Proteases and interleukin-6 gene analysis in 92 giant cell tumors of bone

G. Gamberi1*, M. S. Benassi1, P. Ragazzini1, L. Pazzaglia1, F. Ponticelli1, C. Ferrari1, A. Balladelli1, M. Mercuri2, M. Gigli2, F. Bertoni3 & P. Picci1

Introduction

Giant cell tumor of bone (GCT) is defined as an intramedullary bone tumor, composed of mononucleated cells and osteoclast-like multinucleated giant cells, with a variable and unpredictable potential for growth [1]. GCT was first defined in 1940 by Jaffe, Lichtenstein and Portis, who differentiated it from other bone lesions containing osteoclast-like giant cells, such as histiocytic fibroma, chondroblastoma and aneurysmal bone cyst [2]. GCT usually behaves as a benign tumor, but has a significant tendency to recur locally (10%–40%) and, rarely (2%), may result in the development of lung metastasises. The metastasises have the same histological features as the primary tumor and are frequently curable with resection, but in ~20% of cases they are lethal.

Rarely, a giant cell tumor may transform into a high-grade sarcoma.

The frequency of GCT is approximately 1/million/year, about half the number of cases of osteosarcoma. Treatment is essentially surgical. In contrast to most tumors of bone, GCT has a slight preference for females. It usually occurs in young adults, 15–40 years old. In 90% of cases, GCT is located in the meta-epiphyseal area, the most common sites being the distal femur, proximal tibia and distal radius. Very rarely (<0.5% of cases), GCTs are multicentric in the skeleton.

Histologically, the tumor is very cellular and moderately vascularized. It is composed of round oval or slightly spindle mononucleated cells, and by widespread multinucleated giant cells. The stromal cells have pale cytoplasm with an indistinct border, a nucleus with a distinct nuclear membrane and a usually prominent nucleolus. The multinucleated giant cells contain several nuclei identical to those of stromal cells, and phenotypically and functionally similar to osteoclasts [3].

Background: Giant cell tumor of bone (GCT) is a benign tumor with a significant tendency to recur locally and rarely to produce pulmonary metastases. It is characterized by the presence of multinucleated osteoclast-like giant cells together with mononuclear spindle-shaped cells. Few prognostic markers have been reported to predict the clinical outcome of GCT patients, so is very important to find the factor that can be implicated in its potential aggressiveness.

Patients and methods: Different groups of GCT patients were selected for this study, including patients without evidence of disease and patients who recurred locally or with lung metastasis. The total of 92 tumor samples also included the specimens of the local recurrences and the lung metastasises. By using immunohistochemistry and real-time quantitative polymerase chain reaction techniques, the genetic and proteic analyses were performed on the urokinase-type plasminogen activator (u-PA), its receptor (u-PAR) and its inhibitor (PAI-1), which have been described to be frequently implicated in the process of degradation of the extracellular matrix during the metastatic process. Interleukin-6 (IL-6), a cytokine released by GCT cells, which stimulates resorption of bone, was also analyzed.

Results: IL-6, u-PA, u-PAR and PAI 1 genes were found amplified, respectively, in 7%, 5%, 8% and 12% of total cases (92). In particular, the percentages of amplified genes were higher in the GCT cells that gave rise to metastases (12 cases) and in the samples of lung metastases (nine cases) compared with the disease-free group of patients (60 cases).

Conclusions: These results suggest a possible association of these factors with a higher biological aggressiveness of GCT. Moreover, it appears that increased expression of the IL-6, u-PA, u-PAR and PAI1 proteins might not depend on mutation of the corresponding genes.

Key words: giant cell tumor, interleukin-6, proteases
The course of an untreated GCT is very variable. In the majority of cases the tumor grows rather slowly, but steadily doubles in size in 2–6 months. Occasionally the course is more indolent; exceptionally the tumor may even reach a complete arrest of growth due to extensive spontaneous necrosis and fibrosis. There are also cases of a rapid aggressive growth.

