syk promoter region hypermethylation and loss of its expression in gastric carcinoma

Syk promoter methylation was found in 21 of 61 gastric tumors. They did not show any detectable Syk. In 40 cases without Syk promoter methylation, Syk mRNA expression was found in 13 cases. Syk promoter methylation was significantly related with loss of Syk expression in gastric carcinoma (χ^2=4.2, P<0.05).

DISCUSSION

Syk is ubiquitously expressed in hematopoietic cells and has been extensively studied as effectors of B cell receptor (BCR) signaling[8-10].It has been found to be involved in coupling activated immunoreceptors to downstream signaling events that mediate diverse cellular responses including proliferation, differentiation and phagocytosis[11-16]. Some reports are of opinions that Syk is a candidated suppressor gene.

In this paper, we explored expression of Syk mRNA in gastric cancer and its adjacent non-cancerous tissues. Our results showed that the level and rate of Syk mRNA expression in gastric cancer tissues were significantly lower than those in adjacent non-cancerous tissues, especially invasive gastric cancer tissues did not show any detectable Syk mRNA expression, indicating that loss of Syk mRNA expression may be correlated to gastric carcinogenesis. Our findings were in agreement with the results of Goodman *et al*.[17,18].

Furthermore, we observed that only four gastric cancer tissues from 31 patients with lymph node metastasis had Syk mRNA expression which was negatively correlated to lymph node metastasis. In B cells, Syk activity was a key regulator of Akt kinase activity after BCR engagement, because Syk induced PI3-K-dependent Akt activation and inhibition of apoptosis[18]. Numerous studies have demonstrated deregulated Akt activity in gastric cancer cells. It is tempting to speculate that Syk may also regulate Akt activity in gastric cells. Syk mRNA expression was reduced during human gastric cancer progression, and then it was unlikely that Syk-dependent signaling contributed to the increased Akt activity observed in gastric cancer cells[19-21]. Findings by Okamura *et al*.[23] suggest that Syk mRNA expression was positively correlated to p53 expression. They found that Syk gene expression was repressed in a *p53*-dependent manner in human colon carcinoma cells, indicating that loss of *p*53 function during tumorigenesis could lead to deregulated Syk activity[7,22]. Study by Mahabeleshwar *et al*.[19] suggested Syk suppressed cell motility and inhibited PI-3 kinase activity and uPA secretion by blocking NF-*k*B activity through tyrosine phosphorylation of I*k*Bα. So loss of Syk expression might contribute to gastric oncogensis and metastasis[18,23].

However, the mechanism of Syk expression loss remains unclear. A number of cancer-associated genes have been shown to be inactivated by hypermethylation of CpG islands during gastric tumorigenesis. About 50% of human genes have clusters of CPG dinucleotides (CPG islands) in their 5' -regulatory sequences. Gene silencing through methylation of these sites has been observed in early developmental stages and aging. Aberrant methylation may lead to deregulation of gene expression. Most notably, tumor suppressor genes, mismatched repair genes, and others such as estrogen receptor, E-cadheria, death-associated protein kinase, and thrombospodin-I, were repressed by CPG island hypermethylation in cancer tissues, inhibition of the transcription of these genes could provide an epigenetic mechanism of clonal selection during tumorigenesis[24,25].

In this study, the 5' CpG island methylation status of the Syk gene in gastric cancer tissues was examined. We found Syk 5' CpG hypermethylation in 35%(21/60) of unselected gastric tumors, whereas all of the matched neighboring normal gastric tissues exhibited unmethylated DNA. We found that the rate of methylation of Syk promoter in gastric carcinoma was significantly higher than that in adjacent normal tissues, suggesting that hypermethylation of Syk promoter region may be associated with oncogenesis of gastric cancer. In addition, the rate of Syk promoter region methylation in the patients with lymph node metastasis is significantly higher than that in the patients without lymph node metastasis, indicating that methylation of Syk promoter methylation is related to lymph node metastasis of gastric cancer. The absence of Syk protein was reflected by the loss of its mRNA expression in gastric cancers, suggesting that the loss of Syk mRNA expression occurs at the transcriptional level. 5' CpG hypermethylation of Syk was reported to be associated with loss or reduction of Syk gene expression, which may provide a new way to treat gastric cancer[26,27].

However, we did not observe any significant correlation between Syk methylation and tumor grade. This may be attributable to a relatively small sample size and the complexity of unselected patient population. Additional detailed studies

using patient cohort should be done needed to examine the value of Syk methylation as a diagnostic or prognostic marker[28,29].

The recent identification of Syk as a potent modulator of gastric epithelial cell growth has generated a need for further exploration of the role of nonreceptor tyrosine kinases in gastric cancer progression and metastasis[28-30]. These initial results offer the promise that novel molecular targets may be identified both for the prevention of gastric cancer development and for inhibition of metastatic gastric cancer spread. The continual elucidation of novel targets such as Syk is a critical part of the endeavor to eradicate gastric cancer. These findings may provide promise molecular targets for the prevention of gastric cancer development[31-33].

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