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Central giant cell granuloma of the jaws: assessment of cell cycle proteins

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BACKGROUND: Several reports have demonstrated the presence of a high proliferative activity in central giant cell granuloma, raising the possibility that deregulation of the cell cycle may contribute to its pathogenesis. As we identified alterations of cyclin D1 in giant cell tumor of bone, and as there are histologic similarities between central giant cell granuloma and giant cell tumor, we assessed jaw lesions for the presence of similar alterations.

METHODS: Formalin-fixed, paraffin-embedded tissue from 29 cases of central giant cell granuloma was assessed for the expression of cyclin D1, cyclin B1, and MIB-1 (Ki-67) using immunohistochemistry. In addition, differential polymerase chain reaction (PCR) was used to determine whether there was cyclin D1 gene amplification.

RESULTS: The cyclin D1 gene copy number appeared to be minimally elevated in 31% of the cases. Cyclin D1 protein overexpression was observed in 28 of 29 cases (96.5%). Immunostaining was present predominantly in the nuclei of the giant cells. Cyclin B1 and MIB-1 immunoreactivity was restricted to the mononuclear cells with no staining present in the giant cells.

CONCLUSIONS: Cyclin D1 protein overexpression may be involved in the formation of the giant cells and the pathogenesis of central giant cell granuloma. As the distribution of immunostaining is identical to that observed in giant cell tumor of bone, our results support the possibility that central giant cell granuloma of the jaws and giant cell tumor of bone represent a similar disease process that clinically and histologically may have somewhat different features because of differences in the anatomical site of involvement.

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Introduction

Central giant cell granuloma is a jaw lesion that usually involves the tooth-bearing areas anterior to the first permanent molar and is more common in adolescents and young adults. Histologically, it is characterized by the presence of multinucleated giant cells and mononuclear cells within a fibrous stroma. The clinical behavior of central giant cell granuloma is variable, ranging from a slowly growing, asymptomatic radiolucent lesion to an aggressive process associated with pain, root resorption, cortical bone destruction, and a tendency to recur after treatment (1). Delineation of the lesions that will exhibit a more aggressive course has been problematic. Although there has been two studies suggesting that the size of the giant cells and their fractional surface area may identify tumors with a more aggressive behavior (1, 2), other studies have not identified histologic or immunohistochemical features that are reliably predictive of the clinical course.

The pathogenesis of central giant cell granuloma is not completely understood. There is not even agreement as to whether this is a neoplasm, as some investigators consider central giant cell granuloma as a reactive process (3). The occurrence of giant cell tumor in the jaw is also controversial. Some investigators have suggested that true giant cell tumor of bone is rare in the jaw (3, 4), while others believe that giant cell tumor and central giant cell granuloma represent a continuum of the same disease process, and that giant cell tumor can occur in jaw bones (5, 6).

Recent studies have demonstrated the presence of proliferative activity in the mononuclear cells of central giant cell granuloma based on the expression of the cell cycle protein Ki-67 (7, 8). This raises the possibility that deregulation of the cell cycle may contribute to the pathogenesis of central giant cell granuloma. Cell cycle abnormalities in tumors are often manifested as aberrant expression of the various cyclins, which override the normal regulatory controls and drive progression through the cell cycle. Cyclin D1 is a critical cell cycle regulator that activates the cyclin-dependent kinases, CDK4 and 6, and regulates passage of cells through the G_1/S restriction point. The cyclin D–CDK4/6 complexes phosphorylate the retinoblastoma protein (pRb) and release the E2F transcription factors from

inhibition by pRb, enabling them to transactivate the genes whose products are critical S-phase effectors (9, 10). Deregulation of one or more components of the cyclin D1–pRb pathway is observed in many human neoplasms, suggesting that deregulation of the G_1/S restriction point contributes to uncontrolled cell proliferation. Overexpression of cyclin D1 has been described in breast carcinoma, oral epithelial dysplasia, and carcinoma, and is frequently associated with amplification of the cyclin D1 gene (11). Cyclin B1, another cell cycle regulatory protein, is involved in the regulation of the G_2/M phase of the cell cycle. It controls the activities of CDK1, and deregulation of the cyclin B1–CDK1 complex may promote cell proliferation or cause uncontrolled cell growth. Cyclin B1 overexpression has been detected in gastric and breast carcinomas, and in esophageal and oral squamous cell carcinomas (12–15). Because of their roles in regulating the cell cycle, alterations of cyclin D1 and/or cyclin B1 might contribute to the proliferative activity observed in central giant cell granulomas.

