

## c-kit Expression in Dedifferentiated and Well-differentiated Liposarcomas; Immunohistochemistry and Genetic Analysis

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**Abstract.** *Background:* c-kit expression by immunohistochemistry has been utilized to identify cancer patients who can be treated with imatinib-mesylate. In gastrointestinal stromal tumors (GISTs), an activating mutation in c-kit predicts treatment response; its presence in other soft tissue tumors is unexplored. *Materials and Methods:* We evaluated seven cases of dedifferentiated liposarcomas (DDLs) and compared those with seven well-differentiated liposarcomas (WDLs). Immunohistochemical staining for c-kit was performed using a polyclonal antibody. Using PCR, exons 9, 10-11, 12-13 and 17 of c-kit were amplified and direct DNA sequencing performed. *Results:* Two out of 7 (30%) DDLs showed focal weak immunoreactivity with c-kit; no (0%) WDLs stained with c-kit. Seven out of 7 (100%) DDLs showed an allelic variation in exon 10, with a single base pair substitution (A>C) at codon 541; 3/7 (43%) WDLs showed the same change. *Conclusion:* c-kit immunoreactivity did not correlate with the change in DNA sequence; DDLs showed a consistent allelic variation in c-kit that may have significant prognostic, diagnostic and therapeutic implications.

c-kit is a proto-oncogene located on the long arm of chromosome 4 (1) and encodes a transmembrane glycoprotein (2, 3) with a tyrosine kinase domain. It is essential for hematopoiesis (4), spermatogenesis (5), melanogenesis (6) and gut motility (pacemaker cells) (7). It plays an important role in cell proliferation, differentiation, apoptosis and adhesion (8). Upon binding its ligand, stem cell factor (SCF), c-kit forms an active dimer that autophosphorylates itself and activates a signaling cascade that induces cell growth (9, 10). c-kit is associated with several highly malignant tumors and

has been extensively studied in gastrointestinal stromal tumors (GISTs) (11), dermatofibrosarcoma protuberans (12) and mastocytosis (13, 14). Imatinib-mesylate is a small molecular weight signal transduction inhibitor that blocks the tyrosine kinase activity of c-kit. It has been successfully used to treat GISTs and chronic myelogenous leukemia (15). The efficacy of this agent in other tumors is being studied. An important question is whether other tumors will respond to this therapy. Using immunohistochemistry alone to decide treatment may be misleading. For example, although most GISTs are immunoreactive with c-kit, only half of those have mutations in the c-kit gene (16). It has also been shown that responsiveness to imatinib-mesylate depends on the location of the c-kit or PDGFRA mutation (17). Bauer *et al.* (18) have reported a case with metastatic GIST who responded well to imatinib-mesylate treatment, despite the near absence of c-kit expression in two different samples of his tumor, although an in-frame deletion in c-kit exon 11 was seen.

The expression of c-kit by immunohistochemistry has been studied in many soft tissue tumors and other malignancies; however, mutational analysis has not been performed in most of these. There is little data on c-kit expression in liposarcomas and no report is available on genetic analysis of these tumors. This study evaluated c-kit expression in dedifferentiated liposarcomas with comparison to well-differentiated (adipocytic) liposarcomas and examined the integrity of c-kit using direct sequencing of the gene after PCR amplification.

### Materials and Methods

We selected seven cases each of dedifferentiated and well-differentiated (adipocytic) liposarcomas from the archives at the Department of Pathology, University of Colorado Health Sciences Center, USA, between 1992 and 2003. A summary of cases with dedifferentiated liposarcomas and well-differentiated liposarcomas is given in Tables I and II, respectively.

**Immunohistochemistry.** Immunohistochemical analysis was performed using 4-micron-thick sections of formalin-fixed paraffin-

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Table I. Summary of patients with dedifferentiated liposarcomas.

