Adenomatoid Odontogenic Tumor, Evidence for A Mixed Odontogenic Tumor

Kelcie Barnts , Jian Q. Feng , Chunlin Qin , Hua Zhang , Yi-Shing Lisa Cheng

PII: S2212-4403(21)00730-6
DOI: https://doi.org/10.1016/j.oooo.2021.11.005
Reference: OOOO 4762

To appear in: *Oral Surg Oral Med Oral Pathol Oral Radiol*

Received date: 15 June 2021
Revised date: 22 September 2021
Accepted date: 10 November 2021

Please cite this article as: Kelcie Barnts , Jian Q. Feng , Chunlin Qin , Hua Zhang , Yi-Shing Lisa Cheng , Adenomatoid Odontogenic Tumor, Evidence for A Mixed Odontogenic Tumor, *Oral Surg Oral Med Oral Pathol Oral Radiol* (2021), doi: https://doi.org/10.1016/j.oooo.2021.11.005

Adenomatoid Odontogenic Tumor, Evidence for A Mixed Odontogenic Tumor

Kelcie Barnts[1], Jian Q. Feng[2], Chunlin Qin[2], Hua Zhang[2], Yi-Shing Lisa Cheng[3]*

[1] Department of Oral and Maxillofacial Pathology, Medicine and Surgery, Kornberg School of Dentistry, Temple University, Philadelphia, USA

[2] Department of Biomedical Sciences, Texas A&M University College of Dentistry, Dallas, Texas, USA

[3] Department of Diagnostic Sciences, Texas A&M University College of Dentistry, Dallas, Texas, USA

*Corresponding author:
Yi-Shing Lisa Cheng, DDS, MS, PhD
Professor, Diagnostic Sciences,
Texas A&M University, College of Dentistry
3302 Gaston Ave, Dallas, Texas, USA
Tel: (214)828-8912
Fax: (214)828-8306
Email: ycheng@tamu.edu

Keywords: Adenomatoid odontogenic tumor, nestin, dentin sialophosphoprotein, cytokeratin, vimentin, Sirius red

Running title: Adenomatoid Odontogenic Tumor

Funding: Office of Research and Graduate Studies, and Department of Diagnostic Sciences,

Texas A&M University College of Dentistry

Conflicts of interest disclosures: No conflicts of interest to disclose

Abstract

Objective: Adenomatoid odontogenic tumor (AOT) was classified by WHO as a mixed odontogenic tumor in 1992 and reclassified as an epithelial-only tumor in 2005 without clear rationale. The purpose of this study was to investigate if there was any evidence to suggest AOT might be a mixed odontogenic tumor.

Study Design: Immunohistochemical studies with nestin, dentin sialophosphoprotein (DSPP), cytokeratin, and vimentin were performed using 21 cases of AOT and the staining results were analyzed according to the various morphologic patterns seen in AOT. Sirius red stain was used to detect presence of collagen types I and III in AOT products.

Results: Our results showed that 20/21 (95.23%), 0/21 (0%), 21/21 (100%), and 20/21 (95.23%) cases expressed nestin, DSPP, cytokeratin, and vimentin, respectively. Some cells in rosette/duct-like structures (RDS) expressed nestin, vimentin or both, without cytokeratin. Co-expression of vimentin and cytokeratin, or nestin, cytokeratin, and vimentin were noted in some cells. Sirius red was positive in eosinophilic products in RDS, double-layered spheres and dentinoid.

Conclusion: Although most AOT cells appear epithelial, there is a small population of cells expressing mesenchymal proteins and secreting collagen types I and III. These evidence suggests that AOT is a mixed odontogenic tumor.

Statement of Clinical Relevance

The WHO classification, a guideline for practicing pathology, should be based on solid scientific evidence. The supporting evidence of reclassifying AOT from mixed to epithelial-only odontogenic tumor in WHO 2005 classification was weak. Evidence from our study suggests differently.

Introduction

Adenomatoid odontogenic tumor (AOT) is a benign odontogenic tumor, known as ñthe tumor of two-thirdsò because 2/3 of cases are found in females, the maxilla, and patients in their second decade of life.[1, 2] Although any tooth can be associated with AOT, maxillary canines are the most commonly affected.[2, 3] Histologically, AOT shows multiple cell arrangements, including duct-like structures, double-layered spheres, and rosettes. Calcifications of AOT also show various appearances, including dentinoid and Liesegang ring-like basophilic calcifications.

The classification of AOT by the World Health Organization (WHO) changed between the 1992 and the 2005 editions. In 1992, AOT was categorized as a mixed tumor related to ñodontogenic epithelium with odontogenic ectomesenchyme, with or without dental hard tissue formationò.[4] That classification appeared to represent the majority opinion of the selected international panelists based on the knowledge at that time, as explained in the Preface to that edition. A reference section was intentionally omitted because the authors viewed the classification ñnot intended to serve as textbooks but rather to facilitate the adoption of a uniform terminology that will facilitate and improve communication among cancer workersò. However, some description in the AOT section seemed to justify a mixed odontogenic tumor classification, including description about the epithelial component, ñvarying amounts of acidophilic hyalinized materialò and ñThis hyaline material appears to be dysplastic dentin é , and occasionally a

tubular pattern is seen in some areasò.[4] In 2005, AOT was reclassified as an epithelial-only tumor. The description in the Histopathology section of AOT mentioned that ñOccurrence of a hyaline, dysplastic material or calcified osteodentin may be found in AOTs. It is likely the result of a metaplastic process, as odontogenic ectomesenchyme is not present, and thus should not be interpreted as an induction phenomenon, although in very rare cases dentin-like material containing dentinal tubules may occurò.[5] The WHO 2005 edition did not give a clear explanation or provide any reference for the changing point of view or the reclassification, although there was a Reference section in that edition.[5] However, three years prior to writing the 2005 WHO, one of the authors published a paper titled ñRevision of the 1992-edition of the WHO histological typing of odontogenic tumours. A suggestion,ò[6] which indicated why this reclassification may have taken place. The authors recommended reclassification of several odontogenic tumors, including AOT.[6] In discussing the reclassification of AOT,[6] a study by Gao, et al.[7] was the only report cited. It stated that ñ é Gao et al. who studied immunohistochemically the expression and distribution of bone morphogenetic protein (BMP) in various odontogenic tumours. Tumours characterized by epithelio-ectomesenchymal interactions, and producing dentin, enamel, and/or cementum (like ameloblastic fibrodentinoma and compound odontoma among others) were BMP-positive, whereas ameloblastoma, AOT, and calcifying epithelial odontogenic tumour [CEOT] were BMP-*negative*. These findings have supported the present authors in classifying the AOT under group 1.1.1. rather than under group 1.1.2 as in the WHO classification.ò[6]