Few reliable prognostic markers have been reported to predict the clinical outcome of GCT patients, mostly because of the rarity of this neoplasm, which means that it is difficult to collect a large series of patients. Previous studies have shown that tumor size, anatomic site, presence of pathological fractures at diagnosis, histological grading and DNA content are not related to relapse rate and do not predict the biological aggressiveness of GCT cells [4, 5]. This suggests the need for factors related to tumor progression that may be predictive of clinical outcome in GCT patients.

Tumor growth and metastasis involve molecular interactions between tumor cells and the surrounding normal tissue. Several steps are involved in these processes, but degradation of the extracellular matrix is an essential prerequisite for growth of primary tumors, metastatic spread and neoangiogenesis. In particular, among the proteases involved, the urokinase-type plasminogen activation system has been described as frequently implicated in the process of degradation of extracellular matrix during tumor cell proliferation and metastasis [6, 7].

This system includes the urokinase-type plasminogen activator (u-PA), its receptor (u-PAR) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1). Binding of uPA to the uPAR activates the protease and catalyzes the conversion of plasminogen to plasmin, which subsequently activates type IV collagenase, or directly degrades extracellular matrix proteins such as fibrin, laminins and proteoglycans. The enzymatic activity of uPA is regulated by the PAI-1. u-PA, u-PAR and PAI-1 have been associated with disease outcome as prognostic markers in breast [8–10], lung [11], colon [12–14], kidney, ovarian [15], endometrial [16, 17] and gastrointestinal [18] cancers.

Furthermore, the radiographic picture of GCT is characterized by an intense osteolysis that is associated with a progressive increase in biological aggressiveness [19]. Previous studies on primary cultures obtained from GCT have demonstrated that GCT cells are functionally and phenotypically very similar to normal osteoclasts, and are able to resorb bone in vitro [20–22]. Moreover, more recent findings demonstrated that the osteolytic activity of GCT is modulated by interleukin-6 (IL-6), which enhances bone cell resorption activities of tumor cells, indicating IL-6 as a possible prognostic marker for GCT [23].

In GCT, significantly elevated uPA, uPAR, PAI-1 and IL-6 protein levels were described in a previous report [24], which evaluated 57 tissue specimens of GCT, including 48 primary tumors and nine lung metastases. Given the promising results, in the present study we analyzed the same DNA samples as those in the report by Gamberti et al. [24], plus a series of 35 new specimens (24 primary tumors and 11 local recurrences). After having completed the immunohistochemistry analysis on the 35 new tissue specimens, we performed genetic analysis on all 92 tissue specimens (72 primary GCT, nine lung metastases, 11 local recurrences) to evaluate a possible gene amplification of uPA, uPAR, PAI-1 and IL-6, and to verify whether gene mutations might correlate with the corresponding protein overexpression.

**Patients and methods**

**Tumor samples**

In this study, 92 tissue specimens of GCT, including those from 72 patients with primary tumors (1977–1998), nine with metastases and 11 with local recurrences, were examined. Among the 72 primary tumors, 49 were unselected, consecutive cases obtained from patients who remained disease-free, 11 were from a selected group of patients who relapsed with local recurrence, and 12 were from a group of patients who relapsed with lung metastases.

Specimens were obtained from initial biopsies or from samples collected at the time of surgical excision. All tissue samples were fixed in buffered formalin and embedded in paraffin. Diagnoses were made based upon hematoxylin-eosin-stained sections following conventional criteria [25]. All patients were treated with surgery only.

The protein analysis on 24 primary GCT and 11 local recurrences was performed by immunohistochemistry, while the genetic analysis on all 92 specimens was performed by quantitative polymerase chain reaction (PCR).

**Quantitative PCR**

Quantitative PCR was performed on DNA using the ABI PRISM 7700 Sequence Detector System (Applied Biosystem, Foster City, CA) and the TaqMan reaction technique [26, 27].

Tumor DNA purified from formalin-fixed, paraffin-embedded samples was extracted according to a method described previously [28]. Twenty sections of each sample were treated three times for 10 min with 1 ml of xylene to remove the paraffin, washed in ethanol (100% and 95%), and then incubated overnight at 55°C in 0.8 ml of lysis buffer (100 mM Tris–HCl pH 8.5, 5 mM EDTA, 1% SDS, 20 mg/ml proteinase K). DNA was purified by phenol–chloroform extraction and ethanol precipitation. To verify the integrity level, all DNA samples were electrophoresed on 1.5% agarose gel and marked by ethidium bromide staining. Dosage of DNA was determined using a reference curve created by a series of DNA dilutions with a known concentration, then DNA was amplified for the β-actin chosen as the endogenous, single-copy reference gene.