Case#	Age/ Sex	Site	Size	c-kit by IHC	c-kit DNA sequence change
1.	50y/M	Retroperitoneum	20 cm	Negative	Exon 10*
2.	55y/F	Retroperitoneum	20 cm	Negative	Exon 10*
3.	38y/F	Retroperitoneum	23 cm	Negative	Exon 10*
4.	31y/M	Retroperitoneum	25 cm	Negative	Exon 10*
5.	91y/M	Thigh	15 cm	Negative	Exon 10*
6.	49y/M	Supraclavicular	5 cm	Focal +	Exon 10*
7.	62y/M	Retroperitoneum	45 cm	Focal +	Exon 10*

IHC- Immunohistochemistry, \*A>C at codon 541

Table II. Summary of patients with well-differentiated liposarcomas.

Case#	Age/ Sex	Site	Size	c-kit by IHC	c-kit DNA sequence change
1.	54y/M	Retroperitoneum	27 cm	Negative	No change
2.	61y/M	Abdominal wall	10 cm	Negative	No change
3.	56y/M	Thigh	8.8 cm	Negative	No change
4.	69y/F	Thigh	23 cm	Negative	Exon 10*
5.	43y/M	Retroperitoneum	38 cm	Negative	Exon 10*
6.	77y/F	Retroperitoneum	25 cm	Negative	No change
7.	66y/M	Retroperitoneum	25 cm	Negative	Exon 10*

IHC- Immunohistochemistry, \*A>C at codon 541

embedded tissue. For antigen retrieval, the sections were pretreated and pressure cooked with buffer (pH 9.6) for 20 minutes. The antibody used was polyclonal anti-human antibody c-kit (DAKO, A4502, Carpinteria, CA, USA) at 1:200 dilution for 28 minutes. Slides were developed using Enhanced Dab detection kit (Ventana, Tucson, AZ, USA) with avidin-biotin blocker on an autostainer (Benchmark XT automated Ventana IHC stainer). Slides were then counterstained with Gill's hematoxylin, dehydrated and coverslipped with mounting media. The staining was scored as focal or diffuse and an arbitrary score of intensity as weak, moderate or strong was given. GIST tumor sections were used as controls.

**DNA extraction.** Ten-micron-thick sections were cut from paraffin blocks and deparaffinized in xylene followed by dehydration in alcohol (100%, 95%, 70%). DNA was isolated using the DNeasy kit (Qiagen, Valencia, CA, USA), essentially as suggested by the manufacturer except for the addition of an overnight incubation at 55°C in proteinase K.

**PCR amplification.** The sequence of the PCR primers used to amplify exons 9,10-11,12-13, and 17 of c-kit is listed in Table III. Conditions for PCR amplification were a 10-minute activation period for AmpliTaq Gold and DNA denaturation at 95°C, 14 cycles of 95°C for 30 seconds, touchdown 65°C - 58°C for 45 seconds, and 72°C for one minute were followed by 25 cycles of 95°C for 30 seconds, 58°C for 45 seconds and 72°C for one minute, with a final extension period at 72°C for ten minutes.

**DNA sequencing.** Amplified products were sequenced at the UCHSC sequencing core. Sequencing was done by the dye-terminator fluorescent method on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was conducted in both the forward and reverse direction. Results were then analyzed using BLAST (National Center for Biotechnology Information) software.

Table III. Sequence of PCR primers used to amplify c-kit.

Primer name	Oligonucleotide
c-Kit Exon 9 Forward	5' ATTTATTTTCCTAGAGTAAGC CAGGG
c-Kit Exon 9 Reverse	3' ATCATGACTGATATGGTAGA CAGAGC
c-Kit Exon 10-11 Forward	5' TCTGAGACTCACATAGCTTTG CATCC
c-Kit Exon 10-11 Reverse	3' CACAGAAAACCTCATTGTTCA GGTGG
c-Kit Exon 12-13 Forward	5' ATTTTGAAACTGCACAAATG GTCCT
c-Kit Exon 12-13 Reverse	3' GCAAGAGAGAACAACAGTCT GGGTAA
c-Kit Exon 17 Forward	5' GTGTATTCACAGAGACTTG GCAG
c-Kit Exon 17 Reverse	3'GGACTGTCAAGCAGAGAATGG

## Results

The hematoxylin and eosin-stained sections of dedifferentiated liposarcomas (Figure 1A) showed variable morphology. All of the cases had some areas of myxoid background with scattered lipoblasts and arcuate vasculature. The dedifferentiated areas, appeared as non-lipogenic high-grade sarcomatous areas characterized by spindle cells with scant cytoplasm and round to ovoid nuclei with irregular contours and small to large prominent nucleoli. Scattered large malignant giant cells were present in the dedifferentiated zones in almost all the cases. Easily

