

KIT/PDGFR α Expression and Mutation in Testicular Seminoma and Ovarian Dysgerminoma

Song-Yi Choi¹ · Kwang-Sun Suh^{1,2}
Yong-Beom Kim¹
Hyun-Jeong Lee¹ · Eun-Sun Kim¹
Mee-Ja Park³

Departments of ¹Pathology and ²Research
Institute for Medical Sciences,
Chungnam National University School of
Medicine, Daejeon; ³Department of
Pathology, Eulji University Hospital,
Daejeon, Korea

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Corresponding Author

Kwang-Sun Suh, M.D.
Department of Pathology, Chungnam National
University School of Medicine, 33 Munhwa-ro,
Jung-gu, Daejeon 301-721, Korea
Tel: 042-280-7196
Fax: 042-280-7189
E-mail: kssuh@cnu.ac.kr

Background : KIT and PDGFR α are tyrosine kinase receptors. Stem cell factor/KIT-mediated signaling plays a role in normal spermatogenesis, and the alteration of KIT is important in the pathogenesis of seminomas/dysgerminomas (SD). **Methods :** To determine the role of expression and mutation of the *KIT* and *PDGFR α* genes, we analyzed 16 seminoma cases, 4 spermatocytic seminoma (SS) cases and 8 dysgerminoma cases for KIT and PDGFR α expression and mutation of *KIT* (exons 9, 11, 13, and 17) and *PDGFR α* (exons 12 and 18) using PCR-SSCP methods. **Results :** KIT was immunohistochemically positive in all 24 SD cases, and one of four (25%) SS cases. PDGFR α was immunohistochemically evident in 16 of the 24 (66.6%) SD cases, and two of the four (50%) SS cases. KIT expression was significantly reduced in SS compared with seminoma ($p=0.0035$). Four cases (14.3%) displayed mutation in *KIT* exon 17 or *PDGFR α* exon 12. Distant metastasis was present in three cases (10.7%), one of which had a nonsense mutation in *KIT*. **Conclusions :** These results indicate that KIT is expressed in the majority of SD cases, but not in most SS cases. However, there was no significant correlation between the clinicopathologic features and mutation or expression of *KIT* and *PDGFR α* .

Key Words : Seminoma; Dysgerminoma; Proto-oncogene proteins c-kit; Receptor, platelet-derived growth factor alpha; Mutation

The gene encoding *KIT* and platelet-derived growth factor receptor alpha (*PDGFR α*) genes map to chromosome 4q11-12. *KIT* and *PDGFR α* belong to the type III transmembrane tyrosine kinase receptor family.¹⁻⁷ This protein family is characterized by a specific molecular structure consisting of an extracellular (EC) domain, a juxtamembrane (JM) domain, and a tyrosine kinase (TK) domain.^{1,8} *KIT* and *PDGFR α* are activated by their ligands, which are the stem cell factor (SCF) and the PDGF, respectively.^{1,5,6,9} Binding of the ligands leads to dimerization of the receptors and phosphorylation of the tyrosine in the cytoplasmic TK domains.^{1,3,5,7,9} Phosphorylation results in a phosphorylation cascade of tyrosine residues in multiple downstream targets and activation of signaling transduction pathways.^{1,3}

SCF/KIT-mediated signaling is critical for normal development and maintenance of different cell types, including hematopoietic stem cells, mast cells, melanocytes, gametocytes, interstitial cells of Cajal, and germ cells.^{1-4,6-9} Expression of *KIT* and gain-of-function mutation of the *KIT* gene have been identified in tumors arising from these cell lineages, such as chronic mye-

loid leukemia, human mast cell tumors, GISTs, and germ cell tumors (GCTs).^{3,5-7} KIT-TK activity is regulated by the JM domain, which inhibits KIT kinase activity in the absence of the KIT ligand.¹ The gain-of-function mutations in the *KIT* proto-oncogene result in ligand-independent, constitutive activation of the KIT receptor.^{8,9}

