The *KIT* Exon 11 Stop Codon Mutation in Gastrointestinal Stromal Tumors: What Is the Clinical Meaning?

Angela Michelucci*, Caterina Chiappetta[†], Jessica Cacciotti[†], Norman Veccia[†], Elisa Astri[†], Martina Leopizzi[†], Romana Prosperi Porta[‡], Vincenzo Petrozza[†], Carlo Della Rocca[†], Generoso Bevilacqua*, Andrea Cavazzana*,[§], and Claudio Di Cristofano*,[†]

Division of Surgical, Molecular and Ultrastructural Pathology, Department of Oncology, Pisa University Hospital, University of Pisa, Pisa, [†]Department of Medical Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, [‡]Department of Oncology, Medical Oncology Unit, Pisa University Hospital, University of Pisa, Pisa, and [§]Surgical Pathology Unit, ASL1 Massa-Carrara, Tuscany, Italy

Background/Aims: Gastrointestinal stromal tumors (GISTs) strongly express a receptor tyrosine kinase (RTK, c-KIT-CD117) harboring a KIT mutation that causes constitutive receptor activation leading to the development and growth of tumors; 35% of GISTs without KIT mutations have platelet-derived growth factor receptor alpha (PDGFRA) mutations, and the type of mutation plays an important role in the response to treatment. This study aimed to establish the frequency of stop codon mutations in the RTKs, *KIT*, and *PDGFRA*, in GISTs and correlate this molecular alteration with protein expression and treatment responsiveness. **Methods:** Seventy-nine GISTs were analyzed for both *KIT* and *PDGFRA* mutations. Immunohistochemical expression was studied in tissue microarray blocks. **Results:** We found three rare *KIT* mutations in exon 11 that induced a stop codon, two at position 563 and one at position 589, which have never been described before. All three tumors were CD117-, DOG1-, and CD34-positive. Two patients with a *KIT* stop codon mutation did not respond to imatinib therapy and died shortly after treatment. **Conclusions:** The association between stop codon mutations in *KIT* and patient survival, if confirmed in a larger population, may be useful in choosing effective therapies. **(Gut Liver 2013;7:35-40)**

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal neoplasms of the gastrointestinal (GI) tract.[1] They are thought to derive from or differentiate similar to the GI pacemaker cells, the interstitial cells of Cajal.[1] GISTs occur at any site along the tubular GI tract from the esophagus to the anorectum, but they are more common in the stomach (60% to 70%) and in the small bowel (20% to 30%).[1]

The classification of GISTs has been a source of controversy for many years and they have been variously classified as leiomyoma, leiomyosarcomas, or GI autonomic tumors.

In 1998, Hirota *et al.*[2] published two key observations to clarify the nature of GISTs. The majority of GI mesenchymal tumors are GISTs and are heavily and mostly uniformly KIT positive (CD117, stem cell factor receptor). These tumors harbour mutations of the *KIT* causing constitutive activation of KIT kinase, which finally leads to the tumor's development and growth. Following these observations, the immunohistochemical (IHC) detection of the KIT receptor has become the gold standard method for diagnostic confirmation,[3] however, the antibody against DOG1 has also been suggested another specificity marker of GIST.[4] Drugs bind to the enzymatic site of the receptor, blocking it, inhibiting ligand-induced activation and, moreover, inhibiting activation caused by the mutation. Nevertheless, these drugs will not be able to bind to a receptor with an enzymatic site mutation. Drugs which bind to an enzymatic site mutation will not have any effect on the *KIT* wild type either.[5] Different follow-up studies demonstrate that the mutation of the *KIT* juxtamembrane domain, encoded in exon 11, is the most common alteration in GIST. Other types of mutations have been described in exons 9, 13, and 17, but are less frequent.[6] Moreover, GISTs with *KIT* exon 11 deletions seem to be associated with a worse

prognosis.[7-10]

There are, finally, some cases of familial GISTs described in literature with a germline mutation of *KIT*,[11] but most of them are sporadic carrying only a somatic mutation in tumor cells.[2] Moreover, GISTs carrying the *KIT* mutation constitute a very homogeneous class of tumors from a genetic point of view.[12]

