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EVALUATION OF AMELOTIN EXPRESSION IN ODONTOGENIC TUMORS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Evaluation of Amelotin Expression in Odontogenic Tumors

Master of Science, 2009

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This research project focused on evaluating the spatial distribution of the ameloblast-specific protein Amelotin (AMTN) in clinical cases of odontogenic tumors (OT) and the human ameloblastoma cell line AM-1. Any possible correlation of its expression with specific features of the lesions was expected to provide valuable information regarding the biological function of the protein, which is presently unknown. Immunohistochemistry, RT-PCR, immunocytochemistry, mineralization assays and microarray analysis were performed.

Generally, a strong signal was detected in tooth-like structures found in OT, such as the ameloblast layer and calcifying areas mimicking the enamel. Ghost cells present in odontomas and calcifying odontogenic cysts revealed variable signal. *In vitro*, AMTN was immunolocalized to the cytoplasm of AM-1 cells. A slight inhibitory effect on mineralization and a down-regulation of the ameloblast-associated gene ODAM were observed.

These results indicate that AMTN is re-expressed in odontogenic tumors, and suggest a possible involvement of AMTN in biomineralization.

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Foreword

This thesis has been prepared using a paper style format. It is composed of two papers prepared for journal publication (Chapters 2 and 3) framed by introduction and literature review (Chapter 1), discussion (Chapter 4), conclusions (Chapter 5) and references. Appendices 1, 2, 3, 4 and 5 describe additional experiments that have not been incorporated into the two present manuscripts for publication.

Chapters 2 and 3 are two papers presented as originally submitted for publication except for formatting changes aimed to standardize the paper style format of this thesis.

Chapter 2, "Evaluation of Amelotin expression in odontogenic tumors" to be submitted for publication to Journal of Histochemistry and Cytochemistry in August 2009 with authorship by Stolf DP, Bradley G, and Ganss B.

Chapter 3, "Temporo-spatial expression profiling of murine amelotin and its effect on biomineralization" to be submitted for publication to Mechanisms of Development in November 2009 with authorship by Stolf DP, Ganss B.

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List of Acronyms and Abbreviations

MMP-20	enamelysin
KLK-4	kallikrein 4
EMSP1	enamel matrix serine protease 1
AMTN	amelotin
SIBLING	small integrin-binding ligand, N-linked glycoprotein
SCPP	secretory calcium-binding phosphoprotein
BL	basal lamina
HSPG	heparin sulfate proteoglycan or perlecan
AOT	adenomatoid odontogenic tumor
AF	ameloblastic fibroma
AFD	ameloblastic fibro-dentinoma
AFO	ameloblastic fibro-odontoma
COC	calcifying odontogenic cyst
AMEL	amelogenin gene
mRNA	messenger RNA
RT-PCR	reverse transcription polymerase chain reaction
Sheathlin	analogue of ameloblastin
RANKL	receptor activator of nuclear factor kappa B ligand
OPG	osteoprotegerin
CK	cytokeratins
PCNA	proliferating cell nuclear antigen
FGF	fibroblast growth factor
Wnt	wingless family of genes
ECM	extracellular matrix
RANK	receptor activator of nuclear factor kappa B
H&E	hematoxylin & eosin
Tcf	T cell factor
Lef 1	lymphoid enhancer factor
ODAM	odontogenic ameloblast associated proteins

1. Introduction and Literature Review

1.1. Odontogenesis & Enamel Formation

Tooth development is a complex process that occurs through an intricate chain of interactions between tissues originating from ectoderm and ectomesenchyme (Avery 1992; Maas and Bei 1997; Miletich and Sharpe 2003). In fact, epithelial-mesenchymal interactions are also essential for the formation of several other embryonic organs in the human body, such as hair follicles, mammary and salivary glands, lungs, palate and kidney (Arias 2001; Miletich and Sharpe 2003; Yen and Sharpe 2008).

The ectoderm-derived oral epithelium and the neural-crest derived ecto-mesenchyme combine and inductive signaling pathways mediated through specific genes and transcriptional regulators are responsible for initiating odontogenesis, forming a defined epithelial bud (Miletich and Sharpe 2004; Yen and Sharpe 2008). Progressively, the round epithelial bud gains a concave *forma*, and now enters the cap stage of development (Avery 1992; Eversole et al. 2004). In this period, three different structures can be recognized: the enamel organ, the dental papilla, and the cells surrounding these structures known as the dental follicle (Avery 1992; Thesleff and Sharpe 1997). The enamel organ is responsible for determining the shape of the crown, establishing the dentogingival junction, and the enamel formation of the developing tooth germ (Tsujigiwa et al. 2005). As odontogenesis advances, the cap-shaped structure enlarges and the bottom layer of the epithelium (inner

enamel epithelium) move away from the top layer (outer enamel epithelium). This stage is defined as the early bell stage (Eversole et al. 2004), and an initial differentiation is apparent. The cells from the inner enamel epithelium elongate and differentiate into pre-secretory ameloblasts which turn into the future enamel-forming cells. The same initial differentiation occurs within the peripheral cells of the dental papilla, and they are now called pre-secretory odontoblasts and will function in dentin formation (Avery 1992; Stolf et al. 2007). This event takes place at the interface of the epithelium and mesenchyme and is regulated by interactions between the two tissues (Ruch et al. 1995; Thesleff and Aberg 1997; Thesleff and Sharpe 1997). During the early bell stage, matrix deposition begins. As soon as the now differentiated odontoblasts secrete a predentin matrix, small amounts of enamel proteins are released by differentiating ameloblasts and are thought to act as morphogens. Dentin starts to mineralize and the secretory ameloblasts produce the first layers of aprismatic enamel. Subsequently, cytoplasmic extensions called Tome's processes are formed and allow the cells to organize crystals into the rod and interrod pattern (Miletich and Sharpe 2004; Hu et al. 2007). Enamel proteins are then secreted around hydroxyapatite crystals, forming a mineralization front. Crystals grow in length throughout the secretory stage, and as a result, the whole enamel thickness is defined at the end of this phase (Hu et al. 2007).

The enamel matrix is composed primarily of amelogenins – low molecular weight proteins (Avery 1992). They account for more than 90% of total protein content. Amelogenesis also involves other proteins to a lesser extent, the so-called nonamelogenins (i.e. ameloblastin and enamelin), comprising

approximately 5% and 2% of total protein, respectively. Despite this difference, all three are proline rich and both amelogenin and ameloblastin undergo alternative splicing (Hu et al. 1997; Simmons et al. 1998). The various amelogenin isoforms detected explain its heterogeneity. Moreover, there is an increasing evidence that, additionally to structural roles in enamel development, amelogenins may also act in cell signaling, particularly by up-regulating the β-catenin pathway (Simmer and Hu 2002; Veis 2003; Margolis et al. 2006; Matsuzawa et al. 2009). Experiments involving ameloblastin mutant mice resulted in absence of enamel formation and cell detachment, as well as inhibition of cell proliferation and polarity (Fukumoto et al. 2004; Wazen et al. 2009), whereas the overexpression of this protein in ameloblasts recapitulated amelogenesis imperfecta signs (Paine et al. 2003). Thus, it is evident that ameloblastin plays a pivotal role in maintaining ameloblast differentiation by assuring its attachment to the enamel surface, as well as hold a structural function in crystal formation and mineral accretion (Fukumoto et al. 2004; Margolis et al. 2006). Furthermore, enamelin is normally found associated with growing enamel crystallite in the matrix. Knockout studies have revealed lack of enamel formation, since the mineralization front is absent, confirming a role in matrix organization (Margolis et al. 2006; Hu et al. 2008).

During and after the secretory stage, two specific proteinases are responsible for processing and degrading the previously secreted enamel proteins: the matrix metalloproteinase MMP-20 (Enamelysin), and the serine peptidase Kallikrein 4 (KLK4), formerly designated as enamel matrix serine protease 1 (EMSP1) (Simmer and Hu 2002). Both enzymes function in

processing and degrading the matrix proteins deposited during the secretory phase, although the former is normally produced earlier and it is thought to have a special affinity for amelogenins, while the latter is calcium independent and seems to act as a bulk digestive enzyme (Smith 1998; Sire et al. 2007; Nanci 2008). Knockout MMP-20 mice showing hypoplastic enamel with profoundly affected prism structure suggested that this proteinase is essential for catalyzing crystal augmentation. Alternatively, KLK-4 expression is initiated in the transition stage and continues during maturation. It is believed to account for degradation of enamel proteins in order to allow mineralization (Simmer and Hu 2002). It is during the maturation stage that crystal growth in thickness takes place, giving enamel its characteristic 96% mineral content (Nanci 2008).

Since the complex process of amelogenesis is still not completely understood, efforts in identifying components differentially expressed in various dental tissues have recently led to the discovery of new proteins. Amelotin (AMTN) is an ameloblast-specific gene expressed during the maturation stage of enamel development (Iwasaki et al. 2005; Moffatt et al. 2006; Nanci 2008). It has been predicted to be a glycoprotein and possess few similarities with other enamel proteins such as amelogenin, ameloblastin and enamelin, namely multiple potential serine and threonine phosphorylation sites (Iwasaki et al. 2005; Cheon et al. 2007). Additionally, the chromosomal locus of AMTN, identified for murine, rat and human, is in close proximity to other tooth- and bone-linked genes, such as the above mentioned and the SIBLING family. Thus, AMTN has been considered a member of the secretory calcium-binding phosphoproteins (SCPP) gene cluster (Iwasaki et al. 2005; Moffat et al. 2006).

In spite of these predicted similarities, the expression pattern of AMTN is distinct from other enamel proteins as demonstrated by in situ hybridization and immunohistochemical studies in mouse and rat molars and incisors (Iwasaki et al. 2005; Moffat et al. 2006). Post-translational modifications by O-glycosylation have been identified, and there is a size difference of 9kDa between the native and the bacterially expressed recombinant murine proteins (Cheon et al. 2007). It has also been shown that predominantly maturation-stage ameloblasts express this secreted protein, and its accumulation is mainly seen in the basal lamina-like structure formed between these cells and the enamel matrix during this phase (Iwasaki et al. 2005; Moffat et al. 2006).

The basal lamina (BL) lining the ameloblast layer in the tooth has unique features and roles. This structure is observed during amelogenesis, but has distinct properties during pre-secretory and maturation stages. Its composition also changes; when present around the inner enamel epithelium, it contains laminin, HSPG (heparin sulfate proteoglycan or perlecan), type IV collagen and fibronectin (Sawada and Inoue 2001). This BL is thought to orient collagen fibrils at the predentin surface and also restrain mesenchymal cells so they can differentiate into odontoblasts; moreover, it induces ameloblast differentiation by transferring growth factors and other signaling molecules and may also help to form the interdigitations observed at the enamel-dentin junction (Sawada and Inoue 2001).

During the maturation stage, however, the BL shows a distinct pattern and composition; it is wider and composed of an extra, irregular layer that corresponds to the lamina fibroreticularis observed in a typical BL structure.

However, this layer is often seen in continuity with the lamina densa. Unusual high expression of HSPG was found by immunohistochemistry, immunofluorescence and ultrastructure methods, appearing as double tracks (Sawada and Inoue 2001; Al-Kawas and Warshawsky 2008). Fine filaments showing moderate staining for type IV collagen were also revealed, but the presence of laminin in this structure seems controversial, since both variable and absence of signal were reported (Sawada et al. 1992; Nanci et al. 1993; Al-Kawas and Warshawsky 2008). Studies in undemineralized tissues have shown that the BL protrudes into the enamel and is associated with short rod-like enamel crystals at the early stages of maturation; this association becomes even deeper at later maturation stages. Proposed roles for this unusual structural composition include the assurance of a strong ameloblast-enamel binding, hence its unusual thickness and calcification (Sawada and Inoue 2000; Sawada and Inoue 2001). Additionally, this special arrangement provides a smooth transition between the cells, BL and the enamel, which is critical since in this phase the organic content and water are lost and several ion exchanges regulate the pH at the enamel surface (Sawada and Inoue 2000; Sawada and Inoue 2001).

Thus, the high expression of AMTN found in the BL prompted the possible participation of this protein in cell-to-enamel adhesion. Although preliminary studies in our lab using different cell types in adhesion assays to recombinant AMTN do not support this hypothesis (Cheon et al. 2007), further studies should clarify if native AMTN may mediate cell adhesion. Additionally, considering its unique expression pattern, a role in biomineralization, regulating mineral crystal

growth in thickness may be suggested. Nevertheless, the biological roles of AMTN remain speculative at this time, and ongoing studies in transgenic and AMTN-deficient mice should unravel details regarding this matter.

1.2. Odontogenic Tumors

Odontogenic Tumors are rare, generally benign neoplasms that are believed to be derived from remnants of the normal constituents of the tooth germ. Thus, many of the morphological characteristics and inductive interactions that occur in normal development may be seen, to a greater or lesser extent, in the tumors of the odontogenic apparatus (Kramer et al. 1992).

The most widely used classification of these tumors has been structured by the World Health Organization, and is based on the types of tissue involved in the lesion (Kramer et al. 1992; Barnes et al. 2005). According to the newest version of this classification, lesions containing odontogenic epithelium with mature stroma without odontogenic ectomesenchyme include ameloblastomas - divided into four subtypes -, adenomatoid odontogenic tumors (AOT), and others that are not relevant to this thesis. AOT were formerly categorized into the class of tumors containing odontogenic epithelium and odontogenic ectomesenchyme with or without dental hard tissue formation; however, since the dysplastic dentin production eventually observed is not considered true dentin, but rather metaplastically produced dentinoid, there is no evidence that the stroma is actually constituted by odontogenic ectomesenchyme (Philipsen and Reichart 2002). Thus, the mixed origin category comprises the ameloblastic

fibroma (AF) and fibro-dentinoma tumors (AFD), also divided into two different classes – neoplastic and non-neoplastic; the ameloblastic fibro-odontoma (AFO), complex and compound odontomas and calcifying odontogenic cysts (COC) (Barnes et al. 2005).

1.2.1. Ameloblastoma

Ameloblastoma is the most frequent odontogenic tumor arising from dental epithelium, and is characterized by its histological resemblance to the enamel organ of the developing tooth germ, yet enamel formation or any other identifiable extracellular matrix is not observed (Kramer et al. 1992; Snead et al. 1992; Nagatsuka et al. 2005). These lesions are rare in children and the most prevalent period occurs in the age range of 20 to 50 years, with a similar male/female ratio (Mendenhall et al. 2007). Studies have revealed an incidence ranging from 0.6 to 5.6 new cases per 1 million people per year in Sweden, South Africa and Nigeria (Reichart et al. 1995). They are unique to the jaws and if left untreated, often lead to extensive tissue destruction and deformity. The main area of incidence is the mandible, and over two-thirds occur in the molar-ramus region (Greenberg and Glick 2003; Nagatsuka et al. 2005). The tumor is often asymptomatic due to its slow-growth pattern. Occasionally however, swelling, loose teeth, malocclusion, paresthesia and pain may be observed (Becelli et al. 2002; Mendenhall et al. 2007).

Microscopically, all ameloblastomas show a fibrous stroma, with islands or strands of proliferating epithelium cells arranged in a palisade manner

(Greenberg and Glick 2003; Nagatsuka et al. 2005). There is considerable variation in histological patterns, and classification within this context comprises follicular, plexiform, acanthomatous, granular cell, basal cell and desmoplastic types. Of these, the follicular and plexiform types are the most common (Kumamoto et al. 2005). These histologic variants show no correspondence with either the clinical appearance of the tumor or its behavior, and different sections of the same lesion may show one or the other histologic type (Greenberg and Glick 2003). Although defined as a benign neoplasm, ameloblastomas are locally destructive and a high rate of recurrence is observed if the lesions are not entirely excised. A few cases of distant metastasis have been reported in the literature (Eversole et al. 2004; Huang et al. 2007; Sahoo et al. 2007).

The causative mechanisms that may lead to increased proliferation in these tumors remain unknown. Apparently, tumoral cells do not reach their fully differentiated state and that may be the reason for the absence of matrix formation (Tsujigiwa et al. 2005). Some authors have suggested that the signals that are normally secreted during odontogenesis are altered in tumorigenesis (Snead et al. 1992). Errors that lead to an aberrant proliferation in the early bud stage of tooth development are also taken as a possible etiology, although clinical signs may become apparent only later in life (Sarnat 2006).

The expression of enamel proteins normally secreted by ameloblasts has been analyzed in an attempt to elucidate how these signals may be modified in neoplasms. Indeed, the amelogenin (AMEL) gene was reported to be expressed by ameloblastoma epithelial cells by messenger RNA phenotyping

by RT-PCR in combination with Northern Blot and in situ hybridization analysis of mRNA (Snead et al. 1992). Increased AMEL expression levels, predominantly from the Y-chromosomal locus, were detected in male ameloblastoma samples. Notably, in normal human development the AMEL expression levels are much higher from the X-chromosomal locus. The significance of this alteration remains obscure. The expression of AMEL mRNA was also confirmed in an independent study (Tsujigiwa et al. 2005). However, immunolocalization of the amelogenin protein by immunohistochemistry techniques showed negative results in several investigations (Saku et al. 1992; Tsujigiwa et al. 2005; Mendenhall et al. 2007). Thus, it is currently believed that mutations in the expressed mRNA may interfere with the normal translational process (Tsujigiwa et al. 2005).

Several types of mutations were also found to occur in the ameloblastin gene in ameloblastomas. It is thought that these mutations may cause considerable alterations in protein structure or aberrant splicing of the mRNA, resulting in aberrant signaling in the target tissue, thus impairing the differentiation process of neoplastic cells (Perdigao et al. 2004). It is also interesting to notice that ameloblastin mutant mice have developed tumors of epithelial origin, confirmed by expression of enamel proteins such as amelogenin, enamelin and tuftelin in the ameloblast-like cells identified in the lesions (Fukumoto et al. 2004). Although these findings indicate that the lack of ameloblastin might have been the causative factor, further investigations should corroborate this theory. On the other hand, immunohistochemical studies failed to detect the ameloblastin protein in human ameloblastoma tissue. In the same

line of studies, sheatlin, an analogue of ameloblastin, and enamelin, are also known not to be expressed by neoplastic cells (Saku et al. 1992; Takata et al. 2000a).

