

# Correlation of *KIT* and platelet-derived growth factor receptor $\alpha$ mutations with gene activation and expression profiles in gastrointestinal stromal tumors

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Activating mutations of *KIT* and platelet-derived growth factor receptor  $\alpha$  (*PDGFRA*) are known to be alternative and mutually exclusive genetic events in the development of gastrointestinal stromal tumors (GISTs). We examined the effect of the mutations of these two genes on the gene expression profile of 22 GISTs using the oligonucleotide microarray. Mutations of *KIT* and *PDGFRA* were found in 17 cases and three cases, respectively. The remaining two cases had no detectable mutations in either gene. The mutation status of *KIT* and *PDGFRA* was directly related to the expression levels of activated *KIT* and *PDGFRA*, and was also related to the different expression levels of activated proteins that play key roles in the downstream of the receptor tyrosine kinase III family. To evaluate the impact of mutation status and the importance of the type of mutation in gene expression and clinical features, microarray-derived data from 22 GISTs were interpreted using a principal component analysis (PCA). Three relevant principal component representing mutation of *KIT*, *PDGFRA* and chromosome 14q deletion were identified from the interpretation of the oligonucleotide microarray data with PCA. After supervised analysis, there was at least a two fold difference in expression between GISTs with *KIT* and *PDGFRA* mutation in 70 genes. Our findings demonstrate that mutations of *KIT* and *PDGFRA* affect differential activation and expression of some genes, and can be used for the molecular classification of GISTs.

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## Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract (Lewin *et al.*, 1992; Fletcher *et al.*, 2002). The constituent tumor cells are similar morphologically and immunohistochemically to the interstitial cells of Cajal (Hirota *et al.*, 1998; Kindblom *et al.*, 1998), thus suggesting that GISTs originate from stem cells that differentiate toward ICCs.

Recent molecular genetic studies have demonstrated that gain-of-function mutations of the *v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog* (*KIT*) proto-oncogene, a member of receptor tyrosine kinase III family (Besmer *et al.*, 1986), are the most frequent and important changes in the development of GISTs (Besmer *et al.*, 1986; Hirota *et al.*, 1998; Nakahara *et al.*, 1998). Mutations in *KIT* result in ligand-independent kinase activity and autophosphorylation of *KIT* (Kitayama *et al.*, 1995; Ma *et al.*, 1999). The *KIT* mutation is known to be present in 41 to 92% of GISTs (Lasota *et al.*, 1999; Taniguchi *et al.*, 1999; Kim *et al.*, 2000; Lux *et al.*, 2000; Rubin *et al.*, 2001; Choi *et al.*, 2003). Activating mutations of platelet-derived growth factor receptor  $\alpha$  (*PDGFRA*) have recently been found in a subset of GISTs lacking *KIT* mutations (Heinrich *et al.*, 2003). *KIT* and *PDGFRA* belong to the same subfamily of receptor tyrosine kinase III (Blume-Jensen and Hunter, 2001; Pawson, 2002), and the activating mutations of these two genes are mutually exclusive in GISTs. The mutations of these two genes can thus be regarded as alternative oncogenic mechanisms in GISTs.

Although the mutually exclusive activating mutations of *KIT* and *PDGFRA* appear to contribute to the development of GIST through similar pathways, the specific downstream pathways, and their impact on the gene expression profile have not been evaluated. We therefore carried out a transcriptional gene expression study on 22 GISTs and compared the results with

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respect to the type of mutation status, *KIT* and *PDGFRA*.

## Results

### *Clinicopathologic characteristics and mutation status of KIT and PDGFRA in GISTs*

Among the 22 GISTs, five cases were classified as benign (very low or low risk), 10 were classified as borderline (intermediate risk) and seven were classified as malignant (high risk). Immunoreactivity for KIT was present in 18. Immunoreactivity for CD34 and PDGFRA were found in 21 and 17 cases, respectively. 14q deletion was detected in 13 cases (Table 1). *KIT* mutation was detected in 17 out of 22 cases. In the 17 GISTs with *KIT* mutation, 16 mutations were present in exon 11 and one mutation was present in exon 9. Deletion mutations were most frequent, and these were found in seven cases, while point mutations were found in six cases and insertions were found in four cases. Among the remaining five cases lacking *KIT* mutations, three cases had *PDGFRA* mutation, all of them were being point mutations in exon 18.

### *Unsupervised clustering analysis distinguish a subset of GISTs with lacking KIT mutation*

The relative expression of each gene was evaluated by comparing the degree of the expression to the Universal Human Reference RNA. Previous reports (Allander *et al.*, 2001; Nielsen *et al.*, 2002) have described homogeneous gene expression profiles in GISTs in contrast to the profiles of other types of mesenchymal

