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Original Article

Relationship between gene mutations and protein expressions of PDGFR α and C-kit in gastrointestinal stromal tumors

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Abstract: Objective: To investigate the relationship between gene mutations and protein expressions of PDGFR α and C-kit in gastrointestinal stromal tumors (GIST) and its significance in tumorigenesis. Methods: Single strand conformation polymorphism-polymerase chain reaction (PCR-SSCP), immunohistochemistry and Western blot were used to detect the gene mutations in PDGFR α and C-kit and their protein expressions in 105 cases of GIST specimens. Results: In 105 cases of GIST, PDGFR α gene mutation was found in 12 cases (11.4%), which was common in the stomach- derived spindle cell GIST. C-kit gene mutation was found in 58 cases (55.2%), which was common in the small intestine. Mutations of PDGFR α is in 12 cases of GIST were stronger than the C-kit mutations in GIST, normal gastrointestinal tissues and schwannomas. No significant correlation was found between mutations and C-kit protein expression ($P>0.05$), while the protein expression of PDGFR α was significantly correlated with mutations ($P<0.0001$). Conclusion: Mutations of PDGFR α and C-kit plays an important role in part of GIST tumorigenesis. Mutation sites were related with original sites and histological types. Most protein expressions were closely related to their gene mutations in GIST.

Keywords: GIST, PDGFR α, C-kit, gene mutation, Western blot, SSCP

Introduction

Gastrointestinal stromal tumor (GIST) is one of the most common stromal tumors in gastrointestinal tract, which is completely different from leiomyoma, leiomyosarcoma, schwannoma and other mesenchymal tumors [1]. Most GISTs express a receptor tyrosine kinase (TK), KIT [2], which encoded by proto-oncogene c-kit [3-6]. In 1998, Hirota et al [7] firstly reported the specific expression of C-kit protein and the acquired functional C-kit gene mutations in GIST. Recent studies found that in a small number of GIST, there were also gene mutations of platelet-derived growth factor receptor α (PDGFR α), and PDGFR α expression can be detected at the level of transcription in most GIST [8, 9]. PDGFR α is a kind of single-chain transmembrane glycoprotein, belonging to the same III type tyrosine protein kinase as C-kit. There are few studies regarding the expression of PDGFR α in GIST, and the relationship between PDGFR α and C-kit mutations and protein expressions remains unclear in GIST. Therefore, we detected the mutations and protein expressions of PDGFR α and C-kit in a group of GIST to analyze their relationships and explore their roles in GIST.

Materials and methods

Materials

Between 2010 and 2014, 105 cases of GIST patients with complete data and clear diagnosis were selected, including 55 males and 50 females, aged 21-76 (median 52.5) years. There were 70 cases of gastric stromal tumors, 33 cases of intestinal stromal tumors and 2 cases of mesenteric stromal tumors. According to the maximum diameter, the tumors can be divided into 0-5.0 cm group (54 cases), 5.0-10 cm group (36 cases), ≥10 cm group (15 cases). there were 70 cases of spindle cell type, 12 cases of epithelial cell type, and 23 cases of mixed type. Patients were all treated with surgi-

