Nucleolar Organizing Regions and α -Smooth Muscle Actin Expression in a Case of Ameloblastic Carcinoma

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Abstract Ameloblastic carcinoma is a rare lesion of odontogenic origin. It is defined as a malignant epithelial odontogenic tumor that histologically has retained the features of ameloblastic differentiation and also exhibits cytologic features of malignancy, like atypia and mitotic activity. Although this lesion represents a separate entity, differentiating it from ameloblastoma has been often challenging to pathologists. In this case study reporting a case of ameloblastic carcinoma, we have attempted to verify the previous findings on the use of Argyrophilic nucleolar organizing regions (AgNORs) and immunohistochemical staining for the alpha-smooth muscle actin (alpha-SMA) in differentiating ameloblastic carcinoma from ameloblastoma. It was observed that AgNORs was found to be almost twice in ameloblastic carcinoma as it was in ameloblastoma. A difference between the two lesions in the pattern of expression of alpha-SMA was also observed, with alpha-SMA being expressed in the odontogenic epithelium and the stroma of ameloblastic carcinoma whereas, in the case of ameloblastoma, it was found only in the stromal part. These findings suggest that AgNORs and alpha-SMA expression may be used as adjuncts to the routine histopathologic examination to differentiate ameloblastic carcinoma and ameloblastoma.

Keywords Ameloblastic carcinoma · Smooth muscle actin · AgNORs · Ameloblastoma · Epithelial · Odontogenic

Introduction

Ameloblastic carcinoma is a rare lesion of odontogenic origin, occurring commonly in the mandible [1, 2]. It is currently defined as a rare odontogenic malignancy that combines the histological features of ameloblastoma with cytological atypia, even in the absence of metastases [3]. In comparison, ameloblastoma is a more common clinically significant odontogenic tumor which has been defined by Robinson as ‘usually unicentric, nonfunctional, intermittent in growth, anatomically benign and clinically persistent’ [4].

Although these lesions represent two different entities, differentiating between the two has often been challenging to pathologists. Some researchers have suggested that the use of special stains such as Silver staining nucleolar organizer regions may be used to differentiate between various odontogenic cysts and tumors [5–7]. Recent research works utilizing immunohistochemical methods targeting different tissue markers have suggested a variation in expression patterns of these markers in ameloblastoma and ameloblastic carcinoma. These markers include cytokeratins [8], morphogenesis regulation factor Notch1 [9], Ki-67 [9–11], syndecan-1 [10], and alpha-smooth muscle actin [11].

Nucleolar organizer regions are loops of DNA that transcribe for ribosomal RNA (rRNA) located on the short arm of chromosome 13, 14, 15, 21 and 22 [12, 13]. The nucleolar organizer regions reflect protein synthesis and are known to increase in number during malignancies. According to several researchers, the Argyrophilic nucleolar organizing regions (AgNORs) can be used in differentiating between benign and malignant lesions and, in the opinion of some researchers, it is the morphologic characteristics of AgNORs that is more informative than their absolute numbers [14].

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Tissue integrity is maintained by the stroma in physiology. In cancer however, tissue invasion takes place with the help of stroma. Myofibroblasts and cancer-associated fibroblasts are important components of the tumor stroma [15]. Myofibroblasts are specialized stromal cells that exhibit a hybrid phenotype between fibroblasts and smooth muscle cells, and are characterized by expression of the specific isoform alpha of the smooth muscle actin (alpha-SMA) [16]. In a recent study, it has been reported that the pattern of expression of alpha-SMA may be useful in differentiating ameloblastoma and ameloblastic carcinoma [11].

In this case study, a case of ameloblastic carcinoma is reported where we have attempted to verify the previous findings on the use of argyrophilic nucleolar organizing regions (AgNORs) and immunohistochemical staining for the alpha-SMA as adjuncts to routine histopathologic examination in differentiating ameloblastic carcinoma from ameloblastoma.

Case Report

A 64-year old male patient reported to the Department of Oral Medicine, Yenepoya Dental College, Mangalore, India with a complaint of swelling in the right lower jaw since 3 months. The swelling was characterized by a rapid increase in size and was associated with discomfort during functions and pain in the adjacent teeth. Intra-oral examination revealed a circumscribed, proliferative, smooth-surfaced growth on the buccal alveolar mucosa, measuring approximately 6 cm × 5 cm, extending from mandibular right central incisor to mandibular right second molar, and extending apically into the lower buccal vestibule (Fig. 1). The lesion was pale pink in color with multiple yellowish-white & red areas, with underlying blood vessels visible.

