

Expression and mutation of *c-Kit* in intracranial germ cell tumors: A single-centre retrospective study of 30 cases in China

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Abstract. Although primary central nervous system (CNS) germ cell tumors (GCTs) are one of the most treatable types of malignant brain tumor, a subset of patients remain resistant to standard chemotherapy. Gain-of-function mutations of the *c-Kit* gene, and KIT protein expression, have been observed in a number of GCTs, including testicular seminoma, ovarian dysgerminoma and mediastinal seminoma in various ethnic groups. Although a small number of studies have reported the role of *c-Kit* in CNS GCTs, few have focused on Chinese patients exhibiting CNS GCTs. In the present study, the frequency and location of *c-Kit* mutations and KIT protein expression levels in CNS GCTs were investigated in 30 patients, between January 1994 and October 2014. Immunohistochemical assays suggested that KIT protein expression was present in 59.1% patients (66.7% in males and 42.9% in females); however, no statistically significant correlation was identified between KIT protein expression and patient clinicopathological features. By performing PCR amplification and direct sequencing, 4 mutational hot spots of the *c-Kit* gene (exons 9, 11, 13 and 17) were examined, and *c-Kit* gene mutation was identified in 1/17 (5.9%) CNS germinoma cases. This mutation was located in exon 11 at codon 557-558 WK (Tryptophan-Lysine). No *c-Kit* gene mutations were detected in non-germinomatous GCTs. Imatinib, a tyrosine kinase inhibitor, may be an effective treatment against standard chemotherapy-resistant CNS germinoma patients exhibiting *c-Kit* mutations.

Introduction

Primary central nervous system (CNS) germ cell tumors (GCTs) are a rare heterogeneous group of lesions located in the CNS (1). CNS GCTs occur primarily in children and adolescents, with ~90% of cases arising before the age of 20 years (2). Pathological classification of CNS GCTs, according to the World Health Organization criteria, comprises germinoma, teratoma, yolk sac tumor, embryonal carcinoma, choriocarcinoma and mixed GCT (3). Although CNS GCTs are considered to be one of the most treatable types of malignant brain tumor, and may be treated using neoadjuvant therapy in combination with pre/post-chemoradiotherapy, a subset of patients remain resistant to standard chemotherapy agents, primarily due to the clinical and histological heterogeneity of CNS GCTs (4). Therefore, the development of an understanding of the detailed molecular mechanisms underlying CNS GCTs is imperative, in order to discover novel potential treatments for tumors that demonstrate resistance to traditional therapeutic strategies.

The proto-oncogene *c-Kit* encodes a transmembrane tyrosine kinase (TK) receptor, containing an extracellular domain with five immunoglobulin-like repeats (D1 distal-D5 juxtamembrane), a transmembrane domain, a juxtamembrane domain, and TK 1 and 2 domains (5). The natural *c-Kit* ligand, stem cell factor (SCF), binds distal D1, D2 and D3 domains and activates downstream signaling, including Src family kinase, phosphoinositide 3-kinase and mitogen-activated protein kinase signaling pathways, promoting cell migration, proliferation and apoptosis resistance (6). SCF/KIT signaling has a significant role in a number of normal tissues, including germ cells, melanocytes, mast cells and interstitial cells of Cajal (7-10). The absence of KIT or SCF expression in mice results in death, suggesting an irreplaceable role of SCF/KIT signaling during embryonic or perinatal death (11). In addition, human *c-Kit* mutations in exons 8, 9, 11, 13 and 17 have been identified in 75-80% of gastrointestinal stromal tumors (GISTs) (12). *c-Kit* mutations have additionally been frequently identified in 30% of GCTs, including testicular seminoma, ovarian dysgerminoma and mediastinal seminoma (13-15).

It has been observed that there is significant genetic variation for the same disease among certain ethnic groups (16). Considering the fact that mutation of *c-Kit* in CNS GCTs has been reported in a number of studies (17,18), and no study to the

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best of our knowledge has focused on Chinese populations, the present study investigated 4 mutant hot spots of the c-Kit gene (exons 9, 11, 13 and 17) using polymerase chain reaction (PCR) amplification and direct sequencing, to identify the presence, frequency and location of c-Kit mutations. In addition, KIT protein expression was detected using immunohistochemistry, and its correlation with patient clinicopathological data was analyzed.