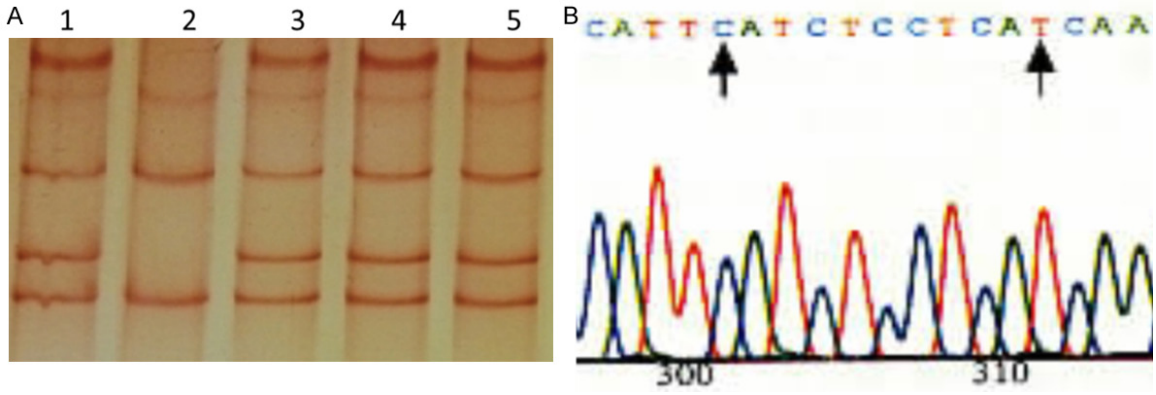

Figure 1. Silver-stained polyacrylamide gel showing the PCR-SSCP patterns of alleles of the C-kit gene amplified from genomic DNA (A), and sequencing results (B).

cal resection. All patients did not receive chemotherapy and radiotherapy before surgery.

Methods

Silver-staining PCR-SSCP

GIST tissue was treated with proteinase K-phenol-chloroform method to extract genomic DNA for PCR. Primer sequences, sizes and annealing temperature referred to the literature (insert) [10]. 10 mL PCR products were mixed with 10 ml 950 g/L formamide denaturing buffer and 30 mL paraffin oil for degeneration. after cooling, the aqueous phase was all loaded on PAGE gel electrophoresis until xylene cyanide reached the bottom of the gel. Gel was immobilized, silver-stained and developed, observed and photographed. Compared with normal controls, SSCP bands with single chain mobility displacement, deletion or multiband indicated the presence of mutations. 50 mL PCR products were recovered, purified and sequenced directly (Shanghai Invitrogen biotechnology Co., Ltd.).

Immunohistochemistry

The paraffin sections were stained by Envision two-step immunohistochemical staining. Rabbit anti-human PDGFR α (1:200) and C-kit polyclonal antibodies (1:100) were purchased from Santa Cruz Company and Dako Company respectively. Envision kit was purchased from Dako Corporation. Positive and negative controls were established in each experiment. Results determination: in the well-organized and clear background, a positive signal was defined as clear yellow or brown, tan particulate staining in the cytoplasm and on the cell membrane of tumor cells. <10% represented negative. 10%-50% represented weakly positive. ≥80% represented strongly positive.

Western blot

Tissues were cracked to extract total protein. Protein quantitation was performed using BCA method. In each lane, 50 mg protein was electrophoresed and transferred to a membrane. Then it was incubated with rabbit anti-human PDGFR α primary antibody and goat anti-human GAPDH primary antibody (1:500, Santa Cruz Inc.) overnight, and then the secondary antibody was added. after membrane-washing, ECL (Santa Cruz Co.) chemiluminescence, exposure to X-ray, development, fixing, and result analysis were performed in order.

Statistical analysis

SPSS 17.0 statistical software was used for the data analysis. The comparison between count data were test using χ^2 test. $P<0.05$ was considered significance.

Results

*PCR-SSCP results (**Figure 1A**)*

The PCR products of PDGFR α exon 18, exon 12 and C-kit exon 11, exon 9 in all specimens were displayed as a single band in 15 g/L agarose gel electrophoresis, and the lengths were 212, 233, 223 and 310 bp. In 105 cases of GIST, PDGFR α mutations were detected in 12 cases (11.4%), including 10 cases of exon 18 mutations and 2 case of exon 12 mutation. C-kit gene mutations were detected in 58 cases

