

## ORIGINAL ARTICLE

## Evaluation of Nuclear Factor of Activated T-cells (NFATc1) Expression in Non-metastatic Osteosarcoma Tissue

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### ABSTRAK

Osteosarkoma, sejenis tumor tulang yang bersifat malignan, merupakan cabaran besar dalam rawatan dan prognosis walaupun terdapat kemajuan dalam terapi. Ekspresi faktor nuklear sel T diaktifkan c1 (NFATc1) dalam tisu osteosarkoma masih kurang diterokai sebagai sasaran terapeutik yang berpotensi. Kajian ini bertujuan untuk menilai ekspresi protein NFATc1 dalam sampel tisu osteosarkoma non-metastatik manusia serta hubung kaitnya dengan kelangsungan hidup pesakit dan subjenisnya. Kedua, kami mengenalpasti hubungan antara butiran demografi klinikal dan kelangsungan hidup pesakit osteosarkoma. Satu kajian keratan rentas telah dijalankan ke atas 31 pesakit osteosarkoma non-metastatik primer. Analisis imunohistokimia digunakan untuk menilai ekspresi protein NFATc1 dalam spesimen tisu yang dikeluarkan secara pembedahan. Data demografi, butiran rawatan dan rekod susulan telah dikumpulkan untuk analisis. Populasi kajian mempunyai purata umur 20.0 tahun, didominasi oleh etnik Melayu. Ekspresi protein NFATc1 adalah positif dalam 25.8%, terutamanya dalam osteosarkoma konvensional (30.4%). Tiada korelasi yang signifikan ditemui antara ekspresi protein NFATc1 dengan kelangsungan hidup pesakit ( $p = 0.685$ ) atau subjenis osteosarcoma ( $p = 0.642$ ). Pesakit dengan NFATc1 positif mempunyai kebarangkalian yang lebih rendah untuk kelangsungan hidup jangka panjang berbanding pesakit NFATc1-negatif (OR = 0.550; 95% CI: 0.106 - 2.860), walaupun perkaitan ini tidak signifikan secara statistik ( $p = 0.477$ ). Dalam kalangan ciri-ciri klinik-demografi yang dikaji, hanya jenis pembedahan (penyelamatan anggota badan vs. amputasi) menunjukkan perkaitan yang signifikan secara statistik dengan kelangsungan hidup ( $p = 0.002$ ), manakala umur, bangsa, jantina, tapak, patah patologi, kemoterapi, dan pembedahan untuk berulang tidak menunjukkan perkaitan yang signifikan. Protein NFATc1 semata-mata tidak berupaya menjadi faktor prognostik dalam osteosarkoma non-metastatik. Kajian lanjut disarankan untuk menerokai NFATc1 dalam kohort yang lebih besar bagi kedua-dua osteosarkoma non-metastatik dan metastatik.

**Kata kunci:** Imunohistokimia; kemandirian; NFATc1; osteosarkoma; prognosis

## ABSTRACT

Osteosarcoma, a primary malignant bone tumour, poses significant challenges in treatment and prognosis despite advancements in therapy. The expression of nuclear factor of activated T cells c1 (NFATc1) in osteosarcoma tissue remains underexplored as a potential therapeutic target. This study aimed to evaluate NFATc1 protein expression in human non-metastatic osteosarcoma tissue samples and its correlation with patient survival and its subtypes. Secondly, we examined the association between clinico-demographic details and survival of osteosarcoma patients. A cross-sectional study was conducted on 31 patients with primary non-metastatic osteosarcoma. Immunohistochemical analysis was used to assess NFATc1 protein expression in surgically resected tissue specimens. Demographic data, treatment details and follow-up records were collected for analysis. The study population had a mean age of 20.0 years, with Malay ethnicity being predominant. NFATc1 protein expression was positive in 25.8% of cases predominantly in conventional osteosarcoma (30.4%). No significant correlation was found between NFATc1 protein expression and patient survival ( $p = 0.685$ ) or osteosarcoma subtypes ( $p = 0.642$ ). NFATc1-positive patients had lower odds of long-term survival compared to NFATc1-negative patients (OR = 0.550; 95% CI: 0.106 - 2.860), although this association was not statistically significant ( $p = 0.477$ ). Among the clinico-demographic characteristics, only the type of surgery (limb salvage vs. amputation) showed a statistically significant association with survival ( $p = 0.002$ ), while age, race, gender, site, pathological fracture, chemotherapy and surgery for recurrence did not. Therefore, NFATc1 alone does not appear to be a useful prognostic biomarker in non-metastatic osteosarcoma. Further study is recommended to explore NFATc1 in larger cohort of both non-metastatic and metastatic osteosarcoma.