To understand the reclassification, the publication by Gao, et al.[7] was examined. Gao, et al.[7] investigated BMP expression by immunohistochemistry in 44 odontogenic tumors, which included two AOTs. Gao, et al.[7] used a BMP antibody (BMPMcAb), which was not clearly

characterized. Based on the information provided, the BMPMcAb antibody most likely detected BMP-1 and BMP-3. Gao, et al.[7] separated the 44 odontogenic tumors into two groups based on the IHC results: BMP-positive tumors and BMP-negative tumors. Most of the BMP-positive tumors formed dentin, cementum, or enamel.[7] The BMP-negative tumors were AOT, ameloblastoma, and CEOT.[7] The conclusion about AOT and CEOT (the BMP-negative tumors that form calcifications) was that ñThis indicates that the mechanism of calcification in AOT and CEOT is possibly different from that in BMP-positive odontogenic tumors.ò[7] No correlation between BMP expression and odontogenic cell type of origin was discussed. Furthermore, BMP-1 and BMP-3 can be expressed by both epithelial and mesenchymal cells.[8] Yet Gaoôs study was the only citation found in the ñRevision of the 1992-edition of the WHO histological typing of odontogenic tumours. A suggestionò[6] to support the reclassification of AOT. This means that AOT was reclassified as an epithelial-only odontogenic tumor based on one IHC study, using an antibody that was not well-characterized and tested only two samples of AOT. The 2017 edition of the WHO maintained the 2005 classification of AOT.[9]

The various cell arrangements and presence of dentinoid calcifications warranted consideration that mesenchymal involvement in AOT was possible. The purpose of this project, therefore, was to investigate whether AOT was an epithelial-only or mixed odontogenic tumor, by immunohistochemical (IHC) studies using nestin, dentin sialophosphoprotein (DSPP), cytokeratin, and vimentin; and the histochemical stain Sirius red. Nestin is an intermediate filament expressed by some mesenchymal progenitor cells (see Discussion). Among odontogenic cells, nestin is expressed by secretory odontoblasts and pulpal cells.[10] DSPP, a critical protein in dentin mineralization, is mainly expressed by odontoblasts.[11] Sirius red identifies the mesenchymal cell products collagen types I and III.[12, 13] Although basement membrane material

secreted by epithelial cells often presents as eosinophilic material resembling ñdentinoidò on histological sections, both collagen types I and III are not found in the basement membrane but are the major organic proteins of dentin (see Discussion).[14-17] A mesenchymal origin of AOT would be suggested by positive results with Sirius red, nestin, DSPP, or vimentin.[18]

Materials and Methods

The study was reviewed and approved by the Texas A&M University Internal Review Board (IRB ID #2019-0749). Formalin-fixed, paraffin-embedded, archived human biopsy tissue was retrieved from the database of the Oral Pathology Division, Department of Diagnostic Sciences, Texas A&M University College of Dentistry. Twenty-one cases diagnosed as AOT were selected as the experimental group. The positive control tissues used for the IHC procedures were odontoma for nestin and DSPP; small intestine for cytokeratin; and leiomyoma for vimentin. Slides with 4 µm sections of the tissues were obtained, deparaffinized by xylene, then rehydrated by serially descending concentrations of alcohol and phosphate buffered saline (PBS).

For nestin IHC investigation, antigen retrieval was done using epitope retrieval citrate buffer (Leica Biosystems, Buffalo Grove, IL, pH 6.0, #RE7113). After endogenous peroxidase inhibition with 3% hydrogen peroxide, the slides were incubated with blocking reagent provided in the MOM system (MOM [Mouse on Mouse]) Elite Peroxidase Kit, Vector Laboratories, Burlingame, CA, #PK-2200). This system was used for blocking, dilution of primary antibody, and secondary antibody detection according to the manufacturerôs recommendations. Slides were then incubated with 1:100 diluted mouse anti-human nestin antibody (clone 10C2, Invitrogen

ThermoFisher Scientific, #MA1-110) for 30 minutes at room temperature followed by detection with the MOM system.

For DSPP, the antigen retrieval, endogenous peroxidase inhibition and blocking was achieved by 0.05% trypsin calcium chloride, 3% hydrogen peroxide and 3% bovine serum albumin/10% goat serum in 0.3% Triton in PBS (PBST), respectively. Slides were then incubated in 1:200 diluted rabbit anti-human DSPP antibody for one hour at room temperature (clone LF-151, Kerafast, Boston, MA), followed by detection with the Vectastain Elite Avidin/Biotin (ABC) Kit (Vector Laboratories, #PK-6100) and DAB system (Vector Laboratories, #SK-4100) according to manufacturerôs instructions.

Both cytokeratin and vimentin IHC were achieved using an automated IHC slide stainer, the Ventana BenchMark Ultra IHC staining module. Primary mouse anti-human pan cytokeratin antibodies (AE1/AE3, Biocare Medical, Pacheco, CA, #CM011C) were used at 1:100 dilution. Mouse anti-human vimentin primary antibody (clone V9, Leica Biosystems, #NCL-L-VIM-V9) was used at 1:80 dilution. Slides were counterstained with hematoxylin.

The Picro Sirius Red Kit (Abcam, Cambridge, MA, ab150681) was used for detection of collagen types I and III. Weigertôs Haematoxylin parts A and B, provided in the Picro Sirius Red kit, were mixed in equal parts. The slides were incubated in this solution for 8 minutes, followed by staining with Picro Sirius Red for 71 minutes.

