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KIT (*c-kit* oncogene product) pathway is constitutively activated in human testicular germ cell tumors

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Abstract

We investigated the expression of KIT (product of *c-kit* oncogene), gain-of-function mutations, and activation of its downstream signal transduction in human testicular cancers. KIT was expressed in 88% (22/25) of seminomas and in 44.4% (4/9) of non-seminomas compared to adjacent normal testicular tissue. Nine of the KIT-expressing seminomas had mutations (40.9%; 9/22) in the *c-kit* gene; two cases in exon 11 and 7 cases in exon 17. Two of these mutations in exon 17 were novel, and the other seven mutations were identical to the already known gain-of-function mutations which cause activation of KIT without ligand stem cell factor. All of the mutant KIT and 53.8% (7/13) of wild-type KIT were phosphorylated (activated) and associated with phosphorylated phosphatidylinositol 3-kinase (PI3K). Akt was also phosphorylated in these seminomas, suggesting that the KIT–PI3K–Akt pathway is activated in seminoma. These findings suggest that the KIT–PI3K–Akt pathway is constitutively activated in testicular germ cell tumors, due to overexpression of KIT protein and/or gain-of-function mutations in the *c-kit* gene.

Keywords: *c-kit*; Gain-of-function mutation; Germ cell tumor; Signal transduction; Testis

Testicular germ cell tumors (TGCT) of adult and adolescent males are the most common cancers found between the ages of 15 and 34 years. Several risk factors for TGCT have been identified, which include a history of undescended testis, testicular dysgenesis, infertility, previously diagnosed TGCT, and a family history of this disease. However, the molecular pathogenesis of testicular germ cell tumors remains poorly understood. Over the past several decades, increased copy number of chromosome 12p has been reported [1]. On the other hand, overexpression of oncogenes such as *RASK2* and *c-kit* has been also reported in TGCT [2–6]. Recently, susceptibility gene for testicular germ cell tumor has been reported to be located on chromosome Xq27 [7].

The *c-kit* protooncogene encodes a type III transmembrane tyrosine kinase receptor (KIT). Binding of the ligand stem cell factor (SCF) to KIT leads to receptor dimerization and autophosphorylation of KIT on its tyrosine residues, resulting in the activation of downstream signal transduction pathways [8,9]. High expression of tyrosine kinase receptor KIT and ligand-independent activation of KIT by the gain-of-function mutation of the c-kit gene has been reported to be implicated in oncogenesis of mastocytosis and gastrointestinal stromal tumors (GIST), a tumor of Cajal cells [10–12]. Testicular germ cell tumor is one of the tumors which express KIT [2–6], but its function remains unknown. Tian et al. [13] first reported that there was a gain-of-function mutation in only 1 of 17 seminomas

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in exon 17. Recently, Sakuma et al. [14] reported that there were gain-of-function mutations in 4 of 29 seminomas in exons 11 and 17, and Kemmer et al. [15] reported that there were gain-of-function mutation in 14 of 54 seminomas in exon 17.

In the present study, we investigated the expression of KIT and mutations in 4 exons of the *c-kit* gene (exons 9, 11, 13, and 17), in which gain-of-function mutations have been found. Furthermore, we investigated activation of KIT and its downstream signal transduction.

Patients and methods

Tissue samples. Tissue samples were obtained from 34 patients who underwent orchiectomy at Osaka University Hospital and affiliated hospitals between 1994 and 2001. Specimens of about 5 mm in diameter of tumor and surrounding normal tissues were dissected individually and immediately frozen and stored at −80 °C until subsequent analysis. The remaining tissues were fixed with buffered formalin for routine histopathologic diagnosis. The characteristics of the patients are described in Table 1.

Genomic DNA was collected from frozen tissue using DNeasy Tissue Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions.

Immunohistochemistry. Paraffin-embedded samples were sectioned at 5 μm and stained with c-19 KIT polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution. After washes in TBS, sections were incubated with HRP-linked anti-rabbit secondary antibody at 1:500, followed by color detection by DAB chromogen according to the manufacturer's instructions (DAKO, Carpinteria, CA).