**Keywords:** Immunohistochemistry; NFATc1; osteosarcoma; prognosis; survival

## INTRODUCTION

Osteosarcoma is a primary malignant bone tumour characterised by the production of osteoid or immature bone matrix by the neoplastic cells (Fletcher et al. 2002). The incidence has a bimodal distribution, with the first peak occurring largely in children and adolescents aged 0 to 24 years, and a secondary peak occurring in individuals aged 60 years and above (Mirabello et al. 2009a). Global rates for the first peak were relatively comparable at an average of 4.3 per million in males and an average of 3.4 per million in females (Mirabello et al. 2009b). However, osteosarcoma in the elderly shows variable incidence rates, mostly arising from secondary pathology (Mirabello et al. 2009a). Primary osteosarcoma commonly arises from the metaphysis of long bones with distal femur being the most common site, followed by proximal tibia and humerus (Faisham et al. 2017). Indeed, treatment in these anatomical areas proves to be challenging as it carries high functional morbidity. The diagnosis largely depends on histological

evidence of varying degrees of osteoid deposition by the malignant osteoblastic-resembling cells (Wadhwa 2014).

Historically, the administration of efficient multiagent chemotherapy drugs in the previously surgical-only treatment of osteosarcoma and multidisciplinary approach have dramatically improved the overall survival rate from less than 20% to around 60-70% (Smeland et al. 2019). A recent large meta-analysis involving 6,661 patients reported an overall 5-year survival rate of approximately 64% (Papakonstantinou et al. 2024). Additionally, local data from various centers have shown 5-year survival rates ranging from 44% to 56% (Faisham et al. 2017; Yasin et al. 2020). Nevertheless, overall survival has remained relatively stagnant for the last few decades despite the advancement in imaging modalities, systemic treatment and surgical techniques. Because of this, in recent years, numerous studies have been largely seeking any potential therapeutic strategies at the molecular levels such as immunotherapy, cellular therapies

and tyrosine kinase inhibitors (Pilavaki et al. 2023).

In most cases, adjacent cortical destruction can be observed at the initial radiological imaging. Tumour tissue analysis showed the presence of active osteoclast within the tumour mass and at the resorption area between tumour and bone junction, suggesting the association between aggressive behaviour of osteosarcoma and enhanced activity of osteoclast (Avnet et al. 2008). Furthermore, receptor activator of nuclear factor kappa-B (RANK) and its ligand (RANKL) play major role in the terminal differentiation of osteoclast in the osteoclastogenesis (Li et al. 2000). Similarly, it has been reported that osteosarcoma cells also expressed RANK and its ligand to a certain degree (Chua et al. 2021). One of the essential downstream intracellular molecules effectively activated by RANKL in osteoclastogenesis is nuclear factor of activated T cells c1 (NFATc1) (Takayanagi et al. 2002).

Nuclear factor of activated T cell (NFAT) proteins is family of transcription factors essential in regulating various biological functions such as immune response, osteoclast, chondroid, cardiac and nervous system (Takayanagi et al. 2002). There are five subtypes of human NFAT protein; NFAT1 (or NFATc2), NFAT2 (also known as NFATc1), NFAT3 (or NFATc4), NFAT4 (or NFATc3) and NFAT5 (Pan et al. 2013; Zawawi et al. 2012). Recently, NFAT has been the main subject of numerous cancer studies, searching for potential therapeutic target in cancer treatment (Lin et al. 2023). NFATc1 and NFATc2 have been largely studied in breast cancer, pancreatic cancer, colorectal cancer, liver cancer, blood malignancies and lung cancer (Lin et al. 2023). They have been implicated for tumour development, invasion and metastasis (Oikawa et al. 2013). In recent literatures, NFATc2 gene had been reported in translocation and fusion gene with ESWR1 or FUS gene in rare aggressive small round sarcoma (Szuhai et al. 2009).

NFATc1 has been extensively studied in the field of osteoclast differentiation and proven to be vital for regulating terminal differentiation of osteoclasts (Zawawi et al. 2012). Although

the relationship between osteoclast and osteosarcoma has been established (Endo-Munoz et al. 2012), and NFATc1 expression had been recently studied to have impact on osteosarcoma cell growth and progression (Huang et al. 2021), the NFATc1 study in osteosarcoma is still limited and further validation in clinical settings is required. Given that NFATc1 functions predominantly as an intracellular transcription factor (Takayanagi et al. 2002), its accurate detection requires tissue-based assays such as immunohistochemistry or molecular analysis of tumour specimens. Although tissue sampling is more invasive than serum assays, it provides cell-specific assessment of pathway activity. Such tissue-level information may inform prognostic stratification and the development of targeted therapies in osteosarcoma. Previous study has shown that NFATc1 may involve in regulating cellular processes critical to cancer progression, including proliferation and migration (Lin et al. 2023). Therefore, it is possible that NFATc1 expression could have both positive and negative implications for osteosarcoma prognosis, depending on the specific context and molecular mechanisms involved.