We have recently described alterations in cyclin D1 gene and/or protein levels in giant cell tumor of long bones (16), and suggested that they could be implicated in tumor pathogenesis. In order to determine whether these alterations are restricted to giant cell tumor or are also present in central giant cell granuloma of the jaws, which would suggest they are biologically related lesions, 29 cases of central giant cell granuloma were examined for cyclin D1 alterations. The presence of cyclin B1 protein overexpression and the proliferative activity in these lesions, as determined by Ki-67 expression, were also assessed.

Materials and methods

Patients and tissues

From the files of the Department of Oral Pathology in the Faculty of Dentistry, University of Toronto, all cases coded as benign giant cell lesion, giant cell granuloma, and giant cell tumor, from 1991 through 2001, were reviewed. Lesions of peripheral location were excluded unless the radiographs or the clinical history clearly indicated a definite intraosseous origin with perforation of the cortex. Forty cases of primary central giant cell granuloma were identified. All tissues had been fixed in formalin and paraffin-embedded. None of the tissues had been decalcified. Seven cases of granulomatous inflammation that contained multinucleated giant cells were also identified for comparison. These included necrotizing, lipid, and suture granulomas (one case each) and four cases of granulomatous inflammation involving lymph nodes. All cases were anonymized.

The histological slides were evaluated for the following features: distribution of the giant cells, presence of coexisting aneurysmal bone cyst-like channels, presence/absence of giant cells in vascular spaces, presence/absence of fibrosis and/or necrosis, presence/absence of foamy macrophages, and presence/absence of bone and/or osteoid in the tumor. When the histologic features varied from field to field, the predominant histologic appearance in the slide was evaluated.

Cell lines

Two cell lines of human breast carcinoma were used as controls in the assessment of cyclin D1 gene amplification. ZR-75-1 (two- to fivefold cyclin D1 amplification; 17, 18) and MDA-MB-231 (no cyclin D1 amplification; 19) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and were grown under standard cell culture conditions in an incubator supplemented with 5% CO_2. Confluent cells were harvested, resuspended in agar, fixed in 10% buffered formalin, and then embedded in paraffin.

Microdissection and DNA extraction

Microdissection and extraction of genomic DNA were done as described previously (20). Tissue sections were cut from the paraffin blocks, placed on glass slides, and briefly stained with hematoxylin and eosin. Tumor was microdissected using a scalpel. Areas of fibrosis, hemorrhage, and oral epithelium were excluded. The lesional tissue was collected and digested overnight at 55°C in proteinase K (20), heated at 95°C for 15 min to inactivate the enzyme, and then stored at 4°C until used.

Semiquantitative differential polymerase chain reaction (PCR)

Cyclin D1 gene (152 bp) was coamplified with the dopamine receptor gene (112 bp). Both genes are located on the long arm of chromosome 11. Table 1 shows the primers used (21) and the PCR conditions. Briefly, differential PCR was performed in a final volume of 15 µl, which contained either 1 or 2 µl of template DNA, PCR buffer (20 mM Tris–HCl

Table 1 Sequences of PCR primers and the amplification conditions used

Primers	*Sense*	*Antisense*
Cyclin D1	5′-ACCAGCTCCTGTGCTGCGAA-3′	5′-CAGGACCTCCTTCTGCACAC-3′
Dopamine receptor	5′-CCACTGAATCTGTCCTGGTATG-3′	5′-GCGTGGCATAGTAGTTGTAGTGG-3′
	Amplification conditions	
Step	Temperature (°C)	Time (min)
Initial denaturation	94	5
30 cycles		
Denaturation	94	1
Annealing	53	1
Extension	72	1
Final extension	72	10