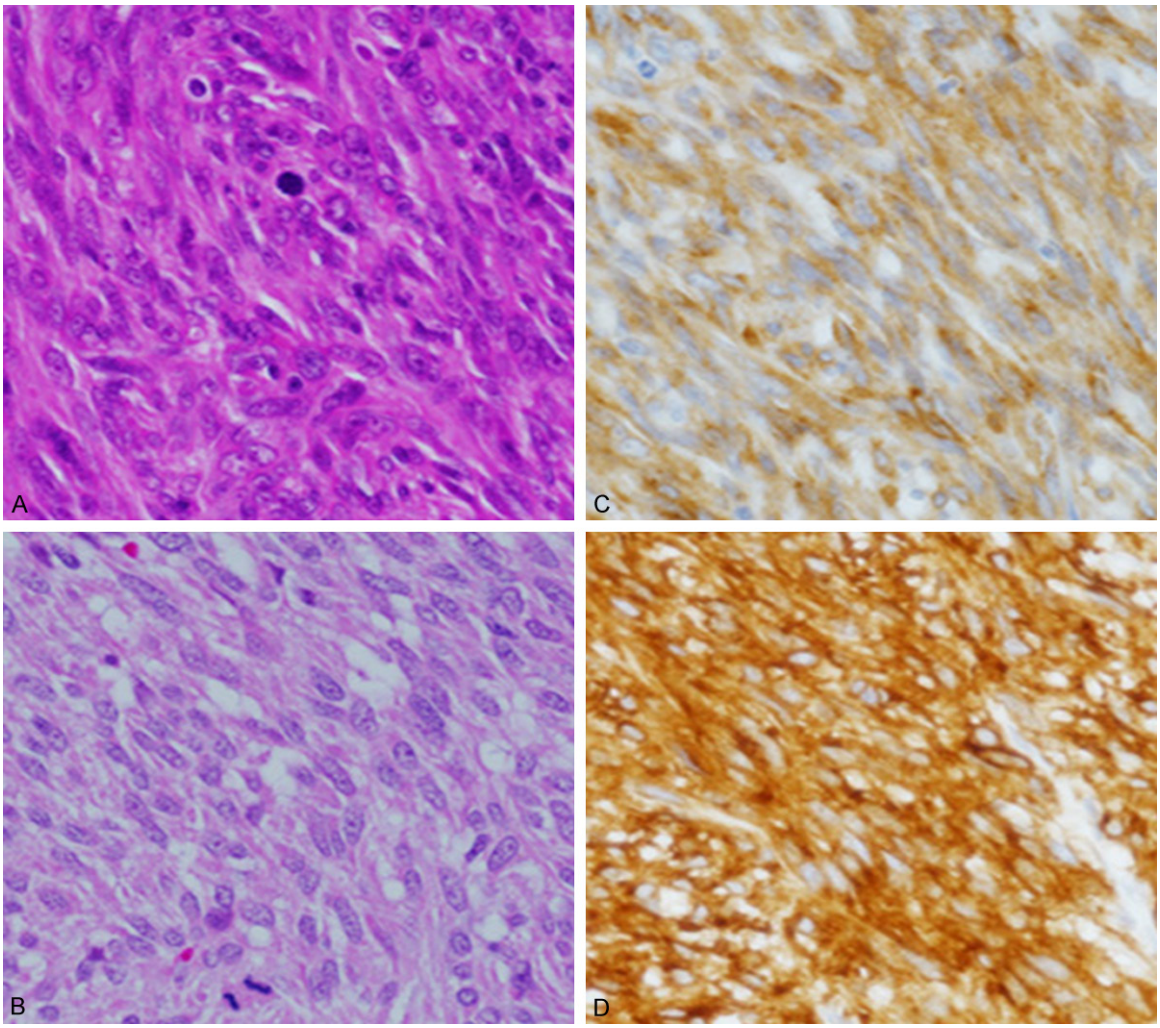

Figure 2. Stromal tumor. A, B: PDGFR α stomach exon 18 the I843S (ATC → TCC) point mutation; C, D: C-kit exon 11 E561K (GAG → AAG), N567I (AAT → ATT), I571L (ATA → CTA) point mutations; A, C: HE; B, D: C-kit.

(55.2%), including 51 cases of exon 11 mutation and 7 cases of exon 9 mutation. No C-kit and PDGFR α mutations had been found in 40 cases of GIST, leiomyoma, nerve sheath tumors and normal gastrointestinal tissues.

