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A COMPARATIVE STUDY ON PHENOTYPICAL AND BIOMOLECULAR CHARACTERIZATION OF GIANT CELL TUMOR OF BONE IN FELINE AND HUMAN SPECIES

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Abstract- Giant cell tumor of bone (GCT_B) is generally an intramedullary tumor with variable and unpredictable potential for growth. GCT_B is a neoplasm formed by a network of spindle-shaped mononuclear stromal cells and multinuclear giant cells similar to osteoclasts. The cellular components interact together with various factors playing also a role in osteoclast function regulation as receptor activator of nuclear factor kappa-B ligand (RANKL), receptor activator of nuclear factor kappa-B (RANK) and osteoprotegerin (OPG) as the key molecular regulation system for bone remodelling. RANKL is the main stimulatory factor for formation of mature osteoclasts and is essential for their survival. Precursors of osteoclasts express the receptor activator nuclear factor kB, RANK and in presence of macrophage colony-stimulating factor (M-CSF) and its ligand, RANKL, it mediates osteoclast formation by increasing the expression of enzymes that dissolve organic and inorganic components of bone, such as MMP-9, uPA proteolytic system. So, these interactions may provide information to develop new approaches for a biological therapy of this tumor. Drugs that target the osteolitic process lower recurrence rate associated to morbidity and mortality and are considered useful for new clinical treatments. The aim of this study was to compare the potential biomarkers involved in bone remodeling and cell proliferation in human and animals GCT_B, in the attempt to define possible common activated pathways or key molecules in the vicious cycle of osteolytic and proliferative process.
Keywords- bone, Tumor, Giant Cell Tumor of Bone, Human, Cats, comparative

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Introduction

Giant Cell Tumor of bone (GCT_B) [6,38] was described first in human specie in 1818 by Sir A. Cooper [6]. Was H.L. Jaffe [20] and coworkers that defined in 1940 a specific criteria to diagnosed GCT_B. Also the histological grading of GCT_B was first established in human species by Jaffe, et al. that related the histological features with the clinical course of many tumors and to predict the outcome on that basis. Enneking, et al. devised a staging system for all benign and malignant tumors of bone, which was based on clinical, radiographic and pathological criteria [11]. Despite consistent benign overall features, GCT_B shows a significant tendency to local recurrence (10%-40%) resulting in limb salvage surgery [10], but rarely develops in lung metastases [12,37]. In fact, human metastasizing GCT_B have been described in 1% to 4% of all cases and 6% of recurrent GCTs [3]. In our knowledge only 1 case was reported in cat by Ferreras in 2005 [12]. Previous studies focusing on a number of clinical, radiographic, histologic and molecular features such as tumor size, presence of pathological fractures, anatomic site, histological grading and DNA content, failed to show a statistically predictive correlation with clinical course [14-16,22]. The present study of a large group of these rare tumors from cats and human had as its purpose the documentation of our experience of cases with modern criteria for an accurate diagnosis and biological characterization of these tumors. Enzymatic activity of the proteolytic system has been associated with outcome and identified as prognostic markers in many human tumors [1,5,9,18]. Given the promising results of the previous papers [24-26], the purpose of the present study was to analyze relationships between clinical parameters, the expression of different biomolecular system components in a large cohort of GCT_B patients, in order to identify a pattern of biological and clinical markers for the selection of a subgroup of patients with increased risk of relapse. MMPs are zinc dependent enzymes implicated in different types of physiological and pathological conditions. The metalloproteinases family is comprised of 11 different members, which are subclassified into four major types according to differences in structure and substrate specificity

