UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
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Programa de Pós-graduação em Genética

*Erica Babeto*

# Análise da expressão gênica no Tumor ósseo de células gigantes

**Tese apresentada para obtenção do Título de Doutorado em Genética.**

**São José do Rio Preto - SP**

**U N E S P** **UNIVERSIDADE ESTADUAL PAULISTA**
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**INSTITUTO DE BIOCIÊNCIAS, LETRAS E CIÊNCIAS EXATAS**
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ERICA BABETO

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Tese apresentada para obtenção do título de Doutora em Genética junto ao Programa de Pós-Graduação em Genética do Instituto de Biociências, Letras e Ciências exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto

**Orientadora: Profa. Dra. Paula Rahal**

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*"Quando se ama as coisas fazem ainda mais sentido"*
***Paulo Coelho***

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*que sentar-se fazendo nada até o final.*

*Eu prefiro na chuva caminhar,*

*que em dias tristes em casa me esconder.*

*Prefiro ser feliz, embora louco,*

*que em conformidade viver..."*

**Martin Luther King**

## RESUMO – ARTIGO 1

O tumor ósseo de células gigantes (TCG) é um tumor benigno, que causa destruição osteolítica, com comportamento biológico incerto e com características particulares como um elevado número de células gigantes multinucleadas e comportamento agressivo. A recorrência local do TCG é freqüentemente observada em 20 a 50% dos casos. Mais agravante que a recorrência, é o fato de que após a recidiva, o paciente muitas vezes também apresenta metástases em outros órgãos, principalmente no pulmão. Dessa forma**,** o objetivo do trabalho foi á investigação da expressão gênica para identificar genes diferencialmente expressos no TCG, que podem estar envolvidos na biologia molecular e desenvolvimento da doença. A hibridização subtrativa rápida (*RaSH*) foi utilizada para identificar novos genes diferencialmente expressos, como *KTN1, NEB, ROCK1* e *ZAK*, que foram validados por PCR quantitativo em tempo real (qPCR) e a imuno-histoquímica em amostras de TCG comparadas ao tecido ósseo normal. A anotação funcional indica que estes genes estão envolvidos em processos celulares relacionadas ao fenótipo tumoral. A expressão dos genes *KTN1* e *ROCK1* encontra-se aumentada e o gene *ZAK* tive sua expressão reduzida nas amostras de TCG analisadas. Pela presença de ilhas CpG na região promotora e baixa expressão no tecido tumoral, o padrão de metilação do gene *ZAK* foi analisado por MSP-PCR. Os genes identificados *KTN1, ROCK1* e *ZAK* pode ser responsável pelas perda de homeostase celular no TCG uma vez que são responsáveis pela várias funções relacionadas com a tumorigênese, como a migração celular, organização do citoesqueleto, apoptose, controle do ciclo celular e, portanto esses resultados poderão contribuir para a compreensão das bases moleculares do TCG, assim ajudando a melhorar o diagnóstico, prognóstico e tratamento dos pacientes.

**Palavras-chave:** tumor ósseo de células gigantes, *RaSH,* expressão gênica, metilação, PCR quantitativo em tempo real (qRT-PCR) *e* imuno-histoquímica.

## RESUMO – ARTIGO 2

O tumor ósseo de células gigantes (TCG), apesar de ser designado como benigno, pode se tornar muito agressivo e apresentar várias características de malignidade, como a alta taxa mitótica, necrose, invasão vascular e metástases. Este tumor tem características únicas, como alta taxa de células multinucleadas, potencial de crescimento variável e comportamento biológico incerto. Neste estudo, buscou-se identificar genes envolvidos na transformação maligna e no desenvolvimento de metástases em TCG, construindo assim um perfil molecular desse tumor. Foi utilizado PCR quantitativo em tempo real (qPCR), imuno-histoquímica e análise de metilação para selecionar genes que são supostamente associados com a progressão do tumor no TCG. A expressão dos genes *ADAM23* e *CDKN2A* (*p16*) mostrou-se diminuída nas amostras de TCG por meio do qPCR e foi visto uma alta frequência de hipermetilação da região promotora. O silenciamento desses genes *ADAM23* e *CDKN2A* (*p16*) pode contribuir para a progressão tumoral e indução de metástase no TCG, uma vez que estão diretamente relacionados à adesão celular e regulação da proliferação. A expressão dos genes *MMP14* e *VIM* foi significativamente maior nos TCGs, mostrando uma associação estatisticamente significativa com a ocorrência de metástase e, portanto são possíveis candidatos a marcadores de prognóstico para pacientes de TCG. Os genes identificados nesse trabalho possibilitam a compreensão das bases moleculares da GCTB, contribuindo assim para melhorar o diagnóstico e prognóstico do paciente.

**Palavras-chave:** tumor ósseo de células gigantes, expressão gênica, metilação, progressão tumoral e imuno-histoquímica.

## ABSTRACT – ARTICLE 1

Giant cells tumors of bone (GCTB) are benign in nature but cause osteolytic destruction, with a number of particular characteristics. These tumors can have uncertain biological behavior, often contain a significant proportion of highly multinucleated cells, and may show aggressive behavior. We have studied differential gene expression in GCTB that may give a better understanding of their physiopathology, and might be helpful in prognosis and treatment. Subtractive hybridization (*RaSH*) was used to identify and measure novel genes that appear to be differentially expressed, including *KTN1, NEB, ROCK1* and *ZAK*, using qRT-PCR and immunohistochemical in the samples of GCTBs compared to normal bone tissue. Normal bone was used in the methodology *RaSH* for comparison with the GCTB in identification of differentially expressed genes. Functional annotation indicated that these genes are involved in cellular processes related to their tumor phenotype. The differential expression of *KTN1*, *ROCK1* and *ZAK* was independently confirmed by qRT-PCR and immunohistochemical. The expression of the *KTN1* and *ROCK1* genes were increased in samples by qRT-PCR and immunohistochemical and *ZAK* had reduced expression. Since *ZAK* have CpG islands in their promoter region and low expression in tumor tissue, their methylation pattern was analyzed by MSP-PCR. The genes identified, *KTN1, ROCK1* and *ZAK* may be responsible for loss of cellular homeostasis in GCTB since they are responsible for various functions related to tumorigenesis such as cell migration, cytoskeletal organization, apoptosis, cell cycle control and thus may contribute at some stage in the process of formation and development of GCTB.

**Keywords:** Giant cell tumor of bone, *RaSH,* gene expression, methylation, Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) and immunohistochemical.

## ABSTRACT – ARTICLE 2

Giant cell tumors of bone (GCTB), despite being designated as benign, can become very aggressive and show several characteristics of malignancy, such as high mitotic rate, necrosis, vascular invasion and metastasis. This tumor has unique histological characteristics, a high rate of multinucleated cell, a variable and unpredictable growth potential, and uncertain biological behavior. In this study, we sought to identify genes involved in malignant transformation and the development of metastasis in GCTB, thus building a molecular profile of tumor. We have combined quantitative real-time polymerase chain reaction (qPCR), immunohistochemistry and analysis of methylation to select genes that are putatively associated with tumor progression in GCTB. The expression of the *ADAM23* and *CDKN2A* genes were decreased in GCTB samples compared to normal bone tissue by qPCR, and a high hypermethylation frequency of the promoter region of *ADAM23* and *CDKN2A* in GCTB was observed. The silencing of genes *ADAM23* and *CDKN2A* may contribute to tumor progression and induction of metastasis in GCTB, since they are directly related to cell adhesion and regulation of proliferation. The expression of genes *MMP14* and *VIM* were significantly higher in CGTBs than in normal bone tissue, confirmed by qPCR and immunohistochemistry, and showed significant statistical association between occurrences of metastasis, supporting the potential use of these genes *MMP14* and *VIM* as a prognostic biomarker for GCTB patients. The set of genes identified here furthers our understanding of the molecular basis of GCTB, thereby helping to improve diagnosis and patient outcome.

**Keywords:** Giant cell tumor of bone, gene expression, methylation, metastasis, tumor progression and immunohistochemistry.

# SUMÁRIO

# *Introdução*

O tumor ósseo de células gigantes (TCG), também conhecido como osteoclastoma (WULLING, DELLING, 2003; MATSUBAYASHI et al., 2009), é um neoplasma osteolítico destrutivo que representa 5 % de todos os tumores ósseos primários e é o 6° tumor ósseo primário mais comum (CAUDELL et al., 2003; KARPIK, 2010; KLENKE et al., 2010). Esse tumor é de linhagem osteoclástica com características histológicas particulares apresentando um elevado número de células gigantes multinucleadas (BALKE et al., 2010). Os TCGs acometem predominantemente jovens adultos entre 20 a 40 anos, de todas as raças (JAMES, DAVIES, 2005; NG et al., 2010) e com uma leve predominância do sexo feminino (TURCOTTE, 2006; KARPIK, 2010).

Clinicamente seu comportamento é agressivo, com crescimento rápido, às vezes em semanas, apesar de oligossintomático, ocasionando afinamento e ruptura da cortical óssea, com invasão das partes moles adjacentes, entretanto, sem invadir e ulcerar a pele e o tecido celular subcutâneo (LEE et al., 2008). TCG normalmente apresenta dor localizada, afetando principalmente as extremidades de ossos longos como fêmur distal, tíbia proximal, rádio distal, úmero proximal e o sacro. Já os sítios mais incomuns são os ossos da mão e do pé, vértebras, costelas, escápula, crânio e trocânter. (CAUDELL et al, 2003; BASSIONY et al., 2009).

A histologia desse tumor é caracterizada pela presença de três tipos distintos de células (Figura 1); as células gigantes multinucleadas, as células estromais e os monócitos semelhantes aos macrófagos (MORGAN et al., 2005; MATSUMOTO et al., 2010). Não há consenso entre a maioria dos autores com relação à verdadeira histogênese dessas lesões, mas as evidências favorecem uma origem mesenquimal e que as células mononucleares seriam progenitoras das células gigantes que designam o tumor (GOLDRING et al., 1987; MATSUBAYASHI et al., 2009). Acredita-se que as células estromais além de darem o suporte para o recrutamento e a formação dos osteoclastos maduros também representam o componente neoplásico do tumor. A atividade de reabsorção óssea dos osteoclastos é a responsável pela osteólise e consequentemente, elevada morbidade observada nos TCGs. (MORGAN et al., 2005; MATSUMOTO et al., 2010). Acredita-se que as células estromais induzem os monócitos a sofrerem fusão para formação das células gigantes multinucleadas, assim causando uma agressiva reabsorção óssea e destruição do esqueleto (LAU et al., 2011). Dessa forma, as moléculas que mediam essa interação podem representar importantes alvos terapêuticos.

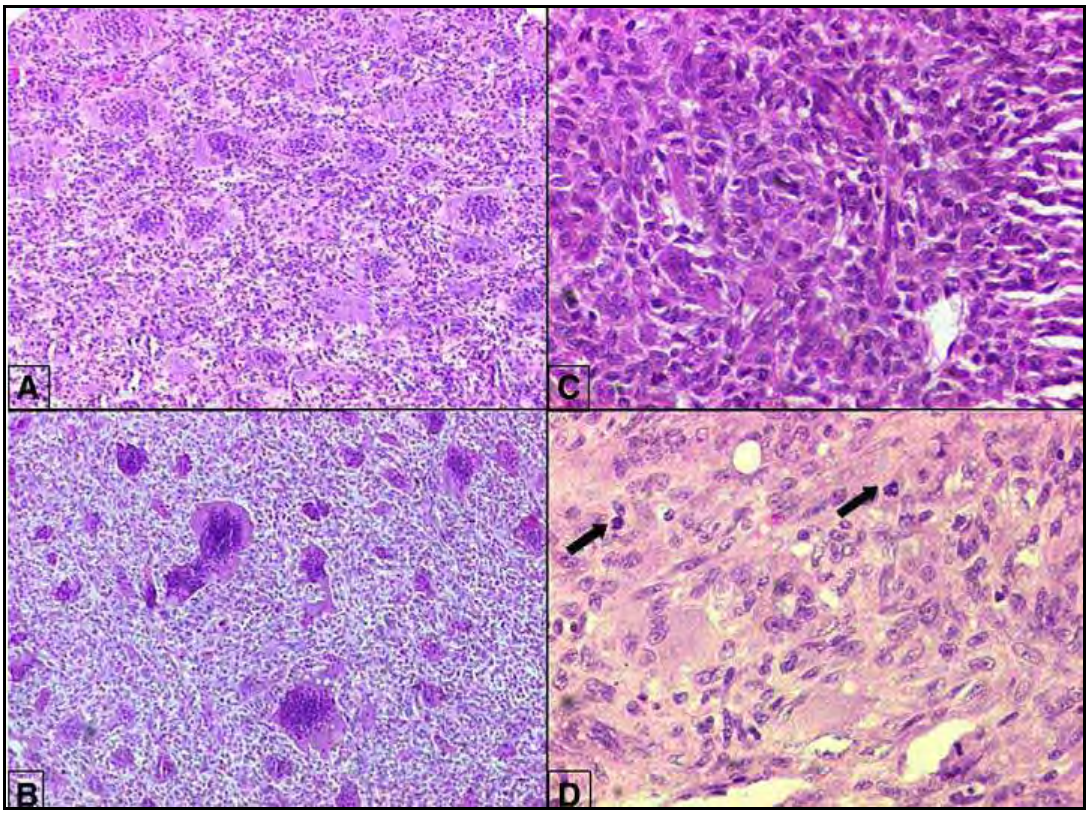


**Figura 1.** Fotomicrografias mostrando as características histológicas de um TCG. Em (A) as células gigantes multinucleadas são distribuídas entre células estromais formando um sincício (100X). Em (B) observe algumas das células gigantes têm um abundante citoplasma eosinofílico homogêneo e muitos núcleos ovais com a membrana nuclear bem definida localizados preferencialmente na região central da célula. As células do estroma aparecem na periferia das células gigantes (40X). (C) As células do estroma têm uma quantidade moderada de citoplasma eosinofílico (200X). (D) Figuras de mitoses (setas) foram observadas nas células estromais e eram escassas e típica (200X). (Modificado de GUPTA et al., 2008)

