THE CELL ORIGINS AND TUMOR PRODUCTS IN

ADENOMATOID ODONTOGENIC TUMOR

A Thesis

by

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ABSTRACT

The purpose of this project was to determine the cell origins and to investigate the histochemical properties of the products present in adenomatoid odontogenic tumor (AOT). In 1992, the World Health Organization (WHO) categorized AOT as a mixed, epithelial and mesenchymal, odontogenic tumor. But in the 2005 edition of the WHO classification, it was reclassified as an epithelial-only tumor. There was no reason provided about this reclassification in the 2005 edition of the WHO classification. Therefore, whether AOT should be classified as a mixed or an epithelial-only tumor remains controversial. In the present investigation, immunohistochemical studies with nestin, dentin sialophosphoprotein (DSPP), cytokeratin, and vimentin were performed in 21 AOT cases and analyzed according to the various cells structures and arrangements seen in AOT. The results showed that 20/21 (95.23%) cases demonstrated nestin expression, 0/21 (0%) cases demonstrated DSPP expression, 21/21 (100%) cases demonstrated cytokeratin expression, and 20/21 (95.23%) cases demonstrated vimentin expression. Cells of the anastomosing cords, intermediate cells, and cells that formed some of the rosette/duct-like structures showed co-expression of vimentin and cytokeratin. In some cases, the cells that constituted the rosette structures showed overlapping expression of nestin, cytokeratin, and vimentin. Histochemical staining with Sirius red demonstrated the presence of collagen types I and III, which are mesenchymal cell products, in some of the tumor products. These results suggest that there is a mixed population of both epithelial and mesenchymal cells present in AOT.

We conclude that AOT is a mixed odontogenic tumor with some products that are secreted by mesenchymal cells. The results of this project provide evidence for the proper classification of AOT.

DEDICATION

To my Grandma Ruby and Grandpa Warren, both of whom passed away during the time I was working on this project. They were both extremely hard working and dedicated people who were great examples for me. They made me so proud.

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This work was supervised by a thesis committee consisting of Dr. Yi-Shing Lisa Cheng of the Department of Diagnostic Sciences and Dr. Jerry Jian Feng, Dr. Chunlin Qin, and Dr. Hua Zhang of the Department of Biomedical Sciences.

All work for the thesis was completed by the student, under the advisement of Dr. Yi-Shing Lisa Cheng of the Department of Diagnostic Sciences.

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NOMENCLATURE

AF	Ameloblastic Fibroma
AOT	Adenomatoid Odontogenic Tumor
BMP	Bone Morphogenetic Protein
CEOT	Calcifying Odontogenic Tumor
CK	Cytokeratin
DSPP	Dentin Sialophosphoprotein
H&E	Hematoxylin and Eosin
IHC	Immunohistochemistry
OF	Odontogenic Fibroma
REE	Reduce Enamel Epithelium
WHO	World Health Organization

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1. INTRODUCTION

1.1 Specific Aims

The purpose of this project was to determine whether adenomatoid odontogenic tumor (AOT) is an epithelial or a mixed epithelial and mesenchymal tumor. The specific aim was to investigate the immunohistochemical and histochemical staining patterns in AOT cells and products. The immunohistochemical stains were chosen to differentiate the epithelial elements from the mesenchymal elements. AOT was classified by the World Health Organization (WHO) as a mixed odontogenic tumor in 1992[1], but it was reclassified as an odontogenic epithelial tumor in 2005 with limited supporting evidence. The goal of this project was to provide additional evidence for the appropriate classification of this tumor in the future.

1.2 Background and Significance

AOT is a lesion that arises from odontogenic tissue and is found exclusively in the tooth-bearing areas of the jaws.[2, 3] The 1903 report of the Japanese surgeon Nakayama, identifying “cystic epithelial tumors” in two young females, may be the first cases with enough documented evidence to confirm these lesions as AOTs, as Nakayama including detailed drawings of the tumor histology in his report.[4] In 1905, Steensland reported an “epithelioma adamantinum,” which likely represents the earliest European publication of an AOT with sufficient documentation.[5] Over the years, many terms have been used to describe AOT. There was a time when AOT was considered to be a variant of ameloblastoma and, as such, the term “adeno-ameloblastoma” was

given to the lesion by Bernier in 1950.[6] In 1969, the term “Adenomatoid Odontogenic Tumor” was introduced by Philipsen and Birn,[7] in order to establish AOT as a distinct entity and to avoid confusion with ameloblastoma, which may have resulted in unnecessarily aggressive treatments.[2, 7] The WHO’s 1971 publication Histological Typing of Odontogenic Tumours, Jaw Cysts, and Allied Lesions also used the term “Adenomatoid Odontogenic Tumor,” and this is now the accepted nomenclature for the lesion.[5, 8]

AOT accounts for about 2-7% of the total number of odontogenic tumors.[5, 9] Although, the word “tumor” is found in the name of this lesion, many would classify AOTs as hamartomatous growths, as opposed to true, benign neoplastic lesions.[5] It has been said that the debate of the true nature of AOTs, as either hamartomatous growths or benign neoplasms, may never be resolved because there are sound arguments to support each of the hypotheses.[2] One piece of evidence that validates AOT as a benign neoplasm is that, while most lesions do not exceed a diameter of 3.0 cm, significantly larger lesions have been reported.[9] The largest lesion that could be found in the literature was reported to be 12.0 cm in diameter, showing that AOT may be capable of continuous growth.[10] Supporting the hamartomatous nature of AOT, recurrences are remarkably rare, reportedly as low as 0.18%, even if the removal of the lesion was incomplete.[3, 11] AOT is the only known epithelial tumor that does not have an identified, malignant counterpart.[4]

Most AOTs are diagnosed in younger people, with 66% of cases being found in patients in the second decade of their lives (between 10 and 19 years old), and it rarely

occurs in patients over 30 years of age.[9] Females are affected by AOT twice as often as males.[9]

Clinically, AOT shows a distinct predilection for the anterior jaws, with 80% of the lesions occurring in incisor/canine regions.[9] Most AOTs are found in the maxilla (64%), and 60% of the cases involve an impacted canine tooth, particularly a maxillary canine tooth.[5, 9] AOT is typically asymptomatic and is discovered either by routine radiography or in the process of investigating a tooth that has not erupted.[9] The permanent first and second molars are the teeth least commonly affected by AOT.[5] Deciduous teeth, as a group, are very rarely associated with this lesion.[5] Radiographically, AOTs are well-delineated radiolucencies, with about 66% of the lesions containing fine calcifications.[5, 9] It was shown that the fine radiopacities of an AOT are more likely to be seen with intraoral radiographs than on panoramic films, which could lead a practitioner toward a clinical diagnosis of AOT.[12] AOTs are more likely to cause the roots of adjacent teeth to be displaced than to be resorbed.[5] Larger intraosseous lesions may cause expansion of the overlying bone.[9] There are three variations of AOT with identical histologic features, classified based on anatomical location or the relationship with associated teeth: follicular, extrafollicular, and peripheral.[2] Follicular AOTs occur around the crowns of unerupted teeth, affecting the follicle.[9] The follicular type is often mistaken for a dentigerous cyst on a radiograph.[5] The follicular type of AOT accounts for 73% of the total AOTs, and is typically diagnosed at about 16 years of age.[5] Sixty-six percent of follicular AOTs arise in the maxilla.[13] Some evidence has shown that if a tooth associated with a follicular AOT is surgically exposed and the lesion is marsupialized, the tooth may continue to erupt and the lesional tissue may regress without further

intervention.[7, 13] Contrarily, other reports have shown that some lesions persist over time and prevent the eruption of the teeth associated with the AOTs, and, eventually, extractions were deemed necessary.[7, 14, 15] It is significant to note that there have been a few cases of AOT that have a fibrous, capsule-like tissue separating crown of the tooth from the tumor tissue, which is not a true dentigerous cyst-type of relationship.[13] It is not uncommon for a follicular AOT to extend apically from cemento-enamel junction, along the root structure of the tooth.[13] In addition, there have been cases of AOT involving more than one tooth.[13] While about 94% of follicular AOTs are associated with only one tooth, 6% are associated with more than one tooth.[2] Extrafollicular AOTs occur within the bone, but are not associated with the follicle of an unerupted tooth.[5] Fifty-eight percent of extrafollicular AOTs are found in the maxilla.[13] Most commonly found between, above, or superimposed over the roots of permanent teeth, extrafollicular AOTs account for 24% of the total number of AOTs.[5, 16-18] Eighty-nine percent of extrafollicular AOTs are associated with the root of a canine tooth.[13] The extrafollicular type of AOT is the AOT that is diagnosed at the latest age, with a mean age of 23 years.[5] The third type of AOT is the peripheral type, which is defined as AOT occurring in gingiva, without intra-osseous involvement.[19] These AOTs make up about 3% of all AOT lesions and tend to be diagnosed early, with the mean age at discovery being 13 years old.[5] The lower mean age at diagnosis may be because the lesion is often evident clinically.[2] It is significant to note that the gender predilection for peripheral AOT, particularly, has a highly significant female skew, with the ratio being 5.3 females to 1 male.[19] Clinically, most peripheral AOTs appear as small, sessile masses on the labial gingiva, overhanging the crowns of the adjacent teeth.[9, 13] These are often mistaken for

gingival fibrous lesions.[5, 9, 13] As many as 88% of peripheral AOTs are found in the anterior maxilla, with 80% affecting the maxillary incisors.[5, 13] This subtype of AOT may sometimes show slight erosion of the cortex of the alveolar bone.[2, 5] As of 2015, only 27 cases of peripheral AOT had been published, leaving a lot left to be learned about this variation of AOT.[20]

The histopathologic features of AOT closely resemble the dental organ.[3] AOTs have thick, fibrous capsules (Figure 1 A), which allow for complete enucleation of the lesions during surgical removal.[9] If an AOT is bisected, grossly, the tumor may be solid or it may show variable degrees of cystic change.[9] The cystic spaces within AOT may or may not contain a yellowish, semisolid material.[2] Microscopically, the tumor cells of AOT display a variety of patterns with scant background stroma (Figure 1 A).[9] The AOT cells can be arranged in sheets (Figure 1 B), anastomosing cords (Figure 1 C), double layered spheres (Figure 1 D), duct-like structures (Figure 1 D), and rosettes (Figure 1 E). Rosettes are a particularly distinctive arrangement of AOT cells.[9] These structures are formed around a central space and may contain an eosinophilic substance.[9] The characteristic duct-like structures of AOT may be plentiful, infrequent, or completely lacking in any given AOT.[9] These areas are made of columnar or cuboidal cells arranged in a ring around a central space.[9] The nuclei of these columnar or cuboidal cells tend to be polarized away from the space.[9] The mechanism of the formation of these duct-like structures is not fully known, but it is believed that these are formed as a result of the secretion of a substance by the proposed pre-ameloblast cells.[9] It is necessary to emphasize that these areas are not true ducts and that AOTs do not contain any true glandular elements.[9] Many AOTs contain small, focal areas of

calcifications throughout the tumor while other AOTs have much larger areas of matrix material or calcifications (Figure 1 F).[9] Some interpret the tumor product to be aborted attempts at enamel formation.[9, 21] The name given to this calcified material was “dentinoid,”[22] not enameloid, although it is believed that the material represents aborted enamel, which would be an epithelial product, not a mesenchymal product. Others have interpreted the calcified material to be cementum.[9]

There are several presentations of calcifications within the scope of AOT.[2] One form is a Liesegang-type pattern of concentric calcifications.[2] These irregular calcified bodies have been interpreted as dystrophic calcification of tumor tissues.[2] There are also globular islands of calcification found within collections of squamoid cells.[2] Yet another type is a hard tissue that may resemble cementum or irregular dentin, superficially.[2] It has been proposed that this is a metaplastic reaction of the background fibrous stroma.[2] Many investigations and studies regarding AOT have focused on the eosinophilic material and the calcified structures.[2] Whether there is amyloid, amyloid-like, or pseudoamyloid materials present in AOT has been controversial.[2] This controversy may have been promoted by the fact that there was not a single, agreed upon definition for amyloid.[2]

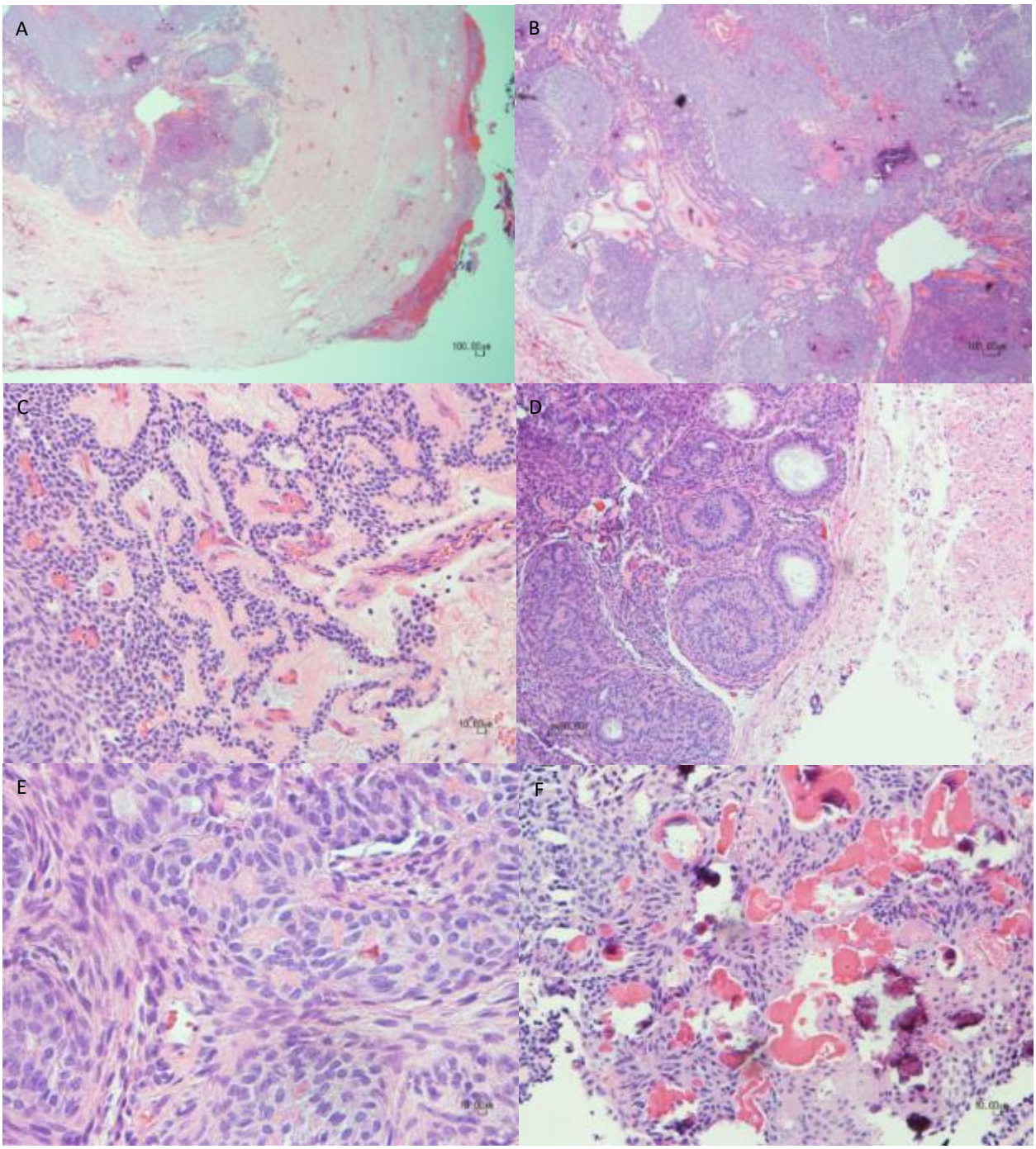

Figure 1 Histologic features of AOT. (A) AOT has a thick, fibrous capsule. (B) AOTs may contain many structures and patterns, giving a heterogeneous histologic appearance. (C) The anastomosing cords of AOT are most commonly found adjacent to the capsule of the lesion. (D) Duct-like structures and double layered spheres are some of the most recognizable structures of AOT. (E) Rosettes with eosinophilic amorphous material are characteristic features of AOT. (F) Calcified materials are found in many AOTs.

The pathogenesis of AOT is not clear. Several hypotheses have been proposed to speculate the origin of the tumor cells.[13] The hypotheses include origins from the dental lamina and its remnants, the enamel organ epithelium, reduced enamel epithelium, or the rests of Malassez.[2, 9] It is known that AOTs occur far more frequently in the successional dentition than in the deciduous dentition.[13] This has led some people to look at the gubernacular cord as a source of cells for the origin of AOT.[23] Successional teeth follow a gubernacular cord in the course of eruption, while this structure is not present in the eruption of deciduous teeth.[13] The gubernacular cord consists of a fibrous band of peripheral nerves, blood and lymphatic vessels, and the epithelial cells or cells clusters of the remnants of the dental lamina.[13, 20] Each successional tooth has a gubernacular cord connecting the pericoronal follicular tissue of successional teeth to the gingival tissue.[13] The gubernacular cord lies within a bony canal called the gubernacular canal, which opens onto the crest of the alveolar bone, just lingual to the deciduous tooth.[13]

The classification of AOT by the WHO has changed over the years, which created some confusion, generated many debates, and provided the opportunity for research to further clarify this lesion. In the second edition of the WHO's Histological Typing of Odontogenic Tumours, Jaw Cysts, and Allied Lesions, published in 1992, AOT was categorized as a tumor arising from odontogenic epithelium with odontogenic mesenchyme, with or without dental hard tissue.[1] In the 2005 edition of the WHO Classification of Tumors Pathology & Genetics Head and Neck Tumours, AOT was reclassified as a tumor of strictly epithelial origin.[24] The AOT section of the 2005 WHO was written by H. P. Philipsen and H. Nikai.[24] In this section of the WHO, there were just

five sources cited, all of which were case reports and/or literature reviews.[7, 15, 21, 23-25] Although this was the first edition of the WHO to reclassify AOT as a purely epithelial tumor, there was no explanation why this reclassification was justified.[24] There were no citations listed in the Definition paragraph in the AOT section of this version, where AOT was defined as being a tumor composed of odontogenic epithelium.[24] Looking into the previous literature, it can be seen that just three years prior to participating in the WHO working group, H. P. Philipsen collaborated with Reichart on an article entitled "Revision of the 1992-edition of the WHO histological typing of odontogenic tumours. A suggestion."[26] In this paper, they recommended that several odontogenic tumors be reclassified and a new edition of the WHO be published.[26] In this 2002 paper, there was a paragraph discussing AOT, and in the sentence where the reclassification of this tumor was being called for, there was a single reference cited, which was a paper published by Gao, et al.[27] in 1997.[26] Philipsen and Reichart[26] stated several pieces of evidence as a basis for reclassifying AOT as an epithelial tumor, such as the dentinoid material found in many AOTs was "generally interpreted as *metaplastically produced dentinoid*" and that the scarce, mature, fibrous stroma of AOT was not the type of stroma that would lead to inductive phenomena. They cited the study by Gao, et al.[27] as the only evidence for stating that AOT should no longer be categorized as a mixed tumor.[26, 27]

In reviewing the study by Gao, et al.,[27] it was found that the purpose of that study was to investigate the protein expression and distribution of bone morphogenetic protein (BMP) by immunohistochemistry in 44 odontogenic tumors, which included two AOTs. The monoclonal BMP antibody (BMPMcAb) was generated by the authors' research

laboratory and the Western blot showed that it reacted to two proteins extracted from human osteosarcoma cells that expressed BMP-1, 2, 3, 4, and 6, but it could not detect human recombinant BMP-2, 4, 6, and 12.[27] It was not clearly stated, but it seems that the antibody used by Gao, et al.[27] recognized BMP-1 and BMP-3, according to the authors. At the time Gao, et al.'s[27] paper was written, there were 15 known subtypes of BMP protein, but the BMP antibody used in the study detected only two proteins. Ameloblastoma, AOTs, and calcifying epithelial odontogenic tumors (CEOTs) were found to be negative for BMP, because they did not react to the two BMP protein subtypes tested, when they may be positive for one or more of the 13 other known subtypes of BMP.[27] There were also several other areas in Gao, et al.'s[27] paper that leave room for questions and doubts. For example, it is known that decalcification during pathology tissue processing can affect downstream immunohistochemical results. It was not specifically stated in Gao, et al.'s study which of the specimens were decalcified, so definite conclusions could not be drawn, considering false negatives might occur in specimens that had been decalcified.[27] In addition, Gao, et al.[27] stated in the text of the Result section that "in both cases of OF [odontogenic fibroma], the tumor cells were almost all negative to BMPMcAb, except for some odontogenic epithelial cells that showed a weak positive reaction." However, the paper listed the result of OF as positive for BMP in their Table 1.[27] If OF had been determined to be negative for BMP, the BMP-negative group of tumors would have consisted of a more diverse group, with ameloblastoma, AOT, CEOT, and odontogenic fibroma. Had this been the group of BMP-negative tumors presented, this would not have led to the conclusion that they were all of only epithelial origin.[27] A conclusion drawn by Gao, et al.[27] was that, because

AOT did not show positive staining for BMPMcAb, the odontogenic epithelial cells might be too immature to synthesize a detectable amount of BMP. This study also found that before the bell stage, all of the odontogenic epithelium of the tooth germ stained positively for BMPMcAb.[27] After the bell stage, the only epithelial cells that stained positively for BMPMcAb were the secretory ameloblasts.[27] This seems to show that the more immature the odontogenic epithelium is, the more likely it will express BMPMcAb, which would contradict their conclusion that the AOT cells did not stain with BMPMcAb because were too immature to express appreciable amounts of the protein.

The article written by Gao, et al.[27] presented several conclusions with regard to AOT. Gao, et al.[27] separated the 44 odontogenic tumors they studied into two group: those that were BMP-positive and form enamel, dentin, cementum, and/or bone and those that were BMP-negative. Because the two cases of AOT did not express BMP, Gao, et al.[27] speculated that these tumors might utilize a different method to form calcifications. While Gao, et al.[27] did not imply that the results indicated the cells of origin of either of these two AOTs, these results were interpreted by Philipsen and Reichart[26] to support the reclassification of AOT, as a lesion that arose from "odontogenic epithelium with mature, fibrous stroma; odontogenic ectomesenchyme not present," because the "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 were BMP-*negative.*"[26] This is all to say that AOT was reclassified from being a mixed odontogenic tumor in the 1992 WHO to an epithelial odontogenic tumor in the 2005 WHO, by the use

of one study that included only one IHC marker and tested only two AOT samples, and possible misinterpretations of the data presented.[24, 26, 27]

After the 2005 edition of the WHO Classification of Tumors Pathology & Genetics Head and Neck Tumours was released, Kumamoto and Ooya[28] conducted a study to investigate the role that BMP might play in odontogenic tumors, apparently in response to the investigation completed by Gao, et al., which was used as the basis for the reclassification of AOT.[24, 27] Kumamoto and Ooya[28] employed both immunohistochemical and reverse transcriptase-polymerase chain reaction (RT-PCR) to detect BMP-2, -4, -7, BMP receptors (BMPR) Ia and II, core-binding factor αI (CBFAI), and osterix in 7 tooth germs, 37 ameloblastomas, 6 AOTs, and 5 malignant ameloblastomas. Using RT-PCR, all of the tested mRNA (BMP-2, -4, -7, BMPR-Ia, BMPR-II, CBFA1, and osterix) was present in AOT.[28] With IHC, BMP-2, -4, -7, BMPRs, and CBFA1 were expressed in 20% to 100% of cells.[28] The authors of the paper made the supposition that BMP and the associated proteins likely played a role in the differentiation of both normal and neoplastic odontogenic tumors through epithelial-mesenchymal interactions.[28] The findings of Kumamoto and Ooya opposed those of Gao, et al., which were previously discussed.[27, 28] Whether BMPs are expressed in AOT is unclear because of the contradictory findings, but this further weakens the basis of the reclassification of AOT in the 2005 edition of the WHO Classification of Tumors Pathology & Genetics Head and Neck Tumours.[24]

There have been many other studies in the literature that used electron microscopy, histochemical, and immunohistochemical methods for investigating the

origins of the cells and products of AOT. The findings of this wealth of information are summarized below.