In this study, we aimed to evaluate the expression of NFATc1 protein in the osteosarcoma tissue and their association with the survival of the patients. Additionally, we also performed sub-analysis on the association of clinico-demographic variables with the survival outcomes of the osteosarcoma patients. We hypothesised the NFATc1 protein will be expressed in osteosarcoma tissue, providing a valuable insight on their involvement in the development of osteosarcoma.

## MATERIALS AND METHODS

### Study Design

We conducted a retrospective cross-sectional study to explore the expression of NFATc1 from the tissue samples taken from surgical resection specimens of osteosarcoma patients. An immunohistochemical analysis was used

to evaluate the NFATc1 expression. This study protocol was approved by Institutional Ethical Review Board (Approval number: USM/JEPeM/18090418; dated 17 December 2018). The informed consent was waived due to the retrospective nature of the study and use of anonymised data.

### Participants

The study population included patients who were histopathologically diagnosed with osteosarcoma through biopsy and received treatment at our institution. These patients received chemotherapy and underwent definitive surgical intervention, which involved either limb salvage surgery or amputation. The surgical resected specimens were sent for final histopathological assessment including definitive final tissue diagnosis, percentage of tissue necrosis and histological margins. We included all age groups and non-metastases at the presentation. The selection of patients followed convenience sampling method. We conducted a search in the electronic database at our institution to identify eligible patients and to check the availability of tissue samples for staining. We excluded patients who had inadequate tissue samples for immunohistochemical staining and those with metastasis at presentation or during follow-up. No minimum follow-up period was specified in this study.

The patients underwent surveillance follow up for a minimum of 5 years with regular interval of computed tomography (CT) scan of lung and whole-body Technetium-99m-MDP bone scan to evaluate metastasis. These imaging surveillance modalities were conducted six-monthly during the first two years and annually for the remaining three years. The patients were considered to have completed their osteosarcoma treatment once they had finished the adjuvant chemotherapy (Faisham et al. 2017).

### Data Collection

We extracted the clinico-demographic data of

the eligible patients from the medical record and surgical database. These include age, ethnicity, gender, site of osteosarcoma, presence of pathological fracture, type of definitive surgery and subtype of osteosarcoma. The patients' survival data was obtained from the clinic follow-up records or, if unavailable, checked from our National Registration Department (NRD). We defined overall survival as the duration of time during which patients remained alive. This was further categorised into short-term and long-term survival, based on a cut-off value of 5 years; survival less than 5 years was considered short-term, and survival of 5 years or more was considered long-term. Subsequently, we performed stratified randomisation between survival groups to ensure a balanced number of patients in both groups for statistical analysis.

### Immunohistochemical Staining

We performed immunohistochemical staining on all representative archive tumour section obtained exclusively from the final resected surgical specimens from each patient to assess the expression of NFATc1. The initial diagnostic biopsy specimens from the eligible patients were not subjected to this staining procedure in this study.

Firstly, the tumour specimens were fixed in 10% formaldehyde solution and embedded in paraffin. Next, serial tissue section of 5-microns thick were cut and stained with haematoxylin and eosin. Subsequently, immunohistochemical techniques were carried out using avidin-biotin peroxidase complex method (Bratthauer 2010), utilising an LSAB2 kit (Dako, Glostrup, Denmark®). The primary antibody used in this study was monoclonal mouse anti-human NFATc1 (clone 7A6, sc-7294) from Santa Cruz Biotechnology, Santa Cruz, CA, diluted to 1:50, followed by counterstaining by hematoxylin. Two independent observers, both blinded to the clinico-pathological details, examined the immunostaining of tumour nuclear area in two randomly chosen microscopic fields using a standard light microscope at 40x100

magnification. Images of both stained and unstained cells were captured. The positivity of NFATc1 expression was confirmed based on the presence of stained cells within these microscopic fields, irrespective of the intensity of their expression (weak, moderate or strong).

### Sample Size

As there were no prior similar studies on NFATc1 expression in osteosarcoma to support formal power calculation, we adopted an exploratory pilot study and used a target sample size of minimum 30 patients, in line with pilot-study recommendation (Browne 1995). The sample size was chosen for feasibility and estimating prevalence and variability rather than formal power calculation.

### Data Analysis

The clinico-demographic characteristics of the patients and presence of NFATc1 were analysed using descriptive statistics. Numerical data were expressed as mean and standard deviation, while categorical data were presented as proportion. Associations between clinico-demographic data and survival status, as well as NFATc1 expression with survival status and osteosarcoma subtypes, were determined using the Pearson chi-square test or Fisher's Exact test. Additionally, binary logistic regression analysis was performed to further examine the association between NFATc1 expression and long-term survival outcomes. All statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) version 24 (IBM Corp, Armonk, Ny, USA), with a  $p < 0.05$  considered statistically significant.

## RESULTS

A total of 192 patients with a histopathologically confirmed diagnosis of primary osteosarcoma were treated at Hospital Pakar Universiti Sains Malaysia between January 1998 and December 2008. Out of 192 patients, 31 patients were eligible for inclusion in this study. The remaining

were excluded due to presence of metastasis on presentation, incomplete medical data and insufficient tissue samples or follow-up records.