tumors. In this study, we initially attempted a molecular pattern analysis to determine whether our spotted-oligoarray system was able to identify different subsets of GISTs by molecular profiling. All of the oligoarray data of the samples described in this study are presented on our web page (<http://www.molpathol.org/data/GIST-arraydata.txt>). With a relevant set of 4693 prefiltered genes (see 'Materials and methods'), we conducted a complete-linkage hierarchical clustering analysis of 22 arrays. A two-way hierarchical clustering analysis did not completely distinguish GISTs with *KIT* mutation and those lacking *KIT* mutation. However, 13 out of 17 GISTs with *KIT* mutations formed a cluster separated by two small clusters. Moreover, among the five GISTs lacking *KIT* mutation, three GISTs with *PDGFRA* mutation and one GIST lacking both *KIT* and *PDGFRA* mutations formed a cluster (Supplementary Figure 1). Next, we applied principal component analysis (PCA) that allow classification of 22 samples with multiple components. The correlations between each component and the corresponding genetic and clinical characteristics were analysed for statistical significance. Component 1 was correlated significantly with the 14q deletion status ( $P=0.0005$ , Mann-Whitney rank-sum test, Table 2, Figure 1a), and component 5 was significantly correlated with the *KIT* and *PDGFRA* mutation status ( $P=0.01$ , Mann-Whitney rank-sum test, Table 2, Figure 1b).

We then tried to identify a robust set of mutation type-related genes by a supervised rank-sum analysis using the Mann-Whitney rank-sum test. To identify the genes that were differentially regulated in GISTs according to the mutation status of *KIT* or *PDGFRA*, we selected 20 GISTs (17 GISTs with *KIT* mutation and three GISTs with *PDGFRA* mutation; two GISTs

**Table 1** Clinicopathological and genetic features of 22 GISTs

Sample no.	Sex	Age	Tumor size (cm)	Tumor grade	Type of Mutation		Immunohistochemistry			Loss of 14q
					<i>KIT</i>	<i>PDGFRA</i>	<i>KIT</i>	<i>PDGFRA</i>	CD34	
1	F	47	5	Borderline	Wild	D842V	-	+	+	Y
2	M	52	7	Borderline	W557R	Wild	+	+	+	Y
3	M	64	2.5	Benign	Wild	D842V	-	+	+	Y
4	F	76	8	Borderline	V560 del	Wild	+	+	+	Y
5	F	57	12	Borderline	V559A	Wild	+	+	+	N
6	F	64	17	Borderline	D579 del	Wild	+	+	+	Y
7	M	45	9	Borderline	T574_R586 ins	Wild	+	-	+	N
8	F	56	5	Borderline	Wild	Wild	-	+	+	Y
9	M	78	17	Malignant	Wild	Wild	+	-	+	N
10	F	36	4	Benign	M552_Y553 del	Wild	+	+	+	N
11	F	68	5.5	Malignant	V559D	Wild	+	+	+	Y
12	M	58	6	Malignant	Y553_Q556 del	Wild	+	+	+	Y
13	M	53	10.5	Borderline	W557_G564 del	Wild	+	+	+	Y
14	M	58	16	Borderline	V559D	Wild	+	+	+	N
15	F	74	4	Benign	D579 del	Wild	+	+	+	Y
16	F	67	8.5	Malignant	K550_Q556 del	Wild	+	-	+	Y
17	F	65	4	Borderline	P573_L589 ins	Wild	+	+	+	N
18	M	52	1.5	Benign	Wild	D842V	-	+	+	N
19	F	43	9.8	Malignant	W557R	Wild	+	-	+	N
20	F	42	33	Malignant	L556_D569 ins	Wild	+	+	+	Y
21	M	65	6.6	Malignant	A502_Y503	Wild	+	-	-	N
22	F	42	3.5	Benign	V559D	Wild	+	+	+	Y

Note: del, deletion; ins, insertion; +, expression; -, no expression; Y, deletion of 14q; N, no deletion

**Table 2** Correlations between PCA components and tumor characteristics

Categories	Mutation status		Deletion of 14q	Tumor Size > 5	Age (60 >, 60 ≤)	Gender	Tumor cell Type	Tumor grade
	PDGFRA	KIT						
Component 1	0.5340	0.8447	0.0005*	0.3059	0.7638	0.2167	0.1965	0.3676
Component 2	0.5987	0.6106	0.8674	0.1722	0.4425	0.3006	0.2712	0.2246
Component 3	0.1654	0.8447	0.1330	0.8378	0.2705	0.9734	0.8110	0.8447
Component 4	0.1965	0.8447	0.0101*	0.8378	0.5258	0.4425	0.6668	0.7244
Component 5	0.0112*	0.0136*	0.7134	0.1014	0.6642	0.8674	0.0498*	0.1704
Component 6	0.1965	0.0256*	0.0663	0.0654	0.5258	0.6642	0.5987	0.2246
Component 7	0.5340	0.0256*	0.1511	0.7848	0.7638	0.1018	0.7377	0.3274
Component 8	0.1654	0.7839	0.8674	0.0880	0.8674	0.7134	0.9618	0.0779
Component 9	0.0397*	0.7839	0.7638	0.4949	0.5258	0.1018	0.6668	0.5054
Component 10	0.1654	0.1266	0.1330	0.9456	0.2426	0.3673	0.2317	0.1961