*Sequencing results (**Figure 1B**)*

Sequencing analysis of 12 cases of PDGFR α mutations showed that 10 cases had exon 18 mutations: 4 cases with 843 mutation of isoleucine mutating into serine (I843S, ATC-TCC). 5 case with 839 mutation of leucine mutating into candied acid (L839P, CTG → CCG). 1 case with the mixed mutation of point and insertion mutations (the point mutation located at the 851site, and the insertion mutation inserted a G base between 833 and 834). There were 2 case had exon 12 mutation, which was an R deletion mutation at the 585 site (AGA → TGA). C-kit exon 11 mutations were detected in 20 cases, and the mutations were all between codons of 556 and 586, including 13 cases of point mutations, 5 cases of deletion mutation or coexisted point mutation and deletion mutation, 2 case of the coexisted insertion mutation, deletion mutation and point mutation.

Mutation, location and types

In the 12 cases of GIST with positive PDGFR α mutations, C-kit mutations were negative. In 58 cases of C-kit mutation-positive GIST, PDGFR α mutations were also negative. in the other 35 cases of GIST, PDGFR α mutations and C-kit mutations were both negative. The 12 cases with PDGFR α mutations were gastric-derived GIST, including 8 cases of spindle cell type and

Table 1. Relationship between gene mutation and protein expression

Mutation	n	C-kit			*P* values	PDGFR α		*P* values
		Strong positive	Weak positive	Negative		Strong positive	Weak positive	
C-kit	58	50	4	4	>0.05	0	58	<0.001
PDGFR α	12	10	0	2		12	0	
Total	70	60	4	6		12	58	

4 case of mixed cell type. C-kit mutations were found in 37 cases of gastric-derived GIST and 21 cases of intestinal-derived GIST, including 40 cases of spindle cell type, 4 cases of epithelial cell type and 6 cases of mixed cell type. and the 4 cases of C-kit exon 9 mutations were all found in intestinal-derived tumors.

Immunohistochemistry

Expression rate of PDGFR α protein was 100%. 105 cases of GIST, leiomyoma, schwannoma and normal gastrointestinal tract tissues all expressed PDGFR α, but the expression degree was different. It was strongly expressed in 12 cases of GIST with PDGFR α mutations. weakly-positive protein expression was found in the other 93 cases of GIST, leiomyoma, schwannoma and normal gastrointestinal tract tissues. Expression rate of C-kit protein was 95.5%, and it was negative in leiomyoma and schwannoma. In 12 cases of PDGFR α mutations, C-kit protein was strongly expressed in 9 cases and negatively expressed in 3 cases (**Figure 2A**, **2B**). In 58 cases of GIST with C-kit mutations, strongly-positive expression of C-kit protein was detected in 50 cases (**Figure 2C**, **2D**). weakly-positive expression was detected in 4 cases and 4 cases had negative expression. There was no significant correlation between mutations and C-kit protein expression (P>0.05), while PDGFR α protein expression was significantly correlated with mutations (P<0.0001, **Table 1**).

Western blot

PDGFR α protein ubiquitously expressed in various tissues, with different levels. mutations of PDGFR α is in five cases of GIST were stronger than the C-kit mutations in GIST, normal gastrointestinal tissues and schwannomas. 12 cases PDGFR α mutations in GIST were stronger than the C-kit mutations in GIST, normal gastrointestinal tract tissues and nerve schwannoma. PDGFR α expression levels in the GIST with C-kit mutations were varied and weaker than that in the GIST with PDGFR α mutations (**Figure 3A**). C-kit protein was highly expressed in most GIST with C-kit mutations, but there were 4 cases of GIST with C-kit mutations without C-kit protein expression. in 12 cases of GIST with PDGFR α mutations, C-kit protein expression was negative or weakly positive (**Figure 3B**).