Fig. 1 Intra-oral photograph showing the extent and appearance of the lesion

Fig. 2 Panoramic radiograph showing well defined radiolucency in relation to mandibular right lateral incisor, canine and premolars

Indentations of the teeth from opposite arch were seen over the surface of the lesion, giving it a lobulated surface like appearance. The swelling was firm in consistency with an intact surface mucosa, and was fixed to the underlying tissues. No pulsation or tenderness was noted on palpation. Cervical lymph nodes were also not palpable. Panoramic radiograph showed a well defined radiolucency extending from the mandibular right lateral incisor to the right first premolar, with faint radiopacities (Fig. 2). The differential diagnosis included squamous cell carcinoma and ameloblastoma and a provisional diagnosis of squamous cell carcinoma was made. Incisional biopsy was performed and the findings of the histopathologic examination were suggestive of a malignant odontogenic lesion. Hence, further investigations were performed to rule out any metastatic lesion, and radiographic examination and CT scan of the chest were performed. Both the investigations were negative for any metastatic lesions.

Patient underwent subtotal mandibulectomy along with excision of the tumor mass under general anesthesia, following which he was suggested radiation therapy. However, the patient was not available for follow-up and no further treatment was possible. The resected tumor measured 5.5 cm × 4.3 cm and was firm in consistency. The cut surface showed creamish white tissue separated by multiple cystic spaces. The excisional specimen underwent routine tissue processing and samples were used for Hematoxylin-Eosin (H&E) staining, AgNORs, and immunohistochemical staining for alpha-SMA. Tissue sections of a specimen of follicular ameloblastoma were also examined by AgNORs and immunohistochemical staining for alpha-SMA to determine whether these diagnostic tests could be used to differentiate ameloblastic carcinoma from ameloblastoma.

The H&E stained sections of ameloblastic carcinoma showed odontogenic epithelial islands of highly irregular

shape spread in a scanty fibrous connective tissue stroma. The periphery of epithelial islands was lined by cuboidal or columnar ameloblast-like cells. The central portion showed cells of both stellate shape and spindle shape. The odontogenic epithelial islands showed abnormal mitotic figures (2–3 per high power field) with proliferating cells. The central cells in few areas showed pleomorphism with an attempt of malignant keratin pearl formation. Slight basilar hyperplasia was observed. The stroma was well vascularized. Necrosis, vascular and neural invasion were not observed. Tiny spicules of calcifications were evident in very few areas. Based on the findings of abnormal proliferating mitotic figures in irregular shaped islands of odontogenic epithelial cells, a diagnosis of ameloblastic carcinoma was made (Fig. 3).

The AgNORs staining was performed as described by Crocker and Nar [17]. Sections of 3 μm thickness of both ameloblastic carcinoma as well as ameloblastoma were deparaffinised in xylene, rehydrated through grades of alcohol, and washed thoroughly in deionised water. Sections were then treated with freshly prepared silver colloidal solution (containing one part by volume of gelatin in formic acid and two parts by volume of aqueous silver nitrate solution) for 30 min at 40°C in a dark environment. Stained sections were washed in deionised water for 5 min, dehydrated through ascending grades of alcohol, and then cleared in xylene and mounted. The brownish black dots inside the nucleus 100 cells in the significant areas of the lesional tissue were counted under 100 $\times$ oil immersion. The AgNORs count in ameloblastic carcinoma was twice the count in ameloblastoma (Figs. 4, 5). The NORs counted in ameloblastoma was 168 in 100 cells and in ameloblastic carcinoma, the count was 342 in 100 cells.

