American Journal of Pathology, Vol. 161, No. 2, August 2002

Correspondence

KIT Gene Mutations in Gastrointestinal Stromal Tumors

More Complex than Previously Recognized?

To the Editor-in-Chief:

Many gastrointestinal stromal tumors (GISTs) contain oncogenic mutations of the *KIT* receptor tyrosine kinase gene. These genomic mutations encode structurally abnormal KIT proteins with constitutive enzymatic activity. In GISTs, most KIT mutations are within the juxtamembrane region (encoded by exon 11). However, smaller numbers of mutations are found in the KIT extracellular region (exon 9), the first part of the split kinase domain (exon 13), or the catalytic activation loop in the second part of the kinase domain (exon 17).[1–8]

Recently, Andersson and colleagues[9] reported a possible novel recurrent *KIT* mutation involving exon 15 and resulting in deletions of SER^{715}. They demonstrated Ser^{715-} *KIT* transcripts in GISTs from 6 of the 11 patients where exon 15 cDNA was evaluated, and 5 of the Ser^{715-} GISTs also had mutations involving the KIT juxtamembrane region. Because the authors regard the Ser^{715-} transcript as mutant, they state that multiple *KIT* mutations in the same tumor, although not previously described in the GIST literature, were quite common in their series. We do not agree with this conclusion, and we are concerned that the authors did not discuss the substantial evidence for Ser^{715-} KIT as a normal variant. We summarize this evidence below.

The Ser^{715-} "mutations" reported by Andersson and co-workers[9] were demonstrated by cDNA sequencing, but no evidence of genomic mutation was provided. Absent a convincing genomic mutation, we see no reason to view these as anything other than normal KIT Ser^{715-} isoforms.[10,11] Such isoforms were reported by Crosier and colleagues in 1993,[10] who found expression of Ser^{715-} and Ser^{715+} KIT in nonneoplastic and neoplastic tissues. These isoforms apparently result from use of an alternate splice acceptor site at the start of exon 15 (Figure 1a). Interestingly, as discussed by Crosier et al,[10] the more 5′ splice acceptor option is not present in murine *KIT* (Figure 1). Therefore, the residue corresponding to human KIT Ser^{715} is not expressed in the mouse (GenBank NM 021099, Y00864, AW762164, BF136330, BF124268, BI134457, BG800962, BE281825, BE949406, BI696285), nor, for similar reasons, in the rat (eg, GenBank NM 022264). KIT Ser^{715} is poorly conserved generally in mammals, and there is an asparagine at this position in the cow, horse, goat, and cat (eg, GenBank AF263827, AJ224645, D45168, S76596). Given that murine KIT lacks SER^{715} and that human Ser^{715-} transcript is found in nonneoplastic cells, we do not believe Ser^{715-} KIT should be viewed as an intrinsic oncoprotein.

We have compared *KIT* cDNA and genomic sequence for the exon 14 and exon 15 splice junctions in seven human primary GISTs. KIT Ser^{715-} and Ser^{715+} transcripts were coexpressed at approximately equal levels in five of the GISTs, whereas the splice junction genomic sequences were normal in all (Figure 1b). No GIST had a genomic mutation affecting the Ser^{715} codon. The genomic and cDNA sequence comparisons show that the mechanism for the Ser^{715-} transcript is not as proposed by Andersson and co-workers,[9] ie, a deletion after the start of exon 15. Rather, this transcript results from an alternate splicing event, exactly as one would surmise from the work of Crosier et al,[10] resulting in omission of the first three nucleotides in exon 15 [163 C, 2164 A, 2165G – GenBank X06182].

