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ORIGINAL PAPER

Central giant cell lesion of the jaws: study of *CCND1* gene amplification and $p16^{INK4a}$ protein levels

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Abstract Central giant cell lesions (CGCLs) are uncommon benign jaw lesions with uncertain etiology and a variable clinical behavior. In neoplasms, alterations in molecules involved in the G1/S checkpoint are frequently found. Loss of $p16^{INK4a}$ expression or overexpression of cyclin D1 may stimulate cell proliferation. The purpose of this study was to analyze *CCND1* gene amplification and the expression of $p16^{INK4a}$ in CGCLs. Structural analysis of the *CCND1* was performed using chromogenic in situ hybridization. Immmunohistochemistry was used to identify $p16^{INK4a}$ protein levels. Statistical analysis correlated the two biomarkers with clinical behavior and between each other. Twenty-four lesions were included, being 11 aggressive and 13 non-aggressive. Moderate/high-level *CCND*1 amplification was found in 12 lesions. Also, immunoreactivity for $p16^{INK4a}$ was present in 12 cases, mainly in mononuclear cells. There was a significantly higher level of $p16^{INK4a}$ expression in mononuclear cells of non-aggressive lesions and lesions with moderate/high-level *CCND*1 amplification in mononuclear cells. It could be speculated that some CGCLs may develop as a true benign neoplasm. The higher expression of $p16^{INK4a}$ in non-aggressive lesions and in cases with moderate/high-level *CCND*1 amplification may show that these molecules have a role in CGCLs.

Keywords Giant cell lesion · CCND1 amplification · $p16^{INK4a}$

Introduction

Central giant cell lesions (CGCLs) are rare intraosseous lesions that occur almost exclusively in the jaws and are more common in adolescents and young adults (Jundt 2005). Microscopically, CGCL is characterized by the

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presence of multinucleated giant cells (MGCs) in a cellular background composed of mononucleated stromal cells (MSCs) with ovoid or spindle-shaped nuclei, which are fibroblastic in origin (Jundt 2005; de Lange and Van den Akker 2005; Kruse-Losler et al. 2006). The giant cells themselves may vary in size, shape, and number, probably representing osteoclasts or macrophages in origin (Regezi 2002; Jundt 2005; de Lange and Van den Akker 2005; Kruse-Losler et al. 2006). Although little is know about pathogenesis of CGCLs, some studies have shown that in the MSCs component are the proliferative cells in CGCLs (O'Malley et al. 1997; Liu et al. 2003).

The CGCL was classified in 2005 by the World Health Organization as an idiopathic benign lesion with a variable clinical behavior 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 (Chuong et al. 1986; Jundt 2005; de Lange and Van den Akker 2005; Kruse-Losler et al. 2006). The pathogenesis of CGCL is not completely understood. There is not even agreement as to whether CGCL is a neoplasm or a reactive process (Souza et al. 1999, 2000; Kauzman et al. 2004; Nogueira et al. 2010; Nogueira et al. 2012). Some authors regard CGCLs as lesions related to giant cell tumors (GCTs) of the bones, representing different ends of a clinical-pathological spectrum of the same diseases process (Auclair et al. 1988; Kauzman et al. 2004).

An imbalance in cell proliferation control is an important characteristic of aggressive lesions, in which molecules involved in the G1/S checkpoint are frequently altered (Malumbres and Barbacid 2001). The late G1 cell cycle checkpoint is controlled by a complex of proteins that include $p16^{INK4a}$, cyclin D1, cyclin-dependent kinases (CDKs) 4/6, and retinoblastoma protein (pRb) (Fang et al. 1998; Diehl 2002). These proteins are components of the pRb cell cycle control pathway; cyclin D1 stimulates the phosphorylation of pRb by association with CDKs, and $p16^{INK4a}$ binds to CDK4/6, blocking their association with D-type cyclins (Fig. 1). Thus, the loss of $p16^{INK4a}$ expression or overexpression of cyclin D1 cause pRb pathway dysfunction and stimulate cell proliferation (Serrano et al. 1993; Mittnacht 1998). While the three D-type cyclins are almost indistinguishable biochemically, only cyclin D1 is frequently overexpressed in cancers. The *CCND1* gene is a proto-oncogene located on chromosome 11q13 and encodes cyclin D1, and the amplification of this gene is a common mechanism that leads to aberrant overexpression of cyclin D1 (Diehl 2002). Only one study (Kauzman et al. 2004) has analyzed the amplification of the *CCND1* gene in CGCLs, and the expression of $p16^{INK4a}$ has never been studied in CGCLs.

