Genetic Analysis of Giant Cell Lesions of the Maxillofacial and Axial/Appendicular Skeletons

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Purpose: To compare the genetic and protein expression of giant cell lesions (GCLs) of the maxillofacial (MF) and axial/appendicular (AA) skeletons. We hypothesized that when grouped according to biologic behavior and not simply by location, MF and AA GCLs would exhibit common genetic characteristics.

Materials and Methods: This was a prospective and retrospective study of patients with GCLs treated at Massachusetts General Hospital from 1993 to 2008. In a preliminary prospective study, fresh tissue from 6 aggressive tumors each from the MF and AA skeletons (n = 12 tumors) was obtained. RNA was extracted and amplified from giant cells (GCs) and stromal cells first separated by laser capture microdissection. Genes highly expressed by GCs and stroma at both locations were determined using an Affymetrix GeneChip analysis. As confirmation, a tissue microarray (TMA) was created retrospectively from representative tissue of preserved pathologic specimens to assess the protein expression of the commonly expressed genes found in the prospective study. Quantification of immunohistochemical staining of MF and AA lesions was performed using Aperio image analysis to determine whether immunoreactivity was predictive of aggressive or nonaggressive behavior.

Results: Five highly ranked genes were found commonly in GCs and stroma at each location: matrix metalloproteinase-9 (MMP-9), cathepsin K (CTSK), T-cell immune regulator-1 (TCIRG1), C-type lectin domain family-11, and zinc finger protein-836. MF (n = 40; 32 aggressive) and AA (n = 48; 28 aggressive) paraffin-embedded tumors were included in the TMA. The proteins CTSK, MMP-9, and TCIRG1 were confirmed to have abundant expression within both MF and AA lesions. Only the staining levels for TCIRG1 within the GCs predicted the clinical behavior of the MF lesions.

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**Conclusions:** MMP-9, CTSK, and TCIRG1 are commonly expressed by GCLs of the MF and AA skeletons. This supports the hypothesis that these lesions are similar but at different locations. TCIRG1 has not been previously associated with GCLs and could be a potential target for molecular diagnosis and/or therapy.

Giant cell lesions (GCLs) are intraosseous benign tumors containing multinucleated giant cells (GCs) within a mononuclear stroma. They can affect both the maxillofacial (MF) and the axial/appendicular (AA) skeletons. Lesions at both sites can vary from small, slow-growing tumors recognized as incidental radiographic findings to large destructive lesions, leading to displacement or resorption of adjacent structures or pathologic fracture.1,2

GCLs of the MF skeleton are more common in females and younger patients (2:1 females to males during the first and second decade) compared with those of the AA skeleton (1.3 to 1.5 females to males during the third to fifth decade).3-9 MF GCLs are more likely to be asymptomatic, and they are often discovered on routine dental radiographic examinations.4,10

Controversy remains regarding the biologic relationship between GCLs of the jaws and giant cell tumors (GCTs) in the AA skeleton.3,5,10-14 This is because subgroups of these tumors (ie, aggressive and nonaggressive) according to biologic behavior have not been differentiated in reported comparisons. Some investigators have postulated that MF and AA GCLs are distinctly separate lesions because of differences in clinical behavior and histopathologic features.5,11,15 Others have supported the hypothesis that these are similar lesions in different locations representing a continuum of the same disease process.3,5,10,17 In each location, the pathogenesis has been hypothesized to involve stromal fibroblasts that recruit monocytes, which then transform into multinucleated GCs.18,19 These GCs have been shown to be phenotypically osteoclasts through immunohistochemistry.20

Clinical and radiographic features can be used to classify GCLs in both locations as aggressive or nonaggressive.5,10,21 Lesions of the MF region are considered aggressive if they are larger than 5 cm, recurrent, or meet 3 of the following 5 criteria: rapid growth, root resorption, tooth displacement, cortical bone thinning, and/or perforation. Nonaggressive lesions grow slowly and are asymptomatic, with a low rate of recurrence after enucleation or curettage. AA lesions are classified according to the classification system of Enneking, also by clinical and radiographic behavior.11,22 Enneking stage 1 (latent) refers to static lesions or those that heal spontaneously. Stage 2 (active) tumors exhibit progressive growth but are limited by natural barriers (ie, cortices). Stage 3 lesions are locally aggressive, with destruction of natural barriers.22 As previously outlined, both the Enneking and the Chuong and Kaban21 MF classification systems, using clinical and radiographic criteria, can be modified to produce a single, biologically consistent binary classification for GCTs in both locations: aggressive and nonaggressive.1,12,13