[5,29,30]. MMPs are interstitial collagenases which cleave the fibrillar collagen types I, II and III. Gelatinases, or type IV collagenases, are responsible for degrading amorphous collagen as well as fibronectin [29,30]. Different authors demonstrated that gelatinase type IV collagenases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) represent a major constituent of the basement membranes of blood vessels and are closely linked to metastatic potential of tumors [33]. MMP-2 and MMP-9, also called type IV collagenases or gelatinases, are related enzymes that break down type IV collagen. Neoplastic invasion and metastasis are complex biological processes with numerous unknown steps [12] [Fig-1], [Fig-2]. The degradation and disruption of extracellular matrix (ECM) is generally considered the initial event of this process. Many proteinases are capable of degrading in vivo extracellular matrix components but the proteinases system primarily responsible for these degradation are Metalloproteinases (MMPs) and Plasminogen Activator (PA) systems [23]. An imbalance between the proteolytic activity of matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9) and the metalloproteinase inhibitor TIMP-2 is responsible for degradation of extracellular components and plays a critical role in tumor invasion and metastasis development [34]. Urochinase Plasminogen Activator (uPA) is a 45-55 kDA serine protease which is secreted as an inactive pro-enzyme (pro-uPA) [2,4]. It seems that activation of pro-uPA mostly occurs after binding to its receptor uPAR. Plasminogen activator inhibitors (PAI-1 and PAI-2) inhibit both receptor-bound and free uPA. uPA is found in cellular structures at the leading edge of migrating cells that are involved in adhesion, migration, invasion and intravasation [35]. The uPA system is consider to be a marker for malignancy in severe types of cancer [17,21,28]. PAI-1 is multifaceted proteolytic inhibitor of uPA that also plays an important role in signal transduction, cell adherence and cell migration. A possible promoting function of PAI-1 in tumor growth is suggested by its potential to modify cell adhesion capacity, which is independent of uPA inhibitor activity. PAI-1 is a clinical marker for poor prognosis in several human cancer [7,8,17,21,28]. Was just reported that an elevated PAI-1 protein level was significantly correlated with tumor size, lymph node involvement, differentiation and invasion in human gastroenteric cancers [34]. Bone's resorption is a very complex process characterized by a great number of events involving the recruitment, transformation and activation of cells precursors from bone marrow in order to from mature osteoclastic cells. The osteoclastic precursors and the osteoclasts themselves present a membrane receptor called RANKL, which once activated by its RANKL ligand (produced by osteoblasts in response to external stimuli) induces the cascade of events that leads to the formation of mature osteoclasts and in the last analysis to bone resorption. OPG (osteoprotegerin) is a molecule belonging to the superfamily of TNF receptors which presents a marked inhibitory activity on osteoclastogenesis, mediated by hits link with RANKL. The balance of this system guarantees correct osteoclastic recruitment [39]. Recently T. Morgan [31] and Collaborators has demonstrated in GCTb that RANKL is higly expressed in giant cells but not in stromal cells. These data suggesting that giant cells component of these tumors in unlikely to be recruited as a results of RANKL production by stromal cells. Osteoclastic differentiation and activation is strongly dependent on TNF (tumor necrosis factor) receptor/TNF-like proteins, OPG and ligand for receptor activator of nuclear factor κB (RANKL). The multinucleated giant cells of GCT_B have some morphologic features resembling osteoclasts [39]. However osteoclasts are formed from the fusion of bone marrow derived circulating mononuclear precursors that express a monocyte/macrophage phenotype that express the ligand for RANK (RANKL) [13]. RANKL stands for Receptor Activator for Nuclear Factor κ B Ligand. This natural and necessary messenger molecule activates osteoclasts, cells involved in bone resorption. Overproduction of RANKL is also implicated in a variety of degenerative bone diseases, like rheumatoid arthritis. Rank is a transmembrane member of the tumor necrosis factor receptor (TNFR) superfamily. The functional expression and biologic effects of RANK have been characterized for osteoclasts. After stimulation of RANK by RANKL, the activated RANK interacts with TNFR-associated factors essential for signal transduction. In bone cells RANK expression can be regarded as a marker of the osteoclastic phenotype [13]. Osteoprotegerin or OPG also known as osteoclastogenesis inhibitory factor (OCIF), is a cytokine and member of TNFR superfamily. Osteoprotegerin inhibits the differentiation and resorption of osteoclasts in vitro and in vivo. Osteoprotegerin, a RANK homologous, works by binding to the RANK-ligand on osteoblast/stromal cells, thus blocking the RANK-RANKL interaction between osteoblast/stromal cells and osteoclast precursors [31]. This inhibits the differentiation of the osteoclast precursor into a mature osteoclast. Recombinant human osteoprotegerin acts on bone specifically, increasing bone mineral density and bone volume. Osteoprotegerin has been used experimentally to decrease bone resorption in women with postmenopausal osteoporosis and in patients with lytic bone metastases [26]. In normal bone, OPG interferes with the binding of RANKL to RANK by acting as a decoy receptor for RANKL and thereby prevents excessive bone destruction. OPG bioavailability is also regulated by its potential sequestration by other factors, such as tumor-necrosis-factor related apoptosis-inducing ligand (TRAIL) and syndecan-1. It was therefore hypothesized that it is the relative abundance of OPG and RANKL that regulates bone resorption [31]. This hypothesis was supported by the finding that RANKL expression in osteoblastic cells and bone marrow stromal cells is increased and OPG expression is reciprocally decreased, by osteotropic factors, including 1,25-dihydroxyvitamin D3, parathyroid hormone-related protein, interleukins (ILs) (in particular, IL-1 and IL -6) and transforming growth factor-β1 [13]. Furthermore, the expression of some of these factors is altered in bone metastasis such that the process of bone resorption is enhanced. Identification and understanding of RANKL/RANK/OPG system has permitted bone research investigators to demonstrate that the cytokines RANKL and OPG are actively implicated in the mechanism of tumor-bone interactions like osteolytic bone metastases and humoral hypercalcemia of malignancy [4,27]. OPG and RANKL expression are modulated by numerous agents that function as bone regulators. OPG espression is positively regulated by estrogens, TNF-α, GH and TGF-β and negatively by PTH and glucocorticoids. RANKL is also modulated by the same agents controlling OPG. The osteoclastic cell lineage failed to regulate its expression even by the most osteotropic factors. These observations reveal that the OPG/RANK/RANKL triad plays a pivotal role in bone biology and that the most osteotropic factors control bone remodelling via the finely regulated balance between OPG and RANKL activities [26]. Cell-cell interactions are also involved in the control of RANKL produc-

tion. GCT_B cells derived from the tumor stroma have been demonstrated to be rich source of RANKL mRNA while tumor giant cells also produce excessive levels of RANK mRNA as compared to the production of these factors by normal osteoclasts. Thus, GCT_B can be viewed as a neoplasm that produces an autonomous and unregulated overexpression of RANKL and RANK, this unregulated increase in osteoclastic activity results in extensive local bone destruction.

