ABSTRACT

**Purpose:** Uterine leiomyosarcomas are rare tumors characterized by their resistance to chemotherapy and radiation treatment. Surgery is the primary method of treatment, but for patients with unresectable disease, alternate therapeutic options are clearly warranted. According to initial observations of c-KIT expression, correlation with a bad prognosis, and the successful therapeutic possibility of STI571 in gastrointestinal stromal tumors, the data have encouraged us to study c-KIT expression in these tumors.

**Experimental Design:** We analyzed the expression of c-KIT and genetic assessment of exon 11 of c-kit gene in 32 uterine leiomyosarcomas.

**Results:** In 17 cases (53.1%), we observed a c-KIT expression in tumor cells. Of the 17 patients with distinct c-KIT-positive immunoreactivity, eight had I or II stage disease and nine had III or IV stage disease. Molecular genetic analysis of exon 11, analyzed by direct DNA sequencing, was performed for all of the c-KIT-positive uterine leiomyosarcomas. No mutations were found.

**Conclusion:** The conventional chemotherapy in leiomyosarcomas appears to be ineffective for patients with metastatic or unresectable disease, and the management of these patients poses a special problem. In these women, new therapeutic strategies are warranted. The treatment with STI571 in leiomyosarcoma patients might be hypothesized, because uterine leiomyosarcomas also express c-KIT.

INTRODUCTION

The c-kit gene is located on the long arm of chromosome 4 (4q11-12) in the human genome. It encodes the Mf, 145000 KIT transmembrane receptor tyrosine kinase. This protein is involved in cell signal transduction. The physiological ligand is the cytokine stem cell factor, also called mast cell growth factor or Steel factor (1). This receptor is important for the development and maintenance of hematopoietic stem cells, mast cells, germ cells, melanocytes, and interstitial cells of Cajal (2).

Uterine leiomyosarcomas are uncommon neoplasias accounting for 0.21–6% of all smooth muscle tumors of the uterus, with a peak incidence in the fifth decade (3). These tumors are characterized by their nearly absolute resistance to chemotherapy (4) and radiation treatment (5) and for their bad prognosis. Surgery is the primary method of treatment for malignant mesenchymal tumors of the uterus, but these tumors represent an incurable malignancy for patients with metastatic or unresectable disease.

Like uterine leiomyosarcomas, gastrointestinal stromal tumors (GISTs) are resistant to classical chemotherapeutic treatment. Moreover, STI571 induces apoptosis in vitro in GIST cells (6), and according to initial clinical studies, the majority of GISTs responds to therapy with a competitive inhibitor of tyrosine kinases, such as STI571, within a few months (7, 8). The successful treatment of patients with advanced GISTs, expressing c-KIT, with a new KIT tyrosine kinase inhibitor encourages the hope that other sarcomas can be treated in a similar way.

Most malignant GISTs have a mutation in c-kit leading to KIT receptor autophosphorylation and ligand-independent activation (9).

The potential of the treatment with STI571 is currently unknown. Several other human cancers may overexpress KIT. Clinical trials evaluating the role of STI571 in the treatment of uterine-malignant stromal tumors are needed.

The aim of this study is the examination of the presence of c-KIT in a series of uterine leiomyosarcoma patients to identify alternative therapeutic options that are clearly warranted.

MATERIALS AND METHODS

**Case Selection**

Files from the years 1984–2001 in the Department of Human Pathology and Oncology of the University of Florence were searched for the diagnosis of malignant smooth muscle tumors of the uterus. Thirty-two uterine leiomyosarcoma patients, aged 39–76 (average 54.68 years, mean 52.5 years), with clinical history and known follow-up, admitted to the Department of Gynecology, Perinatology, and Reproductive Medicine of the University of Florence, were included in this study.

All microscopic slides were reviewed to confirm the histological diagnosis, without knowledge of the clinical outcome. The diagnosis of leiomyosarcoma was based on criteria published previously (10).