In several large published series, each including sequence analyses of more than 40 GISTs, the incidence of KIT V560D mutations has been less than 5%.[3,4,7] We have recently completed analysis of GISTs in an additional group of 220 patients, and found KIT V560D in only 5.9% of these tumors, whereas the overall KIT mutation rate was in excess of 80% (M.C. Heinrich and C.L. Corless, unpublished data). By contrast, 5 of 12 patients (42%) in the series from Andersson and co-workers[9] had GISTs with apparent V560D substitution. There is no clear explanation for the apparent overrepresentation of V560D mutations in this study, but we believe PCR cross-contamination must be considered as a potential confounding factor. We raise this possibility because there is no mention that any of the cDNA mutations were confirmed by PCR amplification of genomic DNA, or by repeating the cDNA sequencing studies from an independent RNA isolate. We believe the interpretation of differing mutations in two metastases from one patient (case 5), only one of these metastases having the V560D mutation, is clouded in the absence of such confirmatory studies. Likewise, the report of differing *KIT* mutations in primary *versus* metastatic GIST (case 4) is unconvincing, because the assumed mutation in this GIST was the exon 15 alternative splicing variant, and no genomic mutation was demonstrated. We have analyzed GIST specimen pairs, either primary and metastasis ($N = 8$), or two metastases ($N = 2$) in 10 patients, and in each patient the identical *KIT* mutation was found in both biopsies (Heinrich, Corless, and Fletcher, unpublished data). The *KIT* mutations in those patients involved exon 11 ($N = 9$) or exon 9 ($N = 1$). Therefore, we disagree with the suggestion that acti-

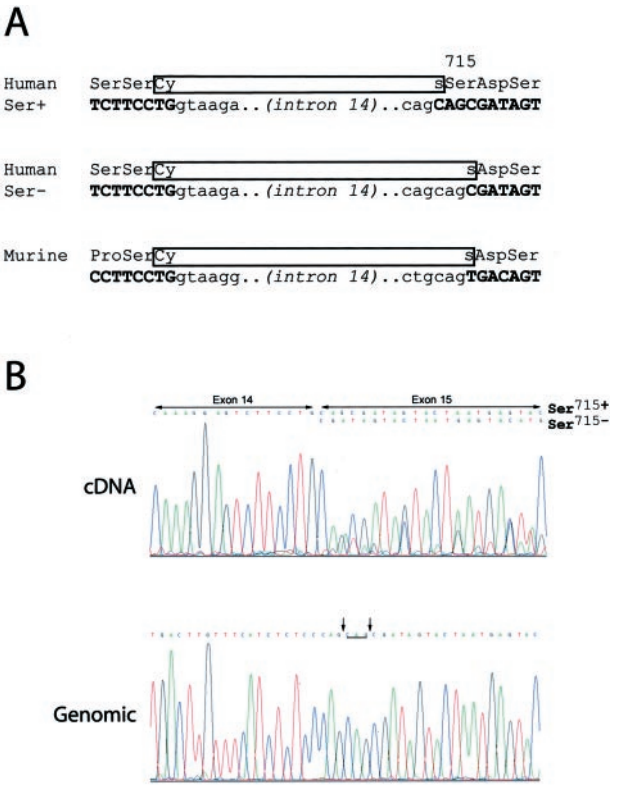

Figure 1.

vating *KIT* mutations, in GISTs, are substantially more complex than previously appreciated.

Jonathan A. Fletcher
Christopher D.M. Fletcher
Harvard Medical School
Boston, Massachusetts

Brian P. Rubin
University of Washington Medical Center
Seattle, Washington

Leonie K. Ashman
University of Newcastle School of Biomedical Sciences
Callaghan, Australia

Christopher L. Corless
Michael C. Heinrich
Oregon Health Sciences University/Portland VA Medical Center
Portland, Oregon