*Note:* This table displays the *P*-values of each correlation between a component (leftmost column) and a tumor characteristic (mutation status, clinical characteristics, top row). Results are shown for the 10 components with the highest impact on global molecular phenotype. Mann–Whitney rank-sum test were used as statistical tests for characteristics with two subgroups, respectively. For each tumor characteristic, the method of grouping tumors and the number of cases in the relevant group is shown (Grouping for *P*-value calculation; *PDGFRA*, GISTs with *PDGFRA* mutation; *KIT*, GISTs with *KIT* mutation; tumor cell type, GISTs with spindle cell type versus GIST with epithelioid and mixed cell type; tumor grade, benign GISTs versus borderline and malignant GISTs). \*Values, significant component-tumor characteristics correlation with *P*-values less than 0.05. Components 1 correlated significantly with loss of chromosome 14q status, while component 5 and 9 correlated significantly with mutation status

lacking both mutations were excluded), and we used a stringent selection criterion (see ‘Materials and methods’). In all, 70 genes were found to be either upregulated (seven genes) or downregulated (63 genes) in 17 GISTs with *KIT* mutation compared to the three GISTs with *PDGFRA* mutation. The genes that were differentially expressed in GISTs with *PDGFRA* mutation are listed in Figure 2A and Supplementary Table 1. In order to examine the reliability of the microarray data, we selected *KIT* and two upregulated genes (*IGFBP2* and *PDGFRA*) in GISTs with *PDGFRA* mutation. The expressions of these genes were analysed by semiquantitative multiplex RT–PCR using the RNA samples used for the microarray analysis. The results of RT–PCR were consistent for all three genes (data not shown).

#### *Mutation of KIT and PDGFRA are directly correlated to the overexpression of active KIT and PDGFRA*

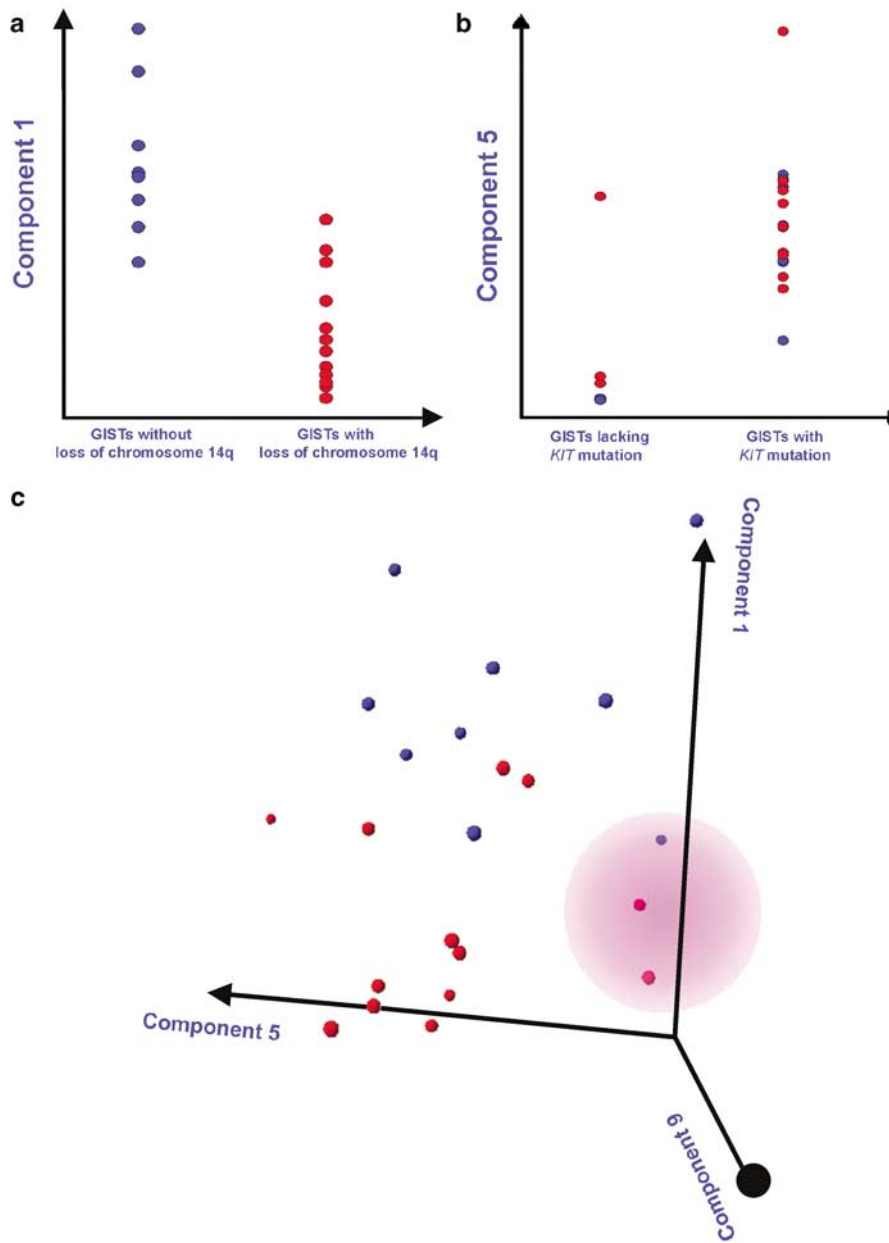
The effect of mutation on the activation of *KIT* and *PDGFRA* was examined at the RNA and protein levels (Figure 2A and B). The levels of *KIT* and *PDGFRA* protein and their phosphorylation status were analysed by Western blotting. *KIT* expression was increased in GISTs with *KIT* mutation (Figure 2B). The average relative expression ratio of *KIT* was 5.6 in 17 cases with *KIT* mutation and 0.1 in five GISTs lacking *KIT* mutation. In contrast, *PDGFRA* expression was found in GISTs regardless of *PDGFRA* mutation status: 14 out of 17 GISTs with *KIT* mutation expressed *PDGFRA* in addition to all three GISTs with *PDGFRA* mutation. To determine whether *KIT* and *PDGFRA* expressions were related to their active forms, we examined their phosphorylation status. Out of 17 GISTs expressing *KIT*, 14 GISTs expressed phospho-*KIT*, whereas phospho-*PDGFRA* was detected in only four out of 17 GISTs expressing *PDGFRA* (Figure 2B). These results indicate that activation of *KIT* and *PDGFRA*