The patients had undergone total abdominal hysterectomy, bilateral salpingo-oophorectomy, and removal of as much tumor as possible in cases with pelvic and/or abdominal spread. Peri-
tonal cytology, omentectomy, and/or peritoneal biopsy were performed to rule out extrauterine disease.

All patients were staged retrospectively, according to a modified staging system of the International Federation of Gynecology and Obstetrics (FIGO) for corpus uteri carcinomas and mixed mesodermal tumors (11). We derived staging information from operative notes and pathology reports.

Tissue Specimens and Immunohistochemistry

The specimens were obtained by surgical resection in all cases and fixed in 10% formalin before being processed in paraffin. H&E-stained sections from each histological specimen were reviewed by two pathologists to confirm the histological diagnosis.

A representative section from each lesion was selected for immunohistochemical analysis. All sections were deparaffinized in Bio-Clear (Bio-Optica, Milan, Italy), hydrated with grade ethanol concentration, and finally hydrated with distilled water.

Antigen retrieval was routinely performed by microwave pretreatment [Microwave MicroMed T/T Mega; Milestone, Sorisole (BG), Italy] in TEC buffer [TRIS-EDTA-citrate (pH 7.8)] for 30'.

As a primary antibody, we used a commercial rabbit polyclonal anti-c-KIT (Ventana Medical Systems, Tucson, AZ) raised against a peptide sequence mapping with the COOH-terminal domain of c-Kit with a molecular weight of M, 145000 of human origin.

All tissue sections were then placed on the Ventana NexES automated stainer using iVIEW DAB Detection kit as revelation system. The primary antibody to c-KIT was placed on the slides and incubated for 32 min according to the protocol suggested by Immunostainer Ventana NexES. When the staining run was complete, the tissue sections were removed from the stainer, counterstained with hematoxylin, dehydrated, and mounted with Permount.

A section containing a c-KIT strongly positive GIST served as the positive external control with each run. Mast cells within the tumor served as positive internal control.

Each tumor was also stained with Giemsa to identify mast cells for comparison with the c-KIT results.

A negative control sample was included with each run by omitting the primary antibody, and each sample yielded no signal.

Evaluation of Immunohistochemical Staining

Positive stains appeared as cytoplasmic and/or membranous stains. The percentage of positive cells and the degree and cellular distribution of the staining were scored. Only cases showing at least 20% moderate-to-strong membranous and/or cytoplasmic positivity were scored as positive, as described previously (12). No background or non-specific staining of tumor stroma or adjacent ovarian parenchyma was encountered, other than positivity in mast cells as expected.

To assess the interobserver variability in the evaluation of c-KIT expression, the evaluations of one author (M. R. R.) were compared with those of another (G. L. T.). Initially the slides were evaluated independently, and those graded diversely were subsequently reevaluated by the two authors together under a discussion microscope. The immunohistochemical analysis was evaluated in a blinded fashion with regard to the clinical data.

Genetic Analysis of Exon 11 in c-Kit Gene

DNA Extraction. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue using the commercial kit CLEAN DNA (AB Analitica, Padoa, Italy) following the manufacturer’s instructions. Total DNA concentration was determined with the Pharmacia GeneQuant spectrophotometer.

DNA Sequencing Analysis. Exon 11 in the c-kit gene was amplified by PCR using the following oligonucleotide primer pair reported previously (9): forward primer, 5'-GATCTATTTTTCCCCCTTTC-3'; reverse primer, 5'-AGCCCCTGTTCATCTGAC-3'. These primers were used to perform both PCR and sequencing reactions.

Three hundred ng of the total DNA were amplified in 50-μl PCR reaction. Samples were treated for 9' at 94°C and then submitted to 35 cycles of amplification at 94°C for 1', 50°C for 1', and 72°C for 90" in a Gene Amp 9700 Thermal Cycler (Applied Biosystems, Foster City, CA).