1.3 Electron Microscopic Studies of AOT

Electron microscopic studies of AOT have given a wide spectrum of findings and interpretations. There have been studies that agreed with previous findings and others that reached conclusions of contradictory results as to the nature of both the tumor products and cell types present in AOT. Despite numerous EM studies having been published, the exact nature of the product or products in AOT is still not clear. Based on the EM findings, researchers have speculated that the product or products in AOT were enamel matrix,[29] pre-enamel,[30] enamel,[31] enameloid, dentin,[29, 31, 32] pre-dentin,[29] cementum,[33] cementoid,[29] bone,[29, 33] amyloid,[31, 32, 34-36] an amyloid-like material,[31, 37] or a material that is similar to the basal lamina.[34, 38, 39] Because the presence of dentin was required to induce the formation of the enamel matrix, the ameloblasts cannot differentiate normally without dentin, and ameloblast development was arrested before the formation of an enamel matrix.[39] Shear[40] reported that tumor globules, which corresponded to amyloid negative–droplets, resembled pre-dentin, and because enamel formation did not occur without formation of dentin, some of the calcified material was dentin that lacked dentinal tubules.[31]

Based on their EM findings, some researchers suggested that the AOT products were enamel or enamel-like material. These researchers did not find collagen in AOT products. Because the matrices of bone, dentin, and cementum consist of collagen and ground substance into which calcium salts were deposited, this strengthened the theory

that the product in AOT was enamel because enamel was unique in that its calcified matrix contained no collagen.[29] During the process of calcification of normal enamel, hydroxyapatite crystals were deposited in a narrow seam of organic matrix.[29] Areas that contained such crystallites and were devoid of collagen were prominent in AOT.[29] Moro, et al.[31] were able to identify some needle-like crystals that were relatively large and resembled enamel crystals in their samples of AOTs. The AOT calcified materials showed an apatite crystal pattern when using electron diffraction.[38] It was determined that the AOT product is consistent with early enamel in some cases.[29] Although not exactly the same, Smith, et al.[34] drew the conclusion that AOT cells might be secreting portions of enamel proteins, so the product could not be enamel, but would be enamel-like. Smith, et al.[34] were not able to identify any true enamel in AOT, but believed that the pre-ameloblast AOT cells could be capable of producing fragments of enamel protein that may account for the amyloid fibrils. Lee[37] interpreted the amyloid-like material differently, stating that the significance of an amyloid-like material in this lesion is unknown, but it could have represented the host's reaction to the lesion.

There was disagreement about whether there were collagenous fibers present in the AOT products, which would influence how the eosinophilic amorphous material is interpreted. Several studies have reported that there were collagen or reticulin fibers present in AOT products,[31, 32, 37-39] while others reported there were no collagen or reticulin fibers found within AOT.[30, 35, 37] The conclusions drawn from these results varied as well. El-Labban[32] determined that the fine filamentous layer perpendicular to the epithelial basal lamina, contained collagen and amyloid, similar to that described in early dentin formation. It was further stated that the majority of the amyloid deposits

contained not only amyloid, but also collagen fibrils in both typical and degraded forms.[32] This suggested that the collagen in these deposits underwent degradation to become amyloid.[32] In separate studies, Lee[37] and Hatekeyama, et al.[39] presented the idea that the eosinophilic amorphous material of AOT was fibrous stroma material, rather than a product made by the AOT cells. The reticulin fibers and hemidesmosome-like structures that connected the tumor cells and the eosinophilic amorphous material found in their studies lent support to the idea that this material is comparable to fibrous stroma and was not a secretory product of the AOT tall columnar duct-like cells.[37, 39] Other studies disagreed with this and stated that the eosinophilic amorphous material was certainly a secretory product of the cells of the duct-like structures.[35, 36] Because Poulson, et al.[36] found secretory granules within the columnar cells of the duct-like structures on EM, they used their presence to strengthen the idea that the eosinophilic amorphous material was of cell origin. Poulson, et al.[36] stated that the duct-like spaces arose from secretory activity by the tall columnar cells forming the duct-like structures. There was a striking accumulation of secretory granules at the pole of the cell nearest to the duct-like space.[36] Schlosnagle and Someren[35] agreed with Poulson, et al.[36] and stated that, in regard to the formation of the duct-like spaces, these are not the result of degeneration because it was not possible to identify cells in the process of degeneration associated with these spaces in the numerous histologic sections taken.

With respect to the origin of the cells of AOT, several investigators believed that the ultrastructure of the tumor cells of AOT were found to be similar to the four layers seen in the enamel organ of a normal tooth germ, in morphology and in the order that the layers are arranged.[35, 39] Some investigators have found that there was a definite

organization to the AOT cells, resembling pre-ameloblasts, stratum intermedium, and stellate reticulum, respectively, from the duct-like spaces outward.[35] This has led to the conclusion that AOT cells arose from the enamel organ epithelium.[34-36, 39, 41] Moro, et al.[31] regarded the tall columnar cells surrounding the eosinophilic amorphous material as corresponding to ameloblasts just prior to the secretory stage. The eosinophilic amorphous material was found intercellularly, between the tall columnar cells, which corresponded to ameloblasts between secretory and maturation stages.[31] Poulson, et al.[36] used the secretory granules present in the tall columnar cells as supporting evidence for attempting to identify the type of cells that comprise AOT. Poulson, et al.[36] found that the material, contained within a dense aggregation of secretory granules, was identified at the luminal pole of a cell. This was interpreted as evidence of secretory activity by the cells surrounding the duct-like spaces.[36] Since elaboration of enamel matrix by ameloblasts involved formation of secretory granules similar in size and shape to those seen in AOT, the tumor cells could possess secretory function and produce both the granules and the intraluminal material.[36] Schlosnagle and Someren[35] disagreed with the view of Poulson, et al.[36] Schlosnagle and Someren[35] stated that there is evidence against these granules playing a significant role in secretion, specifically because of their relatively small numbers and the poorly developed Golgi apparatus.[35] Schlosnagle and Someren[35] do concede that one could argue that the duct-like spaces examined were the result of secretory activity which had been completed, with exhaustion of granules. The cells of the duct-like structures have dense cytoplasm, are rich in mitochondria, tonofilaments, desmosomes, and glycogen granules, and are poor in endoplasmic reticulum and Golgi apparatus.[34-36, 41] These

cells shared common features with pre-ameloblasts: the abundance of free ribosomes and the scarcity of the endoplasmic reticulum.[35, 36] According to Poulson, et al.,[36] there was one cell type found in AOT that was ultrastructurally comparable to cells of the stratum intermedium and stellate reticulum.

Cell junctions have been studied under EM; however, disagreements among studies have also occurred. While Schlosnagle and Someren[35] stated they were able to identify desmosomes between AOT cells, Shimono, et al.[42] found both desmosomes and desmosome-like junctions. The cell junctions could be critical in distinguishing AOT as an epithelial tumor versus a mixed tumor. One difference between ameloblasts and odontoblasts is that ameloblasts had desmosomes, while the odontoblasts had desmosome-like junctions, but not unequivocal desmosomes.[42] The presence of both desmosomes and desmosome-like junctions in AOT suggested that the AOT cells could be both immature ameloblasts and odontoblasts.[42] Tight junctions were not present in the tumors cells, although they were present in normal ameloblasts and odontoblasts,[42] which argued against the AOT cells being either normal ameloblasts or odontoblasts. Tight junctions were considerably dynamic junctions and closely related to the cellular differentiation.[42] Consequently, these junctions cannot be formed in cells that have incomplete membrane differentiation in the apical, lateral and basal directions.[42]

1.4 Histochemical Studies of AOT

AOT cells and products have been investigated extensively with histochemical stains. Forty-two different stains have been used in previous studies of AOT, including 2,2’ dihydroxy-6,6’-dinaphthyl disulphide (DDD),[43] acid mucopolysaccharide (colloid

iron),[44] Alcian blue,[37, 39, 40, 43, 45-47] Alcian blue/Hale's colloidal iron,[48] aldehyde fuchsin/toluidine blue,[48] Alloxan-Schiff,[43] azan blue,[46] celestine blue,[47] Congo red,[31, 32, 37, 49-51] crystal violet,[31] dimethylaminobenzaldehyde (DMAB) nitrite,[31, 43] dinitrofluorobenzene (DNFB) H-acid,[43] Gomori for reticulin,[47] Gomori for alkaline phosphatase,[46] Gomori silver impregnation,[48] Gordon and Sweets,[47] Heidenhain's azan,[48] Lillie's diazo method,[46] Mallory,[40] Mallory's azan,[31] Mallory phosphotungstic acid,[43] Mallory trichrome,[37, 44, 46, 47] Masson trichrome,[39, 43] methyl green-pyronin,[47] methyl violet metachromasia,[37] modified Gomori technique,[32, 37] Morel-Sisley diazotization,[43] mucicarmine,[40, 46] orange G,[47] orcein,[31] p-dimethylaminobenzaldehyde (p-DMAB) nitrite,[51] Pap's silver impregnation,[46] Periodic acid-Schiff (PAS),[31, 37, 39, 40, 43-49] Periodic acid-Schiff with diastase (PAS+D),[44, 48, 49] reticulin,[40] reticulum,[44] silver impregnation,[31, 39] thioflavine T,[32, 37] thioflavin T,[31, 51] toluidine blue,[31, 40, 43, 46] toluidine blue (pH 2.5),[39] toluidine blue (pH 4.4),[39] van Gieson,[31, 32, 37, 39, 40, 44, 46, 47] and von Kossa.[31, 37, 39, 40, 43, 48]

The results regarding AOT cells in these studies have not been consistent and the interpretation of the results also varied. Some investigators have concluded that the tumor cells of AOT were pre-ameloblasts,[31, 40, 48] while others concluded that the AOT cells corresponded with ameloblasts.[45] Several of these studies found enough evidence to support the idea that AOTs arose from tissue of the enamel organ.[45] No investigations found that the AOT cells were similar to odontoblasts, although some studies did indicate the cells may have a mesenchymal origin.[52]

There have been many ideas presented about the nature of eosinophilic amorphous material as well. Studies have concluded that the eosinophilic amorphous

material was similar to mesenchymal fibrous tissue,[46] fibrous stroma,[39] enamel matrix,[43, 53] enamel protein,[49] pre-enamel,[43, 45, 47] dysplastic dentin,[26] dentin,[43] dentin without dentinal tubules,[31] dentinoid,[26, 43] pre-dentin matrix,[40] amyloid,[31, 37, 50] dystrophic calcifications,[47] a product similar to ghost cells,[54] or degenerative or dystrophic products.[43] It has been reported that the product may have arisen from the connective tissue[37, 44] or may be comparable to the basement membrane.[46, 52, 55, 56] Some studies reached the conclusion that the eosinophilic amorphous material was produced by the cells of the duct-like structures. The eosinophilic amorphous material has exhibited staining characteristics similar to that of connective tissue, staining positively with van Gieson and Masson stains, as well as being positive to PAS with diastase and Alcian blue.[37] Some studies used Congo red to show that the tumor product was an enamel protein,[49] while others used Congo red to indicate amyloid. The presence of acid mucopolysaccharide was highlighted by Alcian blue, which stained the eosinophilic amorphous material.[45] There have been suppositions that the alkaline phosphatase in AOT cells might aid in the synthesis and calcification of the homogenous acellular material.[43, 47]

Some authors did not reach specific conclusions as to what they believed the eosinophilic amorphous material was made of, but they ruled out some possibilities. The homogeneous material in the tumors was not pre-enamel, but the material also was not human pre-dentin, because the histochemical staining (especially diastase-PAS) was not compatible with that conclusion.[48] Other studies supported the idea that the eosinophilic amorphous material was pre-dentin and the evidence in favor of this opinion is provided by the positive reactions of this material for reticulin and with

mucicarmine, PAS, Alcian blue, van Gieson and Mallory, as it shows metachromasia.[40] Mori, et al.[43] definitively stated the current histochemical assays prove that the eosinophilic amorphous material is not amyloid.

Collagen has been demonstrated in AOT products, as shown by positive expression with Mallory stain.[44, 47] Some products were identified as reticulin by Gordon and Sweets and Gomori stains.[47] Other studies stated reticulin was not present because the positive-staining fibers appeared to be clumped and amorphous after Gomori silver impregnation, and this is not the normal configuration of reticulin fibers.[48] Spouge[48] stated that because this finding does not support the presence of reticulin, a connective tissue origin for the eosinophilic amorphous material is not corroborated.

1.5 Immunohistochemical Studies of AOT

The results of the immunohistochemical studies of AOT were no less varied than the histochemical and electron microscopic studies. While there have been many papers that investigated the IHC expression of AOT, the following table (Table 1) summarizes the findings of papers in the literature, pertaining to the IHC staining of AOT cells and products. Different authors have proposed different cell types and product compositions, making the idea of AOT being a mixed lesion more possible. The concept of a heterogeneous cell population in AOT seems to present itself when the entirety of the literature is reviewed. Throughout the course of this literature review, it was discovered that there were not always clear descriptions for the cell morphological types that expressed a given marker. Efforts were made in this proposal to preserve the authors' wording, where possible.

Table 1 Summary of the previous immunohistochemical findings in the literature related to AOT cell and product origin, excluding the case reports limited to only one AOT specimen.

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Crivelini, et al.,[57] 2003 n = 4 (Dental Germ, Dental Follicles with reduced enamel epithelium (REE), Ameloblastomas (AB), Calcifying Epithelial Odontogenic Tumors (CEOT), AF, Odontomas (OD))	Cytokeratin 7 (CK7) CK8 CK10 CK13 CK18 CK19 (clones not specified, Dako Corporation)	Negative	
	CK14 (clone not specified, BioGenex Laboratories)	Positive in all tumor cells, including duct-like structures.	All cells of the dental germ were positive for CK14, except for the pre-ameloblasts and secreting ameloblasts, in which CK14 was gradually replaced by CK19. All cells of AOT exhibited CK14, including the duct-like structures. CK19 expression was not seen to replace CK14 in the duct-like structures. Loss of CK14, which indicates secretory activity, was not observed either.
	Vimentin (clone not specified, Dako Corporation)	Positive in fusiform or ovoid cells close to the calcified bodies or darker eosinophilic materials in the cytoplasmic pole near them.	Vimentin-positive tumor cells of AOTs had secretory functions and were related to the reduced enamel epithelium.
Crivelini, et al.,[55] 2005 n = 8 (Dental follicles with REE)	CK7 (clone OV-TL 12/30, Dako Corporation) CK10-13 (clone DE-K13, Dako Corporation) CK18 (clone DC10, Dako Corporation) CK19 (clone RCK108, Dako Corporation) Vimentin (clone VIM3B4, Dako Corporation) P53 (clone DO-7, Dako Corporation)		

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Crivelini, et al.,[55] 2005 n = 8 (Dental follicles with REE), Continued	CK14 (clone LL002, Dako Corporation)	Expression seen in the cords of cubic and peripheral "polyhedrical" cells and in the fusiform and "stellated" cells adjacent to the solid areas; cells forming the adenomatoid structures and solid areas were not positive in two cases and expression varied in others.	Cells of the duct-like and adenomatoid structures were analogous to post-secretory ameloblasts, which were still capable of secreting some enamel proteins. Because it is thought that CK14 is not expressed in secretory ameloblasts, but post-secretory ameloblasts regain their ability to produce CK14, these cells were consistent with post-secretory ameloblasts, in that aspect.
	Laminin (clone LAM-89, Novocastra Laboratories Ltd)	Positive in the luminal surface of cells bordering the adenomatoid structures, in the small intercellular deposits of lighter eosinophilic material interspersed in the epithelial proliferation of spindled areas and cord, and on the borders between the tumor and connective tissue; darker eosinophilic areas were negative.	Some of the intercellular eosinophilic material was immunohistochemically positive for laminin and may have represented basement membrane substance.
	Collagen type IV (clone CIV 22, Dako Corporation)	Discrete expression seen in only the zone of the basement membrane of a few tumors.	
	Proliferating cell nuclear antigen (PCNA) (clone PC10, Dako Corporation	Positivity ranged from weak to strong in most cells of the solid areas, in the adenomatoid areas, cords of cubic and polyhedral cells; absent or weak in the fusiform cells and in the stellated cells interspersed in the solid areas.	The cells in the spindled areas and peripheral cords were the most proliferative and were responsible for tumoral growth.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Crivelini, et al.,[58] 2012 n = 4 (CEOT, Developing ODs, ABs, Calcifying Cystic Odontogenic Tumors (CCOT), AF), Odontogenic Myxomas (OM), Odontogenic Fibromas (OF), and dental follicles with REE and dental lamina, rat incisor teeth)	AMBN (Ameloblastin, produced by Laboratory of Developmental Biology, Nations/Institute of Dental Research, National Institutes of Health. Bethesda, MD. USA)[59]	Only one tumor showed positive in the form of granular deposits in rare groups of fusiform epithelial cells.	
	AMTN (Amelotin, produced by Laboratory for the Study of Calcified and Biomaterials, Quebec, Montreal, Canada) [60]	Variable, but weak, or absent in the mineralized areas; consistently positive in the non-calcified eosinophilic material on the internal surface of the adenomatoid structures; in some cases, it filled the lumen of the gland-like structures and formed small intercellular deposits.	The positive staining of AMTN supported the idea that AOT cells originate from REE and maintained their ability to express AMTN.
	ODAM (Odontogenic Ameloblast-Associated Protein, clone not specified)	Rare, focal, and granular cellular deposits; found in a linear pattern around the calcified material.	"These results suggest that ODAM has a tight relationship with the surface of mineralized tissues rather than their inner structures."
	AMEL (Amelogenin, Abnova Antibody Innovation, Taiwan)	Negative	
de Medeiros, et al.,[61] 2010 n = 10 (ABs, normal mucosa, human placenta)	Fibronectin (clone A 0245, Dako A/S)	Moderate to intense expression scattered throughout the stroma; intense linear labeling seen at the epithelial-mesenchymal junction; a few cases showed sparse foci in AOT cells.	AOT cells, like pre-ameloblasts, were able to synthesize ECM proteins due to the presence of fibronectin inside the luminal spaces of duct-like structures. The focal positivity for fibronectin in epithelial cells of AOT confirmed the capacity of these cells to synthesize this glycoprotein.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
de Medeiros, et al.,[61] 2010 n = 10 (ABs, normal mucosa, human placenta), Continued	Tenascin (clone TN2, Dako A/S)	Variable intensity; focal areas of expression seen close to the tumor parenchyma;	
	Type I Collagen (clone NCL-Coll-Ip, Novocastra Laboratories)	Intense, fibrillar to fibro-reticular labeling throughout the tumor stroma in all cases; some cases showed irregular accumulation of type I collagen between epithelial cells and inside some duct-like structures.	There were different types of ECM associated with AOT, either as luminal content of duct-like structures or in the form of hyaline droplets, consisting of various forms of matrix containing collagen fibrils and their degradation products.
Fujita, et al.,[62] 2006 n = 6 (ABs, Malignant ABs, ODs (compound and complex types), AF, Ameloblastic Fibro-Odontomas (AFO) and Fibrodentinomas, Ameloblastic Fibrosarcomas, and OF)	Nestin (Chemicon International Inc., CA, USA)	5/6 cases showed intense positivity in the cells of the small nodular foci and rosette patterns, localized near the droplets of eosinophilic amorphous material.	AOT cells were not homogenous. A heterogeneous pattern of nestin localization was shown in the AOT. Nestin was expressed in cells near the eosinophilic deposit in the tumor.
Gao, et al.,[27] 1997 n = 2 (AMs, Benign Cementoblastomas (BC), Cementifying Fibromas, Dentinomas, Compound OD, CEOT, OF, and Tooth Germs)	BMP (BMPMcAb, from J. M. Wozney's lab)	Negative	The mechanism of calcification in AOT was possibly different from that in BMP-positive odontogenic tumors. The AOT cells were too immature to synthesize detectable BMP.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Leon, et al.,[50] 2005 n = 39 (No other tissue indicated)	Cytokeratin cocktail (clone AE1/AE3, Dako) Cytokeratin cocktail (clone 34βE12, Dako) CK5 (clone XM26, Novocastra)	Positive in all 39 cases. Expression in cuboidal cells peripheral to the nodules which were composed by basophilic cells with scarce cytoplasm; the epithelial cells covering the duct-like spaces; the trabecular and cribriform areas. Positive staining also found in CEOT-like areas; and in all layers of cystic epithelium when present.	This immunohistochemical pattern showed that certain areas of the tumor were phenotypically variable.
	CK1 (clone 34βB4, Novocastra) CK10 (clone DE-K-10, Dako) CK18 (clone DC10, Dako)	Negative	
	CK6 (clone LHK6B, Novocastra)	56.4% (22/39) of AOT cases had cystic epithelium. Positive in suprabasalar layer of cystic epithelium in 1/22 cases.	
	CK7 (clone OV-TL12/30, Dako)	10/39 cases were positive in the trabecular and cribriform areas; 7/39 cases were positive in the cuboidal peripheral cells of the nodules; 2/39 cases were positive in the fusiform cells; positive in suprabasalar layer of cystic epithelium in 3/22 cases.	Some AOTs had focal positivity for CK7 and CK8, which showed that the AOT was a tumor with variable degrees of differentiation and structural characteristics.
	CK8 (clone 35βH11, Dako)	Positive in 19/39 cases in the trabecular and cribriform areas; 12/39 cases in the cuboidal peripheral cells of the nodules; 5/39 cases were positive in the fusiform and cuboidal cells; positive in suprabasalar layer of cystic epithelium in 2/22 cases.	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Leon, et al.,[50] 2005 n = 39 (No other tissue indicated), Continued	CK13 (clone KS-1A3, Novocastra)	Positive in the suprabasalar layer of the cystic epithelium when present.	
	CK14 (clone NCL-L-LL002, Novocastra)	Positive in all 39 cases. Expression in cuboidal cells peripheral to the nodules, the epithelial cells covering the duct-like spaces, and the trabecular and cribriform areas; positive in the CEOT-like areas; positive in all layers of cystic epithelium, when present; some cases showed negativity in the center of epithelial nodules.	The expression of CK14 supported a probable origin in the reduced dental epithelium. This explained its frequent association with the crown of an embedded tooth, mimicking a dentigerous cyst.
	CK16 (clone LL025, Novocastra)	Positive in suprabasalar layer of cystic epithelium of AOT in 3/22 cases.	
	CK19 (clone RCK108, Dako)	Positive in all cases but less intense expression than AE1/AE3; negative in fusiform cells at the periphery of the nodules and in the CEOT-like areas; positive in the suprabasalar layer of cystic epithelium, when present.	
	Ki67 (clone MIB-1, Dako)	Mean percent of positive cells was 1.66%; (Range 0.5% to 4.6 %)	The low proliferating activity of AOT was in agreement with its benign nature.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Leon, et al.,[50] 2005 n = 39 (No other tissue indicated), Continued	Vimentin (clone Vim 3B4, Dako)	27/39 cases showed positivity; 25 of the 27 positive cases were positive in the trabecular and cribriform areas, as well as in the cuboidal cells peripheral to the nodules; 4 cases were positive in the rosette-like areas; 3 cases were positive in the clear cells in the nodule centers; 3 cases stained in the CEOT-like areas.	
Modolo, et al.,[63] 2010 n=11 (Kidney Tissue)	Biglycan (BGC) (Larry W. Fischer, Matrix Biochemistry Unit/NIH, Bethesda, MD)	Strong expression in eosinophilic amorphous material; Moderate expression in the stroma; tumor cells and calcified material were negative.	The tumor cells probably acted as stimulators of the expression by the stroma. The stroma then acted as an agonist or antagonist of the tumor growth. The amorphous eosinophilic material expressed BGC (which has a role in early calcification process), DEC (which has a role in controlling calcifying mechanisms), OPN (which regulates the deposition of calcium phosphate), BSP (a nucleator of hydroxyapatite (HA)), ONC (a promoter of deposition of mineral calcium phosphate in type I collagen), and OCC (that acts as an inhibitor of HA crystal growth); so it was possible that these regulators of calcification determined