(pH 8.4), 50 mM KCl (Life Technologies, Burlington, Ontario, Canada), 1.5 mM magnesium chloride, 100 μM of each dNTP, 1 U of Platinum® Taq DNA Polymerase (Life Technologies, Burlington, Ontario, Canada), 0.4 μM of cyclin D1 primers, and 0.3 μM of dopamine receptor primers). All samples underwent two separate PCRs to minimize the potential for artifacts. ZR-75-1 and MDA-MB-231 cell lines were used as positive and negative controls for cyclin D1 gene amplification, respectively. In each run, a sample lacking template DNA was used as a negative control for the PCR reaction.

The PCR products were separated by polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. The intensity of the bands was measured using the UVP system and LabWorks Image Acquisition and Analysis software 4.0 (UVP, Upland, CA, USA). The ratio of the cyclin D1 PCR product to the dopamine receptor PCR product (CD1/DR) was calculated. The CD1/DR ratio for ZR-75-1 and MDA-MB-231 cell lines was determined in multiple, independent PCR runs to generate a mean ratio with standard deviation (SD) for each cell line. Cyclin D1 gene amplification was defined as three SDs above the mean CD1/DR ratio obtained for the MDA-MB-231 cell line in which there is no cyclin D1 gene amplification.

Immunohistochemistry

Four-micrometer sections were deparaffinized in xylene, and endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Antigen retrieval was performed by microwaving the sections in 10 mM citrate buffer (pH 6.0) for 18 min at high power setting. The sections were incubated with a blocking reagent (Ultra Streptavidin Detection System, Signet Laboratories, Dedham, MA, USA; 1 : 5 dilution) for 10 min. All primary antibodies were monoclonal and used in the following dilutions: cyclin D1 (clone DCS-6; Dako Diagnostics, Mississauga, Canada), 1 : 25; cyclin B1 (clone 7A9; Novocastra, Newcastle-upon-Tyne, UK), 1 : 20; Ki-67 (clone Mib1; Beckman Coulter, Mississauga, Canada), 1 : 600. Sections were incubated for 1 h at room temperature with the primary antibody followed by 30 min incubation at room temperature with biotinylated horse-antimouse immunoglobulin G (dilution 1 : 200; Vector Laboratories, Burlington, Canada). For visualization of immunoreactivity, the avidin–biotin diaminobenzidine (DAB) Detection Kit (Vectastain Elite ABC Kit, dilution 1 : 2; Vector Laboratories, Burlington, Canada) was used. The sections were counterstained with Mayer's hematoxylin. Sections of a formalin-fixed, paraffin-embedded human lymph node were used as positive controls for the immunostaining. The negative control consisted of replacing the primary antibody with non-immune mouse serum (Dako Diagnostics, Mississauga, Canada). Nuclear staining indicated a positive reaction for cyclin D1 and Ki-67, whereas both nuclear and cytoplasmic staining was considered indicative of positive staining for cyclin B1.

To determine the percentages of positive cells, each slide was evaluated by two investigators (A.K. and R.K.) semi-quantitatively, without knowledge of the gene amplification results. The percentages of positive mononuclear cells and/or giant cells were scored on a scale from 1+ to 3+. Cases with 0–5% positive cells were scored 1+, 6–50% positive cells were scored 2+, and more than 50% were scored 3+. If there was a discrepancy of more than 10% between