All PCR products were purified with Qiaqen PCR purification kit (Qiagen, Valencia, CA) and semi-quantified in a 2% agarose ethidium bromide gel by using DNA molecular weight marker XIV (Roche Diagnostic, Penzberg, Germany). To perform the cycle-sequencing reaction, 20 ng of DNA were then blended with each primer (0.8 μM) in a terminator ready reaction mix containing Big Dye terminators (Applied Biosystems) and submitted to 25 cycles at 96°C for 10', 50°C for 5', and 72°C for 4'. A second purification was then necessary for the Big Dye removal with DyeEx 2.0 Spin kit (Qiagen). Five μl of marked and purified DNA were submitted to sequencing analysis with ABI PRISM 310 Genetic Analyser (Applied Biosystems), and alignments were performed using Sequence Navigator (Applied Biosystems).
Table 1  c-KIT expression in relation to clinical-pathological data

<table>
<thead>
<tr>
<th></th>
<th>c-Kit positive cases (n = 17)</th>
<th>c-Kit negative cases (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 yrs</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>&gt;50 yrs</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>III and IV</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Vascular space involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Present</td>
<td>13</td>
<td>9</td>
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<tr>
<td>Mitoses</td>
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<tr>
<td>≤10 for 10 high-power fields</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>&gt;10 for 10 high-power fields</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

Statistical Analysis

Data analysis was performed using the statistical software SPSS (Chicago, IL). Disease-free interval and cause-specific survival rates were calculated according to the Kaplan-Meier method. The statistical analysis of the c-KIT expression in relation to clinical-pathological data (age, stage, vascular space involvement, mitotic count) lacked statistical correlation; this is shown in Table 1.

RESULTS

Seventeen Cases Had I or II FIGO Stage Disease, and 15 Cases Had III or IVA FIGO Stage Disease. Chemotherapy after surgery was performed in advanced-stage disease cases and in I or II FIGO stage disease cases according to the presence of negative prognostic factors (age > 50 years, presence of vascular space involvement, mitotic count > 10 for 10 high-power fields; Ref. 13). Combination chemotherapy consisted of six cycles of ifosfamide (1500 mg/m², days 1–3) and epidoxorubicin (80 mg/m², day 1).

All patients, at the time of diagnosis, were free of distant metastases (FIGO stage IVB). During the follow-up period, local recurrences and distant metastases were examined with pelvic examination and by routine chest X-ray, ultrasonography, and computed tomography.

In June of 2003, eight patients were alive. One patient (stage III) is living with no evident disease after 48 months, and seven patients (stage I) are living with no evident disease after a follow-up range from 12 to 83 months (mean, 50.28 months; average, 58 months).

Twenty-four patients died during the follow-up period from progression of disease. Twenty-six patients suffered from recurrences or metastases, and these patients received surgical treatment to resect the relapsing masses and then received subsequent chemotherapy for disease.

Immunohistochemistry. In 17 cases (53.1%) we observed a c-KIT expression in tumor cells (Fig. 1). Of the 17 patients with distinct c-KIT-positive immunoreactivity uterine leiomyosarcomas, eight had I or II FIGO stage disease, and nine had III or IV FIGO stage disease. The c-KIT expression in relation to clinical-pathological data (age, stage, vascular space involvement, and mitotic count) lacks statistical relation; this is shown in Table 1.

Among the parameters analyzed, only tumor stage was of prognostic relevance regarding disease-free interval and survival (P = 0.002 and P = 0.004, respectively). The c-KIT expression in relation to the clinical outcome of all uterine leiomyosarcoma patients lacks statistical correlation, and these data remain statistically insignificant when we analyzed separately the I or II stage disease and the III or IV stage disease. We observed only a lower mean disease-free interval and a lower mean overall survival in the c-KIT-positive cases compared with c-KIT-negative cases (Table 2).

Sequencing of Exon 11 of the c-kit Gene. Direct sequencing of exon 11 of the c-kit gene was performed in all of the 17 c-KIT-positive uterine leiomyosarcomas. In all of the cases, DNA extraction and subsequent PCR reaction were successful. None of the analyzed samples revealed a mutation in exon 11.