From a number of follow-up studies, it has been reported that the frequency of somatic mutations in GISTs, which result in a constitutive activation of KIT kinase, is highly variable and a large number of tumors show the wild type *KIT* as well.[13] These observations have led to the study and investigation of some other tyrosine kinase receptors to understand better their potential role in GISTs' pathogenesis.[13] The platelet-derived growth factor receptor alpha (PDGFRA) receptor has been shown to be involved also in GIST carcinogenesis.[13] The PDGFRA receptor activation is important for cellular migration and proliferation and has different roles in embryogenesis.[14] The PDGFRA is necessary for the development of different tissues and in the inflammation and angiogenesis processes. Approximately 35% of GISTs lacking *KIT* mutations have activating mutations in the *PDGFRA* gene;[13] only mutations at exons 12, 14, and 18 have been reported in GISTs.[15] GISTs with *PDGFRA* exon 14 mutations represent a subset of clinically favourable gastric tumors with epithelioid morphology.[15] *PDGFRA* and *KIT* mutations are mutually exclusive.[13,16] KIT and PDGFRA are both members of the PDGFR subfamily of receptor tyrosine kinase (RTK) proteins that have extra cellular Ig-like domains and a domain link to kinase activity.[15]

Imatinib mesylate (Gleevec) is a selective tyrosine kinase inhibitor that binds to the adenosine triphosphate-binding pocket, blocking both kinase activity, and constitutive activation of the receptor. This drug has been largely used for the treatment of chronic myeloid leukemia and is now also used for the clinical management of patients with GISTs. The GISTs that express the constitutively activated mutant isoforms of the KIT or PDGFRA tyrosine kinases are therapeutically targeted by imatinib mesylate. The first observation of the efficacy of Gleevec has been described in a case report of GIST with multiple metastases in which, after 4 weeks of treatment, the metastases were no longer detectable with a positron emission tomography analysis.[17] Two important clinical studies, with the aim of explaining the relationship between mutations in *KIT-PDGFRA* and clinical response, have also demonstrated the efficacy of Gleevec.[16,18]

These studies indicate that the type and locus of mutation of the *KIT* and *PDGFRA* genes predict the response to imatinib therapy. In fact, the presence of KIT exon 11 mutation is the strongest prognostic factor. Furthermore, patients harboring this type of mutation have a better partial response rate to imatinib therapy. The stop codon mutations are rare in the *KIT* gene and have never been reported in primary GISTs but only in the exon 13 at codon 642 in a recurrent duodenal GISTs[19] and in exon 11 at codon 558 in a lymphoma.[20]

The aim of this study was to establish the frequency of stop codon mutations of the RTKs, *KIT*, and *PDGFRA* in GISTs, and correlate this molecular alteration with protein expression and treatment responsiveness.

MATERIALS AND METHODS

1. Patients

During the period from 1996 to 2007, samples from 79 patients with GIST, 40 men and 39 women with a median age of 66 years (range, 40 to 88 years), were collected. The main diagnostic criteria included histological and clinical features compatible with GIST. Primary sites were as follows: stomach (n=56), small intestine (n=14), large intestine (n=6), peritoneum (n=2), and esophagus (n=1). Moreover, IHC analysis of CD117 and CD34 and molecular analysis of *KIT* or *PDGFRA* mutations, were performed in each case to confirm the morphological and clinical diagnoses. The tumors were assessed by two pathologists, and a minimum of one 4-μm-thick hematoxylin-eosin section was examined per centimeter of tumor diameter. The histological grade was assigned according to Fletcher's criteria.[21]

2. Immunohistochemistry

The IHC analysis was performed on tissue micro arrays. The following antibodies were used: CD117 (polyclonal; DAKO Co., Carpinteria, CA, USA), CD34 (QBEnd 10, monoclonal; Ventana Medical System Inc., Tucson, AZ, USA), DOG1 (SP31, monoclonal; Thermo Fisher Scientific Inc., Waltham, MA, USA); SMA (1A4, monoclonal; Ventana Medical System Inc.), S-100 protein (polyclonal; Ventana Medical System Inc.), Vimentin (V9, monoclonal; Ventana Medical System Inc.), and Desmin (D33, monoclonal; Ventana Medical System Inc.). Antigen retrieval was accomplished by microwaving (360 W) the slides for 5 minutes (3 cycles) in 1 mmol/L citrate buffer pH 7. Tumors were considered KIT positive when the tumor cells showed a membranous, cytoplasmic, or Golgi positivity in at least one of the tumor spots.

3. Tissue microarray

Core tissue biopsies 1 mm in diameter were taken from representative regions of each paraffin-embedded tumor (donor block) and arrayed into a new recipient paraffin block (45×20 mm) using ATA-100 (Chemicon International System, Temecula, CA, USA). In order to minimize the influence of tumor heterogeneity, three different core biopsies for each donor block were performed as well as paired normal tissue as an internal control. Each array contained 50 tissue cylinders and there were 10 primary tumors with 10 corresponding normal tissues.