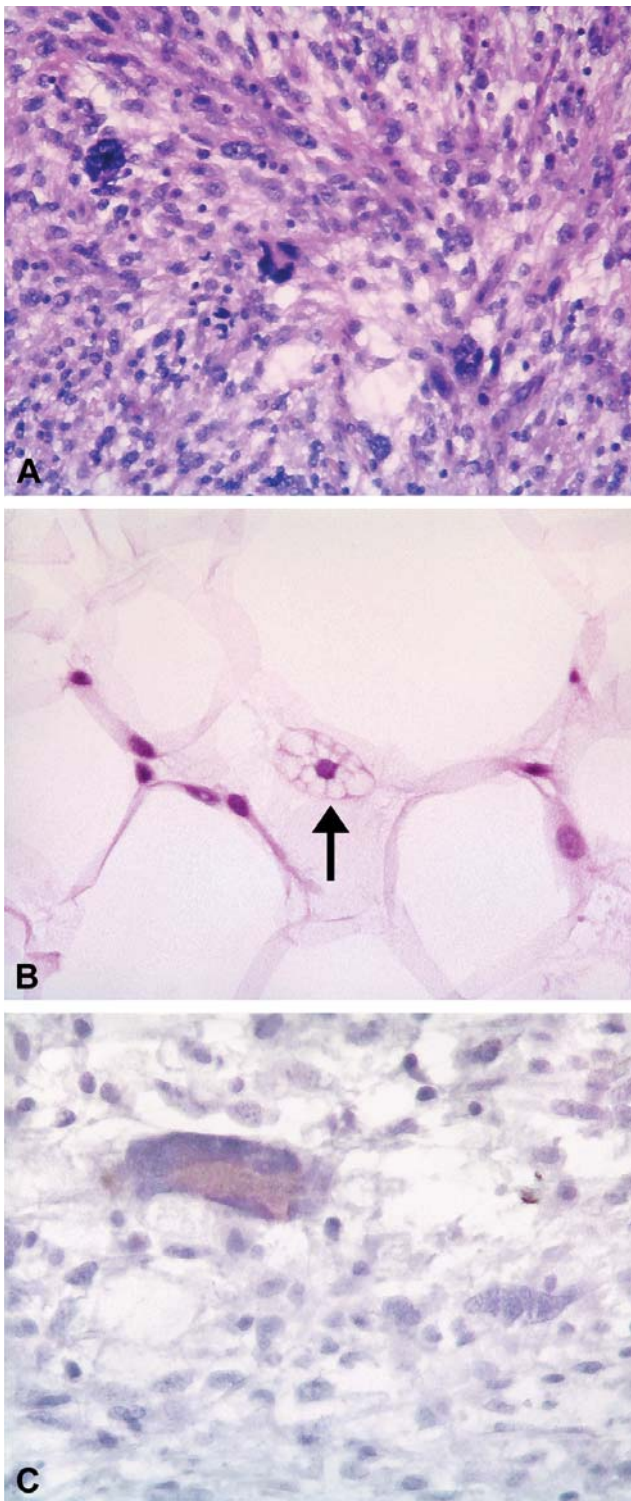


Figure 1. A. An example of a dedifferentiated liposarcoma from our cases showing high-grade sarcomatous area with mitoses. (Hematoxylin & Eosin, 400x). B. An example of a well-differentiated liposarcoma from our cases with variably sized mature adipocytes and a lipoblast (arrow). (Hematoxylin & Eosin, 400x). C. Focal weak immunoreactivity with polyclonal c-kit antibody in a dedifferentiated liposarcoma. (400x).