Results of the IHC markers and the Sirius red stain were analyzed according to the cell arrangements and structures seen in AOT, because it was possible that the cells forming different structures might express proteins differently. The structures in AOT named in this study were described and illustrated in Figure 1. A rosette structure was a central droplet of product surrounded by a single layer of cells (Fig. 1A). A duct-like structure was a single row of cuboidal

to columnar cells around a central lumen (Fig. 1B). A double-layered sphere showed two layers of cuboidal or columnar cells with product between the cell layers (Fig. 1C). Anastomosing cords were strands of interlacing cells (Fig. 1D). A stellate reticulum-like area was made of loosely arranged cells (Fig. 1E). Intermediate cells were spindle-shaped cells found between other structures (Fig. 1F). Condensed collections of cells were epithelial knots (Fig. 1G). Two distinct types of calcifications were defined (Fig. 1H-I). Liesegang ring-like calcifications were globular basophilic calcifications (Fig. 1H). Eosinophilic calcifications were round or irregularly shaped calcifications, previously called dentinoid (Fig. 1I). There were areas that showed these two calcification types integrated together and this combination was called a mixed calcification.

Results

The IHC results were examined qualitatively by light microscopy for localization in AOT and correlation with histological structures. Any positive staining found in any AOT case was counted as a positive result. Overall, 20 of 21 cases showed focal positivity for nestin and vimentin, while 21/21 cases showed diffuse positivity for cytokeratin in most AOT cells. Zero cases showed DSPP positivity (Figures of valid controls used for nestin and DSPP IHC, i.e., odontoma, were shown in Supplement 1A-C).

We found that the appearance of some AOT structures evolved through serial sections for different IHC markers. This issue was then analyzed by obtaining serial sections of an AOT. It showed that small duct-like structures could evolve into epithelial knots and rosettes (Supplement 2A-G). Therefore, it seemed appropriate to re-label the rosette structures as rosette/duct-like structures (RDS). Some large duct-like structures did not evolve into other

structures, so the term ñlarge duct-like structuresò was used. Additionally, double-layered spheres could evolve into rosettes (data not shown).

The RDS staining results showed some variations among the 21 cases. Three different cases of RDS were illustrated (Fig. 2A, F, and K). Nestin expression was seen in RDS cells, showing uniformly weak or focally strong positivity (Fig. 2B, G, and L). Cytokeratin was strongly and diffusely positive, with focal areas of reduced or absent staining (Fig. 2C, H, and M). Vimentin expression ranged from negative to focally or uniformly positive in different RDS (Fig. 2D, I, and N). Some tumor products showed positive results, while others were negative for Sirius red (Fig. 2E, J, and O). Some RDS cells showed nestin and cytokeratin co-expression (Fig. 2B and C), while other RDS cells showed co-expression of nestin and vimentin with reduced or lost cytokeratin expression (Fig. 2G, H, and I). Some RDS cells simultaneously expressed nestin, cytokeratin, and vimentin (Fig. 2L, M, and N).

Large duct-like structures (Fig. 3A) also showed variations in the IHC results. Nestin, DSPP, and vimentin were not expressed by cells of the large duct-like structures (Fig. 3B, C, and F). Cytokeratin expression was seen in cells of the large duct-like structures in every case but one (Fig. 3D and E). Cytokeratin and vimentin, but not nestin and DSPP, were expressed by intermediate cells (Fig. 3D, F, B and C, respectively).

Double-layered sphere cells (Fig. 4A) expressed nestin (Fig. 4B, also shown in 3B) but not DSPP or vimentin (Fig. 4C and E). Cytokeratin positivity was seen in double-layered sphere cells, but staining intensity was reduced (Fig. 4D). The product between the two layers of cells was highlighted with Sirius red (Fig. 4F). Cells of the stellate reticulum-like area did not express nestin, DSPP, or vimentin, but expressed cytokeratin (Fig. 4B-E).

Cells which constituted the anastomosing cords expressed cytokeratin and vimentin but not nestin or DSPP (Supplement 3A-E).

Epithelial knots were seen to evolve into other structures within several serial sections, so they were not specifically described in these IHC results.

Whether the Liesegang ring-like calcifications and eosinophilic products were isolated or found in mixed calcifications, the large eosinophilic products consistently stained positively, and the basophilic products stained negatively for Sirius red (Fig. 5A, F, G, and H). Cytokeratin and vimentin positive staining was seen in the intermediate cells adjacent to the mixed calcifications (Fig. 5B-E).

Discussion

The research question to be answered was whether AOT was an epithelial-only or a mixed epithelial-and-mesenchymal tumor. We hypothesized that AOT had epithelial and mesenchymal cell populations and that was reflected in their products. The IHC and Sirius red findings appeared to support our hypothesis.

Our result showed that 95.23% of the 21 AOT cases expressed nestin in the RDS and double-layered sphere cells. Nestin is a type VI intermediate filament protein found in stem or progenitor cells in central nervous system, pancreatic islets, skeletal muscle, hair follicle, heart, bone marrow and odontoblasts.[10, 19-25] Nestin is expressed in early stage of central nervous system and muscle development; and is lost or down-regulated in mature tissue except during injury and repair.[19, 26-28] Nestin expression is found in secretory odontoblasts, specifically during odontogenesis, and tooth repair after tooth development.[10] Nestin expression is found in pulpal cells but not in ameloblasts. Nestin was used because its expression would indicate a

mesenchymal origin, differentiating toward odontoblasts or pulpal cells. Fujita, et al.[29] investigated nestin expression in several odontogenic tumors, reporting that 5/6 AOTs expressed nestin in the ñsmall nodular foci and rosette patterns.ò Our results appeared to be consistent with their finding, which demonstrated that this was a reproducible finding among different research groups. However, Fujita, et al.[29] did not interpret their finding as an indication of a mesenchymal cell population but interpreted the cells that expressed nestin were ñepitheliumò which ñhas a different immunophenotype.ò In the same study, nestin expression was also found in several mixed odontogenic tumors, such as odontoma, ameloblastic fibroma, and ameloblastic fibro-odontoma, and it was concluded that ñnestin is a useful marker for the odontogenic mesenchyme and odontoblasts in odontogenic tumours,ò It would have been more consistent with Fujitaôs own conclusion if the nestin-positive cells in all odontogenic tumors, including AOT, were considered to have an ectomesenchymal origin or component.