Gathering more information regarding the genetic events involved in CGCL is an important task in understanding this lesion. Due to the role of cyclin D1 and $p16^{INK4a}$ in pRb pathway, and considering that these molecules are frequently involved in tumorigenesis, the purpose of this study was to analyze *CCND1* gene amplification and the expression of $p16^{INK4a}$ in CGCLs of the jaws.

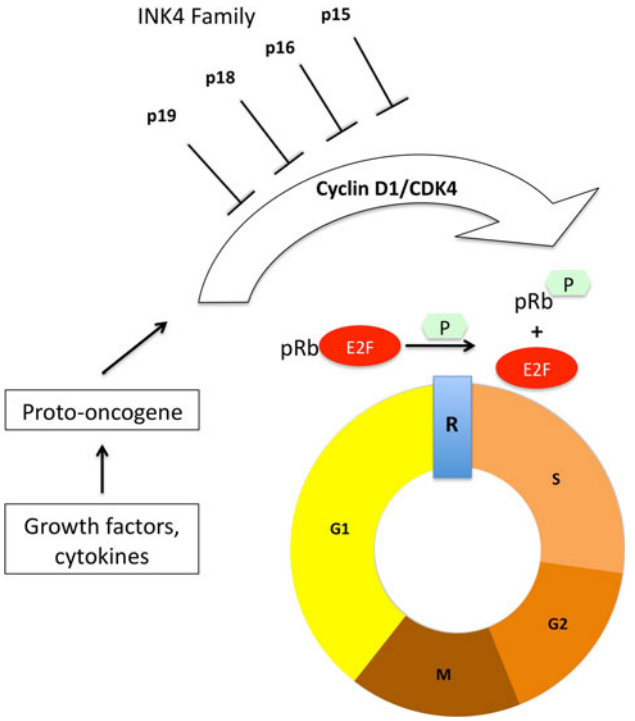

Fig. 1 The pRb pathway. Growth factors lead to the accumulation of cyclin D1 that interacts with CDK4. The complex cyclin d1/CDK4 phosphorylates the pRb, leading to release of the E2F complex, which directs the cell to pass through the restriction point, inducing the transcription of genes of phase S. The $p16^{INK4a}$ protein, a protein of the INK4 family, binds to CDK4 and can interferes with cyclin-CDK interaction, thus inhibiting the phosphorylation of pRb, and restricting the passage of G1–S

Materials and methods

For this study, formalin-fixed paraffin-embedded blocks of 27 consecutive cases of primary CGCLs from patients treated at Memorial Batista Hospital were retrieved from the archives of the Department of Oral and Maxillofacial Pathology, Fortaleza University, Fortaleza, Brazil. Samples were excluded based on associated diagnoses (such as cherubism or hyperparathyroidism, or Brown's tumor), insufficient clinical data to classify the behavior of the lesions as aggressive or non-aggressive, or inadequate specimen for molecular or biochemical analysis. Based on clinic and radiographic data, all cases were classified according to the criteria established by Chuong et al. (1986) (Table 1).

Table 1 Aggressiveness status of CGCL as defined by Chuong et al. (1986)

Aggressive	Non-aggressive
Pain or paresthesia	Asymptomatic
Rapid growth	Slow growth
Cortical perforation	Without cortical perforation
Large dimensions (larger than 5 cm)	Small dimensions
Recurrent lesion	No recurrence
Tooth resorption	Without tooth resorption

The case was considered an aggressive CGCL when presented with one or more of the criteria in the first column

Chromogenic in situ hybridization (CISH) for CCND1 gene amplification

CCND1 gene amplification was performed as previously described (Reis-Filho et al. 2006) using the ready-to-use digoxigenin-labeled SpoT-Light Cyclin D1 amplification probe (Zymed, South San Francisco, CA, USA). Heat pretreatment of deparaffinized sections consisted of incubation for 15 min at 98 °C in CISH pretreatment buffer (SPOT-light tissue pretreatment kit, Zymed) and digestion with pepsin for 7 min at room temperature according to the manufacturer's instructions. An appropriate *CCND1* gene-amplified breast tumor control was included.