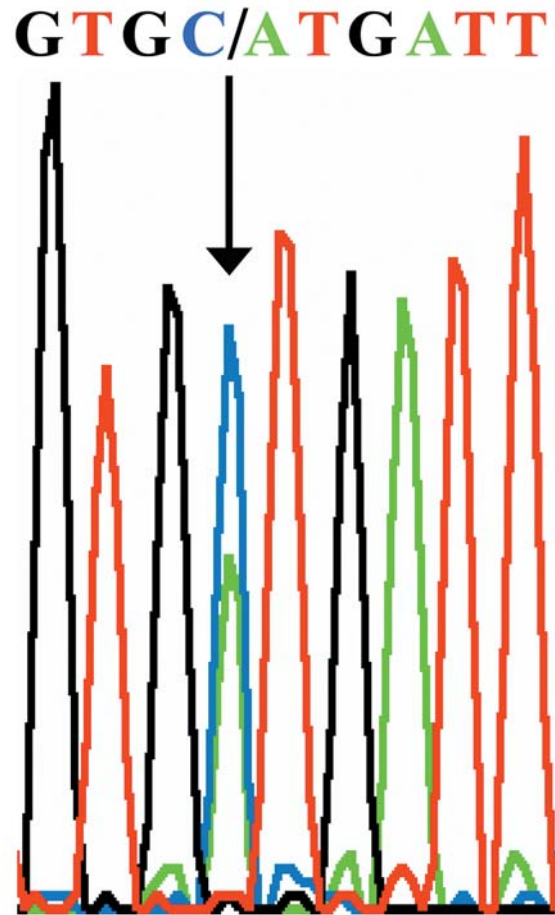


Figure 2. Electropherogram of amplified exon 10 from a dedifferentiated liposarcoma. There is a single base pair substitution A>C at codon 541 (shown by arrow) in one allele.

identifiable mitotic figures, occasionally atypical, were seen in the dedifferentiated areas and the mitotic rate varied from five to fifteen mitoses per ten high-power fields.

The well-differentiated adipocytic liposarcomas (Figure 1B) appeared as sheets of variably sized mature adipocytes with vacuolated cytoplasm and eccentrically pushed nuclei. Scattered few multivacuolated lipoblasts with crenated hyperchromatic nuclei were identified in some of the cases.

**Immunohistochemistry.** Two out of seven (28%) dedifferentiated liposarcomas showed focal weak cytoplasmic staining with c-kit in scattered tumor cells (Figure 1C) comprising less than 5% of the cells. None of the seven (0%) well-differentiated liposarcomas showed any staining with c-kit. Mast cells in the sections served as internal controls and showed strong granular membranous staining.

*Genetic analysis.* Genomic DNA was used to sequence exons 9,10-11, 12-13 and 17 of the c-kit gene using the PCR primers shown in Table III. Exons were amplified and sequenced from the fourteen tumors studied. No mutations or base pair changes were found except in exon 10. Seven out of seven (100%) dedifferentiated liposarcomas had a single base pair change in one allele in the transmembrane domain of c-kit (exon 10). Sequence analysis showed that this was a single base pair substitution at codon 541 from adenine to cytosine (A>C). The electropherogram depicting this change is shown in Figure 2. The resultant protein change was from methionine to leucine. The change found was identified in all the dedifferentiated liposarcomas. In addition, three out of seven (43%) well-differentiated liposarcomas had the same base pair change. As controls, we also sequenced exon 10 in other malignancies that included twenty GISTs, one melanoma and four normal tissues from our data bank. This same change was seen in four out of twenty GISTs, one melanoma and one out of four normal tissues. Normal tissue from our cases was not available for sequencing.

## Discussion

Dedifferentiated liposarcomas are characterized by the tendency to recur locally in at least 40% of cases. These can arise *de novo* in about 90%, while 10% occur in recurrences (19). Aggressive surgical resection is the main treatment modality (20) in these cases; adjuvant radiation and chemotherapy are considered in non-resectable or recurrent cases. Recently, imatinib-mesylate has become widely used in managing these tumors. At this point, little has been reported about the efficacy of imatinib in treating liposarcomas. As noted earlier, knowing the mutational status of the c-kit gene in the tumors is more important than just immunoreactivity to manage these patients effectively. Our study looked into the c-kit gene status in these tumors, however, additional studies will be required to investigate the treatment response of imatinib-mesylate in these tumors.

There have been many reports in the literature that assess the immunohistochemical status of c-kit in soft tissue tumors; however, there have been very few reports on the status of the c-kit gene in these tumors.