Although DSPP expression was not seen in the 21 cases of AOT in our study, this negative result does not completely exclude the possibility of DSPP expression in AOT. There might be two possibilities if DSPP was expressed in AOT but showed a negative result in our study. First, the rabbit anti-human recombinant DSPP antibody recognizes DSPP epitope T132 to D373 (on DSP domain), according to the manufactural information.[30, 31] This particular epitope might be altered or missing in DSPP expressed by the AOT cells. Second, the amount of DSPP expressed by the AOT mesenchymal cells might be small and that trace amount was below the sensitivity of IHC testing. On the other hand, it is also possible that DSPP indeed is not expressed in AOT cells. This also does not necessarily argue against a mesenchymal component in AOT. Collagen type I is the major organic component of dentin and constitutes about 87-89% of dentin matrix.[16, 17] Collagen types III and V account for 1-3% of dental matrix; and non-

collagenous proteins, including small integrin-binding ligand, N-linked glycoproteins (SIBLINGs), proteoglycans and other proteins, account for the rest of 10% or less.[16, 17] DSPP is one of the SIBLINGs family members. Despite DSPP is not expressed in AOT, the positive result of Sirius red in AOT eosinophilic products indicates presence of collagen type I and III, two of the most abundant proteins in dentin matrix, and still supports a mesenchymal component in AOT. In addition, DSPP is immediately cleaved into three proteins after secretion: DSP (dentin sialoprotein), DGP (dentin glycoprotein), and DPP (dentin phosphoprotein).[11] DSPP has been found in polarizing and functional odontoblasts and, to a lesser extent, transiently in pre-secretory ameloblasts.[32, 33] It has been found that odontoblasts expressed and secreted collagen type I before DSPP.[34] Bronkers, et al.[34] reported that collagen type I expression occurred in mesenchymal odontogenic cells prior to final differentiation into odontoblasts, while DSPP/DSP expression was first detected in new odontoblasts. Papagerakis et al.[33] also found that DSPP protein was detected in the cytoplasm of odontoblasts only when they were fully polarized. Our result showed that some RDS cells expressed nestin but not DSPP, and they secreted small eosinophilic products containing collagen types I and III, evidenced by Sirius red positivity. These findings also suggest that those RDS cells may represent pre-odontoblasts or pre-pulpal cells.

The term ñdentinoidò has been defined as ñatypical poorly mineralized dentineò without dentinal tubules in the Introduction section of the WHO 1992 classification;[4] and has been often used to describe the eosinophilic products in AOT in the literature. Our results showed that eosinophilic products (dentinoid), but not basophilic products, stained positively with Sirius red. It indicated that dentinoid, but not the basophilic products, in AOT contained collagen types I and III.[10, 12, 13] Collagen type I is the major organic component of dentin, especially intertubular

dentin, which does not show dentinal tubules.[16, 18] The presence of collagen types I and III in the eosinophilic products suggested the products were most likely aberrant dentin. This finding also justified the usage of ñdentinoidò biochemically. Sirius red also stained positively in larger eosinophilic products found in RDS (Fig. 2E and J), and the linear products in the double layer spheres (Fig. 4F). These findings also supported that the cells surrounding those products were most likely pre-odontoblasts. The basophilic products that were Sirius red-negative could possibly be aberrant enamel. The variations of biochemical properties in AOT products reflected the cells producing them and suggested a mixed cell population.

Our results showed that vimentin expression was found in anastomosing cords, RDS, and/or intermediate cells in 95.23% of cases. Although cells in the anastomosing cords and intermediate cells also expressed cytokeratin (discussed below), scattered RDS cells expressed only vimentin (Fig. 2H and I). Vimentin is an intermediate filament protein, which has been used to identify mesenchymal cells.[35] Previous studies also have reported focal vimentin expression in AOT, although they did not clearly describe the results according to different AOT structures and cell patterns. Crivelini, et al.[36] found vimentin expression in the ñfusiform or ovoid cells close to calcified bodies or darker eosinophilic materials in the cytoplasmic pole near them.ò Leon, et al.[37] described vimentin expression ñin the trabecular and cribriform areas, and also in the cuboidal peripheral cells to the nodules.ò Sudhakara, et al.[38] described vimentin expression in cells ñpresent periphery to Type A cells, internodularly, cribriform pattern in some parts of the tumor; usually spindled-shaped; darkly stained eosinophilic cytoplasm with hyperchromatic nuclei.ò Tatemoto, et al.[39] found vimentin expression in the ñsmall and compact cells at the periphery of the A cell-containing focus.ò Although different terminology was used in these studies, it seemed their findings for vimentin overlapped with our results. Despite vimentin

expression in AOT being a reproducible finding, the interpretations in the previous studies vary. Although not specifically stated, Leon, et al.[37] suggested a possible mesenchymal population in AOT. They interpreted that vimentin expression ñmay indicate the existence of a variable phenotype in certain areas of the tumourò and that ñAOT is a tumour with a variable degree of differentiation and structural characteristics.ò[37] Crivelini, et al.ôs[36] conclusion was that ñvimentin-positive cells of AOTs have secretory functions.ò Sudhakara, et al.[38] interpreted that ñthe heterogenous variable expression of vimentin in AOT suggests it to be a nonspecific marker, but its partial role in histogenesis of mineralized component cannot be negated.ò

Vimentin and cytokeratin co-expression was seen in the anastomosing cords, intermediate cells, and some cells of the RDS. In 1989, Kasper, et al.[40] studied co-expression of vimentin and cytokeratin in developing tooth germs and found transient co-expression of vimentin and cytokeratin in odontogenic epithelial cells of the outer enamel epithelium and stellate reticulum during the bell stage of odontogenesis. Kasper, et al.[40] presented four hypotheses to explain this phenomenon. The first hypothesis was that co-expression was a normal, transient phenomenon occurring during odontogenesis. The second hypothesis was that co-expression indicated proliferating cells. Hypothesis three stated co-expression occurred when cell-to-cell contacts were lost, as seen in the loosely arranged stellate reticulum. The fourth hypothesis was that vimentin and cytokeratin co-expression could indicate cells with secretory or resorptive functions. The AOT cells that co-expressed vimentin and cytokeratin may represent those cells in that transient stage of odontogenesis. Other possibilities are that co-expression could indicate that AOT cells in a proliferative state or with secretory functions, such as those in the RDS.

Some cells of RDS showed co-expression of nestin and cytokeratin (Fig. 2B and C), or co-expression of nestin, cytokeratin and vimentin (Fig. 2L, M, and N). To the best of our knowledge, co-expression of nestin and cytokeratin, or nestin, cytokeratin and vimentin have not been described during odontogenesis in the literature. Whether these phenomena of AOT also occur in normal odontogenesis, or if they represent pathologic processes caused by mutations, as in a benign neoplasm, remains unknown.