Very few studies have addressed the expression of tooth-related proteinases in odontogenic tumors. MMP-20 was not detected by immunohistochemical investigations in ameloblastomas (Takata et al. 2000b). Although mutations in KLK4 have been demonstrated to be associated with the autossomal recessive hypomaturation type of Amelogenesis Imperfecta, attempts to elucidate any role in odontogenic tumors are scarce (Santos and Line 2005).

Extracellular matrix proteins may also play a role in tumorigenesis. It has been demonstrated that proteins such as HSPG are expressed in ameloblastoma samples (Ida-Yonemochi et al. 2002a). Although its function in pathological processes is poorly understood, studies have suggested that HSPG may stimulate the proliferation of stromal cells in neoplastic and inflammatory tissues (Cheng et al. 1995; Ida-Yonemochi et al. 1998). The localization of HSPG molecules in the stellate reticulum-like structures of ameloblastomas in both protein and mRNA levels suggests that they are, at least in part, responsible for the typical loosely arranged phenotype characteristic of stellate reticulum cells. The authors have also suggested that this protein may act as a carrier of nutrients for the neoplastic cells (Ida-Yonemochi et al. 2002a).

Other important constituents of the basement membrane, collagen IV and laminin V appear to be disregulated in ameloblastomas (Salo et al. 1999;

Nakano et al. 2002). The α4 chain of the collagen IV variant was found to be irregularly distributed and expressed in a much lesser extent compared to the other genetically distinct forms (Nakano et al. 2002). Laminin V molecules also stained in a variable pattern in tumor cells of peripheral areas with a weak signal appearing in basement membrane structures around the neoplastic cells (Salo et al. 1999). Both collagen IV and laminin-V, however, were found in invading cell islands and are thought to play a role in cytodifferentiation and tumor progression (Salo et al. 1999; Nakano et al. 2002).

The cause of ameloblastoma local invasiveness remains unknown. It is believed that this process involves the rupture of the basement membrane and the surrounding extracellular matrix with subsequent growth and proliferation of tumor cells. The invasive ability of this lesion is also thought to be related to the release of biologically active molecules produced, such as matrix metalloproteinases, which in turn trigger mitogens to be released randomly, contributing to the cellular proliferation of ameloblastoma cells (Pinheiro et al. 2004; Nagatsuka et al. 2005). Further studies are needed to unravel the mechanisms of oncogenesis, cytodifferentiation, and tumor progression (Kumamoto et al. 2005; Miyake et al. 2006; Stolf et al. 2007).

1.2.2. Adenomatoid Odontogenic Tumor

Adenomatoid odontogenic tumors (AOT) received this name in 1969, when it was proposed that this lesion, formerly considered a variant of

ameloblastoma, presents distinct characteristics and behavior (Philipsen and Birn 1969; Batra and Prasad 2005).

Three types of AOT may be distinguished: follicular, extrafollicular and peripheral. The first is by far the most common variant (73% of cases), characterized by a central lesion related to an impacted tooth, and for that reason it is often confused with a dentigerous cyst (Batra and Prasad 2005). When the intraosseous tumor is not embedded in a tooth it is called extrafollicular, accounting for 24% of cases. Both variants can be seen radiographically as a radiolucency containing radiopaque structures. Since the extrafollicular type is usually located between, above or superimposed to the roots of an erupted tooth, the differential diagnosis may include residual, radicular and lateral periodontal cysts. The relatively rare peripheral variety is often observed as a labial or lingual gingival swelling (Batra and Prasad 2005; Nigam et al. 2005).

This tumor is more frequent in females, with a female to male ratio close to 2:1 (Batra and Prasad 2005). In Asian populations, however, this ratio is even higher (3:1) (Mendis and MacDonald 1990; Toida et al. 1990). More than two thirds of cases are diagnosed in the second decade of life. The anterior maxilla is the most affected area, especially the canine region. Duct-like structures are characteristic, and calcification is sometimes seen and may be extensive. Eosinophilic amorphous material (the so-called tumor droplets), often described as dysplastic enamel, can be found between nodules of epithelial cells that form nests or rosette-like structures (Kramer et al. 1992; Philipsen and Reichart 1998).

Immunohistochemical investigations have suggested the presence of keratin and vimentin in small tumor cells peripheral to ductal, tubular or whorled components (Tatemoto et al. 1988). Vimentin positive cells around calcified bodies and eosinophilic material infer secretory functions (Crivelini et al. 2003). The strong CK14 immunoreaction found in all tumor cells of AOT, including the duct-like structures, suggested that this lesion may arise from the reduced enamel epithelium and that those cells correspond to ameloblasts in the protective stage of odontogenesis (Crivelini et al. 2003). This is contrasted by a broad investigation of enamel proteins and ECM molecules in AOT (Murata et al. 2000). Enzymatic pretreatment and a fixation procedure were utilized and the authors were able to show a dynamic change in the expression of both amelogenin and enamelin. They predict that the former protein is produced first by the cells forming solid nests, with some synthesized molecules reaching the extracellular space and forming hyaline droplets. These deposits are, however, soon degraded by proteinases in the pseudocystic space. Since enamelin was found in the pseudocystic space regardless of protein size, it is suggested that they are produced later and last longer. Moreover, amelogenins would be more susceptible to proteinases than enamelins (Murata et al. 2000).

Expression of amelogenin, enamelin and sheathlin has also been described separately in AOT. The first two proteins were found mainly in small mineralized foci, highly cellular regions forming epithelial nests and hyaline droplets (Saku et al. 1992; Abiko et al. 2001). Sheathlin, on the other hand, was immunolocalized to the eosinophilic substance found in tumor cell nests, thought to be either basement-membrane like material (Courtney and Kerr

1975) or abortive enamel matrix (Mori et al. 1991; Saku et al. 1992), and also in the cytoplasm of surrounding cells (Takata et al. 2000a). MMP-20 was also found to be expressed in the extracellular deposits of AOT. Small hyaline droplets and the majority of calcified areas showed strong immunoreaction for this proteinase (Takata et al. 2000b).

Basement membrane associated molecules were immunolocalized to the luminal space of pseudocystic structures of AOT and showed several similarities to what is found in the structure separating pre-ameloblasts from odontoblasts or pre-dentin in the normal tooth germ (Murata et al. 2000). Type IV collagen, laminin, HSPG, fibronectin and type V collagen co-localize in the basement membrane space and indicate that tumor cells behave like ameloblasts in spite of the obvious differences in their environment. Also, collagens type III and IV located in the stroma corroborate the odontogenic origin of the tumor (Murata et al. 2000).

According to the immunoreactivity observed for RANKL and OPG, the osteolytic potential of AOT seems comparable to that of calcifying odontogenic cyst and unicystic ameloblastomas (Andrade et al. 2008). A greater content of OPG was found in the stromal components when RANKL levels were compared. Since the ligation of RANKL to RANK leads to the activation of osteoclasts and OPG inhibits this interaction, the lack of balance between these molecules has been associated with several diseases such as osteoporosis, rheumatoid arthritis and periodontal disease (Lerner 2004; Belibasakis et al. 2007; Andrade et al. 2008).

1.2.3. Ameloblastic Fibroma / Ameloblastic Fibro-odontoma

The Ameloblastic Fibroma (AF) is usually seen in young patients with an average age of 14 to 15 years, although it is not an uncommon finding over the age of 22. Additionally, a slightly higher predilection for males has been reported (Takeda 1999; Chen et al. 2005; Darling and Daley 2006; Chen et al. 2007), and the majority of cases occur in the posterior area of the mandible (Chen et al. 2007). In many circumstances there is no complaint of the patient in regards to symptoms, albeit hard swelling of the jaw may be present. In some cases, ulceration, pain and drainage were also described (Takeda 1999; Chen et al. 2007). The radiographic characteristics of this tumor are somewhat similar to ameloblastomas, as they present a uni- or multi-locular radiolucency with a sclerotic radiopaque border (Takeda 1999; Chen et al. 2007).

The AF is thought to be a true biphasic tumor since both epithelial and mesenchymal components are neoplastic (Darling and Daley 2006). Microscopically, strands and islands of epithelial cells enclose a small number of cells that resemble the stellate reticulum; the connective tissue is poor in collagen and very cellular, with rounded or angular cells that resemble the dental papilla (Kramer et al. 1992; Chen et al. 2005; Chen et al. 2007). Cell-free areas and juxtaepithelial hyalinization are occasionally detected, but usually no matrix or hard tooth structures are seen (Takeda 1999; Chen et al. 2005; Darling and Daley 2006).

The neoplastic nature of this lesion is confirmed by reports that describe recurrence following surgery and malignant transformation (Kramer et al. 1992;

Chen et al. 2005; Chen et al. 2007). A recent broad review of studies published since 1891 showed that, from the 123 cases described, 33.3% recurred and 11.4% demonstrated malignant transformation with characteristics of Fibrosarcoma (Chen et al. 2007). In those cases, an increase in cellularity of the mesenchymal component is observed, with a concomitant disappearance of the odontogenic epithelium (Takeda 1999; Kobayashi et al. 2005; Chen et al. 2007).

Immunohistochemical studies have demonstrated useful markers that may be employed to detect the malignancy of these tumors. Molecules such as Ki-67 nuclear antigen, p53 protein and proliferative cell nuclear antigen (PCNA), related to cell proliferation, have been detected in fibrosarcomas, but not in AF (Huguet et al. 2001; Kobayashi et al. 2005).

Additionally, bone resorption regulators such as receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG), involved in osteoclast formation, differentiation and activity, were detected mostly in mesenchymal cells of the AF. The authors have suggested that this pathway may be related to the high recurrence and bone resorption potential of the tumor (Andrade et al. 2008).

Uniform distribution of all α-collagen IV chains has also been observed in the basement membrane that separates the epithelial cells from the stroma of AF (Nagatsuka et al. 2002). Collagen type I and procollagen type III were detected at a lower level (Takeda 1999). It has been suggested that the presence of these molecules is related to a protective effect, since the basement membrane is found intact, and thus relating this fact to the benign characteristics of the tumor (Nagatsuka et al. 2002). Cytokeratins (CK) 7, 13

and 14 have been reported to be expressed in AF, exhibiting a similar pattern detected in normal dental lamina, and may regulate the differentiation status of AF cells (Crivelini et al. 2003).

When strong inductive changes are present and lead to the formation of hard tissues, such as dentin and enamel, the lesion is classified as Ameloblastic Fibro-odontoma (AFO). In this case, the odontogenic epithelium is strongly associated with a cell-rich mesenchyme (Kramer et al. 1992; Chen et al. 2005). The lesion is frequently asymptomatic, although swelling and pain may be present. Sometimes, delayed tooth eruption or dislodged teeth are also observed. A slow-growing pattern is characteristic and the neoplasm is well-encapsulated (Miyauchi et al. 1996; Flaitz and Hicks 2001; Yagishita et al. 2001; Oghli et al. 2007).

AFO are relatively rare tumors, constituting 1% among oral biopsies and presenting a reported incidence of 1% to 3% within all odontogenic tumors (Chang et al. 2002). The mean patient age varies from 8.1 years (Slootweg 1981) to 11.5 years (Hooker 1967), and it is a common agreement between authors that patients within the first two decades of life are mostly affected (Kramer et al. 1992; Miyauchi et al. 1996; Chang et al. 2002). A slightly higher predilection for males and the posterior region of the mandible has been reported. When the tumor occurs in the maxilla, the anterior area is favored (Slootweg 1980; Kramer et al. 1992; Flaitz and Hicks 2001).

Radiographically, AFO may have a uni- or multilocular radiolucent appearance with well circumscribed margins and various amounts of radiopaque material of irregular size and form presenting the same density as

tooth structures (Flaitz and Hicks 2001; Yagishita et al. 2001; Chang et al. 2002; Chen et al. 2005). There is little potential for recurrence, although a few cases have been described, but malignant transformation is rare (Friedrich et al. 2001; Chang et al. 2002; Chen et al. 2005). One report depicted a case of AFO that progressed into an odontogenic sarcoma with local metastasis after four recurrences with histological changes (Herzog et al. 1991). Two other cases showing malignant transformation to ameloblastic fibrosarcoma were described (Howell and Burkes1977).

Immunohistochemical investigations have detected a cytoplasmic expression of the amelogenin protein in cuboidal epithelial cells of tooth bud-like projections present in a case of AFO. The stellate reticulum-like cells were also positively stained (Yagishita et al. 2001). On the other hand, sheathlin was negative in both epithelial components forming the characteristic strands and islands and the cellular mesenchyme. However, regions showing immature enamel evidenced a positive signal, as well as the neighboring ameloblast-like cells (Takata et al. 2000a). The same expression pattern was followed by the MMP-20 proteinase, which presented an intense staining only in areas that mimicked the dental enamel. Regions rich in dentinoid materials were negative (Takata et al. 2000b).

Immunolocalization of CK19 in epithelial cells confirms their odontogenic nature (Miyauchi et al. 1996). Distribution of CK14, CK13, 16, and CK18 was observed in the epithelial component even in areas where no epithelial-mesenchymal inductions were present. Allied with the fact that vimentin was co-expressed in peripheral cells of epithelial nests, the authors concluded that the

AFO analyzed presented a differentiation state correspondent to the bell stage of tooth development (Miyauchi et al. 1996).

Other studies that investigated the kinetics of AFO by bromehexidine and PCNA expression have suggested that the ectomesenchymal component is more active than the epithelial apparatus (Sekine et al. 1996; Hegde and Hemavathy 2008).

Moreover, fibroblast growth factors (FGF) are known to act as growth inducers in several tissues from mesenchymal and neuroectodermal origin, taking part in differentiation, proliferation and morphogenesis (Cam et al. 1992). They are particularly important in odontogenesis, actively participating in several stages of tooth development. FGF-2 has been widely shown in stellate reticulum cells and dental mesenchyme. In AFO epithelium, FGF-2 was found in the cytoplasm but was also evidenced by intense nuclear staining in some areas. Since the results are similar to what is found in normal dental physiology, they imply that this molecule is involved in histodifferentiation rather than contributing to any pathological alteration in odontogenic tumor configuration (So et al. 2001).

1.2.4. Odontoma

Odontomas are the most common malformations from mixed odontogenic origin. They resemble tooth structures in a disorganized pattern and are often diagnosed in the first two decades of life (Kaugars et al. 1989; Kramer et al. 1992; Litonjua et al. 2004). Enamel, dentin and pulp are present, but disorderly

arranged. Due to this fact, Odontomas are considered to be hamartomas rather than true neoplasms, although their etiology remains unclear (Kramer et al. 1992; Litonjua et al. 2004).

Histologically, two types of odontomas can be distinguished: complex or compound. The former group comprises lesions containing amorphous hard tissue elements disorderly arranged, where the mandibular premolar or molar areas are mostly affected (Slootweg 1981; Mupparapu et al. 2004; Hidalgo-Sánchez et al. 2008). Conversely, compound odontomas present normal dental tissues altered in size and conformation, appearing as numerous small tooth-like radiopaque structures typically detected in the upper anterior maxilla. They occur more frequently than their counterparts (Gyulai-Gaál et al. 2007; Hidalgo-Sánchez et al. 2008). Odontomas are often casually identified in routine examinations, showing no important signs or symptoms. Sometimes, delayed tooth eruption, persistence of temporary teeth or tooth eruption disturbances are associated (Kaugars et al. 1989; Flaitz and Hicks 2001; Gyulai-Gaál et al. 2007; Hidalgo-Sánchez et al. 2008).

In general, there are a great number of cases diagnosed in the anterior portion of the maxilla. While not considered an odontogenic tumor, the supernumerary tooth is a dental anomaly that closely resembles an Odontoma and it also has a significant predilection for the upper jaw (49.6%) (Kaugars et al. 1989; Cuesta et al. 2003). Also taking into account the histological similarities between both abnormalities, these facts may indicate that they may share the same initiating factor. It is hypothesized that the supernumerary tooth

might represent a more differentiated form of an Odontoma (Kaugars et al. 1989).

Less common conditions have also been reported, such as the finding of an odontoma in the maxillary sinus (Mupparapu et al. 2004) or when the lesion erupts through the soft tissues into the oral cavity (Cuesta et al. 2003; Litonjua et al. 2004; Junquera et al. 2005). Associations with dentigerous cysts, odontogenic keratocysts and calcifying odontogenic cysts have also been described (Kaugars et al. 1989; Buchner 1991; Hirshberg et al. 1994). Surgical removal of the lesion and the surrounding soft tissues is the standard treatment for odontomas, since recurrence is extremely rare (Kaugars et al. 1989; Litonjua et al. 2004; Gyulai-Gaál et al. 2007).

It has already been demonstrated that a disorganized expression of extracellular matrix molecules in the dental mesenchyme leads to inappropriate differentiation of dental tissues (Ida-Yonemochi et al. 1999; Ida-Yonemochi et al. 2002b). As expected, amelogenin and sheathlin have been detected in the enamel matrix observed in odontomas, as well as small foci of mineralized products usually encountered near epithelial cell nests. On the other hand, other structures such as dentin, cementum and pulp presented a negative signal (Takata et al. 2000a; Abiko et al. 2001). These findings suggest that the enamel present in this lesion is immature, since fully mineralized or mature enamel would normally lack such proteins (Inage et al. 1989). Conversely, positive staining for MMP-20 was also identified in both enamel matrix and ameloblast-like cells (Takata et al. 2000b; Väänänen et al. 2004).

Immunolocalization of collagen XVIII was observed in tumoral basement membrane structures and the enamel matrix, in a similar pattern to the normal tooth. However, no positive signal was detected in the ameloblast-like cells (Väänänen et al. 2004). Only CK7 and CK14 were identified in odontomas. The expression in epithelial cells (except secretory ameloblasts) and stellate reticulum-like cells indicate a correlation with tooth germ epithelium origin (Crivelini et al. 2003). Secretory ameloblasts, however, do co-express FGF-1 and FGFR-3, advocating an autocrine and/or paracrine role of these proteins (So et al. 2001).