4. DNA sequencing

Tumor tissue samples were analysed for both *KIT* (NCBI Gene ID, 3815) and *PDGFRA* (NCBI Gene ID, 5156) gene mutations by

direct sequencing analysis of exons 9, 11, 13, and 17 of the *KIT* gene and exons 12, 14, and 18 of the *PDGFRA* gene.

Representative tumor tissue sections (>80% tumor cell) were cut (10 μm thick) and placed directly into a sterile tube. DNA was extracted using QIAamp DNA Mini KIT (Qiagen, Hilden, Germany). All the procedures were performed according to the manufacturer's protocols. In three cases (tumor diameter <1 cm), laser capture microdissection (Leica AS LDM system; Leica Microsystem, Wetzlar, Germany) was used in order to avoid the normal tissue present in the section. About 5,000 microdissected tumor cells were placed directly into a sterile tube containing 60 μL of DNA extraction buffer (Tris-HCl pH 8 100 mM; EDTA 1 mM; Tween-20 1%; Proteinase K 200 to 300 μg/mL) and incubated at 37°C for 12 to 16 hours. The reaction was then inactivated at 95°C for 10 minutes.

The polymerase chain reaction (PCR) was performed using the primers listed in Table 1.

DNAs were amplified in a final volume of 30 μL containing 2 μL of DNA, 2 mM dNTP, 250 ng/μL of each primer, 1.5 mM MgCl$_2$, 1X PCR buffer, and 1U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA). A total of 40 cycles were performed using the Gene Amp PCR System 9700 (Applied Biosystems) at 94°C for 1 minute, specific annealing temperature for 1 minute, 72°C for 1 minute.

The PCR products were then purified using Millipore's Montage PCR96 Cleanup Kit and then sequenced using Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Unincorporated primers and dye terminators were removed using the Montage-SEQ96 Sequencing Reaction Cleanup Kit

Table 1. Primers Used for the Polymerase Chain Reaction

	Primers	Annealing temperature, °C
KIT exon 9	5'-CCAGGGCTTTTGTTTTCTTC-3'	56
	5'-TGGTAGACAGAGCCTAAACATCC-3'	
KIT exon 11	5'-GATCTATTTTTCCCTTTCTC-3'	56
	5'-AGCCCCTGTTTCATACTGAC-3'	
KIT exon 13	5'-TCAGTTTGCCAGTTGTGCTT-3'	56
	5'-AATGTCATGTTTTGATAACCT-3'	
KIT exon 17	5'-TTCTTTTCTCCTCCAACCTAA-3'	56
	5'-TGTCAAGCAGAGAATGGGTA-3'	
PDGFRA exon 12	5'-TCCAGTCACTGTGCTGCTTC-3'	56
	5'-GCAAGGGAAAAGGGAGTCTT-3'	
PDGFRA exon 14	5'-CTCACCTTCTTTCTAACCTTT-3'	56
	5'-ACCCTTATGACCCCATGAACT-3'	
PDGFRA exon 18	5'-ACCATGGATCAGCCAGTCTT-3'	56
	5'-TGAAGGAGGATGAGCCTGACC-3'	

Primers used for the polymerase chain reaction of exons 9, 11, 13, and 17 of the *KIT* gene and exons 12, 14, and 18 of the *PDGFRA* gene and their annealing temperatures.
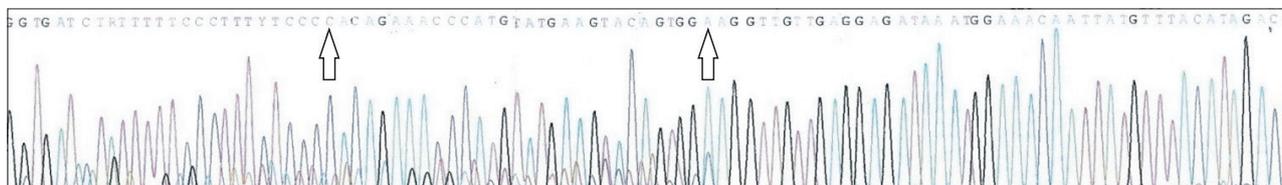
(Millipore, Billerica, MA, USA). Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with 3100 Genetic Analyzer Data Collection software version 1.1. The sequencing and each reaction were performed in triplicate.