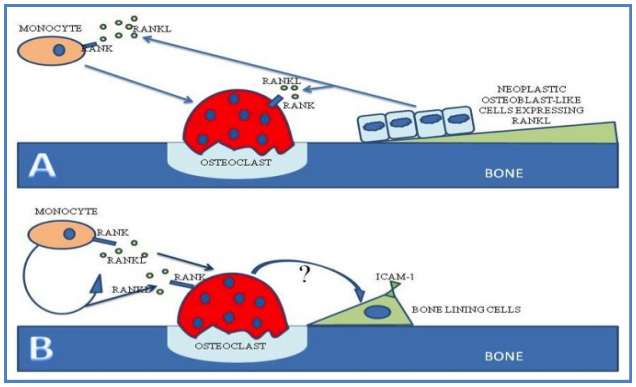

Fig. 1- Histogenesis of Giant Cell Tumor of bone (GCTb) and hypothetical connections between stromal cells and multinucleated giant cells. Neoplastic osteoblast-like stromal cells induce osteoclast activation and differentiation with direct involvement of receptor activator of nuclear factor kB (RANKL) secretion (A). Multinucleated GC/monocytes promote osteoclast activation and differentiation as a result of an autocrine RANKL-mediated circuit, in turn inducing hyperplastic proliferation and activation of osteoblast-like intracellularr adhesion molecule-1-positive bone lining cells (B).

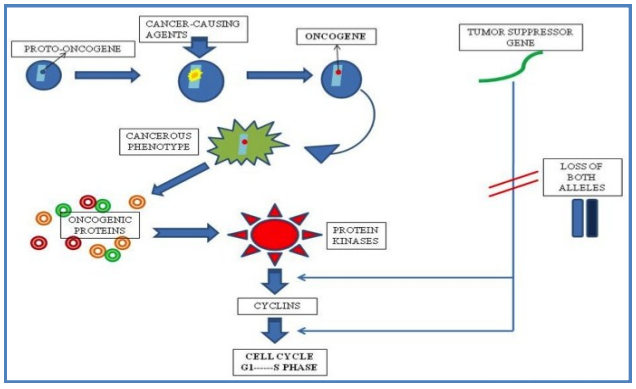

Fig. 2- Neoplasia, or uncontrolled cellular proliferation, can result either from mutations that "turn on" the oncogenes that stimulate growth, or from mutations that result in loss of tumor suppressor genes and their products that inhibit cellular growth.

Materials and Methods

Cases

Ten cases of feline primitive GCT_B, previously diagnosed at the Department of Biopathological Science and Hygiene of Animal and Alimentary Production in Perugia were examined and a total of 448 human patients with GCT of bone were treated at the Rizzoli Institute Orthopaedic Oncology Service [Fig-3], [Fig-4]. For this study, 73 GCT patients with complete histological and clinical documentation were considered. We used Enneking's surgical staging system for GCT of bone [11] that considers clinical, histologic and radiographic findings based on the system by Campanacci, et al [6]. The series included 20 patients who developed lung metastases and 53 with no evidence of lung metastases at a median follow-up of over 10 years. In these two groups, respectively 14 and 16 patients had local recurrences. The 20 metastatic patients represent the entire series of metastatic GCT of our Institution, while the non-metastatic patients were retrospectively investigated and pre-selected with the purpose of identifying risk factors for local recurrences. Specimens were obtained from initial biopsies for primary tumors or from samples collected at the time of surgical excisions for lung metastases. All tissue samples were fixed in buffered-formalin and paraffin-embedded. Diagnosis was defined on hematoxylin-eosin stained sections following conventional criteria [6].

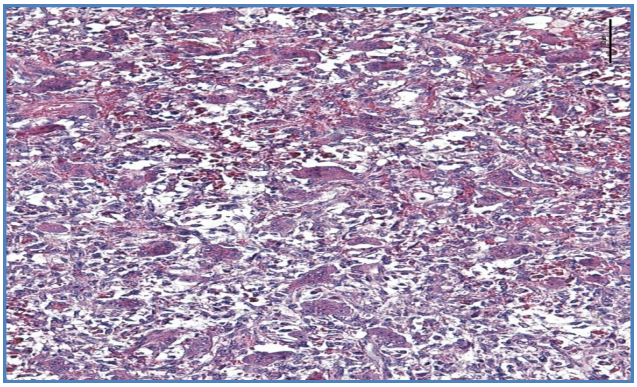

Fig. 3- Human giant cell tumor of bone. Hematoxylin-Eosin stain, 10x.