CISH analysis

The CISH results were evaluated by optical microscopy at high magnification (400×). Only unequivocal signals were counted. Morphologically unequivocal cells were assessed for the presence of the gene probe signals. Amplification was defined as >5 signals per nucleus in more than 50 % of lesion cells or as the presence of large gene copy clusters. High-level gene amplification was defined as more than 10 discrete copies per nucleus or as large gene copy clusters (observed as confluent masses containing more than 10 signals) in more than 50 % of the nuclei evaluated. Low-level amplification was defined as 5–6 copies per nucleus in more than 50 % of the cells. Moderate-level amplification was defined as 7–10 copies per nucleus in more than 50 % of the cells. Unaltered gene copy number was defined as 1–5 copies per nucleus.

Immunohistochemistry

Immunostaining was performed according to a previously described protocol (Faria et al. 2007). For antigen retrieval, deparaffinized sections were pretreated by heating them in a microwave oven in 10 mM citrate buffer, pH 6.0, for 15 min. After cooling, the sections were immersed in PBS containing 3 % hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a humid chamber (4 h, 4^{O}) with a primary antibody against anti-$p16^{INK4a}$ (CINtec Histology®, clone E6H4® dilution, MTM Laboratories, Heidelberg, Germany). After rinsing with PBS, slides were incubated with the secondary antibody followed by the streptavidin-peroxidase complex, both for 30 min at room temperature with a PBS wash between each step (LSAB+ system; DakoCytomation®, Glostrup, Denmark). The slides were developed with diaminobenzidine-H_2O_2 (DAB+ system; DakoCytomation®, Glostrup, Denmark), counterstained with Harry's hematoxylin and mounted. Positive controls consisted of sections of colon cancer; the negative control consisted of replacing the primary antibody with non-immune mouse serum.

Immunostaining analysis

The immunohistochemical staining in the nuclei was assessed using a direct light microscope. A differential count was performed for MSCs and multinucleated giant cells (MGC). Staining was quantified through manual counting of at least 1.000 MSC in 10 different fields at a magnification of 400×. In the same 10 high-power fields, all MGC were counted to a maximum of 1.000 cells. The labeling index (LI) was expressed as the percentage of positive cells with nuclear staining for $p16^{INK4a}$ in each section (Landber and Roos 1993).

Statistical analysis

Data were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as the mean. To compare the marker LI with respect to clinical forms and the treatment result groups, the non-parametric Mann–Whitney test was used. Significance was established at a p value ≤ 0.05.

The present study was approved by the Ethics Committee of the Hospital Complex of the Federal University of Ceará under protocol 13/08, respecting Resolution 196/96 of the National Council of Health—Ministry of Health/Brazil.

Results

Of the 27 selected cases of CGCL, 24 have enough biological material to perform all reactions. Eleven (45.8 %) were in male patients and 13 (54.2 %) in females, with a median age of 19 years (range from 5 to 50 years). Thirteen (54.2 %) lesions were classified as non-aggressive and 11 (45.8 %) as aggressive CGCL. The age, sex, location, and aggressiveness status of the samples are presented in Table 2.

Table 2 Age, gender, aggressiveness and site of CGCL

Case	Gender	Age	Aggressiveness	Location
1	Male	13	Aggressive	Maxilla
2	Female	21	Non-aggressive	Mandible
3	Male	20	Aggressive	Maxilla
4	Male	7	Non-aggressive	Mandible
5	Female	24	Non-aggressive	Maxilla
6	Female	23	Aggressive	Mandible
7	Female	19	Aggressive	Mandible
8	Female	42	Aggressive	Maxilla
9	Male	20	Non-aggressive	Maxilla
10	Male	9	Aggressive	Mandible
11	Male	6	Aggressive	Maxilla
12	Male	5	Aggressive	Maxilla
13	Female	15	Aggressive	Mandible
14	Male	12	Non-aggressive	Mandible
15	Female	24	Non-aggressive	Mandible
16	Male	7	Non-aggressive	Maxilla
17	Male	25	Non-aggressive	Mandible
18	Female	20	Aggressive	Mandible
19	Female	10	Non-aggressive	Mandible
20	Female	18	Non-aggressive	Mandible
21	Female	23	Aggressive	Maxilla
22	Female	50	Non-aggressive	Mandible
23	Female	11	Non-aggressive	Mandible
24	Male	41	Non-aggressive	Mandible