GCTs are neoplasms derived from primordial germ cells. GCTs are broadly divided into seminoma/dysgerminoma (SD) and non-seminoma/non-dysgerminoma categories.⁵ KIT membranous expression is characteristic of most SD, for which alteration of KIT may play a central role in pathogenesis, but is not characteristic of non-seminomas/non-dysgerminomas.³⁻⁵ Several studies have focused on the expression and mutation of KIT in seminoma. However, there is little data concerning correlations between clinicopathologic features and the immunoreactivities or mutations for both KIT and PDGFR α in SD. To date, mutational hot spots in four regions (exons 9, 11, 13, and 17) of the *KIT* gene and two regions (exons 12 and 18) of the *PDGFR α* gene have been found.⁶ The objective of our study was to evalu-

ate the impact of different *KIT* and *PDGFR α* mutations on other clinicopathological factors in these tumors. To elucidate the frequency and locations of the *KIT* and *PDGFR α* gene mutations in SD, we analyzed all mutational hot spots. We screened the mutations of these tumors using single-strand conformation analysis (SSCP). Subsequent direct sequencing of aberrant bands was performed.

MATERIALS AND METHODS

Patient and samples

Sixteen cases of seminoma, four cases of spermatocytic seminoma (SS) and eight cases of dysgerminoma diagnosed from 1989-2007 were retrieved from the surgical pathology profiles of Chungnam National University Hospital and Eulji University Hospital. The cases of seminoma were classified as classic seminoma (CS) and anaplastic seminoma (AS) (Fig. 1). Tumors were staged in accordance with the TNM system and FIGO staging.

Immunohistochemistry

Tissue microarrays were assembled and immunohistochemical staining was performed using a polyclonal anti-CD117 antibody (c-kit) (dilution 1:150, Dako, Carpinteria, CA, USA) and PDGFR α (dilution 1:200, Santa Cruz Biotechnology Inc, Santa

Cruz, CA, USA). Membranous or cytoplasmic staining patterns for c-kit and PDGFR α were considered positive. Intensity of staining was graded as weak, moderate, or strong. Each tumor was considered positive if they had moderate to strong staining in more than 50% of the tumor cells and exhibited at least focal membrane accentuations.

DNA extraction

All tumor samples were taken from formalin fixed, paraffin embedded tissue samples. Hematoxylin and eosin (H&E)-stained sections (4 μ m) were reviewed under a microscope and areas rich in tumor cells were marked. Corresponding areas on unstained sections were scraped from the slides using a scalpel blade. Tumor samples that contained as few non-neoplastic cells as possible (70-90% tumor cellularity) were collected. Three to five dissected 10 μ m sections were incubated at 55°C for one day in 400 μ L of DNA extraction buffer (0.25 μ g/ μ L of proteinase K; Roche, Basel, Switzerland), 20 mM Tris/HCl, pH 8.3 mM MgCl₂ 100 mM KCl, 1% Tween-20, and 1% NP-40). The mixture was boiled for 10 min to inactivate the proteinase K, purified by phenol-chloroform, and concentrated using ethanol precipitation.

PCR amplification of the KIT and PDGFR α genes

Polymerase chain reaction (PCR) primers were designed to amplify exons 9, 11, 13, and 17 of the *KIT* gene and exons 12 and 18 of the *PDGFR α* gene (Table 1). PCR amplification was performed in a total volume of 20 μ L containing 500 ng of template DNA, one unit of ExTaq polymerase (Takara, Shiga, Japan), 1.25 mM of dNTP, 15 pmole of primer, and 2 μ L of 1 $\times$ reaction buffer. PCR cycles consisted of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by one cycle for 7 min at 72°C.

SSCP analysis, silver staining, and direct sequencing

Two microliters of PCR product was mixed with 6 μ L of sample loading buffer containing 95% formide (deionized), 10 mM NaOH, 0.25% Bromophenol blue, and 0.25% Xylene cyanol. The samples were denatured for 3 min at 100°C and quickly chilled on ice. They were then loaded onto a 12% polyacrylamide gel containing 1 $\times$ sample buffer (33 mM Tris-sulfate, 7% Glycerol, pH 8.3), and were electrophoresed at 250 V. After electrophoresis, the gels were disassembled from the glass