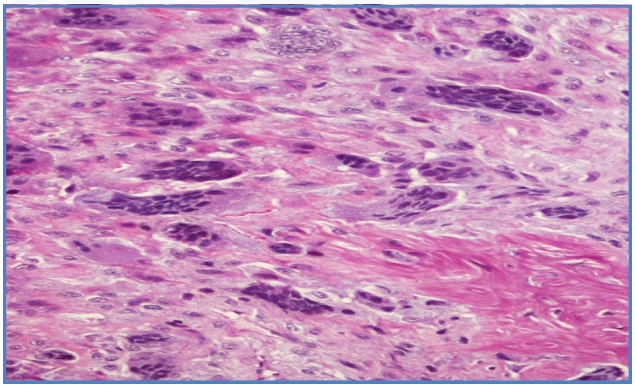

Fig. 4- Bone, feline, giant cell tumor of bone. Neoplastic tissue consists of many multinucleated osteoclast-like giant cells and spindle-shaped mononuclear stromal cells.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin sections. Paraffin was removed with xylene and then dehydrated in sequential diluted ethanol and then rinsed in distilled water. To inhibit endogenous peroxidase activity the tissue sections were with 3% hydrogen peroxide in tris phosphate-buffered saline (PBS). Non specific reactivity was blocked with the use of normal goat serum for 30 minutes. The following primary antibodies were used: anti-RANKL (sc-7628 ; Santa Cruz Biotechnology, Santa Cruz, CA, dil 1:50], anti-OPG (N-20; Santa Cruz Biotechnology, inc., dil: 1:50), anti-CDK4 (sc-260; Santa Cruz Biotechnology, inc., dil. 1:100). For immunodetection of metalloproteinase family (MMP2, MMP9, TIMP2) and urokinase plasminogen activator system (uPA) compo-

nents (u-PA, u-PAR and PAI-1), slides were incubated in a microwave with 10 mM citric-acid solution (pH 6.0) to retrieve the antigen. After washing, the sections were incubated with normal serum and then with the appropriate dilution for each primary antibody. Anti-u-PA polyclonal antibody which reacts with b-chain and inactive 52 kDa precursor form of u-PA for both human and cat samples (C20; Santa Cruz Biotechnology; dilution 1:100). Anti-u-PAR monoclonal antibody which binds with high affinity for both u-PAR and u-PA/u-PAR complex was used for the human samples (CD87;American Diagnostic Inc., Greenwich, UK, dil. 1:50), while anti-u-PAR polyclonal antibody was used for the cats (Santa Cruz Biotechnology; dil. 1:50). Anti-human PAI-1 monoclonal antibody was used as a primary antibody for the human samples (American Diagnostic Inc, dil. 1:25) and anti-PAI-1 polyclonal antibody for the cats (Santa Cruz Biotechnology, dil. 1:25). For both cats and humans, monoclonal anti-MMP9, monoclonal anti-MMP2 (Oncogene Research Products, dil. 1:25 and dil. 1:50 respectively) and anti-TIMP2 (Santa Cruz Biotechnology; dil. 1: 100 for humans, 1:50 for cats) were used. All sections were then incubated with a goat anti-mouse biotinylated antibody, followed by an avidin-biotin-peroxidase complex. Immunostaining was revealed with 3-amino-9ethyl-carbazole and nuclei were counterstained with hematoxylin. Samples with less than 10% positive cells were classified as negative, 10- 25% weakly positive, 26%- 49%, moderately positive and more than 50% strongly positive. Protein was considered over-expressed when sections were strongly positive. Tissue sections of aneurysmal bone cyst, a benign lesion containing both normal osteoclasts and osteoblasts, were used as positive controls. Negative controls were performed by omitting the primary antibody.

Results

Human Species

Clinical characteristics of the 73 GCT human patients are shown in [Table-1]. Median follow-up was 128 months (range 29-312) and minimal follow-up for disease-free patients was set at 60 months. Median age was 30 years (range: 18-56 yrs). Thirty-six patients were male and 37 female. Forty-four cases were of the lower limbs, 13 of the upper extremities and 8 of the spine and pelvis. According to Enneking's surgical staging system [24], 4 cases were stage 1, 35 stage 2 and the remaining 34 were stage 3 lesions. Of the 73 cases, 47 underwent an intra-lesional excision (IL) (curretage) and the remaining 26 an en-bloc resection (EL). Thirty of the 73 patients developed a local recurrence. Of the 30 patients with local recurrence, 19 had one recurrence while 11 had 2 or more local recurrences. One human patient died for tumor related reasons (lung metastases). No significant differences were observed between metastatic and non-metastatic human patients with respect to age, gender, size and site, while stage resulted significantly related to metastatic event. Among the patients with stages 1 and 2, 6/39 (15.4%) developed lung metastasis, versus 14/34 (41.2%) stage 3 (p=0.018). The type of treatment seems also affect the metastasis incidence. Lung metastases incidence was higher among the patients who underwent to curettage (11/26) than in patients who had en bloc resection (9/47) (42.3% versus 19%, p=0.054. Incidence of local recurrence was higher in the metastatic (70%) than non-metastatic (30%) group (p<0.003). Risk of developing lung metastases in patients who had at least 1 local recurrence was 47% and 14% in patients with no local recurrences (p<0.003). Risk of metastasis was 37% after 1st local recurrence and 64% with 2 or more recurrences (p<0.003). Risk of re-recurrence after 1st local recurrence was 5 times higher in the metastatic than non-metastatic group (4% vs. 20%, p<0.007).