Although not constant, one characteristic feature of odontomas is the presence of ghost cells, defined as eosinophilic epithelial cells that usually show aberrant keratinization and represent metaplastic transformation (Sciubba 1985; Tanaka et al. 2007). The function of these peculiar cells is unknown, and there is no correlation with age, gender or location within the tumors (Tanaka et al. 2007). While immunolocalization of Wnt and related molecules that are normally involved in the formation of the tooth bud and amelogenesis has been demonstrated in these cells; the relevance of this association remains to be determined (Stolf et al. 2007; Tanaka et al. 2007).

There has been substantial debate whether the three lesions cited above, namely ameloblastic fibroma, ameloblastic fibro-odontoma and odontoma represent an evolution of the same tumor in different stages. Several authors have shown that the majority of residual or recurrent cases of AF do not exhibit further maturation with features of AFO (Chen et al. 2005; Chen et al. 2007). Conversely, it is not uncommon to find in the literature evidence of AFO growing

into complex odontomas. The comparison of age parameters between the lesions that behave as hamartomas, in some cases, provides additional data supporting this fact. Thus, it appears that some AF and AFO cases could develop further and are hamartomatous lesions in nature, while others may behave differently. Information solely based in histological findings may not be enough to distinguish between these two entities, and the age at the time of diagnosis should be considered a helpful indication (Chen et al. 2005; Chen et al. 2007).

1.2.5. Calcifying Odontogenic Cyst

Due to the diverse histopathological findings relative to the calcifying odontogenic cyst (COC), the lesion has received several designations over the years: mixed odontogenic tumor (Thoma and Goldman 1946), atypical adamantinoma (Maitland 1947), keratinizing and calcifying odontogenic cyst (Gold 1963), keratinizing ameloblastoma (Bhaskar 1965), calcifying ghost cell odontogenic tumor (Fejeskov and Krogh 1972), cystic calcifying odontogenic tumor (Freedman et al. 1975), cholesteastoma of the jaws (Altini and Farman 1975), ghost cell cyst (Farman et al. 1978), dentinogenic ghost cell odontogenic tumour (Praetorius et al. 1981), epithelial odontogenic ghost cell tumour (Ellis and Shmookler 1986) and dentino-ameloblastoma (Shear 1994). It was only in 1962 that Gorlin and colleagues classified the lesion as COC according to some unique features such as the presence of ghost cells, aberrant keratinization and calcifying potential (Gorlin et al. 1962). A close resemblance to the cutaneous

epithelioma of Malherbe was also reported (Mascrès et al. 1990; Hong et al. 1991).

COC may appear closely associated to other odontogenic tumors, such as odontoma (Gorlin et al. 1962; Freedman et al. 1975; Nagao et al. 1982; Keszler and Guglielmotti 1987; Hirshberg et al. 1994; Gallana-Alvarez et al. 2005), ameloblastic fibroma (Lin et al. 2004; Yoon et al. 2004) and ameloblastic fibro-odontoma (Praetorius 1975; Farman et al. 1978). Metastasis and malignant transformation have also been cited in the literature (Altini and Farman 1975; Praetorius et al. 1981; Ikemura et al. 1985; Grodjesk et al. 1987; Castle and Arendt 1999), and the frequency of recurrence is lower in young patients (Mascrès et al. 1990). A general trend constitutes sporadic recurrences detected over five years after the initial treatment, and the majority of case reports found in the literature has described it in elderly patients (Swinborn 1976; Slootweg and Koole 1980; Wright et al. 1984; Daniels 2004). One of the opposite and rare situations include the case of a 11 year-old female patient presenting the same lesion following two years of the initial surgical enucleation (Pullman and Seldin 1971).

Dental hard tissue formation such as enamel and dentin may exist in COC, and these cases are often associated with an odontoma (Kramer et al. 1992). There is similar incidence in the maxilla and the mandible, although ethnic and racial factors may influence this prevalence. Japanese natives have the maxilla more frequently affected than other populations (Saito et al. 1982; Tanimoto et al. 1988; Erasmus et al. 1998). The diagnosis is common in the second decade

of life and there is no obvious gender preference (Sciubba 1985; Mascrès et al. 1990; Hirshberg et al. 1994; Regezi 2002).

Praetorius and colleagues suggested in 1981 a sub-classification of COC into two different entities: cysts and neoplasms. Within the former category, unicystic subtypes comprised simple unicystic type, unicystic odontome-producing type, and cystic ameloblastomatous proliferating type. However, several multicystic cases were reported afterwards (Freedman et al. 1975; Sawyer and Mosadomi 1983; Buchner 1991), and due to the variety of histological patterns found within this lesion, a re-definition of this classification has been formulated by other authors (Buchner 1991; Hong et al. 1991).

The main feature of this cystic lesion is the presence of ghost cells, often located within the epithelial component, although they also occur in odontomas to a lesser extent (Keszler and Guglielmotti 1987; Moleri and Moreira 2002). Some authors have described these cells as derivatives from odontogenic epithelium that underwent metaplastic transformation, and appear as different stages of normal or aberrant keratin formation (Sedano and Pindborg 1975). Although they present different morphological characteristics from the enamel, it was also suggested that these cells may correspond to the result of unsuccessful enamel matrix production from odontogenic epithelium (David and Buchner 1976). It has also been proposed that this enamel-like matrix could be retained within the cells due to their inability to secrete it (Freedman et al. 1975). The ghost cells are characterized by the presence of coarse bundles of tonofilaments, loss of nuclei, and induction of foreign body granulomas. Additionally, they sometimes undergo or induce local dystrophic calcification

(Mascrès et al. 1990; Hong et al. 1991). Thus, another possible explanation for the appearance of these cells is related to the coagulative necrosis of the odontogenic epithelium (Hong et al. 1991).

Strong staining of ghost cells and mineralized material for amelogenin, enamelin and sheathlin have been reported in the literature (Mori et al. 1991; Saku et al. 1992; Takata et al. 2000a; Abiko et al. 2001). Three cases of COC associated with odontomas and presenting enamel matrix also expressed sheathlin intensely. The authors proposed that the abnormal accretion of such enamel proteins may be responsible for the appearance of ghost cells in this lesion (Takata et al. 2000a). As expected, MMP-20 was also found to be strongly expressed in COC. Variable staining in ghost cells, mainly in peripheral areas of the clusters was revealed, as well as in the immature enamel observed in some cases (Takata et al. 2000b). Ghost cells also retained some staining for FGF-1 and FGF-2, although these two factors are not considered relevant for neoplastic transformation or tumor growth (So et al. 2001).

Moreover, similar to AOT, COC presented a higher content of OPG than RANKL in stromal/mesenchymal components, suggesting a lesser reabsorptive activity in these tumors (Andrade et al. 2008).

2. Evaluation of Amelotin Expression in Odontogenic Tumors

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2.1. Abstract

The Amelotin (AMTN) protein is highly and selectively expressed by odontogenic epithelium-derived ameloblasts throughout the maturation stage of enamel formation. Its secreted form is concentrated at the basal lamina interface between the ameloblast cells and the enamel matrix. Odontogenic tumors arise from remnants of the tooth-forming apparatus and are characterized by morphological resemblance to the developing tooth germ. They vary in behavior from slowly expanding encapsulated tumors to locally aggressive and destructive lesions. The aim of this study was to investigate the spatial distribution of AMTN in clinical cases of odontogenic tumors and to correlate its expression with specific features of the lesions, thereby increasing our knowledge of the biology of AMTN. Immunohistochemical staining for AMTN was performed on paraffin sections of human ameloblastoma, ameloblastic fibroma (AF), ameloblastic fibro-odontoma (AFO), odontoma, adenomatoid odontogenic tumor (AOT) and calcifying odontogenic cyst (COC). Generally, ameloblastomas and AF were negative. A strong signal was detected in the ameloblast-like layers found in AFO and odontomas. The epithelial cells that constitute AOT did not stain for AMTN, while calcifying areas of extracellular eosinophilic matrix were intensely stained. Interestingly, ghost cells present in odontomas and COC revealed variable staining, again in

association with foci of calcification. These results suggest a possible involvement of AMTN in the mineralization events that occur at the late stages of enamel formation.

Key Words: Amelogenesis, Amelotin, Odontogenic Tumors

2.2. Introduction

Tooth development is a complex process that occurs through an intricate chain of interactions between tissues derived from ectoderm and ectomesenchyme (Avery 1992; Maas and Bei 1997; Miletich and Sharpe 2003). Different phases of this process can be identified, ranging from initiation and morphogenesis, where the enamel organ is formed, to differentiation of key cells - odontoblasts and ameloblasts and subsequent mineralization of the matrices deposited by them (Avery 1992; Thesleff and Sharpe 1997; Eversole et al. 2004; Tsujigiwa et al. 2005). As soon as differentiated odontoblasts secrete a predentin matrix, small amounts of enamel proteins are released by differentiating ameloblasts and are thought to act as morphogens. Dentin starts to mineralize and the secretory ameloblasts produce the first layers of aprismatic enamel. Subsequently, cytoplasmic extensions called Tome's processes are formed and allow the cells to organize crystals into the rod and interrod pattern (Miletich and Sharpe 2004; Hu et al. 2007). Enamel proteins are then secreted around hydroxyapatite crystals, forming a mineralization front. Crystals grow in length throughout the secretory stage, and as a result, the whole enamel thickness is defined at the end of this phase (Hu et al. 2007).

The enamel matrix is composed primarily of amelogenins – low molecular weight proteins (Avery 1992). They account for more than 90% of total protein content. Amelogenesis also involves other proteins to a lesser extent such as enamelin and ameloblastin. During and after the secretory stage, two families of proteinases are responsible for processing and degrading those proteins: the matrix metalloproteinase family (Enamelysin or MMP-20), and Kalikrein 4 (KLK4). Subsequently, crystal growth in thickness takes place, giving enamel its characteristic 96% mineral content (Nanci 2008).

Since the complex process of amelogenesis is still not completely understood, efforts in identifying components differentially expressed in various dental tissues have recently led to the discovery of new proteins. Amelotin (AMTN) is an ameloblast-specific gene expressed during the maturation stage of enamel development. High levels of the protein are observed exclusively at the transition from secretory to maturation stage ameloblasts, indicating a restricted expression pattern. Immunolocalization of this protein to the basal lamina interface between the ameloblast cells and the enamel matrix throughout the maturation phase was also reported. Nevertheless, its function in tooth development remains speculative at this time, and further studies are needed (Iwasaki et al. 2005; Moffatt et al. 2006; Nanci 2008).

Odontogenic tumors are rare, generally benign neoplasms thought to be derived from remnants of the tooth-forming apparatus. Thus, they resemble many morphological characteristics and inductive interactions that occur in normal tooth development (Kramer et al. 1992). The most used classification of these tumors has been structured by the World Health Organization, and is

based on the types of tissue involved in the lesion (Kramer et al. 1992; Barnes et al. 2005). According to the newest version of this classification, tumors consisting of odontogenic epithelium with mature stroma but without odontogenic ectomesenchyme include ameloblastoma and adenomatoid odontogenic tumor (AOT). Lesions such as ameloblastic fibroma (AF), ameloblastic fibro-odontoma (AFO), complex and compound odontomas and calcifying odontogenic cyst (COC) are classified as mixed tumors, i.e., originating from odontogenic epithelium and ectomesenchyme with or without dental hard tissue formation (Barnes et al. 2005).

Odontogenic tumors are usually slowly growing lesions and many are found during routine radiographic examinations. However, some present aggressive and invasive behavior and rarely, they can show malignant transformation and metastasis, as reported for cases of ameloblastoma, AF, and COC (Altini and Farman 1975; Praetorius et al. 1981; Ikemura et al. 1985; Grodjesk et al. 1987; Kramer et al. 1992; Castle and Arendt 1999; Eversole et al. 2004; Chen et al. 2005; Chen et al. 2007; Huang et al. 2007; Sahoo et al. 2007).

Immunohistochemical studies have detected enamel proteins such as amelogenin, enamelin and sheathlin in several types of odontogenic tumors, as well as the proteinase MMP-20 (Mori et al. 1991; Saku et al. 1992; Murata et al. 2000; Takata et al. 2000a; Takata et al. 2000b; Abiko et al. 2001; Yagishita et al. 2001). Since the tumors of the odontogenic apparatus closely mimic the tissue organization and also the inductive changes that occur in the developing tooth, the study of these proteins may provide relevant information not only for

morphological and classification purposes, but also for the discovery of diagnostic and prognostic markers and the pathological mechanisms that drive tumorigenesis. The aim of this investigation was to study the presence and spatial distribution of the AMTN protein in odontogenic tumors in order to gain insights into the function of AMTN as well as increase our understanding of odontogenic tumor differentiation.

2.3. Materials and Methods

Paraffin blocks of cases of odontogenic tumors were retrieved from the archives of the Departments of Oral Pathology, Faculty of Dentistry, University of Toronto, Canada and Federal University of Santa Catarina, Brazil. The samples comprised 15 cases of ameloblastomas, 1 AF, 2 AFO, 4 odontomas, 4 AOT and 3 COC (Table 2.1). Sections of two cases of a dental follicle containing the reduced enamel epithelium were used as a control. This study has been approved by institutional research ethics board (Appendix 6).

Preparation of Rabbit Anti-Human Polyclonal Antibodies against AMTN

Rabbit polyclonal antibodies against the peptide sequences of human AMTN (AMTN-1 (H2N-RLPTPSGTDDDFAVT-COOH) and AMTN-2 (H2N-PPTKLAPDQGTLPNQ-COOH), both coupled to keyhole limpet hemocyanine (KLH) for immunization) were developed. The antiserum was affinity purified over CNBr-activated sepharose 4B columns (GE HealthCare Bio-Sciences –

Piscataway, NJ) and eluted with a solution containing both 50mM Glycine-HCl and 0.5M NaCl (pH 2.3). Western Blots and peptide competition assays were performed to confirm the specificity of the signal.

Table 1. Number of cases and classification of Odontogenic Tumors analyzed in the study according to WHO*.

Neoplasm	Classification according to WHO	Number of samples analyzed
Ameloblastoma	Odontogenic epithelium and mature stroma without odontogenic ectomesenchyme	15
Adenomatoid Odontogenic Tumor		4
Ameloblastic Fibroma	Odontogenic epithelium with odontogenic ectomesenchyme, with or without dental hard tissue formation	1
Ameloblastic Fibro-odontoma		2
Odontoma		4
Calcifying Odontogenic Cyst		3

* Kramer et al. 1992; Barnes et al. 2005

Immunohistochemistry

5µm sections were cut from formalin-fixed, paraffin-embedded tissue samples and mounted on positively charged slides (Fisher Superfrost Plus, Fisher Scientific - Pittsburgh, PA). Sections were deparaffinized in xylene and rehydrated in a series of graded ethanol dilutions. After washes in distilled water and 0.1% Tween 20 Tris-Buffered Saline (TBS-T) (Sigma Aldrich – St. Louis, MO), the slides were incubated for 5 minutes at room temperature in peroxidase block solution to quench endogenous peroxidase. A standard immunohistochemistry procedure was performed utilizing a commercially

available kit (DakoCytomation, K4010, DAKO – Glostrup, Denmark). The sections were incubated for 30 minutes at room temperature with primary antibody to AMTN diluted 1:1000 in 0.05M Tris-HCl with 1% BSA (Albumin fraction V Powder A-3311, Sigma Aldrich – St. Louis, MO). After several washes with TBST-T, the secondary antibody (Peroxidase Labelled Polymer) was applied for 30 minutes at room temperature. Staining was developed using diaminobenzidine hydrochloride (DAB) for 4 minutes and subsequent counterstain with Methyl Green for 30 seconds, followed by dehydration, mounting and analysis at a light microscope. For the negative control, the primary antibody was replaced by pre-immune serum using the same dilution. Each tissue sample was tested with both anti-AMTN-1 and anti-AMTN-2 antibodies, revealing a similar pattern of immunohistochemical staining in terms of signal strength and localization.

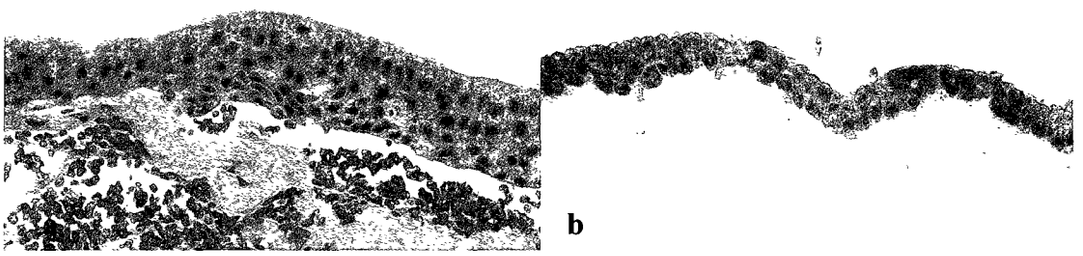
2.4. Results

The immunohistochemical analysis of dental follicle, which was used as a positive control, revealed a strong staining in the cytoplasm of the cells that constitute the reduced enamel epithelium. Sometimes, perinuclear and/or nuclear staining were also observed (Figure 2.1). These cells correspond to the ameloblasts in the final stages of enamel development, and this expression pattern is consistent with the normal profile of AMTN described in the literature (Iwasaki et al. 2005, Moffat et al. 2006).

Figures 2.2 and 2.3 show the immunolocalization of AMTN in all tumor samples tested. An H&E picture of each case was also included to facilitate visualization of the morphological aspects of the sections.

Figure 2.1. Immunohistochemistry results for control samples (dental follicle).

a. H&E staining; b. AMTN signal detected in the reduced enamel epithelium layer.