5. Statistical analysis

The IHC expression of c-KIT has been compared to *KIT* mutations status, using the Fisher's exact test. Two-sided p of <0.05 was considered to represent statistical significance.

RESULTS

Histologically, tumors were classified as low grade (33 cases), intermediate (13 cases), and high grade (22 cases). The remaining 11 cases were submitted to our division for second opinion and molecular studies and no slide was available for correct surgical pathology grading. The median tumor size was 6.7 cm in diameter, ranging from 0.2 to 22 cm in diameter.

1. Molecular results

In total, 68/79 (86%) cases were mutated for *KIT* or *PDGFRA* but the simultaneous presence of both mutations was not observed.

A *KIT* mutation was present in 56/79 (71%) of cases accounting for 82% of all detected mutations (56/68). Mutations were mapped as follows: 51 in the exon 11 (91%), four in exon 9 (7%) (V474I and 3 c.1525_1530dupGCCTAT), and one in exon 17 (2%) (N822K). No mutation was found in exon 13. Deletion was the most frequent mutational event in *KIT* in our series accounting for 61% of cases (34/56) while duplication accounted for 9% of cases (5/56). In three cases, the mutation changed the reading frame and induced a stop codon: c.1648-5_1672del30bp causing a stop codon at amino acid 563, c.1648-2_1672del27bp causing a stop codon at amino acid 563, and c.1733_1758dup26bp causing a stop codon at amino acid 589 (Fig. 1). In the remaining cases a missense mutation was present. A mutational hot spot was defined within codon 550 and 560 of exon 11.

In 12 cases, a mutation was detected in the *PDGFRA* gene (18%): 11 in exon 18 (92%) and one (N659K) in exon 14 (8%). No mutation of exon 12 in *PDGFRA* was present.

2. IHC results and association with molecular analysis

A positive staining was obtained in 63/79 (80%), 77/79 (97%), and 70/79 (89%) cases for CD34, CD117, and DOG1, respectively (Fig. 2). All cases showed a diffuse positivity for vimentin and focal positivity was found in three, four, and eight cases for SMA, desmin and S100 protein respectively. In 70 cases, IHC (CD117) and molecular (*KIT* and *PDGFRA*) results were available for analysis. Among these cases, the CD117 was negative in only two cases, but in these cases DOG1 was positive. No significant correlation was found between KIT expression and *KIT* or *PDGFRA* mutations. In fact, among the 68 cases with

Fig. 1. Results of direct sequencing of *KIT* in exon 11: mutated sequence which causing a stop codon at aminoacid 563.
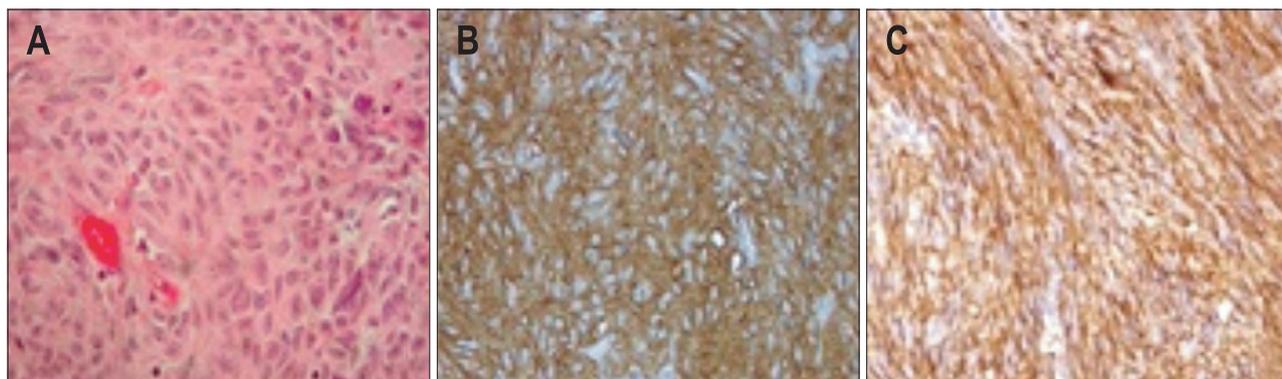

Fig. 2. Morphological and immunohistochemical aspect of gastrointestinal stromal tumors (GIST). (A) Morphological aspect of GIST (H&E stain, ×400). (B) Immunohistochemical expression of CD117 in GIST (×400). (C) Immunohistochemical expression of DOG1 in GIST (×400).

positive CD117 immunostaining and *KIT* or *PDGFRA* mutations, 50/68 (74%) patients had a *KIT* mutation, and 10/68 (15%) cases had a *PDGFRA* mutation. Eight cases (11%) didn't show *KIT* or *PDGFRA* mutations. The two cases without CD117 IHC expression but positive for DOG1 were mutated at exon 11 of *KIT* (c.1663_1677del15bp, codon 555-559) and exon 18 of *PDGFRA* (c.2675_2686del12bp, codon 842-845), respectively.