Table 1- Baseline Demographic and Clinical Characteristics of 73 Humans Patients with Primary Tumor

Sex	No(%)
Male	36
Female	37
Median age-yrs. (range)	30 (18-56)
Follow up-months (range)	128 (29-312)
Surgical Staging	**No(%)**
Stage 1	4
Stage 2	35
Stage 3	34
Surgical Treatment	**No(%)**
Intra-lesional excision (curettage)	47
En bloc resection	26
Common Sites Of Primary Lesion	**No(%)**
Lower extremities	44
Upper extremities	13
Pelvis	8
Spine	8
Clinical Course	**No(%)**
Local recurrences	30
Metastases	20
Disease-free	23
Alive	72
Dead from disease	1

Cats

In counterpart the median age in cats was 12,3 yrs., most females was involved respect the males (6:4) and no data of recurrences was detected in cats after surgical and medical treatments [Table-2] and [Table-3]. The breed distribution of the tumors revealed that European was the most common breed involved and that long bones of the limbs resulted the most frequent sites of primitive localization of all GCT_{BS} investigate in cats.

Table 2- Baseline Demographic and Clinical Characteristics of 10 Cats with Primary Tumor

Sex	No(%)
Male	4
Female	6
Median age-yrs. (range)	12.3 (4-16)
Follow up-months (range)	No data of recurrences

Table 3- Age, Sex, Breed and Localizations of Feline Giant Cell Tumor of Bone

	Age (Yrs.)	Sex	Breed	Localization
Cat	11	M	E	Distal Tibia
Cat	11	M	E	Unknown
Cat	4	M	E	Unknown
Cat	15	F	E	Tibia
Cat	15	F	M.B.	Skull
Cat	12	M (C)	?	Skull
Cat	10	F	E	Femur
Cat	14	F	M.B.	Unknown
Cat	15	F	M.B.	Tibia
Cat	16	F	M.B.	Unknown

M(C): male castrated, E: European, M.B.: mixed breed

Histology

All human and feline tumours were well vascularized and composed of a network of spindle shaped mononuclear stromal cells (MC) that represent the tumour pattern, round mononuclear histiocytic cells and multinucleated giant cells (GC) similar to osteoclasts [Fig-3], [Fig-4]. Mitotic activity was rarely seen in mononuclear cell components. Similarly, haematoxylin-eosin-stained sections of all 10 GCT_B cases had a highly cellular tissue composed of multinucleated giant cells set in a homogenous stroma of mononuclear cells that varied in shape from polygonal to slightly spindle. Reactive bone formation was seen in rare cases and the spicules of metaplastic bone were generally limited to linear condensations of spindle cells. Mitotic figures were occasionally present. In both animals and humans, stroma tumour cells had pale cytoplasm, indistinct cell borders, solitary nucleus with distinct nuclear membrane and an usually prominent nucleolus. The multinucleated giant cells contained several, up to 100, nuclei morphologically identical to those of stromal cells. Scattered areas of degenerative and necrotic tumor cells, haemorrhage and reparative fibrous tissue, foam cells and mild chronic inflammation were observed in some cases both in humans and cats samples.

Immunohistochemistry

In all examined feline cases, mononuclear cells expressed a strong and homogeneous positive membrane staining for RANKL, while the expression of OPG was focal with scarce MC cells presenting cytoplasm immunostaining. This resulted in an increased RANKL/OPG ratio that, in turn, led to excessive development of large multinucleated osteoclasts-like giant cells. A strong association between RANKL, large number of GCT_Bs and MMP9 was seen. In cats, MMP9 and MMP2 were co-overexpressed and exhibited variable staining. MMP9 was strongly and predominantly expressed in giant cells (>50%). MMP2 was present in more than 50% of mononuclear tumor cells in all cases investigated, with staining intensity ranging from moderate to strong [Fig-5]. The metalloproteinase inhibitor,TIMP2, showed different staining patterns only in two cases of feline GCT_B, where some GC (10-20%) appeared faintly positive. The other cases were negative. In humans, RANKL was strongly expressed in the majority of cases including metastatic and locally relapsed, with homogeneous staining distribution (more than 50% of positive cells) in MC membrane. In contrast, OPG was negative or weakly positive (< 25% of positive cells) in the majority of cases studied. Similarly to animals, the overexpression of RANKL appear to be associated with a strong and uniform positivity for MMP9 [Fig-6], but the distribution pattern was different. In fact, in humans the expression of MMP9 was prevalent in the cytoplasm [Fig-7] of MC (> 50% positive cells), while MMP2 staining was distributed across all cell types, including intratumoral osteoclast-like giant cells, with expression patterns, ranging from moderate to strong. Both animals and humans were negative or weakly positive for TIMP2 inhibitor [Table-4]. A different distribution staining in animals ad humans was also seen for uPA system components. The first presented u-PA staining mainly in GC, while PAI-1 was uniformly and strongly expressed in the cytoplasm of both MC and GC. Minimal to no expression of u-PAR was seen in feline GCT_B. Conversely, both u-PA and its receptor u-PAR had strong and uniform expression (>50% positive cells) in MC of 29/73 human clinical samples (40%), associated with weak and focal distribution of their inhibitor, PAI-1, in both MC and GC patterns [Table-4]. Negative controls did not stain in any case.