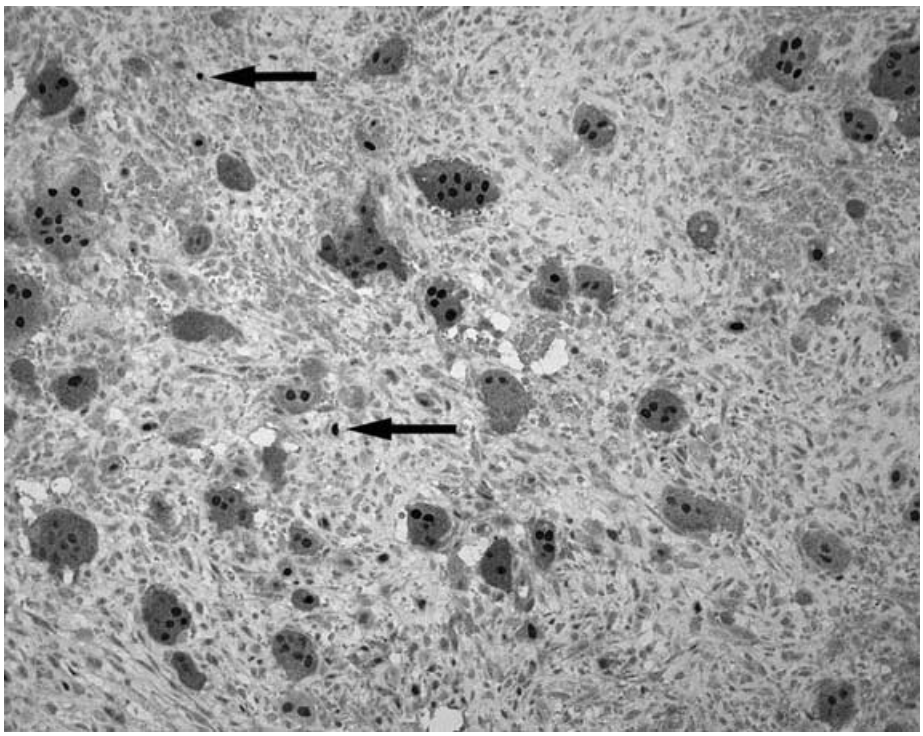

Figure 1 In central giant cell granuloma, cyclin D1 protein expression is seen predominantly in giant cells. Occasional mononuclear cells show staining (arrow). (Immunoperoxidase with hematoxylin counterstain, ×200).

reviewers, the case was re-evaluated by both investigators simultaneously. Immunopositivity in more than 5% of cells was considered indicative of overexpression.

Statistical analysis
The student's *t*-test was used to compare groups. Statistical significance was assigned at $P < 0.05$.

Results

Clinical and histopathological features
Forty cases of central giant cell granuloma were retrieved and, of these, four cases were recurrent lesions, three were hybrid lesions composed of central giant cell granuloma and central odontogenic fibroma, and four had insufficient material for analysis and were excluded from the study. This resulted in a total of 29 cases of central giant cell granuloma available for analysis.

The age of the patients included in the study ranged from 9 to 86 years with a mean of 33.3 ± 19.0 years (mean $\pm$ SD). Most cases occurred during the second and third decades of life; however, two cases occurred in patients younger than 10 years. There were 20 females (71%) and 8 males (29%) with a female to male ratio of 2.5 : 1. There was no statistically significant age difference between the two genders ($P = 0.166$). Twenty-three cases (79%) occurred in the mandible, while only six cases (21%) were present in the maxilla. The location of the lesion relative to the first permanent molar was documented in 19 cases, of which 14 (74%) were anterior and 5 (26%) were posterior to the first permanent molar.

Microscopically, central giant cell granuloma consisted of a variable number of giant cells in a background of ovoid-to-spindle-shaped mononuclear cells. Clustering of the giant cells in areas of hemorrhage was seen in 69% of the cases. In the remaining cases (31%), the giant cells were diffusely distributed throughout the lesion – a distribution similar to that observed in giant cell tumors. Foci of necrosis and clusters of foamy macrophages were not present in any of the lesions examined. In seven cases (24%), aneurysmal bone cyst was present admixed with central giant cell granuloma. Giant cells were seen in vascular channels in four cases (14%), a finding frequently observed in giant cell tumors. Fibrosis was present in 11 cases (38%). Majority of the cases (72%) showed foci of bone and/or osteoid deposition within the lesion.