Studies comparing GCLs of the MF and AA skeletons have been limited by inconsistent terminology, and investigators have typically grouped all lesions together, without considering the clinical or biologic behavior. MF GCLs were first referred to as “giant cell reparative granuloma” by Jaffe11 in 1953. Reports of spontaneous resolution have been published.23,24 However, other MF GCLs are destructive and grow rapidly.10 Additionally, the reluctance to label MF GCLs as “giant cell tumors” can result from the reports of metastases and malignant transformation from AA GCLs.25-27 However, retrospective analyses of cases of lung metastases from AA GCTs have indicated that these might actually be malignant tumors that happen to contain GCs.28 In a recent study, investigators found consistent mutations in histone 3.3 driver variants (H3F3A gene) in the stromal cells of AA GCLs.29 They reported H3,3, H3F3A, and H3F3B in a variety of bone and cartilage tumors.29,30 A subsequent study by a different group of both aggressive and nonaggressive GCLs of the MF skeleton did not find these specific mutations.31 Although this supports that these are different lesions, mutations in histone, the protein that packages DNA into nucleosomes, have been found in a variety of bone and cartilage tumors and in pediatric brain tumors and might not be involved in pathogenesis.32-34

Our group has previously compared the lesions in each location by biologic behavior using the Chuong and Kaban classification21 and a modified version of the Enneking classification1 (ie, aggressive or nonaggressive) and found the lesions to be similar with regard to the phenotypic, clinical, and radiographic appearance.13 Subsequently, it was shown that these lesions are histologically similar and that they could not be differentiated consistently by blinded pathologists.14 These findings support the conclusion that they are similar tumors in different locations.

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The purpose of the present study was to compare the gene and protein expression of GCLs of the MF and AA skeletons. We hypothesized that the gene expression between GCLs at both locations would be consistent and would be confirmed by protein analysis. Second, we hypothesized that the gene expression would have a relationship to the biologic behavior (ie, aggressive vs nonaggressive). Further characterization of the relationship between MF and AA GCLs could provide insight into the etiopathogenesis of these tumors and help direct specific treatment.

Materials and Methods

STUDY DESIGN AND POPULATION

This was a prospective and retrospective cohort study of patients with GCLs treated at Massachusetts General Hospital (MGH) from January 1993 to June 2008. The gene expression analysis was prospective and consisted of data from consecutive patients with AA and MF GCLs treated by us. Patients were included if the lesion was identified as aggressive according to the preoperative clinical and radiographic criteria. For the retrospective protein expression analysis, potential subjects were identified from the MGH Giant Cell Patient Registry. The inclusion criteria were histologically confirmed MF or AA GCLs treated by the Department of Oral and Maxillofacial Surgery or Department of Orthopaedic Surgery and a minimum follow-up period of 6 months. Patients with hyperparathyroidism, cherubism, Noonan syndrome, inadequate histologic samples, follow-up shorter than 6 months, or inadequate documentation were excluded. The MGH institutional review board approved the project (protocol no. 2008P000563).

GENE EXPRESSION ANALYSIS

Study Variables for Protein Expression Analysis

The predictor variables were GCL location (AA vs MF) and tissue type (GCs and stromal cells). The primary outcome variable was the level of gene expression. Biologic behavior was classified preoperatively as aggressive or nonaggressive using standard published criteria, with modification of the Enneking staging systems for AA GCLs into aggressive and nonaggressive lesions, as previously outlined. MF lesions were considered aggressive if they were larger than 5 cm in size, had recurred, or had at least 3 of the following characteristics: root resorption, tooth displacement, or cortical bone thinning or perforation. For AA lesions, all Enneking stage 3 and any stage 2 lesions with pathologic fracture or recurrence were considered aggressive.

Subjects with aggressive lesions provided informed consent for the use of fresh tissue.