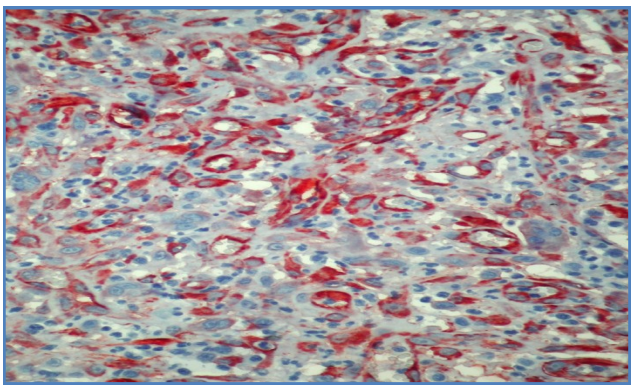

Fig. 5- Femur, cat, giant cell tumor of bone. Immunohistochemical staining of MMP2 in feline GCT_B. Hematoxylin counterstain, 20x.

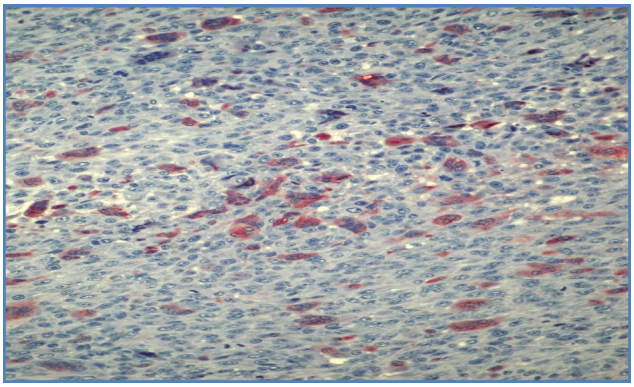

Fig. 6- Femur, cat, giant cell tumor of bone. Immunohistochemical staining of MMP9 in feline GCT_B showed strong expression in osteoclast-like giant cells. Immunohistochemical staining of MMP9. All of the samples investigated were immunohistochemically positive for MMP2 and MMP9 and exhibited variability of staining expression. Hematoxylin counterstain, 10x.

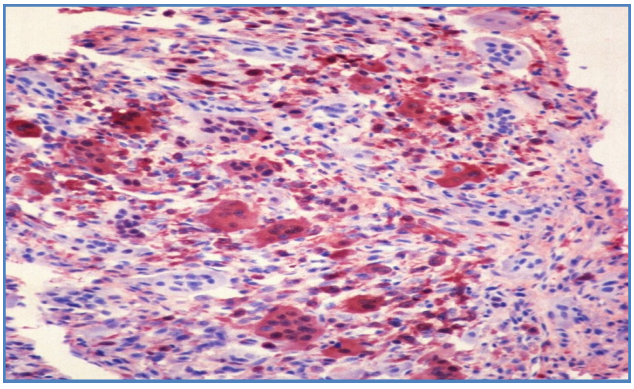

Fig. 7- Tibia, human, giant cell tumor of bone. MMP2 staining was strongly positive in in osteoclast-like giant cells. Immunohistochemical staining of MMP2 in human GCT_B. Hematoxylin counterstain, 10x

Statistical Studies

Interestingly, in humans the co-overexpression of u-PA/u-PAR has been found associated with poor prognosis in terms of local recurrence (17/30 vs. 12/43 without recurrences) and/or metastatic

event (18/20 vs. 11/53 non metastatic), with a Fisher's test statistically significant (90% vs. 21%, p< 0.0005 and 57% vs. 28.0 %, p=0.028 respectively). In addition By Backward Wald logistic regression analysis including variables significant to univariate analysis (immunostaining, local recurrence, stage and treatment), it emerged that the best model to predict metastasis involved overexpression of uPA system components, the presence of local recurrence and treatment. In particular, u-PA/uPAR co-overexpression appeared the strongest prognostic parameter, defining an increased risk for the metastatic event by 94.7% (95% CI, 0.900-0.994; p=0.0005). The ROC curve area obtained by evaluating the metastatic risk with this model was very significant (0,947 95% CI (0.900-0.944; p< 0.0005) [Table-5]. In order to define the intracellular mediators of proliferating pathways affected by osteolytic process in GCT_B, we assessed the expression the cyclin-dependent kinases, CDK4, involved in cell cycle regulation in humans and cats. CDK4 was strongly and uniformly overexpressed (> 50% of positive cells) in human recurrent GCT_B, while localized tumors presented a moderate positivity in 26-49% of stromal cells. In both groups the distribution was predominantly in MC cytoplasm. Conversely, the cats showed positive immunostaining in the cytoplasm of GC (26%-49% of positive cells) [Table-4]. No data of recurrences and/or metastases was detected in the follow up of cats.