was related to the mutation status of *KIT* and *PDGFRA*, respectively, because 13 of 17 GISTs with *KIT* mutation expressed activated *KIT* and all three GISTs with *PDGFRA* mutation expressed activated *PDGFRA*.

Immunohistochemically, *KIT* and *PDGFRA* were detected mainly in the cytoplasm of the tumor cells. *KIT* expression was detected in 17 GISTs with *KIT* mutation and one of five GISTs lacking *KIT* mutation. The cytoplasmic *PDGFRA* expression was detected in all three GISTs with *PDGFRA* mutation, 13 of 17 GISTs with *KIT* mutation and one of two GISTs lacking both *KIT* and *PDGFRA* mutations (Table 1 and Figure 2c).

#### *Expression of activated STAT3, AKT and Erk1/2 in GISTs with KIT and PDGFRA mutation*

Since we found that the mutation status of *KIT* and *PDGFRA* was directly related to the expression of activated *KIT* and *PDGFRA*, we evaluated the expression of the signaling molecules that are known to play key roles downstream of receptor tyrosine kinase III. We selected three GISTs with *PDGFRA* mutation (cases 1, 3 and 18) and five GISTs with *KIT* mutation (cases 5, 12, 15, 19 and 22), and we analysed *STAT3* for the evaluation of JAK/STAT pathway, *Erk1/2* of the MAPK pathway and *AKT* of the PI3K pathway. As expected, all of the above molecules were expressed in their active forms in all of our GISTs; however, the level of their expression differed according to mutation status of *KIT* and *PDGFRA*. The expressions of phospho-*STAT3* and phospho-*AKT* were more intense in GISTs with *KIT* mutation than in those with *PDGFRA* mutation, while expression of phospho-*Erk1/2* was stronger in GISTs with *PDGFRA* mutation. There was no difference in the protein expression level of *STAT3*, *AKT* and *Erk1/2* between our GISTs with *KIT* and *PDGFRA* mutation (Figure 3).