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Modolo, et al.,[63] 2010 n=11 (Kidney Tissue), Continued	Biglycan (BGC) (Larry W. Fischer, Matrix Biochemistry Unit/NIH, Bethesda, MD), Continued	Strong expression in eosinophilic amorphous material; Moderate expression in the stroma; tumor cells and calcified material were negative. Continued	(Continued) a possible relationship between calcified foci and eosinophilic amorphous material. The foci of calcification showed positivity with OPN, a GP [glycoprotein], which suggested relationship of eosinophilic amorphous material to calcified material.
	Decorin (DEC) (Larry W. Fischer, Matrix Biochemistry Unit/NIH, Bethesda, MD)	Moderate expression in eosinophilic amorphous material; Strong expression in the stroma; tumor cells and calcified material were negative.	The presence of BGC and DEC in the stroma of AOT represented a response of the host against the epithelial neoplastic progress, as both showed the ability to inhibit neoplastic growth.
	Osteonectin (ONC) (Larry W. Fischer, Matrix Biochemistry Unit/NIH, Bethesda, MD)	Strong expression in the cytoplasm of the spindle-shaped epithelial cells surrounding the duct-like structures and the cuboidal cells of the periphery of the "nests", moderate staining in the remaining spindle-shaped and cuboidal epithelial cells and faint staining in columnar cells; moderate expression in eosinophilic amorphous material; moderate expression in the stroma; negative in calcified foci.	The presence of ONC, a mineralization stimulator, in neoplastic stroma seemed to represent a local response against the neoplastic proliferation, which influenced reactive bone formation at the periphery of the lesion.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Modolo, et al.,[63] 2010 n=11 (Kidney Tissue), Continued	Osteopontin (OPN) (Larry W. Fischer, Matrix Biochemistry Unit/NIH, Bethesda, MD)	Moderate expression in eosinophilic amorphous material; Faint expression in the stroma; Strong expression was also seen in the calcified foci; tumor cells were negative.	The expression of BSP and OPN in neoplastic stroma seemed to help the neoplastic progression, because they promoted the adhesion of the osteoclasts to the bone surface, which activated the mechanism of resorption.
	BSP (Bone Sialoprotein) (Larry W. Fischer, Matrix Biochemistry Unit/NIH, Bethesda, MD)	Moderate expression in eosinophilic amorphous material; Faint expression in the stroma; tumor cells and calcified foci were negative.	
	Osteocalcin (OCC) (Larry W. Fischer, Matrix Biochemistry Unit/NIH, Bethesda, MD)	Faint expression in eosinophilic amorphous material; Faint expression in the stroma; tumor cells and calcified foci were negative.	The stromal expression of the OCC could help the neoplastic growth, because it had calcium affinity, which lead to the inhibition of the formation and growth of HA crystals.
Mori, et al.,[54] 1991 n = 5 (CEOT, Calcifying Odontogenic Cyst (COC))	Amelogenins (monocolonal antibody, manufacturer not specified)	Positive in high columnar cells forming the duct; Strongly expressed in amyloid-like material; slight positive staining in the cells that show pseudo-glandular and ductal structures; relatively marked staining in tumor cells at the periphery of the pseudo-glandular structures; expression seen in the flattened tumor cells of AOT; calcified materials were negative for amelogenins.	Homogenous material in AOT was similar to that of ghost cells in COC and homogenous acellular material in CEOT.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Murata, et al.,[64] 2000 n= 5	Amelogenin (not specified)	Positivity was mainly localized within tumor cell nests, eosinophilic hyaline droplets, and mineralized materials; found in the cytoplasm of high columnar tumor cells that formed whorled or rosette-like masses; staining of amelogenin did vary based on the size of the duct-like around the crystalloid structures with cubic or round shapes.	The authors speculated that cells forming the duct-like structures were ameloblasts that could not differentiate further because of an increased production of extracellular matrix (ECM) molecules and their retention in the lumina. Because both enamel proteins) and basement membrane-associated ECM molecules were found in the duct-like structures and stromal spaces, it was suggested that the enamel protein and ECM molecules played important roles in the formation of duct-like structures. Amelogenin that was secreted into the extracellular space as eosinophilic hyaline droplets, which was speculated to be degraded by AOT-secreted proteinases. The enamelin, in contrast, was present in the cystic space for a longer period. This was interpreted to mean that enamelin was secreted at a later point in time than amelogenin and that the AOT-secreted enzymes degrade amelogenin, but not enamelin.
	Enamelin (not specified)	Enamelin tended to be localized in the duct-like spaces, eosinophilic hyaline droplets, and mineralized materials; found the inner rim of duct-like structures and inner stromal spaces; columnar cells were negative.	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Murata, et al.,[64] 2000 n= 5, Continued	Collagen type I (provided by Dr. H. Furthmayr, Stanford University, California, USA)	Irregular staining in the mineralized particles; some staining was observed in the stroma; columnar cells were negative.	Positive staining for type I collagen in the mineralized materials showed that the mineralization in AOT is initiated from the extracellular components and that the mineralization in AOT was taking place through a process different than the process of the formation of the AOT-specific structures.
	Collagen type III (provided by Dr. H. Furthmayr, Stanford University, California, USA)	Positive in the fibrohyaline stroma; particularly strong staining around the capillaries; positive in the fibrous capsule of the tumors; columnar cells were negative.	The authors speculated that the enzymatic pretreatment used in this study was the reason some of their IHC markers stain differently than in previous studies.
	Collagen type VI (provided by Dr. H. Furthmayr, Stanford University, California, USA)	Positive in the fibrohyaline stroma; particularly strong staining around the capillaries; columnar cells were negative.	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Murata, et al.,[64] 2000 n= 5, Continued	Collagen type V (provided by Dr. H. Furthmayr, Stanford University, California, USA)	Expressed in luminal space of the duct-like structures, in some hyaline droplets, and some of the inner stromal spaces of whorled or rosette-like foci; the luminal contents, columnar cells, and mineralized materials were negative.	The staining patterns of collagen type IV and V, laminin, HSPG, and fibronectin in the luminal space of the pseudocystic structures is the same as the staining in the lamina basalis ameloblastica (LBA), which is located beneath the line of pre-ameloblasts and separates them from odontoblasts or pre-dentin in the normal tooth germ. AOT may have the ability to synthesize LBA-like ECM, like pre-ameloblasts do. AOTs have a wide range of characteristics, from pre-ameloblasts to mature phase ameloblasts. The staining indicated that the variously shaped inner stroma was developed from hyaline droplets.
	Heparin Sulfate Proteoglycans (HSPG) (not specified) Collagen type IV (provided by Dr. H. Furthmayr, Stanford University, California, USA) Laminin (not specified)	Expressed in luminal contents of the duct-like structures, in hyaline droplets, and in the inner stromal spaces of whorled or rosette-like foci; there was some expression seen in the stroma; no expression was seen in the columnar cells or mineralized particles.	
	Fibronectin (not specified)	Expressed in luminal contents of the duct-like structures, in hyaline droplets, in the inner stromal spaces of whorled or rosette-like foci, and in the stroma; no expression was seen in the columnar cells or mineralized particles.	
	Tenascin (provided by Dr. T. Sakakura, Mie University, Mie, Japan)	Positive in the fibrohyaline stroma; particularly strong staining around the capillaries; no expression observed in other structures.	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Nagatsuka, et al.,[56] 2002 n = 2 (ABs (ordinary and desmoplastic), AF, Ameloblastic Carcinomas, Ameloblastic Fibro-Odontosarcoma, primary intraosseous carcinoma)	Collagen α1 (IV) (H11)/Collagen α2 (IV) (H22)	Expressed in the basement membrane (BM)of cribriform areas and hyaline materials; weakly positive in the BM of epithelial whorls/rosettes/nests and mineralized foci.	The eosinophilic hyaline deposits found in the AOT probably represented excessive altered/abnormal BM constituents sequestered at the specific tumor sites. The cells in cribriform areas phenotypically resembled those in the dental lamina because of similarities in their BM α (IV) compositions. The weak to lacking immunoreactivity for α (IV) chains in the BMs of the other epithelial structures were thought to be due to their constituent cells being histodifferentially more mature, possibly equivalent to the stage just before the secretory phase of odontogenesis, when these BM components disappear.
	Collagen α3 (IV)	Negative	
	Collagen α4 (IV) (H43)	Expressed in the BM of cribriform areas and in hyaline materials.	
	Collagen α5 (IV) (M54)/ Collagen α6 (IV) (M69)	Expressed in the BM of cribriform areas and in hyaline materials; weakly positive in the BM of epithelial whorls/rosettes/nests and mineralized foci.	
Nascimento, et al.,[65] 2017 n = 30 (Solid ABs (SA), Unicystic ABs (UA), lung tissue, skeletal muscle tissue, liver tissue)	Bone Morphogenetic Protein (BMP)-2 (clone 6285, Abcam, Cambridge, USA)	AOT cells showed moderate positivity; variable expression in stromal cells.	BMP-2 participated in the induction of mineralized tissues. Since the formation of mineralized material was common in AOTs, this finding suggested participation of BMP-2 in this tumor.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Nascimento, et al.,[65] 2017 n = 30 (Solid ABs (SA), Unicystic ABs (UA), lung tissue, skeletal muscle tissue, liver tissue), Continued	BMP-4 (clone 81194, Abcam, Cambridge, USA)	AOT cells showed strong positivity; variable expression in stromal cells.	The expression of BMP-4 both in tumor and stromal cells of AOTs suggested that this protein promoted epithelial-mesenchymal interactions that contributed to tumor development, similar to what occurs during odontogenesis.
	BMPR-IA (clone 166707, Abcam, Cambridge, USA)	AOT cells and stromal cells showed strong positivity.	These results suggested the involvement of these molecules and their receptors in the pathogenesis of AOTs.
	BMPR-II (clone 78422, Abcam, Cambridge, USA)	AOT cells and stromal cells showed strong positivity.	
Poomsawat, et al.,[66] 2007 n = 7 (ABs, CCOTs, Oral mucosa)	Laminin 1 (Sigma Chemical Co)	Expressed in the cytoplasm of all tumor cells.	The paired expression of laminin 1 and laminin 5 and the lack of expression of fibronectin supported that the cells within AOT can have a variable degree of differentiation.
	Laminin 5 (Chemicon International, Temecula, CA)	Weak, focal expression in stellate cells and strongly positive in stellate and "squamous cells" around the calcified areas.	Likely, laminin 5 contributed to the initiation of mineralization.
	Collagen type IV (Dako S/A, Glostrup, Denmark)	Never found in the cytoplasm of any tumor cells.	
	Fibronectin (Dako S/A)	Weak, focal expression in stellate cells.	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Poomsawat, et al.,[67] 2012 n = 10 (ABs, UAs (luminal type), CCOT, Keratocystic Odontogenic Tumors (KCOTs), Ameloblastic Carcinomas, Hepatocellular Carcinoma, intestinal tissue)	c-met (clone SC-161, Santa Cruz Biotechnology)	"Squamous cells" displayed moderate to strong positivity; 10/10 stained weakly positive in more than 75% of spindle cells; 9/10 stained weakly positive in more than 75% of duct-like cells; 10/10 stained weakly positive in 5-25% of cells of trabeculae pattern	The HGF/c-met pathway is involved in the differentiation of odontogenic tumors via paracrine or autocrine fashion. This pathway promoted tumor proliferation in odontogenic tumors due to its potent mitogenic effect. The consistent and strong expression of both HGF and c-met in "squamous cells" present in AOT suggested that the HGF/c-met interaction may have an influence on squamous differentiation.
	Hepatocyte Growth Factor (HGF) (clone SC-7949, Santa Cruz Biotechnology)	"Squamous cells" displayed moderate to strong positivity; 10/10 stained weakly positive in more than 75% of spindle cells; 9/10 stained weakly positive in more than 75% of duct-like cells; 10/10 stained weakly positive in 5-25% of cells of trabeculae pattern.	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Saku, et al.,[68] 1991 n = 3 (ABs, CEOT, COC, gingival inflammatory polyps (epulides), OMs, human tooth germs of various stages of development, bovine tooth germs)	Amelogenin (not specified)	Expression in small mineralized product in the whorls or rosettes; positive in hyaline droplets; moderately positive in the cytoplasm of cells around the hyaline droplet; expression found at the periphery of mineralized material with a homogenous appearance; negative in duct-like areas and in areas between duct-like areas.	Small mineralized material in the tumor tissue was dystrophic and was not as highly crystalized as tooth enamel. No immunoreactivity for enamel proteins was found in the large mineralized foci in AOT, which is considered to be due to structural changes of the proteins during progress of mineralization. The proteins changes during mineralization occurred with enamel. Eosinophilic hyaline material on the luminal side of the columnar cells of AOT were not positive for enamel proteins. This has been shown to be mesenchymal matrix by histochemical studies and electron microscopic studies.
	Enamelin (not specified)	Expression was found in the same areas as Amelogenin, but the area Enamelin stain was narrower.	
	Keratin (not specified)	Stained most of the tumor cells; did not stain mineralized or hyaline materials.	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Sudhakara, et al.,[69] 2016 n = 16 (Dentigerous Cysts (DC))	CK14 (not specified)	11/16 were strongly positive, 4/16 were intermediately positive, 1/16 was weakly positive.	CK14 labeling indicated differentiation grades for secretory ameloblasts or ameloblasts in the post-secretory stage in the adenomatoid structures of AOT. These observations formed the basis to explain the presence of enamel-like areas within the tumor cells of AOT.
	Vimentin (not specified)	7/16 cases had positive expression; 9/16 cases showed no expression.	The role of vimentin in the tumourgenesis was negligible. The majority of vimentin positivity expressed by the tumor cells that are smaller, spindle-shaped and present adjacent to the nodules. These cells may have a role in genesis of mineralizing components of AOT.
Takahashi, et al.,[70] 2001 n = 3	Transferrin	3/3 cases were positive in Cell type I (the small compact cells in solid nodules and pseudoglandular cells in duct-like structures), and Cell type II (peripheral elongated cells, spindle-shaped cells in cribriform patterns), and calcified material.	The neoplastic cells of AOT produce transferrin to have a greater availability of intracellular iron for their turnover.
	Ferritin	3/3 cases were positive in Cell type I; and 2/3 cases were positive in Cell type II.	Ferritin is known to be an important iron-storage protein that plays a crucial role in cell metabolism and especially in cell proliferation. This showed that there is iron storage in neoplastic cells of AOT.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Takahashi, et al.,[70] 2001 n = 3, Continued	Lactoferrin	Negative	The lack of staining of lactoferrin in AOT showed that the glandular structures in this tumor were not true ducts.
	α1-antitrypsin (α1-AT)	3/3 cases were positive in Cell type I (small compact cells in solid nodule and pseudoglandular cells in a duct-like structure), Cell type II (peripheral elongated cells and spindle-shaped cells in cribriform pattern), the eosinophilic amorphous material, and the calcified material; Cell type III (metaplastic squamous cells) was negative.	The α1-AT reaction was associated with the inhibition of proteinase degradation in AOT. Based on previous studies, Takahashi, et al.[70] have determined that α1-AT was a useful basal membrane marker. The immunohistochemical findings suggested that the eosinophilic amorphous material could be pooled basal membrane substance. As development of the lesion continued, there was degeneration and eventual foci of dystrophic calcification in the aggregated material.
	α1-antichymotrypsin (α1-ACT)	Negative	The lack of a staining reaction for α1-ACT may indicate that neoplastic cells of this tumor were devoid of glandular differentiation.
	Vimentin	1/3 cases were positive in the peripheral elongated Cell type II.	
	S-100 protein S-100α subunit S-100β subunit	Negative	

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Takata, et al.,[71] 2000 n = 10 (AB, CEOTs, AFOs, COCs, ODs, human fetal tooth germs of various stages of development)	Sheathlin (polyclonal antibodies, manufacture not specified)	Positive staining in the eosinophilic amorphous material found in the solid tumor nests; staining was most intense at the periphery of the eosinophilic material; positive in the cytoplasm of the cells facing the eosinophilic droplets; mineralized foci with or without eosinophilic material were negative; columnar cells that created the duct-like spaces and the small polygonal cells between the duct-like structures were negative; dysplastic dentin-like hyaline material in the stroma was negative; no obvious enamel formation was seen in the cases examined.	Based on comparison to tooth germs, the tumor cells of AOT were considered to differentiate to the degree of functional ameloblasts and secrete sheath proteins in the eosinophilic amorphous material. The mineralized foci in the hyaline materials were negative for sheathlin, while the peripheral area of eosinophilic material and the surrounding cells were positive for the protein. This disappearance of sheathlin production with calcification, as shown by eosinophilic amorphous materials staining positively while mineralized foci were negative, showed that sheathlin is quickly degraded and eliminated from enamel with its maturation, which is what occurs during the normal amelogenesis. The columnar cells of the duct-like spaces are not fully differentiated ameloblasts.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Takata, et al.,[53] 2000 n =23 (ABs, CEOTs, AFOs, COCs, ODs, human fetal tooth germs at various stages of development)	Enamelysin/MMP-20 (203-IC7, Milligen, Biosearch, Division of Millipore, Tokyo, Japan)	Expressed found in hyaline, small, and large mineralized areas; The peripheral areas of the calcified areas stained most intensely; positive in tumor cells around the mineralized areas, but at a weaker intensity; the masses similar to amyloid stained positively; the duct-like structures, as well as the rosette and whorled arrangements were negative; dysplastic dentin-like hyaline materials were negative; some of the epithelial cells within the tumor had appeared to resemble well differentiated ameloblasts.	The AOT cells adjacent to eosinophilic amorphous material and calcified structures were positive for enamelysin expression. The tumor cells surrounding pseudoductal structures lack the ability to differentiate into functional ameloblast-like cells. Enamelysin was consistently observed in the eosinophilic amorphous material, small calcified material, and large mineralized areas, but not in the dysplastic dentin. The hyaline droplets and the small and large mineralized matrices can be regarded as enamel-like substances.
Tatemoto, et al.,[72] 1988 n = 5 (No other tissue indicted)	Keratin, polyclonal antiserum (TK, 41-65 kd) Dakopatts, Copenhagen, Denmark)	Slight positivity in type A cells (spindle or columnar shaped cells and formed solid, ductal, tubular, or whorled structures); slight to moderate positivity in type B cells (small and compact cells at the periphery of the type A cell-containing foci) of the outer or peripheral zone of duct-like structures or rosette-like configurations; positive in the type B cells in large areas of calcification, but cells in areas that showed irregular calcifications were negative; strong positive staining in the one case that contained cystic lining.	It was suggested that the type B cells of AOT possibly arose from stratum intermedium cells, whereas the type A cells corresponded to young or immature ameloblasts which have not fully differentiated. Co-expression of keratin and vimentin in the type B tumor cells was a novel finding among odontogenic epithelial tumors. The thin cystic epithelium in AOT probably originated from undifferentiated odontogenic epithelium.

Table 1 Continued

Author, year of publication number of cases, (control tissue or tumors for comparison, if used)	Stain and clone name, if specified	Result	Authors' interpretation of results
Tatemoto, et al.,[72] 1988 n = 5 (No other tissue indicted), Continued	Keratin, KL1 (Immunotech, Marseilles, France)	Same expression as seen in Polyclonal TK.	As stated above
	Keratin, PKK1 (Labsystem, Helsinki, Finland)	Trace staining in type A cells; slight to moderate positivity in type B cells; strong positive staining in the one case that contained cystic lining.	
	Keratin K8.12 (Bio-yeda, Israel)	Same expression as seen in Polyclonal TK.	
	Vimentin (Dakopatts, Copenhagen, Denmark)	Slight to moderate positivity in type B cells.	
	Desmin (Dakopatts, Copenhagen, Denmark)	Negative in type A and B cells.	
	Keratin, PKK1 + Vimentin (Labsystem, Helsinki, Finland with Dakopatts, Copenhagen, Denmark) Keratin, K8.12 + Vimentin (Bio-yeda, Israel with Dakopatts, Copenhagen, Denmark)	Positive expression in type B cells.	

As demonstrated through this extensive literature review, there have been many people that have attempted to elucidate the origins of the tumor cells and products of AOT, but a definitive conclusion has not been reached. We hypothesize that AOT is a mixed odontogenic tumor that is made of both epithelial and mesenchymal cells and the products types reflects the heterogeneity of the tumor cells.

Because both epithelial and mesenchymal origins have been proposed, both of these conjectures were evaluated. This investigation used a pan cytokeratin (AE1/AE3) marker, dentin sialophosphoprotein (DSPP), nestin, and vimentin

immunohistochemistry, as well as Sirius red histochemistry to evaluate the staining patterns in AOT.

The pan cytokeratin marker was used to identify the cells of epithelial origin. Many studies have investigated cytokeratin markers in AOT, and many have used markers for specific cytokeratins, but this study specifically correlated the cell morphologic types, structures, and locations within the AOT where cytokeratin were expressed. The purpose of using AE1/AE3 was not to sub-classify any of the cells with specific cytokeratin markers, but only to identify which cells were of epithelial origin.

Nestin is a type VI intermediate filament protein.[73] It is a marker that has been used to characterize neuroepithelial stem cells.[73] It is believed that nestin has a role in tooth development and dentin repair.[73, 74] In odontogenesis, nestin expression was seen in the odontoblasts as early as the late bell stage, with the expression of nestin increasing as the odontoblasts mature.[74] About, et al.[74] stated that the increasing expression of nestin indicated that nestin might play a role in the differentiation and function of odontoblasts. In developing third molars, nestin was found in the odontoblastic processes, but not in the cell bodies of the mature odontoblasts under the cusp tips.[74] It was found that nestin was absent in the mature odontoblasts of completely developed normal permanent teeth, so About, et al.[74] concluded that nestin expression is progressively down-regulated in odontoblasts, until there is no longer expression. However, if a tooth was injured by trauma or dental caries, re-expression of the nestin protein was seen in odontoblasts.[74] Nestin has been found to be expressed in the cells of ectomesenchymal origin, which, in AOT, would be considered an indication that the cells were odontoblasts.[74-76] As mentioned above, nestin expression in AOT

was studied by Fujita, et al.[62] That study demonstrated that nestin was expressed in the cells that formed the rosette structures adjacent to amorphous product.[62] The conclusion drawn from that finding was that there was a heterogenous population of epithelial cells that composed AOT.[62] The intention of using nestin in this proposal was not to repeat previous efforts, but to focus on the tumor cells that express nestin and correlate with the expression of DSPP and vimentin, further strengthen the evidence of possible mesenchymal differentiation in AOT.