Laser Capture Microdissection

Fresh specimens from 6 consecutive aggressive GCLs from each location (n = 12) were assessed. The specimens were snap frozen in liquid nitrogen immediately after curettage in the operating room. RNA was extracted using TRI Reagent (Sigma-Aldrich, St Louis, MO) and then subsequently analyzed for quality using the Agilent Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) before being subjected to laser capture microdissection (LCM).

Only specimens with acceptable 18S and 28S peaks were used. LCM was performed using RNase free conditions. The specimens were cut into 8-μm sections and mounted onto uncoated glass slides and kept at −80°F. In preparation for LCM, the specimens were thawed and then fixed using 70% ethanol for 45 seconds. The specimens were stained with methylene blue to aid in the identification of cells using 0.1% methylene blue for 20 seconds. The sections were then gradually dehydrated using ethanol (50, 70, 90, and 100 seconds each for each cycle) before being placed in xylene for 5 to 10 minutes. An Arcturus PixCell II instrument (Applied Biosystems, Life Technologies, Carlsbad, CA) was used for LCM to ensure only tumor cell RNA was used for analysis (Fig 1A,B). In addition, we extracted RNA from stromal cells and multinucleated GCs separately to determine the differences in gene expression.

Extracted RNA was then amplified using Arcturus Picopure RNA isolation kits and amplified using Arcturus RiboAmp RNA amplification kits (Applied Biosystems, Life Technologies). The amplification product was sent to the Harvard Partners Center for Genetics and Genomics facility (Cambridge, MA) to generate biotinylated RNA and subsequent hybridization with GeneChip Human X3P expression arrays (Affymetrix, Santa Clara, CA).

The GeneChip data were compared with 126 primary cell types using the Gene Enrichment Profiler tool, developed by Yair Benita (Xavier Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, MA). The tool allows the gene expression found in each cell type to be compared with the other cell or tissue types to "rank" the gene expression according to the score found during analysis. The higher the score attributed to a gene within a tissue or cell type, the more specific that particular gene is to that particular tissue.

Primary normal tissue and cells were obtained from the National Center for Biotechnology Information Gene Expression Omnibus and ArrayExpress. All samples obtained were profiled using the Affymetrix U133 Plus 2.0 Array platform (Affymetrix). We used
version 14 of the custom transcript definition files provided by Brainarray (Microarray Lab, Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, MI). These files redefine Affymetrix probes by remapping individual probes to the human genome and adjusting to the most up-to-date annotation. The data files were then normalized using the GCRMA module of Bioconductor (available at: https://www.bioconductor.org/), and present and absent cells were calculated for each probe using the MAS5 module. All probes with no cells present were removed. At least 1 sample was required to have an expression value larger than log₂(100).

The gene expression enrichment analysis was performed as previously described. The GCL microarrays were added to the data set of 126 normal tissues and cell types and normalized as a single experiment. Genes scoring greater than 1,000 on the enrichment scale in any of the GCL samples was considered highly enriched in these tissues (Fig 2).

**FIGURE 1.** Photomicrograph of a representative aggressive maxillofacial giant cell lesion A, before and B, after laser capture microdissection to extract giant cells (arrows) (hematoxylin and eosin stain, original magnification ×200).

PROTEIN EXPRESSION ANALYSIS

Study Variables for Protein Expression Analysis

The predictor variables were GCL location (AA vs MF), behavior (aggressive vs nonaggressive), and tissue type (GCs and stromal cells). The outcome variable was the average optical density of staining found on Aperio analysis. Comparisons in optical density were made between locations and between behavior type.

Construction of Tissue Microarray

Paraffin blocks containing GCLs from subjects who had met the inclusion criteria were sectioned into slides (5 μm) and used to mark 3 separate areas containing representative tumor on the paraffin block. The 3 areas of tumor marked were then cored and used to create a tissue microarray (TMA) containing all GCTs from both locations on 1 paraffin block.