Regarding the tumor samples, ameloblastomas were generally negative for AMTN. The typical central mass of loosely connected cells resembling the stellate reticulum did not present any signal, except for a faint label in two cases when cystic changes were present (Figure 2.2c, arrows). Additionally, columnar cells that constitute the tumor islands were negative. The same pattern was followed by the AF case. This sample was negative for AMTN staining, with exception of a small patch of calcification seen in Figure 2.3a'. The two cases of AFO presented a specific and strong positive staining for the ameloblast-like cells and the adjacent enamel matrix. All other cellular content was negative (Figure 2.3b'). Odontoma cases also showed an intense staining in the ameloblast-like layer and patches of enamel matrix, although the signal was not uniform in the latter, being more prominent when calcification was apparent. Interestingly, several patches of ghost cells were also positive for AMTN, even

though variable signal strength could be detected. The staining was stronger when dystrophic calcification was present in those cells (Figure 2.3c', arrows). While the epithelial cells that constitute AOT showed negative staining, focal areas of extracellular eosinophilic matrix revealed a strong signal for AMTN (Figure 2.3d'). A great number of positively stained ghost cells were also found in the COC sections, with the labeling varying in strength (Figure 2.3e', arrows).

Figure 2.2. No AMTN signal was found in the ameloblastoma samples tested, except for a few cases of the follicular type when cystic changes were present (c. arrows). a. H&E staining; b. AMTN staining. Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).

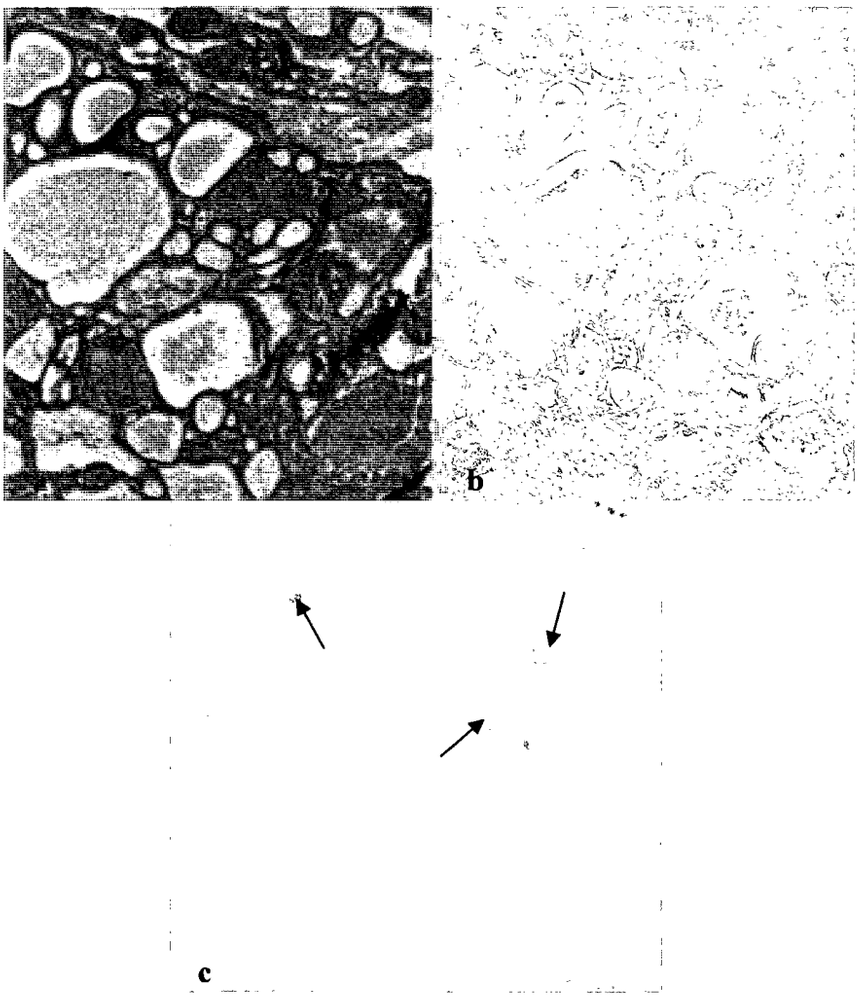
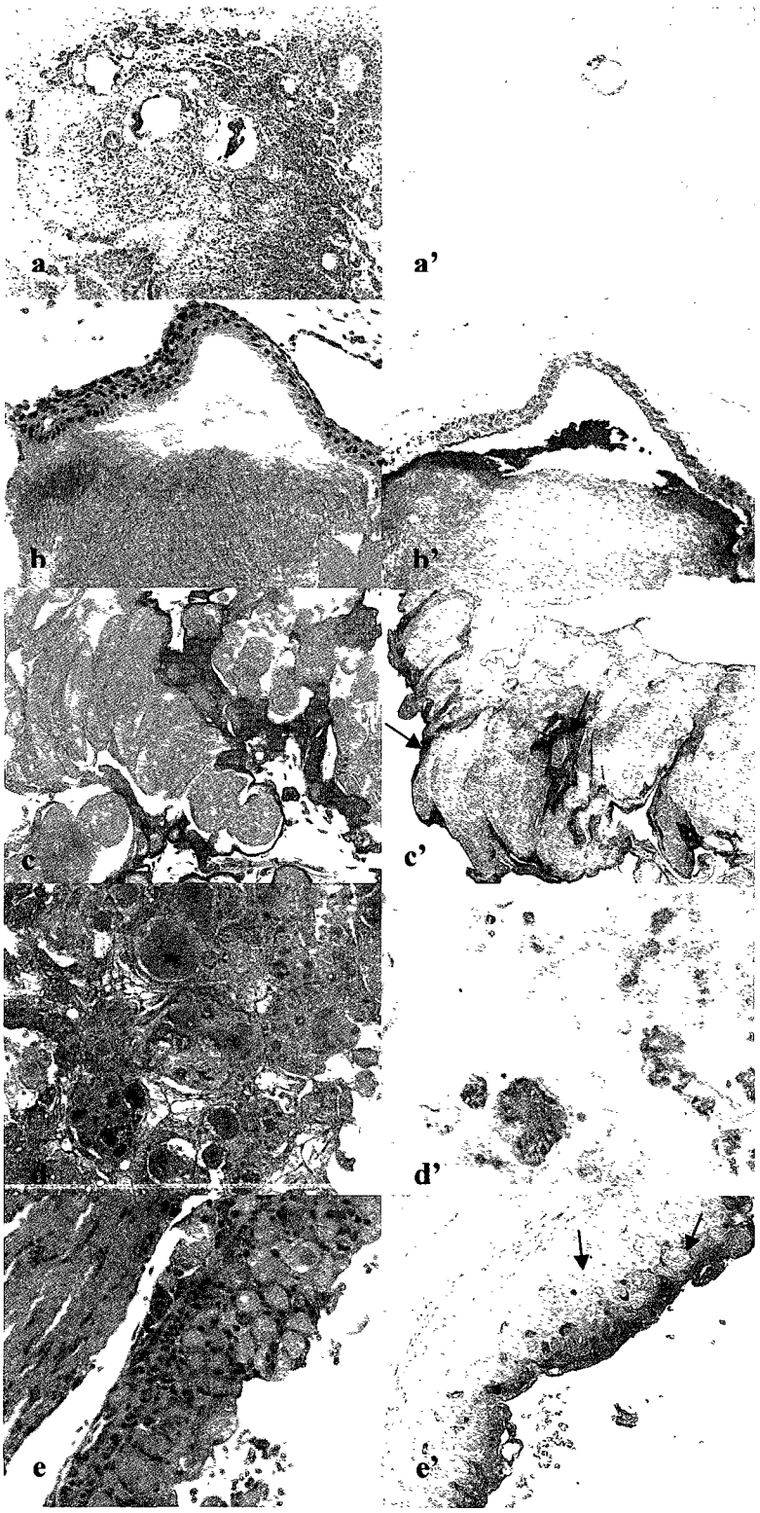


Figure 2.3. H&E staining (left panel) and immunohistochemistry results (right panel) for odontogenic tumors studied.

Ameloblastic Fibroma (a, a'), Ameloblastic Fibro-Odontoma (b, b'), Odontoma (c, c'), Adenomatoid Odontogenic Tumor (d, d'), Calcifying Odontogenic Cyst (e, e'). Arrows

correspond to ghost cells. Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).



2.5. Discussion

We have studied the spatial distribution of AMTN in odontogenic tumors by immunohistochemical staining. Generally, ameloblastomas did not demonstrate a strong evidence of AMTN staining. A faint signal could be observed in a few cases of the follicular type when cystic changes were present. The occurrence of dense granules resembling lysosomes was reported in the cells that resemble the stellate reticulum, and it has been suggested that they might be responsible for the formation of microcysts. Additionally, it has been controversial whether the intrafollicular cysts show a deficiency of absorption and diffusion of nutrients, or if the polarization of epithelial cells facing the 'stellate reticulum' would cause them to remove nutritive elements from the interior of the islands, in an attempt to resemble normal conditions (Campos 1990; Ueno and Matsuo 1991).

Although mRNA phenotyping in combination with Northern Blot and in situ hybridization studies have shown that ameloblastoma epithelial cells express the amelogenin gene, the localization of the protein by immunohistochemistry techniques failed to detect any signal (Saku et al. 1992; Snead et al. 1992; Tsujigiwa et al. 2005; Mendenhall et al. 2007). Thus, it is advocated that structural or functional changes in the expressed mRNA may obstruct the normal translational process (Tsujigiwa et al. 2005). Similar results were obtained for ameloblastin and sheathlin, since these proteins could not be immunolocalized in human ameloblastoma tissue samples (Saku et al. 1992; Takata et al. 2000a).

While ameloblastomas somehow resemble the enamel organ, no enamel formation is observed in the structural organization of the tumor. Apparently, tumoral cells do not reach their fully differentiated state and this may be the reason for the absence of matrix formation (Tsujigiwa et al. 2005). The data obtained in this study supports the evidence that lack of inductive changes prevent the maturation of the ameloblast-like cells, since they are not capable to produce the AMTN protein normally secreted at this stage.

AFO and odontoma are considered hamartomas or developmental malformations. They show evidence of inductive changes in the odontogenic epithelium and ectomesenchyme. Previous studies have demonstrated the expression of amelogenin, sheathlin and MMP-20 in the ameloblast-like cells of AFO, supporting the idea that these tumor cells have undergone ameloblastic differentiation (Takata et al. 2000a, Takata et al. 2000b, Yagishita et al. 2001). These proteins have also been identified in areas mimicking the dental enamel in both AFO and odontomas, suggesting that the matrix present in these lesions is immature since fully mineralized or mature enamel would normally lack such proteins (Takata et al. 2000a, Takata et al. 2000b, Abiko et al, 2001, Väänänen et al. 2004). We observed strong AMTN staining in ameloblast-like cells adjacent to the enamel matrix in these tumors, which is analogous to the expression of AMTN in the maturation stage of enamel formation in the developing tooth. This data indicate that the process of maturation has at least been initiated, and imply that some level of differentiation may be occurring in those cells.

Expression of amelogenin, enamelin and sheathlin has also been described in AOT. The first two proteins were found mainly in small mineralized foci, highly cellular regions forming epithelial nests and hyaline droplets (Saku et al. 1992; Murata et al. 2000; Abiko et al. 2001). Sheathlin, on the other hand, was immunolocalized to the eosinophilic substance found in tumor cell nests, thought to be either basement-membrane like material (Courtney and Kerr 1975) or abortive enamel matrix (Mori et al. 1991; Saku et al. 1992), and also in the cytoplasm of surrounding cells (Takata et al. 2000a). MMP-20 was also found to be expressed in the extracellular deposits of AOT. Small hyaline droplets and the majority of calcified areas showed strong immunoreaction for this proteinase (Takata et al. 2000b).

The staining pattern observed for AMTN in AOT was similar to those reported above. The eosinophilic matrix and small mineralized foci were labeled. However, a very faint staining could be observed in the cytoplasm of some epithelial cells neighboring those areas. The fact that these cells do not present a strong reaction for AMTN, although the protein is localized to the mineralized structures around them, may indicate that the cells actually have reached the end of maturation stage. The time of AMTN expression in their inner secretory machinery may have elapsed and they may subsist in the reduced enamel epithelium phase. Earlier studies suggested that AOT may arise from the reduced enamel epithelium and that the tumor cells correspond to ameloblasts in the protective stage of odontogenesis. This suggestion was supported by the finding of a strong CK14 immunoreaction in all tumor cells of AOT, including the duct-like structures (Crivelini et al. 2003). Additionally, given

the co-existence of amelogenin, enamelin, sheathlin and AMTN in the mineralized areas of the tumor, one may hypothesize that MMP-20 and KLK4 activity is compromised, and thus fails to fully degrade the first three proteins.

Alternatively, a deregulated early and rapid secretion may also be hypothesized, since a very high expression of AMTN is found in the mineralized foci that mimic the dental enamel, which is not seen in the normal development (Moffat el at. 2006). Thus, a disorganized timeline of events may be occurring in these odontogenic tumors.

Although described as a distinctive feature of COC, ghost cells are also found in many odontogenic and non-odontogenic lesions, such as odontomas and craniopharyngiomas, respectively. These cells often keratinize and sometimes undergo dystrophic calcification (Sedano and Pindborg 1975; Sciubba 1985; Keszler and Guglielmotti 1987; Hirshberg et al. 1994; Moleri and Moreira 2002, Regezi 2002). Some authors have described ghost cells as derivatives from odontogenic epithelium that underwent metaplastic transformation, and appear as different stages of normal or aberrant keratin formation (Sedano and Pindborg 1975). Although they present different morphological characteristics from the enamel, it was also suggested that these cells may be the result of unsuccessful enamel matrix production from odontogenic epithelium (David and Buchner 1976). The hypothesis that this defective enamel-like matrix could be retained within the cells due to their inability to secrete it was also reported (Freedman et al. 1975). Ultrastructurally, ghost cells are characterized by the presence of coarse bundles of tonofilaments and loss of nuclei. Additionally, they sometimes undergo or

induce local dystrophic calcification (Mascrès et al. 1990; Hong et al. 1991). Another possible explanation for the appearance of these cells is related to coagulative necrosis of the odontogenic epithelium (Hong et al. 1991).

Strong staining of ghost cells for amelogenin, enamelin and sheathlin have been reported in the literature (Mori et al. 1991; Saku et al. 1992; Takata et al. 2000a; Abiko et al. 2001). Three cases of COC associated with odontomas and presenting enamel matrix also expressed sheathlin intensely. The authors proposed that the abnormal accretion of such enamel proteins may be responsible for the appearance of ghost cells in this lesion (Takata et al. 2000a). As expected, MMP-20 was also found to be strongly expressed in COC. Variable staining in ghost cells, mainly in peripheral areas of the clusters was revealed, as well as in the immature enamel observed in some cases (Takata et al. 2000b).

The labeling detected for AMTN follows the patterns described above. A variable, although sometimes very intense staining observed when calcification is present, indicate that the protein may be related to mineralization events.

2.6. Conclusions

The AMTN protein can be detected by immunohistochemical staining in ameloblast-like cells and the adjacent enamel matrix in AFO and odontoma. These two odontogenic tumors are considered hamartomas and AMTN expression correlates with differentiation of ameloblasts and maturation of enamel matrix. The absence of AMTN staining in the tumor cells of

ameloblastoma suggests that the ameloblast-like cells in this tumor are not mature enough to produce AMTN, which is associated with enamel matrix production. Staining of ghost cells for AMTN is observed and is particularly strong in areas of calcification.

Taken together, these results indicate a possible involvement of the AMTN protein in the mineralization events that occur at the final stages of enamel formation. Additional investigations should elucidate whether a correlation between the localization of AMTN in ameloblast-like cells and nearby calcified material usually found in odontogenic tumors can be linked to tumor cell differentiation and/or maturation and mineralization of enamel-like matrix.

3. Temporo-spatial expression profiling of murine amelotin and its effect on biomineralization

Manuscript to be submitted for publication to Mechanisms of Development

3.1. Abstract

During the maturation stage of amelogenesis, important events provide ideal conditions for crystal growth in thickness that eventually lead to hardening of the enamel matrix: proteinases are secreted and ameloblasts enter a modulation cycle that allow calcium deposition and removal of organic content through a unique basal membrane (BL) formed *de novo*. The newly described Amelotin (AMTN) protein is expressed by maturation stage ameloblasts and accumulates in the BL between these cells and the enamel matrix. Ameloblastomas are aggressive odontogenic tumors that arise from epithelial remnants of the enamel organ. They affect mainly the molar area of the mandible and are characterized by a slow-growth pattern. Through investigations performed in an ameloblastoma cell line (AM-1), we suggest a possible role for AMTN in mineralization. RT-PCR, immunocytochemistry, mineralization assays and microarray analysis were performed. The results have shown that AMTN is localized to the cytoplasm of AM-1 cells and the expression is higher at one week after confluency. A tendency to reduce mineralization by decreasing mineral formation and down-regulating the ODAM gene was observed. Thus, we suggest that AMTN may be related to late events that occur in the maturation stage of amelogenesis by controlling biomineralization.

Key words: Ameloblastoma, Amelogenesis, Biomineralization.

3.2. Introduction

The maturation stage of amelogenesis comprises several events that eventually lead to hardening of the enamel matrix by crystal growth in width and thickness prior to tooth eruption. It is a slow and progressive phenomenon that involves drastic morphological changes in ameloblasts after they have determined the overall thickness of the extracellular layer during the secretory phase (Smith 1998). Variations in the apical end of the cells are responsible for the modulation cycles where ruffle-ended and smooth-ended ameloblasts alternate to enable an ideal environment for mineral accretion and removal of organic content (Smith 1998; Nanci 2008). Ion transport occurs efficiently through a unique basal lamina-like structure (BL) that appears at this stage (Smith and Nanci 1995). It is composed of a cord network expressing unusual high amounts of heparin sulfate proteoglycan (HSPG), as shown by immunohistochemistry, immunofluorescence and ultrastructure methods (Sawada and Inoue 2001; Al-Kawas and Warshawsky 2008). Fine filaments demonstrating moderate staining for type IV collagen were also revealed, but the presence of laminin in this structure seems controversial, since both variable and absence of signal were reported (Sawada et al. 1992; Nanci et al. 1993; Al-Kawas and Warshawsky 2008). It has also been evidenced that this BL is deeply embedded in the enamel and associated to short rod-like enamel crystals (Sawada and Inoue 2000; Sawada and Inoue 2001). Moreover,

concomitant to these modifications, specific proteinases are secreted by the ameloblasts. One of them belongs to the matrix metalloproteinase family, the so-called Enamelysin or MMP-20, and the other is known as Kallikrein 4 (KLK4). Both enzymes function in processing and degrading the matrix proteins deposited during the secretory phase, although the former is normally produced earlier and it is thought to have a special affinity for amelogenins, while the latter is calcium independent and seems to act as a bulk digestive enzyme (Smith 1998; Sire et al. 2007; Nanci 2008). However, much is left to be understood regarding specific events occurring during the process of amelogenesis. Amelotin (AMTN) is an ameloblast-specific gene recently identified (Iwasaki et al. 2005; Moffat et al. 2006). Although it shares similarities with other enamel proteins such as amelogenin, ameloblastin and enamelin, namely multiple serine and threonine phosphorylation sites, little is known about its function (Iwasaki et al. 2005; Moffat et al. 2006; Cheon et al. 2007). In situ hybridization and immunohistochemical studies in mouse and rat molars and incisors showed it is specifically expressed by maturation-stage ameloblasts with accumulation in the BL between these cells and the enamel matrix (Iwasaki et al. 2005; Moffat et al. 2006). Proposed roles for this protein, including the regulation of mineral growth, cell differentiation and/or cell-to-enamel adhesion remain to be proven.