References

1. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y: Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 1998, 279:577–580
2. Ernst SI, Hubbs AE, Przygodzki RM, Emory TS, Sobin LH, O'Leary TJ: KIT mutation portends poor prognosis in gastrointestinal stromal/smooth muscle tumors. Lab Invest 1998, 78:1633–1636
3. Lasota J, Jasinski M, Sarlomo-Rikala M, Miettinen M: Mutations in exon 11 of c-Kit occur preferentially in malignant versus benign gastrointestinal stromal tumors and do not occur in leiomyomas or leiomyosarcomas. Am J Pathol 1999, 154:53–60
4. Taniguchi M, Nishida T, Hirota S, Isozaki K, Ito T, Nomura T, Matsuda H, Kitamura Y: Effect of c-kit mutation on prognosis of gastrointestinal stromal tumors. Cancer Res 1999, 59:4297–4300
5. Lux ML, Rubin BP, Biase TL, Chen CJ, Maclure T, Demetri G, Xiao S, Singer S, Fletcher CD, Fletcher JA: KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. Am J Pathol 2000, 156:791–795
6. Lasota J, Wozniak A, Sarlomo-Rikala M, Rys J, Kordek R, Nassar A, Sobin LH, Miettinen M: Mutations in exon 9 and 13 of KIT gene are rare events in gastrointestinal stromal tumors: a study of 200 cases. Am J Pathol 2000, 157:1091–1095
7. Rubin BP, Singer S, Tsao C, Duensing A, Lux ML, Ruiz R, Hibbard MK, Chen CJ, Xiao S, Tuveson DA, Demetri GD, Fletcher CD, Fletcher JA: KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. Cancer Res 2001, 61:8118–8121
8. Hirota S, Nishita T, Isozaki K, Taniguchi M, Nakamura J, Okazaki T, Kitamura Y: Gain-of-function mutation at the extracellular domain of KIT in gastrointestinal stromal tumours. J Pathol 2001, 193:505–510
9. Andersson J, Sjögren H, Meis-Kindblom JM, Stenman G, Åman P, Kindblom LG: The complexity of KIT gene mutations and chromosome rearrangements and their clinical correlation in gastrointestinal stromal (pacemaker cell) tumors. Am J Pathol 2002, 160:15–22
10. Crosier PS, Ricciardi ST, Hall LR, Vitas MR, Clark SC, Crosier KE: Expression of isoforms of the human receptor tyrosine kinase c-kit in leukemic cell lines and acute myeloid leukemia. Blood 1993, 82:1151–1158
11. Ashman LK: The biology of stem cell factor and its receptor C-kit. Int J Biochem Cell Biol 1999, 31:1037–1051

Author's Reply:

After the completion of our study we became aware of the true nature of the Ser^{715-} KIT transcript in GIST. As pointed out by Dr. Jonathan Fletcher and co-workers, the Ser^{715-} KIT transcript that was detected in 7 of 13 gastrointestinal stromal tumors (GIST) in our series[1] are indeed a normal splice variant. The main reason we interpreted this as a mutation was the fact that this isoform was not recorded in any available public database despite its original description in 1993 by Crosier et al.[2] This Ser^{715-} splice variant has now been submitted to EMBL (Accession number AJ438313). We have subsequently analyzed the distribution of the $Ser^{715+/-}$ isoforms in 18 GIST as well as in normal human bone marrow and fetal liver by RT-PCR and restriction enzyme analyses. Both isoforms were detected and most tumors showed similar expression levels of the two transcripts. In contrast, normal bone marrow and fetal liver showed a clear predominance of the shorter Ser^{715-} isoform (Figure 2). Our misinterpretation of the Ser^{715-} isoform as a mutation could have been avoided by analysis of genomic DNA. However, cDNA-based analyses generally detect what is

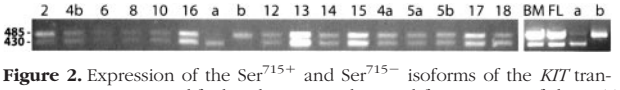

Figure 2. Expression of the Ser^{715+} and Ser^{715-} isoforms of the *KIT* transcript. cDNA was amplified with primers designed for a region of the *KIT* gene including the exons 14–15 border. The PCR products were digested with *Pst*I, a restriction enzyme that only cuts the Ser^{715+} isoform, and separated by gel electrophoresis. Fifteen of the 18 tested tumors are indicated by case numbers. **A** and **B** represent clonal control fragments, **A:** Ser^{715+}; **B:** Ser^{715-}; BM, human bone marrow; FL, human fetal liver

truly expressed in a tumor and are therefore particularly useful in lesions with an admixture of normal cells. The finding of a predominant GNNK-isoform in GIST would not have been possible if the mutation analysis had been performed on genomic DNA alone.