Materials and methods

Patients and specimens. Between January 1994 and October 2014, 21 male and 9 female Chinese patients (male:female ratio, 2.3:1), diagnosed with primary intracranial GCTs at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China) were enrolled in the present study. None of the patients had received chemotherapy or radiotherapy prior to surgery. A total of 39 formalin-fixed paraffin-embedded tumor tissue samples, and one fresh tumor tissue, were collected from primary intracranial GCT patients. All tumor samples were reviewed by two pathologists in order to verify the diagnosis. The basic clinical characteristics of patients are presented in Table I. The present study was approved by the Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China). Written informed consent for the use of patient tissue specimens in the present study was obtained from all patients.

Immunohistochemistry and staining evaluation. Surgical specimens were fixed in 10% formalin (Guanghua Sci-Tech Co., Ltd., Guangdong, China) and embedded in paraffin. Sections (4- μ m) were cut and stained by hematoxylin and eosin (Sangon Biotech Co., Ltd., Shanghai, China). Additional 4- μ m sections were deparaffinized using xylene and rehydrated in a graded series of ethanol. The deparaffinized sections were subsequently incubated with 3% H₂O₂ to inhibit any endogenous peroxidase activity, followed by microwave treatment for antigen retrieval prior to incubation with primary antibody, using a two-step polymer method (EnVision™; Dako, Glostrup, Denmark). The sections were incubated in a humidified chamber at 4°C overnight, following addition of polyclonal rabbit anti-human c-Kit (A4502; Dako) at working dilutions of 1:100. Subsequently, secondary antibody (anti-rabbit IgG; cat no. A042301; Dako) was added following rinsing with phosphate-buffered saline. The sections were incubated at room temperature for 30 min, subsequently immunoreactivity was detected using 3,3-diaminobenzidine (Sangon Biotech Co., Ltd.) for 15 min, and finally counterstained using hematoxylin. GIST tissues were obtained from Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University) and utilized as a positive control for staining. Negative controls were prepared using blocking serum instead of primary antibody. c-Kit expression was assessed based on the intensity and extent of membranous staining. The semi-quantitative scoring system was based on the immunoreactive score (IRS), which was defined as the product of percentage of positive cells (PP) and staining intensity (SI). PP was scored as follows: 0, no staining; 1⁺, 1-25% stained; 2⁺, 26-50% stained; 3⁺, 51-75%, stained; and 4⁺, >75% stained. An IRS of 0, 1⁺ and 2⁺ were defined as weak expression and an IRS of $\geq 3^+$ as strong expression. SI was

scored from 0-3 according to staining colour: 0, no staining; 1, faint yellow; 2, yellow-brown; 3, sepia. The cells were viewed under a microscope (CX31; Olympus Corporation, Tokyo, Japan).

DNA extraction. Genomic DNA was extracted from paraffin-embedded tumor specimens using a QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. The quality and concentration of DNA was assessed using a spectrophotometer (NanoDrop™ 2000; Thermo Fisher Scientific, Waltham, MA, USA) and resolved on a 0.8% TBE gel (Thermo Fisher Scientific).

The amplified DNA fragments were purified using a GFX PCR DNA and Gel Band Purification kit (GE Healthcare Life Sciences, Chalfont, UK) DNA fragments that aligned with exons 9, 11, 13 and 17 were amplified by PCR, using various primers from Invitrogen (Thermo Fisher Scientific; Table II). Each PCR reaction consisted of 5 μ l 10X PCR buffer, 5 μ l magnesium chloride (25 mmol/l), 1 μ l 10 mmol/l deoxynucleotide triphosphates, 0.5 units *Taq* DNA polymerase (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 2 μ l genomic DNA and 1 μ l of each primer (10 μ mol/l), in a final volume of 50 μ l. The cycling conditions were as follows: 94°C for 5 min, 35 cycles at 94°C for 30 sec, at 60°C for 30 sec and 72°C for 30 sec, with a final extension step at 72°C for 10 min. The amplified fragments were purified using a EZ-10 Spin Column PCR Products Purification kit (Bio Basic Canada, Inc., Markham, ON, Canada) and direct sequencing was performed using an ABI Prism® 3100 genetic analyzer (Applied Biosystems; Thermo Fisher Scientific). The sequencing results were analyzed using Chromas Lite software (version 2.1.1; Technelysium Pty Ltd., Brisbane, Australia), with a signal to noise ratio of >98%. Each sample was sequenced at ≥ 2 times.

