OSTEOCLAST-SPECIFIC MARKER NUCLEAR FACTOR OF ACTIVATED T-CELLS, CYTOPLASMIC, CALCINEURIN-DEPENDENT 1 EXPRESSION IN STAGE III GIANT CELL TUMOR OF THE BONE (GCTB)

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ABSTRAK

Pengenalan: Ketumbuhan tumor sel raksasa peringkat III Campanacci boleh menyebabkan komplikasi osteolisis dan osteoklastik (tulang menjadi reput dan hancur) serta mempunyai potensi untuk berlaku ketumbuhan semula dan metastasis pulmonari. Kajian telah menunjukkan RANKL sangat merangsang osteoklastogenesis melalui faktor nuklear sel T yang aktif, sitoplasmik, bergantung kepada 1 kalori (NFATc1) yang mengawal selia beberapa gen spesifik osteoklas. Osteoklastogenesis menjadi terbantut dalam penekanan NFATc1 dan sel stem embrionik yang mengetuk keluar dalam vitro. Pada masa kini, peraturan NFATc1 dalam resorpsi osteoklastik di GCTB masih belum dikaji dan dipelajari terutamanya dalam tahap III GCTB. Adalah penting untuk memahami sepenuhnya patogenesis GCTB yang berkaitan osteoklas untuk mengenal pasti pendekatan terapeutik baru dengan mensasarkan NFATc1 dalam rawatan GCTB. Kami menganalisis ekspresi NFATc1 immuno-histokimia daripada 31 kes berturut-turut tahap III tumor sel raksasa tulang untuk menentukan hubung kait antara klinikal dan patologi.

Metodologi: Ini adalah kajian rentas keratan pemerhatian yang menilai pengwarnaan imunohistokimia untuk NFATc1 dalam kesemua 31 kes berturut-turut peringkat III Campanacci sel gergasi tumor tulang (GCTB) yang dikendalikan dan dirawat di Hospital Universiti Sains Malaysia dari Januari 2004 hingga Disember 2017. Ungkapan NFATc1 telah dinilai oleh pewarna immuno-histokimia di semua bahagian tumor arkib dari setiap pesakit. Saiz siri 5 micrometer tebal dipotong dan teknik immuno-histokimia telah dijalankan. Ekspresi NFATc1 ke atas kawasan sel tumor nuklear diperiksa menggunakan imunohistokimia. Imunohistokimia dinilai dalam tiga kawasan mikroskopik secara rawak menggunakan mikroskop cahaya standard pada 40 x 100 pembesaran oleh pemerhati bebas dua buta. Positif

untuk ekspresi NFATc1 dinilai mengikut peratusan 1000 sel latar belakang menggunakan perisian analisis imej (Olympus - U-RFL-T Cell F). Skor tertinggi dari tiga bidang terpilih diambil untuk analisis statistik menggunakan SPSS versi 25.0. Analisis statistik ditentukan dengan menggunakan ujian t bebas untuk kumpulan yang berbeza dan dianggap signifikan secara statistik apabila nilai p kurang dari 0.05.

Keputusan: Nilai minimum ekspresi NFATc1 yang didapati sebagai peratusan 1000 sel latar adalah 0.81 dengan sisihan piawai 1.48. Julat ini adalah antara 0.0 hingga 6.33 dengan median sebanyak 0.07. Perbandingan ekspresi NFATc1 menunjukkan peratusan yang lebih tinggi dalam kumpulan pengulangan dengan min 1.01 (SD 0.68) berbanding dengan kumpulan tidak berulang dengan min 0.79 (SD 1.55). Perbezaan min ialah 0.22 (-1.06, 1.51). Perbezaan ini secara statistik tidak signifikan dengan p> 0.005. Perbandingan ekspresi NFATc1 menunjukkan nilai min yang lebih tinggi dalam kumpulan metastasis paru-paru iaitu 2.01 (SD 2.49) berbanding dengan 0.58 (SD 1.13) dalam kumpulan metastasis bukan paru-paru. Perbezaan min antara kedua-dua kumpulan adalah 1.43 (-1.63, 4.49) yang secara statistik tidak signifikan dengan nilai p> 0.005.