### Baseline Clinico-demographic Characteristics and Association with Survival Outcomes

The baseline demographic characteristics of primary non-metastatic osteosarcoma patients ( $n = 31$ ) are presented in Table 1. Mean, standard deviation and percentage are used to express the results. The patients had a mean age of 20.0 years, with a standard deviation of 11.74. Our study population showed Malay constituted the largest portion of ethnicity (87.1%). The pathological bone was most frequently seen in the tibia (41.9%), while the subtypes of osteosarcoma yielded conventional osteosarcoma in 74.2% cases. Only the small proportion had pathological fracture (6.5%) and surgery for recurrence (6.5%). Table 2 details the distribution of demographic and clinical characteristics stratified by survival outcomes. Type of surgery showed a statistically significant association with survival ( $p = 0.002$ ), where all the limb-salvage patients survived beyond five years. No other clinico-demographic variable demonstrated significant association with survival.

### Expression of NFATc1 in Osteosarcoma and Comparison among the Subtypes

Out of 31 patients, 23 showed a negative expression of NFATc1 in their tissue samples. Eight patients (25.8%) showed positive NFATc1 expression, in which seven were conventional osteosarcoma subtype and one case of small cell osteosarcoma subtype. The summary of NFATc1 expression findings was presented in Table 3, Table 4, Figure 1 and Figure 2.

### Association of NFATc1 Expression and Survival Status in Osteosarcoma Patients

Chi-square analysis was performed to assess the association between NFATc1 expression and survival status among osteosarcoma patients.

TABLE 1: The baseline clinico-demographic characteristics of the primary non-metastatic osteosarcoma (n = 31)

| Variables                      | Mean (SD)    | n (%)     |
|--------------------------------|--------------|-----------|
| Age                            | 20.0 (11.74) |           |
| Race                           |              |           |
| Malay                          |              | 27 (87.1) |
| Chinese                        |              | 3 (9.7)   |
| Indian                         |              | 1 (3.2)   |
| Gender                         |              |           |
| Female                         |              | 12 (38.7) |
| Male                           |              | 19 (61.3) |
| Survival                       |              |           |
| Short Term                     |              | 16 (51.6) |
| Long Term                      |              | 15 (48.4) |
| Site                           |              |           |
| Acetabulum                     |              | 2 (6.5)   |
| Calcaneum                      |              | 1 (3.2)   |
| Femur                          |              | 8 (25.8)  |
| Fibula                         |              | 1 (3.2)   |
| Humerus                        |              | 3 (9.7)   |
| Ilium                          |              | 1 (3.2)   |
| Radius                         |              | 1 (3.2)   |
| Tibia                          |              | 13 (41.9) |
| Ulna                           |              | 1 (3.2)   |
| Pathological fracture          |              |           |
| No                             |              | 29 (93.5) |
| Yes                            |              | 2 (6.5)   |
| Chemotherapy                   |              |           |
| No                             |              | 1 (3.2)   |
| Yes                            |              | 30 (96.8) |
| Timing of chemotherapy         |              |           |
| Neoadjuvant                    |              | 22 (73.3) |
| Adjuvant                       |              | 30 (100)  |
| Type of surgery                |              |           |
| Amputation                     |              | 8 (25.8)  |
| Limb salvage surgery           |              | 23 (74.2) |
| Surgery for recurrence         |              |           |
| No                             |              | 29 (93.5) |
| Yes                            |              | 2 (6.5)   |
| Subtypes of osteosarcoma (OS): |              |           |
| Conventional                   |              | 23 (74.2) |
| Parosteal                      |              | 1 (3.2)   |
| Small cell                     |              | 1 (3.2)   |
| Chondroblastic                 |              | 4 (12.9)  |
| Fibroblastic                   |              | 2 (6.4)   |

The results showed no statistically significant correlation between NFATc1 positivity and survival group classification (p = 0.685).

Similarly, the association between NFATc1 expression and osteosarcoma histological subtypes was also not statistically significant (p = 0.642). To further explore the relationship between NFATc1 expression and long-term survival, a binary logistic regression analysis was conducted. The analysis showed that patients with positive NFATc1 expression had lower odds of long-term survival compared to those with negative expression (OR = 0.550; 95% CI: 0.106-2.860). However, this association was not statistically significant (p = 0.477). These association analyses were presented in Table 4,5 and 6.