Immunohistochemical findings
All cases of central giant cell granuloma showed immunoreactivity with the antibodies tested. Cyclin D1 positivity was present mainly in the nuclei of the giant cells with few mononuclear cells showing immunopositivity (Fig. 1). Cyclin D1 protein overexpression was present in 28 cases (96.5%), with between 5 and 70% of the giant cells showing cyclin D1 reactivity. When a giant cell was positive, all the nuclei showed staining. In contrast, the giant cells of the granulomatous inflammatory reactions were negative for cyclin D1 staining.

Cyclin B1 was present in mononuclear cells only (Fig. 2). In all cases, the giant cell nuclei were negative for cyclin B1 staining. The percentage of mononuclear cells reactive with the cyclin B1 antibody ranged from 1 to

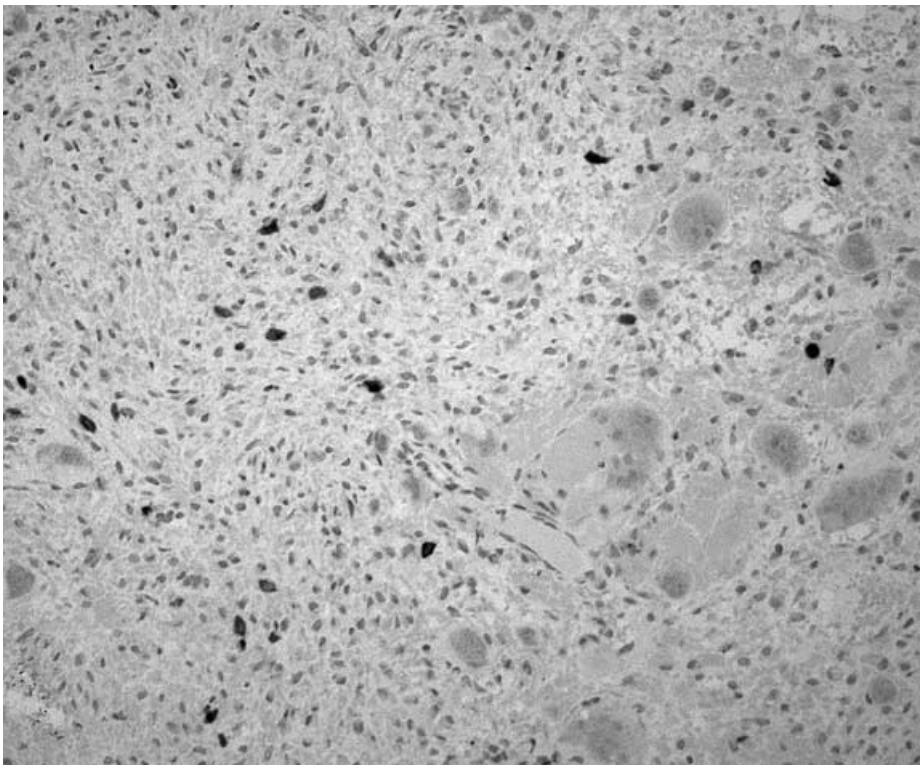

Figure 2 Cyclin B1 protein expression in central giant cell granuloma was present exclusively in mononuclear cells. No immunoreactivity was seen in the giant cells. (Immunoperoxidase with hematoxylin counterstain, ×200).