Protein lysates. Frozen specimens were suspended and homogenized in RIPA buffer 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 1:100 dilution of Phosphatase Inhibitor Cocktail 2 (Sigma, St. Louis, MO). Protein concentration was analyzed using Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Table 1
Patients' characteristics

Histology	Seminoma ($n = 25$)	Non-seminoma ($n = 9$)	
		Yolk sac tumor	3
		Embryonal carcinoma	1
		Immature teratoma	2
		Mixed type	3
Age (years)	21–65 (median 35.5)	1–44 (median 25.5)	
Stage (UICC Stage Grouping)			
IA	6	1	
IB	3		
IS	11	6	
IIA	2		
IIB	1		
IIIA	1		
IIIB	1	2	

Western blotting and immunoprecipitation. For Western blotting, 30 μg of the whole cell lysates was separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electroblotted onto Immobilon-P Transfer Membrane (Millipore, Billerica, MA), and probed with c-19 KIT polyclonal antibody (1:1000 dilution) and with chemiluminescence detection. The expression of the protein was measured by densitometry. The expression of KIT was estimated as '+' when the ratio KIT/β-actin was same or more and less than 2-fold compared to the ratio in normal testis, and estimated as '++' when the ratio was more than 2-fold compared to the ratio in normal testis.

For Akt phosphorylation analyses, 30 μg of the whole cell lysates was separated by 7.5% SDS–PAGE, electroblotted onto Immobilon-P Transfer Membrane (Millipore, Billerica, MA), and probed with 9271 Phospho-Akt (Ser 473) antibody (1:1000 dilution). The membrane was stripped by submerging the membrane in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7) at 50 °C for 30 min, washed for 20 min in T-PBS at room temperature, and restained with 9277 Akt antibody (1:1000 dilution) (Cell Signaling Technology, Beverly, MA). The position of pAkt was confirmed by immunoblotting with Akt antibody after stripping the membrane.

For immunoprecipitation, 500 μg of whole cell lysates was incubated with 2 μg c-19 KIT polyclonal antibody for 2 h, followed by incubation with 20 μl of Protein A–Agarose (Santa Cruz Biotechnology) for 2 h at 4 °C, and then centrifuged at 1000*g* for 30 s. Immunoprecipitates were washed 4 times in PBS and resuspended in 25 μl of sample buffer (1.0 ml glycerol, 0.5 ml β-mercaptoethanol, 3.0 ml of 10% SDS, 1.25 ml of 1.0 M Tris–Hcl, pH 6.7, and 1 mg bromophenol blue) and boiled for 4 min. The immunoprecipitates were resolved by 7.5% SDS–PAGE and blotted to Immobilon-P. The membrane was stained by the PY99 phosphotyrosine monoclonal antibody (Santa Cruz Biotechnology) with chemiluminescence detection. The blots were stripped by the same method mentioned above and were reprobed with c-19 KIT polyclonal antibody (1:1000 dilution), followed by re-stripping and restaining with anti-PI3-Kinase p85 polyclonal antibody (1:2000 dilution) (Upstate Biotechnology, Lake Placid, NY).

Single strand conformation polymorphism (SSCP) analysis of the c-kit. Exons 9, 11, 13, and 17 were amplified using intronic primers as described previously with modifications (Table 2) [16]. Exon 9 encodes the extracellular domain, exon 11 encodes the juxtamembrane domain, and exons 13 and 17 encode the kinase domain. PCRs were performed in 30 μl volumes containing 10 ng of genomic DNA; 1× PCR buffer; 1.5 mM $MgCl_2$; 0.4 μM of each primer; 0.2 mM of dATP, dTTP, dCTP, and dGTP; and 0.375 U *Taq* polymerase. Initial denaturation was at 94 °C for 4 min, followed by 35 cycles and a final extension step (5 min, 94 °C). The cycles included denaturation at 94 °C for 35 s, annealing at each temperature for 35 s, and extension at 72 °C for 40 s.