The strengths of our study include sample size (n=21), an analysis according to histological structures, and correlation with the AOT products. The limitation of our study is that only three mesenchymal markers (nestin, DSPP, vimentin) were used in the IHC investigation. The results provide initial supporting evidence but limited information on characteristics of those mesenchymal cells. Future studies using other proteins expressed by odontogenic mesenchymal cells (odontoblasts, pulpal or stem cells) and/or investigating expression of these mesenchymal proteins and related signal transduction proteins at the mRNA level may provide further evidence and characterization of this small population of mesenchymal cells in AOT. On this regard, it is also worth noting that expression of some odontoblast-related proteins, such as osteopontin, osteonectin, and osteonectin, indeed have been reported previously in AOT cells and/or products.[41]

Conclusion

We conclude that although AOT is predominantly composed of epithelial cells, some cells of the RDS show mesenchymal phenotypes. The eosinophilic products (dentinoid) of AOT contain collagen types I and III, which are mesenchymal products. Our findings suggest that AOT is a mixed odontogenic tumor.

Credit statements

Kelcie Barnts: Conception of work, acquisition, analysis and interpretation of data, drafting and revising the work, final approval.

Jian Jerry Feng: Conception/design of work, interpretation of data, revising the work, final approval.

Chunlin Qin: Conception/design of work, interpretation of data, revising the work, final approval.

Hua Zheng: Conception/design of work, interpretation of data, revising the work, final approval.

Yi-Shing Lisa Cheng: Conception/design of work, interpretation of data, revising the work, final approval.

References

1. Gorlin RJ, Chaudhry AP. Adenoameloblastoma. Oral Surg Oral Med Oral Pathol 1958;11(7):762-8.
2. Philipsen HP, Reichart PA. Adenomatoid odontogenic tumour: facts and figures. Oral Oncol 1999;35(2):125-31.
3. Neville BW, Damm, Douglas D., Allen, Carl M., Chi, Angela C. . Oral and Maxillofacial Pathology. Oral and Maxillofacial Pathology, Fourth Edition. Fourth Edition ed: Elsevier; 2016. p. 664-66.
4. Kramer IRH, Pindborg, J.J., Shear, M. Histological Classification of Odontogenic Tumours. Histological Classification of Odontogenic Tumours. Second Edition ed. New York, New York: Springer-Verlag Berlin Heidelberg 1992. p. 19-20.
5. Philipsen HP, Nikai, H.P. Adenomatoid Odontogenic Tumour. In: Leon Barnes JWE, Peter Reichart, David Sidransky, editor. World Health Organization Classification of Tumours: Pathology & Genetics: Head and Neck Tumours. Third Edition ed. Lyon, France: International Agency for Research on Cancer; 2005. p. 304-05.

6. Philipsen HP, Reichart PA. Revision of the 1992-edition of the WHO histological typing of odontogenic tumours. A suggestion. J Oral Pathol Med 2002;31(5):253-8.
7. Gao YH, Yang LJ, Yamaguchi A. Immunohistochemical demonstration of bone morphogenetic protein in odontogenic tumors. J Oral Pathol Med 1997;26(6):273-7.
8. Ogose A, Motoyama T, Hotta T, Watanabe H. Expression of bone morphogenetic proteins in human osteogenic and epithelial tumor cells. Pathol Int 1996;46(1):9-14.
9. Wright JM. Adenomatoid Odontogenic Tumour. In: El-Naggar AK CJ, Grandis JR, Takata T, Slootweg PJ, editor. WHO Classification of Head and Neck Tumours. 4th Edition ed. France: International Agency for Research on Cancer; 2017. p. 221.
10. About I, Laurent-Maquin D, Lendahl U, Mitsiadis TA. Nestin expression in embryonic and adult human teeth under normal and pathological conditions. Am J Pathol 2000;157(1):287-95.
11. Yamakoshi Y. Dentinogenesis and Dentin Sialophosphoprotein (DSPP). J Oral Biosci 2009;51(3):134.
12. Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 1979;11(4):447-55.
13. Junqueira LC, Cossermelli W, Brentani R. Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy. Arch Histol Jpn 1978;41(3):267-74.
14. Gatseva A, Sin YY, Brezzo G, Van Agtmael T. Basement membrane collagens and disease mechanisms. Essays Biochem 2019;63(3):297-312.
15. LeBleu VS, Macdonald B, Kalluri R. Structure and function of basement membranes. Exp Biol Med (Maywood) 2007;232(9):1121-9.

16. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. Front Biosci (Elite Ed) 2011;3:711-35.

17. Linde A. Dentin matrix proteins: composition and possible functions in calcification. Anat Rec 1989;224(2):154-66.

18. Nanci A. Dentin-Pulp Complex. In: Nanci A, editor. Ten Cate's Oral Histology. Sixth Edition ed. St. Louis, Missouri: Mosby; 2003. p. 192-239.

19. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. Cell 1990;60(4):585-95.

20. Lothian C, Lendahl U. An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. Eur J Neurosci 1997;9(3):452-62.

21. Eberhardt M, Salmon P, von Mach MA, et al. Multipotential nestin and Isl-1 positive mesenchymal stem cells isolated from human pancreatic islets. Biochem Biophys Res Commun 2006;345(3):1167-76.

22. Lobo MV, Arenas MI, Alonso FJ, et al. Nestin, a neuroectodermal stem cell marker molecule, is expressed in Leydig cells of the human testis and in some specific cell types from human testicular tumours. Cell Tissue Res 2004;316(3):369-76.

23. Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. Proc Natl Acad Sci U S A 2005;102(15):5530-4.

24. Kachinsky AM, Dominov JA, Miller JB. Intermediate filaments in cardiac myogenesis: nestin in the developing mouse heart. J Histochem Cytochem 1995;43(8):843-7.

25. Mendez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 2010;466(7308):829-34.

26. Kachinsky AM, Dominov JA, Miller JB. Myogenesis and the intermediate filament protein, nestin. Dev Biol 1994;165(1):216-28.

27. Sejersen T, Lendahl U. Transient expression of the intermediate filament nestin during skeletal muscle development. J Cell Sci 1993;106 (Pt 4):1291-300.