Dr. Fletcher and co-workers suggest that the detection of the V560D mutation of exon 11 in 5 of 14 GIST in our series may be due to PCR cross-contamination, since they have observed this particular mutation in only 5.9% of 220 GIST (M.C. Heinrich and C.L. Corless, unpublished data). We have now repeated the sequence analyses of genomic DNA from the five tumors in which we originally detected the V560D mutation. In three cases (cases 1, 5a, and 9), the mutation was confirmed and in two cases (cases 2 and 10), a normal *KIT* sequence was detected. We were thus able to confirm the existence of a differing mutation pattern in two metachronous metastases (cases 5a and 5b), one of the findings questioned by Dr. Fletcher and co-workers. Our inability to confirm the V560D mutation using genomic DNA in cases 2 and 10 could be due to tumor cell heterogeneity or that the mutation was obscured due to an admixture of non-neoplastic cells in the sample used for extraction of genomic DNA. Although a number of steps were taken to avoid the well known risks of PCR cross-contamination, we cannot entirely exclude this possibility. The reasons for the more frequent occurrence of this particular mutation of exon 11 in our series are not clear. The relatively small size of our series as well as geographic differences could account for the discrepancy. In a subsequent analysis of six additional GIST in which three tumors had mutations in exon 11, we did not detect the V560D mutation in any of the tumors (unpublished data).

Previous studies correlating *KIT* mutation pattern with phenotypic and biological behavior in GIST have been limited to analysis of exon 11 mutations. Several studies have indicated a strong correlation between the existence of exon 11 mutations, a malignant phenotype and aggressive clinical behavior.[3–9] Our recent study demonstrated the occurrence of clinically highly malignant GIST lacking any *KIT* gene mutations as well as *KIT* mutation in a small incidentally detected GIST with an entirely benign histology and clinical course. These findings suggest that the relationship between *KIT* mutations and biological behavior is more complex than previously recognized. In addition, we did not detect any clear correlation between chromosomal aberrations, *KIT* mutations, and phenotypic and biological characteristics in GIST. The recent study of Dr. Rubin and co-workers[10] supports our conclusions regarding the complex relationship between *KIT* mutations and the biological behavior of GIST. Dr. Rubin et al[10] detected *KIT* gene mutations of exons 9, 11, 13 and/or 17 in 44 of 48 GIST, including 10 of 10 benign GIST, 8 of 10 borderline tumors and 26 of 28 malignant GIST. *KIT* mutational status did not correlate with histological subtype or with morphologically determined biological potential. Their findings of *KIT* gene activation without the presence of activating mutations further indicate the complexity of this issue. In view of new therapeutic approaches,[11] further analyses attempting to correlate *KIT* gene function and mutations, biological behavior, and treatment response are essential.

Johanna Andersson
Helene Sjögren
Jeanne Meis-Kindblom
Göran Stenman
Pierre Åman
Lars-Gunnar Kindblom

