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Original Article

Histopathological, immunohistochemical, and image analytic parameters characterizing the stromal component in primary and recurrent giant cell tumor of bone

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ABSTRACT

Giant cell tumor (GCT) of bone is a benign locally aggressive tumor whose biological behavior is unpredictable. Currently, there are no definitive clinical, histological, biochemical, or immunological parameters that can predict its behavior. This study was undertaken to examine whether delineation of reactive and neoplastic stromal component of GCT can help in this regard. 55 cases of GCT (30 primary, 25 recurrent) were subjected to histopathological grading, immunohistochemistry, and image analysis. Spindling of stroma was more frequent in recurrent GCT with 64% cases having more than 50% spindled stroma ($p < 0.001$). Number of mitosis/10 HPF and higher grade were more in recurrent GCT. Mean percentage positivity for CD68 (38.36%) and α 1-ACT (70.86%) was higher in primary than recurrent GCT. PCNA and MiB-1 labeling indices were higher in recurrent (42.62% and 9.18%, respectively) than in primary group (24.75% and 7.7%, respectively). A single numerical parameter encompassing stromal cell population and its proliferation was derived as ratio of PCNA/CD68 and PCNA/ α 1-ACT. Both ratios were higher in recurrent (0.81 ± 0.38 ; 1.58 ± 1.50) than in primary GCT (0.58 ± 0.62 ; 0.34 ± 0.29) ($p = 0.002$; 0.01). On image analysis, parameters significantly different between the two groups were nuclear area and nuclear integrated optical density. It was thus concluded that recurrent GCT shows higher grade, increased mitosis, more spindling, fewer reactive components, and higher proliferation than primary GCT. Delineation of reactive component (α 1-ACT positive) and proliferating component (PCNA positive cells) using immunohistochemistry with calculation of the PCNA/ACT ratio delivers more information than image analysis.

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1. Introduction

Giant cell tumor (GCT) of bone is seen commonly in the 20 to 50-year age group, and is more common in females.¹ It occurs in the epimetaphyseal ends of long bones, with lower end of femur, upper end of tibia, lower ends of radio-ulna, and upper end of humerus being common sites.² The histopathology of GCT is characterized by numerous multinucleated giant cells, mononuclear stromal cells, and frank hemorrhage.³ A range of morphological appearances are seen from specimen to specimen and from section to section.⁴ The histogenesis and the relationship between multinucleated giant cells and mononuclear stromal cells of GCT have been recently better understood with both neoplastic and non-neoplastic reactive cells considered to be contributing to the stromal component. The neoplastic stromal cells are believed to arise from primitive osteoblastic cells while the reactive component is believed to arise from bone marrow-derived monocytic cells. Release of specific cytokines such as RANKL, M-CSF, IL-1, IL-6, and PGE2 by the neoplastic cells recruits reactive cells to the tumor stroma.⁵ Fusion of these reactive mononuclear cells forms the characteristic multinucleated giant cells. The reactive cells express LCA, CD68, α 1ACT, muramidase, MMP-1, MMP-9, and UCHM1, strongly suggesting that they are of hematopoietic origin.⁶

One of the most important characteristics of GCT is its unpredictable biological behavior. GCTs that are deemed to be histologically benign can recur and even metastasize. Many histopathological and radiological characteristics have been evaluated as indicators of prognosis.^{7,8} Previous studies have not found any significant difference in the proliferation of neoplastic cells between primary and recurrent GCT.^{4,9,10}

The present study was undertaken to evaluate whether delineation of reactive and neoplastic component of GCT using immunohistochemistry can help in deriving histopathological performance indicators which differ in primary and recurrent GCT. Nuclear morphometry of immunostained slides by image analysis was used for more accurate measurement and to assess the utility of this approach.