Table 4- Results of Protease Expression in Human GCT_B

	DFS patients			
	MMP9	MMP2	u-PA	u-PAR
1	MC >50%	MC >50%; GC25-50%	MC>50%	MC<25%
2	MC >50%	GC>50%	MC<50%	MC25-50%
3	MC 25%-50%	MC 25-50%	MC25-50%	MC<25%
4	MC 25%-50%	MC 25-50%	MC25-50%	MC25-50%
5	MC >50%	MC25-50%	MC>50%	MC<25%; GC<25%
6	MC >50%	MC >50%; GC<25%	MC<25-50%	MC>50%
7	MC 25%-50%	MC 25-50%; GC 25-50%	MC<25%	MC>50%
8	MC >50%	MC>50%	MC>50%	MC>50%
9	MC25%-50%	GC>50%	MC<25-50%	MC<25-50%
10	MC >50%	MC 25-50%	MC<25%	MC<25%
11	MC<25%	MC<25%	MC>50%	MC>50%
12	MC >50%	MC 25-50%; GC>50%	MC<25%	MC<25%
13	MC >50%	MC<25%	MC25%-50%	MC<25%
14	MC >50%	MC >50%; GC25-50%	MC<25%; GC<25%	MC>50%
15	MC 25%-50%	MC 25-50%; GC 25-50%	MC<25%	MC<25%
16	MC >50%	MC>50%; GC>50%	MC>50%	MC>50%
17	MC >50%	MC >50%; GC<25%	MC<25%	MC<25%
18	MC >50%	MC>50%; GC<25%	MC25-50%	MC>50%
19			MC25-50%	MC<25%
20	MC >50%	MC 25-50%	MC<25%	MC>50%
21	MC 25%-50%	MC >50%; GC<25%	MC>50%	MC<25%
22	MC<25%	MC 25-50%		
23	MC 25%-50%	MC 25-50%	MC>50%	MC>50%

DFS: disease-free survival; MC: mononuclear cells; GC: giant cells

Table 5- The Best Model to Predict Metastasis in Humans by Using by Backward Wald Logistic Regression Analysis

Variables	WALD	OR	95% CI OR
uPA/uPAR co-overexpression	p < 0.0005	12.83	3.069 - 53.639
Local recurrences	p = 0.011	4.533	1.405 - 14.624
Curettage	p = 0.015	4.733	1.357 - 16.506

Discussion

This comparative study on phenotypical and biomolecular characterization of GCT_B in feline and humans species and related data permit to obtain different considerations that never was debate in comparative studies of this rare bone tumor. MMP_2 and MMP_9 were co-expressed in GCT_B and exhibited an intratumor variability of staining diversity [Table-6]. MMP_2 was present in the stromal cells in all cases investigated (with percentage from 20% to 90%), while two cases only from cats investigations showed also scanty affinity for osteoclast-like multinucleated cells. MMP_9 was also highly and predominantly expressed in neoplastic giant cells (>50%) and suggest that this metalloproteinase may contribute, either in humans than in cats to proteolysis associated with different involvement of vascular invasion and local resorption in GCT_B. TIMP2 showed different patterns in staining in only two cases of feline GCT_B where some cells neoplastic giant cells (10%-20%) appeared faintly positive and the other cases were always negative. Our immunostaining studies on MMPs in GCT_B have demonstrated a MMP_2 and MMP_9 activity indicating that MMPs could play an important role in biological invasive behaviour of this tumor. One of the most characteristic features of MMPs is the inhibition of the activities by their common inhibitors know as TIMPs. These data suggest that MMPs production in these tumors could start only when they overtakes in molecular number the level of $TIMP_2$. Multiple factors other then the degradative enzymes for extracellular matrix (ECM) are involved in the metastatic processes of many tumours including GCT_B [32] [Table-6].

Table 6- Results of Protease Expression in Feline GCT_B

	GCT cats			
	MMP9	MMP2	u-PA	PAI-1
1	GC>50	MC>50%	GC 25-50%	MC 25-50%; GC 25-50%
2	GC>50	MC>50%	GC <25%	MC>50%; GC 25-50%
3	GC 25-50%	MC>50%	GC 25-50%	MC> 50%; GC> 50%
4	MC<25%GC>50	MC>50%GC<25%	GC>50%	MC 25-50%; GC 25-50%
5	GC>50	MC>50%	GC>50%	MC<25%; GC 25-50%
6	GC>50	MC>50%	GC 25-50%	MC> 50%; GC 25- 50%
7	MC<25%; GC>50	MC>50%GC<25%	GC25-50%	MC>50%; GC 25-50%
8	GC 25-50%	MC>50%	GC25-50%	MC> 50%; GC> 50%
9	GC>50	MC>50%	GC>50%; MC 25-50%	MC> 50%; GC> 50%
10	MC<25%; GC>50	MC>50%	GC 25-50%	MC> 50%; GC> 50%