Three microliters of PCR products was mixed with an equal volume of formamide dye mixture, heated at 95 °C for 5 min, and chilled on ice immediately. Six microliters of the mixture was electrophoresed on GeneGel Clean (Amersham Biosciences, Piscataway, NJ). These gels were rehydrated by buffer A for exon 11, buffer B for exon 9, and buffer C for exons 13 and 17. Electrophoresis was done according to the manufacturer's instructions. The temperature was kept at 4 °C by the GenePhor DNA Separation System (Amersham Biosciences). After electrophoresis, the gels were silver stained by the DNA Silver Staining Kit (Amersham Biosciences). Aberrant bands were excised, eluted from the gel, and were

Table 2
Primers used for amplification of *c-kit* gene

Exon	Forward primer	Reverse primer
9	5′-*ATTTATTTTCCTAGAGTAAGCCAGGG*-3′	5′- *ATCATGACTGATATGGTAGACAGAGC*-3′
11	5′-*CTATTTTTCCCTTTCTCCCC*-3′	5′-*TACCCAAAAAGGTGACATGG*-3′
13	5′-*ATGCGCTTGACATCAGTTTGC*-3′	5′-*ACAATAAAAGGCAGCTTGGACAC*-3′
17	5′-*TTCACTCTTTACAAGTTAAAATG*-3′	5′-*GGACTGTCAAGGAGAGAATG*-3′

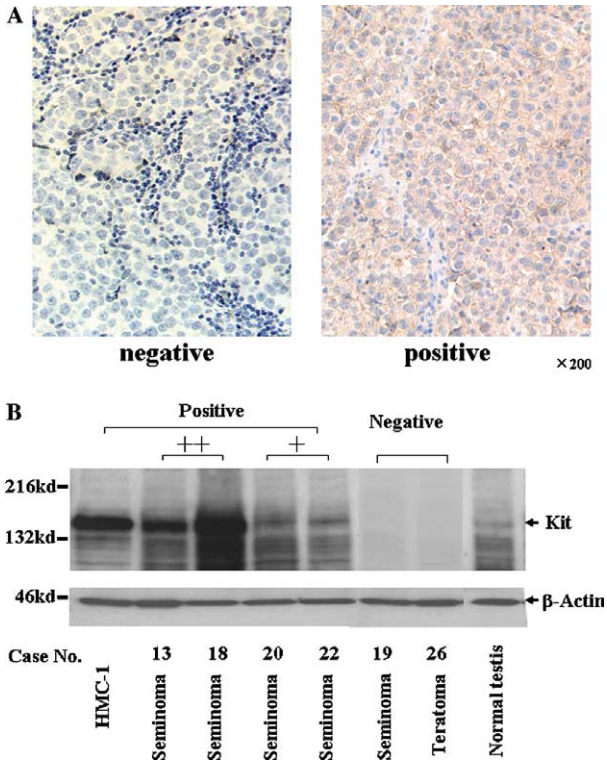

Fig. 1. Expression analysis of KIT in seminomas. (A) Localization of KIT was analyzed by immunohistochemistry. KIT expression was seen in cell membrane of the seminoma cells (right). (B) Intensity of KIT expression was analyzed by Western blotting (HMC-1 is a cell line derived from mastocytosis which expresses KIT and harbors gain-of-function mutations on exon 11 and exon 17 of the *c-kit* gene). The expression of KIT was estimated as '++,' '+' or 'negative' according to Patients and methods.

subjected to a second PCR using the same primers as the first PCR (Table 2). The second PCR products were purified by SUPREC-02 (Takara Bio, Shiga, Japan) and subjected to direct sequencing.

Direct sequencing. Sequence reactions were carried out using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and the products were analyzed by the Genetic Analyzer (ABI PRISM 310; Applied Biosystems). The primers of the reactions were the same as the first PCR primers (Table 2).

Results

Expression of KIT in testicular germ cell tumors

Testicular germ cell tumors were evaluated for KIT expression by immunohistochemical staining and Western blotting. Immunohistochemical staining revealed that KIT was expressed in the cell membrane of the tumor cells (Fig. 1A). KIT (145 kDa) expression was also seen in normal testicular tissues, especially in spermatozoa [17,18]. In Western blotting assay, a case was defined as '+' when the ratio KIT/β-actin was same as or more and less than 2-fold compared to the ratio in normal testis, and estimated as '++' when the ratio was more than 2-fold compared to the ratio in normal testis. There were 13 cases with '+' and 9 cases with '++' (Fig. 1B). In total, KIT expression was seen in 88% (22/25) of seminomas and 44.4% (4/9) of non-seminomas (Table 3). There was no correlation between KIT expression and patients' age, serum marker level, histology or clinical stage (data not shown). Because the KIT overexpression was significantly more frequent in seminomas ($p < 0.01$; χ^2 test), we limited further investigation of KIT to seminoma cases only.