A Organização Mundial de Saúde classifica o TCG como uma lesão potencialmente maligna agressiva (BASSIONY et al., 2009). Clinicamente, TCG apresenta-se como uma lesão benigna, mas muitas vezes agressiva, com tendência à recorrência local. Dependendo do tipo de tratamento e do local do tumor, as taxas de recorrência variam de 0% a 65% (KLENKE et al., 2010). Alguns autores sugerem que o tumor de células gigantes recorrente pode predizer um risco maior de transformação maligna (FAISHAM et al., 2006; HORVAI et al., 2008). A transformação maligna espontânea do TCG é frequentemente observada, ocorrendo com o aparecimento de um sarcoma, osteosarcoma, fibrosarcoma, histiosarcoma maligno ou fibrohistiocitoma maligno ósseo associado a células gigantes pré-existentes (MARUI et al., 2001; BERTONI, BACCHINI, STAALS, 2003; HASHIMOTO et al., 2006).

Esporadicamente, mesmo um TCG com características totalmente benignas pode evoluir com metástases locais, metástases pulmonares, nos gânglios linfáticos e mais raramente ocorrer à transformação maligna (MAK et al., 2009; KARPIK, 2010). O TCG é um daqueles tumores que podem crescer por via intravenosa e dar origem a metástases à distância, com crescimento lento. (FORSYTH et al., 2008; ALBERGHINI et al., 2010). Metástase pulmonar ocorre variavelmente em 1-9% dos casos (VISWANATHAN, JAMBHEKAR, 2009).

O tratamento do TCG é essencialmente cirúrgico e inclui curetagem intra-lesional (com ou sem adjuvantes) e excisão de área afetada, porém a excisão cirúrgica e a reconstrução do membro afetado é bem difícil, pois o tumor envolve geralmente a epífise óssea de pacientes relativamente jovens, limitando assim as opções cirúrgicas (BALKE et al., 2010). Assim, a ressecção ampla é reservada para um pequeno subgrupo de pacientes com tumores biologicamente mais agressivos, recorrentes e extensas (BASSIONY et al., 2009). Historicamente, a radioterapia (RT) tem sido evitada, pois alguns relatos têm sugerido que isso pode resultar em um risco aumentado de transformação sarcomatosa (CAUDELL et al., 2003)

Embora seja clara a importância e a necessidade de investigar os padrões moleculares dos TCGs para descobrir genes que possam estar envolvidos na progressão tumoral, identificando dessa forma, marcadores potenciais para terapias futuras. Relativamente poucos estudos têm sido dirigidos à avaliação dos padrões genéticos dos TCGs. Estudos citogenéticos demonstraram que associações teloméricas e outras anormalidades cromossômicas ocorrem em 50% das células

tumorais na maioria dos pacientes (SCHWARTZ, ESKEW, BUTLER, 2002; FORSYTH et al., 2008).

Alguns trabalhos deram grande ênfase ao estudo de genes relacionados à regulação do ciclo celular. (MATSUBAYASHI et al., 2009). Dentre eles, os mais estudados são o *P53*, o *c-myc* e o *mdm2*, além da pesquisa da proteína ki-67. Kandel e colaboradores em 2005 mostraram que a proteína p21 contribui para o acúmulo da ciclina D1 nas células gigantes, levando a formação dessas células (KANDEL et al., 2005).

Utilizando a metodologia de cDNA *arrays*, Morgan e colaboradores (2005), compararam os padrões de expressão gênica de osteoblastos e osteoclastos com as assinaturas genéticas dos TCGs. Os autores observaram alterações significativas nos padrões de expressão gênica e protéica do receptor no fator nuclear kB (*RANKL*) e concluíram que esse gene atua na formação de osteoclastos contribuindo para a formação do componente neoplásico dos TCGs (MORGAN et al., 2005). Sendo assim, autores sugerem que, o desenvolvimento de novos inibidores da sinalização RANKL pode ser considerado como potenciais agentes terapêuticos para essa doença. Skubitz, et al. (2004) também relatam diferenças na expressão gênica entre TCGs e tecidos ósseos normais obtidos por *arrays*. Os genes *TRAP*, *Lisosomal* $H^+$*-Transporting ATPase*, *MMP3, CSF1, CCR1, OPGL* e *HGK* foram os que apresentaram as maiores diferenças na expressão quando comparados com os padrões observados nos tecidos controles. Os pesquisadores atribuem a superexpressão desses genes à presença de osteoclastos, seus precursores ou ambos no tecido tumoral e correlacionam esse fato com o desenvolvimento da patogênese dos TCGs (SKUBITZ, et al., 2004).

Em outro trabalho Smith e colaboradores (2006) demonstraram por hibridização genômica comparativa (CGH) a amplificação da região 20q 11.1 em 56 casos de TCG. Nessa região amplificada encontra-se o gene *TPX2* cuja expressão alterada também foi detectada por Western Blot. Esse gene é responsável pela codificação de uma proteína essencial na organização dos microtúbulos durante a mitose contribuindo dessa forma para a proliferação celular. Baseando-se nos achados obtidos sobre a expressão gênica em outros tipos de tumores, os autores propõem o TPX2 como candidato a oncogene sendo que a sua expressão pode ser usada como um importante marcador no prognóstico dos TCGs (SMITH, et al., 2006).

O tumor de células gigantes constitui-se assim, pelo exposto, em um tema importante tanto pelos seus desafios, quanto ao seu diagnóstico, que deve ser precoce e correto. A conduta cirúrgica deve ser muita bem planejada e individualizada, devido ao caráter imprevisível do tumor quanto ao seu prognóstico local. Certamente os estudos moleculares abrirão novos caminhos e tornarão o TCG mais previsível quanto ao seu comportamento biológico em um futuro próximo.

# *Objetivos*

Tendo em vista o exposto, o presente trabalho teve como objetivo geral de identificar genes diferencialmente expressos no Tumor ósseo de células gigantes, que possam ser empregados como biomarcadores na patogênese e progressão tumoral, procurando atender os seguintes objetivos específicos:

**Artigo 1**

1. Selecionar genes diferencialmente expressos no tumor ósseo de células gigantes pela metodologia de *RaSH* ;

2. Validar a expressão diferencial dos genes candidatos, por meio da PCR em tempo real (qPCR) e por imuno-histoquímica;

3. Analisar o padrão de metilação na região promotora dos genes que apresentaram baixa expressão gênica no tumor, por meio da técnica de MSP-PCR.

**Artigo 2**

1. Selecionar genes envolvidos na transformação maligna, responsáveis pela formação e desenvolvimento de metástases no tumor ósseo de células gigantes;

2. Avaliar a expressão gênica dos genes selecionados por PCR em tempo real, em amostras de tumor ósseo de células gigantes;

3. Analisar o padrão de metilação na região promotora dos genes que apresentaram baixa expressão gênica, por meio da técnica de MSP-PCR;

4. Correlacionar a hipermetilação de ilhas CpGs com os dados clínicos;

5. Correlacionar os dados de expressão gênica com a presença de metástase.

# *Artigo 1*

Virchows Arch
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ORIGINAL ARTICLE

# Differentially expressed genes in giant cell tumor of bone

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**Abstract** Giant cells tumors of bone (GCTB) are benign in nature but cause osteolytic destruction with a number of particular characteristics. These tumors can have uncertain biological behavior often contain a significant proportion of highly multinucleated cells, and may show aggressive behavior. We have studied differential gene expression in GCTB that may give a better understanding of their physiopathology, and might be helpful in prognosis and treatment. Rapid subtractive hybridization (*RaSH*) was used to identify and measure novel genes that appear to be differentially expressed, including *KTN1*, *NEB*, *ROCK1*, and *ZAK* using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry in the samples of GCTBs compared to normal bone tissue. Normal bone was used in the methodology RaSH for comparison with the GCTB in identification of differentially expressed genes. Functional annotation indicated that these genes are involved in cellular processes related to their tumor phenotype. The differential expression of *KTN1*, *ROCK1*, and *ZAK* was independently confirmed by qRT-PCR and immunohistochemistry. The expression of the *KTN1* and *ROCK1* genes were increased in samples by qRT-PCR and immunohistochemistry, and *ZAK* had reduced expression. Since *ZAK* have CpG islands in their promoter region and low expression in tumor tissue, their methylation pattern was analyzed by MSP-PCR. The genes identified *KTN1*, *ROCK1*, and *ZAK* may be responsible for loss of cellular homeostasis in GCTB since they are responsible for various functions related to tumorigenesis such as cell migration, cytoskeletal organization, apoptosis, and cell cycle control and thus may contribute at some stage in the process of formation and development of GCTB.

**Keywords** Giant cell tumor of bone · RaSH · Gene expression · Methylation · Quantitative real-time polymerase chain reaction (qRT-PCR) · Immunohistochemical

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## Introduction

Giant cell tumor of bone (GCTB) is a rare benign lesion that comprises ~5% of primary bone tumors [1, 2]. They can be locally aggressive and destructive with rapid growth

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leading to thinning and rupture of the cortical bone with invasion of adjacent soft tissues, but without invading and ulcerating the skin and subcutaneous tissue [3, 4]. Generally, GCTBs most frequently occur in young adults between 20 and 40 years of age, with a slight predominance in females. They affect mainly the ends of long bones, and frequently occur in the distal end of the femur and the proximal end of the tibia [5–8].

GCTB is characterized by the presence of numerous osteoclast-like giant cells, and a mononuclear component that includes proliferating mononuclear stromal cells and infiltrating macrophages [9–11]. The mononuclear stromal cell represents the neoplastic component of the tumor, whereas monocytes represent a minor component of the mononuclear cell population [10, 12]. There is consensus among most authors regarding the true histogenesis of these lesions, with the evidence favoring a mesenchymal and mononuclear cells as stem cells of GCTB [13].

Despite its benign histological appearance, GCTB has an unpredictable clinical course [14]. The rate of local recurrence following surgical curettage is relatively high at 18–60%, and some occasionally undergo malignant transformation [15]. Metastasis occurs most commonly to the lung, variously reported in 1–9% of cases [16]. The outcome cannot be predicted on the basis of histological or radiographical criteria. In the absence of a clear histogenetic origin, GCTB is currently classified among lesions with an uncertain derivation and named after its peculiar morphologic appearance [14].

A better understanding of the mechanisms underlying the complex physiopathology of GCTBs, including a characterization of the gene expression profile and the functions of the genes involved in this phenomenon, and the molecular environment that might typify the tumor. We have investigated the molecular biology characteristics of GCTB to determine whether a more directed targeted clinical intervention can be adopted in the future.

The rapid subtraction hybridization approach (*RaSH*) [17] has been used to identify genes involved in tumorigenesis and the development of GCTBs. Subtraction hybridization methods are designed to identify the expression of complementary DNAs (cDNAs) in one of the two analyzed groups, thereby detecting differentially expressed messenger RNAs (mRNAs).

Tumorigenesis is a process of accumulation of genetic and epigenetic abnormalities that lead to cellular dysfunction and malignant transformation. Epigenetic mechanisms involving DNA methylation, histone modifications, and non-coding RNAs regulate and maintain gene expression states. Similar to genetic mutations, alterations in epigenetic regulation can lead to uncontrolled cell division, tumor initiation and growth, and invasiveness and metastasis [18].

The role of DNA methylation in cancer has received much attention; it is accepted that the methylation of the promoter region of many genes is associated with gene silencing [19]. The DNA methylation is the addition of a methyl group for the carbon atom 5′cytosine, present in the dinucleotide CpG, resulting in the formation of a 5-methylcytosine [20]. The methyl groups found mainly on the islands of CpGs promoter regions of genes can influence the changes between the molecule of DNA and proteins, reducing the binding affinity between the promoter regions and transcription factors through mechanisms involving changes in chromatin structure or levels of histone acetylation [21].

Thus, our aim has been to identify and characterize differentially expressed genes in GCTBs compared to normal bone tissue that may be involved in the molecular biology and development of the disease, generating insight for the new prognosis, and treatment and understanding of this tumor.