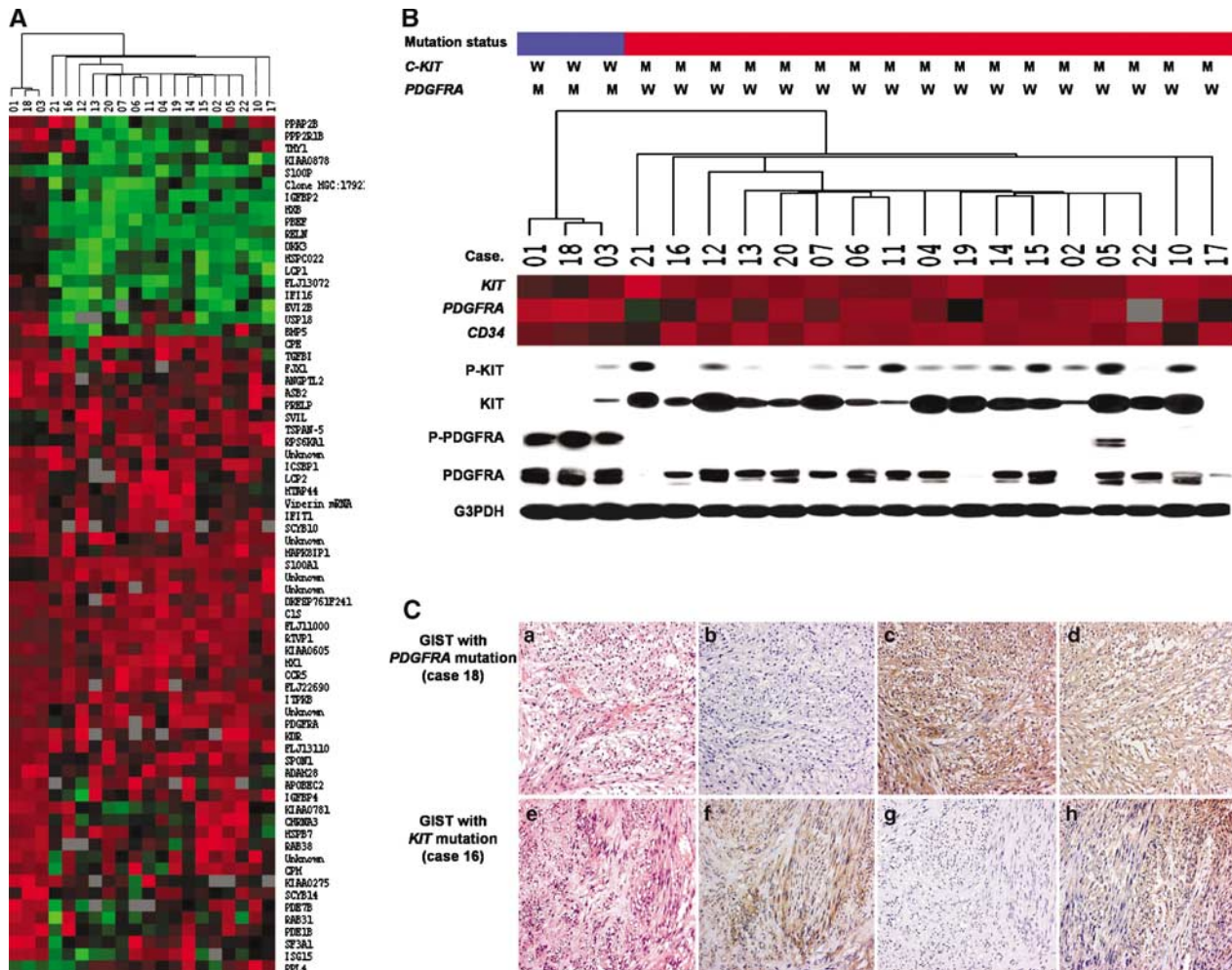


**Figure 1** Scatter plots of the output values in components 1 and 5. (a) One-dimensional scatter plot of component 1 shows that output values for GISTs with 14q deletion were significantly different from those without 14q deletion. (b) One-dimensional scatter plot of component 5 shows that the output values for GISTs with *KIT* mutation were significantly different from the GISTs with *PDGFRA* mutation. (c) Three-dimensional scatter plot of values for component 1 (*Y*-axis), component 5 (*Z*-axis) and component 9 (*X*-axis). Red circles indicate GISTs with the loss of chromosome 14q, and blue circles indicate the GISTs without the loss of chromosome 14q. This plot demonstrates that subcategories of GISTs are present according to 14q deletion and *PDGFRA* mutation status. Three GISTs with *PDGFRA* mutation form a separate cluster on the plot (marked by an ellipse)

## Discussion

In this study, we have analysed the mutations of *KIT* and *PDGFRA* in our 22 GISTs, and compared the gene expression profiles according to the mutation status of these two genes. We found some differences in the gene expression profiles of GISTs according to the *KIT* and *PDGFRA* mutations. These findings suggest that GISTs may be classified at the molecular level according to the mutation status of *KIT* and *PDGFRA*.

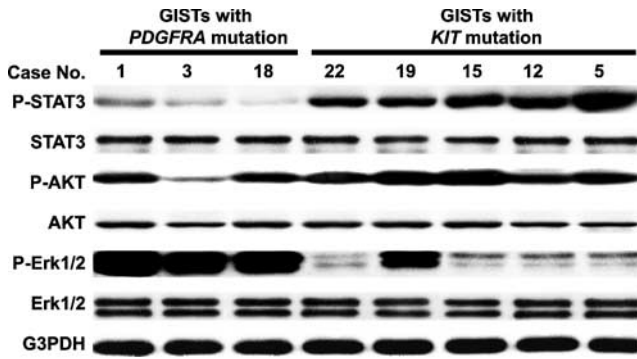
Mutation of *KIT* has been implicated as a major genetic event in the tumorigenesis of GISTs, because most GISTs have shown gain-of-function mutation of *KIT* and germline mutation of *KIT* has been found in familial GISTs (Lasota *et al.*, 1999; Taniguchi *et al.*, 1999; Hirota *et al.*, 2000; Lux *et al.*, 2000; Maeyama *et al.*, 2001). Recently, the mutation of *PDGFRA* has been considered as another causative genetic event (Heinrich *et al.*, 2003; Hirota *et al.*, 2003), as *PDGFRA* mutations were found in most of the GISTs lacking *KIT*



**Figure 2** Expression of *KIT* and *PDGFRA* at the RNA (A) and protein levels (B and C). (A) Supervised hierarchical cluster analysis of gene expression patterns, and the mutation status of *KIT* and *PDGFRA* of 20 GISTs. To detect differentially expressed genes in a given subclass, we ranked the genes using the Mann–Whitney rank-sum test. Outlier genes responsible for mutation status were selected by  $P < 0.01$ . In addition, significant outlier subset genes were further narrowed by filtering genes showing greater than  $\pm 2$ -fold expression changes in GISTs with *KIT* mutation compared to the average values from GISTs with *PDGFRA* mutation. Cases 1, 3 and 18 showed *PDGFRA* mutation (marked as blue), and the other 17 cases showed *KIT* mutation (marked red). (B) RNA expressions of *KIT* and *PDGFRA* were correlated to the mutation status of *KIT* and *PDGFRA* (M, mutated type; W, wild type). In contrast, homogeneous CD34 expression was noted regardless of *KIT* or *PDGFRA* mutation. In all, 17 GISTs with *KIT* mutation showed an overexpression of *KIT* and activated *KIT* protein, while only one (case 3) of three GISTs (cases 1, 3 and 8) with *PDGFRA* mutation expressed *KIT* and showed activated *KIT* protein. *PDGFRA* was expressed in 14 of 17 GISTs with *KIT* mutation and all three GISTs with *PDGFRA* mutation; however, only one (case 5) out of 17 GISTs with *KIT* mutation and all three GISTs (cases 1, 3 and 18) with *PDGFRA* mutation demonstrated activated *PDGFRA*. (C) Example of histological (a and e), immunohistochemical analysis of *KIT* (b and f), *PDGFRA* (c and g) and CD34 (d and f). Overexpression of *KIT* and *PDGFRA* was directly correlated to the mutation status of *KIT* and *PDGFRA*, respectively