Ameloblastomas are the most frequent odontogenic tumors arising from remnants of dental epithelium and histologically resemble the enamel organ of the developing tooth germ, yet enamel formation is not observed (Kramer et al. 1992; Snead et al. 1992; Nagatsuka et al. 2005). These lesions are rare in

children and the greatest period of prevalence occurs in the age range of 20 to 50 years, with a similar male/female ratio (Mendenhall et al. 2007). They are unique to the jaws and if left untreated, often lead to extensive tissue destruction and deformity. The main area of incidence is the mandible, and over two-thirds occur in the molar-ramus region (Greenberg and Glick 2003; Nagatsuka et al. 2005). The tumor is often asymptomatic due to its slow-growth pattern. Occasionally however, swelling, loose teeth, malocclusion, paresthesia and pain may be observed (Becelli et al. 2002; Mendenhall et al. 2007).

Although defined as a benign neoplasm, ameloblastomas are locally destructive and a high rate of recurrence is observed if the lesions are not entirely excised. A few cases of distant metastasis have been reported in the literature (Eversole et al. 2004; Huang et al. 2007; Sahoo et al. 2007), and the causative mechanisms that unchain the formation of these tumors remain unknown.

This study aimed to identify a possible association of the AMTN protein with the mineralization events that occur during the maturation stage of amelogenesis, utilizing an ameloblastoma cell line (AM-1).

3.3. Materials and Methods

Ameloblastoma (AM-1) cell line

The ameloblastoma cell line AM-1 was gently provided by Dr. Takeshi Mitsuyasu, First Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Kyushu University, Japan. It is derived from a 20 year-old woman presenting a mural, plexiform ameloblastoma (Harada et al. 1998). The cells were seeded and cultured using a Defined Keratinocyte – Serum Free Media (Gibco – Invitrogen – Carlsbad, CA), and media changes occurred every 2 days. All cultures were maintained at 37C in a humidified atmosphere of 5% CO_2-air (Harada et al. 1998).

Reverse Transcriptase Polymerase Chain Reaction – RT-PCR

Total RNA was isolated using a commercially available kit (Absolutely RNA Microprep Kit – Stratagene – La Jolla, CA). Cell lysates were obtained and received DNase treatment following elution. RNA was quantified and cDNA synthesized using 50μM oligo$(dT)_{20}$ Primer (SuperScript™ III Reverse Transcriptase, Invitrogen – Carlsbad, CA). A plasmid containing human AMTN cDNA was used as a positive control (Forward (5'): GGAATTC CAT ATG TTA CCA CAG CTC AAA CCT GCT; Reverse (3'): CCG CTC GAG TTA CTG AAT TCC ATT TGC TGA TTC). Sequences for the human AMTN primers utilized were the following: Forward (5'): ACT CGG TCA TTA CCA CAGCT; Reverse

(3'): TAG GTT TCC TCG TGT GTA CG. Forty cycles of amplification were performed, initially with a denaturing step at 94C for 2 min, followed by cycles at 94C for 30 s, 70C for 30 s and 72C for 10 s. Final extension was performed at 72C for 5 min. The RT-PCR amplification products were analyzed in 1.5% agarose gel. Four different groups were tested: AM-1 cells grown at confluency, as well as one, two and three weeks after confluency.

Immunocytochemistry

Affinity purified antibodies against two sequences of the AMTN peptide (AMTN-1 and AMTN-2) were utilized. Cells were grown in chamber slides coated with Poly-L-lysine (Sigma Aldrich – St. Louis, MO) and fixed in ice-cooled Methanol for 10 minutes. After several washes with PBS, cells were blocked with PBS containing 0.2% BSA (Albumin fraction V Powder A-3311, Sigma Aldrich – St. Louis, MO) and 0.2% Triton-X100 (Sigma Aldrich – St. Louis, MO) for 15 minutes. The primary antibodies were diluted 1:100 (AMTN-1) and 1:500 (AMTN-2) and incubated at 37C for 30 minutes. The secondary antibody used was the fluorescein (FITC)-conjugated goat anti-rabbit IgG, fragment specific (Jackson Immuno – West Grove, PA), diluted 1:100. All antibodies were diluted in PBS containing 0.2% BSA and 0.2% Triton X100. DAPI stain (Sigma Aldrich – St. Louis, MO) was applied for 5 minutes for nuclear blue color development according to manufacturer's instructions (Invitrogen, 2006).

Mineralization Assay

Fifty µg/ml ascorbic acid and 10^{-8}M dexamethasone were freshly added to the usual culture medium. When cells reached confluency, 10mM ß-glycerophosphate was supplemented and the treatment with human recombinant AMTN was initiated. Three groups were designated: control, addition of human recombinant AMTN at 10ng/ml and 100ng/ml. Medium changes occurred initially every second day, or every day as the cells became confluent.

At the 35th day in culture, cells were fixed and further divided into 2 groups according to the staining method employed: Von Kossa (cells fixed with 4% paraformaldehyde in PBS overnight at 4C) or Alizarin Red (cells fixed with 10% formaldehyde at room temperature for 15 minutes). Following the former technique, which identifies inorganic phosphate, 2.5% silver nitrate staining was applied for 30 minutes in the dark. After several washes, FC Buffer containing 25% formaldehyde and 5% sodium carbonate was added for 3 minutes, cells were washed extensively with tap water and dried (modified from Dhore et al. 2008). Cells stained with the second method for identification of calcium-rich deposits were submitted to 40mM of Alizarin Red solution for 20 minutes with gentle shaking, washed extensively with distilled water and dried.

Microarray Analysis

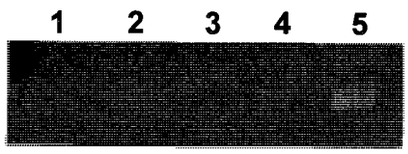
Cells were grown to confluency in 100mm plates. Subsequently, human recombinant AMTN treatment was performed at concentrations of 10ng/ml and 100ng/ml. Twenty-four hours after treatment, total RNA isolation was performed (as described above) and the samples were sent for Microarray Analysis with Two-Color Microarray-Based Gene Expression (Agilent Technologies, Santa Clara – CA) at the Microarray Centre, University Health Network (Toronto, ON, Canada). Samples were prepared, hybridized, washed and scanned according to manufacturer's protocol (Agilent Technologies, 2008).

3.4. Results

RT-PCR results are shown in Figure 3.1. A slight increase of AMTN expression is seen for cells grown until one week after confluency. However, after two and three weeks the signal diminishes, being similar to cells grown until confluency. Confocal microscopic pictures revealed that the signal for AMTN is mainly located at the cytoplasmic portion of the cells (Figure 3.2).

Figure 3.1. RT-PCR showing AMTN expression in different timings for the AM-1 cell line. No increase in expression was found.

1. Cells grown to confluency; **2**. Cells grown to 1 week after confluency; **3**. 2. Cells grown to 2 weeks after confluency; **4**. Cells grown to 3 weeks after confluency; **5**. Positive control (plasmid containing human AMTN cDNA).



Figures 3.3 and 3.4 show results for the mineralization assay. The 6 well-plate stained for Von Kossa and Alizarin Red indicated a slight decrease in mineralization in treated groups when compared to the control. This is clearer for the group treated with human recombinant AMTN at the concentration of 100ng/ml.

Microarray analysis has shown a 3-fold decrease in expression for the ODAM gene in the group of cells treated with human recombinant AMTN protein at 100ng/ml. Differences in down- or up-regulation for other genes were not significant (Table 3.1; Figures 3.5 – 3.7).

Figure 3.2. Immunolocalization of the AMTN protein in the AM-1 cell line. Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).

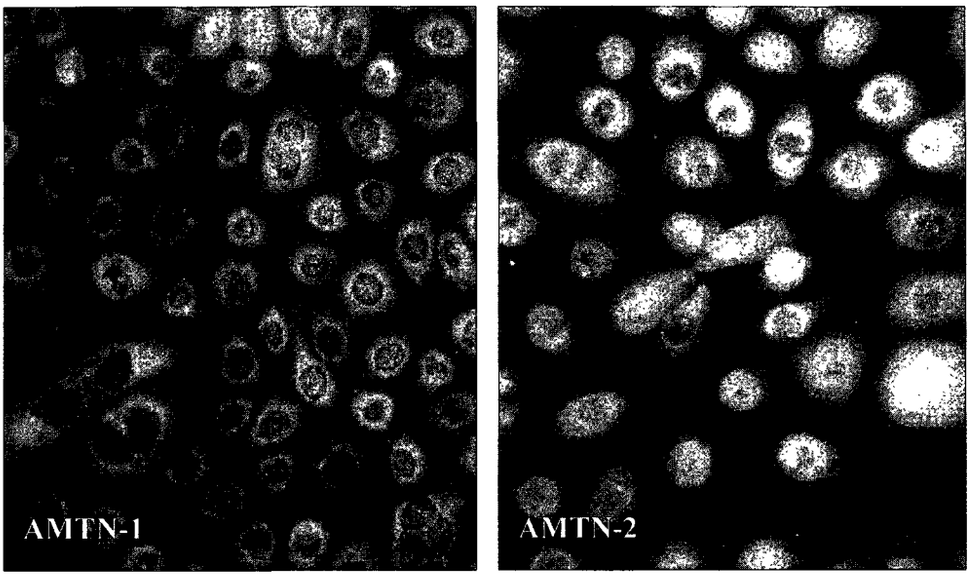


Figure 3.3. Mineralization assay showing results for Von Kossa (row **A**) and Alizarin Red stainings (row **B**).

Columns 1. control; **2**. AMTN treatment at10ng/ml; **3**. AMTN treatment at 100ng/ml.

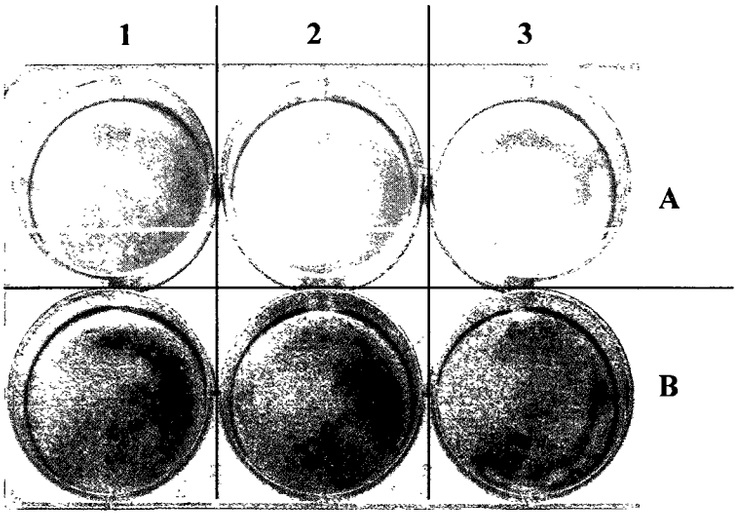


Figure 3.4. Panel showing AM-1 cells treated with osteogenic medium at different timings. Von Kossa and Alizarin Red stainings were performed after 35 days in culture.

A. control (no AMTN treatment), **B**. AMTN treatment at 10ng/ml, **C**. AMTN treatment at 100ng/ml.

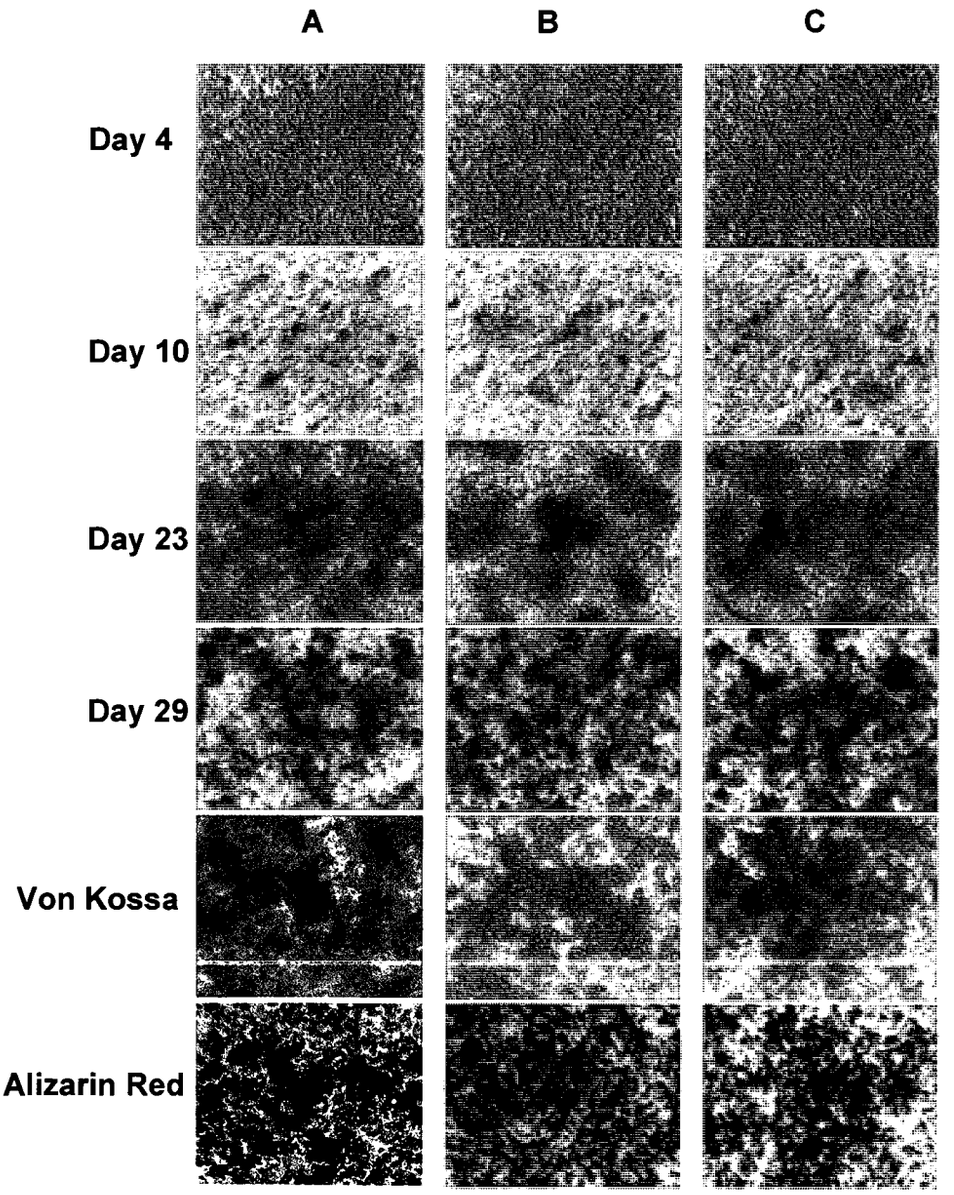
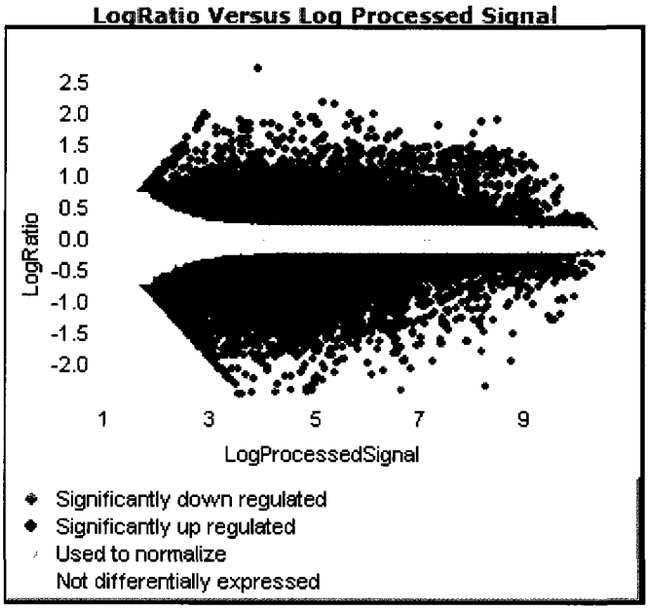


Table 3.1. Intensities were loaded into the software R version 2.8.1 (2008-12-22) with limma_2.16.4 using gProcessedSignal and rProcessedSignal as background subtracted signals for the two channels. Results for ODAM at the different groups tested for Red (R) and Green (G) channels are summarized below.

	Probe ID	Control	100ng/ml	10ng/ml
R	A_23_P58228	25.61255	8.405545	23.7
G	A_23_P58228	247.7421	229.3741	165.9

Figure 3.5. Probes detecting significantly more Cy3- (red) or Cy5- (green) labeled targets are shown, along with the remaining probes which were either used (blue) or not used (yellow) for normalization. (A) Data for samples treated with 100ng/ml AMTN; (B) Data for samples treated with 10ng/ml AMTN.