2. Material and methods

The study was conducted in the form of a retrospective and prospective evaluation including 55 cases of GCT (30 primary; 25 recurrent GCT) in the Departments of Orthopedic Surgery and Pathology, All India Institute of Medical Sciences, from 2004 to 2008. The cases were segregated into primary and recurrent GCT. The first group included primary cases presenting for the first time and without evidence of aggressive behavior in whom either a curettage or en-block resection was performed. The second group constituted recurrent cases of GCT in which a primary resection had been performed earlier at some other center and the tumor had recurred. Histopathological details of primary tumor resection were not available. A minimum follow-up of 6 months was available after the surgery was performed.

2.1. Histopathological analysis

All the H&E stained slides were reviewed by two observers for morphological features and presence of extra skeletal invasion. Nuclear grading was done (Grade 1 with stromal cells lacking pleomorphism and hyperchromasia, Grade 2 having stromal cells showing nuclear enlargement and pleomorphism without significant hyperchromasia or atypical mitosis, Grade 3 showing marked pleomorphism accompanied by hyperchromasia and presence of occasional atypical mitosis).⁴ The number of mitotic figures was counted per 10 high power fields in the areas with highest mitotic activity. Percentage of spindle cells in the stroma was calculated by counting 500 cells under high power with examination of at least 10 fields.

2.2. Immunohistochemistry

Sections were cut from representative blocks of formalin fixed paraffin embedded tissue on poly-L-lysine coated slides. Sequential slides cut from the same block were subjected to immunohistochemistry for CD68, α 1-Antichymotrypsin (α 1-ACT), MIB-1, PCNA, VEGF, and p53 with LSAB2 streptavidin-biotin horseradish peroxidase detection with Diaminobenzidine (DAKO corporation, USA). Positivity was assessed by counting a minimum of 500 cells for each marker.

2.3. Image analysis

Morphometry was performed using Image Pro-Plus Analysis Software version 7.0 (Media Cybernetics Corporation, USA) and a 12-bit digital camera (Media Cybernetics Corporation, USA with inbuilt image grabber card). Multiple representative images were captured from α 1-ACT and CD68-stained slides of the primary and recurrent cases of GCT. Enough images were captured in each case to permit analysis of at least 50 nuclei each of positive and negative immunostained cells per case. The software delivered the values of various parameters, which are enumerated in [Table 1](#).

2.4. Statistical analysis

Univariate analyses for all parameters were done by parametric 'Students t-test' and 'Mann-Whitney' non-parametric test. *p*-Value for proportion was calculated by applying 'Fishers exact test'. Logistic regression was done by 'Chi-square test' to calculate the 'odds ratio' with 95% confidence limit and its *p* value. Multivariate analyses by multiple logistic regressions were employed to identify independent predictors of recurrence in GCT.

3. Results

3.1. Clinical characteristics

Both the groups had comparable age distribution with mean age in primary GCT of 29 years (range 15–59 years) and that in recurrent GCT of 27.8 years (range 15–45 years). Male:female ratio in primary GCT was 2:1, whereas in recurrent GCT it was 1:1.08. Overall male:female ratio was 1.39:1. The sites of

Table 1 – Histopathological, immunohistochemical, and image analytic parameters in primary and recurrent giant cell tumor of bone.