CISH analysis

Normal or low-level *CCND1* gene amplification in mononuclear cells (Fig. 2) was found in 14 (58.4 %) lesions and moderate or high-level amplification in 10 (41.6 %) CGCLs (Table 3). No statistical significance was found ($p = 0.697$) with respect to association of *CCND*1 amplification in mononuclear cells and aggressiveness of the CGCL (Table 4). Also, moderate or high-level CCND1 amplification in multinucleated giant cells was found in 10 (41.6 %) of CGCL, again no association was found with aggressiveness ($p = 0.697$) (Table 5). Eight of the cases with *CCND1* amplification in mononuclear cells also presented amplification in multinucleated giant cells.

Immunostaining analysis

Immunoreactivity for $p16^{INK4a}$ was detected in 12 cases (50.0 %) in mononuclear cells and only in 5 cases (20.8 %) in multinucleated giant cells (Fig. 3; Table 3). The non-parametric Mann–Whitney test revealed a significantly ($p = 0.044$) higher level of expression of $p16^{INK4a}$ in mononuclear cells in non-aggressive CGCLs (Table 6). Considering only immunostainig in multinucleated giant cells, 4 positive cases occurred in non-aggressive CGCL, and 1 case in an aggressive lesion.

Correlation between the biomarkers

Analyzing the correlation between *CCND*1 gene amplification and $p16^{INK4a}$ immunoreactivity revealed that there was significantly ($p = 0.012$) higher $p16^{INK4a}$ expression in mononuclear cells of CGCLs with moderate/high-level *CCND*1 gene amplification in mononuclear cells (Table 7). Although only 5 cases showed positive immunoreactivity for $p16^{INK4a}$ in multinucleated giant cells, 3 of these cases occurred in CGCL with *CCND1* gene amplification in multinucleated giant cells ($p = 0.615$) (Table 8).

Discussion

Some authors have stated that CGCL is an uncommon reactive process that affects the jaw bone and that may be related to trauma, such as dental extraction (Unal et al. 2006). Aggressive CGCL, with rapid growth and high recurrence rates, are believed to be neoplastic in nature, but the etiopathognesis is still uncertain (Nogueira et al. 2012). Analyses of cell cycle regulatory proteins have been used to distinguish neoplastic from reactive conditions and to predict the biological behavior of tumors (Souza et al. 1999). Few studies regarding cell cycle protein alterations in CGCL have been published (O'Malley et al. 1997; Souza et al. 1999, 2000; Kauzman et al. 2004). The identification of molecular markers characteristic of a lesion and understanding the nature and behavior of a lesion may allow clinicians to better classify and eventually treat such lesions.

In this study, moderate to elevated *CCND*1 gene amplification in MSC and/or MGC was found in 12/24 cases. Although the authors knows that gene amplification doesn't necessarily, but usually, leads to high protein levels, overexpression of cyclin D1 and *CCND*1 gene amplification were previously demonstrated by Kauzman et al. (2004) in CGCL, and this overexpression could be implicated in the pathogenesis of CGCL and in the formation of giant cells. Although no statistical significant difference in amplification was found between aggressive and non-aggressive lesions, the amplification of the *CCND*1 gene could indicate that CGCL may be true neoplastic in nature.

Kandel et al. (2006) demonstrated *CCND*1 gene amplification and cyclin D1 and p21 overexpression in cultured giant cells of GCTs of bones. Kauzman et al. (2003) also found low-level *CCND*1 gene amplification in 19/31 cases of GCT. In the same study, cyclin D1 and D3 overexpression in multinucleated giant cells and cyclin B1 overexpression and Ki-67 immunoreactivity were detected in