Statistical analysis. Statistical analyses were performed using SPSS for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA). The χ^2 or Fisher's exact tests were used for categorical and ordinal variables. $P < 0.05$ was considered to indicate a statistically significant difference, and all reported P-values were two-sided.

Results

Variable clinical characteristics of the patients. The mean age of diagnosis of intracranial GCTs was 17.2 years (range, 7-31 years). The duration of symptoms prior to diagnosis ranged from 5 days to 5 years, with a mean duration of 9.7 months. A total of 23 patients (76.7%), including all of the female patients investigated in the present study, were younger than 20 years at diagnosis. Intracranial GCT sites included the sellar region, pineal gland, hypothalamus, third ventricle, basal ganglia and others. The sellar region and pineal gland were the most common sites for intracranial GCT occurrence. Clinical presentations were dependent on the location and size of the tumor in the intracranial region. Symptoms varied at diagnosis, and included headaches, visual disturbances, signs of increased intracranial pressure and endocrine abnormalities (Table I).

KIT protein is expressed in intracranial GCT. KIT protein, which was primarily expressed in the cytoplasmic membrane,

Table I. Clinicopathological data, c-Kit gene mutations and c-Kit IHC staining in 23 intracranial germ cell tumors.

Case number	Age, years	Gender	Location	Histological type	Size, cm	c-Kit IHC	c-Kit mutation
1	12	F	Sellar	G	3.5	2 ⁺	Wild-type
2	14	M	Pineal	G	1.5	4 ⁺	Wild-type
3	9	M	Pineal	G	5.0	3 ⁺	Wild-type
4	17	M	Third ventricle	G	2.5	3 ⁺	Wild-type
5	7	M	Third ventricle	IT	2.5	-	Wild-type
6	15	M	Pineal	G	3.0	4 ⁺	557,558
7	16	M	Hypothalamus	G	5.0	3 ⁺	Wild-type
8	30	M	Sellar	G	2.0	2 ⁺	Wild-type
9	17	F	Hypothalamus	G	3.0	4 ⁺	Wild-type
10	8	M	Basal ganglia	G	5.5	2 ⁺	Wild-type
11	13	F	Sellar	G	3.0	2 ⁺	Wild-type
12	11	F	Suprasellar	G	3.0	1 ⁺	Wild-type
13	11	F	Sellar	G	4.5	3 ⁺	Wild-type
14	16	M	Sellar	G	2.0	-	Wild-type
15	18	M	Hypothalamus	G	3.0	4 ⁺	Wild-type
16	30	M	Hypothalamus	G	2.5	3 ⁺	Wild-type
17	25	M	Third ventricle	G	5.0	3 ⁺	Wild-type
18	31	M	Pineal	MT	3.0	-	Wild-type
19	18	M	Hypothalamus	MT	5.0	-	Wild-type
20	24	M	Pineal	MGCT	3.0	2 ⁺	Wild-type
21	11	M	Pineal	G	3.0	4 ⁺	Wild-type
22	9	F	Pineal	MGCT	3.5	1 ⁺	Wild-type
23	12	F	Sellar	EC	4.0	-	Wild-type

IHC, immunohistochemistry; G, germinoma; MT, mature teratoma; IT, immature teratoma; EC, embryonal carcinoma; MGCTs, mixed germ cell tumors; F, female; M, male.

Table II. Summary of primer sequences utilized for amplification and sequencing of c-Kit exons 9, 11, 13 and 17.