Parameters assessed	Primary GCT (N = 30)	Recurrent GCT (N = 25)	p-Value
Histopathological analysis (percentage of cases)			
Spindled stroma >50% of tumor area	13.33%	64.0%	<0.001
Nucleoli in >50% of stromal cells	13.33%	32%	0.41
Mitotic count			
0–4/10 hpf	86.66%	68%	0.05
5–9/10 hpf	13.33%	20%	
>10/10 hpf	0%	12%	
Histological grade			
Grade 1	46.67%	20.00%	0.03
Grade ≥ 2	53.33%	80.00%	
Immunohistochemistry (mean percentage positivity in stromal cells)			
CD 68	38.36%	30.32%	0.19
α1-Antichymotrypsin	70.86%	58.41%	0.05
MIB-1	7.7%	9.18%	0.56
PCNA	24.75%	42.62%	0.03
PCNA/CD68 ratio >0.56	25%	81.25%	<0.002
PCNA/α1-ACT ratio >0.47	25%	87.50%	<0.01
Image analysis (mean value of group)			
Nuclear area in μm^2			
CD 68 +ve cells	32.62	35.22	0.24
CD 68 –ve cells	35.63	38.14	0.15
ACT +ve cells	33.65	34.79	0.45
ACT –ve cells	36.75	37.48	0.55
Nuclear integrated optical density			
CD 68 +ve cells	4883	8407	NS
CD 68 –ve cells	6406	9694	NS
ACT +ve cells	4671	3767	0.04
ACT –ve cells	6032	5155	0.07

involvement in primary and recurrent groups were distal femur (8 and 6, respectively), proximal tibia (9 and 9, respectively), proximal femur (2 and 4, respectively), distal radius (7 and 4, respectively), and others (4 and 2, respectively).

3.2. Histopathological analysis

Spindle cells predominated in recurrent cases, comprising more than 50% of all stromal cells in 64% of the cases. The percentage of spindled stromal cells in primary GCT (32.33 ± 20.80) was lower than in recurrent GCT (51.20 ± 22.05) ($p < 0.001$). The percentage of spindle cells was separately scored as 1+ for less than 25%, 2+ for 25–49%, and 3+ for 50% or more which highlighted the difference between primary and recurrent GCT (Fig. 1). The recurrent group had a higher rate of mitosis with the mean number of mitosis/10 HPF (4.00 ± 3.82) being higher than the primary GCT group (2.5 ± 1.53), which was statistically significant ($p = 0.05$). Recurrent GCT also showed higher grades. Grade 3 was only seen in recurrent GCT (Fig. 2). Statistical analysis showed that lower grade (Grade 1) was more likely in primary GCT and higher grade (Grades 2 and 3) were more likely in recurrent GCT ($p = 0.03$).

Fig. 1 – Stacked bar chart showing percentage of primary and recurrent GCT cases showing spindled stroma.

3.3. Immunohistochemistry

Primary GCT showed higher percentage of CD68 and α1-ACT positive stromal cells (38.36 ± 22.15 ; 70.86 ± 18.13 , respectively) than the recurrent group (30.32 ± 22.35 ; 58.42 ± 28.09 , respectively); however, the difference ($p = 0.19$; $p = 0.05$, respectively) was significant only for α1-ACT. The mean MIB-1 labeling index in primary cases was lower at 7.7% (range 1–21%). In recurrent cases, it was 9.18% (range 2–32%). The difference was not significant ($p = 0.56$). PCNA positivity in primary group was lower (mean 24.75%) than recurrent GCT (mean 42.62%), which was significant ($p = 0.02$). Percentage positivity of p53 in both primary and recurrent cases was less than 10% in all Grade 1, Grade 2, and Grade 3 tumors and was considered as negative. VEGF expression was similar between primary and recurrent cases and was not significant. The mean of PCNA/CD68 ratio in recurrent GCT was 0.81, which was significantly higher compared to primary GCT with mean ratio of 0.58 ($p = 0.002$). PCNA/α1-ACT ratio in recurrent group (mean = 1.58) was also higher compared to primary group (mean = 0.34) and was statistically significant ($p = 0.01$). MIB-1/CD68 ratio in recurrent group (mean = 1.06) was higher than primary group (mean = 0.95) but was not significant ($p = 0.40$). MIB-1/α1-ACT in recurrent group (mean = 0.27) was significantly higher than in primary (mean = 0.12) group ($p = 0.02$) (Fig. 3a–c).

Fig. 2 – Stacked bar chart showing percentage of cases with histological Grades 1, 2, and 3 in primary and recurrent GCT groups.