## Materials and methods

### Patients and tissues

GCTB tissue samples were obtained from the tumor bank at The Pio XII Foundation/IBILCE-UNESP, São Paulo, Brazil. The use of all patient-derived material was approved by institution's research ethics board, The Pio XII Foundation—Cancer Hospital Barretos, and informed consent was obtained individually from the patients. The diagnosis of GCTB was established by biopsy prior to surgical excision by pathologists. Tissues were obtained at surgery from patients undergoing tumor resection. Microdissection was performed to collect samples.

Fifty-six samples were collected from primary GCTB, 23 fresh frozen samples of GCBT, 24 paraffin-embedded samples of GCTB, and 9 fresh histologically normal medullar bone tissue evaluated by a pathologist for confirmation of any morphological alteration. (Table 1 gives the clinicopathologic features of these tumors).

The study population included 43 patients. Twenty-one (48%) were male and 22 (52%) female, and the minimum and maximum ages were 13 and 74 years, respectively, mean 35.6 years±14.9. Main localizations of the GCTBs were femur (26%), tibia (21%), and radius (14%). Eight patients (17%) had recurrence between 11 and 42 months after surgery and six patients (12, 7%) had metastases between 1 and 65 months after surgery.

### RNA extraction and RT-PCR

Total RNA was isolated from GCTB tissue and normal tissue using the TRIzol reagent (solution for extraction of RNA, Life Technologies, Grand Island, NY) according to the

**Table 1** Epidemiological, clinical, and pathological characteristics of 23 fresh samples of GCTB and 24 paraffin-embedded samples of GCTB

| Sample | Age (years) | Sex | Anatomic location | Recurrence (date) | Metastasis (date) |
|---|---|---|---|---|---|
| 1F | 37 | M | Proximal tibia L | No | 19 months |
| 2F | 41 | F | Distal radius R | No | No |
| 3F | 24 | M | Proximal femur L | No | No |
| 4F/P | 52 | F | Distal femur L | No | No |
| 5F | 17 | M | Scapula L | No | No |
| 6F | 32 | F | Olecranon R | 11 months | No |
| 7F/P | 52 | M | Distal femur L | 11 months | No |
| 8F/P | 74 | F | Distal femur L | 19 months | No |
| 9F | 35 | M | Distal femur L | No | No |
| 10F | 33 | M | Proximal tibia R | 20 months | No |
| 11F | 21 | F | Proximal tibia R | No | No |
| 12F | 37 | F | Proximal tibia L | No | No |
| 13F | 13 | F | [a] | No | 41 months |
| 14F/P | 28 | F | Distal femur R | 19 months | No |
| 15F | 19 | F | Sacrum | No | No |
| 16F | 22 | F | Distal fibula R | No | No |
| 17F | 16 | F | Ischium R | No | No |
| 18F | 22 | M | Distal fibula L | No | 20 months |
| 19F | 27 | M | Proximal humerus L | No | No |
| 20F | 69 | F | Occipital | No | No |
| 21F | 24 | M | Proximal tibia R | 22 months | No |
| 22F | 46 | F | Distal radius L | No | No |
| 23F | 58 | F | Hemipelvis R | No | No |
| 24P | 55 | M | Proximal tíbia L | No | No |
| 25P | 22 | F | Ischium R | No | 65 months |
| 26P | 57 | M | Proximal tíbia L | 42 months | No |
| 27P | 34 | M | Proximal tíbia R | No | No |
| 28P | 24 | M | Distal radius L | No | No |
| 29P | 27 | M | Distal femur L | No | No |
| 30P | 51 | F | Scapula L | Yes[a] | 07 months |
| 31P | 31 | M | Wrist R | No | No |
| 32P | 22 | F | Distal radius R | No | No |
| 33P | 41 | M | Distal femur L | No | 01 months |
| 34P | 24 | F | Wrist L | No | No |
| 35P | 41 | M | Distal femur R | No | No |
| 36P | 55 | M | Distal radius L | No | No |
| 37P | 41 | F | Distal radius L | No | No |
| 38P | 35 | M | Thumb L | No | No |
| 39P | 39 | M | Proximal fíbula L | No | No |
| 40P | 53 | M | Proximal tibia R | No | No |
| 41P | 38 | F | Distal femur L | No | No |
| 42P | 19 | F | Distal fêmur L | No | No |
| 43P | 23 | F | Forefinger R | No | No |

*F* fresh tumor, *P* paraffin-embedded tumor, *F* female, *M* male, *R* right, *L* left,

[a] No information was obtained

manufacturer's instructions. RNA integrity post-purification was checked using the Agilent 2100-Bioanalyser, giving a minimal RIN value of 5.5.

For quantitative real-time PCR (qRT-PCR), ~5 μg of total RNA from each sample were used to synthesize cDNA with a high-capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. The quality of the cDNA was checked by PCR of the housekeeping gene, *β-ACTIN*. The primer sets were 5′ to 3′ GGCATCGTGATG GACTCCG and GCTGGAAGGTGGACAGCG. The PCR

products were analyzed by electrophoresis on 1% agarose gel and stained with ethidium bromide.

### Rapid subtractive hybridization

For this methodology, paired samples of the same patient (2F) were used, a sample of GCTB and a sample of histologically normal bone tissue. RaSH cDNA libraries were performed as described previously [17] with modifications. From the 25 μg total RNA pool, cDNAs were synthesized and digested with MboI (Invitrogen Life Technologies) at 37°C for 1 h and extracted with phenol–chloroform followed by ethanol precipitation. The digested cDNAs were mixed with 20 mmol/L of primers XDPN-14 (50CTGATCACTCGAGA30) and XDPN-12 (50GATCTCTCGAGT30) in 30 μL of 1X T4 DNA Ligase Buffer (Invitrogen Life Technologies), heated at 55°C for 1 min, and cooled to 14°C within 1 h. Ligation was carried out overnight at 14°C After adding 9 units of T4 DNA ligase to the mixtures individually.

The mixtures were diluted to 100 μL and >40 mL of the mixtures were used for PCR amplification with primer XDPN-18 (5′CTGATCACTCGAGAGATC′3). Portions (10 μg) of the tester PCR products (CGT or normal tissue) were digested with 20 units of XhoI and purified with phenol–chloroform extraction and ethanol precipitation. The fragments were inserted into XhoI-digested pZERO plasmid (1 μg/μl) at 16°C for 3 h. The constructs were introduced into the TOP10 competent cells. Two RaSH cDNA libraries were prepared, one using cDNA from the CGT as tester with the normal bone as driver, and the other using cDNA from the normal bone as tester with cDNA from the CGT as driver.

All bacterial colonies were analyzed by PCR with use of the M13 forward and M13 reverse primers to verify those with an insert. The sequences of these clones were determined with DNA sequencer (ABI PRISM 377, Applied Biosystems) and DYEnamic ET dye terminator sequencing kit (Amersham Biosciences). The sequences were analyzed using an annotation pipeline that had 4 steps: (1) quality checking, phred base-calling, cutoff 0.05; (2) vector trimming and removal of undesirable sequences, such as bacterial, mitochondrial, and rRNA sequences; (3) masking of repetitive elements and screening of low-complexity regions by Repeat Masker, using the default settings; and (4) annotation against existing databases, using BLASTN with default parameters. Significant hits were determined by using an *E* value threshold of 10–15 for searches against nucleotide sequence databases [22].

### Validation by quantitative real time

The qRT-PCR was used to assess the expression of genes found by the RaSH method in all fresh samples of CGTB individually. For analysis, we used 23 fresh samples of CGTBs and a pool of total RNA from a subset of nine fresh tissue of normal bone, defined as normal reference. Gene-specific primers for qRT-PCR were designed for optimal hybridization kinetics, using the Primer 3.0 program (provided by the Whitehead/MIT Center for Genome Research, Cambridge, MA).

Quantitative real-time PCR was performed using an ABI prism 7300 sequencer detector system and Sybr Green PCR Core Reagent (Applied Biosystems), following the manufacturer's protocol. In brief, the reaction mixture (20 μl total volume) contained 25 ng of cDNA, gene-specific forward and reverse primers for each gene, and 10 μL of 2× quantitative Sybr Green PCR Master Mix. Relative quantification is given by the CT values, determined for triplicate reactions for GCTB samples and reference sample from each gene, and for the endogenous control (glyceraldehyde 3-phosphate dehydrogenase; *GAPDH*). The primer sequences are given in Table 2.

Thus, the relative expression of each specific gene was calculated by using the formula: $R = (E\ \text{target})^{\Delta \text{Ct target}(\text{control}-\text{sample})} / (E\ \text{endogenous})^{\Delta \text{Ct endogenous}(\text{control}-\text{sample})}$, as previously described [23].

### DNA extraction

DNA samples of fresh tissue were isolated using TRIzol Reagent (Life Technologies). For the extraction of DNA from

**Table 2** Primer sets used for validation by qRT-PCR amplification

| Gene | Primers 5′–3′: | Primers concentration (μM) | Base pair |
|---|---|---|---|
| *GAPDH* | 5′ ACCCACTCCTCCACCTTTGA<br>5′CTGTTGCTGTAGCCAAATTCGT | 0.4 μM | 79 pb |
| *KTN1* | 5′GTTTCCCCAGAAACGGAGTC<br>5′TGTGAGCTGTTGGTTTACCG | 0.5 μM | 150 pb |
| *NEB* | 5′GAAGTGGCCAAGAAGCAAAG<br>5′TGTGGCCTTCTTGATGTCTG | 0.5 μM | 102 pb |
| *ROCK1* | 5′CTGGTTTTGTTCGTGCTTCC<br>5′GTAGCATCCCACACGATTCC | 0.5 μM | 142 pb |
| *ZAK* | 5′TATGGAGGCTCCTGTCAAGG<br>5′TCCAACCAAGGACATGTGTG | 0.5 μM | 139 pb |

paraffin, samples were first deparaffinized with xylene, and the tissue samples digested in a buffer (100 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8.0, 25 mmol/L ethylenediamine tetraacetic acid [EDTA], and 1% sodium dodecyl sulfate) containing 20 mg/mL proteinase K at 50°C for 3 days. Total DNA was isolated by phenol–chloroform extraction and ethanol precipitation. The DNA pellets were suspended with 20 mL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and stored at −20°C until PCR amplification.

### Methylation-specific PCR

Genomic DNA from both tumoral and normal tissues was treated with sodium bisulfite to modify unmethylated cytosine to uracil [24]. After the DNA conversion, hypermethylation in CGTs was determined by the MSP method [25], to analyze the methylation pattern of the *ZAK* gene. The primers used for PCR reaction were specific for methylated and unmethylated DNA (Table 3). PCR was individually performed in 25-μL reaction volumes, containing 1 × Platinum Taq buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.4 μmol/L of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen), and 1 μL of treated DNA. In vitro methylated DNA (IVD) was used as a positive control, and DNA from lymphocytes of healthy donors were used as negative controls. PCR products were separated on silver-staining 8% non-denaturing polyacrylamide gels. PCR amplification conditions are available on request.

### Statistical analysis of quantitative real-time PCR data

Statistical analysis was performed using the Minitab Student 14 software, and the significance level was set at $p \leq 0.05$. Relative expression levels detected by qRT-PCR for the four genes in CGTs samples were transformed into natural logarithms. The Wilcoxon Signed Ranks Test was applied to compare the gene expression levels in tumor tissue and normal bone tissue.

### Immunohistochemical staining

Immunohistochemistry was used to assess the expression of proteins found by the RaSH method in paraffin-embedded samples of CGTB individually. For analysis, we used 22 paraffin-embedded samples of CGTBs.

Immunohistochemical staining was performed on 4-μm sections obtained from formalin-fixed, paraffin-embedded blocks. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. Antigen retrieval was carried out in citrate buffer (10 mM, pH 6) for 30 min at 95°C in a Pan Steam. Polyclonal antibodies used were rabbit anti-human KTN1, rabbit anti-human ROCK1, and polyclonal rabbit anti-human ZAK (Sigma, St. Louis, MO, USA) antibodies at 1:100 were applied incubated at 4°C overnight. Afterward, sections were incubated with a biotinylated secondary antibody and then exposed to a streptavidin complex (HRP ready-to-use, DakoCytomation, Carpinteria, CA). Positive reactions were visualized with 3,3′ diaminobenzidine tetrahydrochloride (DAB, Signet® Laboratories, Dedham, MA, USA), followed by counter-staining with hematoxylin.

Normal testicle was used as a positive control for KTN1 and normal liver were used as the positive control to ROCK1 and ZAK. Sections treated without primary antibodies were used as negative controls. Immunoreactivity was evaluated blindly by two observers, who independently assessed the immunostainings with a semi-quantitative grading system (− no staining; + weak staining in 10–30% of cells; ++ moderate staining in 31–65% of cells; +++ strong staining of more than 66% of cells) [15].