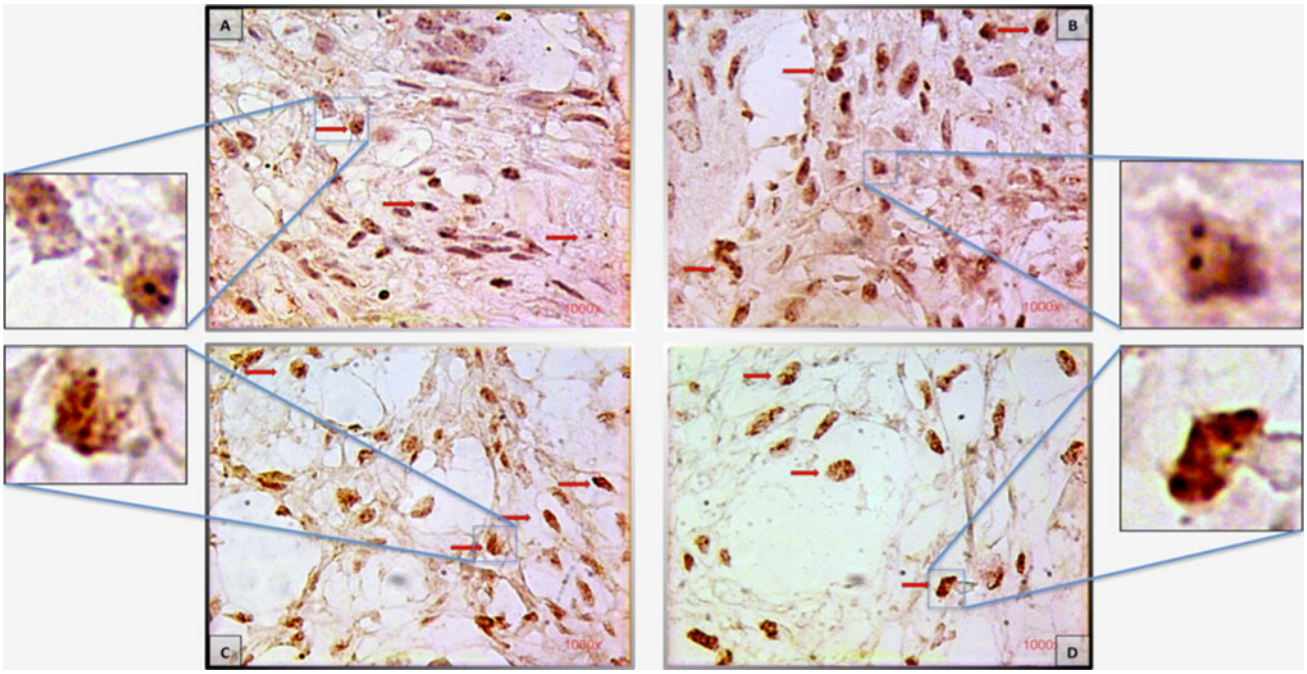

Fig. 2 CISH for cyclin D1, all in ×1,000. **a** Non-aggressive CGCL: normal amplification. Only two gene copies per nucleus (*arrow* and *box*). **b** Aggressive CGCL: low-level amplification, 5–6 spots per nucleus in more than 50 % of the cells (*arrow*), compared with normal amplification (*box*). **c** Aggressive CGCL: high-level amplification, more than 10 spots or clusters in more than 50 % of the cells (*arrow* and *box*). **d** Non-aggressive CGCL: high-level cyclin D1 amplification (*arrow* and *box*)

Table 3 *CCND1* gene amplification and $p16^{INK4a}$ expression in CGCL

	Normal *n* (%)	Low-level *n* (%)	Moderate-level *n* (%)	High-level *n* (%)
*CCND*1 gene amplification				
MSC	9 (37.50 %)	5 (20.83 %)	5 (20.83 %)	5 (20.83 %)
MGC	10 (41.67 %)	4 (16.67 %)	8 (33.33 %)	2 (8.33 %)

Cell type	Negative *n* (%)	5–25 *n* (%)	26–75 *n* (%)	>75 *n* (%)
$p16^{INK4a}$ expression (LI)				
MSC	12 (50.00 %)	6 (25.00 %)	6 (25.00 %)	–
MGC	19 (79.17 %)	3 (12.50 %)	2 (8.33 %)	–

MSC monomuclear stromal cells, *MGC* multinucleated giant cells

Table 4 Correlation between *CCND*1 gene amplification in MSC and aggressiveness of CGCL (Fisher's exact test)

Aggressiveness	Amplification		Total *n* (%)	*p*
	No/low-level *n* (%)	Moderate/high-level *n* (%)		
Aggressive	7 (63.6)	4 (36.4)	11 (100.0)	0.697
Non-aggressive	7 (53.8)	6 (46.2)	13 (100.0)	