Dentin sialophosphoprotein (DSPP) is a protein that is essential in the process of dentin mineralization.[77] Type I Collagen and DSPP are the most abundant proteins in dentin.[77] In fact, 90% of the non-collagenous proteins comprising dentin are DSPP and its cleavage products.[78, 79] DSPP has been located in polarizing and functional odontoblasts, predominantly, and, to a lesser extent, it is transiently expressed in pre-secretory ameloblasts.[78] DSPP is cleaved intercellularly into three proteins with different compositions and functions: DSP (dentin sialoprotein), DGP (dentin glycoprotein), and DPP (dentin phosphoprotein).[77] These proteins may represent unstable, intermediate proteins in the extracellular degradation process of DSPP, but these cleaved proteins also play important roles in the dentin mineralization process.[77] Dentin is the tissue that has the highest concentration of DSPP, but DSPP is expressed in other tissues, like bone, cementum, as well as non-mineralized tissues.[79] DPP is thought to promote the mineralization of dentin by binding to calcium and presenting it to collagen fibers at the mineralization front.[79] DPP was found to be secreted at the mineralization front in dentin and its expression is maintained in mineralized dentin.[79] The function of DSP has not been as clearly defined.[79] A study by Suzuki, et al.[80] indicated that DSP may be

involved in the initiation of the mineralization of dentin, but not in the maturation processes DSP has been found to be secreted into the pre-dentin.[79] This study utilized an antibody for DSPP, which recognizes DSPP as well as DSP.[81]

Vimentin is a class III intermediate filament protein that is found in most cells of mesenchymal origin.[82, 83] Expression of vimentin has been related to changes in cell shape and motility.[84, 85] Vimentin also has been found in some epithelial cells, co-expressed with the intermediate filament of cytokeratin.[86] In fact, the de novo expression of vimentin in epithelial cells may be required in order to propagate the migratory ability of cells during development.[86] Vimentin has been used as a marker of mesenchymal cells, neoplasms, and sarcomas, but it is also known that vimentin can be found in some carcinomas.[83] In early embryogenesis, vimentin has been found to be expressed in many of the cells, beginning with the migratory cells at the time the mesodermal cleft is established.[83, 87] The co-expression of vimentin and cytokeratin is a known phenomenon, occurring in the process of fetal development, including tooth development, and has also been seen in reactive proliferations and neoplasms of the adult tissues.[88-91] The simultaneous expression of vimentin and cytokeratins is thought to occur during mesenchymal-to-epithelial or epithelial-to-mesenchymal transformation during organ development.[86] Several researchers have investigated vimentin expression and co-expression with cytokeratin in the structures of a developing tooth. Epithelial cells on the aboral front of the dental lamina expressed both vimentin and cytokeratin as the cells began to migrate away from the dental crest.[92] In the developing tooth germ, Heikenheimo, et al.[93] showed that there was expression of vimentin found in the dental papilla as well as in the stellate reticulum cells of the developing enamel

organ in the bell stage and these findings were confirmed by Kasper, et al.[89] A study by Kero, et al.[86] looked at the expression of vimentin and cytokeratin at several times points during odontogenesis. Kero, et al.[86] found that during the seventh to eighth developmental week, vimentin and cytokeratin were both expressed in the dental crest and in the tooth bud, with greater expression of vimentin in the epithelial components of tooth initiation that in the underlying mesenchymal tissues. During the twelfth week of development, there was strong expression of vimentin in the dental papilla and dental sac, while the epithelial components of the developing tooth showed only mild expression.[86] By the fourteenth week of development, which would correspond to teeth at the advanced bell stage, moderate expression of vimentin was seen in the stratum intermedium, inner enamel epithelium, and in the dental papilla, with mild expression of vimentin being observed in the other epithelial components of the tooth germ.[86, 94] In the twentieth week of development, vimentin and cytokeratin co-expression was seen in the pre-ameloblasts, the stellate reticulum, and the mesenchymal components of the forming tooth.[86] Of a particular note was that the pre-ameloblasts at the interface with the stratum intermedium expressed cytokeratin as well as vimentin.[86] The twentieth week of development falls into the time period of matrix secretion and tooth formation.[94] During that time period, it was observed that pre-odontoblasts also co-expressed vimentin and cytokeratin.[86] Pre-odontoblasts showed cytoplasmic expression of vimentin, but odontoblasts have vimentin expression confined to the apical pole of the cell.[95] Lesot, et al.[95] reported that there was no expression of cytokeratin in any of the pulpal tissue.

Vimentin expression in AOT has been investigated in several previous studies, with some of those studies focusing on the co-expression of vimentin and cytokeratins.[50, 55, 57, 69, 70, 72] Tatemoto, et al.[72] reported the co-expression of cytokeratins and vimentin in AOT cells as a novel finding in 1988. Takahashi, et al.[70] reported finding vimentin expression in the peripheral elongated cells in one out of the three cases of AOT they investigated. In a study published in 2003, Crivelini, et al.[57] reported expression of vimentin in the fusiform or ovoid cells near the calcified bodies or eosinophilic material. Their interpretation was that the vimentin expression indicated secretory function of the AOT cells.[57] However, in a follow up study conducted by Crivelini, et al.,[55] it was reported that no vimentin positivity was seen in AOT in the eight cases studied. The lack of expression of vimentin in this investigation led them to conclude that AOT should be considered as an odontogenic epithelial neoplasm without ectomesenchyme.[55] Leon, et al.[50] reported 27 of 39 cases of AOT showed vimentin expression in “the trabecular and cribriform areas,” “cuboidal peripheral cells,” and, less commonly, in “the the rosette-like areas,” “clear cells located in the nodules centres,” and “the CEOT-like areas.” This group concluded that because of varibility in the staining of the AOT cells with vimentin, that there could be more than one cell phenotype present in some areas of AOT.[50] In 2016, Sudhakara, et al.[69] published a study, describing the finding of 9/16 AOT showing expression of vimentin. The conclusion was that vimentin did not play a role in the tumorgenesis of AOT, because the majority of cells did not express this marker.[69] It is also mentioned that the vimentin expression in the cells surrounding the mineralized material could mean that these cells played a role in the formation of the material.[69] The current study sought to investigate

not only vimentin expression in AOT but also to correlate its expression with the expression of other mesenchymal proteins in AOT in order to explore possible mesenchymal involvement in AOT.

Collagen type I is typically synthesized by fibroblasts but is not made by epithelial cells. Sirius red staining in AOT products showed evidence supporting cells of mesenchymal origin. Sirius Red is a histochemical staining method that stains collagen types I and III red. It was possible to differentiate between collagen types I and III by using polarized lenses to view Sirius red-stained slides.[96, 97] Collagen type I showed yellow-orange birefringence and collagen type III showed green birefringence under polarized light.[96, 97]

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cases to be Investigated

The study was conducted using formalin-fixed, paraffin-embedded, archived human tissue and the corresponding demographic and clinical information 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. Because most commercially available primary antibodies were not been validated in decalcified tissue and the biopsy material for AOT sometimes required decalcification during tissue processing, a preliminary study to validate the purchased primary antibodies on positive controls was conducted before testing on experimental group was initiated. AF, an odontogenic tumor typically did not require decalcification, and odontoma, an odontogenic hamartoma typically requiring decalcification during processing, was used for validation purposes as well as the positive controls for this project. Both AF and odontoma without primary antibody incubation were used as negative controls in the IHC procedures in this project. No negative controls were necessary in the Sirius Red studies.

2.1.1.1 Immunohistochemistry

2.1.1.1.1 Primary antibodies

2.1.1.1.1.1 Pan-Cytokeratin (AE1/AE3, catalog #CM011C, Biocare, 1:100 dilution, monoclonal mouse anti-human antibody)

2.1.1.1.1.2 Dentin Sialophosphoprotein (DSPP) (clone LF-151, Kerafast, polyclonal rabbit anti-human antibody)

2.1.1.1.1.3 Nestin (clone 10C2, Invitrogen, monoclonal mouse anti-human antibody)

2.1.1.1.1.4 Vimentin (clone V9, catalog #NCL-L-VIM-V9, Leica, 1:80 dilution, monoclonal mouse anti-human antibody)

2.1.1.2 Histochemistry

2.1.1.2.1 Picro Sirius Red Kit (ab150681, Abcam)

2.2 Method of Investigation

2.2.1 Immunohistochemistry Protocol

2.2.1.1 Pan cytokeratin

Sections of tissue, 4 micrometers in thickness, were obtained from the selected AOT cases and positive controls. The sections were deparaffinized using xylene, then rehydrated with serially decreasing concentrations of reagent alcohol followed by phosphate buffered saline (PBS). The slides were loaded into a Ventana BenchMark Ultra IHC staining module. The slides were incubated for 8 minutes at 100ºC in Ultra Cell Conditioning (Ultra CC1, Roche Tissue Diagnostics, #950-224). This was followed by a 20-minute and a 36-minute wash with Ultra CC1. Blocking of non-specific staining was accomplished using casein (Ventana Antibody Diluent with Casein, Roche Tissue Diagnostics, #760-219) for a 4-minute incubation. The primary antibody of pan cytokeratin (AE1/AE3, Biocare, catalog #CM011C) was diluted 1:100 in Da Vinci Green diluent (Biocare Medical, #PD900) and the slides were incubated for 32 minutes at 37ºC. The slides were incubated in UltraBlock (Biocare Medical, V-Blocker #BRI4001)

for 4 minutes. Detection was accomplished using UltraView Universal DAB Detection Kit (Roche Tissue Diagnostics, #760-500). A four-minute hematoxylin incubation was used to counterstain the slides. The slides were dehydrated in increasing concentrations of reagent alcohol, then washed twice in xylene before mounting with coverslips using Permount mounting media (Fisher Scientific, #SP15-100).

2.2.1.2 Dentin sialophosphoprotein (DSPP)

Sections of tissue, 4 micrometers in thickness, were obtained from the selected AOT cases and the positive and negative controls. The sections were deparaffinized using xylene, then rehydrated with serially decreasing concentrations of reagent alcohol followed by PBS. Antigen retrieval was accomplished by incubating the slides in trypsin buffer (0.05% trypsin calcium chloride) for 15 minutes at 37˚C, then rinsed with tap water for 5 minutes. The slides were washed for 3 minutes three times in 0.3% Triton in PBS (PBST). Endogenous peroxidases were quenched using 3% hydrogen peroxide for 10 minutes in the dark. The slides were incubated for one hour in 3% bovine serum albumin, 10% goat serum (Abcam, #ab7481), diluted in PBST. The primary antibody solution was made by diluting DSPP (clone LF-151, Kerafast) to 1:200, with 2% goat serum and PBST. The negative control eliminated the primary antibody and contained only 2% goat serum and PBST. Incubation with the primary antibody took place for one hour in a humidified chamber at room temperature. The secondary antibody (biotinylated goat anti-rabbit IgG, Vector #BP-9100) was diluted to 1:200 in 2% goat serum and PBST and incubation was for one hour. The Vectastain Elite Avidin/Biotin (ABC) Kit (Vector, #PK-6100) was used. Component A and component B were mixed in a 1:1 ratio and diluted in 2% goat serum and PBST. ABC was mixed 30 minutes before

use. Incubation with ABC was 30 minutes. Color development was completed with the DAB system (Vector Laboratories, #SK-4100). DAB 1, DAB 2, DAB 3 were mixed in a 1:2:1 ratio and diluted in PBST. The slides were incubated in DAB for 10 minutes. The slides were counterstained with hematoxylin 1:4 dilution. The slides were processed through increasing concentrations of reagent alcohol, then in xylene. Coverslips were mounted with Permount mounting medium (Fisher Scientific, #SP15-100).

2.2.1.3 Nestin

Sections of tissue, 4 micrometers in thickness, were obtained from the selected AOT cases and the controls. The sections were deparaffinized using xylene, then rehydrated with serially decreasing concentrations of reagent alcohol followed by PBS. Antigen retrieval was accomplished by incubating the slides in epitope retrieval citrate buffer (Leica Biosystems, pH 6.0, #RE7113) for 15 minutes at 95-100˚C in a steamer, then rinsed with tap water for 5 minutes. The slides were washed for 3 minutes three times in PBST. Endogenous peroxidases were quenched using 3% hydrogen peroxide for 10 minutes in the dark. The MOM system (MOM [Mouse on Mouse]) Elite Peroxidase Kit, Vector Laboratories #PK-2200) was used. MOM protein concentrate diluted in PBS to make three measures of MOM diluent. Blocking was accomplished by incubating the slides in one measure of MOM diluent for five minutes. The primary antibody solution was made by diluting nestin (clone 10C2, Invitrogen ThermoFisher Scientific, #MA1-110) to 1:100, using the second measure of MOM diluent. The negative control eliminated the primary antibody and contained only MOM diluent. Incubation with the primary antibody took place for 30 minutes at room temperature. The secondary antibody was a 1:250 dilution of MOM Biotinylated Anti-Mouse IgG

Reagent and the third measure of the MOM diluent. The MOM component A and component B were mixed in a 1:1 ratio and diluted in PBS. ABC was mixed 30 minutes before use. Incubation with ABC was 30 minutes. Color development was completed with the DAB system (Vector Laboratories, SK-4100). DAB 1, DAB 2, DAB 3 were mixed in a 1:2:1 ratio and diluted in PBST. The slides were incubated in DAB for 10 minutes. The slides were counterstained with hematoxylin 1:4 dilution. The slides were processed through increasing concentrations of reagent alcohol, then in xylene. Coverslips were mounted with Permount mounting medium (Fisher Scientific, #SP15-100).

2.2.1.4 Vimentin

Sections of tissue, 4 micrometers in thickness, were obtained from the selected AOT cases and the controls. The sections were deparaffinized using xylene, then rehydrated with serial decreasing concentrations of reagent alcohol followed by phosphate buffered saline. The slides were loaded into a Ventana BenchMark Ultra IHC staining module. The slides were incubated at 100ºC for four minutes in Ultra CC1. There were sequential incubations of 8 minutes, 16 minutes, and 24 minutes with Ultra CC1. Pre-primary peroxidase inhibitor (Ventana-Roche, #760-700) was used to quench the endogenous peroxide. Vimentin (clone V9, Leica Biosystems, #NCL-L-VIM-V9) primary antibody was diluted to to 1:80 with antibody diluent (Leica, #AR9352). Detection was accomplished using UltraView Universal DAB Detection Kit (Roche Tissue Diagnostics, #760-500). A four-minute hematoxylin incubation was used to counterstain the slides. The slides were dehydrated in increasing concentrations of

reagent alcohol, then washed twice in xylene before mounting with coverslips using Permount mounting media (Fisher Scientific, #SP15-100).

2.2.2 Histochemistry Protocol

The Picro Sirius Red Kit (ab150681, Abcam) was used for the staining of collagen types I and III. Weigert's 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 washing under running water for 10 minutes. Slides were stained for 71 minutes in Picro Sirius Red. The slides then were washed in two changes of acidified water. The slides were dehydrated in increasing concentrations of reagent alcohol, then washed twice in xylene before mounting with coverslips using Permount mounting media (Fischer Scientific, #SP15-100).

2.3 Method of Analysis

The expression of AE1/AE3, DSPP, nestin, vimentin, and Sirius Red in each of the control cases and the AOT cases were analyzed under light microscopy. The expression of AE1/AE3, vimentin, DSPP, and nestin were described according to tumor cell morphology and structure. The staining pattern of Sirius red in the eosinophilic amorphous material and calcified material were described. Based on previous studies, we would expect AE1/AE3 to be expressed in the cytoplasm of the cells of epithelial origin. Tumor cells expressing vimentin, DSPP, or nestin indicated mesenchymal origin. Sirius red positive staining in any tumor products indicated collagen type I and III in their components, evidence suggesting the surrounding tumor cells were mesenchymal in origin. In addition to viewing Sirius Red under light microscopy, the results were also

viewed under polarized light to analyze the birefringence of the stained products. The representative areas were recorded and photographed.

Because that AOT demonstrates many structures and patterns, to avoid miscommunication when comparing our results with those reported in previously published studies, the following photomicrographs were used to provide the definition of each terminology used in this study and its corresponding structures or arrangements in AOT (Figures 2 to 12). Any particular AOT may demonstrate one, many, or all of the following cell structures, patterns, and/or forms of calcifications. The method of analysis was to correlate the immunohistochemical results with the tumor cells in these histological structures.

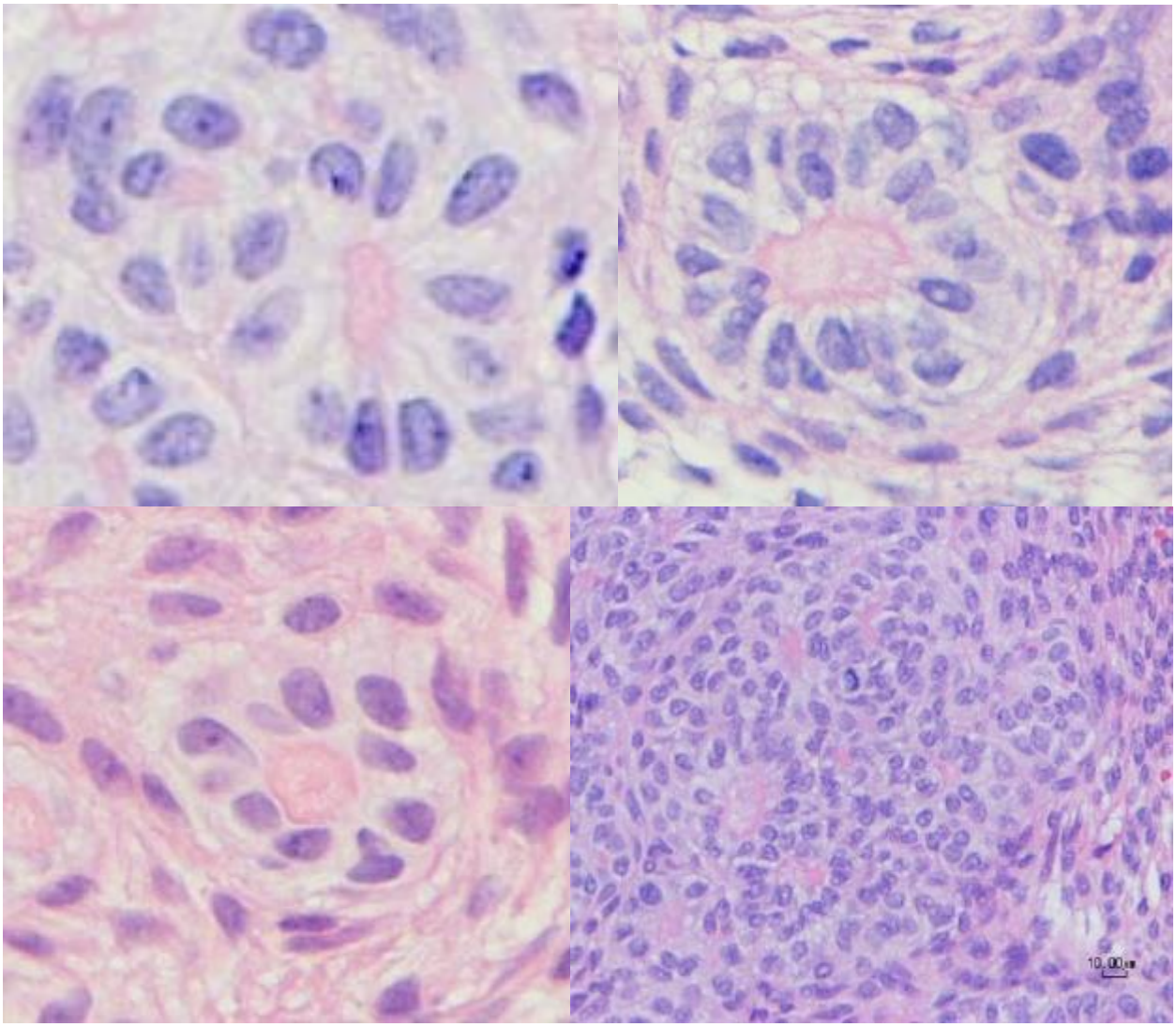

Figure 2 Histology of rosette structures. These photomicrographs showed a structure of elongated to cuboidal cells arranged in a circular pattern around a central droplet of product. These structures were referred to as rosettes, in this study. For a structure to be called a rosette, an amorphous product had to be present in the center. Rosettes tended to be small in size. If the central droplet was absent and there was a lumen in the center, the structures was no longer a rosette, but a duct-like structure (H&E staining).

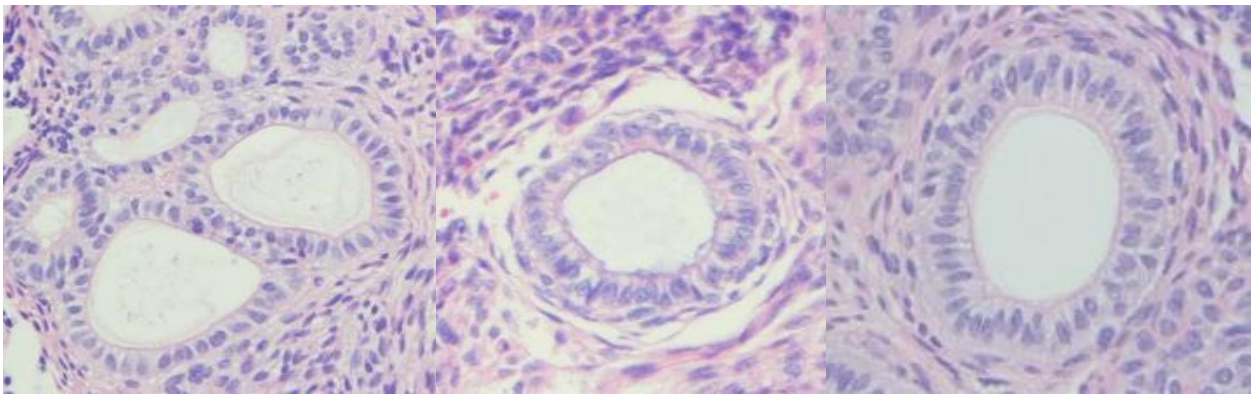

Figure 3 Histology of duct-like structures. These photomicrographs show what were referred to, in this study, as duct-like structures. These structures were made of a single layer of cells that ranged from columnar to cuboidal cells with nuclei polarized away from a central space. These structures were defined by the lumen that the cells create. There was a variable amount of product present on the luminal surface of the cells. These structures ranged in size, from small to quite large (H&E staining).

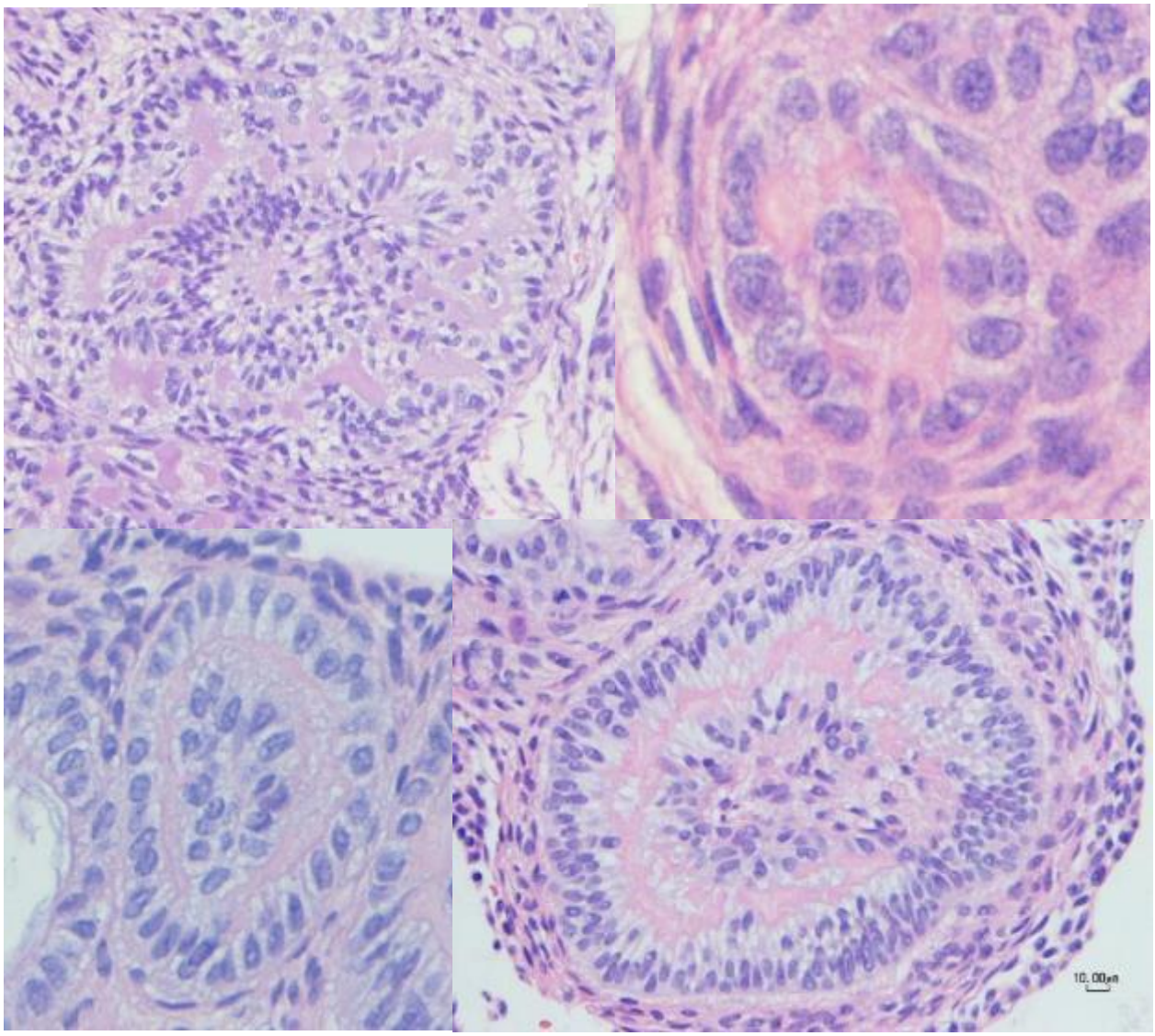

Figure 4 Histology of double-layered spheres. These photomicrographs showed structures that looked somewhat like a telescoping duct or a duct in a duct without a central lumen. This structure was made of two circular layers of columnar to cuboidal shaped cells, with nuclei that polarized away from the center of the structure. At times, there was some eosinophilic amorphous material found between the two cell layers. These structures were referred to as double layered spheres, in this study. These structures were present in various sizes but tended to be larger to accommodate the two-layer arrangement. This structure differs from the duct-like structure because it lacked the central lumen (H&E staining).