mutation, and both *PDGFRA* and *KIT* belong to the same subfamily of receptor tyrosine kinase III (Blumenjensen and Hunter, 2001; Pawson, 2002). Thus, it has been speculated that the mutations of these two genes facilitate the transformation and progression of GISTs through the same pathway. We confirmed here that the mutations of *KIT* and *PDGFRA* are mutually exclusive in our GISTs. Furthermore, our data demonstrated that the mutations of *KIT* and *PDGFRA* correlated with their activation. The overexpression of *KIT* was found in 16 out of 17 GISTs with *KIT* mutation and one out of five GISTs lacking *KIT* mutation by Western blotting

analysis. We have also demonstrated the presence of phosphorylated *KIT* in most GISTs with *KIT* mutation, and thus provided direct evidence for the role of *KIT* mutation in tumorigenesis. In contrast, the overexpression of *PDGFRA* was detected in most of GISTs regardless of *PDGFRA* mutation. *PDGFRA* was detected in 14 out of 17 GISTs with *KIT* mutation, in all three GISTs with *PDGFRA* mutation and in one out of two GISTs lacking both *KIT* and *PDGFRA* mutations. These findings are similar to a previous report where a subset of GISTs with *KIT* mutation expressed *PDGFRA* (West *et al.*, 2004). In this study, we found



**Figure 3** Expression level of activated STAT3, AKT and Erk1/2. Expressions of phospho-STAT3 and phospho-AKT were more intense in GISTs with *KIT* mutation than in those with *PDGFRA* mutation, while expression of phospho-Erk1/2 was stronger in GISTs with *PDGFRA* mutation. No difference in the protein expression level of STAT3, AKT and Erk1/2 was noted between five GISTs with *KIT* mutation and three GISTs with *PDGFRA* mutation

that the level of *PDGFRA* expression was higher in GISTs with *PDGFRA* mutation, in which activated *PDGFRA* was detected in only four out of 18 cases, and three of these exhibited the *PDGFRA* mutation. These findings indicate that the mutation of *PDGFRA* directly correlates with the activation of *PDGFRA*, and may be involved in the tumorigenesis of GISTs.

*KIT* and *PDGFRA* are members of the receptor tyrosine kinase III family that are known to transduce their signals through the JAK/STAT pathway (Ihle and Kerr 1995; Schindler and Darnell, 1995; Weiler *et al.*, 1996; Brizzi *et al.*, 1999; Mui, 1999), Ras/ERK pathway (Porri and McCormick, 1996; Burack and Sturgill, 1997), PLC- $\gamma$  pathway (Zhang *et al.*, 1996; Barker *et al.*, 1998), PI3K pathway (Timokhina *et al.*, 1998) and PDK and AKT (Schlessinger, 2000) pathways. All of these pathways have been suggested to contribute to tumor development, as they have been shown to regulate the cell cycle and cell differentiation through the activation of transcription factors (Miettinen *et al.*, 2002). Our oligonucleotide microarray analysis allowed us to evaluate 15 genes (*AKT*, *GRB*, *JAK*, *MAP2K*, *MAPK*, *PDK*, *PI3K*, *PIP3*, *RAF*, *RAS*, *SHC*, *SRC*, *STAT1*, *STAT3*, *STAT5*) that are known to be involved in these signal transduction pathways. There were no remarkable differences in their RNA expression levels in our GISTs with *KIT* mutation and *PDGFRA* mutation. However, we did not find differences in the expression levels of activated proteins related to the JAK/STAT, PI3K and MAPK signaling pathways between the GISTs with *KIT* mutation and those with *PDGFRA* mutation. The expressions of phospho-STAT3 and phospho-AKT were more intense in the GISTs with *KIT* mutation than in those with *PDGFRA* mutation, while expression of phospho-Erk1/2 was stronger in GISTs with *PDGFRA* mutation. These findings suggest that there are some quantitative differences in the activation of the proteins downstream of receptor tyrosine kinase III between GISTs with *KIT* mutation

and those with *PDGFRA* mutation, and that these differences may be associated with differential gene expression.