DISCUSSION

Osteosarcoma remains a significant clinical challenge, as the overall survival rate for high-grade osteosarcoma has plateaued despite advances in treatment over recent decades (Pilavaki et al. 2023). The recurrence rate has been reported to be as high as 30-35%, either as local or distant metastasis (Bielack et al. 2009) with five year overall survival of 28% (Bacci et al. 2005). Given this, the current research emphasises the development of targeted therapies to identify potential therapeutic targets for both newly diagnosed and recurrent osteosarcoma cases. In this study, we investigated the expression of NFATc1 using immunohistochemical analysis in the surgically resected tissue specimens from a cohort of non-metastatic osteosarcoma patients, aiming to correlate NFATc1 expression with survival outcomes and clinico-demographic variables. We found that NFATc1 protein expression was limited in 25.8% of cases, primarily within the conventional osteosarcoma subtype, and demonstrated a negative association between NFATc1 positivity and the survival and osteosarcoma subtypes.

NFATc1 is a transcription factor that plays a crucial role in the regulation of immune response and bone homeostasis (Takayanagi et

TABLE 2: Clinico-demographic characteristics of non-metastatic osteosarcoma patients and their association with survival status (short-term &lt;5 years vs. long-term ≥5 years)

| Variables               | Total, n (%) | Survivals <sup>§</sup>        |                              | p-value <sup>†</sup> |
|-------------------------|--------------|-------------------------------|------------------------------|----------------------|
|                         |              | Short term, n (%)<br>(n = 16) | Long term, n (%)<br>(n = 15) |                      |
| Age (years)             |              |                               |                              | 0.704*               |
| Below 20                | 21 (67.7)    | 10 (62.5)                     | 11 (73.3)                    |                      |
| Above 20                | 10 (32.3)    | 6 (37.5)                      | 4 (26.7)                     |                      |
| Race                    |              |                               |                              | 1.000*               |
| Malay                   | 27 (87.1)    | 14 (87.5)                     | 13 (86.6)                    |                      |
| Non-Malay               |              |                               |                              |                      |
| Chinese                 | 3 (9.7)      | 2 (12.5)                      | 1 (6.7)                      |                      |
| Indian                  | 1 (3.2)      | 0 (0.0)                       | 1 (6.7)                      |                      |
| Gender                  |              |                               |                              | 0.552                |
| Female                  | 12 (38.7)    | 7 (43.7)                      | 5 (33.3)                     |                      |
| Male                    | 19 (61.3)    | 9 (56.3)                      | 10 (66.7)                    |                      |
| Site                    |              |                               |                              | 0.226*               |
| Extremities             |              |                               |                              |                      |
| Femur                   | 8 (25.8)     | 2 (12.5)                      | 6 (40.0)                     |                      |
| Tibia                   | 13 (41.9)    | 8 (50.0)                      | 5 (33.3)                     |                      |
| Fibula                  | 1 (3.2)      | 0 (0.0)                       | 1 (6.7)                      |                      |
| Calcaneum               | 1 (3.2)      | 1 (6.25)                      | 0 (0.0)                      |                      |
| Humerus                 | 3 (9.7)      | 2 (12.5)                      | 1 (6.7)                      |                      |
| Radius                  | 1 (3.2)      | 0 (0.0)                       | 1 (6.7)                      |                      |
| Ulna                    | 1 (3.2)      | 0 (0.0)                       | 1 (6.7)                      |                      |
| Pelvis                  |              |                               |                              |                      |
| Acetabulum              | 2 (6.5)      | 2 (12.5)                      | 0 (0.0)                      |                      |
| Ilium                   | 1 (3.2)      | 1 (6.25)                      | 0 (0.0)                      |                      |
| Subtype of osteosarcoma |              |                               |                              | 0.433*               |
| Conventional            | 23 (74.2)    | 13 (86.7)                     | 10 (66.7)                    |                      |
| Others                  |              |                               |                              |                      |
| Parosteal               | 1 (3.2)      | 1 (6.3)                       | 0 (0.0)                      |                      |
| Small cell              | 1 (3.2)      | 0 (0.0)                       | 1 (6.7)                      |                      |
| Chondroblastic          | 4 (12.9)     | 2 (12.5)                      | 2 (13.3)                     |                      |
| Fibroblastic            | 2 (6.5)      | 0 (0.0)                       | 2 (13.3)                     |                      |
| Chemotherapy            |              |                               |                              | 0.484*               |
| Yes                     | 30 (96.8)    | 16 (100.0)                    | 14 (93.3)                    |                      |
| No                      | 1 (3.2)      | 0 (0.0)                       | 1 (6.7)                      |                      |
| Pathological fracture   |              |                               |                              | 0.484*               |
| Yes                     | 2 (6.5)      | 2 (12.5)                      | 0 (0.0)                      |                      |
| No                      | 29 (93.5)    | 14 (87.5)                     | 15 (100.0)                   |                      |
| Type of surgery         |              |                               |                              | 0.002*†              |
| Limb salvage            | 23 (74.2)    | 8 (50.0)                      | 15 (100.0)                   |                      |
| Amputation              | 8 (25.8)     | 8 (50.0)                      | 0 (0.0)                      |                      |
| Surgery for recurrence  |              |                               |                              | 0.484*               |
| Yes                     | 2 (6.5)      | 2 (12.5)                      | 0 (0.0)                      |                      |
| No                      | 29 (93.5)    | 14 (87.5)                     | 15 (100.0)                   |                      |

\*Fisher Exact Test was applied due to cell counts &lt;5 in one or more groups.