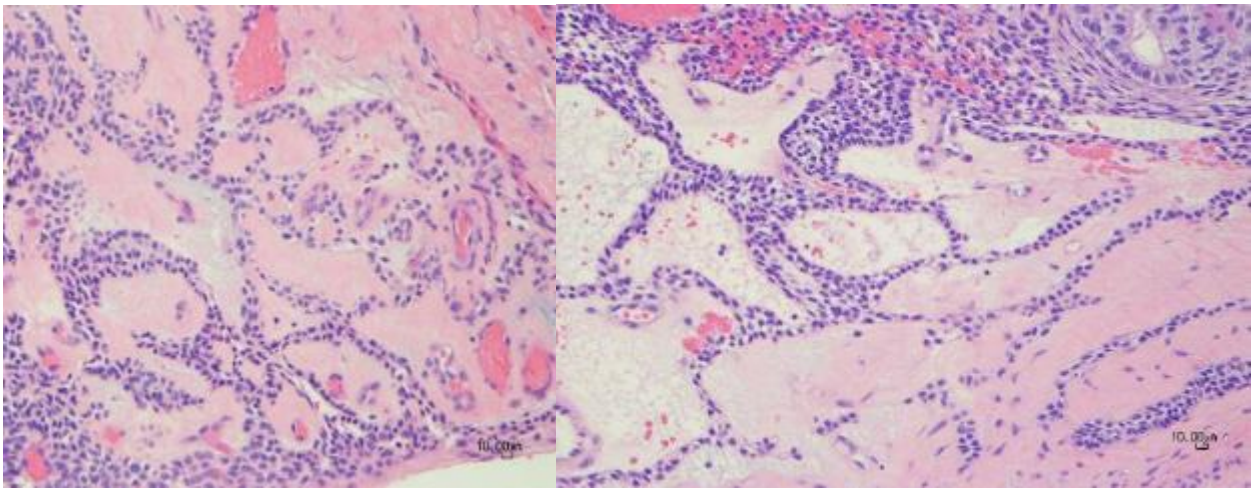

Figure 5 Histology of anastomosing cords. These photomicrographs demonstrated arrangements of cells that were seen at the periphery of the tumor, near the capsule and throughout the tumor. The cells were arranged in cords that interconnect and this pattern was referred to as the anastomosing cords, in this study (H&E staining).

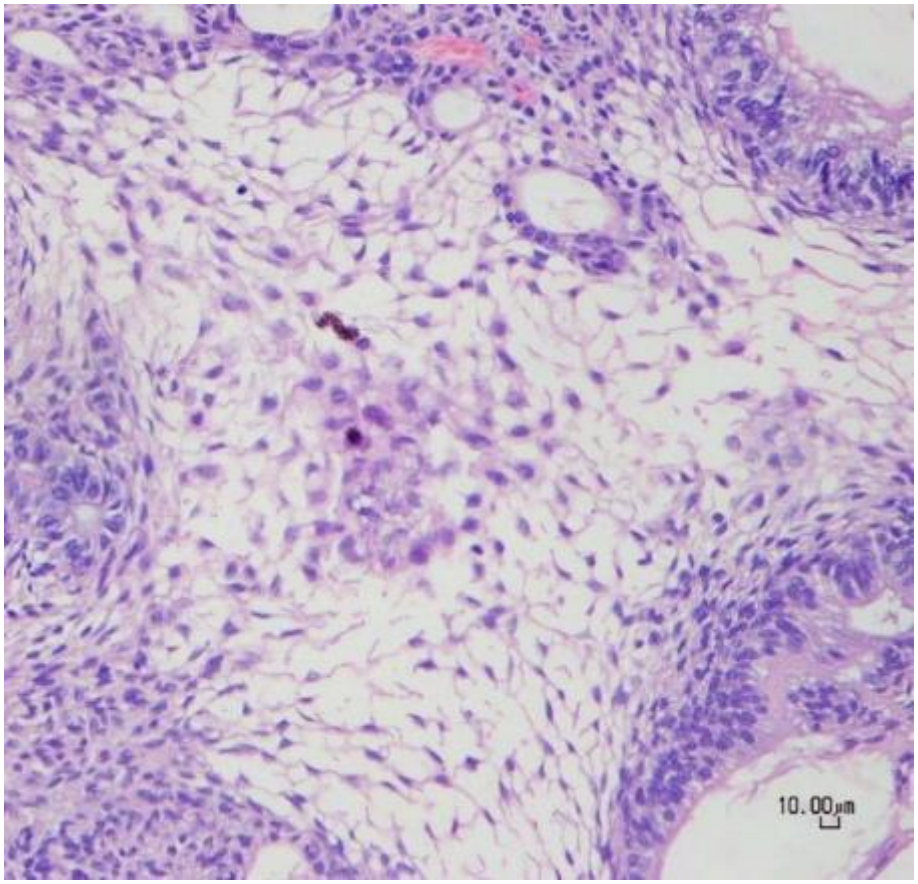

Figure 6 Histology of a stellate reticulum-like area. This photomicrograph showed an area of AOT that looked similar to a stellate reticulum. The cells were loosely arranged and stellate in shape, with little cytoplasm. The area was relatively hypocellular. Areas like this were called stellate reticulum-like areas (H&E staining).

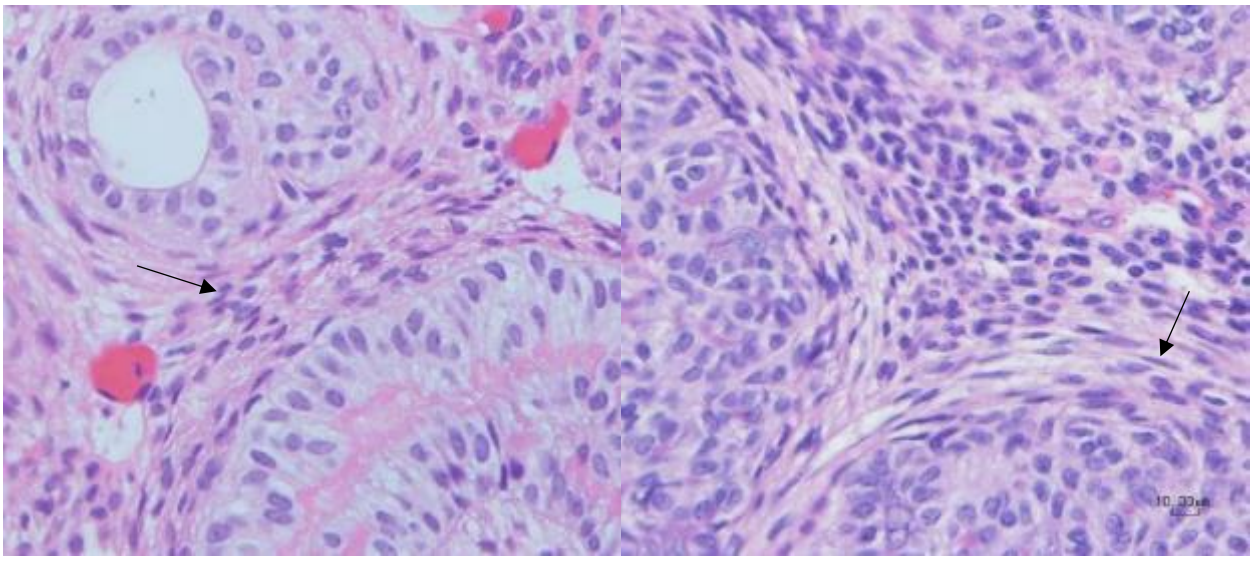

Figure 7 Histology of the intermediate cells found in AOT. The above photomicrographs showed the intermediate cells, as they were called in this study. These AOT cells were found between more organized structures. These areas varied in cellularity and the cell shapes ranged from polygonal to spindled in shape, with eosinophilic cytoplasm and indistinct cell borders (H&E staining).

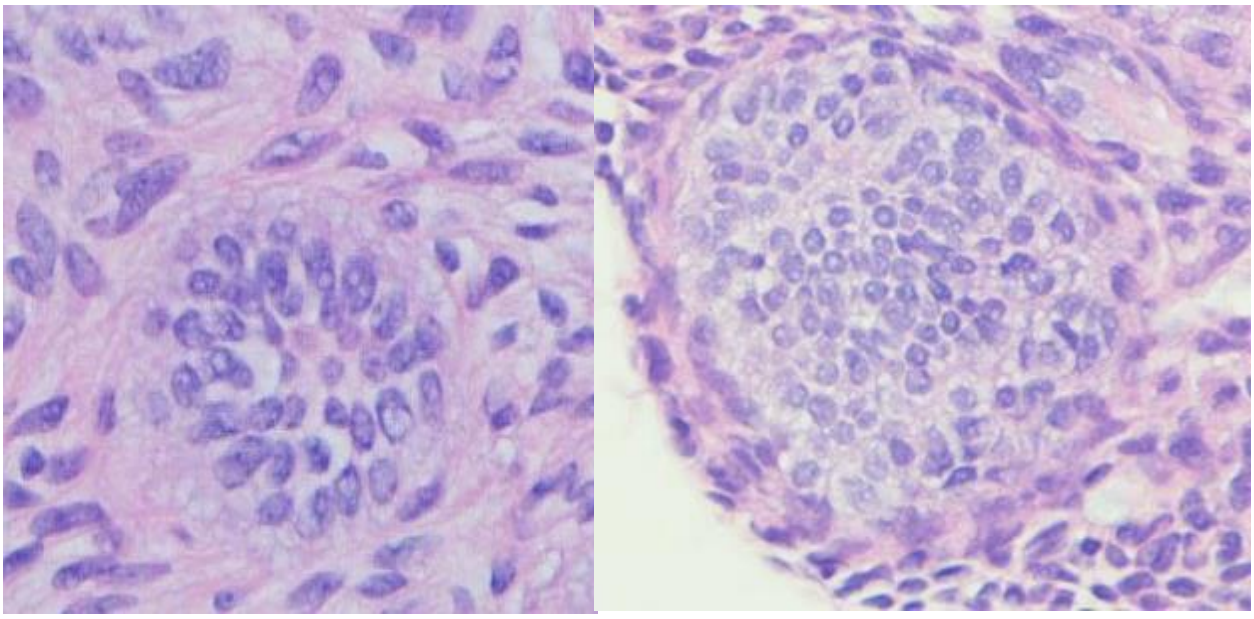

Figure 8 Histology of the epithelial knots. These photomicrographs demonstrated what were called epithelial knots in this study. These areas look like they may be located at the periphery of one of the organized structures of AOT. Because these knots may be only superficial cuts of one of the structures and it was not possible to determine which structure they were part of, they were put in their own category (H&E staining).

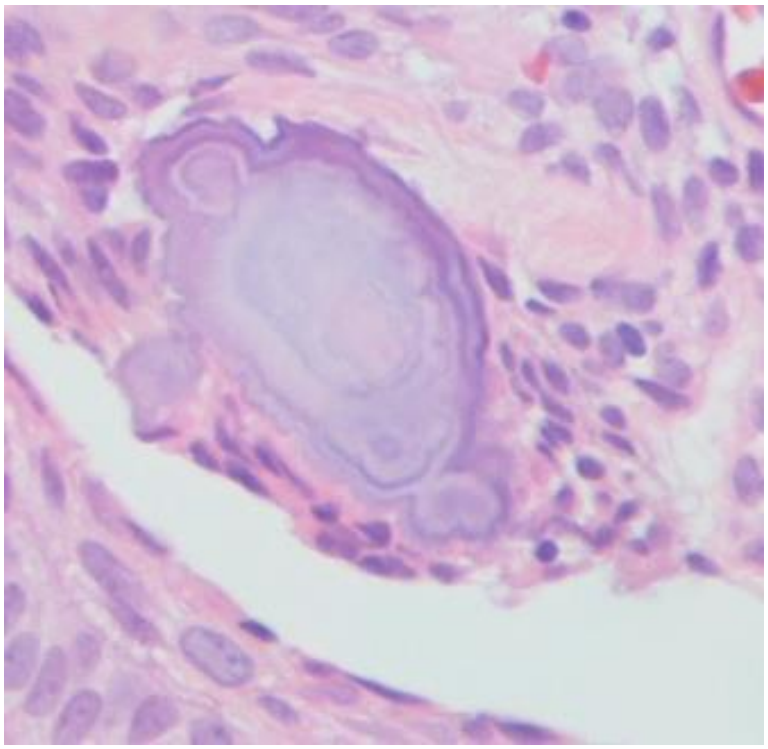

Figure 9 Histology of Liesegang ring-like calcifications. This photomicrograph showed material that has a concentric ring configuration. This study used the term that others have used for these structures, which were Liesegang ring-like calcifications. These areas demonstrated a basophilic, purple staining, of varying shades (H&E staining).

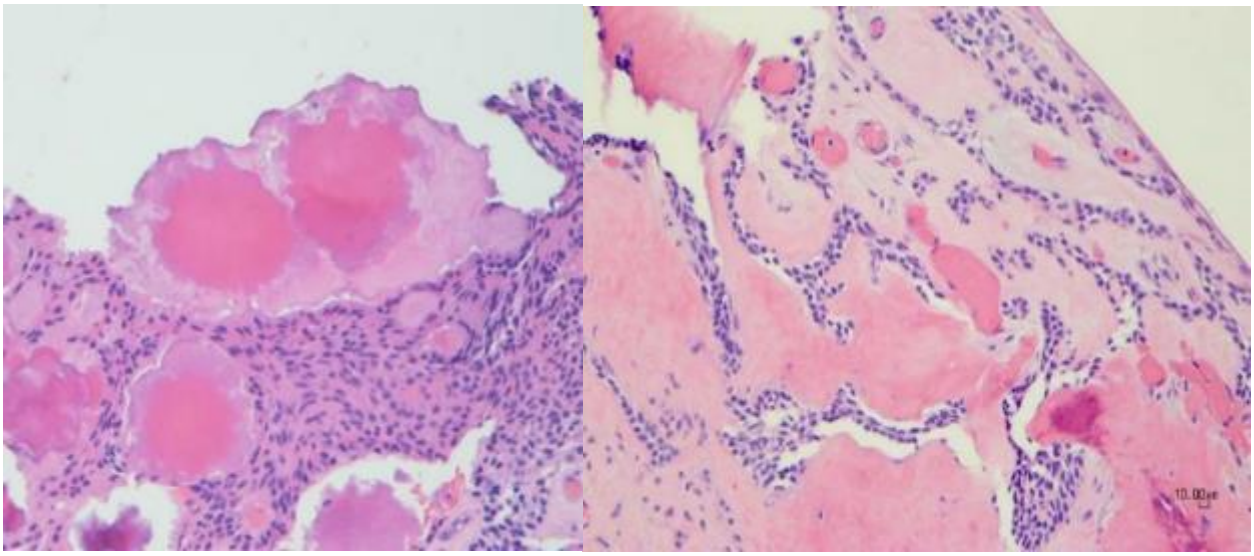

Figure 10 Histology of eosinophilic products (dentinoid). This photomicrograph showed what this study called large mineralized foci. These areas showed eosinophilic staining, of varying colors of pink. These areas have been called dentinoid in some investigations (H&E staining).

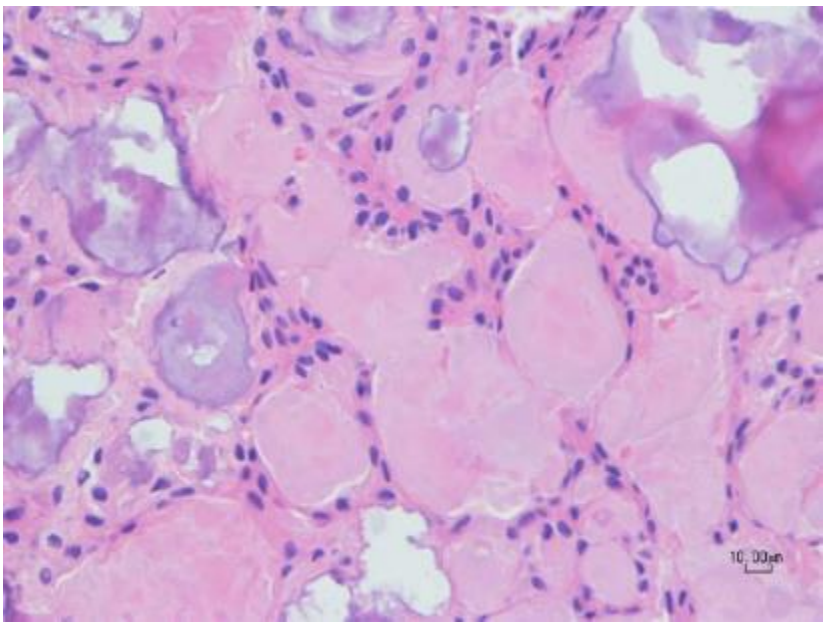

Figure 11 Histology of an area of mixed calcifications. This area showed a mixed population of both eosinophilic and basophilic calcifications. These smaller areas of eosinophilic calcified product were referred as small eosinophilic calcifications, in this study. Other authors have called areas such as this photomicrograph demonstrated CEOT-like areas. In this study, an area like this was referred to as a mixed calcification, further described in Figure 12 (H&E staining).

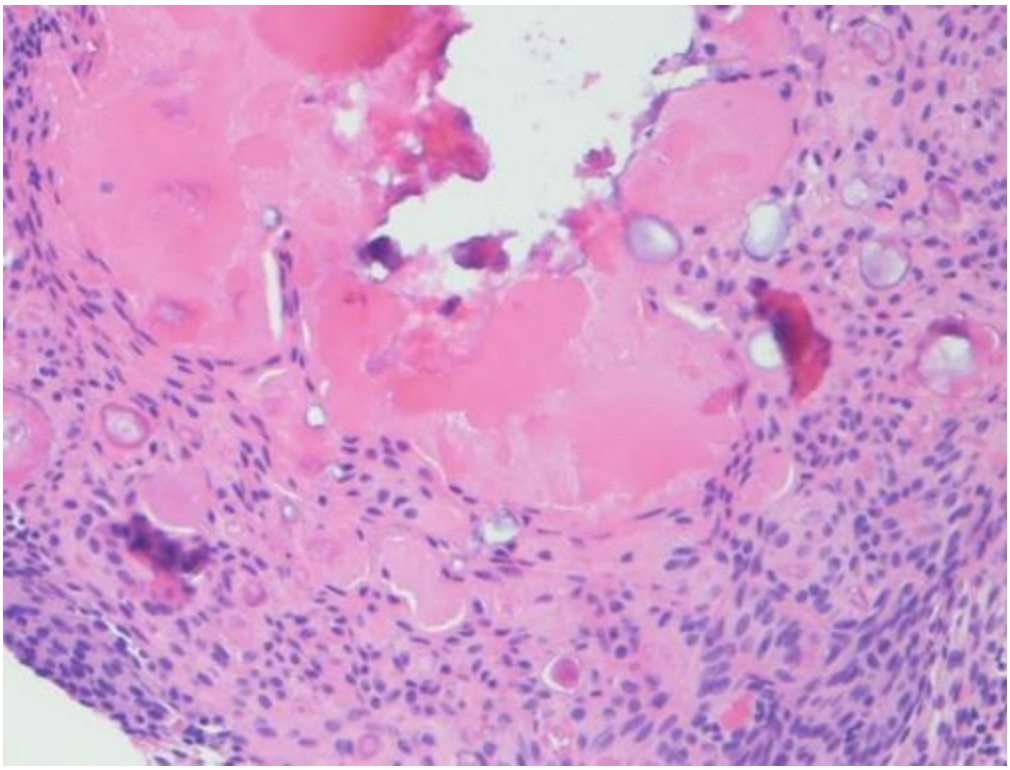

Figure 12 An area of mixed calcifications, showing a large mineralized focus adjacent to Liesegang ring-like calcifications. This photomicrograph showed an area of mixed calcifications. There were areas of eosinophilic large mineralized foci with intermixed basophilic Liesegang ring-like calcifications and small eosinophilic calcifications. These areas were referred to as mixed calcifications, but to specifically define the areas that were stained, an attempt was made to compare the H&E stained slides with the Sirius red slides to identify whether the areas staining were the eosinophilic large mineralized foci or the basophilic Liesegang ring-like areas (H&E staining).

3. RESULTS

The results will first be presented with the general trends that were seen throughout the 21 cases of AOT being investigated, then the individual AOT structures, defined in the Method of Analysis section, will be examined.

A summary of the immunohistochemical results is presented in Table 2 and shown with a representative AOT case in Figure 15. Of the 21 cases of AOT, 20 cases were at least focally positive for nestin (Table 2 and Figure 15 B). Dentin sialophosphoprotein (DSPP) was negative in the tumors, with none of the cases showing expression (Figure 15 C). Cytokeratin expression was found in all of the cases included in this study (Figure 15 D). Vimentin was at least focally positive in 20 of the 21 (95.23%) cases of AOT (Figure 15 E). The case that showed negative for nestin was not the same case that was negative for vimentin.

Table 2 Results of Immunohistochemical stains.

	Nestin (clone 10C2)	DSPP (clone LF-151)	Cytokeratin (clone AE1/AE3)	Vimentin (clone V9)
Number of AOTs that express marker	20/21	0/21	21/21	20/21
Percent of AOTs that express marker	95.23%	0%	100%	95.23%

A tumor was counted as positive if there was any positivity seen in the tumor cells.

While investigating the IHC findings in individual structures, we found that it was difficult to interpret the results of a few structures because they showed an evolution in subsequent sections. In order to examine this, 4 um-thick serial sections were made

and stained with H&E. There was an evolution of histological features, with some areas showing a defined structure evolving into another defined structure in subsequent sections. Figure 13 A-G showed a duct-like structure converting into an epithelial knot then into rosette structures in seven consecutive sections. Based on this information, it was not possible to interpret the IHC results of duct-like structure or epithelial knots as isolated structures because they were not present in multiple serial sections, which were used for IHC after the H&E stain was performed. Figure 14 A-D showed a higher magnification view of the evolution of a duct-like structure into a rosette structure in four consecutive sections. It was because of this reason, in our IHC investigation of the rosette structures, there were figures that appeared as rosettes in the area of interest on H&E, while the same area showed duct-like structures in some of the sections shown in the IHC results.

Figure 15 A showed the most common IHC findings, as described in Table 2, in a representative AOT case. Nestin showed focal positivity, with the positively stained cells usually being found in small clusters (Figure 15 B). These nestin-positive clusters typically contained some tumor products. In most sections of the AOT, DSPP expression was not seen (Figure 15 C). Pan cytokeratin stained all of the cells that formed the structures of AOT, including the duct-like structures, rosettes, anastomosing cords, and intermediate cells (Figure 15 D). Cytokeratin expression was reduced or absent staining in focal areas in AOT, which will be described in detail later. Vimentin was expressed in cells at the periphery of the tumor nodules and in the anastomosing cords (Figure 15 E).