Fig. 3 Hematoxylin & Eosin stained section of ameloblastic carcinoma under 20 $\times$ magnification showing abnormal mitotic figures (in circle)

Fig. 4 Silver staining nucleolar organizing regions in ameloblastoma under 100 $\times$ oil immersion magnification

Fig. 5 Silver staining nucleolar organizing regions (indicated by arrows) in ameloblastic carcinoma under 100 $\times$ oil immersion magnification

The immunohistochemistry for alpha-SMA was performed at the Department of Pathology, Kasturba Medical College, Manipal University, Karnataka. Antigen retrieval was done in a microwave oven, followed by immunohistochemical staining in which mouse monoclonal antibodies to alpha-SMA and Super Sensitive Detection Kits (BioGenex Laboratories Inc, San Ramon, California, USA) were used for determining the expression of alpha-SMA in ameloblastic carcinoma as well as ameloblastoma. Internal controls were used during the immunohistochemical procedure. In order to block endogenous peroxidase activity, the sections were treated with Peroxidase block (3% Hydrogen Peroxide) for 15 min and the slides were placed in the humid chamber. After this, the slides were drained and rinsed in two changes of TRIS buffer (wash buffer), each of 5 min duration. Nonspecific protein–protein

interactions were blocked by treating and incubating the tissue sections with power block (casein in phosphate buffered saline) for 10 min duration in a humid chamber. After this, the remaining solution was drained from the slides. The sections were then incubated in the pre-diluted primary antibody (alpha-SMA) at room temperature in humid chamber for 60 min. Again, the remaining solution was drained from the slides and rinsed in two changes of TRIS buffer as mentioned earlier. Sections were then treated with reagent for enhancing the staining for 30 min, followed by rinse in two changes of TRIS buffer. Sections were then incubated with secondary antibody solution (biotinylated anti-immunoglobulins) for 30 min. This was followed by rinsing with wash buffer and treatment with horseradish peroxidase-conjugated streptavidin for 30 min. Subsequently, the sections were covered with drops of 3,3'-diaminobenzidine chromogen (DAB) and DAB buffer and allowed to stand in the humid chamber for 8–10 min. During this period, brown staining was visible with varying intensity on different sections. Then the slides were rinsed first in distilled water followed by running tap water. The sections were then counterstained with hematoxylin solution for 20 s, rinsed in running tap water, and dehydrated and mounted with di-n-butyl phthalate in xylene.

In the case of ameloblastoma, the immunohistochemical staining appearing dark brown was well appreciated all along the endothelial cells of blood vessels and partially also in close approximation with the epithelial islands, in the stroma (Fig. 6).

In the case of ameloblastic carcinoma, definite immunohistochemical positivity with brown stain was appreciated all along the endothelial cells of blood vessels and partially also in close approximation with the epithelial islands, in the stroma. Similar staining was also seen in the

Fig. 6 Expression of α -smooth muscle actin (indicated by arrows) in ameloblastoma under 10 $\times$ magnification

Fig. 7 Expression of α -smooth muscle actin (indicated by arrows) in ameloblastic carcinoma under 20 $\times$ magnification

stellate reticulum like cells of the odontogenic islands (Fig. 7).

Discussion

Ameloblastic carcinoma is a rare malignant odontogenic tumor which affects males more frequently, particularly in the older age groups. It occurs more commonly in the mandible than in the maxilla. The most common presenting symptoms include swelling, pain, and rapid growth. According to the current definition of ameloblastic carcinoma, it is a lesion which combines the histologic features of ameloblastoma with cytological atypia.

In the present case study, the clinical and histopathologic features were suggestive of ameloblastic carcinoma. Factors such as the patient's age, rate of growth and the loss of bone suggest the possibility of a malignant lesion. More aggressive proliferation was observed in certain areas in the H&E stained sections of ameloblastic carcinoma. Irregular islands of odontogenic epithelium were also appreciated in ameloblastic carcinoma which could help in differentiating a malignant lesion from a benign one.

In the present case study, we attempted to verify whether AgNORs, which is increased during malignancies, and pattern of expression of alpha-SMA, which is associated with poor prognosis, can be used to differentiate ameloblastic carcinoma from ameloblastoma.

As this rare lesion may pose a diagnostic difficulty, researchers have suggested different methods to differentiate benign and malignant odontogenic lesions. These methods rely on detection of features suggestive of dysplastic or aggressive tumor behavior as well as the use of various immunohistochemical markers in determining the aggressive and malignant potential of odontogenic lesions.

The various immunohistochemical markers which have been studied include cytokeratins [8], Notch1 [9], Ki-67 [9–11], syndecan-1 [10], and alpha-smooth muscle actin [11].