## Results

The RaSH approach was adopted to identify differentially expressed genes in GCTBs compared with the normal bone tissue. A total of 619 cDNA clones were sequenced, 169 clones were obtained from the reverse library (down-regulated genes) and 450 clones were obtained from the upregulated genes library. To analyze the sequences, phred and phrap software were used to select the best quality sequences according to the following pattern: phred cutoff of 0.09, minmatch 10, and minscore 20; sequences with >100 base pairs of quality were accepted. The best quality sequences were submitted to the BLAST alignment

**Table 3** Primer set used for methylation-specific polymerase chain reaction

| Gene | Primer sequence | | T (°C) | Size of product M |
|---|---|---|---|---|
| | Methylated sequence (5′-3′) | Unmethylated sequence (5′-3′) | | |
| *ZAK* Primer 1 | F: GGGTCGTTGTCGTTTTAATTTTCGTC<br>R: CGAAACGTAAACAACAACCGAAAAACG | F: GTTGTGTGGGTTGTTGTTGTTTTAATTTTTGTTG<br>R: TCCCAAACAAAACATAAACAACAACCAAAAACA | 67 | 76 pb |
| *ZAK* Primer 2 | F: GAGGGCGGTTTAGTCGTTTC<br>R: TCCGCCTCTCTACACGACG | F: GGGAGGGTGGTTTAGTTGTTTTG<br>R: TTAAAAATTAAACATTCCACCTCTCTACACAACA | 64 | 86 pb |

*T* annealing temperature (°C), *U* unmethylated sequence, *M* methylated sequence

program [26]. After alignment with the RefSeq database, the sequences that presented >90% of the target sequence length at alignment were selected. These included *KTN1*, *NEB*, *ROCK1*, and *ZAK*.

We compared the relative expression levels of four genes by qRT-PCR, using triple determination and normalization based on the GAPDH level. In the validation of the target genes, GCTBs samples were used and normal bone tissue as reference (group control).

*KTN1* and *ROCK1* were significantly upregulated in 100% tumor tissues compared to normal bone tissue ($p<0.000$). *ZAK* was significantly downregulated in 100% tumor tissues compared to normal bone tissue ($p<0.001$). *NEB* gene was not differentially expressed in GCTBs compared with normal bone tissue ($p=0.964$). Relative gene expression data are presented in Fig. 1.

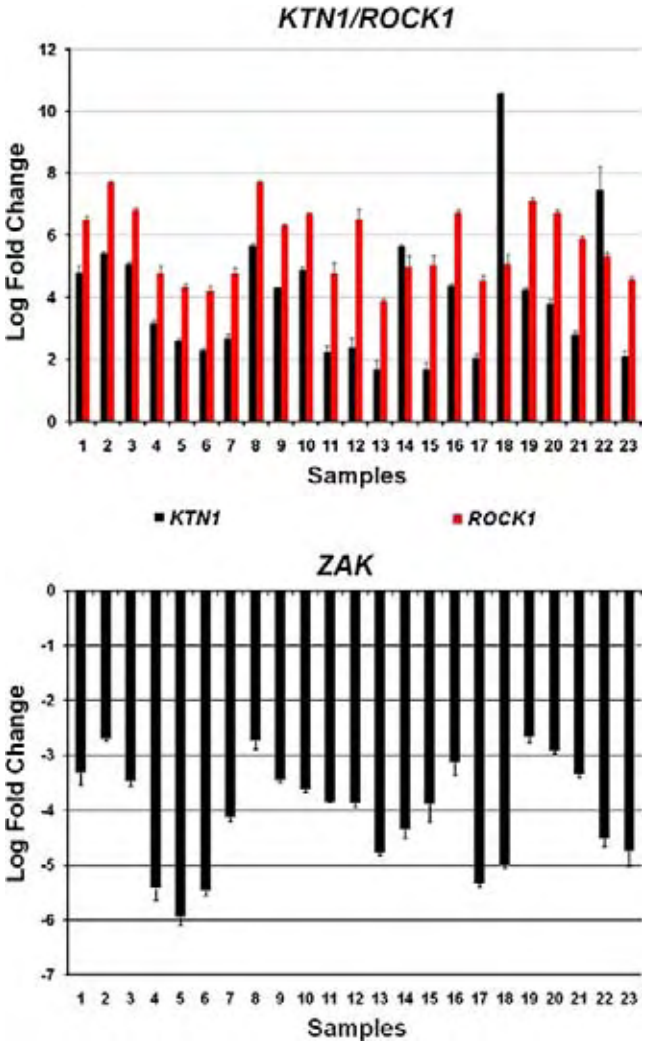


**Fig. 1** Relative expression values of the selected genes for validation by qRT-PCR. Expression of gene *ZAK* was significantly lower in GCTB compared to normal reference (normal tissue bone). *ROCK1 and KTN1* are overexpressed in GCTB. The samples used correspond to the samples 1F–23F Table 1

The methylation pattern of the promoter region of *ZAK* by MSP-PCR was investigated after gene expression analysis. Two pairs of primers for the gene *ZAK* were designed. *ZAK* hypermethylation was detected in 18.2% (4/22) ($p=0.82$) of the samples with the P1 primers and none ($p=1.000$) of the samples when the P2 primer was used (Fig. 2). The sets of primers for the gene *ZAK* did not indicate hypermethylation in the lymphocytes analyzed. The methylation pattern data are presented in Table 4.

We used immunohistochemistry to evaluate protein expression of genes found by RaSH in different cell types due to the heterogeneity of histological CGTBs. Immunohistochemical staining of GCTB samples confirmed the presence of KTN1 and ROCK1 in the GCTB microenvironment. KTN1 immunostaining revealed cellular strong staining in 90% (20/22) of GCTB tissue samples, localized mainly in the multinucleated giant cells and stromal cells and occasionally monocytic cell (Fig. 3a). ROCK1 staining was strongly positive in 59% (13/22) of the samples in the cytoplasm of multinucleated giant cells, stromal cells, and monocytic cells (Fig. 3b). In contrast, the expression of ZAK was weakly localized mainly in 82% (18/22) of the samples in the cytoplasm of the multinucleated giant cells and stromal cells, and occasionally in some of the monocytic cells (Fig. 3b). In normal bone tissue, there

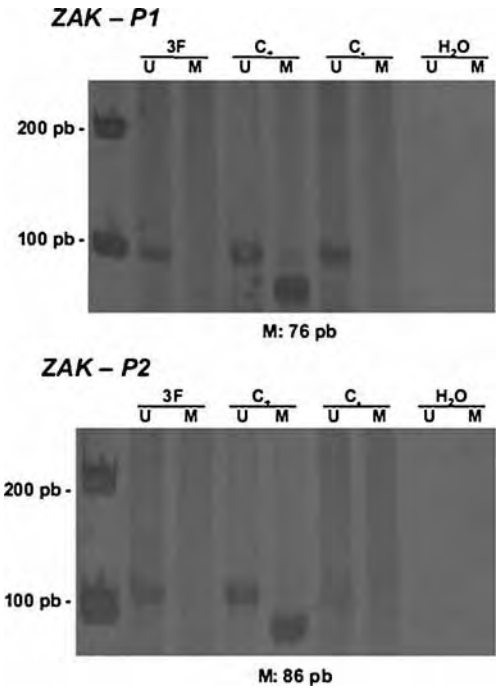


**Fig. 2** Representative examples of MSP reaction for gene *ZAK* P1 and *ZAK* P2. Tumor (3F) of a GCTB patient. *Lanes U* and *M* correspond to unmethylated and methylated reactions, respectively. In each case, *C−* indicates DNA from lymphocyte, *C+* indicates IVD, $H_2O$ indicates negative PCR control. On the *left*: molecular weight marker; and *below*: the size of methylated PCR product

**Table 4** Methylation pattern of *ZAK* in 27 tumor samples of GCTB

| Sample | *ZAK* (P1) | *ZAK* (P2) |
|---|---|---|
| 3F | ○ | ○ |
| 4F | X | ○ |
| 7F | ● | ○ |
| 8F | ○ | ○ |
| 9F | ● | ○ |
| 10F | ○ | ○ |
| 11F | ○ | ○ |
| 12F | ○ | ○ |
| 13F | ○ | ○ |
| 14F | ○ | ○ |
| 15F | ● | X |
| 18F | ○ | ○ |
| 19F | ○ | ○ |
| 23F | ○ | ○ |
| 24P | X | X |
| 25P | ○ | ○ |
| 28P | X | X |
| 29P | ○ | ○ |
| 30P | ○ | ○ |
| 35P | X | X |
| 37P | ○ | ○ |
| 38P | X | X |
| 39P | ○ | ○ |
| 40P | ○ | ○ |
| 41P | ○ | ○ |
| 42P | ● | ○ |
| 43P | ○ | ○ |

*Solid circles* methylated genes, *hollow circles* unmethylated genes, *x* not amplified

was no immunohistochemical staining in different cell types for the three genes analyzed.

The results showed that genes *ROCK1* and *KTN1* were upregulated in GCTB, confirmed by qRT-PCR and immunohistochemistry, *ZAK* was significantly lower, by qRT-PCR and immunohistochemistry, in the GCTB samples, and that the *NEB* gene was not differentially expressed by qRT-PCR.

## Discussion

GCTB does not fit well into a strictly defined category. Therefore, it has been approached in the context of its morphological definition of reactive rounded mononuclear cells, reactive osteoclastic giant cells, and neoplastic mononuclear spindle-shaped cells. It is clinically defined as a benign bone tumor, but it has a tendency to recur and can behave aggressively in a local manner, occasionally metastasizing to the lungs. Although both morphologic and radiologic examination can lead to the correct diagnosis in most cases, diagnosis of GCTB can often be difficult, with no clear diagnostic marker currently being available.

RaSH in allowing us to analyze genes that are expressed differentially in two samples, including genes that have not previously been characterized and rare transcripts, led to the identification of specific genes and signaling pathways involved in the regulation of the disease process. This method has simpler hybridization and subtraction steps than other subtractive hybridization methods.

Three potential biomarkers of giant cell tumor of bone were identified, validated by qRT-PCR and immunohistochemistry, and proved to have distinct differences in expression between tumor and non-neoplastic samples. The markers were genes *KTN1, ROCK1*, and *ZAK,* the first two being significantly upregulated and the last downregulated. The expression of genes *ROCK1* and *KTN1* were significantly high in GCTBs than in normal bone tissue and *ZAK* was significantly lower in tumor tissues than in the non-tumor adjacent tissue.

*ROCK1* located in 18q11.1, encodes a protein associated with Rho kinase and belongs to a family of serine/threonine kinases activated via interaction with Rho GTPases. *ROCK* is involved in a wide range of fundamental cellular functions such as regulation the reorganization of the actin cytoskeleton, formation of the focal adhesion, smooth muscle contraction, cell migration, gene expression, and apoptosis [27, 28]. Rho-associated serine-threonine protein kinase, *ROCK*, one of the best characterized downstream effectors of Rho, is activated when it selectively binds to the active GTP-bound form of Rho. Activated *ROCK* interacts with the actin cytoskeleton to promote stress fiber formation and assembly of focal contacts [29]. There is considerable evidence to suggest that Rho–ROCK signalling is deregulated in cancer, and thereby contributes to invasive and metastatic behavior [30].

Upregulation of Rho–ROCK signaling in tumors is well-known to be linked to increased invasion and metastatic potential, which may occur as a result of increased *RhoA, RhoC*, or *ROCK1* expression [30]. Cell migration is pivotal in metastasis, and rearrangements in the actin cytoskeleton are certainly involved. Overexpression of Rho has been linked to progression of human cancers, and therefore the Rho/ROCK pathway is a major player in cancer progression through its regulation of actin cytoskeleton reorganization; indeed, a specific ROCK inhibitor can suppress the tumor growth and metastasis [29], and therefore could be a prime molecular target for the prevention of cancer invasion and metastasis [30].