Table 5 Correlation between *CCND*1 gene amplification in MGC and aggressiveness of CGCL (Fisher's exact test)

Aggressiveness	Amplification		Total *n* (%)	*p*
	No/low-level *n* (%)	Moderate/high-level *n* (%)		
Aggressive	7 (63.6)	4 (36.4)	11 (100.0)	0.697
Non-aggressive	7 (53.8)	6 (46.2)	13 (100.0)	

mononuclear cells, indicating that the mononuclear cells are the proliferative component of GCT (Kauzman et al. 2003). Expression of Ki-67 and PCNA in the mononuclear cells of CGCLs also indicates that these cells are the proliferative cells in CGCLs (Souza et al. 1999, 2000; Liu et al. 2003; Kauzman et al. 2004).

Considering $p16^{INK4a}$ immunoreactivity, this suppressor was detected mainly in mononuclear cells, and a higher level of expression was detected in non-aggressive lesions ($p = 0.044$). Because $p16^{INK4a}$ is a negative regulator of cell division that slows down the progression of the cell cycle by inactivating cyclin dependent kinase, the higher

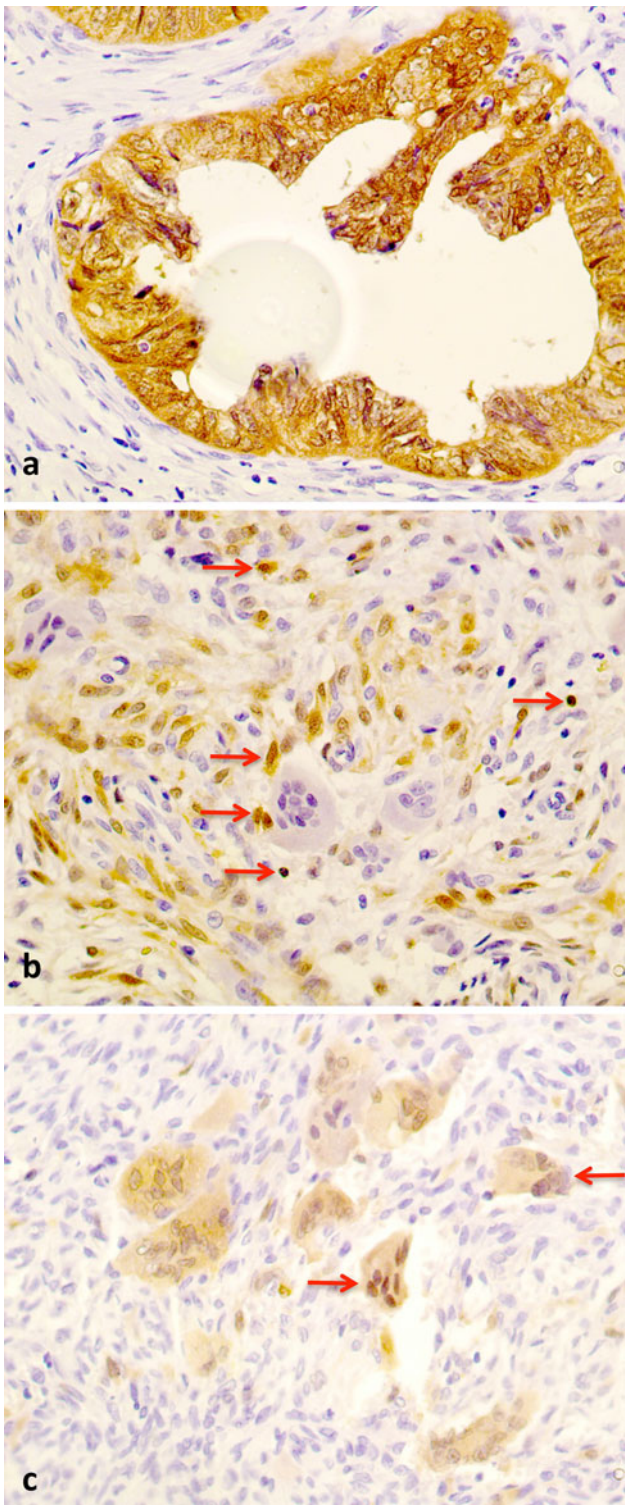

Fig. 3 Immunoreactivity for $p16^{INK4a}$, all in ×400. **a** Colon cancer, positive control. **b** Non-aggressive CGCL with nuclear staining in mononuclear cells (*arrow*). **c** Aggressive CGCL with negative staining in mononuclear cells and positive cytoplasmic and nuclear staining in multinucleated giant cells (*arrow*)