Commercially available antibodies to the proteins corresponding to the commonly over-expressed genes were then optimized using positive and negative controls under the direction of a board-certified pathologist. Antibodies to the following proteins were analyzed: matrix metalloproteinase-9 (MMP-9; mouse monoclonal; catalog no. MAB3309; clone 56-2A4; Millipore, Billerica, MA), cathepsin K (CTSK; mouse monoclonal; catalog no. ab90238; Abcam, Cambridge, MA), T-cell immune regulator-1 (TCIRG1; rabbit polyclonal; catalog no. NBP1-88110; Novus Biologicals, Littleton, CO), stem cell growth factor (SCGF; zinc-finger 836, mouse monoclonal; catalog no. ab90238; Abcam), and C-type lectin domain family 11 (CLEC11A; rabbit polyclonal; Abgent Inc, San Diego, CA).

Immunohistochemistry

Sections (5 μm) of the TMA were stained using the following immunohistochemical techniques. For MMP-9, immunohistochemistry was performed manually using Decloaking Chambers (Biocare Medical, Concord, CA) for antigen retrieval using citrate (pH 6.0) solution. The primary antibody was incubated at room temperature for 1 hour at a 1:1,500 dilution. Mouse Envision-HRP reagent (Dako, Carpinteria, CA) was used to detect antigen, followed by development in diaminobenzene for 5 minutes. For cathepsin-K (1:100) and TCIRG1 (1:400), CLEC11A (1:50), and SCGF antibodies (1:50), Leica’s BOND-III staining platform (Leica Biosystems Inc, Buffalo Grove, IL) was used with the Bond Polymer Refine Detection Kit (Leica Biosystems Inc). Antigen retrieval was performed online using Bond Epitope Retrieval Solution 1 (cathepsin-K and SCGF) or 2 (TCIRG1, CLEC11A; Leica Biosystems Inc) for 30 minutes.

Quantification of Immunohistochemical Staining

The level of staining was quantified in each lesion using Aperio (Leica Biosystems Inc) digital pathology scanning and analysis using a color deconvolution
The average optical density of the GCs and stroma were calculated using 3 different areas within each specimen. Positive controls for each antibody were included in the TMA. Omitting the primary antibody served as the negative control.

**STATISTICAL ANALYSIS**

Data were entered into a statistical database (SPSS, version 22.0; SPSS Inc, Chicago, IL) for analysis. Descriptive statistics were calculated. For comparisons between optical density by location and behavior, an independent *t* test was used. The Bonferroni correction was used given the multiple comparisons (*P* ≤ .00625 was considered significant).

**Results**

**GENE EXPRESSION ANALYSIS**

The 4 tissue types studied (GCs and stroma from each location) were clustered together on an unsupervised analysis of gene expression. The genes with the highest enrichment scores for each of the 4 tissue types are provided (Table 1). Five genes were shared by all 4 mentioned tissue types: MMP-9, CTSK, TCIRG1, CLEC11A, and ZNF836. Of these, 3 (MMP-9, CTSK, and TCIRG1) were in the top 8 expressed genes of each tissue type (Table 1). Genes scoring above 1,000 for any of the GCT samples were selected, and a heat map was generated for the entire data set (Fig 3). Genes enriched in stroma and GCs were clearly distinct from those in other tissues.

**ANALYSIS OF PROTEIN EXPRESSION**

**Demographic Data**

The study sample included 88 subjects (40 MF and 48 AA) who met the inclusion criteria. A modified classification system identified 32 and 28 aggressive lesions of the MF and AA skeletons, respectively; 8 MF and 20 AA were classified as nonaggressive.

**Immunohistochemistry**

Staining using the antibodies for MMP-9 and CTSK was positive in both GCs and stroma (Table 2, Figs 3, 4). TCIRG1 showed positive staining in GCs but less so in the stroma (Table 2, Fig 5). CLEC11A and ZNF836 did not show positive staining in either tissue type.

The difference in the optical density of staining according to behavior and location is summarized in Table 2. No statistically significant difference was found for MMP-9 or CTSK with regard to behavior. TCIRG1 had greater staining intensity of the GCs of nonaggressive MF lesions compared with aggressive MF lesions (*P* < .001).

**Discussion**

In this set of experiments, we compared the genetic and protein expression of GCLs of the MF and AA skeletons. We hypothesized that a common genetic expression would be present between GCLs at both locations that could be confirmed by protein expression analysis. The intensity of antibody staining was hypothesized to be related to the biologic behavior.