GISTs show unique chromosomal, morphological and immunohistochemical characteristics. Recently, there have been two studies on the gene expression of GIST, and both have demonstrated that the gene expression profile of GISTs is different from those of the other types of mesenchymal tumors (Allander *et al.*, 2001; Nielsen *et al.*, 2002). Although these data indicated that GISTs are unique and homogeneous tumors, they show some diverse intertumoral variations at the clinical, morphological and genetic level. Clinically, GISTs are classified as benign, borderline or malignant tumors. Histopathologically, they exhibit two distinct forms, spindle and epithelioid, and mixed patterns are detected in some cases. In addition, several peculiar histologic patterns, such as alveolar pattern, have been detected in some GISTs. The genetic characteristics of GISTs are also diverse. The loss of the long arm of chromosome 14 was detected in 69 to 80% of GISTs (El-Rifai *et al.*, 1998, 2000; Kim *et al.*, 2000), and *KIT* mutation was found in 41 to 92% of GISTs (Lasota *et al.*, 1999; Taniguchi *et al.*, 1999; Kim *et al.*, 2000; Lux *et al.*, 2000; Rubin *et al.*, 2001; Choi *et al.*, 2003). In this study, we attempted to classify GIST at the molecular level according to the overall gene expression profiles. Our data suggest that GISTs may be categorized into subsets according to the mutation status of *KIT* and *PDGFRA*. The direct link between molecular classification and the activating mutations should be regarded as an important finding. Different gene expression patterns of the GISTs according to the anatomic tumor site and the different gene mutation status have recently been reported (Antonescu *et al.*, 2004; Subramanian *et al.*, 2004). When we correlated the molecular classification with the clinical, pathological and genetic characteristics by the PCA model, 14q deletion, *KIT* mutation and *PDGFRA* mutation had strong impacts. Furthermore, our data reveal that 70 genes are expressed differentially between the GISTs with the *KIT* and GISTs with the *PDGFRA* mutation. Most of these genes are overexpressed in GISTs with *PDGFRA* mutation. Some of these genes have known functions in signal transduction (*ASB2*, *IGFBP2*, *IGFBP4*, *PDGFRA*, *RPS6KA1* and *S100A1*) and development (*RELN* and *ANGPTL2*). Further analysis in a larger series of cases and determination of the possible functional mechanism of these differentially expressed genes should clarify the molecular characteristics of *KIT* and *PDGFRA* mutation.

## Materials and methods

### Patients and tissue samples

In all, 22 GISTs of the stomach were included in this study. All cases were identified prospectively and consecutively in the Department of Pathology at Yonsei University Medical Center from September 1995 to November 2002 for molecular marker studies. Authorization for the use of those tissues for research

was obtained from the Institutional Review Board. Among these 22 GISTs, 12 had previously reported for chromosomal and proteome analysis (Kim *et al.*, 2000; Choi *et al.*, 2003). Information on demographic features and tumor sites was obtained from hospital chart and clinicians. The subjects of study included 13 females and nine males ranging in age from 36 to 78 years (Table 1). Conventional pathologic parameters (tumor size, number and grade) were examined prospectively without prior knowledge of the molecular data. The tumor grade of GISTs was divided into three groups according to the criteria of Lewin (Lewin *et al.*, 1992; Fletcher *et al.*, 2002).

For DNA and RNA extraction, fresh tumors were obtained immediately after surgical excision, and stored at  $-70^{\circ}\text{C}$  before use. To enrich the tumor cell population, areas containing more than 90% of tumor cells were sampled from hematoxylin–eosin-stained slides using the cryostat microdissection technique. Genomic DNA was prepared using the sodium dodecyl sulfate–proteinase K and phenol–chloroform extraction method.

#### Mutation analysis of *KIT* and *PDGFRA*

Somatic mutations in exons 9, 11, 13 and 17 of *KIT* and mutations in exons 12 and 18 of *PDGFRA* were analysed in our 22 GISTs using PCR-based assay as described previously (Lasota *et al.*, 1999; Taniguchi *et al.*, 1999; Lux *et al.*, 2000; Rubin *et al.*, 2001; Heinrich *et al.*, 2003). PCR products were separated on 6% polyacrylamide gels, followed by autoradiography for single-strand conformational polymorphism analysis. The PCR products were also sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

#### Microarray formulation

High-density spotted oligonucleotide microarrays were manufactured at the array core facility at Genome Institute of Singapore. The human Oligolibarray™ was purchased from Compugen/Sigma-Genosys. It consisted of 18 861 oligonucleotides, representing 18 664 LEADS™ clusters and 197 controls (GAPDH). Sixtyimers of synthesized oligomers were robotically printed and processed.

#### RNA preparation and hybridization

Total RNA was extracted from microdissected frozen tissues using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (20  $\mu\text{g}$ ) was used as input for cDNA target synthesis as described previously (Joseph *et al.*, 1997). The targets and Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) were hybridized to an oligonucleotide microarray containing 18 664 probe sets representing 18 664 unique (LEADS™) genes, and the array was scanned using GenePix scanners. Expression values for each gene were calculated by using the GenPix Pro 4.0 analysis software.

#### Agglomerative hierarchical clustering

Unsupervised hierarchical clustering analysis was used to classify the 22 GISTs according to the gene expression. We used a data set of genes that satisfied the filtering criteria: genes having more than 70% of log-transformed ratio values (presenting in across all arrays) were taken and genes with less than 0.35 standard deviations of log-transformed ratio were discarded. The selected gene data set was then applied to complete-linkage hierarchical clustering analysis using the uncentered correlation similarity metric method in Cluster

version 2.20. The results of expression map were visualized with Treeview version 1.60 software (<http://rana.lbl.gov/EisenSoftware.htm>).