Discussion

PDGFR α gene mapping is very similar to C-kit, both of which are located in the human 4q12-13. Its product: PDGFR α served as a single chain transmembrane glycoprotein, with molecular mass of 185 kDa. As well as C-kit, it belonged to III tyrosine kinase family and the structure was similar. When PDGFR α combined with PDGF ligand, they can stimulate phosphorylation of tyrosine residues. Thereby they regulated cell growth, proliferation, adhesion, metastasis, differentiation and apoptosis [11]. Now, most studies suggested that, mutant activation of receptor tyrosine kinase C-kit was an important molecular event for most GIST genesis and development [12]. Recently, in a few cases, PDGFR α functional mutations were found [8, 9]. Its mutant was similar with C-kit, which was concentrated in the membrane proximal region and kinase domain, indicating that PDGFR α mutation may be another cause of GIST, especially in the C-kit mutations in GIST tumors negative formation, it played an important role.

Mutation types of PDGFR α were various, such as functional mutations and non-functional mutations. They were not only in tumor tissue, but also in normal tissues. Corless et al [13] studied and summarized 1105 cases of GIST mutation type, the most common of which was exon18 D842V (accounting for 62.6%). exon18 Del DIMH842-845 and exon12 V561D (total 14.9%) were followed. In this group, with 12 cases of PDGFR α mutations, 4 cases were exon 18 of 843 point mutations from Ile to Ser. 2 cases of exon 12 with 585 deletion mutant. It has not been reported in the literature of these two types, and the rest mutations were not in the position of the mutation hot spot which may

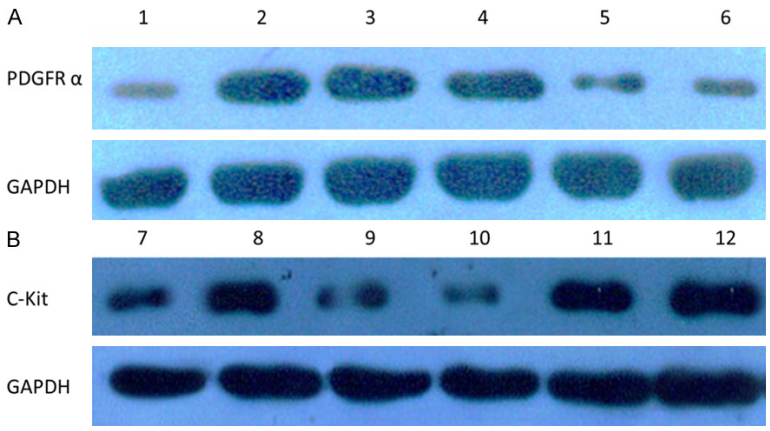

Figure 3. Western blotting results, A: PDGFR α, 1: Schwannoma; 2: PDGFR α exon 18 mutation; 3: PDGFR α exon 12 mutation; 4, 5, 6: C-kit exon 11 mutation. B: C-Kit, 7, 8, 9, 11, 12: C-kit exon 11 mutation; 10: PDGFR α exon 18 mutation.

be related to race. It was unclear that 12 cases of mutations in this group were functional mutations. Vitro PDGFR α functional analyses were required to confirm. Mutation type and condition of C-kit in this group were basically the same as that reported in literatures. The codons of 20 cases of detected exon 11 mutations were between 556-586. Currently, STI571 is the first-line therapy drug for inoperable GIST patients. It selectively inhibited the proto-oncogene abl, Abl-Bcr, C-kit and PDGF receptor tyrosine kinases and thereby blocked signal transduction in order to achieve the purpose of treatment [14]. Studies have shown that mutation detection may be useful in predicting drug efficacy [1], such as mutant GIST of C-kit exon 11 and PDGFR α exon 12, compared with C-kit exon 9 and PDGFR α exon 18, had a good effect on STI571. In addition, effect of treatment was also relevant to mutation. For example, Debiec-Rychter et al [15] and Weisberg et al [16] found that PDGFR α-D842V mutant was resistant to STI571 therefore it led to new studies on PKC412 and other new drugs.