Rho kinase (ROCK) through its regulating of cytoskeletal events increases with the degree of malignancy, and is

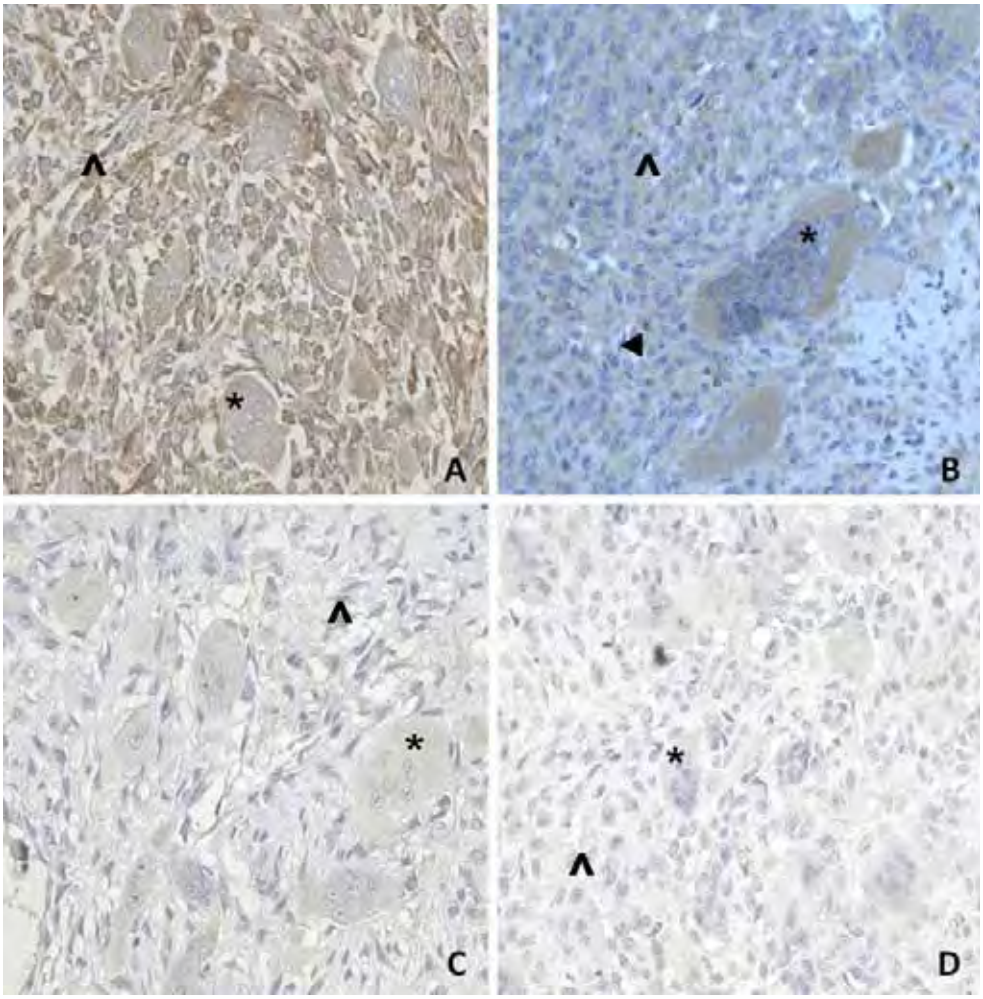


**Fig. 3** Immunolocalization of KTN1, ROCK1, and ZAK in human specimens of primary GCTB samples. **a** KTN1; **b** ROCK1; **c** ZAK; **d** negative control (original magnification 400×). These are representative figures from 22 GCTB patient tissue samples. The staining patterns for KTN1, ROCK1, and ZAK were generally consistent among all the patient samples. The presence of multinucleated giant cells is marked with *stars*, mesenchymal stromal cells with *caret*, and monocytes cell with *arrow*. Positive stainings of the corresponding antigens show *brownish color* in various degrees, in contrast to the *bluish color* in negative stainings

known to contribute to the invasion of hepatocellular carcinoma [31], bladder cancer [29], melanoma [30], and to be upregulated in anaplastic astrocytomas and glioblastomas [32]. Since ROCK1 was upregulated in multinucleated giant cells, stromal cells, and monocytic cells of GCTBs, we believe that overexpression in these tumors may contribute to their occasional invasion, metastasis, and increased malignancy.

*KTN1* gene is located in the region 14q22.1 and encodes the full kinectin, membrane receptor for kinesin that is found in the endoplasmic reticulum and is responsible for the transport of vesicles along microtubules. It is expressed in the brain, liver, ovarian, and hematopoietic cells [33, 34]. The kinectin has an important role in vesicle transport as a receptor for kinesin and dynein, thereby aiding the movement of motor proteins within the cell. However, the exact mechanism by which cells control the transport of vesicles remains unclear [35]. According to Santama et al. [36], kinectin was demonstrated in vivo by overexpression and RNA interference assay, and is selectively involved in the transport of specific types of organelles.

Besides interacting with the kinesin, the kinectin also interacts with other proteins, such as Rho protein, which forms the complex Rho-kinectin responsible for transport of microtubules between the actin filaments and the elongation factor of translation-1δ [37, 38]. The latter plays an important role in regulating the synthesis of proteins that participate in the elongation phase of mRNA translation.

The exact function of *KTN1* and its role in tumor progression remain unclear. There are no reports in the literature about the relationship of *KTN1* gene in tumors, but this gene was highly expressed in all GCTBs.

*ZAK* gene is located in the region 2q24.2 and belongs to the family of mixed lineage kinase, which comprises a group of serine/threonine kinases that works with *MAP3K* [39, 40]. It is expressed in most tissues and is regulated by environmental stresses [39]. The expression of *ZAK* arrests cells in G2, due to the effect that its protein somehow decreases the expression of cyclin E [41].

In addition to this important function for example, overexpression can induce apoptosis in hepatocyte cell line [39. The gene *NF-κB* belongs to a gene family of

transcription factors regulating the transcription of several genes related to immune response, cell proliferation, apoptosis, and the progression and maintenance of the status factor [42, 43]. *NF-κB* super can regulate the expression of proteins that interfere with the receptor via apoptosis, and induce the expression of apoptosis inhibitors and some family members of anti-apoptotic Bcl-2 [44]. Another way of induction of apoptosis by *ZAK* gene is via the JNK/SAPK, which belongs to the MAPK family and is activated by many types of cellular stresses or extracellular signals. The activation of this pathway is associated with regulation of cell proliferation, survival, apoptosis, and immune response by regulating the gene expression of cytokines. Furthermore, this protein is associated with the regulation of mRNA stabilization, cell migration, and integrity of the cytoskeleton [44, 45].

The low expression of the *ZAK* gene can therefore lead to poorer inhibition of cell proliferation by cycle arrest in tumor cells and more sustained growth of the cells of CGTBs.

Regarding the methylation pattern of gene *ZAK* presenting island CpGs in their promoter region, we found that this gene was downregulated in GCTBs, although no methylation occurred in the promoter region. Other mechanisms of gene silencing might be eliminating their activities, such as microRNAs and RNA interference.

In conclusion, differentially expressed genes in the CGTBs include those whose deregulation is potentially associated with disease progression. Methylation of the promoter region is not responsible for the downregulation of gene *ZAK*. These findings will contribute to the understanding of the molecular basis of GCTB, thus helping to improve diagnosis, treatment, and patient outcome. Further studies are required to evaluate whether the identified genes are specifically altered in GCTB or might also be deregulated in other tumors.

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# *Artigo 2*

# EXPRESSION OF *ADAM23, CDKN2A (P16), MMP14* AND *VIM* AND ITS ASSOCIATION WITH TUMOR PROGRESSION IN GCTB

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**ABSTRACT**

Giant cell tumors of bone (GCTB), despite being designated as benign, can become very aggressive and show several characteristics of malignancy, such as high mitotic rate, necrosis, vascular invasion and metastasis. This tumor has unique histological characteristics, a high rate of multinucleated cell, a variable and unpredictable growth potential, and uncertain biological behavior. In this study, we sought to identify genes involved in malignant transformation and the development of metastasis in GCTB, thus building a molecular profile of tumor. We have combined quantitative real-time polymerase chain reaction (qPCR), immunohistochemistry and analysis of methylation to select genes that are putatively associated with tumor progression in GCTB. The expression of the *ADAM23* and *CDKN2A* genes were decreased in GCTB samples compared to normal bone tissue by qPCR, and a high hypermethylation frequency of the promoter region of *ADAM23* and *CDKN2A* in GCTB was observed. The silencing of genes *ADAM23* and *CDKN2A* may contribute to tumor progression and induction of metastasis in GCTB, since they are directly related to cell adhesion and regulation of proliferation. The expression of genes *MMP14* and *VIM* were significantly higher in CGTBs than in normal bone tissue, confirmed by qPCR and immunohistochemistry, and showed significant statistical association between occurrences of metastasis, supporting the potential use of these genes *MMP14* and *VIM* as a prognostic biomarker for GCTB patients. The set of genes identified here furthers our understanding of the molecular basis of GCTB, thereby helping to improve diagnosis and patient outcome.

**Keywords:** Giant cell tumor of bone, gene expression, methylation, metastasis, tumor progression and immunohistochemistry

## 1. INTRODUCTION

Giant cell tumor of bone (GCTB), also known as osteoclastoma [1, 2], accounts for ~5% of all primary bone tumors and is the sixth most common primary bone tumor [3, 4]. GCTB is an expansile osteolytic tumor that most often arises at the end of a long bone in a skeletally mature patient [5]. This neoplasm usually affects young adults; about two-thirds of the patients are between 20 and 40 years of age, and all races are affected [6, 7]. It most commonly involves the distal femur, proximal tibia, distal radius, proximal humerus and the sacral bone [8, 9], with a slight predominance in females [9, 10].

Histologically, GCTB comprises 3 distinct cell types: multinucleated osteoclast-like giant cells, monocytic round-shaped macrophage-like cells, and spindle-shaped, fibroblast-like stromal cells. The stromal cells of GCTB are the primary neoplastic cells; they are the only proliferating cell component in long-term culture [11, 12]. Although their exact origin has yet to be determined, they may come from either an osteoblastic lineage or bone marrow mesenchymal cells, which might regulate the formation of multinucleated osteoclast-like giant cells in the neoplasm [13]. The stromal cells of GCTB may drive the macrophage-like cells to undergo fusion to form multinucleated osteoclast-like giant cells, and the latter eventually cause aggressive bone resorption and skeletal destruction [14].

The World Health Organization has classified GCTB as an aggressive, potentially malignant lesion [8]. Clinically, GCTB have been considered benign, but often becoming an aggressive lesion with a tendency to local recurrence. Depending on the type of treatment and the local presentation of the tumor, recurrence rates range from 0 to 65% [15]. Some reports suggest that recurrent giant-cell tumor may predict a higher risk of malignant transformation [16, 17]. GCTB is one of the rare benign tumors that can grow intravascularly and give rise to distant metastases, with slow growth [18, 19]. Lung metastasis occurs in 1- 9% of cases [20].

Although numerous attempts have been made to predict the behavior of GCTB, there are no definite biological or histological parameters that determine the prognosis or aggressiveness of this lesion [21]. Because very few studies have investigated the genetic profile of GCTBs, we have tried to identify differentially expressed genes involved in malignant transformation that are responsible for the formation and development of metastasis in GCTB, thus building a molecular profile of tumor.

## 2. MATERIAL AND METHODS

### 2. 1 GCTB sample collection

The saomple collection comprises a total of 47 cases of primary GCTB, including 23 fresh samples of GCTB, 24 paraffin-embedded samples of GCTB, and 9 fresh histologically normal medullar bone tissue confirmed by pathologists Tissues were obtained from Tumor Bank at The Pio XII Foundation/IBILCE-UNESP, São Paulo, Brazil. The use of all patient-derived material was approved by institution's Research Ethics Board, The Pio XII Foundation – Cancer Hospital Barretos, and informed consent was obtained individually from the patients. The diagnosis of GCTB was established by biopsy prior to surgical excision. Tissues were obtained at surgery from patients undergoing tumor resection, and the diagnosis of GCTB was verified post-operatively by a histopathologist. Microdissection was done in collected samples. Table 1 gives the clinicopathologic features of these tumors.

### 2.2 RNA Extraction

Total RNA was isolated from GCTB tissue and normal tissue using the TRIzol reagent (Life Technologies, Grand Island, NY, USA) following the protocol instructions. For qPCR, ~5 μg of total RNA from each sample were used to synthesize cDNA with a High-Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. The quality of the cDNA was checked by PCR of the reference gene, *β-ACTIN (ACTB)*. The primer sets were 5'- to 3'-GGCATCGTGATGGACTCCG and GCTGGAAGGTGGACAGCG. The PCR products were analyzed by electrophoresis on 1% agarose gel and stained with ethidium bromide.

### 2.3 Quantitative Real-time Polymerase Chain Reaction (qPCR)

Twenty genes related to tumorigenesis, progression, cell migration and tumor malignancy were selected (Table 1 in supplementary material). We used 23 fresh samples of CGTBs and a pool of total RNA from a subset of 9 fresh tissue samples of normal bone, defined as the normal reference. Gene-specific primers for qPCR were designed for optimal hybridization kinetics using the Primer 3.0 program (provided by the Whitehead/MIT Center for Genome Research, Cambridge, MA, USA).

Quantitative Real-time PCR involved an ABI prism 7300 sequencer detector system and SybrGreen PCR Core Reagent (Applied Biosystems). In brief, the reaction mixture (20 μl total volume) contained 25 ng of cDNA, gene-specific forward and reverse primers for each gene, and 10 μL of 2x Quantitative Sybr Green PCR Master

Mix. Relative quantification is given by the CT values, determined for triplicate reactions for GCTB samples and reference sample from each gene, and for the endogenous control (glyceraldehydes-3-phosphate dehydrogenase*; GAPDH)*. The primer sequences are available on request. Thus, the relative expression of each specific gene was calculated by using the formula: $R = (E\ target)^{\Delta Ct\ target\ (control\ -\ sample)}/(E\ endogenous)^{\Delta Ct\ endogenous\ (control\ -\ sample)}$ [22]. The cutoff for analysis of gene expression was equal to or greater than 2 for both increase and decrease of expression. A value below this cutoff was taken as indicating not significant change in expression.