A



B

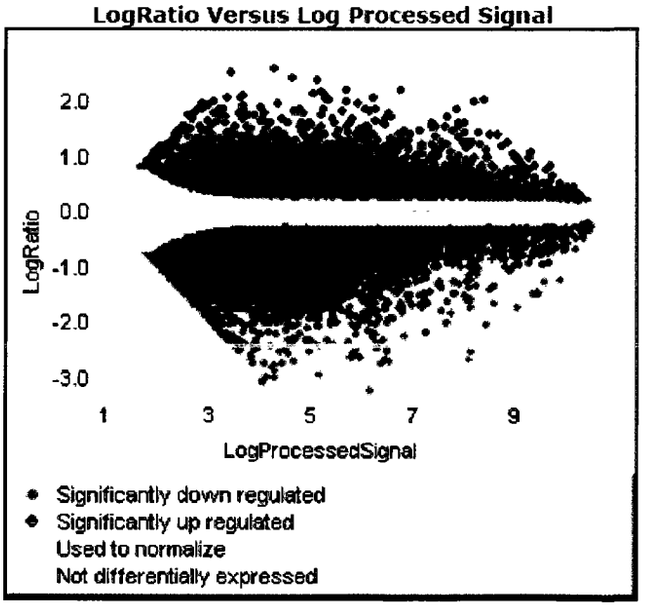


Figure 3.6. Log2 intensities boxplots generated for Red (R) and Green (G) channels. (1) control 2; (2) group treated with 100ng/ml AMTN; (3) group treated with 10ng/ml AMTN.

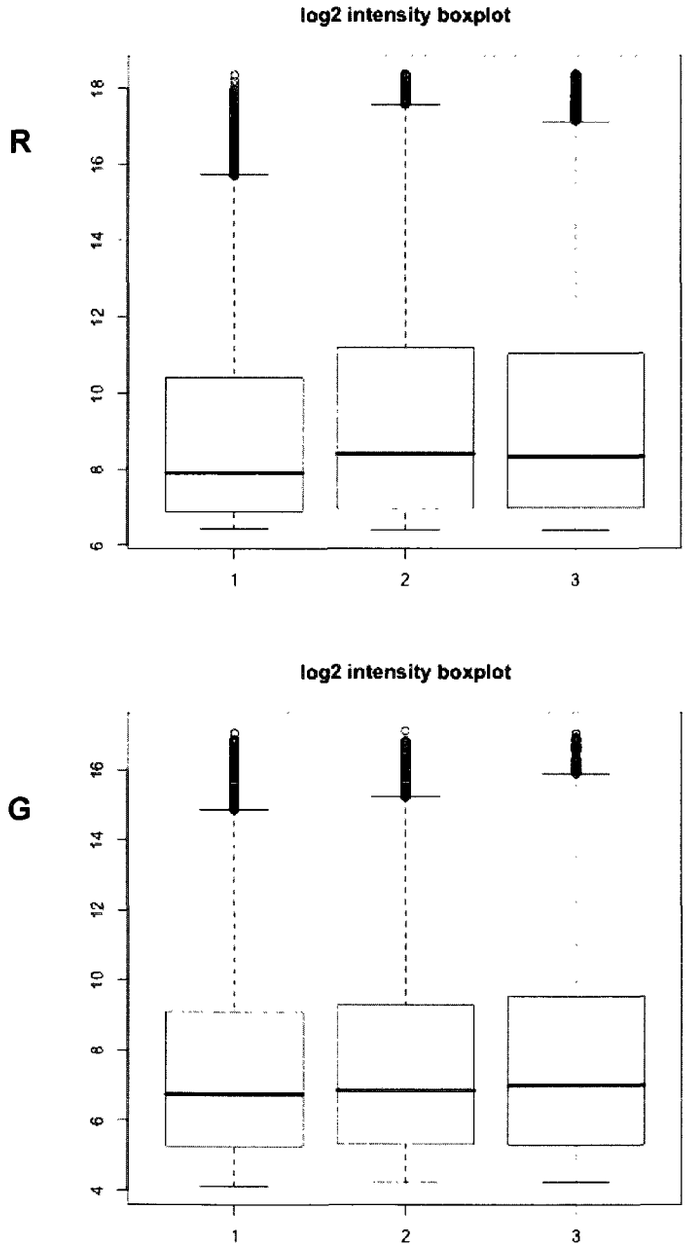
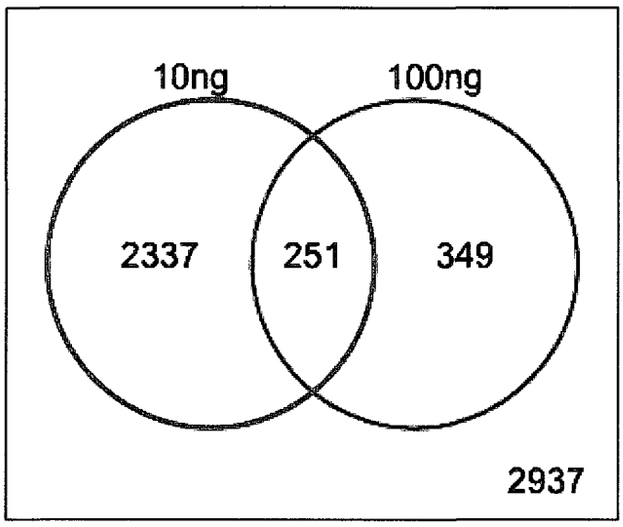


Figure 3.7. Venn Diagram showing correlation of probes for the groups tested (10ng/ml and 100ng/ml AMTN).



3.5. Discussion

Expression of AMTN is for the first time demonstrated in ameloblastoma cells in culture, as evidenced by RT-PCR and immunofluorescence methods. It is interesting to notice that, although these tumors have a close resemblance to the enamel organ, it is widely believed that an undifferentiated state predominates. The epithelial cells that constitute the classic islands or strands are often designated ameloblast-like cells, since they mimic the behavior of those in normal tooth development (Kramer et al. 1992; Snead et al. 1992; Nagatsuka et al. 2005). Nonetheless, it is often advocated that these cells do not undergo differentiation due to their inability to express enamel matrix proteins such as amelogenin, ameloblastin and enamelin. Northern Blot and In situ hybridization analysis of messenger RNA however, have detected the

amelogenin gene in those cells (Snead et al. 1992). Increased levels of this gene, particularly the Y copy, were also detected in male human ameloblastoma samples by RT-PCR and in situ hybridization techniques (Tsujigiwa et al. 2005). Since immunohistochemical studies failed to reveal any signal in tissue samples, it is believed that structural or functional changes in the expressed mRNA may obstruct the normal translational process (Saku et al. 1992; Tsujigiwa et al. 2005; Mendenhall et al. 2007). Another indication that these cells do not progress into an advanced differentiated state is the absence of MMP-20 (Takata et al. 2000b).

One recent report, however, identified expression of ODAM not only in ameloblastomas, but also in gastric, lung and breast tumors (Kestler et al. 2008). This protein, alternatively called Apin, has a very similar expression pattern to AMTN. It is strongly expressed by maturation-stage ameloblasts (Park et al. 2007; Moffat et al. 2008), although its secretion was also found to a lesser extent in the surface of enamel during the secretory phase (Park et al. 2007). Another interesting observation is that both over- and under-expression of ODAM had affected MMP-20 mRNA production, up- and down-regulating it, respectively. The predicted immunolocalization of this protein in normal ruffle-ended ameloblasts also indicate a possible relevant role in ion transport (Park et al. 2007).

A possible explanation for the absence of AMTN signal in ameloblastoma tissue samples by immunohistochemical studies (shown in Chapter 2) is that the AMTN translation may be compromised in a similar way to amelogenin. On the other hand, the fact that AM-1 cells showed evidence of cytoplasmic

expression in culture may be explained by environmental conditions, which can alter the gene expression profile of the system. The induction of changes in the expression of genes when different methodologies are used is not new (Pennanen et al. 2009; Swanson et al. 2009). A clarifying experiment using ameloblastoma tissue samples would be an in situ hybridization analysis to further elucidate whether AMTN mRNA is indeed present, but protein translation is impaired. One may speculate that the AM-1 cell line would also express ODAM in culture; additionally, since they retain the cytoplasmic expression of AMTN, it may be hypothesized that maturation or even secretion of an identifiable matrix could occur if mesenchymal stimulus were present. Further studies should unravel details regarding this matter.

As demonstrated by the mineralization assay, the AM-1 cell line was able to mineralize upon stimulation with osteogenic media. Von Kossa and Alizarin Red stainings further identified calcium and phosphate-rich deposits that retain similarities to the composition of normal enamel. Further investigations should reveal if an increase in the concentration of exogenous AMTN in the culture media would have any deeper effect in retarding mineralization. It is however difficult to predict the actual amount of protein secreted by the cells in vivo. Such dose/response experiments could provide hints whether AMTN is in fact involved in regulating the rate of extracellular matrix mineralization. The possible interaction and relation of AMTN with other proteins, expressed in a similar fashion, such as ODAM, should also be investigated.

An acute effect on the overall gene expression profile was also analyzed by microarray analysis following 24 hours of AMTN treatment. Notably, a 3-fold

decrease for the ODAM transcript was observed in the group treated with 100ng/ml of exogenous AMTN. This could be of functional significance given the possible regulation of MMP-20 by ODAM (Park et al. 2007) and the similar pattern of distribution of ODAM and AMTN (Moffat et al. 2008). Both proteins are highly expressed throughout the maturation stage of amelogenesis, although AMTN is found in the basal lamina between ameloblasts and the enamel surface, while ODAM is diffusely localized in the apical surface of ameloblasts (Moffat et al. 2008). Possible interactions between them are yet to be confirmed. Interestingly, these two proteins also share several similarities with the members of the secretory calcium-binding phosphoprotein (SCPP), which also include other extracellular matrix proteins such as amelogenin, ameloblastin and enamelin (Sire et al. 2007). In fact, it is suggested that these five ameloblast genes are derived from a common ancestor, although the largest part of the sequences diverge and characterize the specific functions of each protein (Sire et al. 2007). The fact that the ODAM mRNA levels appeared reduced after only 24 hours of AMTN exposure may imply that this regulation, if confirmed, is rather direct.

Finally, an important functional consideration is that the bacterially expressed recombinant human AMTN used in these experiments carries an N-terminal His_6-tag and does not contain any post-translational modifications. Several studies in the literature have demonstrated that (poly)histidine tags can affect protein properties and interfere with its bioactivity (Schmeisser et al. 2006; Sayari et al. 2007). The lack of post-translational modifications such as phosphorylations and O-glycosylations may induce a non-physiological

response to the protein in culture; the native protein would arguably present additional and/or more biologically relevant outcomes in this case.

3.6. Conclusions

Amelotin was immunolocalized to the cytoplasm of ameloblastoma AM-1 cells in culture. RT-PCR procedures revealed an expression peak for cells cultured up to one week after confluency, returning to basal levels following three weeks of confluency.

We have also shown that the AM-1 cell line does mineralize upon stimulation, and this might be a useful tool for mineralization studies in the future. The effect of overexpressing AMTN in the cell culture environment, performed by addition of human recombinant AMTN protein into the media, was a slight decrease in mineralization. An acute effect assessed by microarray analysis was demonstrated by a down-regulation of the ODAM gene, indicating a close association between them. Further investigations should clarify the role of AMTN in the later events that control enamel mineralization and maturation.

4. Discussion

4.1. Spatial distribution of AMTN in odontogenic tumors from epithelial origin

4.1.1. Ameloblastomas

As shown in Chapter 2, ameloblastomas did not demonstrate evidence of AMTN expression. Exceptions can be made for two cases of the follicular type presenting cystic changes, where a very faint staining could be observed. Studies that have identified the appearance of dense granules resembling lysosomes in the stellate reticulum-like cells suggest they could be related to the formation of microcysts. Additionally, it has been controversial whether the intrafollicular cysts show a deficiency of absorption and diffusion of nutrients, or if the polarization of epithelial cells facing the 'stellate reticulum' would cause them to remove nutritive elements from the interior of the islands, in an attempt to resemble normal conditions (Campos 1990; Ueno and Matsuo 1991).

Despite the fact that ameloblastomas resemble the enamel organ to some extent, no enamel formation or any other identifiable matrix is observed in the structural organization of the tumor. Assumptions for this particularity rely on the fact that tumoral cells do not reach their fully differentiated state (Tsujigiwa et al. 2005). In fact, the epithelial cells that constitute the classic islands or strands are often designated ameloblast-like cells, since they mimic the behavior of those in normal tooth development (Kramer et al. 1992; Snead et al. 1992;

Nagatsuka et al. 2005). A morphological analysis of a simple plexiform ameloblastoma has identified central cells corresponding to two distinct phases: some were characterized by tonofilaments and resemblance of stellate reticulum and stratum intermedium, probably representing a supporting role. Others showing extensive Golgi complexes and granular endoplasmic reticulum would be related to a secretory function (Matthiessen et al. 1980). However, regardless of these similarities, it is generally believed that ameloblastoma cells do not achieve the state of secretory ameloblasts. Some authors emphasize that the synthesis of extracellular proteins is possible only when both intra- and extracellular conditions are appropriate for their release. Thus, since in normal development enamel matrix secretion is dependent on the deposition of the first few micrometers of dentin, ameloblastoma cells cannot proceed to this phase since this matrix is not produced by the tumor (Saku et al. 1992).

Additionally, several immunohistochemical investigations failed to identify enamel proteins such as amelogenin, enamelin, ameloblastin and sheathlin in tumor samples (Saku et al. 1992; Takata et al. 2000a), with the exception of one study that reported amelogenin expression in peripheral cells and cystic areas of follicular ameloblastomas (Mori et al. 1991). Another indication that these cells do not progress into a fully differentiated state is the absence of MMP-20 (Takata et al. 2000b). On the other hand, transcripts for the amelogenin gene were detected in ameloblastoma epithelial cells through mRNA phenotyping by RT-PCR, in combination with Northern Blot and in situ hybridization analysis (Snead et al. 1992; Tsujigiwa et al. 2005). A possible explanation for this fact is that the normal translational process is hindered by

mutations and/or structural changes in the expressed mRNA (Tsujigiwa et al. 2005). The authors have also suggested that the signaling events occurring between the stroma and epithelial cells are able to induce the transcription of the amelogenin gene, but absence of essential molecules or disturbed pathways may be responsible for the impaired production of matrix in ameloblastomas (Snead et al. 1992).

Increased levels of the amelogenin transcript, particularly from the Y-chromosomal locus, were also detected in male human ameloblastoma samples by RT-PCR and in situ hybridization techniques (Tsujigiwa et al. 2005). Notably, normal development is characterized by higher expression levels from the X-chromosomal locus. It is speculated that the sex chromosomes undergo epigenetic changes that may account for the formation of the tumor. Moreover, it is also possible that the detected transcripts constitute a pseudogene sharing homologies with the amelogenin Y-copy that would not yield a functional protein or would inhibit the translation of the normal amelogenin mRNA (Tsujigiwa et al. 2005).

Nevertheless, although there is a general agreement that ameloblastoma cells do not achieve differentiation, one recent report has shown intriguing results in regards to a newly-discovered protein, ODAM. Alternatively called Apin, it was initially isolated from amyloid substances found in a human calcifying epithelial odontogenic tumor (Solomon et al. 2003). It presents a very similar expression pattern to AMTN in normal amelogenesis, being strongly expressed by maturation-stage ameloblasts (Park et al. 2007; Moffat et al. 2008), although its secretion was also found to a lesser extent in the surface of

enamel during the secretory stage, adjacent to the Tome's processes (Park et al. 2007). Junctional epithelium cells also revealed the presence of this protein, corroborating the evidence that the reduced enamel epithelium constitute the origin of this tissue. Thus, ODAM may also be a useful marker to distinguish the junctional epithelium in the oral mucosa. Furthermore, the predicted immunolocalization of this protein in normal ruffle-ended ameloblasts indicate that it may play a relevant role in ion transport and removal of organic content. Another remarkable observation is that both over- and under-expression of ODAM had affected MMP-20 mRNA production, up- and down-regulating it, respectively (Park et al. 2007). However, the significance of this finding is debatable, since MMP-20 is specifically produced by secretory stage-ameloblasts in a moment that ODAM is rather weakly expressed or absent (Moffat et al. 2008).

In addition to its presence in the enamel organ or tooth-related structures, the expression of ODAM was also detected in nasal and salivary glands and the epididymis of rats (Moffat et al. 2008). In pathological samples, the protein was found not only in epithelial cells of ameloblastomas, but also in gastric, lung and breast tumors (Kestler et al. 2008, Moffat et al. 2008), although the significance of this finding is unknown. Additional studies in odontogenic tumors should reveal more details about its distribution and possible correlation to tumoral status.

Hence, the data obtained by immunohistochemistry in this study supports the evidence that lack of inductive changes prevent the maturation of the ameloblast-like cells, since they are not capable to produce the AMTN protein

normally secreted at this stage. However, in the light of the new discoveries reported above, further evidence should corroborate these results. One may also speculate that AMTN translation may be compromised in a similar way to amelogenin, hence the absence of AMTN signal in ameloblastoma tissue samples by immunohistochemical methods. A clarifying experiment using ameloblastoma tissue samples would be an in situ hybridization analysis to further elucidate whether AMTN mRNA is indeed present, but protein translation is impaired. However, the availability of tissue samples suitable for such studies is extremely limited.

4.1.2. Adenomatoid Odontogenic Tumor

The expression of enamel proteins has also been described in AOT. Small mineralized foci, hyaline droplets located near epithelial cells and the cells itself were labeled with anti-amelogenin and anti-enamelin antibodies (Saku et al. 1992; Murata et al. 2000; Abiko et al. 2001). Large mineralized foci, however, did not present any signal for those proteins, a fact explained by their possible degradation and structural changes believed to occur in neoplastic tissue, recapitulating normal development (Saku et al. 1992). Sheathlin was found at high levels in the periphery of the eosinophilic substance found in tumor cell nests and also in the cytoplasm of cells facing these droplets (Takata et al. 2000a). The eosinophilic material is thought to be either basement-membrane like material (Courtney and Kerr 1975) or abortive enamel (Mori et al. 1991; Saku et al. 1992), since real enamel matrix is rarely seen in these tumors

(Takata et al. 2000b). The absence of sheathlin in mineralized foci is thought to be related to its rapid degradation as the normal secretory stage advances, and this fate would be mimicked in tumoral samples (Takata et al. 2000a). MMP-20 was also found to be expressed in the extracellular deposits of AOT. It is particularly interesting to notice that large calcified areas presented an intense signal, confirming the hypothesis of other studies that the degradation of enamel proteins may occur in a mostly normal fashion (Takata et al. 2000b).