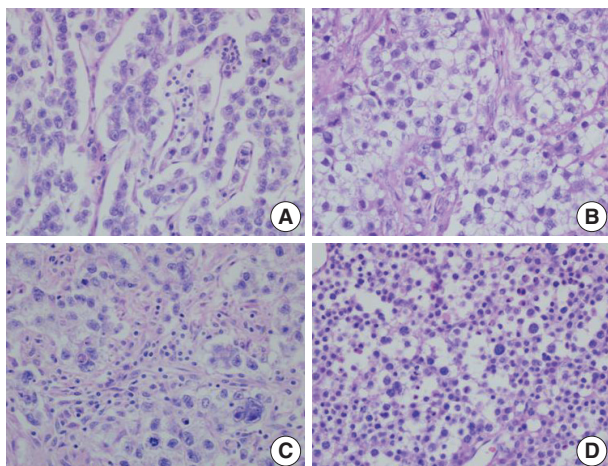


Fig. 1. Histologic features of dysgerminoma (A), seminoma (B, C) and spermatocytic seminoma (D). Dysgerminoma (A) and classic seminoma (B) consist of uniform tumor cells with abundant clear cytoplasm and distinct cell membrane. Anaplastic seminoma (C) exhibits marked pleomorphism and frequent mitosis. Spermatocytic seminoma (D) shows three cell types: giant cells, medium-sized cells and small lymphocyte-like cells.

Table 1. Amplification primers for SSCP/PCR mutational analysis of the KIT and PDGFRA genes

Exon/primer	Sequence (5'-3')	Size (bp)	Temp (°C)
KIT 9a-F	TTT CCT AGA GTA AGC CAG GGC	180	57
KIT 9a-R	GTT GTA TGC CTT ACA TTC AAC CG		
KIT 9b-F	AGT GCA TTC AAG CAC AAT GG	146	57
KIT 9b-R	GAC AGA GCC TAA ACA TCC CC		
KIT 11-F	CTA TTT TTC CCT TTC TCC CC	193	55
KIT 11-R	TAC CCA AAA AGG TGACAT GG		
KIT 13-F	CCT GTA TGG TAC TGC ATG CG	226	52
KIT 13-R	GAC AGA GCC TAA AGA TCC CC		
KIT 17-F	ATG GTT TTC TTT TCT CCT CC	243	55
KIT 17-R	TAC ATT ATG AAA GTC ACA GG		
PDGFRA 12-1-F	CCA GTT ACC TGT CCT GGT CAT	141	53
PDGFRA 12-1-R	TGG AAA CTC CCA TCT TGA GTC		
PDGFRA 12-2-F	AAA TTC GCT GGA GGG TCA TT	143	53
PDGFRA 12-2-R	GGA GGT TAC CCC ATG GAA CT		
PDGFRA 18-F	AGT GTG TCC ACC GTG ATC TG	146	53
PDGFRA 18-R	GTG AGG GAA GTG AGG ACG TA		

plate, then stained using a Silver Stain Plus kit (Bio-Rad, Hercules, CA, USA) followed by air drying. Samples with abnormal bands were sequenced automatically using a genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Group comparisons of categorical variables were evaluated using the χ^2 test or Fisher's exact test. Comparisons of average means were performed with the Mann-Whitney U-test. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 14.0 statistical software program (SPSS, Chicago, IL, USA).

RESULTS

Clinical and pathologic characteristics

Among the 20 seminoma and SS cases, 16 cases underwent unilateral orchiectomy and four cases underwent unilateral orchiectomy with adjuvant radiotherapy. The 16 seminoma cases were classified as CS (n=9) and AS (n=7). One CS case was associated with cryptorchidism. Among the eight dysgerminoma cases, five underwent unilateral salpingo-oophorectomy. One case underwent hysterectomy with bilateral salpingo-oophorectomy. The remaining two cases were diagnosed using biopsy samples from both the ovary and neck lymph node, and both were treated using chemotherapy. The clinical stages of SD and

Table 2. Patient demographics and clinicopathologic characteristics

	Seminoma ^a (n=16)	Spermatocytic seminoma (n=4)	Dysgerminoma (n=8)
Mean age, years (range)	32.5 (23-48)	50.6 (32-69)	27.4 (16-44)
Tumor size, cm (range)	5.5 (2.2-12)	7.0 (4-9)	19.2 (10-33)
Stage no. (%)			
IA	2 (12.5%)	0	3 (37.5%)
IB	13 (81.3%)	4 (100%)	0
IIB	0	0	3 (37.5%)
IIC	1 (6.25%)	0	0
IV	0	0	2 (25.0%)
Tumor recurrence	2 (12.5%)	1 (25.0%)	0

^aCases include classic and anaplastic seminomas.