Many studies have reported that, the mutation rate of C-kit exon was 40%-90% and exon 11 mutation rate was the highest. Previous studies showed that C-kit exon 11 mutation rate was 40.4% [17]. PDGFR α had two main mutation exons, and the mutation rate were 5.6 % (exon18) and 1.5% (exon12). the remaining approximately 12% of GIST did not have these two mutations [18]. In this group, GIST mutation rate was 55.2%. PDGFR α mutation rate was 11.4%, indicating that these two mutations were important molecular mechanism for the occurrence of GIST, but it was not the only mechanism, and other mechanisms existed. Miettinen et al [19] and Andersson et al [20] reported that, in children, adolescents, and with neurofibromatosis type I (NF1) of GIST, C-kit exon9, 11, 13, 17 and PDGFR α exon12, 18 mutations were not detected, indicating that different pathogenesis existed. However, further research on the pathogenesis of the GIST has not yet carried out.

We found that, if PDGFR α mutation was found in GIST, there would be no C-kit mutation, which was consistent with most findings [21]. Wasag et al [22] found that, PDGFR α mutation mostly occurred in the stomach, and the pathological were almost epithelial and hybrid cells. C-kit mutation usually occurred in the small intestine, and the pathological was mainly spindle cell. At the same time, PDGFR α mutations were usually found in mucus epithelioid GIST [23]. 12 cases of PDGFR α mutations were derived from gastric tumors in this group. But PDGFR α mutation was not detected in the epithelial type of GIST, which was not consistent with the foreign literatures. It may be related to race. In Chinese GIST research reports, epithelial type of GIST was rare [24]. Compared with the common C-kit proto-oncogene mutations of GIST, the tumorigenic mechanism changed from C-kit to PDGFR α in PDGFR α mutations of GIST. Thus C-kit expression was reduced and PDGFR α expression was increased.

In this group, PDGFR α immunohistochemical results suggested that it was not a specific marker for GIST. However, expression intensity was associated with PDGFR α gene mutation ($P<0.0001$). Pauls et al [25] found that, under normal circumstances, PDGFR α were expressed in myenteric and Schwann cells of neural guitar body, but it did not expressed in the stromal Cajal cells. When PDGFR α mutated and their protein would be expressed in the stoma. Furthermore, it was also related with the poor specificity of the antibody. Currently

many researchers [26] have not found meaningful and repeatable commercial antibodies. Therefore, PDGFR α antibody bad not been used clinically yet. This study showed that the relationship between C-kit protein immunohistochemistry and its mutation and protein expression ($P>0.05$) was the same as previously reported literatures [7, 27], indicating that C-kit gene mutation generally did not affect the expression of protein product.

Semi-quantitative analysis of protein expression in this study was consistent with the reports of Hirota et al [9], Sokurai et al [23] and Kang et al [28], indicating that the protein expression was closely related with the mutations of corresponding genes. therefore, the PDGFR α mutation is directly related to the levels of PDGFR α expression. the different levels of activated proteins also play an important role in the downstream of III type tyrosine kinase family, so that we can guide clinical treatment based on the mutations predicted by protein expression levels.

In addition, we also found that some gene mutations and protein expressions were not synchronized. C-kit protein was also expressed in the GIST with PDGFR α mutations, while there was no C-kit protein expression in two cases of GIST with C-kit mutations. This was consistent with the findings of some scholars [11]: although two mutations occurred independently and their protein expressions were closely related with their gene mutations, their protein expression sometimes overlapped. so the expression of the protein cannot be entirely representative of gene mutations, which can only be an adjunct to the diagnosis of GIST.

In short, PDGFR α mutation rate was 11.2%, which is more common in the spindle cell type gastric-derived GIST, and PDGFR α and C-kit mutations were independent in GIST. In most GIST, PDGFR α protein expression was closely related to its gene mutations, but PDGFR α and C-kit protein expressions were mostly co-existed in tumors with PDGFR α mutations. Therefore, protein expression cannot be entirely representative of gene mutation, which can only be an adjunct to the diagnosis of GIST.

Disclosure of conflict of interest

None.

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