Lau et al. observed a negative cytokeratin expression by ameloblastoma cells of the ameloblastic carcinoma and suggested that this may be helpful in determining the aggressive and malignant potential of the lesion [8]. Nakano et al. reported that the expression of Notch1 in ameloblastoma was localized in the most proliferating odontogenic epithelial cells where as in the case of ameloblastic carcinoma, it was detected in most of the neoplastic cells. They also reported a variation in the expression of the Ki-67 between the two neoplasms, with few Ki-67 positive cells observed in the proliferating tumor nests in the case of ameloblastoma and increased number of Ki-67 positive cells being detected in almost all tumor cells in the case of ameloblastic carcinoma [9]. Bologna-Molina et al. reported an increased expression of Ki-67 and a decreased expression of syndecan-1 in ameloblastic carcinoma when compared to various ameloblastomas [10].

In a recent study investigating the utility of various epithelial and stromal markers in differentiating ameloblastic carcinoma from ameloblastoma, it was found that expression of alpha-SMA was found in epithelial islands in the former but not in the latter. Based on their findings, the authors suggested that the presence of alpha-SMA within epithelial islands is highly predictive of ameloblastic carcinoma [11].

Tumor progression occurs within a microecosystem, where cancer cells and myofibroblasts exchange proteinases and cytokines that promote growth directly through stimulation of proliferation and survival, as well as invasion through local proteolysis of the extracellular matrix and stimulation of motility [18]. Through expression and secretion of cytokines, chemokines, growth factors, extracellular matrix molecules, adhesion molecules and receptors, myofibroblasts promote epithelial-mesenchymal interactions, including neoplastic growth and invasion [19]. Studies on oral squamous cell carcinoma have suggested that increased stromal myofibroblasts as assessed by alpha-SMA immunoreactivity is associated with poor prognosis [20].

Quantitative evaluation of odontogenic cysts and tumors has shown that the stroma of these lesions are composed of myofibroblasts as reflected by alpha-SMA-positive cells [21]. Furthermore, it has been clearly shown that the mean number of myofibroblasts in well-recognized aggressive odontogenic lesions (e.g., Solid follicular ameloblastoma and Odontogenic keratocyst-Parakeratinised) was high and did not differ significantly from that in oral squamous

cell carcinoma. In contrast, known non-aggressive lesions (e.g., Unicystic ameloblastoma, Ameloblastic fibroma, Odontogenic keratocyst-Orthokeratinised and dentigerous cyst) showed significantly lower results compared to solid follicular ameloblastoma and/or odontogenic keratocyst-parakeratinised. Thus, a positive link was suggested between the number of myofibroblasts present in the stroma and the aggressive behavior of the odontogenic cyst/tumor [21].

AgNORs technique has been a useful method in diagnostic pathology, especially for the differentiation of benign and malignant tumors [22]. Neoplastic cells generally exhibit a rise in the synthesis of normal and abnormal products and thus frequently feature a significant rise in AgNORs material [23]. However, the exact significance of changes in AgNORs counts from one lesion to another is not fully understood. AgNORs counts rise with increased cell ploidy, with increased transcriptional activity and in stages of active cell proliferation [24]. Moreover, AgNOR counts may not be a good indicator of cell proliferation but rather represent variations in metabolic or transcriptional activity [7]. Another disadvantage of this technique is that, unlike in immunohistochemistry where the visibility of the DAB staining will reveal the presence of the required marker, the counting of NORs under 100 $\times$ is more difficult.

We stained both the lesions with AgNORs and counted AgNORs and found it to be almost twice in ameloblastic carcinoma in comparison with ameloblastoma. We also used immunohistochemical method for expression of alpha-SMA in both ameloblastic carcinoma and ameloblastoma, in order to distinguish the two lesions on the basis of alpha-SMA distribution. We found the alpha-SMA expression was present only in the stroma of ameloblastoma in close association with the odontogenic epithelium along with the endothelial expression. However, in the ameloblastic carcinoma, it was observed that the alpha-SMA expression was present in both the odontogenic epithelium as well as the stroma. In the epithelial part, the expression was seen more in the stellate reticulum-like cells rather than the tall ameloblast-like cells. The reason for the myofibroblasts being associated with neoplastic epithelium is still not clear except for an association with distant metastasis and an epithelial-mesenchymal transition [11].

To conclude, the findings of the present case study suggest that the difference in the AgNORs and expression of alpha-SMA between the two lesions, apart from the routine H&E staining, may be helpful to differentiate the benign and the malignant lesions. Although few investigators have reported similar results [11], further studies need to be carried out with adequate sample size to more clearly determine the role of AgNORs and alpha-SMA in the diagnosis of ameloblastic carcinoma.

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