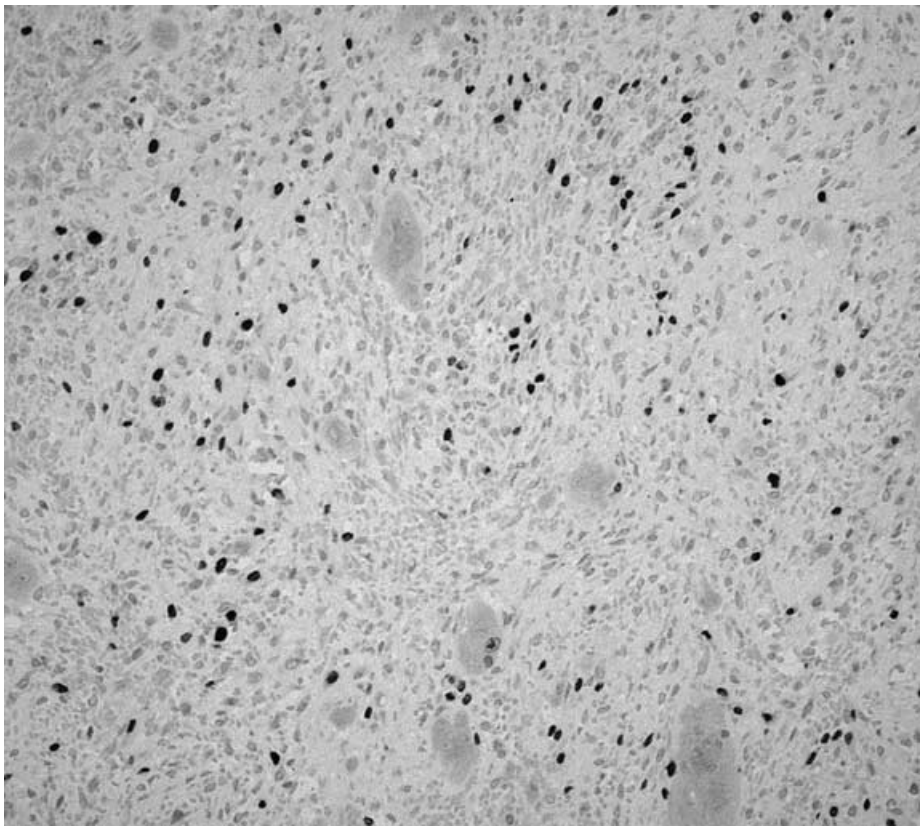

Figure 3 Immunohistochemical staining for Ki-67 in central giant cell granuloma showed immunoreactivity exclusively in mononuclear cells. All giant cells were negative. (Immunoperoxidase with hematoxylin counterstain, ×200).

10%, and cyclin B1 protein overexpression was present in only one case. Ten to thirty per cent of the mononuclear cells showed Ki-67 staining. No immunoreactivity was seen in the nuclei of the giant cells (Fig. 3). Cytoplasmic staining was present in some giant cells and was considered non-specific, as it was also present in the negative controls. Table 2 summarizes the results of staining in giant and mononuclear cells.

Table 2 Summary of immunohistochemical results

		Number of cases showing immunopositivity		
Antibody	*Cell type*	*1+ (%)*	*2+ (%)*	*3+ (%)*
Cyclin D1	GC	1 (3)	11 (38)	17 (59)
	MC	25 (86)	4 (14)	0 (0)
Cyclin B1	GC	0 (0)	0 (0)	0 (0)
	MC	28 (97)	1 (3)	0 (0)
Ki-67	GC	0 (0)	0 (0)	0 (0)
	MC	2 (7)	27 (93)	0 (0)

Central giant cell granuloma cases were immunostained with antibodies reactive with cyclin D1, cyclin B1, and Ki-67 ($n = 29$ cases). The percentages of giant cells (GC) and mononuclear cells (MC) showing immunoreactivity were estimated (1+, 0–5% positive cells; 2+, 6–50% positive cells; 3+, >50% positive cells). The number of cases in each category is given (the numbers in parentheses indicate the percentage of the total number of cases).

Differential PCR findings

The sensitivity of the differential PCR assay used to assess cyclin D1 gene amplification in this study has been demonstrated previously (22). The mean CD1/DR ratio of the ZR-75 cell line, which has two- to fivefold amplification of the cyclin D1 gene, was 1.41 ± 0.15 (mean ± SD; 95% CI: 1.35–1.47). The MDA-MB-231 cell line, which has no cyclin D1 gene amplification, had a mean ratio of 0.61 ± 0.09 (mean ± SD; 95% CI: 0.55–0.67). Based on this, a value of 0.9 (three SDs above the mean CD1/DR ratio of the negative control) or greater was considered indicative of cyclin D1 gene amplification.