Hornick *et al.* (21) performed immunohistochemical stains for c-kit expression in 365 soft tissue tumors that included ten dedifferentiated liposarcomas. None of the dedifferentiated liposarcomas showed any staining with the polyclonal (A4502; no antigen retrieval) antibody. Their conclusion was that immunohistochemical staining of c-kit was seen in a limited number of soft tissue tumors and its diagnostic utility in tumors other than GISTs is limited. They also pointed out later (22) that immunohistochemical techniques, as well as interpretation criteria, should be standardized.

Went *et al.* (23) did a large study consisting of 3000 tumors of 120 tumor categories that included various solid tumors and performed mutational analysis of c-kit on only those tumors that expressed c-kit by immunohistochemistry. They concluded that c-kit activating mutations in exon11 were seen in only 50% of the GISTs and one of two kit-expressing melanomas, however no mutational analysis was performed in tumors negative for c-kit by immunohistochemistry. Hou *et al.* (16) and Wardelmann *et al.* (24) have also reported that c-kit mutations in exon 11 are seen more commonly in malignant GISTs as compared to benign GISTs.

The results of the study performed by Smithey *et al.* (25) on pediatric soft tissue tumors showed strong c-kit immunoreactivity in synovial sarcomas, osteosarcomas and Ewing's sarcomas; however, no mutational analysis was performed in the positively- or the negatively- staining tumors.

Fiorentini *et al.* (26) reported a case of retroperitoneal liposarcoma with positive c-kit staining who was treated with imatinib-mesylate and showed considerable improvement. No mutational analysis was performed in this case.

Miettinen *et al.* (27) also performed immunohistochemical analysis of GISTs and other tumors and saw focal weak immunoreactivity with c-kit in liposarcomas.

We have reached similar conclusions to others about immunoreactivity with c-kit and found it to be weak and focal in the dedifferentiated liposarcomas, precluding its diagnostic utility. Further, we believe that relying on the immunohistochemical results for initiating therapy with imatinib-mesylate should only be done with caution. Ours is the first study to examine in detail the integrity of the c-kit gene in dedifferentiated liposarcomas. In our study, however, we found a single base pair substitution in exon 10 of the c-kit gene (transmembrane domain) in these tumors. We could find no correlation between c-kit expression by immunohistochemistry and this genetic change.

The single nucleotide polymorphism (SNP) at codon 541 from A>C at nucleotide 1642 with the amino acid change from methionine to leucine has previously been reported by Nagata *et al.* (28). In their report, this SNP was found to occur with a frequency of 9% in sixty-four unrelated healthy subjects. They also analysed two generations with this polymorphism and suggested that this SNP does not result in disease. Yavuz *et al.* (29) also reported this and another polymorphism in the c-kit gene. This second SNP (81517C>T) in intron 17 was found to be similar in patients with mastocytosis and in healthy subjects.

The frequency of this base pair change A>C in the SNP database is, however, apparently less than that found by Nagata's group. Indeed, no figure is given, although it is noted that A>G at this site has a frequency of 0.1% while the presumed wild-type 'AA' allele represents 99.9% in the general population.

Inheritance of 'loss of function', another polymorphic mutation at position 1513A>C in the P2X7 gene, has been shown to contribute to the pathogenesis of chronic lymphoid leukemia (30). Activation of the P2X7 receptor is an important mechanism of apoptosis in the cells of the immune system. The presence of one inactive allele reduces the function of the gene by half, and two inactive alleles abolish the function. It is also known that SNPs can affect susceptibility to diseases. The fact that this polymorphism was found in all seven dedifferentiated liposarcomas and only in a small percentage of controls has led us to conclude that this may be a marker of dedifferentiation. As noted previously, 10% of dedifferentiated liposarcomas arise in recurrent well-differentiated liposarcomas and the presence of this same SNP in 43% of well-differentiated liposarcomas in our series may throw some more light on the natural history of these tumors and may better predict those well-differentiated liposarcomas that may dedifferentiate. Whether this is a germline or somatic polymorphism needs to be determined by more studies in these patients. Large studies are required to see if this genetic change is an activating mutation and if these tumors would respond to imatinib- mesylate therapy.

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