GCLs of the AA and MF skeletons share common genetic expression of MMP-9, CTSK, and TCIRG1, supporting the hypothesis that they are likely similar lesions at different anatomic locations. The common genetic expression was confirmed by assessment of protein expression using a TMA to quantify immunoreactivity.

Both MMP-9 and CTSK have been shown to be expressed in GCLs in previous studies. They are thought to be involved in collagen degradation and hence osteolysis. Mutations in TCIRG1 have been associated with osteopetrosis, which is thought to

<table>
<thead>
<tr>
<th>Table 1. TOP 10 MOST ENRICHED GENES FOR EACH OF THE 4 TISSUE TYPES STUDIED</th>
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<tbody>
<tr>
<td>Maxillofacial</td>
</tr>
<tr>
<td>Giant Cells</td>
</tr>
<tr>
<td>MMP-9*</td>
</tr>
<tr>
<td>ACP5</td>
</tr>
<tr>
<td>CTSK</td>
</tr>
<tr>
<td>TCIRG1</td>
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<tr>
<td>HYAL1</td>
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<tr>
<td>CCR1</td>
</tr>
<tr>
<td>TNFRSF11A</td>
</tr>
<tr>
<td>SF3A2</td>
</tr>
<tr>
<td>CLEC11A</td>
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<tr>
<td>SLCO4A1</td>
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Abbreviations: ACP5, acid phosphatase 5; ARRB1, arrestin beta 1; ASPN, asporin; CCR1, C-C motif chemokine receptor 1; CLEC11A, C-type lectin domain family 11; COL24A1, collagen type XXIV alpha 1; CTHRC1, collagen triple helix repeat containing 1; CTSK, cathepsin K; FNDRC1, fibronectin type III domain containing 1; HOXC9, homeobox C9; HYAL1, hyaluronoglucosaminidase 1; ITGA10, integrin subunit alpha 10; LOC653390, hypothetical protein LOC100292228 or RRN3 RNA polymerase I transcription factor homolog (S. cerevisiae) pseudogene; MMP-9, metalloproteinase-9; MSX1, Msh homeobox 1; MXRA5, matrix-remodeling associated 5; OMD, osteomodulin; S100A2, S100 calcium binding protein A2; S3A2, splicing factor 3a subunit 2; SLCA2A10, solute carrier family 2 member 10; SLCO4A1, solute carrier organic anion transporter family member 4A1; TCIRG1, T-cell immune regulator-1; TNFRSF11A, tumor necrosis factor receptor superfamily member 11a; ZNF, zinc finger protein.

* Found in all 4 tissue types.

FIGURE 3. Tissue microarray staining of matrix metalloproteinase-9 (>200) for A, maxillofacial and B, axial/appendicular giant cell tumors. Staining is intense in both the giant cells (arrows) and the cytoplasm of mononuclear stromal cells (arrowheads).


### Table 2. COMPARISON OF ANTIBODY STAINING BY LOCATION AND BEHAVIOR

<table>
<thead>
<tr>
<th>Protein</th>
<th>Maxillofacial</th>
<th>Axial/Appendicular</th>
<th>P Value</th>
<th>Maxillofacial</th>
<th>Axial/Appendicular</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aggressive</td>
<td>Nonaggressive</td>
<td></td>
<td>Aggressive</td>
<td>Nonaggressive</td>
<td></td>
</tr>
<tr>
<td>CTSK</td>
<td>0.1476</td>
<td>0.1261</td>
<td>.35</td>
<td>0.1394</td>
<td>0.1359</td>
<td>.19</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.1058</td>
<td>0.1042</td>
<td>.87</td>
<td>0.1026</td>
<td>0.1036</td>
<td>.35</td>
</tr>
<tr>
<td>TCIRG1</td>
<td>0.1361</td>
<td>0.1075</td>
<td>&lt;.0001†</td>
<td>0.1305</td>
<td>0.1093</td>
<td>.96</td>
</tr>
</tbody>
</table>

Data presented as optical density.

Abbreviations: CTSK, cathepsin K; GC, giant cell; MMP-9, matrix metalloproteinase-9; TCIRG1, T-cell immune regulator-1.