Kesimpulan: Kajian ini menunjukkan tidak semua 31 kes GCTB tahap III yang agresif positif diwarnai dengan antibodi NFATc1 dengan kemungkinan osteoklas bukanlah faktor utama yang bertanggungjawab dalam pemusnahan tulang di dalam kes GCTB. Nilai min ekspresi NFATc1 didapati secara statistik tidak signifikan apabila diuji terhadap risiko metastasis pulmonari dan penyakit berulang, menjadikannya penanda yang tidak berguna untuk meramalkan risiko ketumbuhan semula dan metastasis pulmonari dalam tumor sel tumor gergasi yang agresif. Kajian ini menunjukkan bahawa terdapat kemungkinan laluan lain yang terlibat dalam kemusnahan tulang di dalm kes GCTB yang memerlukan penyelidikan lanjut.

ABSTRACT

Introduction: Osteoclastic bone resorption and osteolysis with tendency for local recurrence and pulmonary metastases are a common complication of stage III Campanacci giant cell tumour of the bone (GCTB). Studies have shown RANKL highly stimulates osteoclastogenesis through nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), which regulates a number of osteoclast-specific genes. Osteoclastogenesis is retarded in NFATc1 suppression and knock-out embryonic stem cells *in vitro.* To our knowledge, the regulation of NFATc1 in osteoclastic resorption in GCTB has not been studied in stage III GCTB. It is important to fully understand the osteoclast-associated GCTB pathogenesis to identify a new therapeutic approach by targeting NFATc1 in GCTB treatment. We analyzed NFATc1 expression immuno-histochemistry of 31 consecutive cases of stage III giant cell tumour of the bone to determine the clinico-pathological correlation.

Methodology: This observational cross-sectional study evaluated the immunohistochemical staining for NFATc1 expression in 31 consecutive cases of stage III Campanacci giant cell tumour of bone (GCTB) operated and treated at Hospital Universiti Sains Malaysia from January 2004 to December 2017. Expression of NFATc1 was assessed by immuno-histochemical staining in all representative archive tumour sections from each patient. Serial sections of 5 μm was cut and underwent immuno-histochemical staining. NFATc1 expression over nuclear area of tumor cells was examined using immunohistochemistry. Immunostainings were evaluated in three randomly chosen microscopic fields using a standard light microscope at 40 x 100 magnification by two-blinded independent observers. Positivity for NFATc1 expression was assessed according to percentage of 1000 background cells using an image analysis software (Olympus – U-RFL-T Cell F). The average score from three selected field was taken for statistical analysis using SPSS version 25.0. Statistical analysis was determined

using independent t-test for different group and considered statistically significant when p values were less than 0.05.

Results: The mean value of NFATc1 expression obtained as a percentage of 1000 background cells was 0.81 with standard deviation of 1.48. The range was between 0.0 to 6.33 with a median of 0.07. Comparison of NFATc1 expression showed higher percentage in recurrence group with mean of 1.01 (SD 0.68) compared to non-recurrence group with the mean of 0.79 (SD 1.55). The mean difference was 0.22 (-1.06, 1.51). This difference was statistically not significant with $p > 0.005$. A comparison of NFATc1 expression showed higher mean value in lung metastases group which was 2.01 (SD 2.49) compared to 0.58 (SD 1.13) in non-lung metastases group. The mean difference between the two groups were 1.43 (-1.63, 4.49) which is statistically not significant with the p value > 0.005.

Conclusions: This study shows not all 31 cases with aggressive GCTB stage III were positively stained with NFATc1 antibody showing the possibility osteoclast may have not been the main cells responsible in the bone destruction in GCTB condition. The mean value of NFATc1 expression was found to be statistically not significant when tested against the risk of pulmonary metastases and recurrence disease, making it not a useful marker to predict the risk of recurrence and pulmonary metastases in aggressive type of GCTB. This study suggested that there must be further research to be carried out to understand other different pathways of bone resorption and osteolysis in GCTB.