Table 3
Summary of the seminoma cases

Case No.	Age	Histology	Stage (UICC Stage Groupings)	*c-kit* mutation	KIT expression	KIT phosphorylation
1	27	Seminoma	I	L576P (exon 11)	++	+
3	36	Seminoma	I	D816A (exon 17)	++	+
5	37	Seminoma	I	D816H (exon 17)	++	+
7	52	Seminoma	I	D816V (exon 17)	+	+
2	33	Seminoma	I	D816V (exon 17)	++	+
4	36	Seminoma	I	D820V (exon 17)	++	+
6	51	Seminoma	I	Δ57 bp (codon555-573) (exon 11)	++	+
8	27	Seminoma	IIA	D816H (exon17)	+	+
9	42	Seminoma	IIA	D816V (exon 17)	+	+
10	23	Seminoma	I		–	Not estimated
11	23	Seminoma	I		+	+
12	28	Seminoma	I		+	–
13	30	Seminoma	I		+	+
14	32	Seminoma	I		++	+
15	34	Seminoma	I		++	+
16	35	Seminoma	I		+	–
17	35	Seminoma	I		+	–
18	36	Seminoma	I		+	–
19	37	Seminoma	I		–	Not estimated
20	43	Seminoma	I		+	+
21	65	Seminoma	I		+	–
22	36	Seminoma	IIA		+	+
23	21	Seminoma	IIB		–	Not estimated
24	33	Seminoma	IIIA		+	–
25	55	Seminoma	IIIB2		++	+

Mutations of the c-kit gene

We performed polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis for exons 9, 11, 13, and 17 in the *c-kit* gene to detect gain-of-function mutations. Aberrant bands were seen in exons 11 and 17 (Fig. 2A). These bands were excised and subjected to direct sequencing (Fig. 2B). In total, 9 cases had mutations (40.9%; 9/22). No mutations were found in normal testicular tissues. Patients who had *c-kit* mutations are summarized in Table 3. KIT was expressed in all of the cases that possessed mutations. KIT mutations did not correlate with clinical features (age or stage) of the seminomas. Seven of these mutations were identical to mutations previously reported as gain-of-function mutations in several malignancies including testicular germ cell tumors [10,13,19]. The other two mutations (D816A, D820V) were novel.

Activation of KIT and PI3K in seminoma

Cell lysates from the KIT-expressing seminoma were subjected to immunoprecipitation with anti-KIT antibody, and these precipitates were immunoblotted with anti-phosphotyrosine antibody, anti-KIT antibody, and anti-PI3K antibody (Fig. 3A). KIT was phosphorylated in 72.7% (16/22) of the cases that expressed KIT. There was no correlation between KIT phosphorylation and any clinical features. All of the KIT protein from the cases which had