Quantitative PCR is based on the use of a fluorogenic probe designed to hybridize the gene target. The probe consists of an oligonucleotide with a fluorescent reporter dye, FAM (6-carboxy-fluorescein), covalently linked to the 5′ end of the target sequence. The probe is annealed to a second primer with a quencher dye (TAMRA, 6-carboxy-tetramethyl rhodamine), located at the 3′ end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by FRET energy transfer [29].

During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites, and the 5′–3′ exonuclease activity of the Taq polymerase cleaves the probe between the reporter and the quencher (Table 1). Cleavage generates an increase in fluorescence emission of reporter dye. This process occurs in each PCR cycle, leading to an increase in fluorescence signal proportional to the concentration of target sequences in the initial sample. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, only specific amplification is not detected.

The ABI PRISM 7700 Sequence Detection System was used to monitor the increase in reporter fluorescence following PCR. With this instrument, a 96-well microplate thermal cycler is connected by fiber optic cables to a CCD camera detector. Laser excitation (488 nm) and fluorescence detection (between 520 and 660 nm) are performed every 7 s throughout PCR cycling. The signal attributable to the 5′ nuclease reaction is expressed as an Rn value,
Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>u-PA</td>
<td>5′-TGCAGCGTGGCCAC-3′</td>
<td>5′-TGCCTGGAAGAAACAGGTT-3′</td>
<td>5′-CCCTACTCCTCCACTCCCTCCCTCGC-3′</td>
</tr>
<tr>
<td>u-PA</td>
<td>5′-TGGGCACTGTCGACAGGTTG-3′</td>
<td>5′-TGGGCACTGTCGACAGGTTG-3′</td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>5′-AGCCGTGGGACCAGCTGAC-3′</td>
<td>5′-CCACCCCGCTTCGAC-3′</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-GACTCAGGGAAAAATCCCAAACA-3′</td>
<td>5′-CCACCCCGCTTCGAC-3′</td>
<td></td>
</tr>
</tbody>
</table>

IL-6, interleukin-6; u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; PAI-1, u-PA inhibitor type 1.

which represents the reporter signal normalized against the emission of passive reference (ROX) minus the baseline signal established in first cycles of PCR (conventionally from three to 15). This value increases during PCR because the amplicon copy number increases until the reaction approaches a plateau. At the same time, the algorithm determines the cycle threshold (CT). The CT represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. The cycle number at which the fluorescence of each sample reaches the CT is inversely proportional to the number of target copies present in the initial sample.

Target gene relative quantity is calculated by comparison with a calibrator. As a calibrator, we used DNA extracted from healthy human lymphocytes. The following mathematical formulae allow quantification of the number of gene copies of the target compared with the calibrator and with β-actin, chosen as an endogenous, single-copy reference gene.

\[
\Delta CT_{\text{sample}} = CT_{\text{gene}} - CT_{\beta-\text{actin}}
\]

\[
\Delta CT_{\text{calibrator}} = CT_{\text{sample}} - CT_{\text{calibrator}}
\]

\[
\Delta \Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{calibrator}}
\]

\[
2^{-\Delta \Delta CT} = \text{number of gene copies}
\]