DISCUSSION

Prognosis of patients with metastatic uterine leiomyosarcomas is generally poor. The chemotherapy in leiomyosarcoma appears to be ineffective for patients with metastatic or unresectable disease, and the management of these patients poses a particular problem. In these women, new therapeutic strategies are warranted.

The report about the efficacy of therapy with STI571 in a

<table>
<thead>
<tr>
<th></th>
<th>No. cases</th>
<th>Mean DFI* ± SE</th>
<th>P</th>
<th>Mean OS ± SE</th>
<th>P</th>
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<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>I and II</td>
<td>17</td>
<td>37 ± 8</td>
<td>0.004</td>
<td>50 ± 8</td>
<td>0.002</td>
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<tr>
<td>III and IV</td>
<td>15</td>
<td>10 ± 3</td>
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<td>18 ± 4</td>
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<td>c-Kit expression</td>
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<td></td>
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<tr>
<td>Positive</td>
<td>17</td>
<td>20 ± 7</td>
<td>0.28</td>
<td>28 ± 7</td>
<td>0.16</td>
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<tr>
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<td>15</td>
<td>29 ± 7</td>
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<td>42 ± 9</td>
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<td>Stage I and II c-Kit expression</td>
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<tr>
<td>Positive</td>
<td>8</td>
<td>35 ± 13</td>
<td>0.58</td>
<td>45 ± 12</td>
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<td>9</td>
<td>38 ± 8</td>
<td></td>
<td>54 ± 11</td>
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<tr>
<td>Stage III and IV c-Kit expression</td>
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<td></td>
<td></td>
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<tr>
<td>Positive</td>
<td>9</td>
<td>9 ± 3</td>
<td>0.72</td>
<td>16 ± 5</td>
<td>0.33</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>13 ± 7</td>
<td></td>
<td>20 ± 5</td>
<td></td>
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</tbody>
</table>

*DFI, disease-free interval; OS, overall survival.
patient who had a metastatic GIST with c-KIT immunohistochemically positive staining and a c-kit mutation from exon 11 (8) encouraged us to search the c-KIT expression also in uterine stromal malignant tumors.

The c-KIT expression has been observed also in other malignancy such as small cell lung cancer (14) and sarcomas (15).

Now we have demonstrated, in accordance with Wang et al. (16), that even uterine leiomyosarcomas express c-KIT, and this expression suggests therapeutic targeting opportunities. Whether uterine malignant stromal tumors will respond to KIT inhibition remains to be established.

These results provide the rationale to move forward with clinical testing of STI571 as anticancer therapy for advanced uterine leiomyosarcoma patients who express immunohistochemically the c-KIT. Additional studies in this area will help clarify this therapeutic possibility.

Our encouraging immunohistochesmical results and the results of lack of exon 11 mutations suggest the opportunity to examine other exons in addition to exon 11.

Lasota et al. (17) asserted that lack of c-kit mutation in malignant GISTs suggests that the mutation in the exon 11 of c-kit is not the only mechanism related to tumorigenesis and malignancy and that other molecular mechanisms must exist. Such mechanisms may include mutations in other regions of the c-kit. Further investigation into sequencing analysis of other c-kit exons is on-going in our laboratory.

Deregulated autocrine or paracrine loops between KIT and its ligands provide an alternative mechanism in which STI571 may interfere (18).

We do not know if c-KIT-positive uterine leiomyosarcomas will respond to therapy with a KIT inhibitor, in particular with the STI571. This important point remains to be established. Moreover, patients with metastatic unsectable uterine leiomyosarcomas who do not respond to chemotherapy pose a particular problem. In these patients, alternate therapeutic options are clearly warranted; our findings suggest that clinical trials with STI571 are needed in these aggressive and chemoresistant tumors.

REFERENCES


c-Kit Expression in Patients with Uterine Leiomyosarcomas: A Potential Alternative Therapeutic Treatment

Maria Rosaria Raspollini, Gianni Amunni, Alessandro Villanucci, et al.


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