In our study, the staining pattern observed for AMTN in AOT was similar to those reported above, since small mineralized foci and extracellular eosinophilic matrix were labeled. However, a very faint staining could be observed in the cytoplasm of some epithelial cells neighboring those areas. The fact that these cells do not present a strong signal for AMTN, although the protein is localized to the mineralized structures around them, may indicate that the cells actually have reached the end of the maturation stage. The time of AMTN expression in their inner secretory machinery may have elapsed and they in fact may subsist in the reduced enamel epithelium phase. This would be in accordance with earlier studies suggesting that AOT may arise from the reduced enamel epithelium and that those cells correspond to ameloblasts in the protective stage of odontogenesis. This suggestion arose from the findings of a strong CK14 immunoreaction in all tumor cells of AOT, including the duct-like structures (Crivelini et al. 2003). Additionally, given the co-existence of amelogenin, enamelin, sheathlin and AMTN in the mineralized areas of the tumor, one may hypothesize that MMP-20 and KLK4 activity is compromised, and thus fails to fully degrade the first three proteins.

Alternatively, a deregulated early and rapid secretion may also be hypothesized, since a very intense signal of AMTN was found in the mineralized foci that mimic the dental enamel, which is not seen during normal development (Moffat el at. 2006). Thus, a disorganized timeline of events may be occurring in these odontogenic tumors.

4.2. Spatial distribution of AMTN in odontogenic tumors from mixed origin

4.2.1. Ameloblastic Fibroma, Ameloblastic Fibro-odontoma and Odontoma

In Chapter 2, it is evident that the ameloblastic fibroma case investigated in this study presented a negative signal when tested with anti-human AMTN antibodies. The only small patch of mineralization encountered in the case showed a moderate staining. Although conceptually this tumor usually does not present any extracellular matrix formation, this finding only contributes to the bulk evidence from this work that only mineralized, enamel-like matrices in odontogenic tumors express the AMTN epitope.

The main discussion concerning AF in the literature indicates that it may represent the initial step for the development of ameloblastic fibro-odontomas, proceeding to odontomas (Chen et al. 2007). However, several investigations have revealed that the majority of AF does not present any evidence of maturation, and recurrent lesions lack characteristics of AFO or odontomas (Chen et al. 2005; Chen et al. 2007). Additionally, the age registered for AF

cases were far beyond the range in which AFO occurred, another fact that weaken this hypothesis (Slootweg 1981; Chen et al. 2005). The few exceptions reported were particularly diagnosed in younger patients (under the age of 19 years) in the study of 123 cases available in the English-language literature since 1891 (Chen et al. 2007); and a case study of a 6 year-old boy with a recurrent tumor, described in a retrospective investigation from 1949-2002 in China (Chen et al. 2005). Thus, dissimilarities in clinical information between AF and AFO exclude the possibility of an evolution from one to the other. Additionally, the tendency of the former to recur and undergo malignant transformation also indicates its neoplastic nature, greatly differing from AFO, since the latter usually presents a less aggressive behavior (Sootweg 1981, Chen et al. 2005). However, more consistent data (i.e. age, sex and predilection site) indicate a possible relation between AFO and odontomas, and it is believed that at least a number of these lesions may represent a continuous development. Nevertheless, it is generally believed that only the complex odontomas share these similarities, since the compound variety usually occurs at a lower age range (mean 14.8 - 15.1 years, compared to 20.3 - 25.9 years for complex odontoma) and the anterior maxilla is mostly affected. It is important to notice that the differentiation of odontogenic tissues occurs at an earlier period on this site (Slootweg 1981; Chen et al. 2005).

Therefore, it is generally agreed that a majority of AF present a neoplastic behavior with no tendency to develop further into AFO. Conversely, cases diagnosed earlier in life may represent the initial step of an evolutionary path that culminates in the formation of complex odontomas. Due to their

hamartomatous nature, AFO are more likely to differentiate and advance in this process; yet, information solely based on histological findings may not be sufficient to distinguish between these entities, and the age at the time of diagnosis should be considered a helpful indication (Slootweg 1981; Chen et al. 2005; Chen et al. 2007).

Concerning the expression of enamel proteins in AFO, immunohistochemical investigations have localized the amelogenin protein in the cytoplasm of cuboidal epithelial cells of tooth bud-like projections and stellate reticulum-like cells (Yagishita et al. 2001). Immature enamel, together with neighboring ameloblast-like cells showed a positive signal for sheathlin, but epithelial strands and cellular mesenchyme were negative (Takata et al. 2000a). The same expression pattern was followed by the MMP-20 proteinase, which presented an intense staining only in areas that mimicked the dental enamel. Regions rich in dentinoid materials were negative (Takata et al. 2000b).

In a similar fashion, amelogenin and sheathlin have been detected in odontomas. The enamel matrix, as well as small foci of mineralized products usually encountered near epithelial cell nests showed intense staining. Other structures such as dentin, cementum and pulp, however, were not labeled (Takata et al. 2000a; Abiko et al. 2001). Positive staining for MMP-20 was also identified in both enamel matrix and ameloblast-like cells (Takata et al. 2000b; Väänänen et al. 2004).

We observed strong AMTN staining in ameloblast-like cells adjacent to the enamel matrix in AFO and odontomas, which is analogous to the expression of AMTN in the maturation stage of enamel formation in the developing tooth. This

data indicate that the process of maturation has at least been initiated, and imply that some level of differentiation may be occurring in those cells.

4.2.2. Calcifying Odontogenic Cyst

The main finding from our immunohistochemical analysis in Calcifying Odontogenic Cysts was the remarkable, although variable AMTN expression in ghost cells. Much controversy and discussion is found in the literature regarding this cell type, whose function or significance in these tumors is unknown. It has been suggested that these cells derive from the metaplastic transformation of the odontogenic epithelium, representing different stages of normal or aberrant keratin formation (Sedano and Pindborg 1975). Other assumptions propose that ghost cells could be the result of unsuccessful enamel matrix production from the odontogenic epithelium (David and Buchner 1976), or that they somewhat retain this matrix-like material due to an impaired secretory system (Freedman et al. 1975). A closer look into the morphology of these cells reveals cytoplasmic fibrillar components, recognized as tonofilaments, and loss of nuclei. Furthermore, calcified deposits have often been found, and it is believed that they may induce local dystrophic calcification (Kerebel and Kerebel 1985; Mascrès et al. 1990; Hong et al. 1991). It was suggested that ghost cells passively undergo this calcifying process, becoming embedded in a material containing hydroxyapatite-like crystals (Kerebel and Kerebel 1985). Ischemia and coagulative necrosis of the odontogenic epithelium have also been cited as possible causes for the appearance of these cells. Although the latter is

supported by the assumption that COC may undergo central liquefaction necrosis of odontogenic epithelial clusters that could cause coagulative necrosis, there is no concrete evidence to confirm these hypotheses (Kerebel and Kerebel 1985; Hong et al. 1991).

Although it is known that ghost cells produce keratin, this process is thought to be aberrant or incomplete. The potential of epithelium cells located nearby dentinoid material to undergo metaplasia and become ghost cells was also highlighted (Anneroth and Hansen 1982; Yamamoto et al. 1988; Mascrès et al. 1990). Thus, ghost cells are best described as cells that have undergone metaplasia and are somewhat unable to activate proper developmental and inductive processes (Kerebel and Kerebel 1985).

Ghost cells were found to express high levels of amelogenin, enamelin and sheathlin (Mori et al. 1991; Saku et al. 1992; Takata et al. 2000a; Abiko et al. 2001). Three cases of COC associated with odontomas and presenting enamel matrix also expressed sheathlin intensely. Considering the intense labeling of such enamel proteins in ghost cells, authors have suggested that the abnormal protein accretion in the lesion could be responsible for the appearance of the cells (Takata et al. 2000a). An intense, variable signal was also found for MMP-20 in ghost cells of COC. The labeling was concentrated in peripheral areas of clusters, as well as in the immature enamel that was observed in some cases (Takata et al. 2000b).

The signal detected for AMTN in this investigation follows the patterns described above. A variable yet sometimes very strong staining observed when calcification is present indicates that the protein may be related to mineralization

events. Similarities between the ultrastructural appearance of ghost cells and the degenerative alterations observed in oral squamous epithelium after surgical trauma have been reported. Furthermore, needle-like crystals representing dystrophic calcification were also connected to these alterations in arteriosclerotic aortas (Fejerskov and Krogh 1972; Anneroth and Hansen 1982). Degenerated mitochondria, which have a high calcium uptake stored as amorphous $CaPO_4$, have been associated with calcifications seen in ghost cells since they were localized nearby these areas (Mimura et al. 2002).

Finally, the co-expression of hard keratins and Wnt related molecules, namely, beta catenin, Tcf (T cell factor) and Lef 1 (lymphoid enhancer factor), in odontomas indicate that this signaling pathway may be active in ghost cell formation (Tanaka et al. 2007). Wnt signaling is known to participate in the formation of the tooth bud and also in the process of amelogenesis (Thesleff and Sharpe 1997; Kumamoto and Ooya 2005; Stolf et al. 2007). Additionally, the promoter domains of amelogenin, enamelin and hard keratin genes all contain Lef-1 binding sites, suggesting a direct regulation by the Wnt pathway (Tanaka et al. 2007).

4.3. Expression pattern of AMTN in AM-1 cells; mineralization and microarray assays

In Chapter 3 we have shown the expression of AMTN in ameloblastoma cells (AM-1) in culture by RT-PCR and immunofluorescence methods. The protein was immunolocalized to the cytoplasm of the cells, and had a similar

distribution for both anti-human AMTN antibodies used. The fact that the epitope was detected in cells in culture, but not in tissue samples may be explained by environmental conditions, which can alter the gene expression profile of the system. The induction of changes in the expression of genes when different methodologies are used is not new (Pennanen et al. 2009; Swanson et al. 2009). Alternatively, epitopes in archival, fixed tissue samples may not be as well preserved as in cultivated cells.

As demonstrated by the mineralization assay, the AM-1 cell line was able to mineralize upon stimulation with osteogenic media. Von Kossa and Alizarin Red stainings identified calcium and phosphate-rich deposits that retain similarities to the composition of normal enamel. Further investigations should reveal if an increase in the concentration of exogenous AMTN in the culture media would have any greater effect in retarding mineralization. It is however difficult to estimate the actual amount of protein secreted by the cells in vivo. Such dose/response experiments could provide hints whether AMTN is in fact involved in regulating the rate of extracellular matrix mineralization. The possible interaction and relation of AMTN with other proteins, expressed in a similar fashion, such as ODAM, should also be investigated. One may speculate that the AM-1 cell line would also express this protein in culture; additionally, since they retain a cytoplasmic expression of AMTN, it may be hypothesized that maturation or even secretion of an identifiable matrix could occur if mesenchymal stimulus were present. Further studies should unravel details regarding this matter.

An acute effect on the overall gene expression profile was also analyzed by microarray analysis following 24 hours of AMTN treatment. Notably, a 3-fold decrease for the ODAM transcript was observed in the group treated with 100ng/ml of exogenous AMTN. This could be of functional significance given the possible regulation of MMP-20 by ODAM (Park et al. 2007) and the similar pattern of distribution of ODAM and AMTN (Moffat et al. 2008). Both proteins are highly expressed throughout the maturation stage of amelogenesis, although AMTN is found in the basal lamina between ameloblasts and the enamel surface, while ODAM is diffusely localized in the apical surface of ameloblasts (Moffat et al. 2008). Possible interactions between them are yet to be confirmed. Interestingly, these two proteins also share several similarities with the members of the secretory calcium-binding phosphoprotein (SCPP), which also include other extracellular matrix proteins such as amelogenin, ameloblastin and enamelin (Sire et al. 2007). In fact, it is suggested that these five ameloblast genes are derived from a common ancestor, although the largest part of the sequences diverge and characterize the specific functions of each protein (Sire et al. 2007). The fact that ODAM mRNA levels appeared reduced after only 24 hours of AMTN exposure may imply that this regulation, if confirmed, is rather direct.

Finally, an important functional consideration is that the bacterially expressed recombinant human AMTN used in these experiments carries an N-terminal His_6-tag and does not contain any post-translational modifications. Several studies in the literature have demonstrated that (poly)histidine tags can affect protein properties and interfere with its bioactivity (Schmeisser et al.

2006; Sayari et al. 2007). The lack of post-translational modifications such as phosphorylations and O-glycosylations may induce a non-physiological response to the protein in culture; the native protein would arguably present additional and/or more biologically relevant outcomes in this case.

5. Conclusions

This investigation demonstrated the expression of the AMTN protein in odontogenic tumors of epithelial (AOT) and mixed origin (AFO, odontoma, COC). The protein was immunolocalized to the ameloblast-like layer found in the structural organization of these tumors and also in the matrix-like components that mimics normal enamel. However, since the tumoral attempt to recapitulate normal odontogenesis is usually unsuccessful, and the structures that constitute the tumors are rather disorganized, the expression pattern is also irregular. Nevertheless, further studies should unravel whether AMTN has any causative role in the development or progression of these tumors.

On the other hand, ameloblastomas did not show evidence of AMTN expression. This fact supports the notion that the epithelial components are not true ameloblasts since they do not attain differentiation, and thus, are not able to produce enamel matrix. However, the newly discovered gene ODAM, which has a pattern of distribution similar to AMTN in normal development, was found in the epithelial cells of ameloblastomas by immunohistochemical methods. Further investigations should confirm these results and clarify whether the expression of ODAM is rather triggered earlier or if the cells actually reach a certain level of differentiation. In this case, AMTN expression may be impaired. These assumptions warrant further exploration.

Additionally, the AM-1 cell line was shown to mineralize upon stimulation, and this might be a useful tool for mineralization studies in the future. Cells were affected by the overexpression of exogenous AMTN in different timings: in a

long-term period, a slight decrease in mineralization was observed; the acute effect was a 3-fold decrease of ODAM expression levels assessed by microarray analysis.

Taken together, these results indicate a possible involvement of the AMTN protein in the mineralization events that occur at the final stages of enamel formation. Further studies should clarify whether this protein has any function related to controlling biomineralization.

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7. Appendices

Appendix 1 - Expression analysis of AMTN in ameloblastomas by immunohistochemistry using crude and partially purified antibodies and validation of affinity purification

Immunohistochemistry

The initial immunohistochemical study performed in this investigation utilized clinical cases of human ameloblastomas and crude anti-AMTN peptide antisera against two sequences of the protein. The paraffin sections were retrieved from the archives of the Department of Oral Pathology, Faculty of Dentistry, University of Toronto. Sections of a case of ameloblastic fibro-odontoma were used as a control. The commercially available kit DakoCytomation - K4010 (DAKO – Glostrup, Denmark) was utilized for the immunohistochemistry procedures; rabbit polyclonal antibodies against the peptide sequences of human AMTN (AMTN-1 (H2N-RLPTPSGTDDDFAVT-COOH) and AMTN-2 (H2N-PPTKLAPDQGTLPNQ-COOH)) were applied at a dilution of 1:1000 (Figure A1.1). In order to enhance the signal, antigen retrieval was performed utilizing citric acid buffer (pH=6.0). This method comprised intermittent microwave heating for a total of 5 minutes, starting with 2 minutes at 50% power and subsequent intervals of 1 minute. Color development was achieved using diaminobenzidine hydrochloride (DAB) diluted in the buffer supplied by the manufacturer (1:100) for 4 minutes and subsequent

counterstain with undiluted Methyl Green for 30 seconds; each step was followed by quick washes in distilled water. For the negative control, the primary antibody was replaced by pre-immune serum from the same rabbit at the same dilution.

In general, the expression of AMTN was clearly marked in the nucleus of the cuboidal or columnar epithelial cells that constitute the tumor islands. The central mass of loosely connected cells or cystic areas showed negative staining, and the fibrous stroma presented a faint signal (Figure A1.2). The most striking observation from the data shown was the unforeseen nuclear expression of AMTN in the epithelial cells of ameloblastomas, since during normal tooth development AMTN is identified in the cytoplasm of ameloblast cells as well as in the basal lamina interface between those and the enamel matrix, given that it is a secreted protein. Despite also being an odontogenic tumor, the positive control showed a pattern of expression comparable to normal tissues, since inductive changes usually lead to the formation of enamel matrix. Thus, most of the components responsible for amelogenesis show a quite normal morphology and function in this lesion (Figure A1.3).

Regarding the antigen retrieval technique, it provided good conditions for unmasking the epitope AMTN-2 in control specimens; however, no great variation on signal strength could be observed for AMTN-1 epitope (Figure A1.3).

Figure A1.1. Amelotin protein sequence. Note the correspondent sequences used for the development of both antibodies used in this study: AMTN-1 (dotted line) and AMTN-2 (full line).

Figure A1.2. Expression pattern of AMTN in ameloblastoma sections. **a.** sample stained with anti-AMTN-1 antibody, **a'.** sample after antigen retrieval treatment; **b.** expression pattern of epitope AMTN-2, **b'.** staining after antigen retrieval treatment. Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).