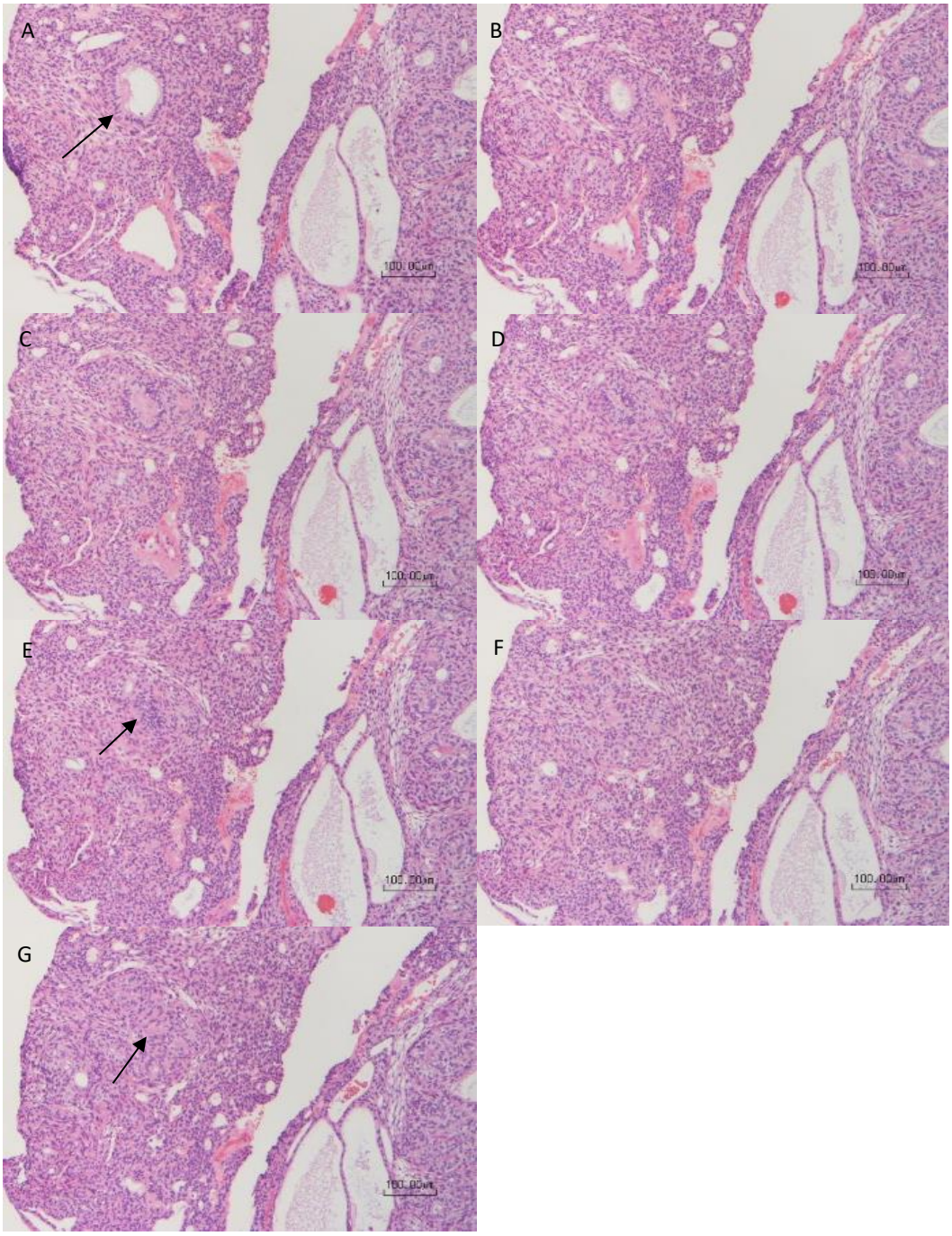

Figure 13 Serial sections of AOT showing a duct-like structure evolving into an epithelial sphere and then into rosettes. (A) A duct-like structure was noted (arrow). (B) The luminal space became smaller. (C) The luminal space was not present and the cells constituting the duct-like structure showed nuclei arranged away from the original luminal space. (D) No hint of duct-like structure was seen. (E) The same area appeared to be an epithelial knot (arrow). (F) The epithelial knot disappeared and the cells in the same area showed rosette arrangement, with eosinophils product in the middle (arrow). (G) A rosette structure, not duct-like structure or epithelial knot, was seen in the same area in this deeper section (arrow, H&E stain, original magnification x 70).

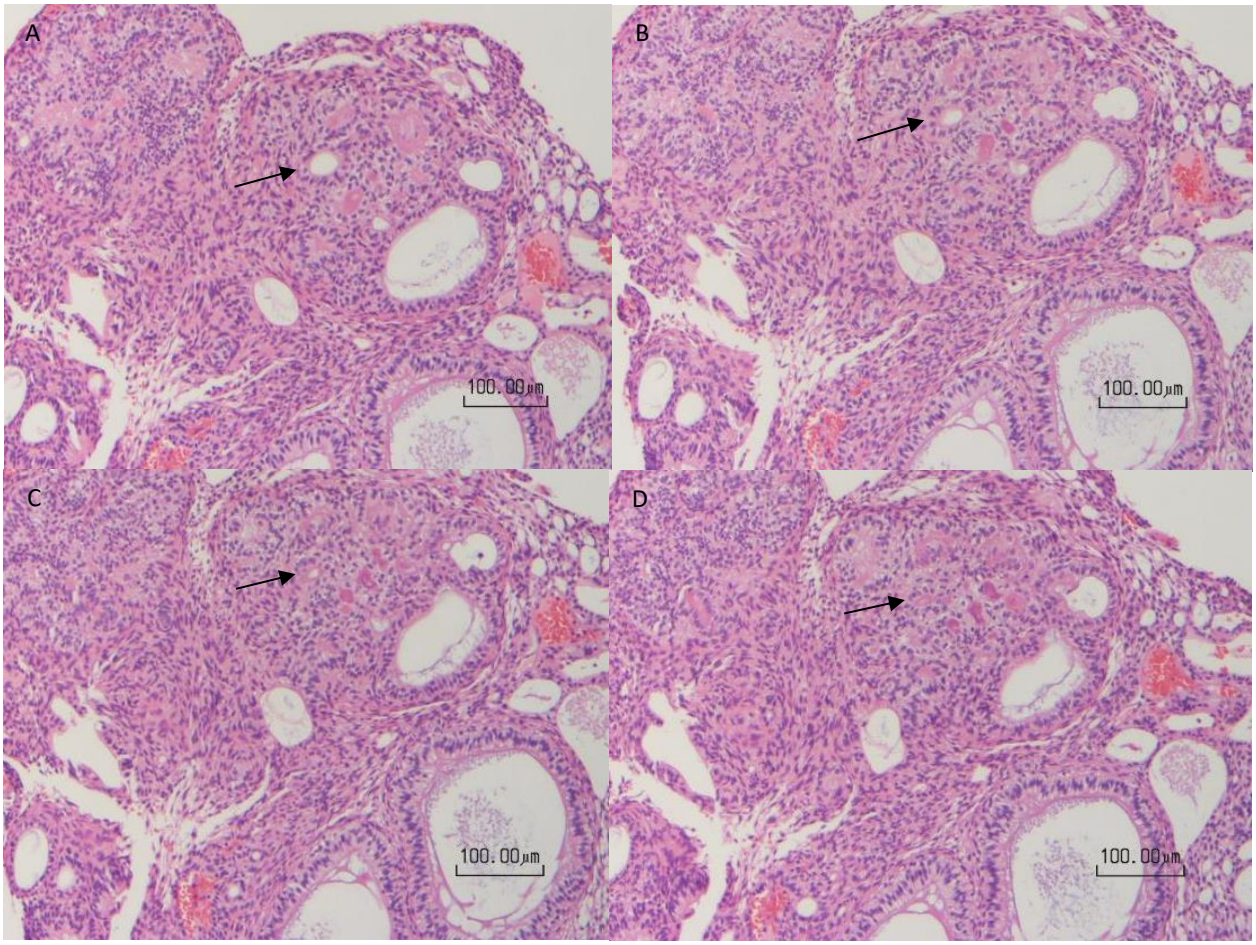

Figure 14 Serial sections showing the evolution of a small duct-like structure into a rosette in AOT. (A) There was a duct-like structure, indicated by the arrow. (B) In the next section, the duct-like structure had become smaller (arrow). (C) The duct-like structure decreased in size to the point that the lumen was less than one-quarter of the size it had been in Figure 14 A (arrow). (D) In the subsequent section, the duct-like structure became a rosette structure (arrow, H&E staining, original magnification x 70).

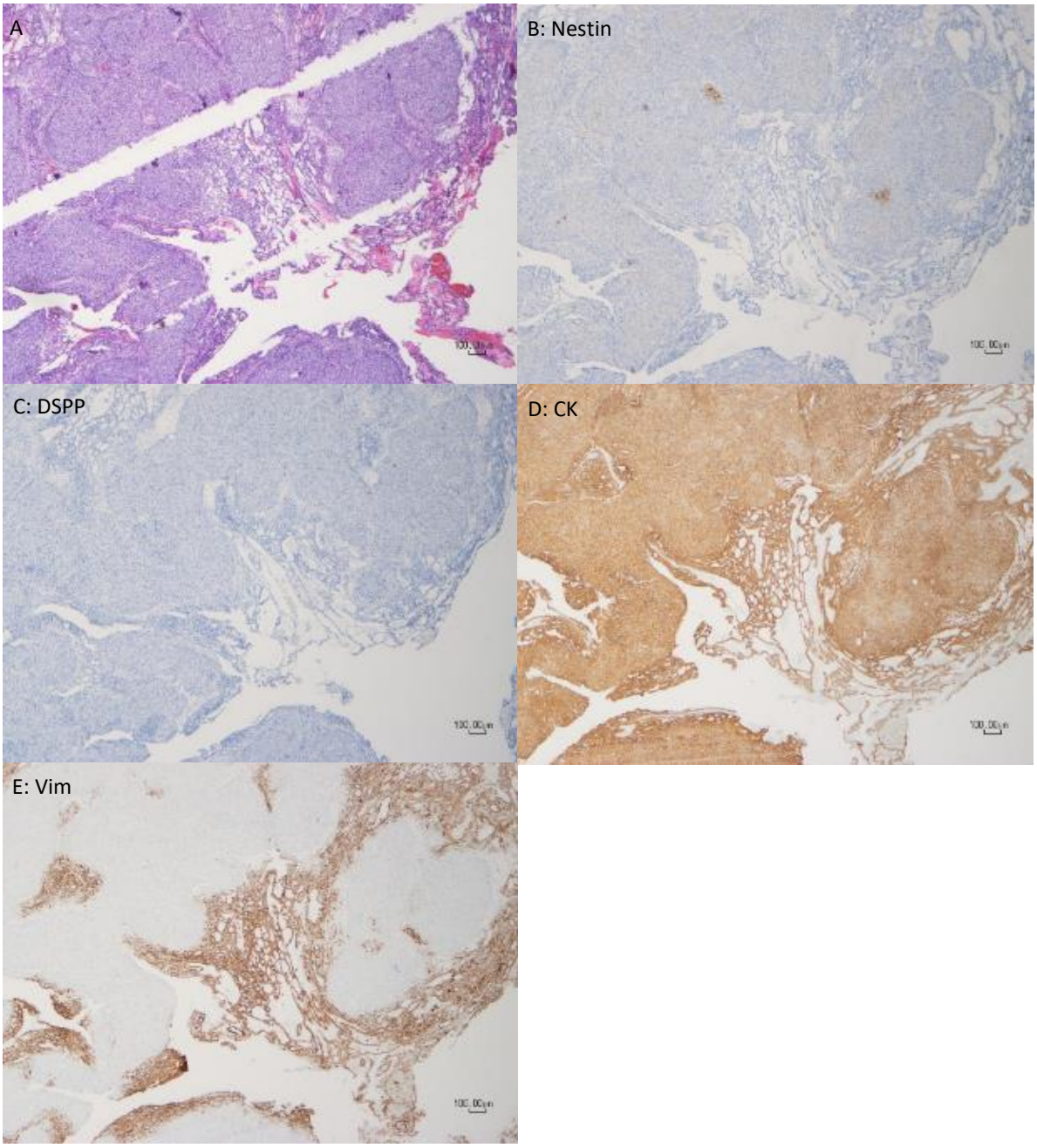

Figure 15 The H&E staining, IHC, and Sirius red results in a representative AOT case. (A) AOT consisted of nodules and cords of tumor cells (H&E staining, original magnification x 28). (B) There were focal areas that showed a moderate intensity of staining for nestin (Nestin IHC stain, original magnification x 28). (C) Most areas of the cases of AOT did not show expression of dentin sialophosphoprotein (DSPP) (DSPP IHC stain, original magnification x 28). (D) Pan cytokeratin showed strong, diffuse staining throughout the AOT (AE1/AE3 pan cytokeratin IHC stain, original magnification x 28). (E) Vimentin was expressed in the intermediate cells at the periphery of the nodules and in the anastomosing cords (Vimentin IHC stain, original magnification x 28).

The individual structures of AOT are described in the following sections.

3.1 Rosette/Duct-like Structures

The first structures that will be discussed are the rosette/duct-like structures. In this study, we have observed the evolvement of rosette structures into small duct-like structures, as described in Figure 14. Therefore, the term rosettes in Method of Investigation was modified to rosettes/duct-like structures to reflect this fact.

Figures 16, 17, 18, and 19 demonstrated that there was variability in the IHC results in the rosettes/duct-like structures. Most AOT cases showed faint positive staining for nestin in the cells which constituted the rosette/duct-like structures (Figure 16 B and 18 B), but a few cases showed strong positive staining in those cells (Figure 17 B). Nestin expression was also seen in the endothelial cells of the blood vessels included in the specimen (Figure 16 B, arrow), a finding that has been previously reported in the literature.[98] DSPP was not found in any of the products or cells in the rosettes (Figure 16 C, 17 C, 18 C). Pan cytokeratin showed strongly, diffusely positive staining in most areas of the AOT, but seemed to show a reduced intensity or no staining in some of the cells that formed the rosette/duct-like structures (Figure 16 D and 17 D). In some cases, the cells which formed the rosettes/duct-like structures did not express vimentin (Figure 16 E). However, there were also cases that showed strong positive cytoplasmic staining for vimentin in some or all of the cells that formed the rosette structures (Figure 17 E and 18 E, respectively). Sirius red staining of the AOT products in rosettes showed variability. Sirius red demonstrated that the product at the center of the rosettes stained red (Figure 16 F), orange to yellow, (Figure 17 F), or

yellow (Figure 18 F). Polarized lenses showed an orange birefringence of the red product stained by the Sirius red, which indicated that this product contained collagen type I (Figure 16 G and 17 G). When the product was yellow with Sirius red staining, there was no birefringence with polarized lenses (Figure 18 G). Higher magnification pictures of the rosette/duct-like structures stained with H&E and Sirius red were included in order to show the heterogeneity of the product (Figures 16 H and I, 17 H and I, and 18 H and I). With the Sirius red stain (Figures 16 I, 17 I, and 18 I), the product demonstrated different ratios of red-staining product to yellow staining product. In Figure 16 I, the product stained red. The product in Figure 17 I was composed partially of red staining product and partially yellow staining product. Figure 18 I showed a product that was primarily yellow, but had a border that stained red with Sirius red.

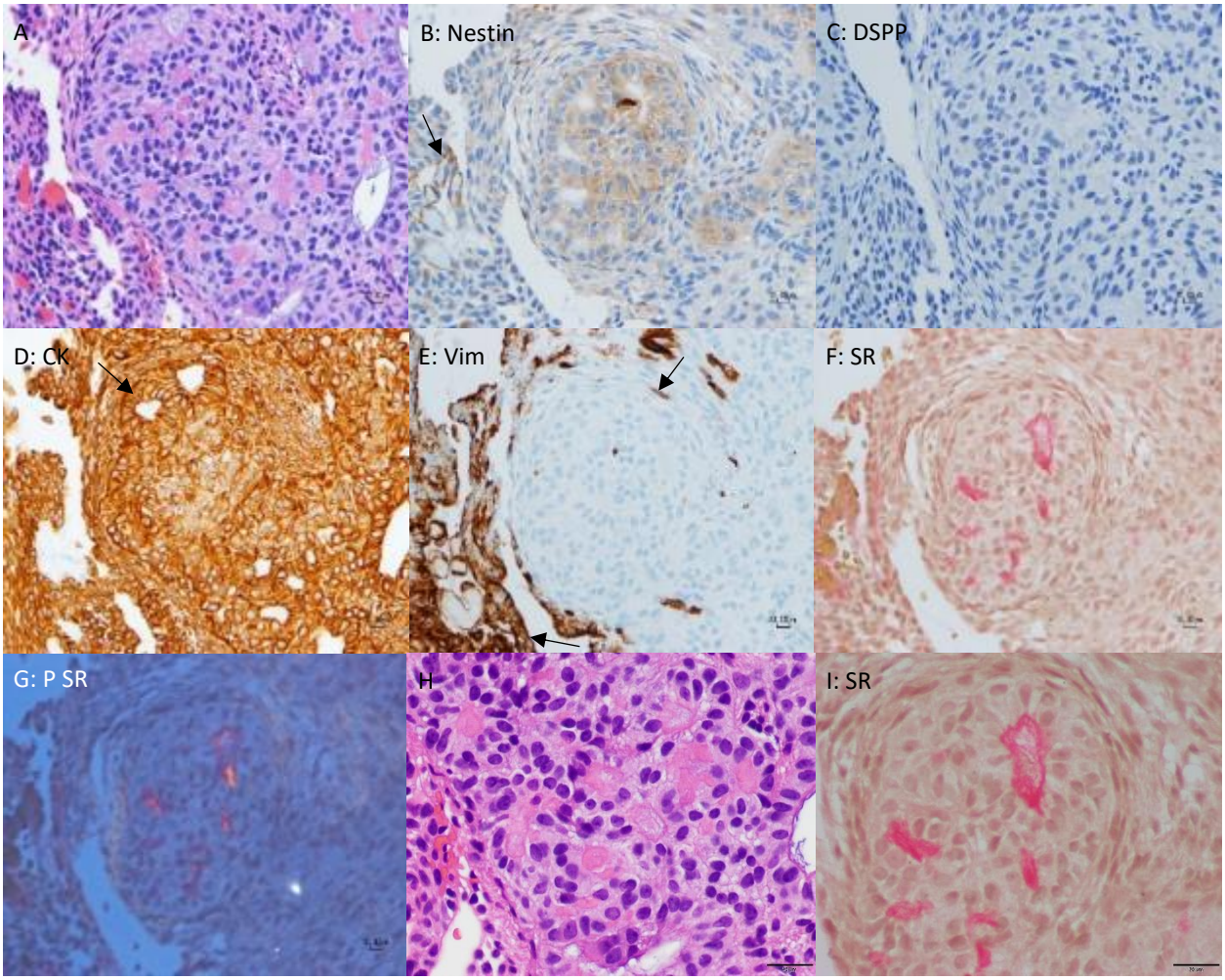

Figure 16 The H&E, IHC, and Sirius red results in a representative case of AOT rosettes. (A) Multiple rosettes with eosinophilic products were seen in focal areas of the AOTs. These rosettes, as a group, formed a somewhat ovoid nodule (H&E stain, original magnification x 280). (B) There was expression of nestin in the cytoplasm of the columnar to cuboidal cells that formed the rosette/duct-like structures. Nestin positivity was also seen in the blood vessels in this section (arrow), which was reported previously (see text) (Nestin IHC stain, original magnification x 280). (C) DSPP was not expressed in the rosettes of AOT (DSSP IHC stain, original magnification x 280). (D) Although all tumor cells showed positive staining for pan cytokeratin, reduced staining intensity was noted in some of the cells that formed rosettes. There were strongly staining cells that formed duct-like structures (arrow), as well as the intermediate cells that were surrounding this tumor nodule (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) Vimentin stained a few cells within the area of the rosette/duct-like structures, although the rosettes did not show vimentin expression. Vimentin expression was also found in the intermediate cells surrounding the AOT nodule which contained the rosettes (arrows) (Vimentin IHC stain, original magnification x 280). (F) Sirius red stained the product within the rosettes (Sirius red histochemical stain, original magnification x 280). (G) With the use of polarized lenses, orange birefringence was seen in the product (Sirius red histochemical stain with polarized lenses, original magnification x 280). (H) These rosette/duct-like structures showed the true pink eosinophilic product at the center of the rosette structures (H&E stain, original magnification x400). (I) Sirius red showed that the product in the center of the rosettes in stained bright red (Sirius red histochemical stain, original magnification x400).

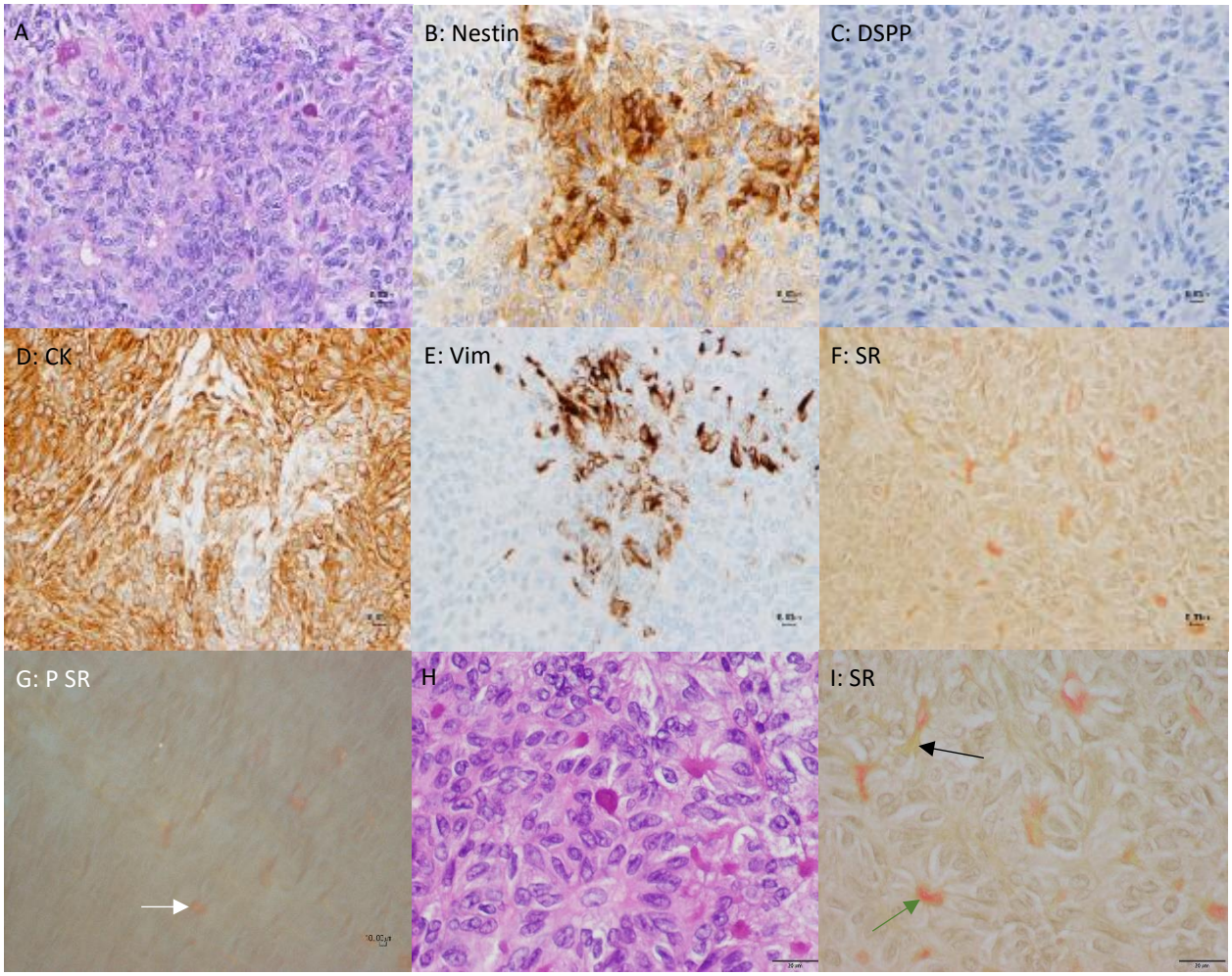

Figure 17 A variation of the IHC and histochemical results in the rosette/duct-like structures. (A) Multiple poorly formed rosettes were present in this section. The product that was present here was magenta in color (H&E stain, original magnification x 280). (B) There was intense cytoplasmic staining for nestin in many of the cells that formed the rosettes, but other cells demonstrated a moderate to faint expression (Nestin IHC stain, original magnification x 280). (C) No positive staining of dentin sialophosphoprotein (DSSP) was seen in the rosettes (DSSP IHC stain, original magnification x 280). (D) The expression of cytokeratin in the area of rosettes was reduced or negative (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) Several cells in the area of rosettes stained strongly positive for vimentin (Vimentin IHC stain, original magnification x 280). (F) Sirius red stained the product in the rosettes a light red to orange color (black arrow) or yellow color (Sirius red histochemical stain, original magnification x 280). (G) With the use of polarized lenses, there was reduced birefringence observed in this product (white arrow) compared to the birefringence seen in Figure 16 G (Sirius red histochemical stain with polarized lenses, original magnification x 280). (H) In the second example of rosettes, the product stained a magenta color, which appeared to be a combination of eosinophilic and basophilic portions (H&E stain, original magnification x400). (I) Sirius red staining in this area showed products with variable combinations of both red (green arrow) and yellow (black arrow) color (Sirius red histochemical stain, original magnification x 400).