Keyword; *NFATc1, Giant Cell Tumour Bone, Osteolysis*

Chapter 1

INTRODUCTION

1.1 INTRODUCTION

Giant cell tumor of bone (GCT) is a locally, aggressive, benign bone tumor with high incidence of recurrence rate and potential of pulmonary metastases [1]. They are one of the most common neoplasms, accounting for 5% to 10% of all the primary bone tumours. Additionally, they are one of the few osseous bone lesions with female predominance, with a female-to-male ratio of 1.3-1.5 to 1. [2] The tumour was thought to be malignant initially and was often treated with amputation until it was coined the phrase "giant cell tumour of bone," by Bloodgood in 1912, that the non-malignant behaviour of this lesion was first established. [3]

Aggressive lesions are common in Asian population compared to western population and they have been shown to have higher rate of recurrence and pulmonary metastases [4, 5]. The reason behind this prevalence disparity remains unclear. Studied done by Turcotte et al [6] and Arnold et al [7] showing high prevalence and aggressive presentations in Southern India and China, where it represents 20% of all biopsied primary tumors of bone. In Malaysia, Faisham et al [5] and Ismail et al [8] have reported that most of cases presented were staged III GCT which were locally aggressive tumours and have high rate of metastasis to lung which range from 19.4% to 30%. The disease typically affects skeletally mature adults aged 20 to 40 years of age instead of skeletally immature patients which are accounts for fewer than 5% of GCTS of bone and patients aged more than 65 years which are accounts for less than 10% [9]. Most of the tumours occur around the knee joint (50%) followed by distal radius and proximal humerus. [10, 11]. A small percentage of tumors occur in the axial skeleton, especially in the spine and sacrum region.

GCTs of bone usually presented as solitary lesions except in fewer than 1% of of tumors are multicentric. [12] Multicentric GCT of bone tend to be presented in younger patients and the tumors often involve the small bones of the hands and feet with more likely to be confined to the metaphysis and diaphysis when a long bone is involved.

Radiographic appearance of GCT bone shows an osteolytic lesion with well-defined border, eccentrically around the epiphyseal-metaphyseal region which is closed to juxta-articular location, no mineralized tumour matrix and occurs in patients with closed physes [6, 7, 13]. However, GCT of bone may be centered in the metaphysis and may abut the physis in children and adolescent with open physes. MRI is crucial for the detection of extraosseous soft tissue extension, intra-articular and marrow involvement which is important as part of local staging of the disease. Campannaci et al. have classified GCT into 3 stages. Stage 3 lesions are aggressive in nature. They are rapidly growing symptomatic lesions and are always associated with spontaneous fracture [4]. The tumour has fussy borders, with rapid permeative growth and often bulges into the soft tissue. On MRI, Stage 3 lesions characterized by breakage of bone cortex and local soft tissue extension whereby in MRA (Magnetic Resonance Angiography) showed local increase in vascularity. According to Rock et al [14], there is an increased risk of recurrence in stage 3 tumour. However, many other studies do not regard these staging systems as the predictive of the prognosis [1, 15, 16] because of no correlation found between aggressiveness seeing in radiography and local recurrence of GCT.

Macroscopically, GCTs of bone are characteristically homogeneous, pale brown, firm

though friable, and solid with spongy appearance. However, secondary changes GCT presents as a hemorrhagic, soft mass eroding bone. Microscopically, GCT of bone is a biphasic lesion composed of multinucleated, osteoclast-like giant cells and mononuclear stromal cells with a variable and unpredictable potential for growth. The tumor features a usually bland stromal cell population made of osteoblast lineage which are considered as a neoplastic factor in GCT, and a second population of monocytes and eponymous multinucleated giant cells that are capable for bone resorption.

The stromal cells in the GCT of bone are the neoplastic component of the tumour, releasing chemokines (macrophage chemoattractant protein-1 and interleukin (IL)-8) and proosteoclastogenic cytokines (macrophage colony stimulating factor (M-CSF), IL-1, IL-6, IL-11 and IL-17) to promote osteoclast formation and differentiation [17]), which leads to osteoclastogenesis and eventually brings on to osteolytic destruction of bone which are a common complication of giant cell tumour of the bone (GCTB) [18-20].