3. Clinical, morphological, IHC findings in patients with stop codon mutations of *KIT*

The three patients showing a *KIT* stop codon mutations were clinically followed with mean follow-up of 27 months (range, 12 to 48 months). These patients were two male and one female aged 64, 75, and 77 years, respectively. Two patients died of the disease after surgical and imatinib therapy 26 and 20 months after diagnosis, respectively. In these latter patients, the tumors were both located in the stomach and the size of the lesions were 7.5×7 and 5×4.3 cm, respectively. The surviving patient is well with no evidence of disease 52 months after diagnosis. The tumor was located in the duodenum, being 11.5×10 cm in size and with epitheliod features. All three tumors were CD117, DOG1, and CD34 positive.

DISCUSSION

Currently, the histopathological diagnosis of GIST is performed combining the morphological aspect with positive IHC test results for CD117.[21,22] In our series, the morphological diagnosis of GISTs was confirmed with CD117, CD34, and DOG1 IHC analysis with positive test results in 97%, 80%, and 89% cases, respectively.

The uncontrolled cellular growth is the basis of tumor formation and in GISTs it is probably connected to somatic mutations of the *KIT* and *PDGFRA* genes that lead to the constitutive activation of receptors, c-KIT or PDGFRA, causing the independent activation of the receptor in the absence of ligands.[23]

In our series, 68/79 (86%) cases were mutated for *KIT* or *PDGFRA*. *KIT* mutations were present in 56/79 (71%) of cases accounting for 82% of all detected mutations (56/68), in 12/79 cases (15%) the *PDGFRA* gene was mutated, in accordance with reported data.[24]

The type of mutations in the *KIT* gene were highly variable and included deletion, duplication, and missense mutation leading to a gain-of-function of KIT. But, we found three mutations that changed the reading frame and induced a stop codon, two at 563 and one at 589 level, in exon 11 which has never been described until now. In fact, stop codon mutations are rare in the *KIT* gene and have never been reported in primary GIST but only in the exon 13 at codon 642 in a recurrent duodenal GIST[19] and in exon 11 at codon 558 in a lymphoma.[20]

The three cases with stop codon mutations were positive for CD117 and these types of mutations did not show a different immunoreactivity to our CD117 antibody or different clinical-pathological characteristics. All the stop codon mutations detected were in exon 11 of *KIT* that encodes for the regulatory juxtamembrane helix. The structure of the receptor KIT comprised an extracellular domain, a juxtamembrane domain and an intracellular catalytic part of the kinase domain. Mutations in exon 11 affect the juxtamembrane domain of the receptor (the portion just inside the cell membrane) while the CD117 antibody

recognizes the extracellular domain of the receptor and thus the positivity of CD117 in association with a stop codon mutation in exon 11 can be explained by the different location of the mutations and the binding site of CD117. The real state of c-KIT protein or the biological effect of stop codon mutations in GIST is not known. The presence of a truncated protein would not be able to justify the activation of a tyrosine kinase pathway.

The responsiveness to imatinib mesylate appears to correlate to the tumor's genotype and a better clinical response is obtained in patients showing exon 11 *KIT* mutations. Interestingly, in our study, two patients with *KIT* stop codon mutations died after imatinib therapy, this type of mutation could influence targeted therapies. These findings suggest that also the type of mutation affecting exon 11 may influence the response to therapy. In fact, mutations causing the formation of stop codons, although they are exceedingly rare, appear to confer resistance to therapy. This finding necessitates further investigation to understand the rarity of this mutation.

CONFLICTS OF INTEREST

ACKNOWLEDGEMENTS

REFERENCES

1. Agaimy A. Gastrointestinal stromal tumors (GIST) from risk stratification systems to the new TNM proposal: more questions than answers? A review emphasizing the need for a standardized GIST reporting. Int J Clin Exp Pathol 2010;3:461-471.

2. Hirota S, Isozaki K, Moriyama Y, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 1998;279:577-580.

3. Miettinen M, Lasota J. Gastrointestinal stromal tumors: definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. Virchows Arch 2001;438:1-12.