28. Holmin S, Almqvist P, Lendahl U, Mathiesen T. Adult nestin-expressing subependymal cells differentiate to astrocytes in response to brain injury. Eur J Neurosci 1997;9(1):65-75.

29. Fujita S, Hideshima K, Ikeda T. Nestin expression in odontoblasts and odontogenic ectomesenchymal tissue of odontogenic tumours. J Clin Pathol 2006;59(3):240-5.

30. Kerafast. Rabbit anti- human DSPP antibody LF-151 datasheet: Kerafast

31. Fisher LW Antisera. National Institute of Dental Craniofacial Research "https://www.nidcr.nih.gov/research/archive/intramural-lab/antisera#sialoprotein". Accessed June 21, 2019.

32. Begue-Kirn C, Krebsbach PH, Bartlett JD, Butler WT. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. Eur J Oral Sci 1998;106(5):963-70.

33. Papagerakis P, Berdal A, Mesbah M, et al. Investigation of osteocalcin, osteonectin, and dentin sialophosphoprotein in developing human teeth. Bone 2002;30(2):377-85.

34. Bronckers AL, D'Souza RN, Butler WT, et al. Dentin sialoprotein: biosynthesis and developmental appearance in rat tooth germs in comparison with amelogenins, osteocalcin and collagen type-I. Cell Tissue Res 1993;272(2):237-47.

35. Franke WW, Schmid E, Osborn M, Weber K. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc Natl Acad Sci U S A 1978;75(10):5034-8.

36. Crivelini MM, de Araujo VC, de Sousa SO, de Araujo NS. Cytokeratins in epithelia of odontogenic neoplasms. Oral Dis 2003;9(1):1-6.

37. Leon JE, Mata GM, Fregnani ER, et al. Clinicopathological and immunohistochemical study of 39 cases of Adenomatoid Odontogenic Tumour: a multicentric study. Oral Oncol 2005;41(8):835-42.

38. Sudhakara M, Rudrayya SP, Vanaki SS, et al. Expression of CK14 and vimentin in adenomatoid odontogenic tumor and dentigerous cyst. J Oral Maxillofac Pathol 2016;20(3):369-76.

39. Tatemoto Y, Tanaka T, Okada Y, Mori M. Adenomatoid odontogenic tumour: co-expression of keratin and vimentin. Virchows Arch A Pathol Anat Histopathol 1988;413(4):341-7.

40. Kasper M, Karsten U, Stosiek P, Moll R. Distribution of intermediate-filament proteins in the human enamel organ: unusually complex pattern of coexpression of cytokeratin polypeptides and vimentin. Differentiation 1989;40(3):207-14.

41. Modolo F, Biz MT, Martins MT, Machado de Sousa SO, de Araujo NS. Expression of extracellular matrix proteins in adenomatoid odontogenic tumor. J Oral Pathol Med 2010;39(3):230-5.

Figure Legends

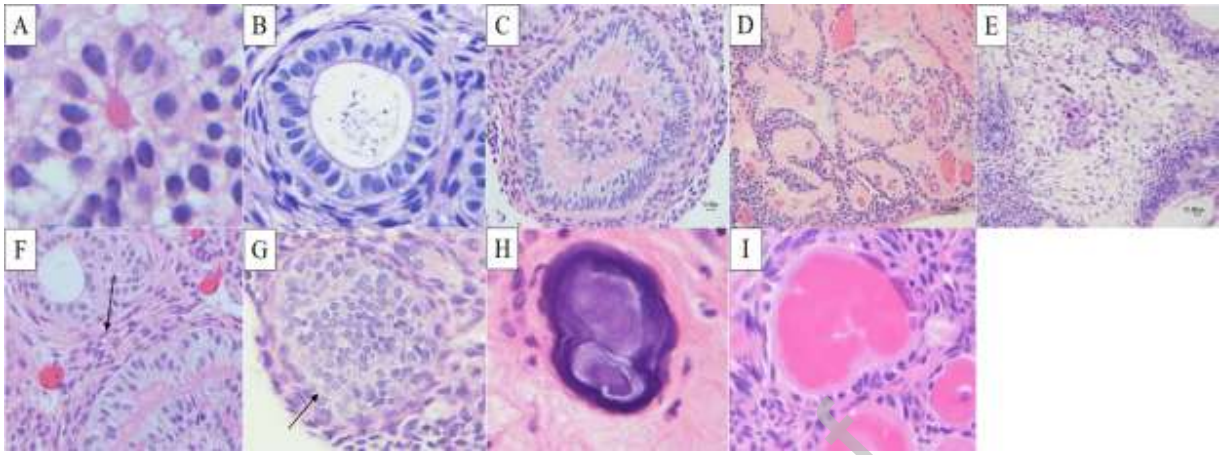

Figure 1. The illustration of various structures found in AOT and analyzed in this study. **A)** Rosette structure. **B)** Duct-like structure. **C)** Double-layered sphere. **D)** Anastomosing cords. **E)** Stellate reticulum-like area. **F)** Intermediate cells (arrow). **G)** Epithelial knot (arrow). **H)** Liesegang ring-like basophilic calcification. **I)** Eosinophilic products or dentinoid.