$p16^{INK4a}$ expression in non-aggressive lesions may be the reason why they exhibit slower growth. Additionally, the higher $p16^{INK4a}$ expression found in CGCLs with moderate to elevated *CCND*1 gene amplification ($p = 0.012$) support this hypothesis and justify the clinical behavior of this lesion. It is possible that cyclin D1 overexpression could induce $p16^{INK4a}$ transcription.

Alternatively, it could be postulated that, in both aggressive and non-aggressive CGCLs with a neoplastic nature, an epigenetic alteration in mononuclear cells, such as *CCND*1 amplification, could change the cell cycle. CGCLs with cyclin D1 amplification could have a higher proliferative rate and a more aggressive behavior. However, when there is a negative cell cycle regulator, such as $p16^{INK4a}$ or another tumor suppressor protein, this lesion could present a slower proliferative rate and thus slower growth. To our knowledge, this is the first report of the study of $p16^{INK4a}$ expression in CGCLs; other tumor suppressor genes should be investigated to understand the etiopathogenesis of this lesion. Some authors have previously evaluated p53 protein expression in CGCLs, but no immunoreactivity was found (O'Malley et al. 1997; Souza et al. 1999, 2000). Supporting the neoplastic nature of CGCL, Carinci et al. (2005) analyzed the genetic profile of two cases of CGCL of the jaws and found some up-regulated and down-regulated genes. In addition, the gene SH3BP2 has been investigated in the pathogenesis of CGCL, Carvalho et al. (2009) described a mutation in the SH3BP2 gene in a case of non-familial CGCL, but Teixeira et al. (2011) investigating 30 cases of CGCL, analyzed all exons known to be involved in cherubism, none of them were mutated.

Although *CCND*1gene amplification has been observed in CGCLs, no difference was observed between the clinical forms of this lesion. However, the expression of $p16^{INK4a}$ seems to play a role in aggressiveness of the lesion, those lesions with increased expression of $p16^{INK4a}$ in MSC may have a more indolent clinical behavior. Also, the findings of the present study corroborate the hypothesis that at least some CGCLs may have a neoplastic origin. These lesions do not fit neatly into the concept of either reactive or neoplastic process; instead, CGCLs exhibit features of both (Regezi and Pogrel 2004). It could be speculated that CGCLs may develop in two different ways, one being a reactive process and other appears to be a true benign neoplasm, and that *CCND*1 amplification may contribute to the tumorigenesis of CGCLs. Further studies focused on clinical and molecular analysis, including new studies about cell cycle protein, particularly cyclin D1 protein levels, could help better the understanding of the pathogenesis of CGCL.

Table 6 Parameters used for the calculation of Mann–Whitney *U* test for the evaluation of the expression of $p16^{INK4a}$ in CGCL, according to CGCL aggressiveness

Aggressiveness	*n*	Median	Q_{25}–Q_{75}	Mean of ranks	Sum of ranks	*U*	*p*
Aggressive	11	0	0–14	9.45	104.00	38.00	0.044
Non-aggressive	13	22	4.5–34	15.08	196.00		

Table 7 Parameters used for the calculation of Mann–Whitney *U* test for the evaluation of the expression of $p16^{INK4a}$ in CGCL, according *CCND*1 gene amplification in MSC

Amplification *CCND*1	*n*	Median	Q_{25}–Q_{75}	Mean of ranks	Sum of ranks	*U*	*p*
No/low-level	14	0	0–16	9.54	133.50	28.50	0.012
Moderate/high-level	10	23	11–36.75	16.65	166.50		

Table 8 Correlation between *CCND*1 gene amplification in and $p16^{INK4a}$ expression in MGC (Fisher's exact test)

Amplification	$p16^{INK4a}$ expression		Total *n* (%)	*p*
	Negative (LI < 5) *n* (%)	Positive (LI > 5) *n* (%)		
No/low-level	12 (85.7)	2 (14.3)	14 (100.0)	0.615
Moderate/high-level	7 (70.0)	3 (30.0)	10 (100.0)	

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