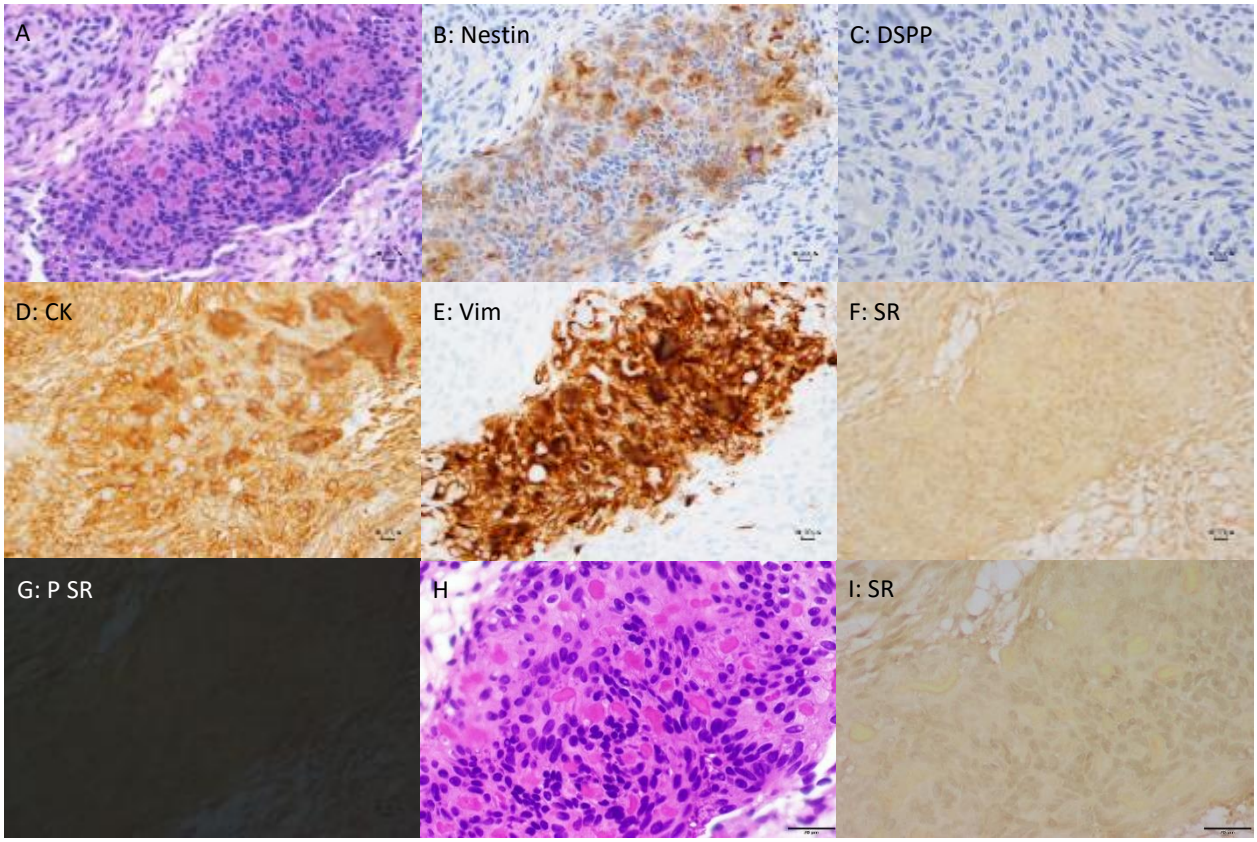

Figure 18 Another case showing variations in IHC and histochemical results in AOT rosettes. (A) There was a well-defined nodule of AOT that showed several rosette structures with magenta-staining product (H&E stain, original magnification x 280). (B) Nestin showed positive staining in a moderate to strong intensity in all of the cells that formed the rosettes (Nestin IHC stain, original magnification x 280). (C) Dentin sialophosphoprotein (DSPP) expression was not seen (DSPP IHC stain, original magnification x 280). (D) Many of the cells forming the rosette structures expressed cytokeratin at the same intensity as in the cells that surrounded this nodule. Within the nodule, there were cells that expressed cytokeratin at a higher intensity (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) There were many rosette cells in this nodule that expressed vimentin in the cytoplasm (Vimentin IHC stain, original magnification x 280). (F) The product in the rosettes stained only with counterstain with Sirius red (Sirius red histochemical stain, original magnification x 280). (G) With the use of polarized lenses, there was no birefringence observed in this section (Sirius red histochemical stain with polarized lenses, original magnification x 280). (H) Another case of AOT showed products stained in magenta color within the rosette structures (H&E stain, original magnification x400). (I) The product only had a limited amount of red stain with Sirius red. There was a red border around the yellow product, but the majority of the product stains yellow (Sirius red histochemical stain, original magnification x400).

3.2 Duct-like Structures and Intermediate Cells

The duct-like structures had a single row of columnar to cuboidal cells around a central lumen, and the intermediate cells are found between the duct-like structures (Figure 19 A). Lumens of some of the duct-like structures contained basophilic debris. In most of the examples of duct-like structures found in the AOTs, the cells that created the duct-like spaces did not show nestin expression. There was also no expression of nestin seen in the intermediate cells (Figure 19 B and 20 B). DSPP expression was not seen in the duct-like structures (Figure 19 C and 20 C). Cytokeratin was strongly expressed in the cells of the duct-like structures and in the intermediate cells between the structures in most cases (Figure 19 D), except that one case did not express cytokeratin in the duct-like structures and the expression was faint in the intermediate cells (Figure 20 D). Vimentin positivity was not seen in the cells of the duct-like structure in any of the cases (Figure 19 E) except one, which showed positive staining in scattered ductal cells (Figure 20 E). However, vimentin expression was consistently seen in the intermediate cells between the duct-like structures (Figure 19 E and 20 E).

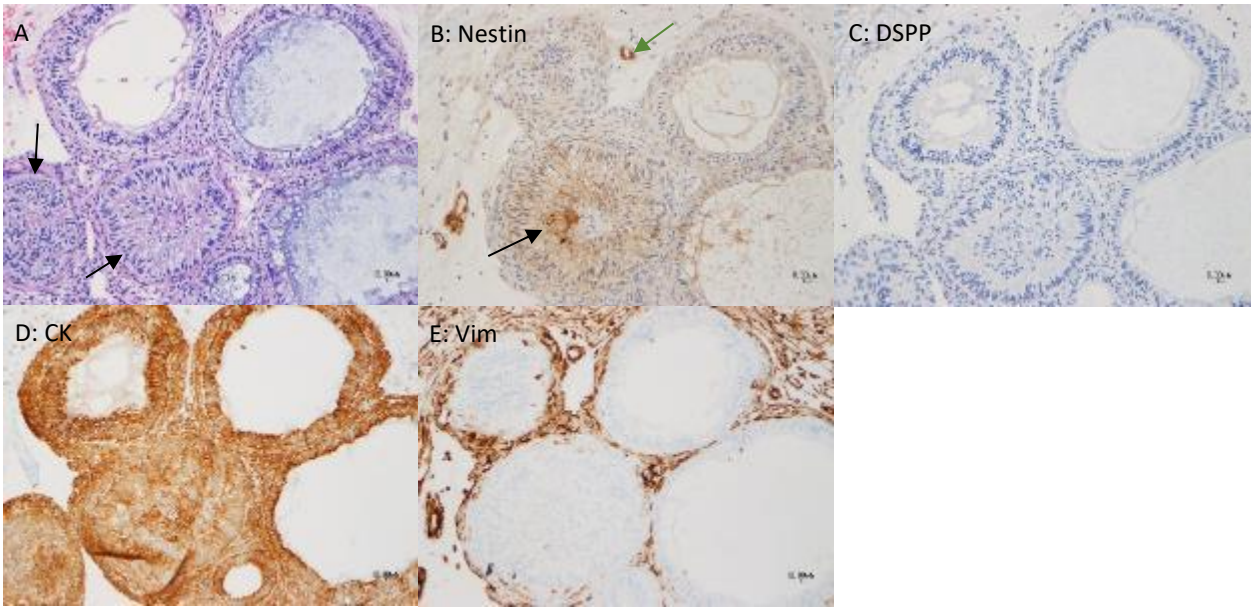

Figure 19 The presentation of the H&E and IHC expression in the duct-like structures of AOT in all but one case. (A) There were several duct-like structures as well as two double-layered spheres (arrows, H&E stain, original magnification x140). (B) There was no expression of nestin in the cells that are making up the duct-like structures, but some expression of nestin was seen in the double-layered sphere (black arrow) and the blood vessels (green arrow, Nestin IHC stain, original magnification x 140). (C) Dentin sialophosphoprotein (DSPP) expression was absent from the duct-like structures and intermediate cells (DSPP IHC stain, original magnification x 140). (D) There was strong, diffuse staining of pan cytokeratin in the duct-like structures, double-layered sphere, and intermediate cells (AE1/AE3 pan cytokeratin IHC stain, original magnification x 140). (E) Vimentin expression was seen in the intermediate cells surrounding the duct-like structures, but there was no positive expression found in the cells that formed the duct-like structures (Vimentin IHC stain, original magnification x 140).

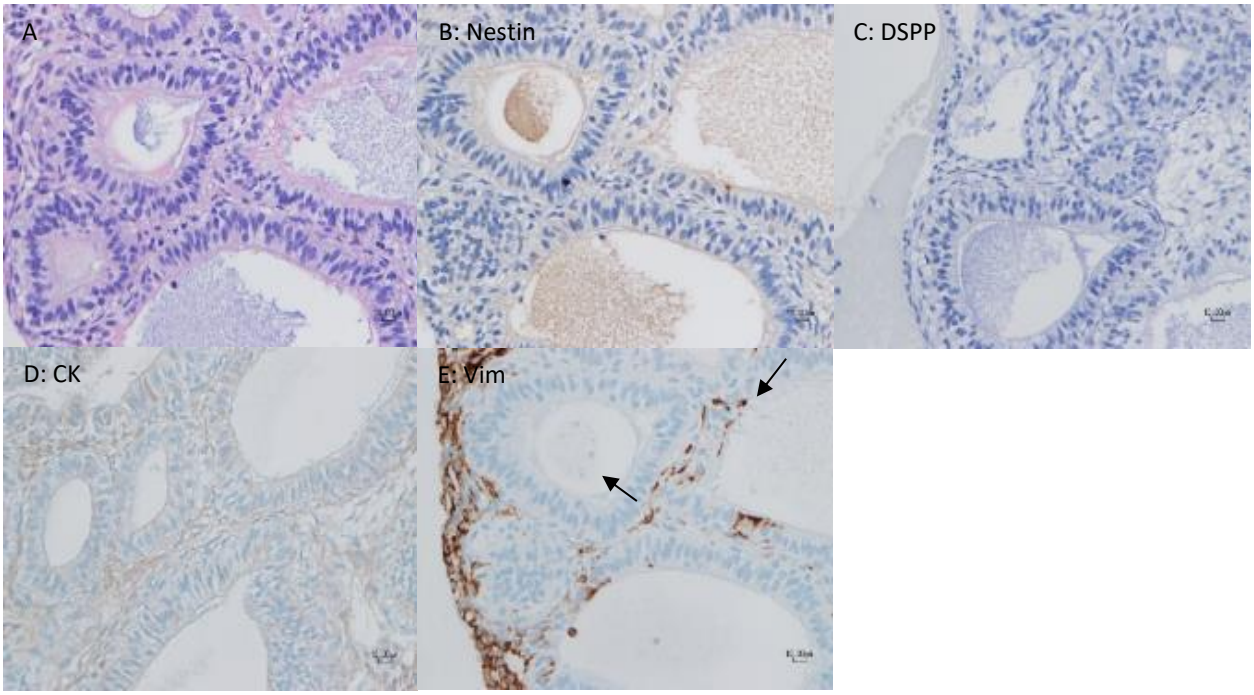

Figure 20 One case that showed different IHC results for the duct-like structures and intermediate cells in AOT. (A) There were multiple duct-like structures with central lumens in this section. The intermediate cells were intervening between and adjacent to the duct-like structures (H&E stain, original magnification x 280). (B) Nestin was not expressed in the duct-like structures. Non-specific binding was noted in the debris within the luminal spaces, (Nestin IHC stain, original magnification x 280). (C) Dentin sialophosphoprotein (DSPP) did not show any expression in the duct-like structures or intermediate cells of AOT (DSPP IHC stain, original magnification x 280). (D) Pan cytokeratin was negative in cells that formed these duct-like structures. There was faint positive staining in the intermediate cells (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) Vimentin showed positive cytoplasmic staining in scattered columnar cells in duct-like structures (arrows) and more diffusely positive in the intermediate cells (Vimentin IHC stain, original magnification x 280).

3.3 Double-Layered Spheres and Stellate Reticulum-Like Areas

A double-layered sphere was composed of a double layer of columnar cells arranged around a central area of tumor cells (Figure 21 A). Many times, there was an eosinophilic product present between two rows of columnar cells. There was a stellate reticulum-like zone (Figure 21 A, arrow) composed of loosely arranged, spindle shaped cells present between the columnar cells and the polyhedral cells in the center. Nestin

was expressed in the cytoplasm of the tall columnar cells that formed the double-layered sphere, and occasional cells in the center (Figure 21 B). There was no appreciable staining of cells or products with DSPP in this structure (Figures 21 C). Cytokeratin showed strongly and diffusely positive staining in the stellate-reticulum-like cells, in the polyhedral cells at the center of the structure, and in the intermediate cells outside the sphere. The columnar cells that formed the double-layered sphere showed reduced expression of cytokeratin, when compared to the intermediate cells surrounding this structure (Figure 21 D). None of the cells that formed the double-layered sphere expressed vimentin; but its expression was found in the intermediate cells adjacent to the double-layered sphere (Figure 21 E, black arrow) and, at times, in scattered cells in the center of the double-layered sphere (Figure 21 E, green arrow). The product between the two layers of tall columnar cells in the sphere stained red with Sirius red (Figure 21 F) and showed orange birefringence under polarized microscopy (Figure 21 G).

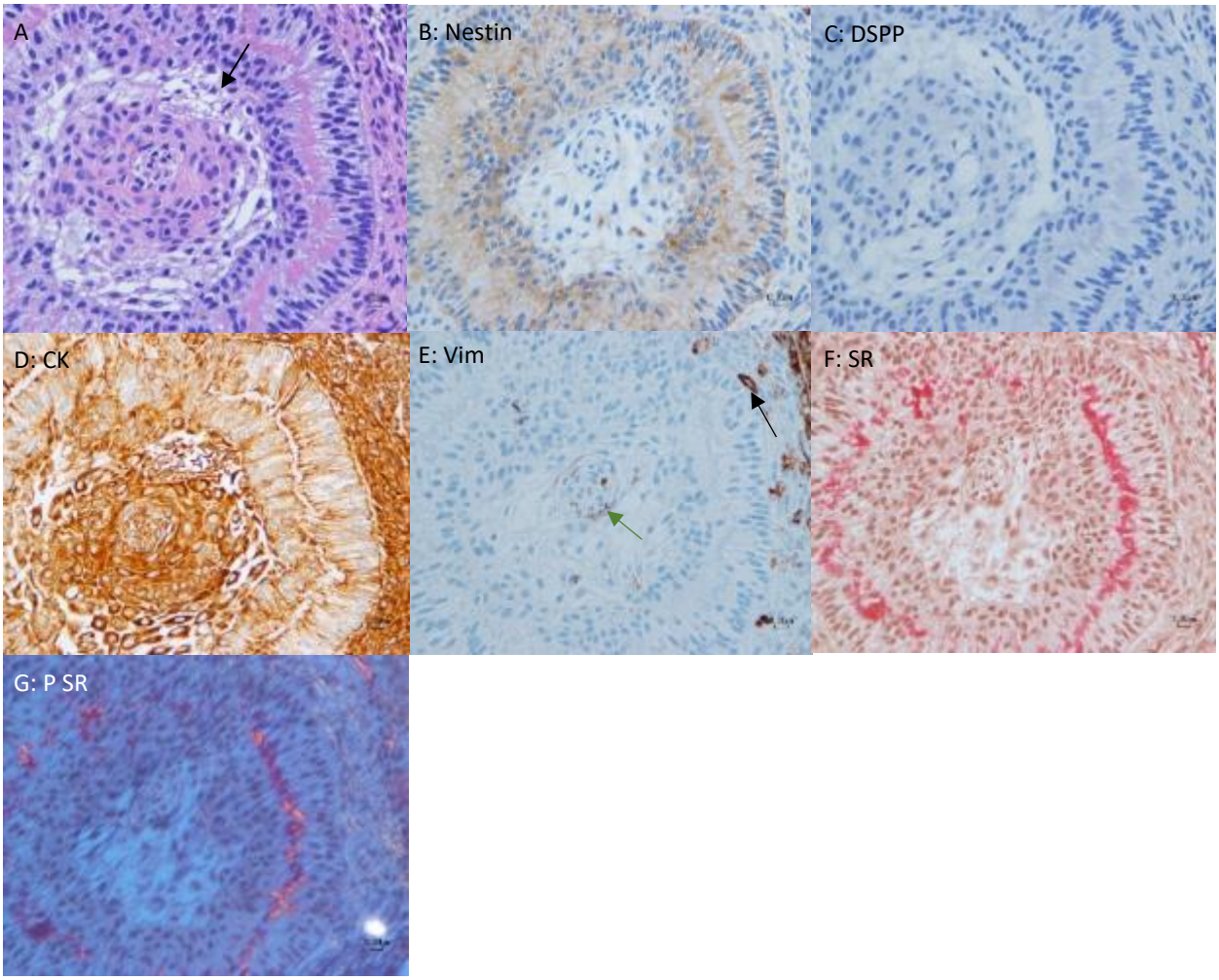

Figure 21 Expression patterns in a double-layered sphere. (A) Between the two layers of tall columnar cells, there was an eosinophilic product present. Inside of the double layer of columnar cells, there were polyhedral cells in the center surrounded by loosely arranged, spindle-shaped cells, which compose the stellate reticulum-like area (arrow, H&E staining, original magnification x 280). (B) The columnar cells in the sphere expressed nestin in their cytoplasm (Nestin IHC stain, original magnification x 280). (C) No expression of DSPP was found within the columnar cells of the double-layered sphere or the cells within this structure (DSPP IHC stain, original magnification x 280). (D) There was faint positive cytoplasmic staining of cytokeratin in the columnar cells that made up the double-layered sphere. Strong positive staining for cytokeratin was seen in the intermediate cells outside of the double-layered sphere and in scattered cells in the center of the sphere (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) Vimentin expression was seen in scattered intermediate cells outside of the double-layered sphere (black arrow), in a few spindle-shaped cells, and in the polyhedral cells inside the sphere (green arrow). There was no expression of vimentin in the columnar cells that formed the double-layered sphere (Vimentin IHC stain, original magnification x 280). (F) The product between the rows of columnar cells stained red with Sirius red (Sirius red histochemical stain, original magnification x 280). (G) When using polarized lenses, the product showed yellow-orange birefringence (Sirius red histochemical stain with polarized lenses, original magnification x 280).

3.4 Anastomosing Cords

The structures called anastomosing cords are often found at the periphery of the AOT. These cords can weave into the fibrous capsule but are also found in the body of tumors (Figure 22 A). There was no expression of nestin or DSPP in the cells that constituted anastomosing cords (Figure 22 B and C). Both pan cytokeratin and vimentin were strongly positive in the cells which formed these anastomosing cords (Figure 22 D and E).

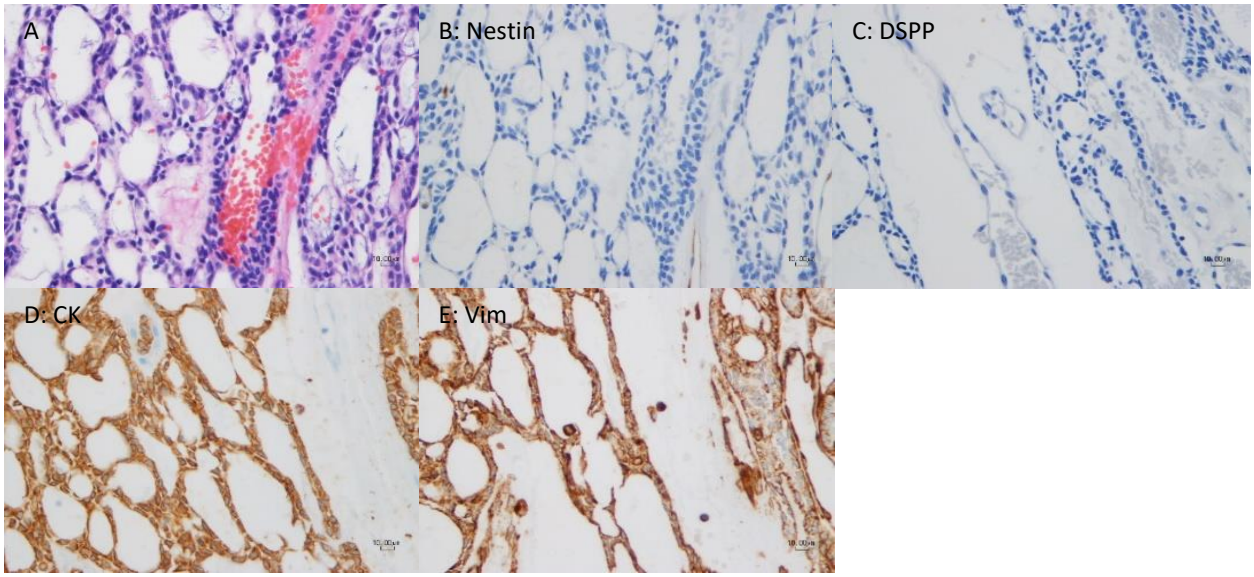

Figure 22 IHC results for the anastomosing cords of AOT. (A) The anastomosing cords of AOT were composed of delicate, weaving strands of AOT cells (H&E stain, original magnification x 280). (B) Tumor cells of the anastomosing cords did not express nestin (Nestin IHC stain, original stain x 280). (C) There was no expression of dentin sialophosphoprotein (DSPP) seen in the anastomosing cords (DSPP IHC stain, original stain x 280). (D) Strong and diffuse expression of cytokeratin was seen in the anastomosing cords (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) Vimentin showed strong and diffuse cytoplasmic staining of the cells of anastomosing cords (Vimentin IHC stain, original magnification x 280).

3.5 Mixed Calcifications

In this study, a “mixed calcification” was defined as a calcified material that showed areas of both eosinophilic and basophilic staining (Figures 23 A and 24 A). They, presumably, represented a type of product found in AOT. Figures 23 and 24 demonstrated two types of mixed calcifications in AOT. The first type of mixed calcification was a combination of eosinophilic and globular basophilic product (Figure 23 A). The second type of mixed calcification also contained both eosinophilic and basophilic staining on H&E, but the basophilic stained area did not show a globular pattern (Figure 24 A and B). Scattered cells adjacent to the mixed calcifications showed positive cytoplasmic staining for nestin (Figure 23 B). DSPP did not show any positive staining (Figure 24 C). Both cytokeratin and vimentin expression were seen in the tumor cells adjacent to the mixed calcifications (Figure 23 D and E). The Sirius red staining of the products showed areas of red and yellow, appeared to correspond to the staining pattern seen with H&E, with the eosinophilic areas stained red with Sirius red, while the basophilic areas stained yellow with Sirius red (Figure 23 F and Figure 24 C and E). The birefringence pattern of the Sirius red showed a distinct difference based on the color of the product: the red product showed yellow-orange or green birefringence, while the yellow product did not show any birefringence (Figure 23 G and Figure 24 D and F). The red product on Sirius red showed birefringence, but the yellow staining product did not, which was consistent with the birefringence pattern seen in the rosette/duct-like structures (Figures 16-18). The mixed calcifications appeared to represent a mixture of two biochemically different products.