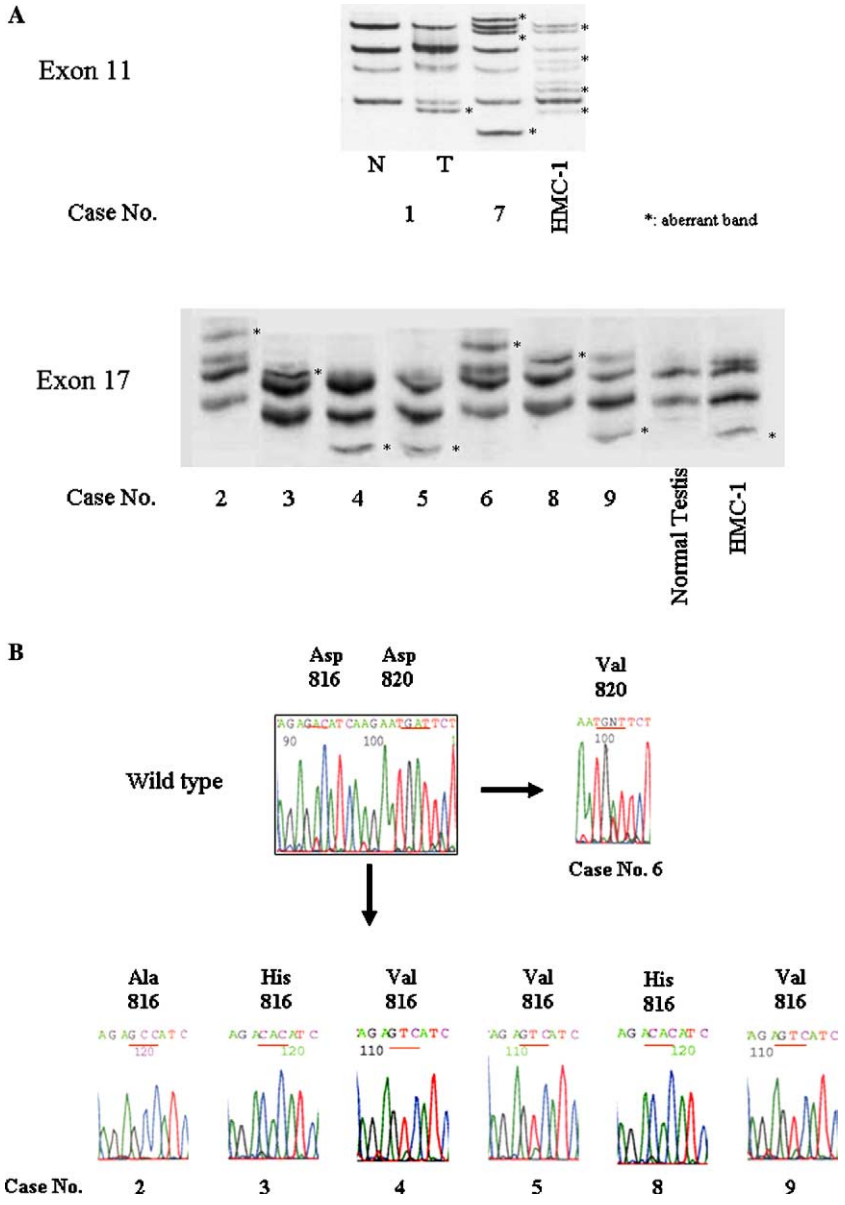

Fig. 2. Mutation analysis of the *c-kit* gene. (A) SSCP analysis of KIT-expressing seminoma. Aberrant bands were seen in exon 11 and exon 17. Aberrant bands were detected by comparing to the bands seen in normal testis. N, normal testicular tissue from Case No.1. Normal testis was obtained from the patient with epididymoorchitis. (B) Aberrant bands were directly sequenced (Results were summarized in Table 3). N, normal T, tumor.