Immunohistochemistry
The expression levels of IL-6, u-PA, uPAR and PAI-1 proteins were assessed on paraffin-embedded tumor sections using the avidin–biotin–peroxidase complex method. After dewaxing, endogenous tissue peroxidase activity was blocked by treatment with 0.3% H2O2 for 30 min at room temperature. For immunodetection of IL-6, u-PA, u-PA and PAI-1 proteins, the slides were incubated in a microwave with 10 mM citric-acid solution (pH 6.0) to retrieve the antigen. After washing, sections were incubated with normal serum for 15 min at room temperature and then overnight at 4°C with the appropriate dilution of each primary antibody. The primary antibodies used were: anti-human IL-6 monoclonal antibody (dilution rate 1:30; R&D System, Minneapolis, MN); anti-u-PA polyclonal antibody C-20 (dilution rate 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), which reacts with b-chain and the inactive 52 kDa precursor form of u-PA of human origin; anti-human uPAR monoclonal antibody CD87 (dilution rate 1:50; American Diagnostic Inc., Greenwich, UK), which binds with high affinity both uPAR and uPA/uPAR complexes; and anti-human PAI-1 monoclonal antibody (dilution rate 1:25; American Diagnostic Inc.), which recognizes both PAI-1 and uPA–PAI-1 complexes. To reveal immunoreaction, sections were incubated with a goat anti-mouse biotinylated antibody and then with an avidin–biotin–peroxidase complex.

Figure 1. Percentage of cases with gene amplification. IL-6, interleukin-6; u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; PAI-1, u-PA inhibitor type 1; PRI-NED, primary GCT with no evidence of disease; PRI-MET, primary GCT with lung metastasis.

Immunostaining was revealed with 3-amino-9-ethyl-carbazole and nuclei were counterstained with hematoxylin.

According to the percentage of positive cells, positive samples were scored as negative (no positive cells), weakly positive (≤25% positive cells), moderately positive (25%–50% positive cells) or strongly positive (>50% positive cells). Overexpression of each protein was considered only in the presence of strong positivity.

Tissue sections of aneurysmal bone cyst, a benign lesion that contains both normal osteoclasts and osteoblasts, were used as positive controls. Negative controls were performed by omitting the primary antibody.

Statistical analysis
Two-tailed Fisher’s exact test was used to estimate the association between the different groups.

Results

Morphological analysis of GCT tissue sections
All cases showed the typical features of GCT, with multinucleated cells and a mixed population of spindle-shaped mononuclear cells. The 12 primary GCTs of patients who developed lung metastases presented the same histological features of tumors obtained from disease-free patients.

Gene analysis
Gene analysis was performed on all 92 samples. The gene was considered amplified only when its copy number was at least three times that of the β-actin gene, which was chosen as a single-copy reference gene.

The IL-6 gene was found to be amplified in 17% of patients who developed metastasis, and in 5% of disease-free patients. With respect to lung metastases, the IL-6 gene was found to be amplified in 11% of cases.

With respect to the proteases, u-PA and u-PAR were found to be amplified in 17% of patients who developed metastases, and in 3% and 5% of disease-free patients, respectively.

The PAI-1 gene was found to be amplified in 25% of the metastatic GCT and in 8% of disease-free patients.

In the cases of lung metastases, u-PA, u-PAR and PAI-1 genes were found to be amplified in 11%, 22% and 33% of samples, respectively.
The local recurrence samples presented no amplified cases for any of the genes studied (Figure 1; Table 2).

Protein expression analysis

IL6, u-PA, u-PAR and PAI-1 protein analyses were performed on the 35 specimens (24 primary tumors from disease-free patients and 11 local recurrences). These proteins had a variable degree of positivity in the two GCT groups studied. IL-6 protein was found to be overexpressed in two cases of primary GCT (8%), while u-PAR and PAI1 proteins were found to be overexpressed in one case of primary GCT (4%). Only in one case of local recurrence were u-PA and PAI-1 proteins found to be overexpressed (4%). In positive tumors, immunostaining was present in both mononuclear tumor cells and multinucleated, osteoclast-like giant cells, without any significant differences between the two cell populations (Table 3; Figure 2).

Discussion

Giant cell tumor of bone is a benign neoplasm characterized by the presence of mononuclear cells together with multinucleated giant cells that resemble normal osteoclasts [30]. It may exhibit considerable local aggressiveness, often associated with intense osteolytic activity. Rarely, GCT may developed lung metastases, suggesting that some tumors may acquire an aggressive phenotype [31]. This tumor is rare, and it is very difficult to collect data for a large number of cases; only the Rizzoli Institute, which sees the majority of Italian cases, is capable of gathering sufficient data. Therefore, in this study, the number of cases for every group is small, but is representative of this rare tumor.