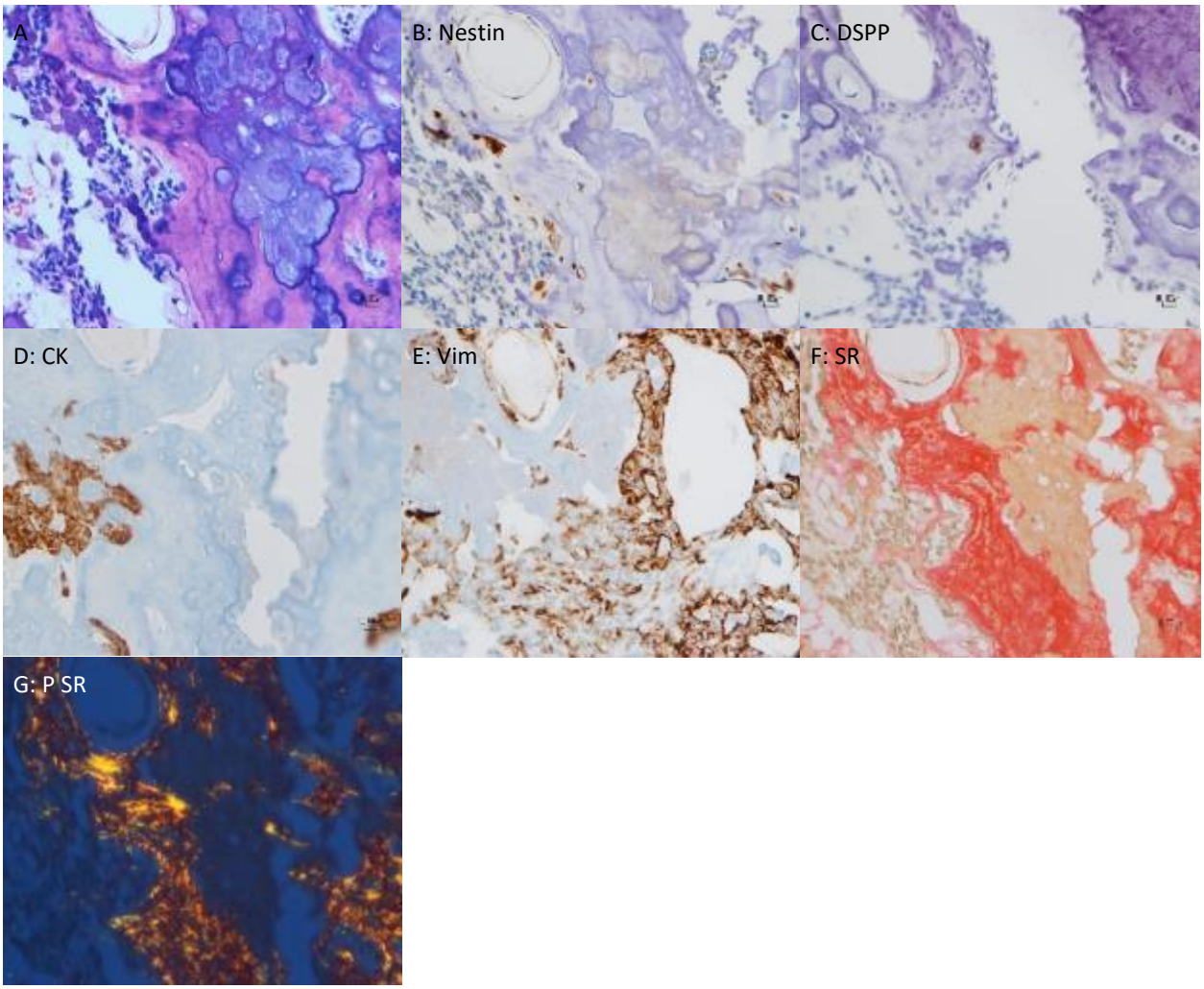

Figure 23 IHC and Sirius red results in mixed calcifications and surrounding tumor cells. (A) The mixed calcified material consisted of both eosinophilic and basophilic areas. Some of the basophilic areas demonstrated Liesegang ring-like pattern (H&E stain, original magnification x 280). (B) There were scattered cells that stained positive for nestin at the periphery of the calcified material (Nestin IHC stain, original stain x 280). (C) Dentin sialophosphoprotein (DSPP) did not show any positivity in the tumor cells adjacent to the mixed calcified material (DSPP IHC stain, original stain x 280). (D) Cytokeratin stained strongly positive in the tumor cells adjacent to the mixed calcifications (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) There was positive cytoplasmic staining for vimentin in the cells at the periphery of the mixed calcified material (Vimentin IHC stain, original magnification x 280). (F) Sirius red showed a staining pattern in the mixed calcifications that corresponded with the staining pattern with H&E. It appeared that the basophilic area in H&E stained yellow with Sirius red, while the eosinophilic area in H&E stained red with Sirius red (Sirius red histochemical stain, original magnification x 280). (G) When polarized lenses were used to view the Sirius red stain, the red product demonstrated yellow-orange birefringence. The yellow product did not have any birefringence (Sirius red histochemical stain with polarized lenses, original magnification x 280).

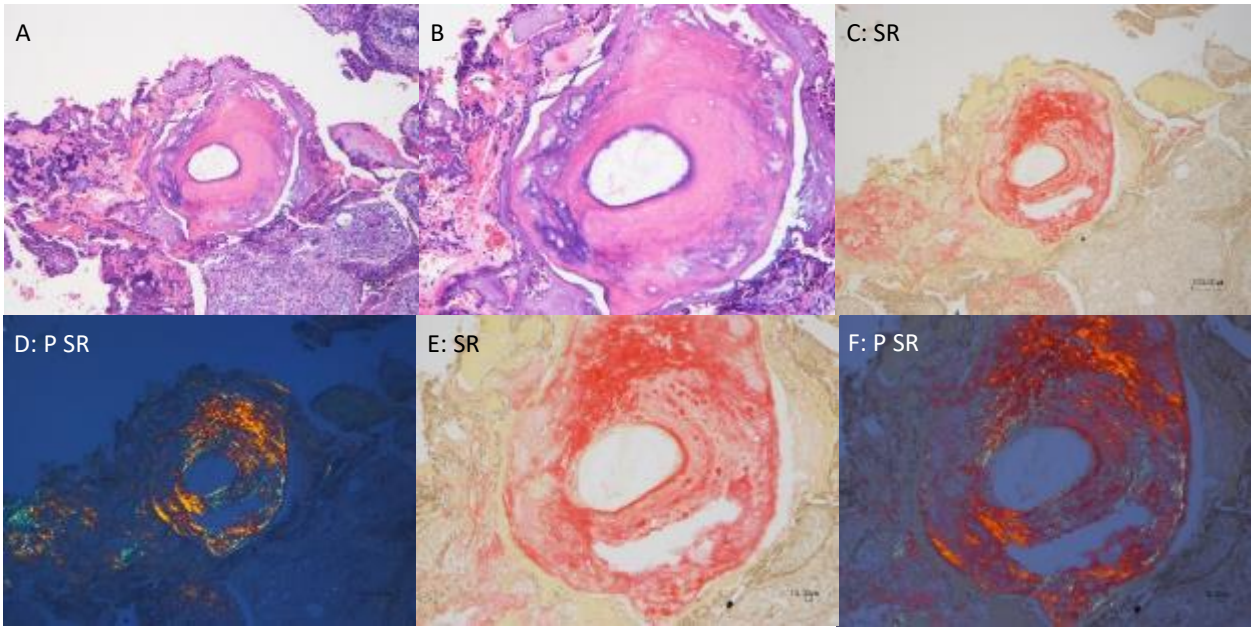

Figure 24 Sirius red staining result of the second type of the mixed calcification. (A) A large mixed eosinophilic and basophilic calcification was seen surrounded by tumor cells (H&E stain, original magnification x 70). (B) Higher magnification showed that the calcification was primarily eosinophilic with focal areas of basophilic material without a globular pattern. In addition to the smaller basophilic areas, there was basophilic product that encircled the entire eosinophilic calcification (H&E staining, original magnification x 140). (C) The Sirius red showed red in the eosinophilic area, and yellow in the basophilic areas of this product (Sirius red histochemical stain, original magnification x 70). (D) With the use of polarized lenses, both the eosinophilic and the basophilic areas showed yellow-orange or green birefringence (Sirius red histochemical stain with polarized lenses, original magnification x 70). (E) The higher magnification of Sirius red demonstrated details of the delineation of the red product and yellow product (Sirius red histochemical stain, original magnification x 140). (F) The closer view of Sirius red under polarized lenses made it clear that the red product showed both yellow-orange and green birefringence. The surrounding yellow product did not demonstrate birefringence (Sirius red histochemical stain with polarized lenses, original magnification x 140).

3.6 Eosinophilic Product (Dentinoid) and Surrounding Cells

The large eosinophilic products, often referred to as dentinoid when discussing AOT, may contain scattered spindle cells and could be seen surrounded by columnar cells (Figure 25 A). Positive cytoplasmic staining for nestin was found in the columnar cells as well as the spindle cells within the product (Figure 25 B). No staining for DSPP was observed (Figure 25 C). There was a consistent, strong, and diffuse expression of cytokeratin in the cytoplasm of the columnar cells (Figure 25 D). Vimentin expression was not seen in the columnar cells but was seen in the intermediate cells (Figure 25 E, black arrow). The Sirius red stained red for the eosinophilic product (Figure 25 F). When using polarized lenses, orange birefringence was seen (Figure 25 G).

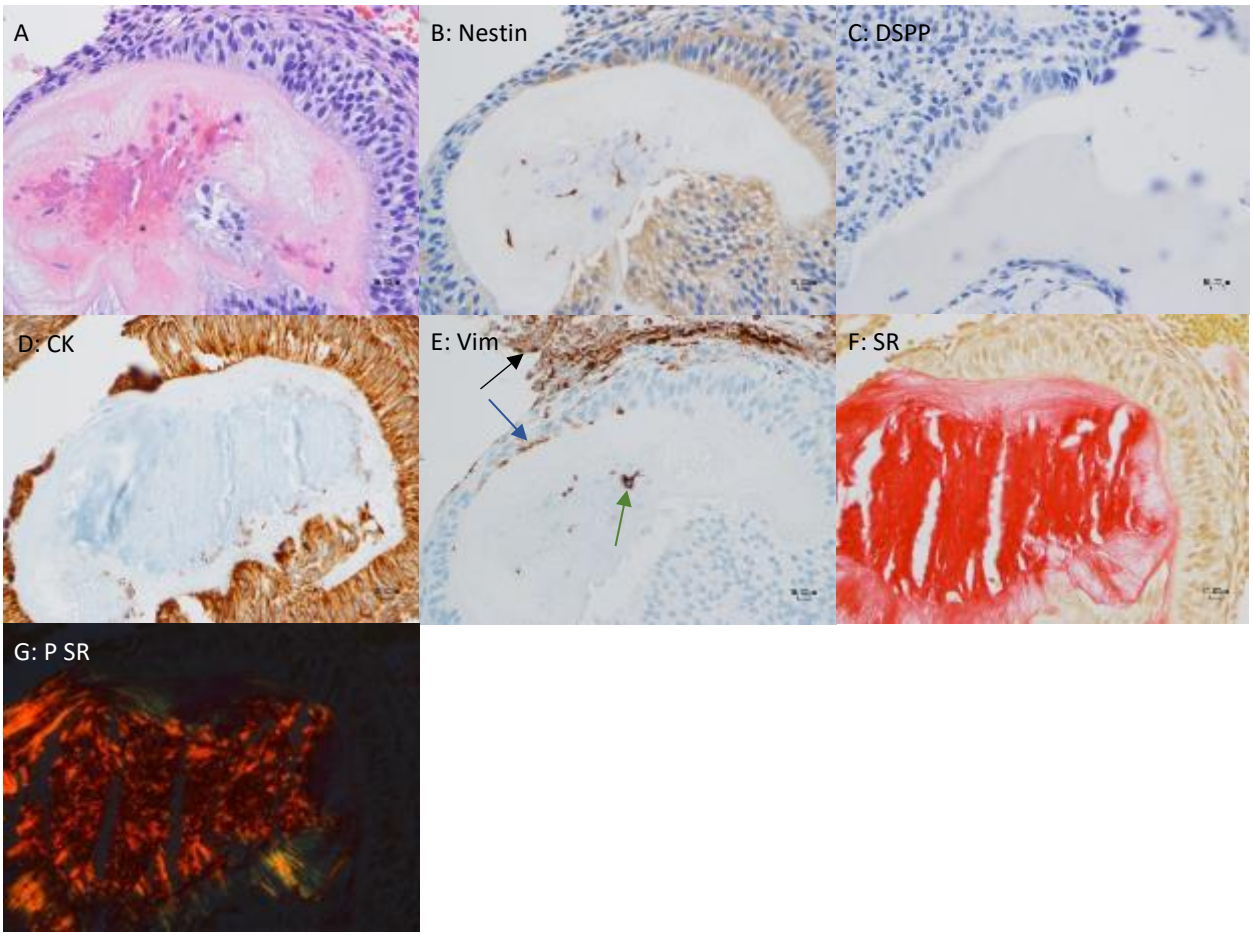

Figure 25 The IHC and Sirius red results for the eosinophilic product. (A) The eosinophilic product contained spindle cells and was surrounded by columnar to cuboidal cells (H&E stain, original magnification x 280). (B) There was cytoplasmic expression of nestin in the columnar lining cells. There were a few, scattered nestin-positive cells found within the tumor product (Nestin IHC stain, original stain x 280). (C) There was no expression of dentin sialophosphoprotein (DSPP) in the eosinophilic product (DSPP IHC stain, original stain x 280). (D) Cytokeratin showed intense and widespread expression in the columnar cells surrounding the product (AE1/AE3 pan cytokeratin IHC stain, original magnification x 280). (E) Vimentin was positive in the intermediate cells (black arrow). There was expression of vimentin in a few, scattered spindle or dendritic cells within the dentinoid product (green arrow). There were several of vimentin-expressing cells found adjacent to the product (blue arrow, Vimentin IHC stain, original magnification x 280). (F) The product stained positive for Sirius red (Sirius red histochemical stain, original magnification x 280). (G) When polarized lenses were used to view the Sirius red stain, the product demonstrated red birefringence. Outside of the red birefringence, there was some yellow-orange birefringence (Sirius red histochemical stain with polarized lenses, original magnification x 280).

4. DISCUSSION

The purpose of this project was to determine whether AOT is an epithelial or a mixed epithelial and mesenchymal tumor. We hypothesized that there were both epithelial and mesenchymal components that contributed to the cell population and to the products of AOT. The immunohistochemical (IHC) and histochemical findings in this study appeared to support our hypothesis. The immunohistochemical results showed that, although more than 90% of the AOT cells stained positively for cytokeratin, 20 of the 21 AOTs also showed positivity for nestin and vimentin. Because nestin and vimentin are known to be mesenchymal intermediate filament proteins, their expression in AOT provide evidence to support that AOT has a mesenchymal component. Both positive and negative Sirius red staining results were found in the AOT products, which suggests that the material produced by the AOT cells is heterogeneous in nature. There were portions of the tumor product that stained positively (red) with Sirius red histochemical stain and showed orange to yellow birefringence under polarized light. This finding demonstrates that collagen type I is present in one type of AOT product.[96-98] The results from this study suggest that, while AOT is predominantly epithelial, there are cells that are mesenchymal in origin and support AOT being classified as a mixed odontogenic neoplasm.

Our results showed nestin expression was present in the cells that formed the rosette/duct-like structures. Our study is not the first study to identify nestin expression in AOT. Fujita, et al.[62] described the expression of nestin in AOT and in several mixed odontogenic tumors, including odontomas, ameloblastic fibromas, ameloblastic fibro-

odontomas, ameloblastic fibro-dentinomas, ameloblastic fibrosarcomas, and odontogenic fibromas, as well as in ameloblastomas, malignant ameloblastomas, and myxomas.[62] This study described the most intense nestin positivity being seen in the ectomesenchymal cells found adjacent to the odontogenic epithelium in the mixed odontogenic tumors.[62] Similar to our findings, Fujita, et al.[62] reported that nestin was expressed in the spindled and rosette cells in the areas near the amorphous product of AOT. In that study, there were 5/6 (83.3%) AOTs positive for nestin, while our study showed positivity in 20/21 (95.2%).[62] The authors of that study concluded that these nestin-positive AOT cells were epithelial cells, but of a different immunophenotype than the other AOT epithelial cells.[62] While this would be one interpretation of the findings, we think an alternative interpretation would be that the expression of nestin in the AOT cells forming the rosette/duct-like structures suggests that those cells are showing odontoblast differentiation. This interpretation seems more consistent with Fujita, et al.'s[62] findings in the other mixed odontogenic tumors as well as the findings reported by others that in odontogenic cells, nestin expression was found only in odontoblasts during tooth development and dentin repair.[74]

The rosette structures of AOT contained droplets of tumor product, which is presumably produced by the surrounding rosette cells. Our Sirius red results showed that these droplets were heterogeneous in nature (Figure 16 F, 17 F, 18 F), which could be explained as aberrant product formation in neoplastic conditions. Some droplets stained positive for Sirius red, a feature indicating presence of collagen type I and III, which both have been found in dentin.[74, 96, 97, 99] Because collagen type I is the major organic component of dentin, especially in intertubular dentin, our finding also suggests

that the product in rosettes is most likely to be aberrant intertubular dentin.[99, 100] This further supports our IHC findings that the cells that formed the rosette structure in AOT are most likely to be odontoblasts. There have been studies that used Sirius red to investigate the collagen present in the connective capsule of AOT and in the AOT as a whole, without separating the findings in the capsules from the findings in the product or calcified material.[101, 102] To the best of our knowledge, no previous studies have specifically investigated AOT products with Sirius red stain.

Our study used an antibody that recognizes both DSPP and DSP; and our results showed an absence of expression of these two proteins in AOT cells. The three cleaved products of DSPP, which are DSP, DGP, and DPP, play an critical role in dentin mineralization and maturation.[77, 79, 80, 101, 102] Expression of DSPP was not observed in the cytoplasm of any of the cells or in any type of AOT product. When combined with the result of nestin detection, the negative result of DSPP suggests that the nestin-positive cells are most likely pre-odontoblasts or early-stage odontoblasts, because they were not actively producing DSPP.

Our investigation found vimentin expression in 20 out of 21 AOTs examined. Although most of these vimentin-positive cells showed simultaneous positive staining for cytokeratin (Figure 18 D and E), there were tumor cells in rosettes and duct-like structures that showed positive staining only for vimentin and not for cytokeratin (Figure 17D and 20D). This finding is consistent with the results that were found in the nestin investigation, which indicates that the rosettes cells are most likely pre-odontoblasts or early-stage odontoblasts and are, therefore, are mesenchymal in origin. Vimentin expression has been reported in AOT in several studies previously.[50, 55, 69, 72] The areas

of the AOT that showed vimentin expression in the studies by Leon, et al.[50] and Tatemoto, et al.[72] were described as equivalent to the anastomosing cords in our study (Figures 13 E, 18 E, and 21 E), although in our study these cords also showed co-expression with cytokeratin. Despite vimentin being an intermediate filament protein found most commonly in mesenchymal cells, these authors did not interpret their findings as evidence supporting a possible mesenchymal component in AOT. Leon, et al.[50] explained that vimentin expression in AOT showed "certain areas of the tumour are phenotypically variable." In the 2003 study by Crivelini, et al.,[57] the areas that were positive for vimentin were the "fusiform or ovoid cells adjacent to calcified bodies and darker eosinophilic material." This description seems to be equivalent to the positivity we found in the cells forming the rosette/duct-like structures adjacent to the eosinophilic material (Figures 15 E and 16 E). Crivelini et al.,[57] believed that the vimentin expression in AOT cells demonstrated the epithelial cells with secretory function and showed that the cells were related to the reduced enamel epithelium.[57] The paper written by Sudhakara, et al.[69] concluded that the variable expression of vimentin in AOT suggested that vimentin was not a specific marker for epithelial tumors, such as AOT. Sudhakara, et al.[69] stated that vimentin could possibly play a role in the formation of the mineralized material because vimentin was present in the rosette cells surrounding the material.

Our results showed that vimentin and cytokeratin were co-expressed in the intermediate cells surrounding the rosettes/duct-like structures and double-layered spheres, in the anastomosing cords, and in some of the areas of rosettes/duct-like structures in AOT (Figure 18 D and E, Figure 19 D and E, Figure 22 D and E). There

are reports of cytokeratin and vimentin co-expression in several odontogenic tumors.[38, 50, 57, 69, 72] Some of the studies concerning co-expression of cytokeratin and vimentin have used antibodies to a single cytokeratin and made conclusions based on the expression of the that specific cytokeratins.[38, 50, 57, 69, 89] The pan cytokeratin antibody clone that was used in our study was AE1/AE3. The AE1/AE3 contains antibodies for cytokeratins (CK) 1, 2, 3, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17, 18, and 19.[103, 104] Therefore, we are unable to discuss any specific cytokeratin and can only speak about the staining results of the pan cytokeratin. During the process of normal human enamel organ development, Kasper, et al.[89] found transient co-expression of cytokeratin and vimentin in the outer enamel epithelium and stellate reticulum cells during the bell stage. While the reason for this co-expression was still unknown, Kasper, et al.[89] proposed four hypotheses as possible explainations for this phenomenon. The first hypothesis was that the co-expression occurred as a temporary phenomenon during odontogenesis, because there are several other tissues that showed transient co-expression of cytokeratin and vimentin during embryognesis.[89] The second hypothesis was that the co-expression of vimentin and cytokeratin indicated a proliferative state of the epithelial cells, because it had also been found in proliferative mesothelial cells,[105] proliferative endometrial glandular cells,[106, 107] and regenerating kidney-tubule epithelial cells.[89, 108] The vimentin and cytokeratin co-expression was detected in the stellate reticulum during a proliferative phase of the enamel organ. Kasper, et al.[89] cited a study by Ben-Ze'ev, et al.[109] in their third hypothesis. Ben-Ze'ev, et al.[109] reported that there was an increased expression of vimentin in epithelial cells when cell-to-cell contacts were lost. Kasper, et al.[89] proposed this as an explanation for the co-expression in the cells of the

stellate reticulum, which were spindled in shape and loosely arranged. The final hypothesis offered by Kasper, et al.[89] was that the co-expression might be related to the secretory or resorptive functions of non-glandular epithelial cells around cavities containing low-protein body fluids, or in cells submerged in low-protein environment. This hypothesis was based on: 1) co-expression of vimentin and cytokeratin was seen in the cells of Wharton's jelly of the umbilical cord; 2) the stellate reticulum cells appeared to submerged in a proteoglycan-rich (low-protein) environment; and 3) a complex secretory process was seen in this stage of tooth development. These four hypotheses may not be mutually exclusive. Although Tatemoto, et al.[72] first reported that co-expression of vimentin and cytokeratin as a novel finding in 1988 and two additional studies found the same phenomenon since then, no new information has been added to the knowledge regarding this subject, and the reason for this co-expression in odontogenic cells remains unknown.

The products of odontogenic tumors were presumed to be aberrant secretory products of odontogenic cells, i.e., enamel or dentin. Based on our Sirius red stain result, there were at least two products present in the mixed calcifications in AOT (Figure 23 F and Figure 24 C and E). The product that showed eosinophilic stain with H&E stained positively (red) with Sirius red, which indicated the presence of collagen. This type of product showed orange, yellow, and, in some areas, green birefringence. According to Junquiera, et al.,[96, 97] collagen type I displayed orange to yellow birefringence, while collagen type III showed green birefringence, demonstrating that this product contained collagen type I and III. The product that was basophilic with H&E stained yellow (counterstain) with Sirius red and did not show birefringence (Figure 23

G and Figure 24 D and F), which indicated that collagen types I and III were not present in the second product. These results suggest that both aberrant dentin and aberrant enamel were seen in AOT, which is additional evidence supporting a heterogeneous population of tumor cells, which includes both epithelial and mesenchymal odontogenic cells.

5. CONCLUSION

AOT cells show a heterogeneous population involving both epithelial and mesenchymal components. This heterogeneity is also reflected in the products of AOT.

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