Exon	Primer	Sequence 5'→3'	Annealing temperature, °C	Fragment size, bp
c-Kit 9	F	TCCTAGAGTAAGCCAGGGCTT	54	284
	R	TGGTAGACAGAGCCTAAACATCC		
c-Kit 11	F	CTGAGACAATAATTATTA AAAAGGTGA	55	227
	R	TTATGTGTACCCAAAAAGGTGACA		
c-Kit 13	F	GCTTGACATCAGTTTGCCAG	54	193
	R	AAAGGCAGCTTGGACACGGCTTTA		
c-Kit 17	F	TACAAGTTAAAATGAATTTAAATGGT	53	228
	R	AAGTTGAAACTAAAAATCCTTTGC		

F, forward; R, reverse.

was detected in 21/22 germinoma (95.5%) and 2/2 mixed GCT cases. However, expression of KIT protein was not observed in any of the teratoma (4/4), embryonal carcinoma (1/1) or choriocarcinoma (1/1) cases. High levels of staining were observed in 13/22 (59.1%; 66.7% in males and 42.9% in females) germinoma cases (Fig. 1). As presented in Table III KIT protein expression did not correlate with mutation of the c-Kit gene or any patient clinicopathological parameters, including age, gender, tumor size, tumor location and prognosis.

c-Kit gene mutation was observed in a single germinoma patient. A total of 23 specimens (17 germinoma and 5 non-germinomatous cases) were screened for mutations in the c-Kit gene. Among 17 intracranial germinoma cases, no gene mutations was identified in 16 patients (Fig. 2) and only one c-Kit gene mutation was identified in one patient (5.9%). The observed mutation was located in exon 11, which encodes the juxtamembrane, and was classified as an in-frame deletion at codon 557-558 WK [Tryptophan (Trp)-Lysine (Lys)]. The mutation was considered to

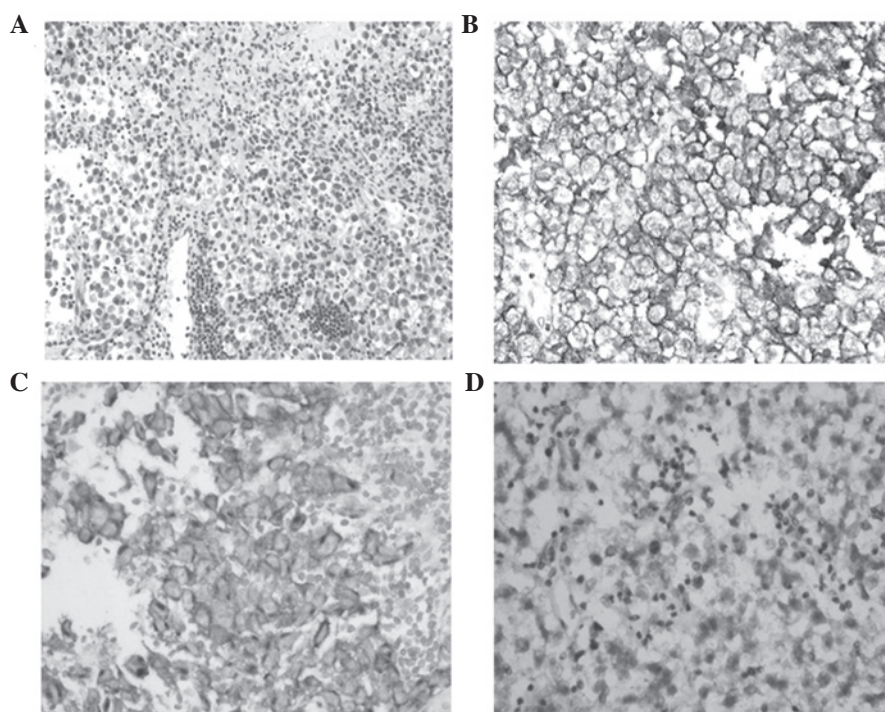


Figure 1. Representative results of c-Kit immunohistochemical staining of tumor cells in intracranial germinomas (magnification, x200). (A) H&E staining, (B) and (C) strong c-Kit expression and (D) weak c-Kit expression. H&E, hematoxylin and eosin.