Fig. 3 – (A) Primary giant cell tumor of bone showing positive staining for α 1-ACT in >25% of stromal cells (IHC 400 $\times$). (B) Recurrent giant cell tumor of bone showing positive staining for CD 68 in <25% of stromal cells (IHC 400 $\times$). (C) Recurrent giant cell tumor of bone for showing positive staining for PCNA in >25% of stromal cells (IHC 200 $\times$). (D) Nuclei of ACT negative cells outlined for image analysis.

3.4. Image analysis

CD 68 as well as α 1-ACT positive and negative stromal cells showed similar nuclear size parameters (nuclear area, perimeter, diameter, fractal dimension, axis major/axis minor, aspect ratio, radius ratio) in both primary and recurrent groups. CD 68 negative stromal cells showed slightly more nuclear density than CD 68 positive cells in both primary and recurrent groups but this was not significant. Similar results were observed with α 1-ACT positive and negative cells. Other features analyzed, such as fractal dimension, heterogeneity, and clumpiness, were not significant (Fig. 3d).

3.5. Multivariate analysis

Multivariate linear regression analysis was performed for the parameters, which were found to be significant. PCNA/ α 1-ACT ratio and PCNA/CD68 ratio were the two most important independent factors, which were different between primary and recurrent GCT. When PCNA/ α 1-ACT ratio alone was >0.47, the odds of it being a recurrent tumor were 12.3 with 95% confidence interval 1.80–83.59 ($p=0.01$). Similarly, when PCNA/CD68 ratio alone was >0.56, the odds of it being a recurrent tumor were 6.7 with 95% confidence limit 1.06–42.27. When PCNA/ α 1-ACT ratio >0.47 and PCNA/CD68 ratio >0.56 together were taken as classification criteria, 84% of tumors

could be correctly classified with a sensitivity of 85.71% and specificity of 81.82%. All the other parameters, which were significant in univariate analysis, were interdependent and were not individually significant on multivariate analysis between the two groups.

4. Discussion

GCT represents 5% of bone tumors in which recurrence, malignant transformation, and metastasis can occur.¹¹ Recurrence is common being seen in 33–50% in earlier studies and is higher with simple curettage and grafting as the treatment method.^{12,13} Radiologically, it is usually lytic and expansile without prominent peripheral sclerosis and periosteal reaction.¹⁴ Radiological classification is the most important prognostic determinant with little value of histopathological factors being identified. There is marked subjectivity in the histopathological grading systems and many reports on the correlation of histological features and prognosis have concluded that it is impossible to judge whether or not a GCT of bone is likely to recur from its histological characteristics.^{4,15} At present, no grading system has proved to have prognostic relevance.

Recent understanding of the cellular components of GCT and its molecular and cytokine-mediated derivation has shed

new light on the nature of GCT. However, this improvement in understanding has not resulted in any difference in histopathological analysis of GCT, which might have clinical importance. The present study was designed to attempt immunohistochemical delineation of the stromal component and compartmentalized derivation of parameters, which might influence behavior.

As a first approximation, two groups of primary and recurrent GCT were chosen for this study. Ideally, the initial specimen of a case which subsequently showed recurrence would have been better; however, in our referral center, the earlier specimens from surgery done in other hospitals were not available. Hence, the recurrent tumor itself was taken to evaluate the validity of the pathological approach.

Two cellular constituents can be identified in GCT. Multinucleated giant cells are abundant and their size and number as well as the nuclei vary significantly. The size, shape, and appearance of the nuclei have been reported to be similar in the giant and stromal cells. However, it is now clear that the giant cells are true osteoclasts created in situ by the fusion of bone marrow-derived reactive mononuclear cells. These reactive cells are immunopositive with markers of mononuclear phagocyte system like CD68 and α 1-ACT. The true neoplastic cells are negative for these markers being of primitive osteoblastic or fibroblastic lineage. As such, positive markers for the true neoplastic cells are few and inconsistent.^{16,17} In the present study, the approach was to mark the reactive components and analyze the marker positive (reactive) and marker negative (neoplastic) cells separately. However, it was seen that on morphometry, reactive and neoplastic cells of GCT resembled each other on most image analytic nuclear parameters. Spindling when present was also a feature of both the compartments as well.