### 2.4 DNA Extraction

DNA samples of fresh tissue were isolated using TRIzol Reagent (Life Technologies, Grand Island, NY, USA). For the extraction of DNA from paraffin, samples was deparaffinized with xylene, and the tissue samples digested in a buffer (100 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8.0, 25 mmol/L ethylenediamine tetraacetic acid (EDTA) and 1% sodium dodecyl sulfate) containing 20 mg/mL proteinase K at 50°C for 3 days. Total DNA was isolated by phenol-chloroform extraction and ethanol precipitation. The DNA pellets were suspended with 20 mL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and stored at -20°C until PCR amplification. To determine the quality of the DNA extracted, all samples were submitted to PCR amplification of the 315-bp β-globin gene (HBB), using the G73/G74 primers. The samples that were positive for the HBB gene were later processed by bisulfite treatment. The primer sets were 5'- to 3'- G73: GAAGAGCCAAGGACAGGTAC and G74: CAACTTCATCCACGTTCACC

### 2.5 Bisulfite modification of DNA and methylation-specific PCR

Genomic DNA extracted from tissues was modified by bisulfite treatment according to Calmon et al. [23]. DNA methylation status in the CpG island of promoter was determined by the previously described MSP procedure [24], which used primers specific for the methylated (M) or unmethylated (U) sequences of the bisulfite-modified DNA. The primers used for each gene in the PCR reaction were specific for methylated and unmethylated DNA (Table 2). Bisulfite-modified DNA from peripheral blood lymphocytes of a healthy individual previously treated and untreated with SssI methyltransferase (New England Biolabs, Ipswich, MA, USA) served as a positive control for hypermethylated and unmethylated DNA, respectively. A blank control containing all the PCR components, except template DNA, was also included in all the experiments. Reaction products were separated by electrophoresis on an 8% polyacrylamide gel, and stained with silver nitrate.

### 2.6 Immunohistochemistry

Unstained 4.5 µm sections were cut from each paraffin block, deparaffinized and rehydrated by routine techniques. Endogenous peroxidase activity was blocked with 0.3% $H_2O_2$ for 30 min in citrate buffer (10 mM, pH-6) at 95 °C. Monoclonal antibodies used were anti-human CDH1 (Dako, Carpinteria, CA, USA) at a 1:100 dilution, anti-human MMP14 (Abcam Inc., Cambridge, MA, USA) at a 1:50 dilution, anti-human TIMP2 (Chemicon/Millipore) at a 1:150 dilution, anti-human VIM (Dako) at a 1:100 dilution and polyclonal rabbit anti-human MKK3 (Abcam Inc.) antibodies at 1:50 were applied incubated at 4°C overnight. Afterward, sections were incubated with a biotinylated secondary antibody and exposed to a streptavidin complex (HRP Ready-to Use, DakoCytomation, Carpinteria, CA, USA). Positive reactions were visualized with 3,3' diaminobenzidine tetrahydrochloride (DAB, Signet® Laboratories, Dedham, MA, USA), followed by counterstaining with hematoxylin.

Sections treated without primary antibodies were used as negative controls. Immunoreactivity was evaluated blindly by two observers independently assessed by semi-quantitative grading (− no staining; + weak staining in 10-30% of cells; ++ moderate staining in 31-65% of cells; +++ strong staining of >66% of cells) [25].

### 2.7 Statistical analysis

Statistical analysis was performed using the Minitab Student 14 software, with significance set at $P \leq 0.05$. Relative expression levels detected by qPCR in CGTBs samples were transformed into natural logarithms. The Wilcoxon Signed Ranks Test was applied to compare the gene expression levels in tumor tissue and normal bone tissue. The Kruskal-Wallis Test was used for determine whether there was an association between the gene expression and recurrence/metastasis. For analysis of protein, expression by immunohistochemistry used Fisher's exact test, which was also used for determine whether there was any association between the protein expression and recurrence/metastasis, and between methylation pattern and recurrence/metastasis.

## 3. RESULTS

The study population involved 43 patients. Twenty-one (48%) were male and 22 (52%) female, the minimum and maximum ages being 13 and 74 years, respectively, mean 35.6 years ± 14.9. Main localizations of the GCTBs were femur (26%), tibia (21%) and radius (14%). Eight patients (17%) had recurrence between 11 and 42

months after surgery, and 6 patients (12, 7%) had metastases between 1 and 65 months after surgery.

For the detection of differentially expressed genes in GCTBs potentially involved in the malignant transformation and development of these tumors, the gene expression profiles of 23 fresh samples of primary GCTBs were recorded. This looked at the relative expression levels of 20 genes by qPCR, using triple determination and normalization based on the *GAPDH* level. In the evaluation of the target genes, GCTBs samples were used, while normal bone tissue evaluated as the reference (group control).

The relative gene expression data are given in Table 3, which shows that genes *ADAM10, CXCL14, CDH2, CDK4, FN1, MAP2K3, MMP14, SERPINE1, PTEN, SNAI1, TGFβ1, TIMP2, TIMP3, TWIST1, VIM,* and *ZEB1* were overexpressed in the GCTB samples compared to the sample reference ($P$<0.001), the value of gene expression ranging from 2.03 to 6.13. *ADAM23, CDH1* and *CDKN2A (p16)* were significantly downregulated in tumor tissues compared to normal bone tissue ($P$<0.001), the value of gene expression ranging from -6.46 to -4.70, and the *NFKβ1* gene was not differentially expressed. The genes MAP2K3, *MMP14, TIMP2* and VIM showed association between increased gene expression and metastasis, with $P$=0.036, $P$=0.028, $P$=0.036 and $P$=0.045, respectively.

After gene expression analysis, the methylation patterns of the promoter region of genes *ADAM23, CDH1* and *CDKN2A (p16)* by MSP-PCR were analyzed. This involved a designed single pair of primers for the *ADAM23, CDH1* and *CDKN2A (p16)* genes, and an external pair of primers for the *CDKN2A (p16)* gene.

Hypermethylation in the 5' region of the *ADAM23* gene was detected in 92% (23/25) of the GCTBs ($P$<0.001) (Figure 1). Hypermethylation of the promoter region of the gene *CDKN2A (p16)* was found in 80% (16/20) of the tumors analyzed ($P$= 0.012; Figure 1). However, methylation of the *CDH1* gene was not significant in GCTB samples ($P$=0.791). The sets of primers for the genes gave no indication of hypermethylation in the normal lymphocytes analyzed (pattern data given in Table 4). No statistically significant association could be found between the presence of hypermethylation in the promoter region of genes *ADAM23* and *CDKN2A (p16)* and clinico-pathological parameters (recurrence and metastasis). Hypermethylation in the promoter region of genes *ADAM23* and *CDKN2A (p16)* in GCTB samples was detected.

Protein expression of genes *MAP2K3, MMP14, TIMP2* and *VIM* showed immunohistochemically a statistically significant association between gene expression and the occurrence of metastasis in different cell types, due to the heterogeneity of

CGTBs. Immunohistochemical staining of GCTB samples also confirmed the presence of MMP14 and VIM in the GCTB microenvironment. MMP14 staining was strongly positive in the cytoplasm of multinucleated giant cells, stromal cells and monocytic cells in 86.3% (19/22) (*P*=0.004) of the samples (Figure 2A), and VIM staining was strongly positive in the cytoplasm of multinucleated giant cells, stromal cells and monocytic cells in 81.8% (18/22) (*P*=0.017) (Figure 2B). The MAP2K3 and TIMP2 proteins were not differentially expressed by immunohistochemical (*P*=0.134). there was no immunohistochemical staining of the 4 genes analyzed in different cell types of normal bone tissue.

The results show that genes *MMP14* and *VIM* were overexpressed in GCTB, with a significant association between occurrence of metastasis (*P*=0.028 and *P*=0.045, respectively).

## 4. DISCUSSION

GCTB is a tumor that despite being designated as benign has become very aggressive and presented high rates of local recurrence after curettage and metastasis [9, 26, 27]. This tumor has unique histological characteristics, consisting of mononuclear cells and multinucleated giant cells with a variable and unpredictable growth potential [18]. Thus, it has several characteristics of malignancy such as high mitotic rate, necrosis, vascular invasion and metastasis [17]. Although both morphologic and radiologic examination can lead to the correct diagnosis in the majority of cases, the prognostic of GCTB can be difficult in some cases and no useful marker is currently available clinically to aid in its diagnosis and outcome [26].

Cancer cells are characterized by changes in gene expression and transcriptional inactivation due to epigenetic events that can initiate the expansion of pre-malignant cells during the early stages of tumorigenesis [28, 29]. Methylation is major epigenetic modification in human cells, and changes in methylation pattern have an important role in the genesis of tumors as a mechanism of transcriptional inactivation [30]. DNA methylation of the methyl group C-5 in 5'-cytosine results in the formation of 5-methylcytosine [31, 32]. The methyl groups decreases the binding affinity between the promoter regions of DNA and transcription factors. [33].

Hypermethylation of the promoters of the genes *ADAM 23* and *CDKN2A (p16)* is a way of silencing these genes, as seen in other tumor types [34, 35]. Profiles of the genes *ADAM23* and *CDKN2A (p16)* showed they were hypermethylated in tumor tissue.

The *ADAM23* gene is located on chromosome 2q33 and encodes ADAM domain 23; this constitutes an Adam family of type I transmembrane glycoproteins that have a common structural organization, including a metalloprotease and disintegrin domain. This possesses a potent adhesion domain and is involved in cell-cell and cell-matrix interactions [23, 36], which. Contact between cells can prevent growth by contact inhibition, especially of normal cells. Some studies have suggested that this is a metastasis suppressor gene [37].

Analysis of the *ADAM23* gene promoter suggests that methylation is an active epigenetic event in silencing of gene activity. Hypermethylation of the promoter region of *ADAM 23* gene has been associated with advanced breast cancer [38], brain, gastric cancers and head and neck cancer [23]. We found a high methylation frequency in *ADAM23* promoter region in GCTB, which could lead to tumor progression and metastasis should cancers cells lose their contact inhibition, i.e. lead to cell proliferation in an uncontrolled manner.

*CDKN2A* gene, also known as $p16^{INK4}$, is located in chromosome region 9p21, and is regarded as tumor suppressor gene because it is frequently silenced by deletion or an inactivating mutation in human cancers [39]. The product of *CDKN2A*, p16, is a potent cyclin-dependent kinase inhibitor and a critical negative G1-specific regulator that halts progression at the G1-S phase boundary in the cell cycle; loss of function can lead to uncontrolled cell proliferation [40]. p16 induces cell cycle arrest and prevents cell division by inhibiting the cyclin-dependent kinases, CDK4 and CDK6, and also CDK-mediated phosphorylation of the retinoblastoma gene [41, 42]. *CDKN2A* gene protects cells from undergoing malignant transformation and the genome from mutagenic events, induces apoptosis in cells that escape the control of cell cycle, and inhibits cell migration and metastasis [43].

Methylation of cytosine residues at CpG sites in the *CDKN2A* gene promoter resulting in silencing p16 expression occurs in many cell lines, including colorectal cancer and some primary carcinomas of varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma [35]. Aberrant inactivation of *CDKN2A* gene by methylation is a frequent early event in multiple human cancers [44], making it of interest as a biomarker of pre-malignant alterations [45]. In this study, the *CDKN2A* gene expression could possibly have been suppressed due the DNA methylation, because of the high methylation frequency in its promoter region. The loss of *CDKN2A* activity by mutations or promoter hypermethylation is a common step in the development and tumor progression [43], inactivation allowing tumor cells to pass through the G1 cell cycle checkpoint [46].

From gene expression and immunohistochemical analyses, 2 potential biomarkers of metastasis of giant cell tumor of bone have been identified, and shown to have distinctive differences in expression from non-neoplastic samples. The expression of genes *MMP14* and *VIM* were significantly higher in CGTBs than in normal bone tissue, and showed a statistically significant association with the occurrence of metastasis.

Membrane type-1 metalloproteinase, also known as *MMP14*, is located in chromosome region 14q11-q12, and is a member of the matrix metalloproteinases (MMPs), which are Zn(2+)-binding endopeptidases that degrade components of the extracellular matrix. Metalloproteinases have been implicated in a number of physiological and pathological processes, including tissue remodeling processes, wound repair, angiogenesis, cellular migration, invasion and metastasis of tumor cells [47, 48]. *MMP14* is directly involved in cell locomotion and matrix degradation, as also in the activation of soluble MMPs. The presence of invasion-promoting *MMP14*, either in tumor cells or the surrounding stroma, is linked to tumorigenesis, cell migration, angiogenesis and metastasis [48-50].

Invasion and metastasis require the disruption of several collagen-endowed tissue barriers, basement membrane and vascular endothelial cells that constitute a continuous physical obstacle to tumor metastasis [51]. *MMP14* is a potent collagenase highly expressed in human and mouse breast carcinomas, and localized to both stromal and tumor epithelial cells [52]; it is also responsible for degrading and remodeling the fibrin matrix and participates in the formation of new vessels within a collagen rich-matrix [53]. *MMP14* functions therefore as an oncogene, interferes in the control of tumors, and promotes invasive behavior of cells [50]. Our study shows the *MMP14* gene is overexpressed in multinucleated giant cells, stromal cells and monocytic cells of GCTBs, and this is associated with metastasis. The data support the hypothesis of Dong et al. [54] that the *MMP14* gene serves as a marker of the extent of metastasis and dissemination of the neuroblastoma. High levels of *MMP14* may also play a role in invasiveness and metastasis of breast cancer [51].