SS cases were: IA, (n=5; 17.5%); IB, (n=17; 60.7%); IIB, (n=3; 10.7%); IIC, (n=1; 3.6%); and IV, (n=2; 7.1%) (Table 2). SS, compared with seminoma (including CS and AS), tended to occur at a more advanced age ($p=0.042$, Mann-Whitney U-test). Dysgerminoma exhibited a larger size (mean tumor size of 19.2 cm) than both seminoma and SS ($p=0.002$ and $p=0.014$, respectively, Mann-Whitney U-test). Based on the radiologic modalities, three of 28 cases (10.7%) showed distant metastasis (Table 2). One AS case showed lung metastasis 15 months post-operatively. One CS case showed hepatic metastasis 68 months post-operatively. One SS case showed pericardial metastasis 79 months post-operatively, which proved lethal.

KIT and PDGFRA expression by immunohistochemistry

Immunohistochemical staining for KIT was observed in eight of eight (100%) dysgerminomas, 16 of 16 (100%) seminomas, and one of four (25%) SS (Fig. 2). PDGFRA was positive in six of eight (75.0%) dysgerminomas, 10 of 16 (62.4%) seminomas, and two of four (50%) SS (Fig. 2). KIT expression was reduced in SS compared with seminoma ($p=0.0035$, Fisher's exact test) (Table 3). PDGFRA expression was not statistically correlated with the histologic type or expression of KIT ($p>0.05$, Fisher's exact test).

KIT and PDGFRA gene mutation in GCTs

For 15 of the 16 cases of seminoma, all four cases of SS and seven of the eight cases of dysgerminoma, we were able to amplify the specific exons using PCR. The products were screened for mutations using SSCP. Aberrant bands were identified in KIT exon 17 of two seminomas and one dysgerminoma, and in PDGFRA exon 12 of one SS. These four samples were subjected to

direct sequencing and all of them exhibited a single nucleotide substitution (Table 4). Three point mutations in *KIT* exon 17 and one point mutation in *PDGFRA* exon 12 were observed. One CS (case 1) and one dysgerminoma (case 21) showed a missense mutation in *KIT* exon 17. These mutations were 2466T → G and 2470 G → T substitutions, leading to Asn822Lys and Val824Leu at the protein level, respectively (Fig. 3). Case 19 was an AS exhibiting a nonsense mutation at codon 796 in *KIT* exon 17. One SS (case 16) showed a 1834A → G substitution leading to no amino acid change.

Correlation between clinicopathologic features and mutation or expression of *KIT* and *PDGFRA*

One CS (case 1) that displayed a missense mutation in *KIT* exon 17, was associated with cryptorchidism. One AS (case 19) with a nonsense mutation in *KIT* exon 17 showed lung metastasis 15 months post-operatively. This patient was treated with chemotherapy after recurrence and lived for 29 months from the date of primary diagnosis. In addition, the other three cases with mutations showed no evidence of metastasis or recurrence

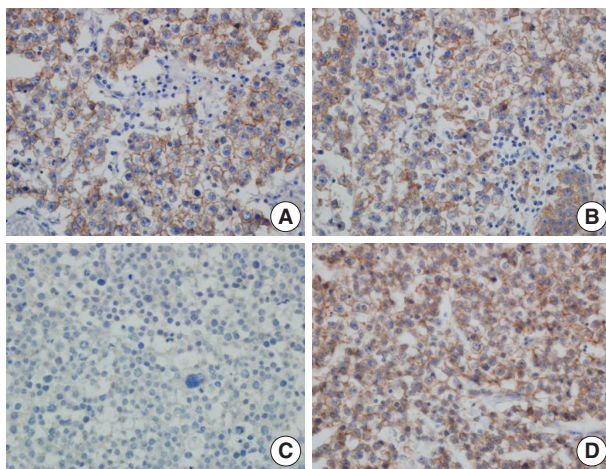


Fig. 2. Immunohistochemical expression of KIT and PDGFRA in dysgerminoma and seminoma. Membranous staining of c-kit in dysgerminoma (A) and classic seminoma (B). Negative reactivity for KIT in spermatocytic seminoma (C). Membranous staining of PDGFRA in classic seminoma (D).