§Percentages in short-term and long-term columns are column percentages.

†Statistically significant at p &lt; 0.05

TABLE 3: NFATc1 expression in the osteosarcoma tissue (n = 31)

| NFATc1                                         | n (%)     |
|------------------------------------------------|-----------|
| Negative                                       | 23 (74.2) |
| Positive                                       | 8 (25.8)  |
| NHATc1: Nuclear factor of activated T cells c1 |           |

TABLE 4: Association between NFATc1 expression and osteosarcoma subtypes

| Osteosarcoma Subtype        | NFATc1 Expression |                 | p-value <sup>†</sup> |
|-----------------------------|-------------------|-----------------|----------------------|
|                             | Negative, n (%)   | Positive, n (%) |                      |
| Conventional (n = 23)       | 16 (69.6)         | 7 (30.4)        | 0.642*               |
| Others <sup>a</sup> (n = 8) | 7 (87.5)          | 1 (12.5)        |                      |

NHATc1: Nuclear factor of activated T cells c1  
<sup>a</sup>Others include parosteal, small cell, chondroblastic, and fibroblastic osteosarcoma  
\*Fisher Exact Test was applied due to cell counts <5 in one group  
<sup>†</sup>Statistically significant at p < 0.05

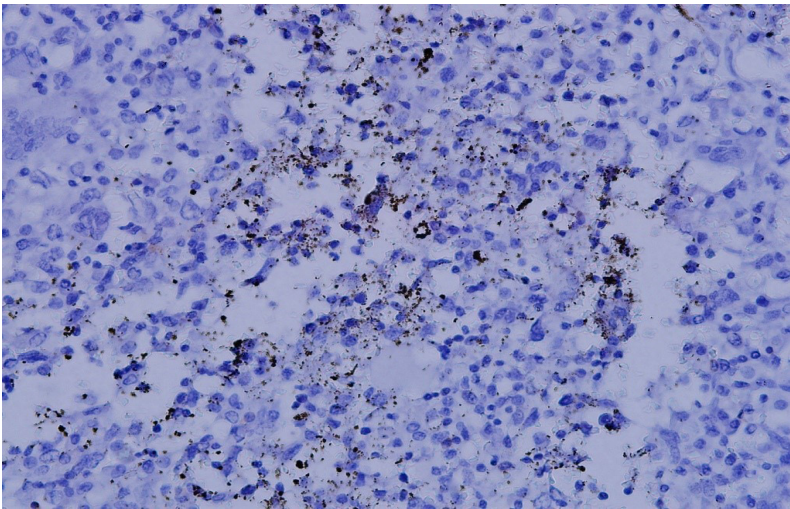


FIGURE 1: Positive NFATc1 from a 16-year-old boy with osteoblastic osteosarcoma distal femur. After the surgical resection, he underwent 6 courses chemotherapy with 95% necrosis. NFATc1 was found to be positive as shown, and he survived 10 years disease free (NFATc1: Nuclear factor of activated T cells c1)

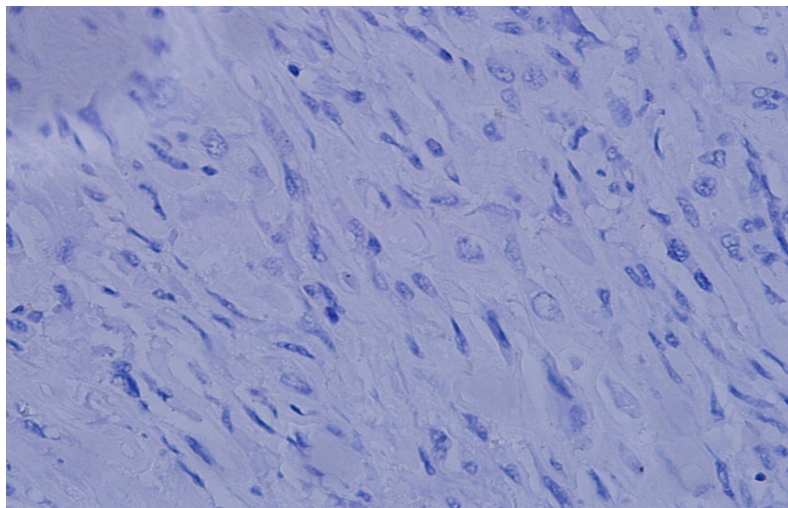


FIGURE 2: Negative NFATc1. A 16-year-old girl with proximal humerus osteoblastic osteosarcoma. After the surgical resection, she underwent 6 courses of chemotherapy and wide resection margin and endoprosthesis. Died 2 years later due to multiple pulmonary metastases (NFATc1: Nuclear factor of activated T cells c1)