4. Liegl B, Hornick JL, Corless CL, Fletcher CD. Monoclonal antibody DOG1.1 shows higher sensitivity than KIT in the diagnosis of gastrointestinal stromal tumors, including unusual subtypes. Am J Surg Pathol 2009;33:437-446.

5. Longley BJ, Reguera MJ, Ma Y. Classes of c-KIT activating mutations: proposed mechanisms of action and implications for disease classification and therapy. Leuk Res 2001;25:571-576.

6. Rubin BP, Singer S, Tsao C, et al. KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. Cancer Res 2001;61:8118-121.

7. Kontogianni-Katsarou K, Dimitriadis E, Lariou C, Kairi-Vassilatou E, Pandis N, Kondi-Paphiti A. KIT exon 11 codon 557/558 deletion/insertion mutations define a subset of gastrointestinal stromal tumors with malignant potential. World J Gastroenterol 2008;14:1891-1897.

8. Heinrich MC, Owzar K, Corless CL, et al. Correlation of kinase genotype and clinical outcome in the North American Intergroup Phase III Trial of imatinib mesylate for treatment of advanced gastrointestinal stromal tumor: CALGB 150105 Study by Cancer and Leukemia Group B and Southwest Oncology Group. J Clin Oncol 2008;26:5360-5367.

9. Heinrich MC, Maki RG, Corless CL, et al. Primary and secondary kinase genotypes correlate with the biological and clinical activity of sunitinib in imatinib-resistant gastrointestinal stromal tumor. J Clin Oncol 2008;26:5352-5359.

10. Andersson J, Bümming P, Meis-Kindblom JM, et al. Gastrointestinal stromal tumors with KIT exon 11 deletions are associated with poor prognosis. Gastroenterology 2006;130:1573-1581.

11. Nishida T, Hirota S, Taniguchi M, et al. Familial gastrointestinal stromal tumours with germline mutation of the KIT gene. Nat Genet 1998;19:323-324.

12. Allander SV, Nupponen NN, Ringnér M, et al. Gastrointestinal stromal tumors with KIT mutations exhibit a remarkably homogeneous gene expression profile. Cancer Res 2001;61:8624-8628.

13. Heinrich MC, Corless CL, Duensing A, et al. PDGFRA activating mutations in gastrointestinal stromal tumors. Science 2003;299:708-710.

14. Jones AV, Cross NC. Oncogenic derivatives of platelet-derived growth factor receptors. Cell Mol Life Sci 2004;61:2912-2923.

15. Lasota J, Stachura J, Miettinen M. GISTs with PDGFRA exon 14 mutations represent subset of clinically favorable gastric tumors with epithelioid morphology. Lab Invest 2006;86:94-100.

16. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 2003;21:4342-4349.

17. Stroobants S, Goeminne J, Seegers M, et al. 18FDG-Positron emission tomography for the early prediction of response in advanced soft tissue sarcoma treated with imatinib mesylate (Glivec). Eur J Cancer 2003;39:2012-2020.

18. Debiec-Rychter M, Wasag B, Stul M, et al. Gastrointestinal stromal tumours (GISTs) negative for KIT (CD117 antigen) immunoreactivity. J Pathol 2004;202:430-438.

19. Vu HA, Xinh PT, Kikushima M, et al. A recurrent duodenal gastrointestinal stromal tumor with a frameshift mutation resulting in a stop codon in KIT exon 13. Genes Chromosomes Cancer 2005;42:179-183.

20. Choe YS, Kim JG, Sohn SK, et al. c-kit expression and mutations in peripheral T cell lymphomas, except for extra-nodal NK/T cell lymphomas. Leuk Lymphoma 2006;47:267-270.

21. Fletcher CD, Berman JJ, Corless C, et al. Diagnosis of gastrointestinal stromal tumors: a consensus approach. Hum Pathol 2002;33:459-465.

22. Kindblom LG, Remotti HE, Aldenborg F, Meis-Kindblom JM.

Gastrointestinal pacemaker cell tumor (GIPACT): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. Am J Pathol 1998;152:1259-1269.

23. Judson IR. Prognosis, imatinib dose, and benefit of sunitinib in GIST: knowing the genotype. J Clin Oncol 2008;26:5322-5325.

24. Steigen SE, Eide TJ, Wasag B, Lasota J, Miettinen M. Mutations in gastrointestinal stromal tumors: a population-based study from Northern Norway. APMIS 2007;115:289-298.