Nine cases (31%) of central giant cell granuloma showed a CD1/DR ratio ≥ 0.9. However, none of the cases reached the value of 1.35, which is the lower limit of the 95% CI of the positive control (ZR-75), suggesting that if the cyclin D1 gene was amplified in these nine cases, it was at a very low level. Of the nine central giant cell granulomas with a CD1/DR ratio of ≥ 0.9, three were histologically similar to giant cell tumor. Figure 4 shows the distribution of the CD1/DR ratios in central giant cell granuloma.

Discussion

Previous reports have demonstrated the presence of a higher proliferative activity in central giant cell granuloma of the jaws compared to giant cell tumor of long bone (23). As we identified alterations of the cell cycle regulator, cyclin D1, in

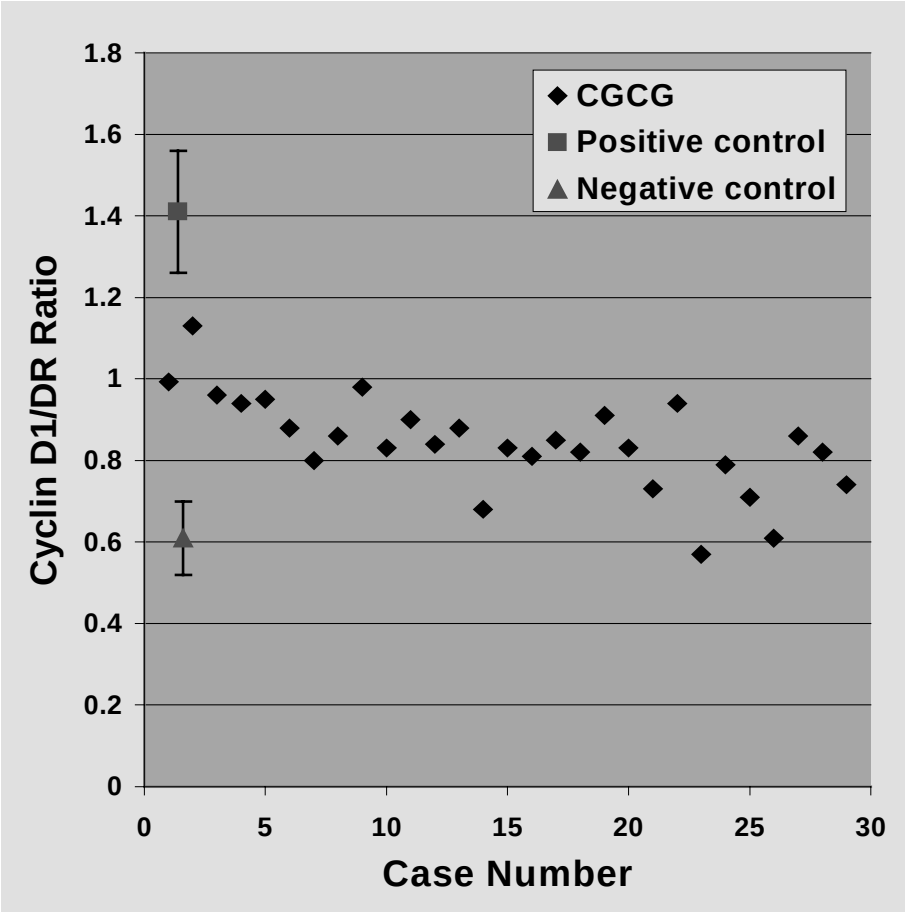

Figure 4 Amplification of the cyclin D1 gene was evaluated by differential PCR in 29 cases of central giant cell granuloma as described under Materials and methods. Each case was analyzed twice and the average ratio between cyclin D1 PCR product and dopamine receptor PCR product (CD1/DR) was calculated and is shown in the graph (◆). The mean CD1/DR ratio was also determined for the positive (ZR-75-1, $n = 20$; ■) and the negative (MDA-MB-231, $n = 20$; ▲) controls and expressed as mean ± SD. These are shown on the left of the graph for comparison.