Göteborg University
Göteborg, Sweden

References

1. Andersson J, Sjögren H, Meis-Kindblom JM, Stenman G, Åman P, Kindblom LG: The complexity of KIT gene mutations and chromosome rearrangements and their clinical correlation in gastrointestinal stromal (pacemaker cell) tumors. Am J Pathol 2002, 160:15–22
2. Crosier PS, Ricciardi ST, Hall LR, Vitas MR, Clark SC, Crosier KE: Expression of isoforms of the human receptor tyrosine kinase c-kit in leukemic cell lines and acute myeloid leukemia. Blood 1993, 82: 1151–1158
3. Ernst SI, Hubbs AE, Przygodzki RM, Emory TS, Sobin LH, O'Leary TJ: KIT mutation portends poor prognosis in gastrointestinal stromal/smooth muscle tumors. Lab Invest 1998, 78:1633–1636
4. Lasota J, Jasinski M, Sarlomo-Rikala M, Miettinen M: Mutations in exon 11 of c-Kit occur preferentially in malignant versus benign gastrointestinal stromal tumors and do not occur in leiomyomas or leiomyosarcomas. Am J Pathol 1999, 154:53–60
5. Moskaluk CA, Tian Q, Marshall CR, Rumpel CA, Franquemont DW, Frierson Jr HF: Mutations of c-kit JM domain are found in a minority of human gastrointestinal stromal tumors. Oncogene 1999, 18:1897–1902
6. Taniguchi M, Nishida T, Hirota S, Isozaki K, Ito T, Nomura T, Matsuda H, Kitamura Y: Effect of c-kit mutation on prognosis of gastrointestinal stromal tumors. Cancer Res 1999, 59:4297–4300
7. Nishida T, Hirota S: Biological and clinical review of stromal tumors in the gastrointestinal tract. Histol Histopathol 2000, 15:1293–1301
8. Nishida T, Nakamura J, Taniguchi M, Hirota S, Ito T, Kitamura Y, Matsuda H: Clinicopathological features of gastric stromal tumors. J Exp Clin Cancer Res 2000, 19:417–425
9. Pidhorecky I, Cheney RT, Kraybill WG, Gibbs JF: Gastrointestinal stromal tumors: current diagnosis, biologic behavior, and management. Ann Surg Oncol 2000, 7:705–712
10. Rubin BP, Singer S, Tsao C, Duensing A, Lux ML, Ruiz R, Hibbard MK, Chen CJ, Xiao S, Tuveson DA, Demetri GD, Fletcher CD, Fletcher JA: KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. Cancer Res 2001, 61:8118–8121
11. Joensuu H, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, Silberman SL, Capdeville R, Dimitrijevic S, Druker B, Demetri GD: Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med 2001, 344:1052–1056

Apparent KIT Ser^{715} Deletion in GIST mRNA Is Not Detectable in Genomic DNA and Represents a Previously Known Splice Variant of KIT Transcript

To the Editor-in-Chief:

Gain-of-function mutations leading into ligand-independent activation of KIT and having a transforming effect *in*

vitro have been documented in gastrointestinal stromal tumors (GISTs).[1] A majority of these mutations affects juxtamembrane KIT domain, although mutations in extracellular and kinase domains have also been reported in the subsets of GISTs.[2–4] However, only one type of KIT mutation can be identified in any given tumor. The presence of two different somatic mutations in one tumor was reported once[5] and seems to be an extremely rare event since has not been seen in other studies.[2,4]

Recently, an RNA-based study found deletions resulting in loss of Lys^{704} and Asn^{705} in the first KIT kinase domain (exon 14) and loss of Ser^{715} in the interkinase KIT domain (exon 15) in GISTs carrying mutations of juxtamembrane KIT domain.[6]

We have selected DNA and RNA samples from KIT-positive GISTs (74 samples from 69 patients) to evaluate molecular alterations in *KIT* exons 14 and 15 using PCR and RT-PCR amplification. There were 30 gastric, 16 small intestinal, 7 colonic, and 6 rectal primary tumors, 11 recurrent, and 4 tumors of unknown origin. *KIT* exon 11 and exon 9 mutations were present in 42 and 9 samples, respectively. Direct sequencing of exon 14 and 15 showed wild-type *KIT* sequences in 31 and 47 analyzed samples respectively. Similarly, one peak consistent with the presence of wild-type *KIT* only was seen in 13 cases analyzed by capillary gel electrophoresis (Figure 1A). However, capillary gel electrophoresis of the RT-PCR products revealed the presence of two peaks in 16 of 21 analyzed tumors (Figure 1B). Direct sequencing of the PCR products confirmed the presence of wild-type *KIT* and another, 3 bp shorter *KIT* transcript lacking Ser^{715} (Figure 1C). The ratio between the two forms of *KIT* transcripts was unequal with preferential expression of the wild-type *KIT*. This observation and a previously published studies on different types of KIT isoforms in normal and malignant hematopoietic cells[7] clearly indicate that the deletion of Ser^{715} recently reported by Anderson and co-authors[6] does not represent a somatic mutation involved in GISTs tumorigenesis, but rather a splice variant of *KIT* transcript.