Although numerous attempts have been made to predict the behavior of GCT of bone, there has been no definitive clinical, biological or histological parameter to predict the aggressiveness of the tumour in terms of potential for local recurrence and to determine its metastatic potential [5].

Immunohistochemical studies give additional useful information in predicting the behavior of GCT. Researchers found that the increasing rate of proliferating might increase the probability of local recurrence. There are various proliferation markers such as p53, Ki-67, PCNA, MMP-2 and MMP-9 (Matrix Metalloproteinase), RANKL, VEGF and recently

serum Tartrate-Resistant Acid Phosphatase 5b that had been studied with intention to correlate with the aggressiveness of GCT [8, 21-23].

Study of p53 expression of 50 consecutive cases of stage III GCTB in our institution showing p53 expression is a good prognostic factor to predict the risk of local recurrence and lung metastasis [22]. Studies by others [17, 24, 25] and from our group have shown the importance of receptor activator of nuclear factor κB ligand (RANKL) expression to directly stimulate osteoclastogenesis resulting in the osteoclast-mediated bone destruction in GCTB [26]. Treatment using a natural RANKL antagonist, osteoprotegerin (OPG) [27] a monoclonal antibody directed against RANKL, denosumab [28-30] and an anti-resorptive drug, bisphosphonate [31], have shown reduced osteoclast resorption. These support the understanding that targeting osteoclast formation and resorption is likely effective to modulate the osteolysis associated with GCTB [32].

Studies also have shown RANKL highly stimulates osteoclastogenesis through nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) [33, 34] which regulates a number of osteoclast-specific genes [35-37]. Osteoclastogenesis is retarded in NFATc1 suppression [38] and knock-out embryonic stem cells *in vitro [28, 34]*. Apart from the RANKL/RANK-NFATc1 system, immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway involving osteoclast-associated receptor (OSCAR) has been identified as an important co-stimulatory pathway in osteoclasts [39]. OSCAR has a specific role in co-stimulating osteoclastogenesis [39-42] and they may be a positive feedback loop involved in the interaction between OSCAR and NFATc1 [39, 43].

We evaluated 40 archived tissue specimens diagnosed with GCT of bone, for positivity of NFATc1 in hope to find its expression. To our knowledge, the regulation of NFATc1 in osteoclastic resorption in GCTB has not been studied. It is important to fully understand the osteoclast-associated GCTB pathogenesis to offer a novel therapeutic approach in the treatment of this locally destructive primary bone tumor.

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Chapter 2

OBJECTIVES OF THE STUDY

2.1. General Objectives

1. To assess the expression of NFATc1 in GCTB patients

2.2. Specific Objectives

1. To identify the expression of NFATc1 in GCTB patients by immunohistochemistry
2. To determine the NFATc1 expression in GCTB in newly diagnosed cases, recurrent or non-recurrent cases, lung metastastasis or without lung metastasis, and different grades of GCTB.

Chapter 3

MANUSCRIPT

3.1. TITLE: OSTEOCLAST-SPECIFIC MARKER, NUCLEAR FACTOR OF ACTIVATED T-CELLS, CYTOPLASMIC, CALCINEURIN-DEPENDENT 1 EXPRESSION IN STAGE III GIANT CELL TUMOR OF THE BONE

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3.2 ABSTRACT

Introduction: Osteoclastic bone resorption and osteolysis with tendency for local recurrence and pulmonary metastases are a common complication of stage III Campanacci giant cell tumour of the bone (GCTB). Studies have shown receptor activator of nuclear factor κB ligand (RANKL) highly stimulates osteoclastogenesis through nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), which is involved in the regulation of a number of osteoclast-specific genes. Osteoclastogenesis is retarded in NFATc1 suppression and knocked-out embryonic stem cells *in vitro*. To our knowledge, the regulation of NFATc1 in osteoclastic resorption in GCTB has not been studied in stage III GCTB. It is important to fully understand the osteoclast-associated GCTB pathogenesis to identify a new therapeutic approach by targeting NFATc1 in GCTB treatment. We analyzed NFATc1 expression by immunohistochemistry technique in 31 consecutive cases of stage III giant cell tumour of the bone to understand the clinico-pathological correlation.