To understand the enhanced biological aggressiveness of this tumor better, we analyzed gene amplification and protein expression of factors involved in bone resorption and metastatic dissemination.
in samples from disease-free GCT patients, metastatic GCT patients, patients with lung metastasis from GCT, and those with local recurrences from GCT.

Invasion and metastasis of solid tumors require proteolytic enzymes that degrade the extracellular matrix and basement membranes [32].

The factors analyzed in this paper were some of the proteases of the urokinase-type plasminogen activation system and interleukin-6. The system includes: the osteolytic enzyme plasmin, its precursor plasminogen, the urokinase-type plasminogen activator (u-PA) and the plasminogen activator inhibitor type 1 (PAI-1), which modulates catalytic action of u-PA [33]. Moreover, the action of plasminogen activators is facilitated by the presence of receptors for u-PA (U-PAR) on the cell surface.

High levels of u-PA, u-PAR and PAI-1 have been reported to be correlated with poor prognosis in many different human tumors. In particular, u-PA was the first proteinase to be reported as a prognostic factor in breast cancer and other tumors, including lung, bladder, stomach, colorectum and brain [8, 10, 12–15, 17].

IL-6 can be considered one of the most important regulators of bone remodeling by inducing osteoclast precursor differentiation and increasing osteoclast-mediated bone resorption [34, 35]. The ability of GCT cells to produce IL-6 [19] might therefore influence the local osteolysis by stimulating normal osteoclasts, as well as the GCT cells that are capable of bone resorption activity [22].

Given the promising results of the previous study [24], in the present study we analyzed a series of 35 new specimens (24 primary tumors and 11 local recurrences) to complete the IL-6, u-PA, u-PAR and PAI-1 protein analysis. We then performed a genetic analysis on all 92 tissue specimens (72 primary GCTs, nine lung metastases, 11 local recurrences) to evaluate the amplification of the corresponding genes. Protein analysis performed using immunohistochemistry confirmed the data obtained in the previous study. The percentages of overexpressed proteins are very similar to those obtained previously [24] (Table 3; Figures 3(a) and 3(b)). These data thus confirm that increased protein expression of these molecules could be a factor implicated in the increased aggressiveness of GCT, and that the analysis of IL-6, u-PA, u-PAR and PAI-1 at diagnosis could be useful in identifying those GCTs that are potentially at higher risk of metastases.

Genetic analysis was performed using real time PCR. This system simultaneously provides the desired information on the quantitative evaluation and qualitative recognition of the amplified target. In fact, the presence of a specific internal probe carrying the signal generating system guarantees the specificity of the PCR product to be measured. Furthermore, the high sensitivity of the detection of the fluorescent signal allows the detection of PCR products starting from low quantities of DNA templates, even after a limited number of PCR cycles.

The percentages of amplified genes (for all four genes studied) were higher in the GCTs that yielded metastases (12 cases) and in the samples of lung metastases (nine cases) compared with the disease-free group of patients (60 cases) (Table 2; Figure 3), suggesting a possible association of these factors with a higher biological aggressiveness. However, statistical analysis of these data showed no significant differences among the groups of
patients studied. The genetic results obtained in this study could therefore indicate that increased expression of the IL-6, u-PA, u-PAR and PAI1 proteins might not depend upon the mutation of the corresponding genes, but probably upon another biological mechanism, such as the absence or slowed down cell catabolism of the same protein or RNA messenger, or from increased transcription of the RNA messenger. Further investigations are necessary to evaluate the reason behind the overexpression of this protein.

With respect to local recurrences, no gene was found to be amplified in the 11 cases studied, and in only one case were u-PA and u-PAR proteins found to be overexpressed. This could indicate that local aggressiveness in GCT does not depend upon a biological factor, but an inadequate surgery.

Acknowledgements

The authors thanks Gris tina Ghinelli, medical artist, for graphic work. This study was supported by grants from the Associazione Italiana Ricerca sul Cancro (AIRC), the Centro Interdipartimentale Ricerca sul Cancro (CIRC), the Ricerca Finalizzata del Ministero della Sanita, and the CNR-MIUR.

References