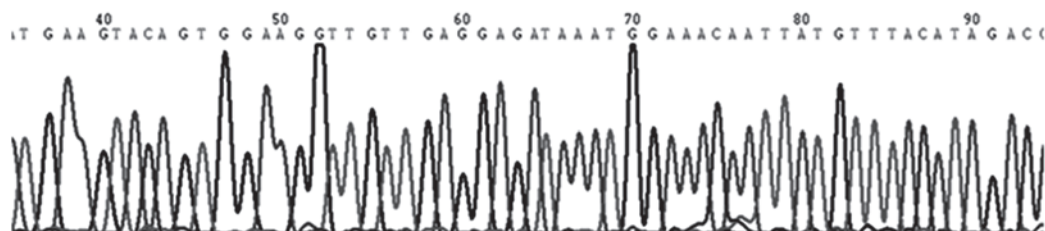


Figure 2. Genomic sequencing of exon 11 of the c-Kit gene, demonstrating a wild-type sequence.

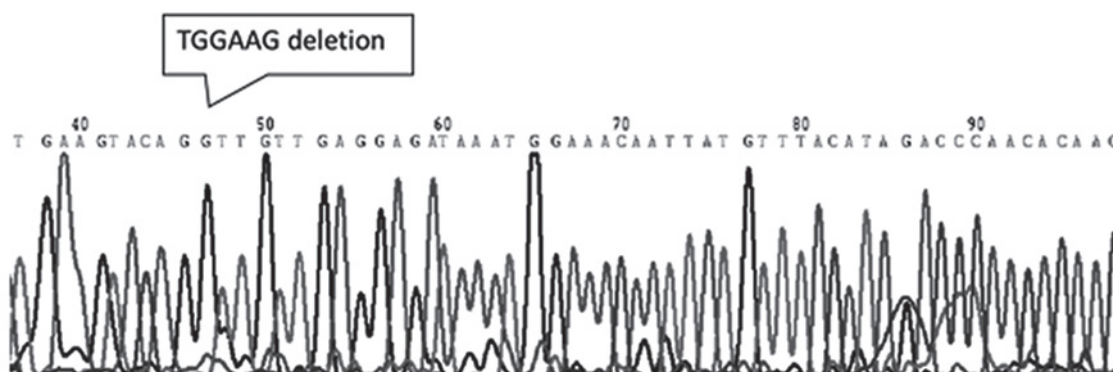


Figure 3. Genomic sequencing of exon 11 of the c-Kit gene. This case exhibited an in-frame deletion mutation (deletion 6 bp, TGGAAG) at codon 557-558 W K (Tryptophan-Lysine). The mutation was considered to be a gain-of-function type.

a be gain-of-function type (Fig. 3). None of the investigated germinoma cases exhibited mutations in exons 9, 13 or 17 of the *c-Kit* gene. No mutations were detected in exons 9, 11, 13 or 17 of the *c-Kit* gene in any of the non-germinomatous GCT cases, including 3 teratomas and 2 mixed GCTs, in the present study.

Discussion

Due to significant advances in treatment options for CNS germinoma patients, it has become essential to develop novel therapeutic strategies for patients that achieve no response

to standard chemotherapeutic agents. Due to its expression and mutation in CNS GCTs, *c-Kit* is a significant potential therapeutic target (18). KIT protein expression in intracranial germinoma cases has been reported in a number of previous studies (17,18). Sakuma *et al* (17) identified that 100% of intracranial germinoma cases investigated demonstrated membranous KIT protein expression. Similar to these previous findings, the results of the present study revealed that KIT protein expression was detectable in 95.5% of intracranial germinoma cases. However, KIT protein expression was not observed to significantly correlate with *c-Kit* gene mutation or patient clinicopathological parameters, including age, gender, tumor size, tumor location and prognosis, which was in accordance with multiple types of tumors with the exceptions of the GISTs (19).