Methodology: This observational cross-sectional study evaluated the immunohistochemical staining for NFATc1 expression in 31 consecutive cases of stage III Campanacci giant cell tumour of bone (GCTB) operated and treated at Hospital Universiti Sains Malaysia from January 2004 to December 2017. Expression of NFATc1 was assessed using immuno-histochemical staining method in all representative archive tumour sections. Serial sections of 5 μm was cut and underwent immuno-histochemical staining. NFATc1 expression over nuclear area of tumor cells was examined and evaluated in three random microscopic fields using a standard light microscope at 40 x 100 magnification by two-blinded independent observers. Positivity for NFATc1 expression was assessed according to percentage of 1000 background cells using an image analysis software (Olympus – U-RFL-T Cell F). The average score from three selected field was taken for statistical analysis using SPSS version 25.0. Statistical

analysis was carried out using independent t-test for different groups and considered statistically significant when p values were less than 0.05.

Results: The mean value of NFATc1 expression obtained as a percentage of 1000 background cells was 0.81 with standard deviation of 1.48. The range was between 0.0 to 6.33 with a median of 0.07. Comparison of NFATc1 expression showed higher percentage in recurrence group with a mean of 1.01 (SD 0.68) compared to non-recurrence group with a mean of 0.79 (SD 1.55). The mean difference was 0.22 (-1.06, 1.51). This difference was statistically not significant with $p > 0.005$. A comparison of NFATc1 expression showed higher mean value in lung metastases group which was 2.01 (SD 2.49) compared to 0.58 (SD 1.13) in non-lung metastases group. The mean difference between the two groups were 1.43 (-1.63, 4.49) which is statistically not significant with the p value > 0.005.

Conclusions: This study shows not all 31 cases with aggressive GCTB stage III were positively stained with NFATc1 antibody showing the possibility osteoclast may have not been the main cells responsible in the bone destruction in GCTB condition. The mean value of NFATc1 expression was found to be statistically not significant when tested against the risk of pulmonary metastases and recurrence disease, as expected due to lack expression in GCTB tissue samples making it not be able to predict the risk of tumour recurrence or lung metastases. This study suggested that there must be further research to be carried out to understand other different pathways of bone resorption and osteolysis in GCTB .

Keyword: *NFATc1, Giant Cell Tumour Bone, Osteolysis*

3.3 INTRODUCTION

Giant cell tumour of bone (GCTB) is a benign bone tumour but locally aggressive with high incidence of recurrence and potential of pulmonary metastases [1]. High prevalence rate was observed in Oriental population especially in Southern India and China, where GCTB represents 20% of all primary bone tumour [2]. Aggressive lesions are common in Asian population compared to western population. In Oriental population, the reported cases of GCTB is associated with a higher rate of recurrence and pulmonary metastases [3, 4]. In Malaysia, most of the cases reported were locally aggressive, stage III GCTB with a high rate of metastasis to lung ranging from 19.4% to 30% [4, 5].

Previous studies [6-8] and from our group [9] have shown the importance of receptor activator of nuclear factor κB ligand (RANKL) expression to directly stimulate osteoclastogenesis resulting in the osteoclast-mediated bone destruction in GCTB [9]. The key pathway in RANKL/RANK osteoclastogenesis is NFATc1.

We evaluated 31 archived tissue specimens diagnosed with stage III Campanacci GCTB. To our knowledge, the regulation of NFATc1 in osteoclastic resorption in GCTB has not been studied. It is important to fully understand the osteoclast-associated GCTB pathogenesis to offer a novel therapeutic approach in the treatment of this locally destructive primary bone tumor. This study aims to identify the expression of NFATc1 by immunohistochemistry in stage III Campanacci GCTB and its association with recurrent and non-recurrent, lung metastasis or without lung metastasis.