In particular the distinct immunostaining patterns observed for MMPs and TIMPs within GCT_B are consistent with their involvement in tumor growth and diffusion and the correlated expression in neoplastic neo-vasculature, mononuclear stromal cells and multinucleated giant cells emphasises the importance of the tumor microenvironment in the pathogenesis of GCT_B. Thus an unbalance in this proteolytic system was seen in both groups, suggesting the main role of the tumor microenvironment in the pathogenesis of GCT_B through the regulation of factors affecting both proliferation and osteolysis. This results agree with others just described in human GCT_{BS} that demonstrate strong correlations through incidence of matrix invasion and related molecules involved such also uPA system [3,19]. We also investigated the expression of uPA system components either in humans and cats species. A higher incidence of uPA and uPAR overexpression was found in the relapsed than in non relapsed group from human species. Our data support the evidence that the targeting of the uPA system may

have, especially in humans species, a therapeutic potential. Conversely in cats uPAR was never expressed and uPA is prevalently positive in giant cells with an osteoclast-like pattern. Interesting data was the diffuse and uniform positivity of GCT_B to PAI-1 in cats that conflicts with the data in humans where PAI-1 almost never was expressed and it suggest that excess or absence of PAI-1 may deregulate plasmin-mediated proteolysis, inhibiting or promoting progression depending on cellular source. GCT_B is characterized morphologically by the presence of numerous multinucleate giant cells, some of which mimics osteoclasts, arise from well vascularized bone marrow stromal cells of the medullary cavity of bone organs formerly it was consider by some authors that tumor cells of GCT_B arise from macrophage precursors of the bone marrow-derived mononuclear/macrophage cells. Our studies used immunohistochemical staining techniques focused also on the biological functions of RANKL, RANK and OPG, participants in normal bone metabolism and remodelling activities, demonstrate that tumor cells in GCT_B could arise from bone marrow stroma. Was also demonstrated that direct contact between mononuclear stromal cells and osteoclast precursors is not required for osteoclast formation suggesting that GCT_B stromal cells can induce osteoclastogenesis by producing releasing soluble RANKL. Our findings indicate that the accumulation of multinucleated tumor giant cells occurs by a RANKL-dependent mechanism and that resorption by these cells may be controlled by inhibitors of osteoclast activity [36]. RANKL was expressed immunohistochemically on the surfaces of pre-osteoblastic/stromal cells. The observation that RANKL is higly expressed in multinucleated tumor giant cells of GCT_B holds that these components could are passively recruited by the production of RANKL by the stromal neoplastic components. Different molecules was identified and extensively characterized that are capable of regulating proliferation, differentiation, fusion, activation and apoptosis of osteoclasts. Osteoclast differentiation and activation is critically dependent on the tumor necrosis factor (TNF) receptor/TNF-like proteins, osteoprotegerin (OPG) and ligand for receptor activator of nuclear factor kB (RANKL). RANKL expression has been observed in GCT-derived stromal cells and these cells support osteoclast formation. These and other observations have led to widely accepted proposition that the expression of RANKL by the neoplastic stromal cell causes the recruitment of osteoclasts [13]. GCT_B cells derived from the tumor stroma have been demonstrated to be rich source of RANKL mRNA while tumor giant cells also produce excessive levels of RANK mRNA as compared to the production of these factors by normal osteoclasts. Thus, GCT_B can be viewed as a neoplasm that produces an autonomous and unregulated overexpression of RANKL and RANK, this unregulated increase in osteoclastic activity results in extensive local bone destruction. While the activity of proteases has been related to extracellular matrix degradation, the first event in metastatic process [29], cell cycle deregulation appear to be the first hallmark in cell growth deregulation following proliferative signals [19]. Regulatory function of both positive and negative factors on cell cycle progression is achieved by selective phosphorylation-dephosphorylation processes of critical substrates involved in G1/S and G2/M transition. Cyclin D1 acts at mid-G1 phase activating Cdk4 that in turns phosphorylates and inhibits the product of RB tumor suppressor gene (pRb), releasing the transcriptional factors, that induce transcription of genes which positively control cell progression [18]. In our previous studies [26], we found also overexpression of CDK4 in human GCT_B with strong and uniform immunostaining more evident in MC of relapsed cases. In cats a major involvement of GC is present and the activation of RANKL pathway appear to lead to activation of both MMP9 and CDK4, thus contributing to vascular invasion and local resorption. Multiple factors other then the degradative enzymes for ECMs are involved in the metastatic processes of a lot of tumors enclosed GCT_B. In particular the distinct immunostaining patterns observed within GCT_B are consistent with their involvement in tumor growth and diffusion and the correlate expression in neoplastic neo-vasculature, mononuclear stromal cells and multinucleated giant cells emphasises the importance of the tumor microenvironment in the pathogenesis of GCT_B. More investigations must be performed to clarify better these first results and related close mechanisms involved in behaviour and biological pattern of this rare bone tumor.

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