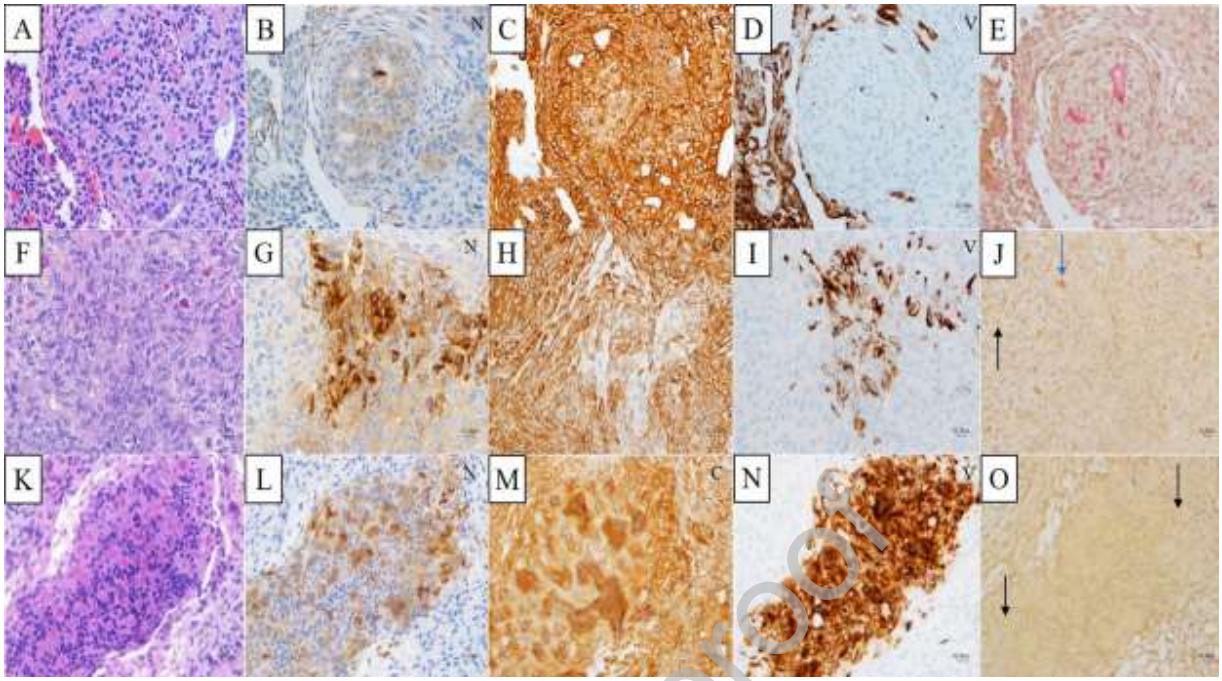

Figure 2. Representative cases of the Sirius red staining and the IHC results for nestin, cytokeratin, and vimentin found in RDS. A-E, F-J, and K-O represented three different cases. **A, F, K)** RDS seen in three cases of AOT (H&E stain, original magnification x 280). **B, G, L)** Nestin-positive staining was found in most of the cells of the RDS (Nestin IHC stain, original magnification x 280). **C, H, M)** Cytokeratin-positive staining was found in nearly all the cells of the RDS, except some focal areas, as in H (AE1/AE3 IHC stain, original magnification x 280). **D, I, N)** Vimentin expression in the RDS varied from no expression (D) to expression in scattered cells (I), to expression in most cells (N) (Vimentin IHC stain, original magnification x 280). **E, J, O)** Sirius red staining showed positive (E, and blue arrow in J) in some products, and negative (black arrows in J and O) in other products of rosettes (Sirius red stain, original magnification x 280).

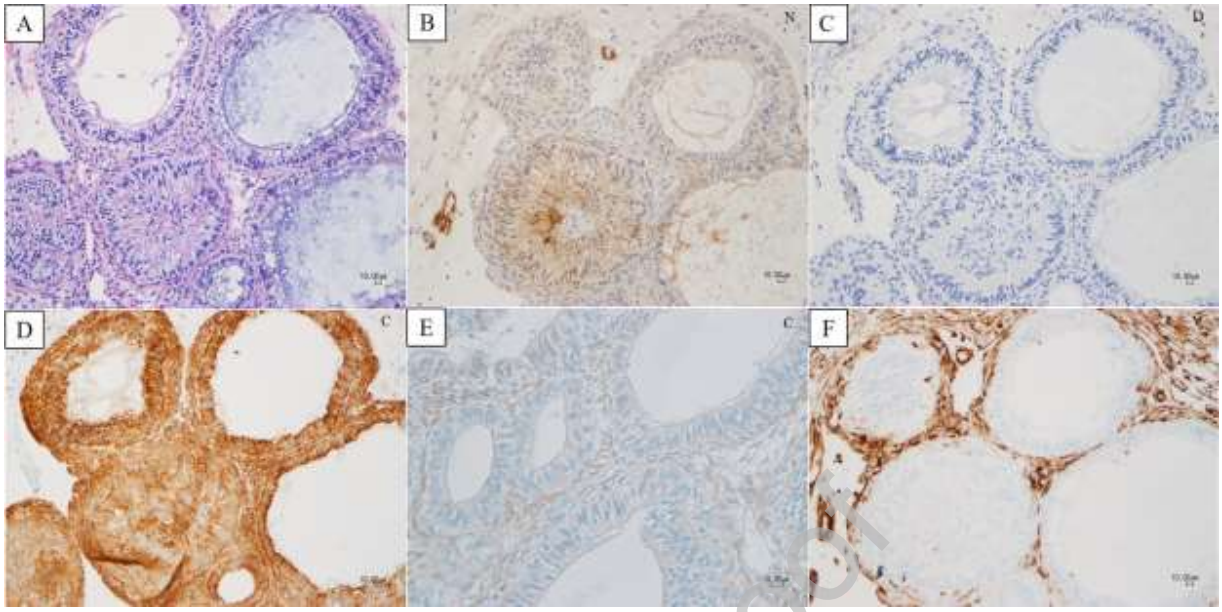

Figure 3. The IHC results of the large duct-like structures and intermediate cells showed variations in cytokeratin expression. **A)** Large duct-like structures and intermediate cells seen in AOT (H&E stain, original magnification x 140). **B)** Nestin expression was not seen in the cells of the large duct-like structures or the intermediate cells but could be seen in the cells of the double-layered sphere (Nestin IHC stain, original magnification x 140). **C)** DSPP was not expressed in the cells of the large duct-like structures or in the intermediate cells (DSPP IHC stain, original magnification x 140). **D)** Cytokeratin was strongly and diffusely expressed in the cells of the large duct-like structures and intermediate cells (AE1/AE3 IHC stain, original magnification x 140). **E)** Cytokeratin expression was not seen in the cells of the large duct-like structures in one case, although faint expression of cytokeratin was seen in the intermediate cells (AE1/AE3 IHC stain, original magnification x 280). **F)** Vimentin was not expressed in the cells of the large duct-like structures but could be seen in the intermediate cells among the large duct-like structures (Vimentin IHC stain, original magnification x 140).