Differences were identified between the primary and recurrent group. Overall, there were more reactive components in primary GCT and less of reactive cells in recurrent GCT. Spindling, higher mitosis and higher expression of both proliferation markers were more frequently seen in recurrent than in primary GCT. Previous reports have not found the mitotic count to be different in primary and recurrent GCT or in cases with malignant transformation.¹⁸

In this study, there were only three cases of high-grade (Grade-3) lesions, all of which were recurrent. For statistical analysis intermediate-grade (Grade-2) GCT and high-grade (Grade-3) GCT were clubbed together. Grading was found to be statistically significant and on logistic regression Grade 2 or Grade 3 GCT was 3.5 times more likely to be recurrent as compared to Grade 1 GCT. The number of giant cells was similar in primary and recurrent groups as seen previously.⁴ Other features like stromal cell nucleoli score, giant cell nucleoli score, stromal cell groove score, and giant cell groove score were also not significantly different in primary and recurrent GCT.

Reports from the literature regarding immunostaining for CD 68 have shown similar findings to our observations with the CD68 positive mononuclear cells in GCT accounting for 30% of total mononuclear cells.¹⁹ Mean percent positivity of α 1-ACT was found to be higher in both primary and recurrent GCT group when compared to CD68 staining in primary and recurrent GCT group respectively. α 1-ACT is therefore a better

marker for complete delineation of reactive components. α 1-ACT positivity was significantly different between primary and recurrent GCT with less reactive component in recurrent GCT. Hence, there is progressive decrease of the stromal reactive component with recurrence.²⁰

We found the mean PCNA labeling index in primary group to be 24.75%, while it was much higher at 42.62% in recurrent GCT. Comparable results were seen in previous studies where PCNA expression was associated with aggressiveness. An inverse correlation was present between CD68 and α 1-ACT on one side and MIB-1 and PCNA on the other side in this study, indicating that it is the neoplastic component, which is mainly responsible for the proliferation, as reported earlier.^{21–23} MIB-1 labeling was lower compared to PCNA and this can be explained by specificity of Ki-67 nuclear antigen expression on all proliferating cells during late G1, S, M, and G2 phase of the cell cycle while PCNA is essential for cellular DNA synthesis and reacts with all proliferating cells in general. Derivation of a score calculated as the ratio between PCNA percent positivity and CD68 percent positivity derived a single numerical parameter for comparing stromal cells in GCT. The PCNA/CD68 ratio captures the information on proliferation as well as reactive components. Tumors with higher score have higher proliferation and lower reactive components. Tumors with lower scores have lower proliferation and higher reactive component. Multivariate analysis identified PCNA/ α 1-ACT ratio and PCNA/CD68 ratio as the two most important independent factors related to recurrence in GCT. When both the parameters (PCNA/ α 1-ACT ratio >0.56 and PCNA/CD68 >0.47) are satisfied, it has sensitivity of 85.71% and specificity of 81.82% to correctly classify a given case in the primary or recurrent group. Image analysis failed to add any value to immunohistochemistry since significant nuclear changes are occurring in the reactive cells as well. While neoplastic cell do show some differences from reactive cells, these are subtle and difficult to identify objectively even on image analysis.

Our results therefore confirm that delineation of the stromal components of GCT for reactive and proliferating components is a useful procedure. This information can be encapsulated by derivation of a simple ratio of proliferating component to reactive component. It is however necessary to conduct similar evaluations on the initial specimens of a recurrent case for meaningful prognostic information to be derived.

Conflicts of interest

The authors have none to declare.

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