The *Vim* gene, located in 10p13, is one of the most familiar members of intermediate filaments (IFs), characteristic of mesenchymal cells. IFs, actin microfilaments and microtubules are 3 major structural components of the cytoskeleton in charge of contraction and migration of cells [55]. IFs have an important role in adhesion and cell-cell interactions through their association with hemi-desmosomes and desmosomes, stabilizing the architecture of the cytoplasm. Vimentin also has a key role in adhesion by regulating integrin functioning [56, 57], possibly by regulating the trafficking and cell surface expression of β1 integrins [58]. Vimentin is highly

abundant in human monocytes and activated macrophages, especially multinucleated giant cells. Increased expression of vimentin is a late event in the differentiation of human monocytic cells [57].

Numerous studies have shown that vimentin is a metastasis-associated factor in multiple malignancies, such as prostate cancer, breast cancer, gastric cancer, and gallbladder cancer [55]. This suggests that vimentin should play an important role in tumor progression and serve as a potential biomarker for the metastasis. Our results show that increased expression of vimentin in multinucleated giant cells, stromal cells and monocytic cells of GCTBs is significantly associated with metastasis in GCTB and therefore the increased expression of vimentin might be seen as a novel metastatic indicator for GCTB. Zhao et al. [59] demonstrated that vimentin affects prostate cancer cell motility and invasion, and vimentin could be a predictor of those tumors that might progress to metastatic disease. Another study [55] showed that vimentin is not only a diagnostic marker, but also a hematogenous predictor of metastasis in human melanomas.

In summary, hypermethylation of the promoter region of *ADAM23* and *CDKN2A* occurs in GCTB. Their silencing may contribute to tumor progression and metastasis, since the genes are directly related with adhesion domains involved in cell-cell and cell -matrix interactions that can negatively regulate cell growth. The study also shows a correlation between the overexpression of genes *MMP14* and *VIM* and metastasis, but studies with larger numbers of samples of GCTB will be required for the validation of these genes as potential markers for predicting metastasis. The findings contribute to to a better understanding of the tumorigenesis of GCTB, and may therefore help to improve diagnosis and patient outcome.

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**Conflict of interest**

The authors declare that they have no conflict of interest with the organization that sponsored the research.

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**Table 1**. Epidemiological, clinical and pathological characteristics of samples of GCTB

| Sample | Age(y) | Sex | Anatomic location | Outcome | |
|---|---|---|---|---|---|
| | | | | Recurrence (date) | Metastasis (date) |
| 1F | 37 | M | Proximal tibia L | ned | 19 months |
| 2F | 41 | F | Distal radius R | ned | ned |
| 3F | 24 | M | Proximal femur L | ned | ned |
| 4F/P | 52 | F | Distal femur L | ned | ned |
| 5F | 17 | M | Scapula L | ned | ned |
| 6F | 32 | F | Olecranon R | 11 months | ned |
| 7F/P | 52 | M | Distal femur L | 11 months | ned |
| 8F/P | 74 | F | Distal femur L | 19 months | ned |
| 9F | 35 | M | Distal femur L | ned | ned |
| 10F | 33 | M | Proximal tibia R | 20 months | ned |
| 11F | 21 | F | Proximal tibia R | ned | ned |
| 12F | 37 | F | Proximal tibia L | ned | ned |
| 13F | 13 | F | * | ned | 41 months |
| 14F/P | 28 | F | Distal femur R | 19 months | ned |
| 15F | 19 | F | Sacrum | ned | ned |
| 16F | 22 | F | Distal fibula R | ned | ned |
| 17F | 16 | F | Ischium R | ned | ned |
| 18F | 22 | M | Distal fibula L | ned | 20 months |
| 19F | 27 | M | Proximal humerus L | ned | No |
| 20F | 69 | F | Occipital | ned | ned |
| 21F | 24 | M | Proximal tibia R | 22 months | ned |
| 22F | 46 | F | Distal radius L | ned | ned |
| 23F | 58 | F | Hemipelvis R | ned | ned |
| 24P | 55 | M | Proximal tíbia L | ned | ned |
| 25P | 22 | F | Ischium R | ned | 65 months |
| 26P | 57 | M | Proximal tíbia L | 42 months | ned |
| 27P | 34 | M | Proximal tíbia R | ned | ned |
| 28P | 24 | M | Distal radius L | ned | ned |
| 29P | 27 | M | Distal femur L | ned | ned |
| 30P | 51 | F | Scapula L | Yes* | 07 months |

| | | | | | |
|---|---|---|---|---|---|
| 31P | 31 | M | Wrist R | ned | ned |
| 32P | 22 | F | Distal radius R | ned | ned |
| 33P | 41 | M | Distal femur L | ned | 01 months |
| 34P | 24 | F | Wrist L | ned | ned |
| 35P | 41 | M | Distal femur R | ned | ned |
| 36P | 55 | M | Distal radius L | ned | ned |
| 37P | 41 | F | Distal radius L | ned | ned |
| 38P | 35 | M | Thumb L | ned | ned |
| 39P | 39 | M | Proximal fibula L | ned | ned |
| 40P | 53 | M | Proximal tibia R | ned | ned |
| 41P | 38 | F | Distal femur L | ned | ned |
| 42P | 19 | F | Distal fêmur L | ned | ned |

*Abbreviations: f: fresh tumor; p: paraffin-embedded tumor; f: female; m: male; r: right; l: left*; ned: no evidence of disease, * = no information was obtained.

**Table 2.** Primer sequences used in methylation-specific polymerase chain reaction

| Gene | External Primer | T°C | Primer sequence | | T°C | Size of product M |
|---|---|---|---|---|---|---|
| | | | Methylated sequence (5'-3') | Unmethylated sequence (5'-3') | | |
| ***ADAM23*** | | | F:ATTGTTTTTTGGTTAGAATGTCG | F:ATTGTTTTTTTGTTAGAATGTTG | 59 | 100 |
| | | | R:TAAAAAAAACACAAAAAACCGAACG | R:TAAAAAAAACACAAAAAACCAAACA | | |
| ***CDH1*** | | | F: TTAGGTTAGAGGGTTATCGCGT | F: TAATTTTAGGTTAGAGGGTTATTGT | 60 | 86 |
| | | | R: TAACTAAAATTCACCTACCGAC | R: CACAACCAATCAACACA | | |
| ***CDKN2A (p16)*** | F:GGAGAGGGGGAGAGTAGGT | 60 | F:CGGGGAGTAGTATGGAGTCGGCGGC | F:TGGGGAGTAGTATGGAGTTGGTGGT | 64 | 81 |
| | R:CTACAAACCCTCTACCCACCT | | R: GACCCCGAACCGCGACCGTAA | R: CAACCCCAAACCACAACCATAA | | |

T= annealing temperature (°C), U= Unmethylated sequence, M= Methylated sequence.

**Table 3**. Relative expression values of the selected genes for evalution by qPCR in GCTB

| Gene | Expression | Ratio* | *P* value |
|---|---|---|---|
| *ADAM 10* | 100% overexpression | 7.13 | <0.001 |
| *ADAM23* | 100% downexpression | -4.70 | <0.001 |
| *CXCL14* | 95% overexpression | 3.90 | <0.001 |
| *CDH1* | 95% downexpression | -5.99 | <0.001 |
| *CDH2* | 100% overexpression | 3.99 | <0.001 |
| *CDK4* | 100% overexpression | 2.49 | <0.001 |
| *FN1* | 100% overexpression | 5.85 | <0.001 |
| *MAP2K3* | 100% overexpression | 5.30 | <0.001 |
| *MMP14* | 100% overexpression | 6.58 | <0.001 |
| *CDKN2A (p16)* | 100% downexpression | -6.46 | <0.001 |
| *SERPINE1* | 100% overexpression | 10.66 | <0.001 |
| *PTEN* | 100% overexpression | 11.94 | <0.001 |
| *SNAI1* | 95% overexpression | 2.03 | <0.001 |
| *TGFβ1* | 100% overexpression | 5.69 | <0.001 |
| *TIMP2* | 95 % overexpression | 2.82 | <0.001 |
| *TIMP3* | 91% overexpression | 2.19 | <0.001 |
| *TWIST1* | 100% overexpression | 13.06 | <0.001 |
| *VIM* | 100% overexpression | 4.71 | <0.001 |
| *ZEB1* | 100% overexpression | 5.07 | <0.001 |

*Ratio: Mean among gene expression levels in GCTB compared to normal reference (normal tissue bone).

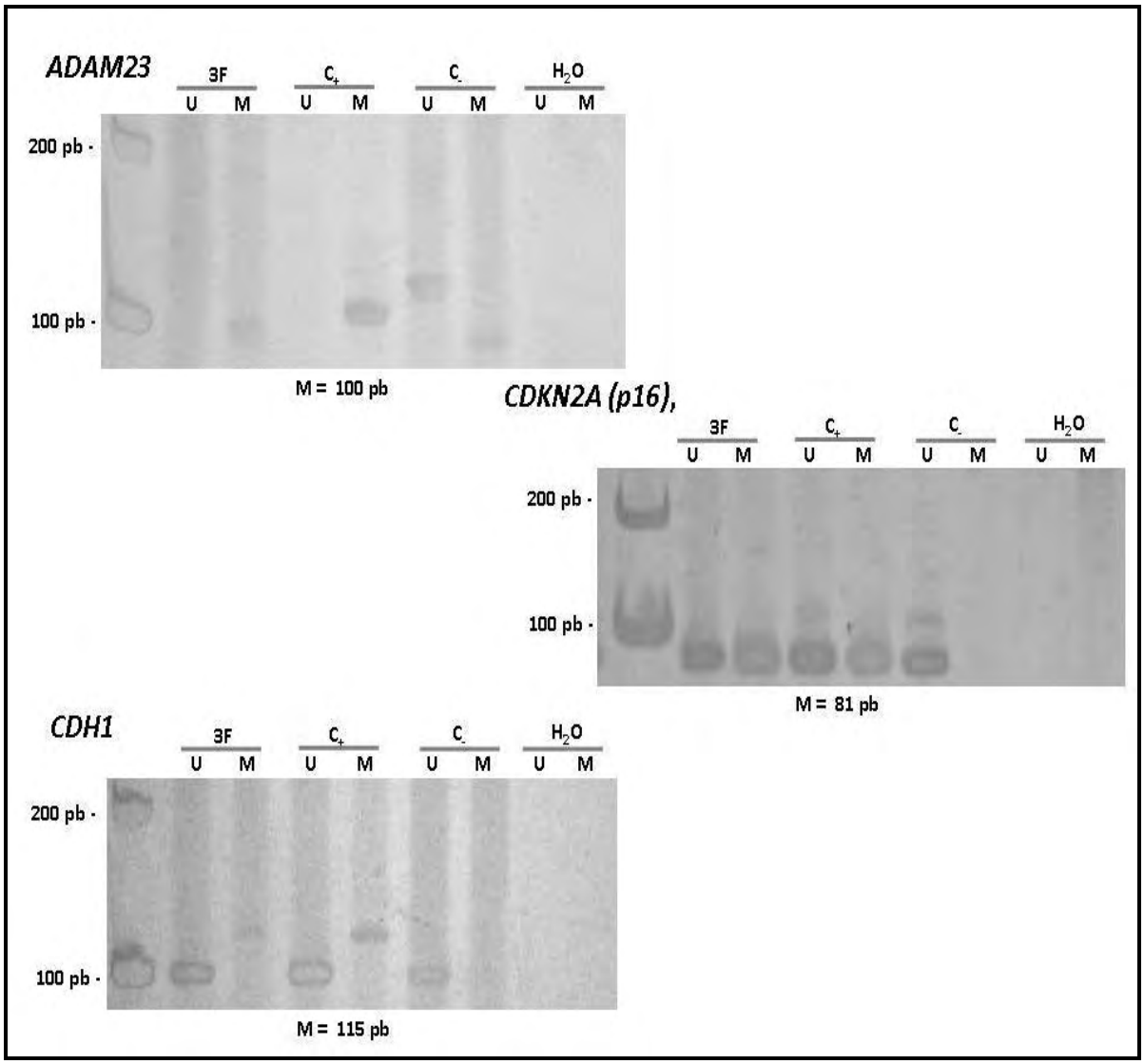


**Figure 1.** Representative examples of MSP reaction for genes *ADAM23, CDH1* and *CDKN2A (p16)*. Tumor (3F) of a GCTB patient. Lanes U and M correspond to unmethylated and methylated reactions, respectively. In each case, C- indicates DNA from lymphocyte, C+ indicates in vitro methylated DNA (IVD), $H_2O$ indicates negative PCR control. On the left: molecular weight marker; and below: size of methylated PCR product.