In the present study, no mutations were detected in exons 9, 11, 13, and 17 of the *c-Kit* gene in non-germinomatous GCT cases, including 3 teratomas and 2 mixed GCTs, which was a similar finding to the results of a number of previous studies (5,20-22). The present study identified that 1/17 (5.88%) CNS germinoma cases exhibited a *c-Kit* gene mutation in exon 11, which encodes the juxtamembrane, and this mutation was classified as an in-frame deletion at codon 557-558 WK (Trp-Lys). The results of the present study differed from previous studies, which reported that *c-Kit* mutations were identified in 4/16 (25%) and 3/13 (23%) Japanese patients exhibiting germinomas (17,18). In addition, these previous studies demonstrated that 75% of germinoma cases exhibiting mutations possessed a point mutation at exon 17 (D816V, D820V and N822Y), and only 25% of gene mutations were observed to occur at exon 11 (17,18). There may be several reasons for the discrepancy observed between the results of the present and previous studies. The present study was performed on a cohort of Chinese patients, while the previous studies were performed on Japanese patient cohorts (17,18). Therefore, the differences between these populations may be one of the most plausible explanations underlying the discrepancy observed between the results of the present study and previous studies. Furthermore, as CNS GCTs are rare tumors, it is often only possible to study small sample sizes, which may also be responsible for the inconsistencies in results. Therefore, studies aiming to enroll increased numbers of CNS GCT patients are required, and the association between population factors and *c-Kit* gene mutations in CNS GCTs requires further investigation.

Gain-of-function mutations of *c-Kit* have been identified in a number of neoplasms, including GIST (23), mastocytosis (24) and hematologic malignancies (25). In addition, GIST patients exhibiting *c-Kit* mutations in exon 11, which encodes the juxtamembrane domain, have been reported to exhibit unfavorable prognosis (26). In agreement with the results of a number of previous studies, in the present study, the patient exhibiting a *c-Kit* mutation encountered recurrence 8 months subsequent to primary surgical excision, which suggested that *c-Kit* mutation may be a gain-of-function type mutation responsible for refractory intracranial germinomas. However, there was no statistically significant correlation observed between *c-Kit* protein expression and gene mutation in the present study.

Imatinib, a TK inhibitor, has been utilized in order to block the activated *c-Kit* receptor TK (27). A previous study

Table III. Comparison of clinicopathological data between germinoma patients with high and low KIT protein expression.

Variable	KIT expression		P-value
	High, n=13	Low, n=9	
Median age (range)	17 (9-30)	15 (8-30)	1
Gender (male:female)	10:3	5:4	0.376
Maximum size, cm	3.45 ± 1.21	3.17 ± 1.29	0.611
Location			
Sellar	1	4	0.395
Pineal	4	1	
Third ventricle	2	1	
Hypothalamus	4	1	
Basal ganglia	1	1	
Suprasella	1	1	1
Alive and well	13	9	
<i>c-Kit</i> mutation	1	0	

Statistics were obtained by χ^2 or Fisher's exact test.

has demonstrated that imatinib is able to exert a significant suppressive effect on the activation of the mutational *c-Kit* gene (28). CNS GCT cases exhibiting a mutant *c-Kit* gene within exon 11 have been reported to demonstrate sensitivity to imatinib treatment (29). However, other studies identified that a mutant *c-Kit* gene, which exhibited a codon 816 mutation at exon 17, resulted in resistance to imatinib treatment in GISTs (27,30). Therefore, it is possible that sensitivity to imatinib may depend on the type and site of the *c-Kit* mutation, implying that imatinib may not be a suitable treatment for certain patients exhibiting intracranial GCT. In the present study, a missense mutation in exon 11 of the *c-Kit* gene was detected in a CNS GCT patient. This patient was diagnosed with germinoma exhibiting no notable clinicopathological features, and experienced recurrence 8 months subsequent to primary surgical excision. As the patient was harboring an activated mutation, they may have demonstrated resistance to traditional anticancer therapy and sensitivity to treatment with imatinib. Germinoma patients exhibiting high levels of KIT protein expression may benefit from gene mutation analysis, which may assist in determination of whether imatinib treatment is appropriate.

In conclusion, KIT protein expression was detected in 95.5% of germinoma cases and was not observed to correlate with *c-Kit* gene mutation or clinicopathological parameters. In addition, the present study identified that 1/17 (5.9%) patients possessed mutations at exon 11 of the *c-Kit* gene, which differed from the results of a number of previous studies based on Japanese patient cohorts (17,18). No mutations were detected in non-germinomatous GCT cases. To the best of our knowledge, the present study was the first investigation into the expression and mutation of *c-Kit* in Chinese patients exhibiting CNS GCTs. Additional studies investigating increased numbers of intracranial GCT patients are required, and whole exome sequencing of *c-Kit*

may additionally be conducted in order to identify additional mutant locations.

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