* Comparing aggressive and nonaggressive lesions.

† Statistically significant.

result from defective osteoclast function. This is relevant to GCLs because the multinucleated GC have been shown to be phenotypically osteoclasts. Further study of TCIRG1 in GCLs might shed light on the still poorly understood pathogenesis of GCTs.

Both CLEC11A, a protein associated with monocytes, and ZNF836, a transcription factor not studied in any tissue type, had common genetic expression between GCLs at each location. The commercial antibodies to these proteins were difficult to optimize as a first step and resulted in negative staining of the TMA and its positive controls. The relative obscurity of these proteins limits commercial availability, and confirmation studies do not exist.

Only TCIRG1 had a statistically significant association with biologic behavior. TCIRG1 staining intensity was greater in nonaggressive MF lesions. Not enough is known about the role of TCIRG1 in these lesions to make conclusions. One would expect MMP-9 and CTSK to have greater expression in aggressive lesions; however, this was not seen in the present study.

A recent study that performed whole genome sequencing of GCLs of the AA skeleton found that 92% (49 of 53) of GCTs of bone harvested histone 3.3 variants exclusively in H3F3A (p.Gly34 Trp or p.Gly34 Leu mutations). They also found consistent mutations in this histone gene at a different location for 95% (73 of 77) of chondroblastomas and to a much lesser extent in osteosarcoma and 2 variants of chondrosarcoma. The mutations were restricted to the stromal cell population and not detected in osteoclasts or their precursors. Gomes et al in a subsequent study did not find identical mutations in the H3F3A gene of 9 GCLs (6 aggressive) of the MF.

**FIGURE 4.** Tissue microarray staining of cathepsin K (×200) for A, maxillofacial and B, axial/appendicular lesions. Staining is intense in both the giant cells (arrows) and mononuclear stromal cells (arrowheads).

skeleton using direct sequencing of an amplified polymerase chain reaction product. This discrepancy supports the theory that GCLs of the AA skeleton are different from MF lesions, but it is possible that other specific histone mutations would be uncovered with whole genome sequencing or perhaps by separating stromal cells from GCs as the first group did. The relationship of mutations in genes coding for histones to the pathogenesis of GCLs is unclear, and these studies did not confirm the significance of the mutations by assessing the corresponding protein expression. It has been thought that dysfunctional H3 histone disrupts epigenetic post-translational modifications in other genes involved in tumorigenesis. Other studies that compared AA and MF GCLs did not compare them according to biologic behavior (ie, aggressive vs nonaggressive). We believe that similarities exist between aggressive GCLs of the MF skeleton and the classic GCTs of the AA skeleton. The nonaggressive variant seems less related to AA GCLs. Our studies have consistently shown that when separating the lesions by behavior, clinically, radiographically, histologically, and now genetically, the lesions exist on a continuous spectrum.

The present study had several limitations. First, because our center is a tertiary referral center, most of the MF GCLs in the present study were of the aggressive subtype. This is in contrast to previous published studies, in which most GCLs would be classified as nonaggressive. Given the relative paucity of nonaggressive MF lesions, conclusions regarding histologic comparisons with the other groups might not be significant because of inadequate power to detect differences.

It is unclear how the ability of proteins in formalin-fixed paraffin-embedded specimens to bind antibody
changes or diminishes over time. Many specimens included in the present study were more than 10 years old. In addition, quantification of immunohistochemistry is often unreliable in decalcified specimens. We minimized these limitations with the use of a TMA.

A TMA has many advantages over standard immunohistochemical staining of individual specimen slides. All included subjects’ lesions were added to a single paraffin block, from which sections provide an annotated cohort of lesions to be stained together under the same conditions. The cores used to create the TMA were taken only from areas identified by the pathologist as being tumor, simplifying scoring.

In conclusion, GCLs of the AA and MF skeletons share expression of MMP-9, CTSK, and TCIRG1. This supports our hypothesis that these tumors represent similar lesions in different locations. TCIRG1 has not been described in GCLs and represents a potential target for molecular diagnosis or therapy.

Acknowledgment

The authors acknowledge Brad Tricomi for his assistance with scoring the TMA and performing the statistical analyses.

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