**Table 4**. Methylation pattern of *ADAM23, CDH1* and *CDKN2A (p16)*, in 26 tumor samples of GCTB

| Samples | *ADAM23* | *p16* | *CDH1* |
|---|---|---|---|
| 3F | ● | ● | ○ |
| 4F | ● | ● | ● |
| 7F | ● | ● | ● |
| 9F | ● | ● | ○ |
| 10F | ● | ● | ○ |
| 11F | ● | ● | ○ |
| 12F | ● | ● | X |
| 13F | ● | ● | ○ |
| 14F | ● | ● | ○ |
| 15F | ● | ● | ● |
| 18F | ● | X | X |
| 19F | ● | ● | ● |
| 23F | ● | ● | ● |
| 24P | ● | X | x |
| 25P | ● | X | X |
| 28P | ● | X | X |
| 29P | ○ | X | X |
| 30P | ● | ○ | X |
| 35P | ● | ○ | ● |
| 37P | ● | ● | X |
| 38P | X | ● | X |
| 39P | ● | ● | X |
| 40P | ● | ○ | X |
| 41P | ● | ● | ● |
| 42P | ○ | X | X |
| 43P | ● | ○ | ● |

legend: ●: methylated genes; ○: unmethylated genes and x: not amplified.

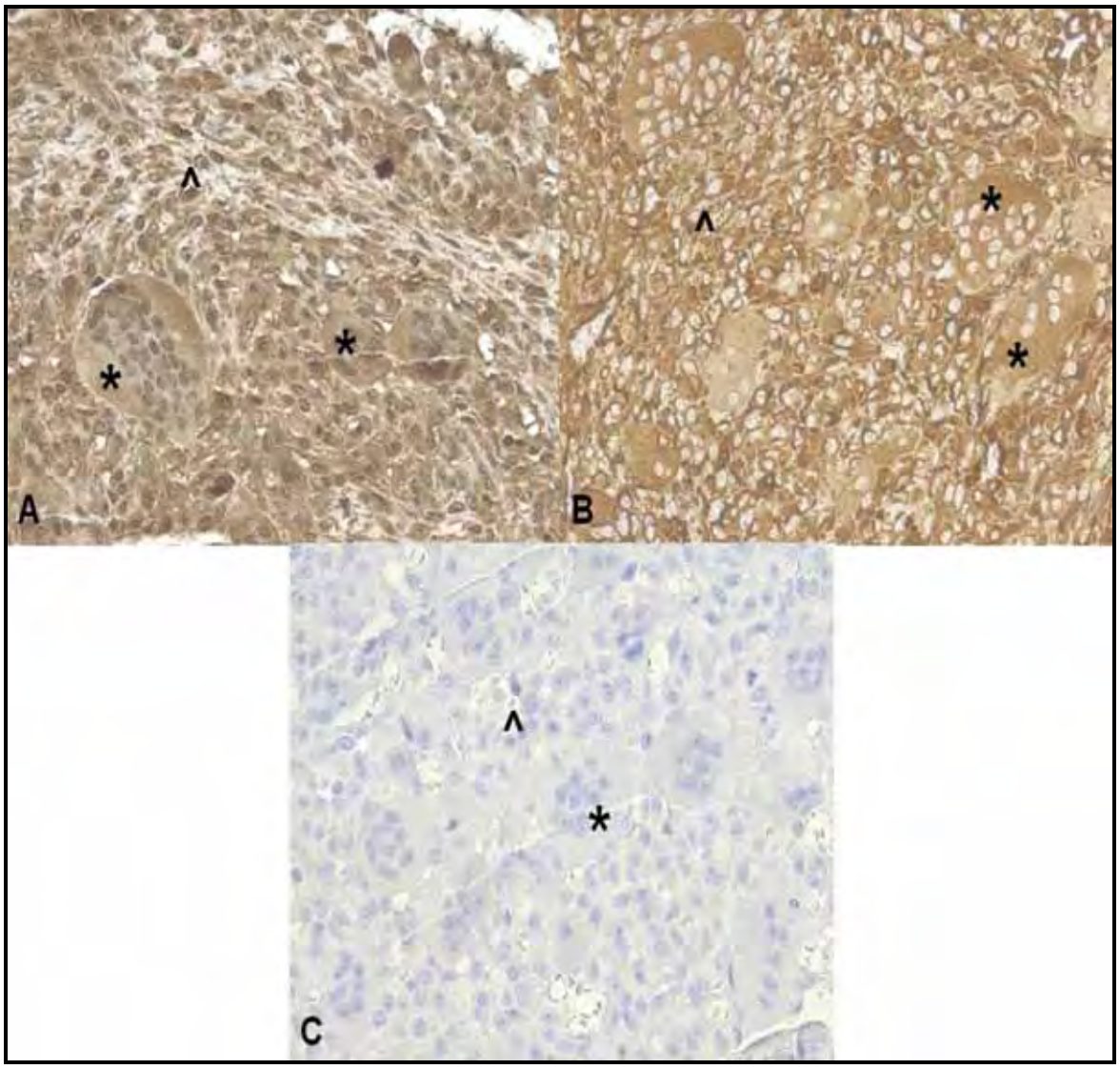


**Fig 2.** Immunolocalization of MMP14 and VIM in primary samples of GCTB. (A) MMP14; (B) VIM1; (C) negative control (original magnification 400X). These are representative figures from 22 GCTB patient tissue samples. The staining patterns for MMp14 and VIM were generally consistent among all the samples. The presence of multinucleated giant cells is marked with stars, mesenchymal stromal cells with ^. Positive staining of the corresponding antigens show brownish color in varing degrees, in contrast to the bluish color in negative staining.

## Supplementary data

**Table 1**. Summary of selected genes for expression analysis in GCTB

| Gene Symbol | Name of Gene | Gene ID | GO molecular function description | GO biological process description | GO cellular component description |
|---|---|---|---|---|---|
| *ADAM 10* | ADAM metallopeptidase domain 10 | 102 | Metalloendopeptidase activity | Monocyte activation, negative regulation of cell adhesion, positive regulation of cell proliferation, positive regulation of cell growth | Plasma membrane, citoplasm and nucleus |
| *ADAM23* | ADAM metallopeptidase domain 23 | 8745 | Metalloendopeptidase and metallopeptidase activity | Cell adhesion | Extracellular region and Plasma membrane |
| *CXCL14* | Chemokine (C-X-C motif) ligand 14 | 9547 | Chemokine activity | Cell-cell signaling, signal transduction | Extracellular region, Golgi apparatus |
| *CDH1* | Cadherin 1, type 1, E-cadherin (epithelial) | 999 | Cell adhesion molecule binding, transcription activator activity, protein binding | Cell-cell adhesion, negative regulation of cell-cell adhesion, regulation of caspase activity | Plasma membrane, citoplasm, cell junction |
| *CDH2* | Cadherin 2, type 1, N-cadherin (neuronal) | 1000 | Protein binding | Cell adhesion, cell migration, cell-cell junction organization, regulation of Rho protein signal transduction | Plasma membrane, cell-cell junction |
| *CDK4* | Cyclin-dependent kinase 4 | 1019 | Protein binding, nucleotide binding | Cell division, positive regulation of apoptosis, signal transduction, positive regulation of cell proliferation, regulation of cell cycle, regulation of gene expression | Citoplasm, cytosol, nucleus |
| *FN1* | Fibronectin 1 | 2335 | Extracellular matrix structural constituent, collagen binding, | Angiogenesis, cell adhesion, cell migration, regulation of cell shape, response to wounding | Extracellular region, extracellular matrix |

| | | | | | |
|---|---|---|---|---|---|
| | | | protein binding | | |
| *MAP2K3* | Mitogen-activated protein kinase kinase 3 | 5606 | Transferase activity, protein binding, MAP kinase kinase activity, nucleotide binding | Activation of MAPK activity, positive regulation of protein kinase activity, signal transduction | Cytosol, nucleoplasm |
| *MMP14* | Matrix metallopeptidase 14 (membrane-inserted) | 4323 | Peptidase activator activity, protein binding, metalloendopeptidase activity | Angiogenesis, cell migration, endothelial cell proliferation, ossification, tissue remodeling, negative regulation of focal adhesion assembly | Cytoplasm, extracellular matrix, membrane |
| *NFKβ1* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | 4790 | DNA binding, protein binding, transcription activator activity | Apoptosis, signal transduction, positive regulation of transcription, positive regulation of macrophage derived foam cell differentiation | Mitochondrion, cytoplasm, nucleus, cytosol, nucleoplasm |
| *CDKN2A (p16)* | Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) | 1029 | Protein binding, transcription activator activity | Activation of caspase activity, cell cycle, induction of apoptosis, negative regulation of cell growth, negative regulation of cell-matrix adhesion | Cytoplasm, cytosol, nucleolus, nucleoplasm, nucleus |
| *SERPINE1* | Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 5054 | Protein binding, peptidase inhibitor activity | Negative regulation of apoptosis, negative regulation of cell migration, positive regulation of angiogenesis | Extracellular matrix, extracellular region, plasma membrane |
| *PTEN* | Phosphatase and tensin homolog | 5728 | Protein binding, protein tyrosine phosphatase activity | Angiogenesis, cell proliferation, cell migration, induction of apoptosis, negative regulation of apoptosis, negative regulation of epithelial cell proliferation | Mitochondrion, cytoplasm, nucleus, cytosol |
| *SNAI1* | Snail homolog 1 (Drosophila) | 6615 | Protein binding, transcription activator activity, sequence-specific DNA binding, zinc ion binding | Osteoblast differentiation, positive regulation of cell migration, epithelial to mesenchymal transition, trophoblast giant cell differentiation | Cytoplasm, nucleus, |
| *TGFβ1* | Transforming growth factor, | 7040 | Protein binding, transcription | Cell cycle arrest, cell-cell junction organization, | Cytoplasm, nucleus, |

| | | | | | |
|---|---|---|---|---|---|
| | beta 1 | | activator activity, transforming growth factor beta receptor binding, growth factor activity | cellular response to growth factor stimulus, cell death, epidermal growth factor receptor signaling pathway | extracellular region |
| *TIMP2* | TIMP metallopeptidase inhibitor 2 | 7077 | Integrin binding, protein binding, metalloendopeptidase inhibitor activity | Negative regulation of cell proliferation, regulation of MAPKKK cascade, regulation of camp metabolic process | Basement membrane, cell surface, extracellular region |
| *TIMP3* | TIMP metallopeptidase inhibitor 3 | 7078 | Protein binding, metalloendopeptidase inhibitor activity, enzyme inhibitor activity | Tissue regeneration, negative regulation of membrane protein ectodomain proteolysis, aging, response to mechanical stimulus | Cytoplasm, extracellular region, colocalizes_with extracellular matrix, basement membrane |
| *TWIST1* | Twist homolog 1 (Drosophila) | 7291 | Protein binding, DNA binding, transcription regulator activity | Anatomical structure morphogenesis, apoptosis, negative regulation of cell differentiation, negative regulation of transcription from RNA polymerase II promoter, skeletal system development | Nucleus |
| *VIM* | Vimentin | 7431 | Protein binding, protein kinase binding, structural constituent of cytoskeleton | apoptosis, cellular component movement, intermediate filament-based process | Cytoplasm, cytoskeleton, cytosol, intermediate filament |
| *ZEB1* | Zinc finger E-box binding homeobox 1 | 6935 | Protein binding, transcription factor binding, sequence-specific DNA binding, chromatin binding | Cell proliferation, negative regulation of epithelial cell differentiation, negative regulation of transcription, regulation of mesenchymal cell proliferation | Cytoplasm, nucleus, |

# *Conclusões*

O Artigo 1 permitiu estabelecer as seguintes conclusões:

1. Foram encontrados 3 genes diferencialmente expressos no TCG, pela metodologia de *RaSH, , K*TN1, *ROCK1*e *ZAK;*

2. Os genes *KTN1* e *ROCK1* apresentaram evidencia de aumento de expressão e o gene *ZAK* apresentou evidencia de baixa expressão no TCG comparado ao tecido ósseo normal pelas técnicas de qPCR e pela imuno-histoquímica;

3. Não há evidência de metilação da região promotora do *ZAK.*

4. Os genes diferencialmente expressos no TCG são associados com a progressão da doença e contribuirão para compreensão das bases moleculares dessa doença, assim ajudando a melhorar o diagnóstico, prognóstico e tratamento dos pacientes.

O Artigo 2 permitiu estabelecer as seguintes conclusões:

1. Os genes *ADAM10, CXCL14, CDH2, CDK4, FN1, MAP2K3, MMP14, SERPINE1, PTEN, SNAI1, TGFβ1, TIMP2, TIMP3, TWIST1, VIM,* e *ZEB1* apresentaram evidencia de aumento de expressão no TCG, confirmado pelo PCR quantitativo em tempo real (qPCR);

2. Os genes *ADAM23, CDH1* e *CDKN2A (p16)* apresentaram evidencia de baixa expressão no TCG comparado ao tecido ósseo normal pelas técnicas de qPCR;

3. Os genes *ADAM23* e *CDKN2A (p16)* encontram-se hipermetilado em freqüência elevada, nas amostras de TCG;

4. Não houve evidência de associação entre a metilação da região promotora do *ADAM23* e *CDKN2A (p16)* e os dados clínicos dos pacientes;

5. Há evidência de associação entre o aumento da expressão dos genes *MMP14* e *VIM*, em amostras tumorais, e a presença de metástase;

6. Os genes *MMP14* e *VIM* são possíveis candidatos a marcadores de metástase para o tumor ósseo de células gigantes.

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