TABLE 5: Association between NFATc1 expression and survival status

| Survival Status <sup>b</sup> | NFATc1 Expression |                 | p-value <sup>†</sup> |
|------------------------------|-------------------|-----------------|----------------------|
|                              | Negative, n (%)   | Positive, n (%) |                      |
| Short-term (n = 16)          | 11 (68.7)         | 5 (31.3)        | 0.685*               |
| Long-term (n = 15)           | 12 (80.0)         | 3 (20.0)        |                      |

NHATc1: Nuclear factor of activated T cells c1  
<sup>b</sup>Survival status defined as short-term : survival less than 5 years; long-term : survival 5 years or more  
<sup>\*</sup>Fisher's Exact Test was applied due to cell counts <5 in one group  
<sup>†</sup>Statistically significant at p < 0.05

TABLE 6: Binary Logistic Regression for predicting long-term survival based on NFATc1 Expression

| Predictor       | Long-term Survival <sup>c</sup> |               | p-value* |
|-----------------|---------------------------------|---------------|----------|
|                 | Odds Ratio (OR)                 | 95% CI for OR |          |
| NFATc1 Positive | 0.550                           | 0.106 – 2.860 | 0.477    |

NHATc1: Nuclear factor of activated T cells c1; OR: Odds ratio; CI: Confidence interval  
<sup>c</sup>Long-term survival defined as 5 years or more; reference category = NFATc1 negative  
<sup>\*</sup>Statistically significant at p < 0.05

al. 2002). The activation of most NFAT proteins is dependent on the calcium-mediated calcineurin/NFAT signaling pathway. This pathway is initiated by cell surface receptor activation, which leads to phospholipase C activation, intracellular calcium ion ( $\text{Ca}^{2+}$ ) release, calcineurin activation, dephosphorylation of NFATc1, and its subsequent nuclear translocation to regulate gene expression for various biological functions (Lin et al. 2023). Previous research has indicated that aberrant NFATc1 expression may be associated with the development of cancer (Buchholz et al. 2006; Neal & Clipstone 2003). It has been shown that continuous and unregulated NFATc1 activity can inhibit adipocyte differentiation and induce tumorous characteristics in non-immune cell types (Neal & Clipstone 2003). Another study on pancreatic cancer tissues found that NFATc1 protein was significantly overexpressed, and the  $\text{Ca}^{2+}$ /calcineurin/NFATc1 signaling pathway was ectopically activated, leading to the downstream effect of c-myc upregulation, which caused cell proliferation and enhanced malignant potential (Buchholz et al. 2006). Another recent research has shown that NFATc1 plays a crucial role in cancer cell proliferation through an alternative NFAT-MDM2-p53 signaling pathway. In their study, decreased FKBP52 protein was found to downregulate MDM2 expression via NFAT proteins, specifically NFATc1 and NFATc2. This, in turn, led to upregulation of the p53 protein, ultimately reducing cancer cell proliferation (Hanaki et al. 2024).

NFAT proteins, including NFATc1 and NFATc2, have been found to play a pivotal role in the tumorigenesis, invasion, and metastasis in various cancers, such as breast carcinoma, blood malignancies, pancreatic cancer, colorectal and gastric cancer (Lin et al. 2023; Oikawa et al. 2013; Shen et al. 2021; Szuhai et al. 2009; Wang et al. 2024). Recent evidence also implicates NFAT proteins in sarcoma, with ESWR1-NFATc2 fusion genes identified in aggressive sarcoma cells correlating with poor clinical outcomes (Giuliani et al. 2014; Perret et al. 2020; Szuhai et al. 2009). While some studies suggest NFATc1 involvement in osteosarcoma development and progression,

the evidence remains limited of this specific type of sarcoma (Giuliani et al. 2014; Huang et al. 2021). Huang et al. (2021) reported increased NFATc1 expression in human osteosarcoma specimens and demonstrated RCAN1.4 suppresses osteosarcoma growth and metastasis via calcineurin/NFAT signaling pathway. Similarly, Giuliani et al. (2014) found that P2X7 receptor activation significantly elevated nuclear NFATc1 levels. In contrast to these findings, our study observed limited NFATc1 expression (8/31 cases, 25.8%) in non-metastatic osteosarcoma specimens. This discrepancy may stem from differences in the analysed cohorts, as prior studies did not specify the metastatic status of their osteosarcoma samples (Giuliani et al. 2014; Huang et al. 2021). Indeed, a study on viral-induced osteosarcoma cells showed NFATc1 activation promotes a metastatic phenotype through matrix metalloproteinase (MMP)-2 upregulation, which displayed invasiveness and metastatic capability (Velupillai et al. 2010). This aligns with our findings and suggests that NFATc1 expression may be temporarily and differentially regulated during osteosarcoma progression, potentially increasing during the transition to a metastatic state. Additionally, a study on temporal gene expression in osteoclastogenesis has shown consistently high NFAT2, also known as NFATc1, levels within 24 hours of RANKL stimulation, further supporting the context-dependent nature of NFATc1 expression (Ishida et al. 2002).