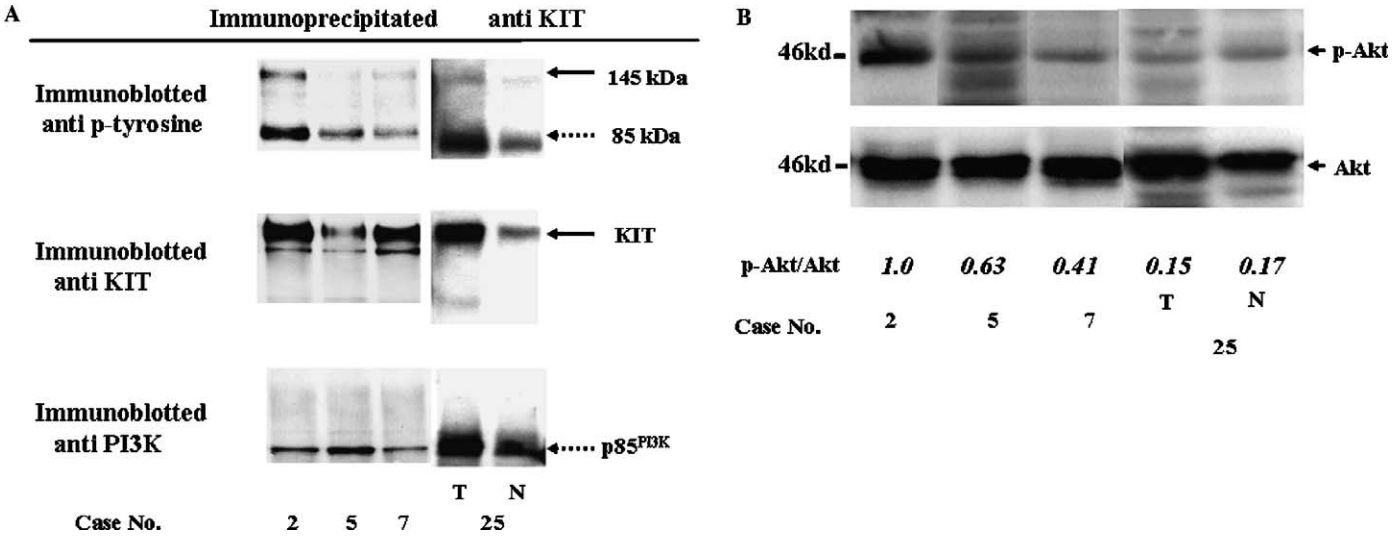

Fig. 3. Activation of KIT in KIT-expressing seminomas. (A) Cell lysates from KIT-expressing seminomas were immunoprecipitated by anti-KIT antibody and immunoblotted by anti-phosphotyrosine antibody (top), anti-PI3-kinase p85 antibody (middle), and anti-KIT antibody (bottom). In the top panel, 145 and 85 kDa bands represent phosphorylated KIT and phosphorylated PI3K p85 subunit, respectively. Sixteen of 22 KIT-expressing seminomas had phosphorylated KIT, and they were associated with PI3K p85 subunit. (B) Cell lysates were immunoblotted against phosphor-Akt and Akt. Akt was phosphorylated in all of the cases that had phosphorylated KIT and PI3K. N, normal testicular tissue; T, testicular tumor tissue.

known gain-of-function mutations (Fig. 3A, case No. 2) and novel mutations (Fig. 3A, case No. 5, 7) were phosphorylated. Interestingly, 7 out of 13 cases (53.8%) without mutation in the *c-kit* gene had phosphorylated KIT, suggesting the existence of the alternative mechanisms other than *c-kit* gene mutation to phosphorylate KIT. The association between KIT expression, *c-kit* gene mutation, and KIT phosphorylation in all seminomas is summarized in Fig. 4. In the immunoprecipitation assay, we found that phosphorylated PI3K p85 subunit ($p85^{PI3K}$) is associated with phosphorylated KIT, suggesting that PI3K is activated through KIT activation.