giant cell tumor of bone, and as there are histologic similarities between these two lesions, we examined central giant cell granuloma for similar alterations. Cyclin D1 protein overexpression was present in 28 of 29 cases (96.5%) of central giant cell granuloma. Most interestingly, protein overexpression was present primarily in the giant cells. In order to evaluate whether these changes were present in other giant cell-containing lesions, seven cases of granulomatous inflammation were examined for cyclin D1 protein expression. None of the giant cells in these lesions showed positive staining for the cyclin D1 protein (data not shown). This suggests that the mechanism(s) regulating the formation of the giant cells in reactive lesions may differ from those in central giant cell granuloma.

In order to determine whether cyclin D1 overexpression in the giant cells was associated with cell proliferation, we examined the expression of cyclin B1, which would indicate transition from the G_2 to the M phase of the cell cycle, and for the presence of Ki-67 protein, which is expressed in all phases of the cell cycle but not in quiescent cells. None of the giant cells expressed these proteins, indicating that they are not proliferating. The presence of cyclin D1 staining in the giant cells in the absence of detectable cell proliferation implicates aberrant expression of this protein in the pathogenesis of central giant cell granuloma and possibly in the formation of the giant cells. Cyclin B1 protein overexpression was present in only one case of central giant cell granuloma, suggesting that this cell cycle protein is likely not involved in the pathogenesis of central giant cell granuloma.

As an identical distribution of immunohistochemical staining was observed in giant cell tumor of bone (16) and in central giant cell granuloma of the jaws, this raises the possibility that the latter is a tumoral lesion that is related to giant cell tumor of bone. de Souza et al. (23) also observed that giant cell granuloma and giant cell tumor of bone showed similar patterns of immunohistochemical staining for other cell cycle regulatory proteins, MDM2 and p53. In their series, there was diffuse expression of MDM2 in mononuclear cells, while both tumors were negative for p53 staining.

Differential PCR identified nine cases of central giant cell granuloma that had cyclin D1 gene copy numbers slightly higher than the negative controls, but lower than those detected in ZR-75-1, which has a low level of amplification of the cyclin D1 gene. This was similar to that observed in

giant cell tumor of bone. As the gene levels were quite low and only 9 of 29 cases showed this change, it is likely that mechanisms other than gene amplification are involved in the overexpression of the cyclin D1 protein observed in the giant cells. The dopamine receptor, used as the reference gene in the differential PCR assay, was selected because similar to the cyclin D1 gene, it is located on the long arm of chromosome 11 and, therefore, this assay will be unaffected by alterations of chromosome 11 or its long arm (21). However, this results in a limitation of this method in that if extra copies of chromosome 11 (or parts of the chromosome) are the cause of the cyclin D1 protein overexpression, this assay will not identify such a change.

Our study, similar to others, found that there is an overlap between the histological features of central giant cell granuloma of the jaws and giant cell tumor of bone (24–26). One-third of the cases showed diffuse distribution of the giant cells throughout the lesion, a feature frequently observed in giant cell tumor of bone. This observation, as well as the similar alterations of the cyclin D1 gene and/or protein for both central giant cell granuloma and giant cell tumor, and the identical distribution of immunohistochemical staining for cyclin D1, cyclin B1, and MIB-1, would argue against the suggestion that these are different entities. The absence of necrosis and foamy macrophages in giant cell granuloma suggests that these lesions might be discovered at an earlier stage of development and, thus, these degenerative changes, commonly observed in giant cell tumor of bone, do not occur. It is more likely that central giant cell granuloma and giant cell tumor represent a similar disease process that may have different clinical and histological features because of differences in the anatomical site of involvement. This could have implications for the management of patients diagnosed with central giant cell granuloma.

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