#### Principal component analysis

PCA is one of the unsupervised approach techniques. In contrast to hierarchical clustering, PCA permits sample classification into multiple independent components. PCA searches for key components in a multidimensional data set to explain differences among the observations (Raychaudhuri *et al.*, 2000). Initially, the set of genes that had 100% of log-transformed ratio values presented in all arrays) were selected. The data of the selected genes were applied to PCA by using Cluster version 2.20. The data of 22 components and eigenvalues for each component were obtained. We compared each component with clinicopathological data, the status of *KIT* and *PDGFRA* mutation and deletion of long arm of chromosome 14.

#### Identification of differentially expressed genes according to the mutation status of *KIT* and *PDGFRA*

To determine whether GISTs can be classified according to the mutation status of *KIT* or *PDGFRA*, 20 cases (17 with *KIT* mutation and three with *PDGFRA* mutation) of GISTs were assessed using the two-way hierarchical clustering analysis. To detect differentially expressed genes according to the mutation status of *KIT* or *PDGFRA*, we ranked the genes using the Mann–Whitney rank-sum test. Outlier genes responsible for GISTs with *KIT* or *PDGFRA* mutation were selected by  $P < 0.01$ . In addition, significant outlier subset genes were further narrowed down by filtering genes showing greater than  $\pm 2$ -fold expression changes in GISTs with *KIT* mutation compared to the average values from GISTs with *PDGFRA* mutation (Table 2).

#### Western blot analysis

Whole lysates from tumor specimens were prepared by using lysis buffer (50 mM Tris (pH 7.4), 1% Triton X-100, 5 mM EDTA, 1 mM KCl, 140 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1% aprotinin, 1  $\mu\text{M}$  leupeptin and 1 mM sodium *ortho*-vanadate). Total protein lysates (20  $\mu\text{g}$ ) were loaded into each lane, size-fractionated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane that was blocked with Tris-buffered saline–Tween 20 containing 5% skim milk. Primary antibodies, *KIT* (Santacruz Biotech, Santa Cruz, CA, USA), *PDGFRA* (Santacruz Biotech), glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Trevigen, Gaithersburg, MD, USA), Erk1/2 and phospho-Erk1/2 (Santacruz Biotech), STAT3 and phospho-STAT3 (Cell signaling, Beverly, MA, USA), AKT and phospho-AKT (Cell signaling) and phosphotyrosine-HRP (Amersham Pharmacia Biotech, UK) were incubated for 1 h at room temperature. After washing, membranes were incubated with HRP-conjugated secondary antibody (Santacruz Biotech), washed and then developed with ECL-Plus (Amersham Pharmacia Biotech).

#### Immunoprecipitation

Tissue lysates (500  $\mu\text{g}$ ) were precleared and gently rocked on an orbital shaker with immunoprecipitating antibodies, *KIT* (Santacruz Biotech) and *PDGFRA* (Santacruz Biotech) at  $4^{\circ}\text{C}$ . The immune complexes were collected by centrifugation and boiled to dissociate the immunocomplexes from the beads. The beads were collected by centrifugation and protein

separation was performed by SDS-PAGE with the supernatant fraction.

#### Semiquantitative RT-PCR

First-strand cDNA was synthesized from 1 µg of total RNA using random hexamer primers (Qiagen) and M-MLV Reverse Transcriptase (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. cDNA (20 ng) from each sample was used in each reaction. All the RT-PCR primer sets were designed to contain an exon-exon junction, and they were as follows: *KIT*, 5'-AGTGAGGCCAGCCTTCAA-3' and 5'-GGTTTGGGAATGCTTCATA-3'; *PDGFRA*, 5'-TTCAGGAAATAAGGTATCGAAGC-3' and 5'-GATGGTCTCCTCCACTTGG-3'; *IGFBP2*, 5'-GCAGACAATGGCGATGACC-3' and 5'-GTTGGGGTTCACACACCAG-3'; and *β-Actin*, 5'-ACAGACTTGCCTCAGGAG-3' and 5'-TGATGCCTCTGGTCGTACCAC-3'. *β-Actin* was used as a quantitative internal control. After RT-PCR, 5 µl aliquots of the products were subjected to 2% agarose gel electrophoresis and subsequently stained with ethidium bromide.

#### Immunohistochemical analysis

The tissue array was constructed from formalin-fixed and paraffin-embedded tissues (Petagen Inc., Seoul, Korea) and

used for the immunostaining. Deparaffinization and rehydration were performed using xylene and alcohol. The sections were treated with 0.3% hydrogen peroxidase for 3 min and blocking antibody for 30 min. The antibodies used were as follows: *KIT* (polyclonal, DAKO; 1:1000 (v/v)), *PDGFRA* (polyclonal, Santa Cruz Biotech; 1:200 (v/v)) and CD34 (monoclonal, DAKO; 1:50 (v/v)). The avidin-biotin complex methodology was employed. The chromogen was diaminobenzidine and counterstaining was carried out with methyl green. The staining of *KIT*, *PDGFRA* and CD34 were categorized as either expressed and absent: cases with definite staining in more than 10% of the tumor cells were categorized as expressed, and cases with staining in less than 10% of the tumor cells were categorized as absent.

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Supplementary Information accompanies the paper on Oncogene website (<http://www.nature.com/onc>)