The present study focused on a cohort of the non-metastatic osteosarcoma samples, aiming to assess the potential of NFATc1 protein as an early biomarker signaling the transition to metastatic disease. However, our findings indicate that NFATc1 protein expression, in isolation, may not serve as a reliable early prognostic biomarker in non-metastatic osteosarcoma. We observed a non-significant association between NFATc1 expression and both patient survival and osteosarcoma subtypes. Nevertheless, PROSER2, a proline- and serine-rich protein, has been reported as a potential negative prognostic biomarker that modulates NFATc1 activity in the Wnt/ $\text{Ca}^{2+}$ /NFATc1 signalling pathway (Li et al.

2022). We highlighted PROSER2 because it has direct prognostic data in osteosarcoma cohorts and mechanistic link to NFATc1 signalling (Li et al. 2022), aligning with our study aim. This raises the possibility that NFATc1 could function as an indirect biomarker, reflecting PROSER2-related activity in osteosarcoma progression due to their functional relationship. This also suggests that the role of NFATc1 may be more subtle, potentially acting in concert with other factors in the tumour microenvironment (Liu et al. 2022). Future research, incorporating larger patient cohorts including both non-metastatic and metastatic cases and exploring the interplay between NFATc1 and other relevant proteins, such as PROSER2 and NR4A1, is important to fully define the utility of NFATc1 as a prognostic indicator in osteosarcoma (Li et al. 2022; Liu et al. 2022).

In our cohort, the most prevalent subtype was conventional osteosarcoma, further classified into osteoblastic, fibroblastic and chondroblastic subgroups based on the predominant matrix morphology (Fletcher et al. 2002). We observed NFATc1 positivity in seven out of eight conventional osteosarcoma cases, with approximately 30% of our 23 conventional osteosarcoma samples displaying NFATc1 expression. NFATc1 is a key downstream effector of RANKL signalling, leading to osteoclast differentiation and bone resorption in the tumour microenvironment (Takayanagi et al. 2002). RANK ligands are produced by both osteoblasts and osteosarcoma cells (Chua et al. 2021). While conventional osteosarcoma exhibits osteoblastic features, including RANKL production (Akiyama et al. 2008), our findings suggest the need for further investigation into the potential interplay mechanisms governing NFATc1 expression in this context. Specifically, future studies should investigate potential feedback loops or alternative signalling pathways that may modulate NFATc1 activity in conventional osteosarcoma.

We also examined the association of NFATc1 with survival outcomes and histological subtypes and found no statistically significant association. To further explore this relationship, NFATc1-

positive patients had lower odds of long-term survival compared to NFATc1-negative patients, though this finding did not reach statistical significance. This suggests that, in our sample, NFATc1 expression may not serve as a reliable prognostic marker for survival in osteosarcoma. The absence of statistical significance is likely due to the limited sample size, which restricts the power to detect potentially meaningful associations. In contrast, our study found a statistically significant association between surgical type and survival status, with patients undergoing limb salvage surgery demonstrating better long-term survival than those who had amputation. This aligns with meta-analysis by Papakonstantinou et al. (2024) reporting that osteosarcoma patients treated with limb salvage surgery had nearly double the 5-year overall survival rate (Papakonstantinou et al. 2024). This likely reflects the surgical selection and tumour biology, as limb salvage is typically chosen in localised and chemotherapy-responsive tumour.

This study has some limitations that could be addressed in future research. Firstly, the study population was limited to non-metastatic cases at initial diagnosis, so the findings should be interpreted in the context of non-metastatic disease and may not apply to metastatic counterparts. Secondly, although we met our feasibility target ( $n = 31$ ), the study was not powered for between-group analyses; the small sample size (particularly the NFATc1-positive cases) may have limited the ability to detect statistically significant associations. Moreover, NFATc1 undergoes transient, stimulus-dependent nuclear activation, such as during osteoclast differentiation (Takayanagi et al. 2002); therefore, single time-point tissue sampling may underestimate the prevalence of active NFATc1 and partly explain the low positivity rate observed in our study. Additionally, analysing tissue samples collected at different time-points, such as initial biopsy and surgical resection, may reveal temporal and differential patterns of NFATc1 expression over the course of the disease.

## CONCLUSION

This study found that NFATc1 protein expression in this non-metastatic cohort was infrequent (25.8%, 8/31 samples). Although most NFATc1-positive cases were conventional subtype, distributions by subtype and survival did not differ significantly. NFATc1 alone does not appear to be a useful prognostic biomarker, and given the small number of positive cases, these findings should be considered exploratory. Further research in larger cohorts, including both non-metastatic and metastatic cases, is recommended to better elucidate the role of NFATc1 in osteosarcoma.

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