Lack of mutation in exon 14 in our studies would not exclude the possibility that deletion of Lys^{704} and Asn^{705} could be a rare, possibly secondary, random molecular change. To investigate the relationship of alternative splicing between exon 14 and 15 and exon 11 pathological changes in GISTs, mutant, and wild-type KIT transcripts were amplified separately in three cases using the allele-specific primers from exon 11 and the common primer from exon 15. The wild-type KIT allele was amplified by a primer homologous to the deleted sequences, while the mutant KIT allele was amplified by a primer homologous to specific sequences created by the deletion. RT-PCR amplification of the *KIT* exon 11–15 generated products of 538–541 bp and 529–531 bp for wild-type and mutant KIT, respectively. Because separation of 3 bp different fragments in this size range is beyond resolution of capillary gel electrophoresis, a five cycle-long semi-nested PCR amplification was performed to obtain a shorter product. The specificity of this RT-PCR has been confirmed by amplification of KIT Gly-Asn-Asn-$Lys^{510–513}$ isoforms using forward exon 9 primer and allele-specific reverse primers. In this case, smaller size RT-PCR products fractionated by capillary gel electrophoresis revealed predicted allele-specific sizes. Using this strategy, we have detected KIT Ser^{715-} and KIT Gly-Asn-Asn-$Lys^{510–513+}$ transcripts in GISTs from the wild-type and mutant *KIT* mRNA therefore supporting a naturally occurring variation in the splicing mechanism.

Two types of KIT isoforms have been reported, KIT Gly-Asn-Asn-$Lys^{510–513+/-}$ alternatively splices 12 bp located immediately downstream to the extracellular KIT domain following exon 9 sequences and KIT $Ser^{715+/-}$ alternatively splices first 3 bp of exon 15, an interkinase KIT domain.[7,8] Expression of these isoforms has been documented in normal human hematopoietic cells, leukemic cell lines, acute myeloid leukemia blasts, and GISTs.[6–8] However, no evidence has been found suggesting that up-regulation of KIT isoforms contributes to the neoplastic process.[7,8]

More recent studies showed that KIT Gly-Asn-Asn-Lys^{-} and Gly-Asn-Asn-Lys^{+} differ in activation of downstream KIT signaling pathways and show different transforming activity when expressed in NIH3T3 cells; only cells expressing the Gly-Asn-Asn-Lys^{-} isoform were tumorigenic in nude mice.[9] Modification of KIT in GISTs by alternative splicing events involving exon 9 and exon 15 may contribute to different biological function of mutant