Table 4. Mutations in the *KIT* and *PDGFRA* genes

Case No.	Gene	Exon	Tumor type	Base change	Amino-acid change	Consequence	Follow up (mon)
1	<i>KIT</i>	17	CS	AAT → AAG	N822K	Missense	NED (25)
19	<i>KIT</i>	17	AS	AGA → TGA	R796 → stop	Nonsense	Lung metastasis (15)
21	<i>KIT</i>	17	DG	GTG → TTG	V824L	Missense	NED (22)
16	<i>PDGFRA</i>	12	SS	CCA → CCG	P567P	Silent	NED (29)

CS, classic seminoma; AS, anaplastic seminoma; SS, spermatocytic seminoma; DG, dysgerminoma; NED, no evidence of disease.

during the mean follow-up duration of 29.3 months. There was no statistically significant correlation between a mutation and recurrence or survival. All three cases with *KIT* mutation were positive for both *KIT* and *PDGFRA*, but a SS case with *PDGFRA* mutation was negative for *KIT* and *PDGFRA*. However, there was no significant correlation between the mutation and expression.

DISCUSSION

The expression of *KIT* has been demonstrated in a variety of tumors, including GCTs such as seminoma and dysgerminoma, which are both dysgerminoma analogous tumors arising from primordial germ cells.^{5,7,10} Previous studies have shown

Table 3. Correlation between *KIT* expression and the seminoma histologic type

IHC		Seminoma ^a (n=16)	Spermatocytic (n=4)	p-value (Fisher's exact test)
C-kit	+	16 (100%)	1 (25%)	0.0035
	-	0 (0%)	3 (75%)	
PDGFRA	+	10 (62.4%)	2 (50%)	NS
	-	6 (37.6%)	2 (50%)	

^aCases include classic and anaplastic seminomas.

NS, indicates not significant.

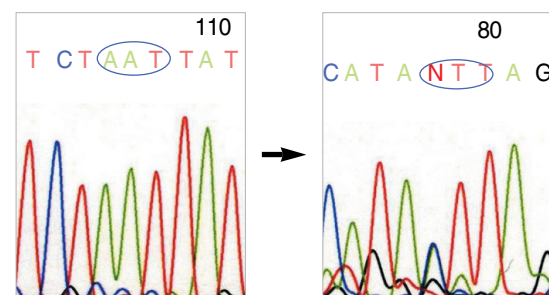


Fig. 3. Chromatograms showing a single nucleotide change at codon 822 in exon 17 of the *KIT* gene. Forward sequence of non-mutated case (A) and reverse sequence of mutated case (B) showing 2466T → G.

KIT expressions of 80-100% in both seminomas and dysgerminomas.^{3,5,7,11,12} There have been only a few large-scale studies of dysgerminoma because of its rarity. Sever *et al.*⁷ reported KIT expression in 26 of 30 (87%) cases of dysgerminoma. Herein, we detected KIT expression in 16 of 16 (100%) cases of seminoma and eight to eight (100%) cases of dysgerminoma. These results are consistent with previous reports of KIT expression in gonadal GCTs.⁷ These similar KIT expression results support the idea that both tumors arise from the same pathogenetic process.

SS is not a variant of seminoma, but is a distinct disease with a different pathogenesis.^{13,14} Presently, we observed a significant difference in KIT expression between seminoma and SS, indicating that these two diseases should be considered as distinct entities. The diagnostic criteria of AS, which displays dedifferentiated features, can be confusing. It has been reported that anaplastic variants demonstrate a lower frequency of KIT expression than CS.³ However, we did not observe any significant difference between AS and CS cases.

Accurate diagnosis of dysgerminoma is important because this tumor shows a better prognosis with proper treatment.⁷ Our finding of KIT expression in 100% of dysgerminoma cases indicates that this marker should be useful for differential diagnosis. However, a wide range of KIT expressions has been observed in other ovarian tumors, such as serous carcinomas, granulosa cell tumors, and Brenner tumors.¹⁵⁻²⁰

There have been a number of studies of KIT and PDGFRA expression in a variety of tumors, including GISTs, ovarian tumors, Merkel cell tumors, Ewing sarcomas, and chordoma.¹⁷⁻²² However, there has not hitherto been a study regarding PDGFRA expression in gonadal GCTs. Presently, we detected expression of PDGFRA in 10 of 16 (62.4%) seminomas and six of eight (75%) dysgerminomas. In contrast to the KIT expression, PDGFRA showed no significant difference between seminoma and SS cases.