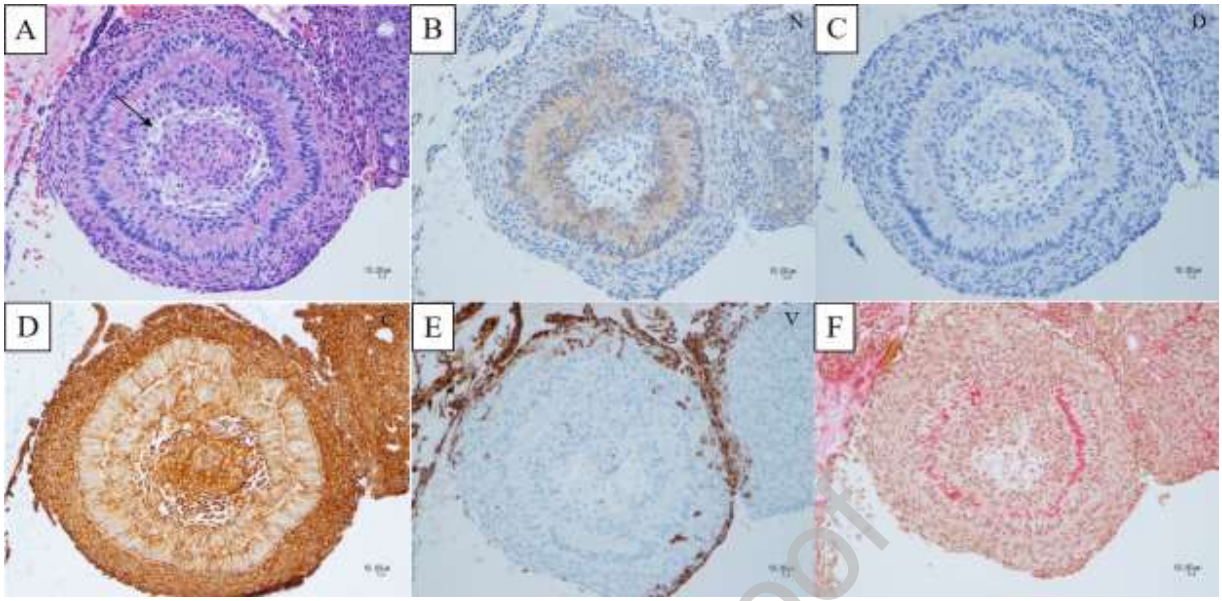

Figure 4. The IHC results of double-layered spheres and stellate reticulum-like area. **A)** A double-layered sphere was seen with a stellate reticulum-like area in the center (arrow, H&E stain, original magnification x 140). **B)** Nestin was expressed in the cells that formed the double-layered sphere, but not in the cells of the stellate reticulum-like area within or the intermediate cells outside of the double-layered sphere (Nestin IHC stain, original magnification x 140). **C)** No expression of DSPP was seen (DSPP IHC stain, original magnification x 140). **D)** Cytokeratin-positive staining was seen in the cells that constituted this structure, but the intensity of the staining was reduced compared to the intermediate cells around this structure or the stellate reticulum-like area in the center of the structure. There was strong expression of cytokeratin in cells of the stellate reticulum-like area and the intermediate cells (AE1/AE3 IHC stain, original magnification x 140). **E)** Vimentin was not expressed in the cells that formed the double-layered sphere or the stellate reticulum-like area but was positive in scattered intermediate cells (Vimentin IHC stain, original magnification x 140). **F)** Sirius red highlighted the products found between the two cell layers of the double-layered sphere (Sirius red stain, original magnification x 140).

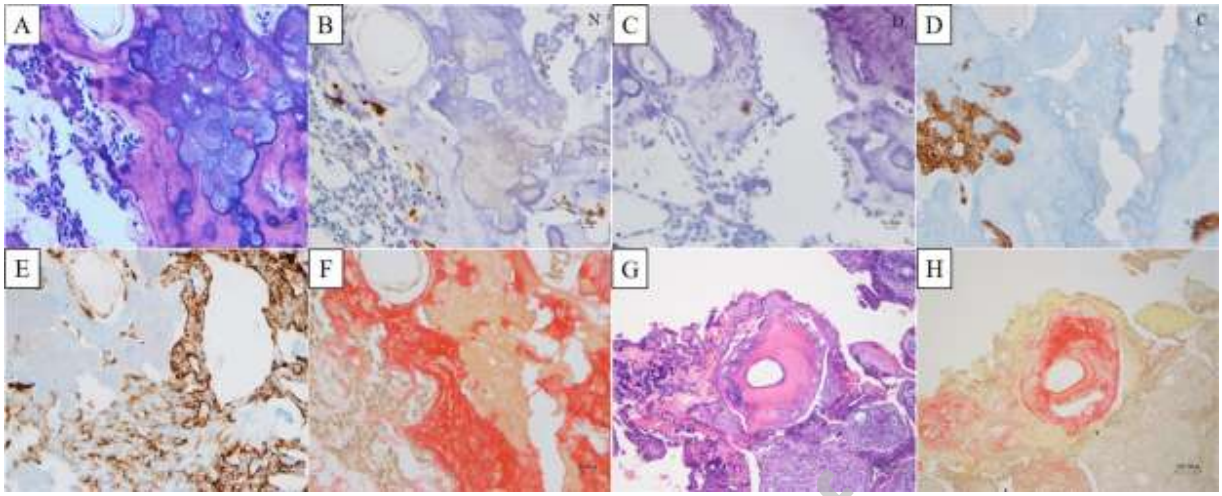

Figure 5. Sirius red staining results in the mixed calcifications of AOT and the IHC results in the cells adjacent to the mixed calcifications. A-F and G-H represented two different cases. **A, G)** Mixed calcifications that showed eosinophilic products and basophilic calcifications adjacent to each other (H&E stain, A: original magnification x 280, G: original magnification x 70). **B)** A few scattered cells adjacent to the mixed calcifications or trapped in the calcifications showed nestin expression (Nestin IHC stain, original magnification x 280). **C)** DSPP expression was not seen in either the products or the adjacent cells (DSPP IHC stain, original magnification x 280). **D)** The cells adjacent to the calcifications expressed cytokeratin (AE1/AE3 IHC stain, original magnification x 280). **E)** Vimentin was expressed in the cells adjacent to the calcifications (Vimentin IHC stain, original magnification x 280). **F, H)** The eosinophilic products in the mixed calcifications stained positively, while the areas that were basophilic stained negatively for Sirius red (Sirius red stain, F: original magnification x 280, H: original magnification x 70).