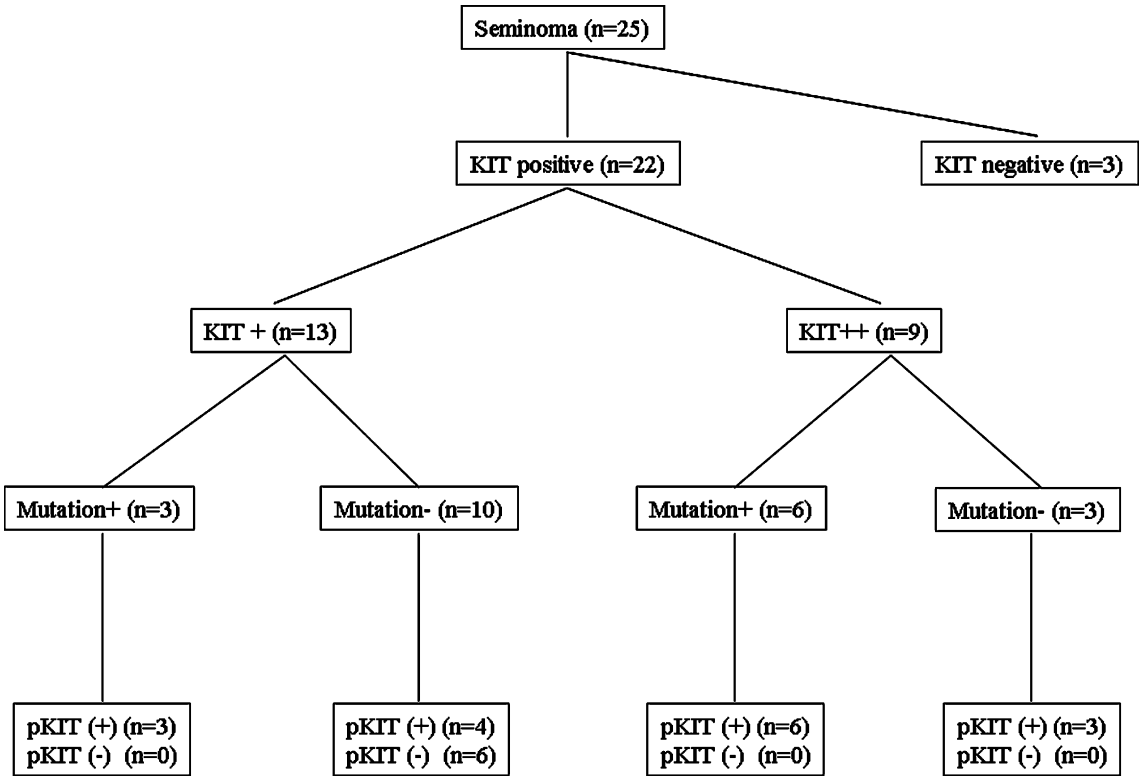

Fig. 4. Study flowchart of all the seminomas. The status of c-kit mutations, KIT expression, and KIT phosphorylation in all the seminoma cases were summarized. pKIT (+), KIT phosphorylation positive; pKIT (−), KIT phosphorylation negative.

We confirmed the activation of KIT–PI3K pathway by analyzing the phosphorylation of Akt, which is one of the downstream targets of activated PI3K (Fig. 3B). Phosphorylation of Akt was analyzed by measuring the ratio of phosphor-Akt (pAkt/Akt). The phosphorylation of Akt was seen in all of the cases that have phosphorylated KIT and PI3K. The extent of the phosphorylation of Akt tended to be higher in the cases that had mutation in the *c-kit* gene (Fig. 3B, case Nos. 2, 5, and 7) than the case that did not have *c-kit* mutation (Fig. 3B, case No. 25).

Discussion

The *c-kit* proto-oncogene encodes the transmembrane tyrosine kinase receptor KIT whose ligand is SCF. SCF binds to KIT and leads to receptor dimerization and autophosphorylation of KIT on its tyrosine residues, resulting in activation of downstream signal transduction [8,9]. Because KIT and SCF are required for hematopoiesis and normal development of germ cells [18,19], abnormal expression of KIT may lead to oncogenesis of testicular germ cell tumors. As expected, expression of KIT was detected in 88% (22/25) of seminomas and 44.4% (4/9) of non-seminomas. The expression rate of 88% in seminomas in this study is compatible with those reported previously [3–6], but the expression rate of 44.4% in non-seminomas is higher than previous reports. This may reflect the fact that teratomas frequently express the *c-kit* gene product [6]. In fact, more than half of the cases (55.6%; 5/9) of non-seminomas in this study contained teratomatous areas.

KIT consists of an extracellular domain, a transmembrane domain, a juxtamembrane domain, and two kinase domains which are divided by a kinase insert. Gain-of-function mutations in the kinase domain reportedly cause ligand-independent activation of KIT. Therefore, we next investigated mutations in the *c-kit* gene and found nine cases (40.9%; 9/22) with mutation in the *c-kit* gene. Seven of them were identical to the known gain-of-function mutation and two were novel mutations. There are only a few previous publications that reported gain-of-function mutations in testicular germ cell tumors [13]. According to these reports, the prevalence of gain-of-function mutation in seminomas is 5.9–14%. In comparison with these reports, the mutation rate of 40.9% in our study is surprisingly high. This difference may be due to the difference in the method to detect mutations. In the previous studies, they directly sequenced the PCR product of the *c-kit* gene or cDNA, which may cause contamination from the normal allele. To avoid contamination from the normal allele, we performed SSCP analysis and purified the aberrant band, and then directly sequenced. Our procedure may lead to high sensitivity to detect a mutation and resulted in high mutation rate in seminomas. We also showed the mutations in exon 11 in testicular germ cell tumors, however, the two novel mutations (D816A, D820V) in exon 17 found in this study remain to be investigated whether they are gain-of-function mutations or not.