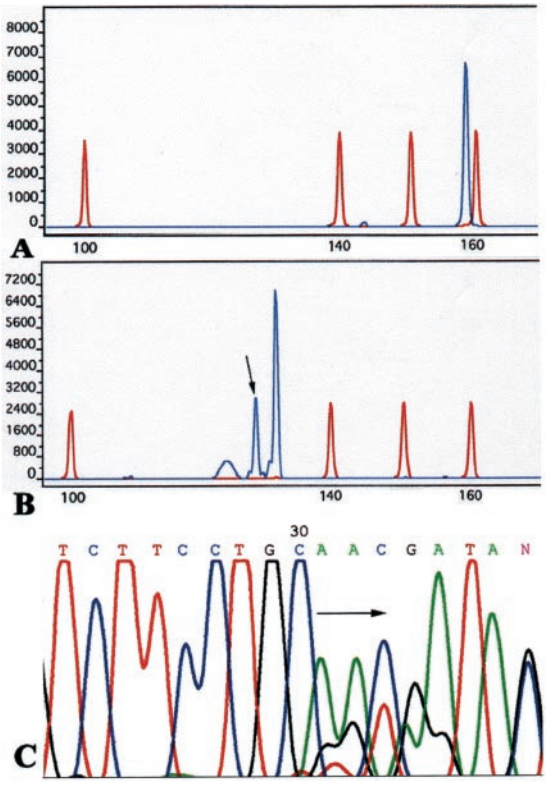

Figure 1. Capillary gel electrophoresis fragment analysis and direct sequencing of *KIT* PCR amplification products. PCR amplification of exon 15 genomic sequences (**A**) and RT-PCR amplification of exon 14/15 junctional sequences (**B**). Blue peaks represent PCR products. Red peaks indicate molecular size markers, values in bp are shown below. **Arrow** indicates *KIT* Ser^{715-}. Direct sequencing of RT-PCR product from B shows presence of *KIT* $Ser^{715\ +/-}$ (**C**). The horizontal **arrow** indicates shift of allelic sequences.

KIT and should be further studied using *in vitro* and *in vivo* models.

Authors' Note: The opinions and assertions contained herein are the expressed views of the authors and are not to be construed as official or reflecting the views of the Departments of the Army or Defense. This study was partly supported by the American Registry of Pathology.

Jerzy Lasota
Janusz Kopczynski
Mourad Majidi
Markku Miettinen

Armed Forces Institute of Pathology
Washington, DC

Maarit Sarlomo-Rikala

Haartman Institute
Helsinki, Finland

References

1. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Tunio GM, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y: Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 1998, 279: 577–580
2. Lux ML, Rubin BP, Biase TL, Chen C-J, Maclure T, Demetri G, Xiao S, Singer S, Fletcher CDM, Fletcher JA: KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. Am J Pathol 2000, 156:791–795
3. Lasota J, Wozniak A, Sarlomo-Rikala M, Rys J, Kordek R, Nassar A, Sobin LH, Miettinen M: Mutations in exons 9 and 13 of KIT gene are rare events in gastrointestinal stromal tumors. Am J Pathol 2000, 157:1091–1095
4. Rubin BP, Singer S, Tsao C, Duensing A, Lux ML, Ruiz R, Hibbard MK, Chen C-J, Xiao S, Tuveson DA, Demetri GD, Fletcher DMF, Fletcher JA: KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. Cancer Res 2001, 61:8118–8121
5. Sakurai S, Oguni S, Hironaka M, Fukayama M, Morinaga S, Saito K: Mutations in c-kit gene exons 9 and 13 in gastrointestinal stromal tumors among Japanese. Jpn J Cancer Res 2001, 92:494–498
6. Andersson J, Sjögren H, Meis-Kindblom JM, Stenman G, Åman P, Kindblom L-G: The complexity of KIT gene mutations and chromosome rearrangements and their clinical correlation in gastrointestinal stromal (pacemaker cell) tumors. Am J Pathol 2002, 160:15–22
7. Crosier PS, Ricciardi ST, Hall LR, Vitas MR, Clark SC, Crosier KE: Expression of isoforms of the human receptor tyrosine kinase c-kit in leukemic cell lines and acute myeloid leukemia. Blood 1993, 82: 1151–1158
8. Piao X, Curtis JE, Minkin S, Minden MD, Bernstein A: Expression of the Kit and Kit A receptor isoforms in human acute myelogenous leukemia. Blood 1994, 83:476–481
9. Caruana G, Cambareri AC, Ashman LK: Isoforms of c-kit differ in activation of signaling pathways and transformation of NIH3T3 fibroblasts. Oncogene 1999, 18:5573–5581