It is important to understand *KIT* and *PDGFRA* gene mutations because of their potential therapeutic usefulness. Several tyrosine kinase (TK) inhibitors, in particular imatinib mesylate (Gleevec), have proven to be effective for treatment of chronic myelogenous leukemia and GISTs.^{1,7,8} Dysgerminoma has a high cure rate of approximately 95% with a high radiosensitivity.^{7,23} However, subsequent reproductive difficulty is a common side effect due to the young age of patients.²³ Therefore, identification of a treatment modality that can maintain fertility is a pressing current issue in therapeutic development.⁷ Responsiveness to the TK inhibitors is probably correlated with the location of

the gene mutation.^{1,3} Unfortunately, prediction of the mutation is not possible based on immunohistochemical expression and morphology.⁷ In agreement with previous reports, there was no correlation between the mutation and immunohistochemical expression or histologic type in this study.

KIT gene mutation in GCTs has been recognized primarily in exons 11 and 17.^{3,4,6} In the literature, 27 of 123 seminoma cases were demonstrated to have a *KIT* gene mutation with occurrence rates ranging from 0-40.9%.^{3,4,6,24} In this study, three of the 28 SD cases (10.7%) showed mutation in exon 17 of *KIT*. In the previous studies, the majority of *KIT* mutations were located in exon 17 and only four cases were identified in exon 11.^{3,6,15,24} Hoie-Hansen *et al.*¹¹ reported that five of 17 cases of dysgerminoma had a point mutation at codon 816 in *KIT* exon 17. One other single case report of metastatic dysgerminoma also showed D816V mutation of *KIT*.²⁵ This D816V mutation is thought to cause resistance to imatinib mesylate.³ However, the mutation at codon 816, the predominant type of *KIT* mutation in GCTs, was not observed in our cases. The mutations of *KIT* observed in this study were two neighboring missense mutations and a nonsense mutation in exon 17. No nonsense mutation has been reported in previous studies in GCTs.

In our study, the D822K mutation of *KIT* in CS was identical to the previously reported gain-of-function mutation in testicular GCTs.^{3,26,27} In addition, this case was associated with cryptorchidism, an important risk factor of testicular GCTs.¹⁰ GCTs in cryptorchidism are thought to be caused by a defect in testicular development and cellular differentiation.²⁸ In a previous study, two of five cases of seminoma with *KIT* mutation were associated with undescended testis.²⁹

The V824L mutation of *KIT* in dysgerminoma in our cases has not previously been reported in ovarian GCTs. Even though the mutation at codon 816 in *KIT* exon 17 is the predominant type and its effect on target therapy has been reported, the variability of other mutation locations have been documented. Therefore, further detection of mutation locations and understanding their functions are necessary for development of targeted therapies with reduced toxicities.

PDGFRA mutations have been described in a number of GISTs studies. Most of these mutations have been identified in the vicinity of codon 842 in exon 18.¹ Our study is the first consideration of the *PDGFRA* mutation in GCTs. Presently, one SS showed a synonymous variant at codon 567 in exon 12, which was documented in dbSNP. This SS case with *PDGFRA* mutation showed no expression for KIT or PDGFRA. All three cases with *KIT* mutation showed immunoreactivity for both KIT and

PDGFR α . No SS case showed *KIT* mutation. These results also indicate that the pathogenesis of SS is different from both seminoma and dysgerminoma.

As mentioned above, one AS case with *KIT* mutation showed lung metastasis, which occurred earlier (15 months post-operatively) than the other two metastatic cases without mutation (68 and 79 months post-operatively). However, there was no significant correlation between the clinicopathologic features and mutation or expression in this study. Rapley *et al.*²⁹ reported that *KIT* mutation could predict bilateral testicular cancer, however there is little data concerning the clinical outcomes in GCTs with mutation. It is unclear whether the *KIT* or *PDGFR α* gene mutations are associated with clinicopathologic features. Thus, larger scale studies considering the impact of *KIT* and *PDGFR α* mutation on clinicopathological factors are necessary.

In conclusion, *KIT* expression was observed in a majority of SD cases and tended to be negative in SS. These findings indicate that *KIT*-mediated signaling plays a role in the pathogenesis of SD but not of SS. There was no correlation between the clinicopathologic features and mutation or expression of *KIT* and *PDGFR α* . Even though *KIT* and *PDGFR α* mutations have a low diagnostic value due to multiple locations and low frequencies, understanding of these mutations is important because of their influence on therapy. Responsiveness to TK inhibitors probably depends on the location of the gene mutation, so further understanding of *KIT* and *PDGFR α* mutations in these tumors will lead to targeted therapies with reduced toxicities.

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