There are a number of molecules and pathways associated with KIT signal transduction, including PI3K–Akt [20], JAK/STAT [21,22], the Src family [23], and the Ras-Raf-MAP kinase [24]. PI3K is a heterodimer which consists of an 85 kDa regulatory subunit ($p85^{PI3K}$) and a 110 kDa catalytic subunit ($p110^{PI3K}$). Autophosphorylation of KIT causes recruitment of $p85^{PI3K}$ to the kinase insert domain of KIT [19,25], and $p85^{PI3K}$ tyrosine residue is phosphorylated [26]. Once $p85^{PI3K}$ is phosphorylated, its regulatory activity is relieved leading to activation of catalytic activity of $p110^{PI3K}$. Activated PI3K produces phosphatidylinositol (3,4,5) triphosphate which promotes the recruitment of Akt to the plasma membrane, resulting in phosphorylation of Akt on Thr308 and Ser473, and triggering a cascade of responses such as cell growth, proliferation, survival, and motility leading to tumor progression [27,28].

Finally, we investigated the activation of KIT and its downstream signal transduction in seminomas. All of the mutant KIT and 53.8% (7/13) of wild type KIT were phosphorylated (activated) and associated with $p85^{PI3K}$. There are three possible mechanisms of the phosphorylation of wild type KIT. (1) Mutations in other exons caused phosphorylation of KIT (i.e., mutation in exons 8 and 10 seen in AML [29]); (2) overexpression of the ligand SCF, which is reported in some testicular cancer, caused phosphorylation of KIT [4]; (3) mutation of other tyrosine kinase receptor phosphorylated KIT (i.e., mutation of PDGF receptor α activates KIT in GIST [30]). Akt was also activated in seminomas. These results suggest that Akt activation by PI3K may be involved in tumorigenesis of seminoma. In fact, Meng et al. [31,32] had reported that activation of PI3K–Akt pathway by glial cell line-derived neurotrophic factor (GDNF) in mouse promoted malignant testicular tumors which mimic human seminoma. This report may support the importance of activation of Akt in the tumorigenesis of seminoma.

Many kinds of tyrosine kinases are related to malignancies, and agents that block tyrosine kinase activity have attracted attention as new anti-cancer agents. STI571 is an ATP-competitive inhibitor of Abl, KIT, and PDGF-R. Reports have shown its effects on chronic myelogenous leukemia, which is associated with constitutive Abl activation by the Bcr-Abl fusion protein, and GIST, which is associated with constitutive KIT activation by gain-of-function mutations of the *c-kit* gene [33–35]. Longley et al. [19] divided the gain-of-function mutations of the *c-kit* gene into two types; 'regulatory type' mutations and 'enzymatic pocket type' mutations. 'Regulatory type' mutations, which are associated with exons 9 and 11, cause ligand-independent dimerization of KIT resulting in constitutive activation of KIT. 'Enzymatic pocket type' mutations, which are associated with exons 13, 14, and 17, cause alteration of the amino acid sequence forming the enzymatic site, resulting in activa-

tion of KIT without dimerization. STI571 inhibits ligand-induced and 'regulatory type' mutation-induced activation of KIT, but it does not always inhibit KIT activation induced by 'enzymatic pocket type' mutation. Therefore, it is important to clarify the type of mutation to predict the effect of STI571. In the present study, we found a high rate of gain-of-function mutation (40.9%) in seminoma, 2 of these mutations being found in exon 11. We have not yet examined the growth-inhibitory effect of STI571 on human testicular germ cell tumors because we do not have testicular cancer cell lines possessing 'regulatory type' mutation in the *c-kit* gene so far, but our results suggest that STI571 may have anti-cancer activity in some types of seminomas.

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