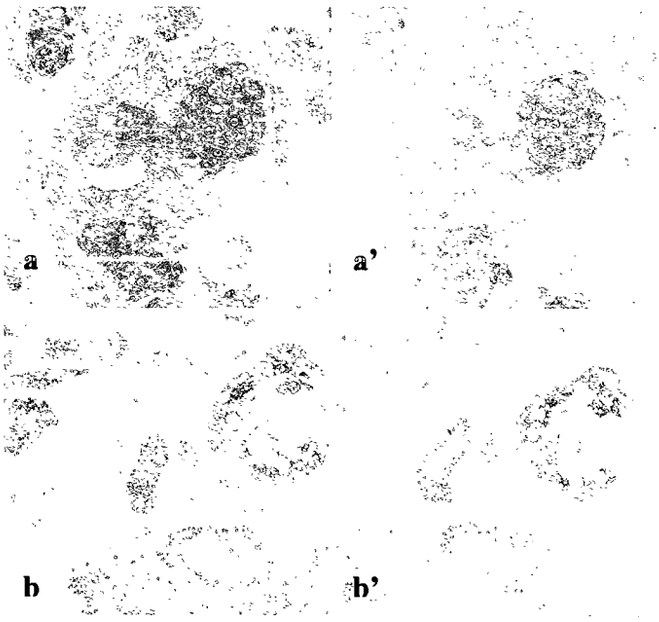
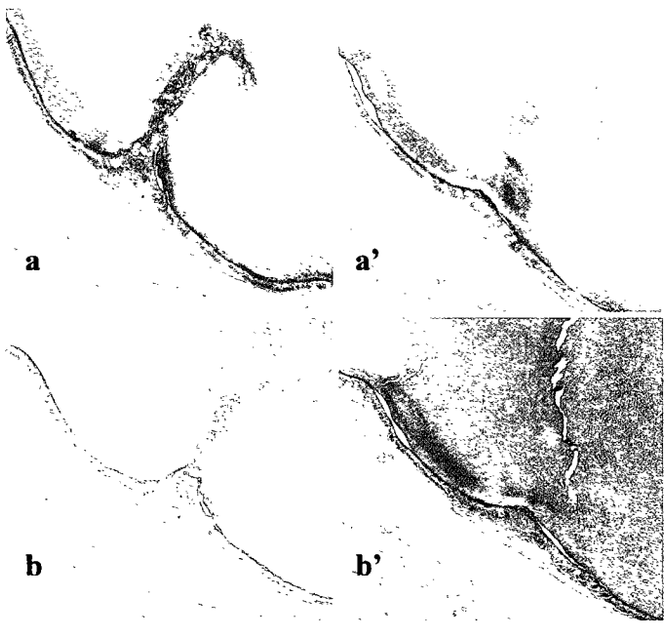


Figure A1.3. Ameloblastic fibro-odontoma sections used as a control. **a.** sample stained with anti-AMTN-1 antibody, **a'.** sample under antigen retrieval treatment; **b.** expression pattern using epitope AMTN-2, **b'.** staining after antigen retrieval treatment.



Antibody purification with Recombinant protein G agarose (IgG purification)

Since the results presented above were unexpected, it was suspected that much of the staining was artefactual, thus further antibody purification was performed to improve the specificity of staining.

Recombinant protein G agarose (Invitrogen, 15920-010 - Carlsbad, CA) was packed to a small column (BioRad, 731-1550 - Hercules, CA) and equilibrated with Binding buffer (0.01M sodium phosphate (pH7.0); 0.15M sodium chloride). After loading the samples (3ml of crude rabbit polyclonal antibodies against human AMTN-1 and AMTN-2 after filtration through 0.22µm

filter in a syringe to remove particles), the column was washed once with 10ml binding buffer and eluted IgG was obtained with elution buffer (6ml of 0.1M glycine hydrochloride (pH2.6)), The pH of the eluted fractions (0.5ml each) was immediately adjusted to pH 7.0 with 1M Tris-Cl buffer (pH 9.0). Optical density of eluted samples was checked using the spectrophotometer (DU-640 UV/VIS Scanning Spectrophotometer, Beckman Coulter, Fullerton, CA) at the wavelength 280nm, to estimate protein concentration. 20microL of each of the eluted fractions was subsequently separated by electrophoresis on a 12% polyacrylamide gel, which was then stained with coomassie blue to confirm the protein concentration and purity (Figure A1.4). The immunohistochemical technique was repeated for the ameloblastoma tissue sections, using the purified antibody (combination of three fractions presenting the higher optical density values) at a dilution of 1:1000, but no differences were found compared to the unpurified samples. The signal was still strong and concentrated in the nucleus of the epithelial cells (Figure A1.5).

Figure A1.4. Polyacrylamide gel (12%) showing protein bands for IgG purified antibodies against AMTN-1 and AMTN-2 in comparison to BSA standards.

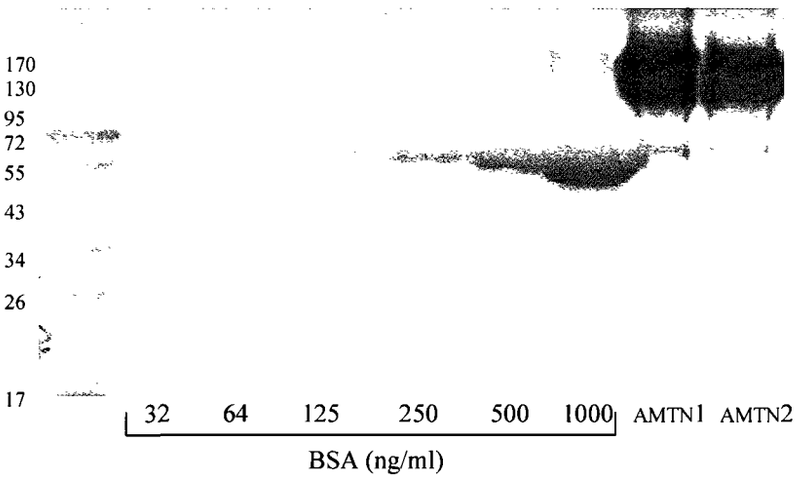
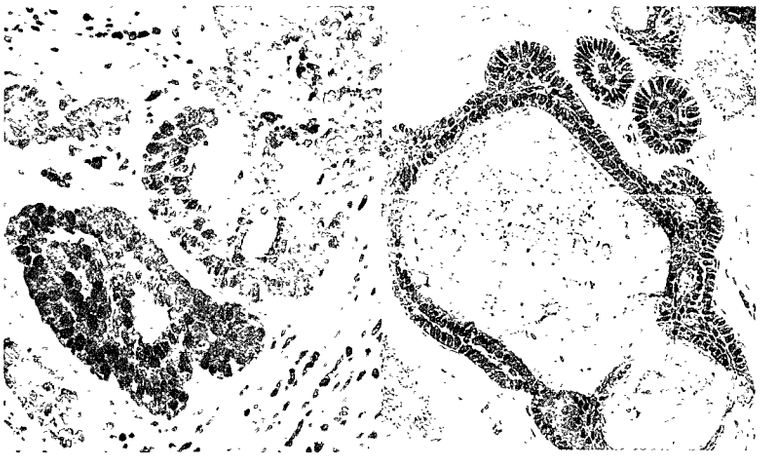


Figure A1.5. Ameloblastoma sections stained with anti-AMTN-1 and AMTN-2 IgG purified antibodies, respectively.

Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).



Affinity purification

Since the data obtained with the partially purified antibody, shown above, did not improve the previous results, the antisera were further purified via affinity chromatography. Two CNBr-activated sepharose 4B columns (GE HealthCare Bio-Sciences – Piscataway, NJ) were utilized for the purification of each antibody: one prepared with BSA (Albumin fraction V Powder A-3311, Sigma Aldrich – St. Louis, MO) and the other with the respective antigenic peptide (AMTN-1 or AMTN-2) coupled to BSA. After 5ml of blocking buffer was added, the columns were incubated for 3 hours with gentle rotation at room temperature. Subsequently, eight washes with 5ml of washing buffers, alternating between the two, were performed (100mM Tris, 500mM sodium chloride (pH8.0); 100mM glycine, 500mM sodium chloride (pH3.5), respectively) to discard any unbound material. The antibodies were filtered through a 0.22µm filter in a syringe and applied to both columns, twice through the BSA column and three times through the respective AMTN column. The columns were then washed with 10ml of PBS and the antibodies eluted with a solution containing both 50mM Glycine-HCl and 0.5M NaCl (pH 2.3). Ten fractions of 0.5ml were obtained, and optical density of eluted samples was checked using the spectrophotometer (DU-640 UV/VIS Scanning Spectrophotometer, Beckman Coulter, Fullerton, CA) at the wavelength 280nm, to estimate protein concentration. 20microL of each of the eluted fractions was subsequently separated by electrophoresis on a 12% polyacrylamide gel, stained with coomassie blue confirmed the presence of high protein concentration and purity

(Figure A1.6). Western blots were performed in order to further verify the specificity of the affinity purified antibodies. Two types of positive control were employed: the antigenic peptides AMTN-1 and AMTN-2, each coupled to BSA (Figure A1.7, lanes c and d); and a whole cell lysate obtained from the AM-1 cell line (demonstrated in Figure A1.7, lane b) spiked with 250ng of human bacterially expressed, recombinant AMTN protein (24kDa) (Figure A1.7, lanes d and f). The results validated the specificity of both antibodies. The immunohistochemistry procedure was then repeated and the results reported in Chapter 2.

Cryosections

One case of ameloblastoma obtained from a patient operated at Mount Sinai Hospital (Toronto, ON, Canada) (please see Appendix 6 for ethics approval) was also utilized for immunohistochemistry. The tissue was excised, preserved in liquid nitrogen, and cryosections were obtained. Samples were air dried for 15 minutes at room temperature, fixed in 100% acetone at -20C for 10 minutes and air dried for 30 minutes at room temperature. Washings, application of peroxidase block, primary and sencondary affinity purified antibodies and colour development, were performed as described in Chapter 2. No AMTN staining was observed in these sections, corroborating the results obtained with ameloblastoma paraffin samples (not shown).

Figure A1.6. Validation of anti-*AMTN* affinity purified antibodies used in the study. **A., B.** Coomassie blue stained gels showing purity and amount of AMTN-1 and AMTN-2 antibodies, (red), compared to increasing concentrations of BSA (ng/ml).

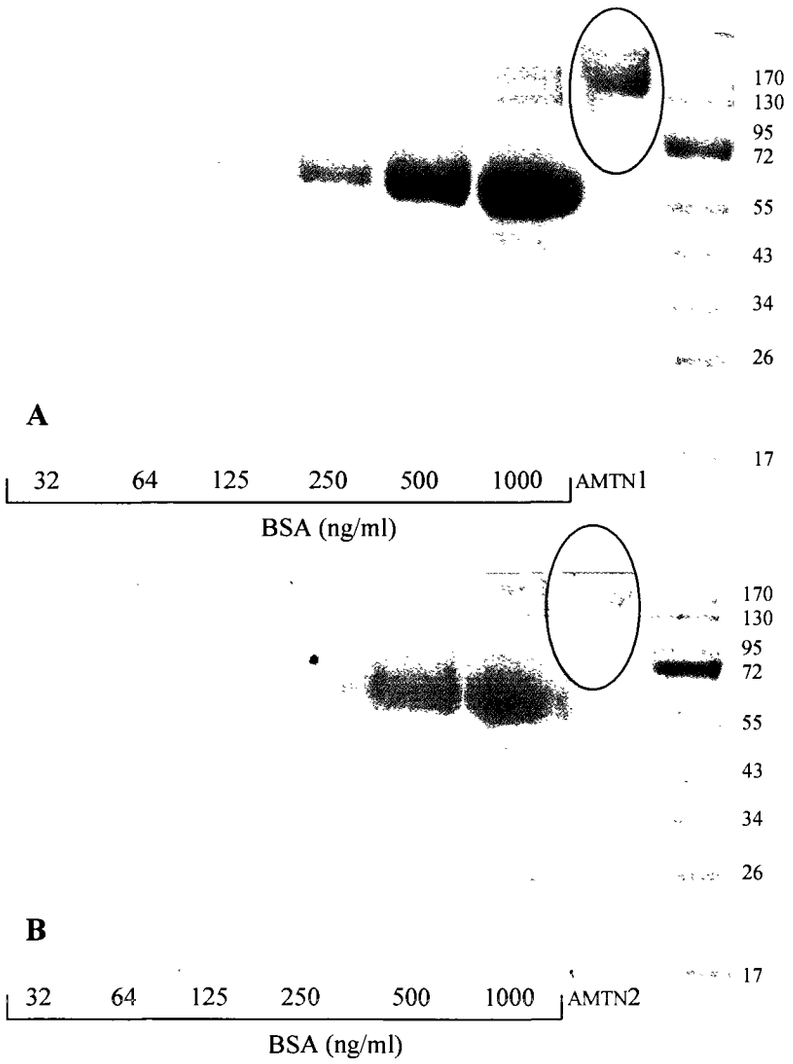
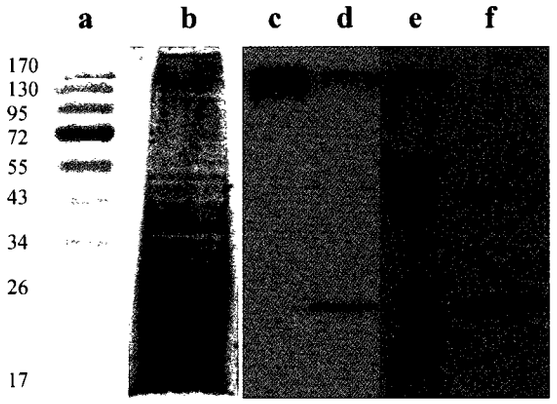


Figure A1.7. Coomassie blue stained gel showing molecular weight protein marker. **b.** Silver stained gel of a whole cell protein lysate prepared from the human Ameloblastoma cell line. The whole cell lysate has been spiked with 250ng of the Human Recombinant *AMTN* protein (Mw=24 kDa), and probed with anti-*AMTN-1* and anti-*AMTN-2* antibodies in lanes **d** and **f**, respectively. Antigenic peptides AMTN-1 and AMTN-2, each coupled to BSA, were also probed with anti-*AMTN-1* and anti-*AMTN-2* antibodies in lanes **c** and **e**, respectively.



Appendix 2. Peptide competition assay

As shown in Chapter 2, ameloblastomas generally presented negative results when tested with both anti-human, affinity purified AMTN antibodies. However, one exception is the single case presented below. This plexiform ameloblastoma showed a strong signal in the dense edematous stroma (Figure A2.1b'). Surprisingly, this pattern was not seen in the other plexiform cases tested. It may be suggested that the nature of stromal constituents may influence the signal, since the cases showing absence of signal presented a looser and more porous stroma.

In order to confirm the veracity of the signal, a peptide competition procedure was performed and showed that the staining is specific. This method is based on the incubation of the antibody with two different substrates: the antigenic peptide (human AMTN-1 (H2N-RLPTPSGTDDDFAVT-COOH) and human AMTN-2 (H2N-PPTKLAPDQGTLPNQ-COOH)), which it should specifically recognize, and a non-related peptide (mouse Zfp60 (H2N-EGDANRNITNKKE-COOH)). The incubation is held overnight, with gentle shaking at 4C. After centrifugation, the solutions are applied as the primary antibodies in an immunohistochemistry procedure. The antibody incubated with its competing antigenic peptide should provide a negative result, while the presence of a non-competing peptide should allow positive staining to the referred protein.

The results are shown in Figure A2.2. This procedure confirmed that the signal was abolished by competition with the specific antigenic peptide only.

However, since this pattern was not followed by any other plexiform case tested, and the staining is not concentrated in any epithelial cells of the tumor – considered the main active components of the lesion –, further experiments are warranted to verify the reproducibility of this result.

Figure A2.1. No AMTN signal was found in the ameloblastoma samples tested with the affinity purified antibodies, except for one plexiform case showing staining in the edematous stroma (**b'**). **a., b.** H&E staining; **a', b'.** AMTN staining. Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).

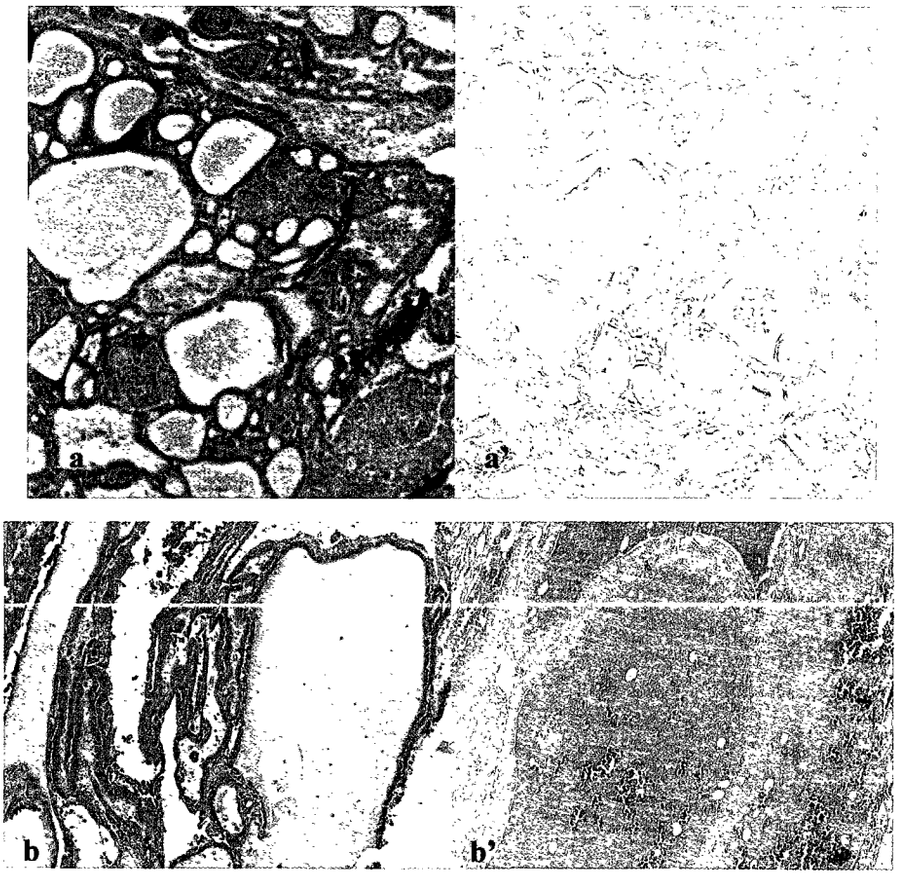
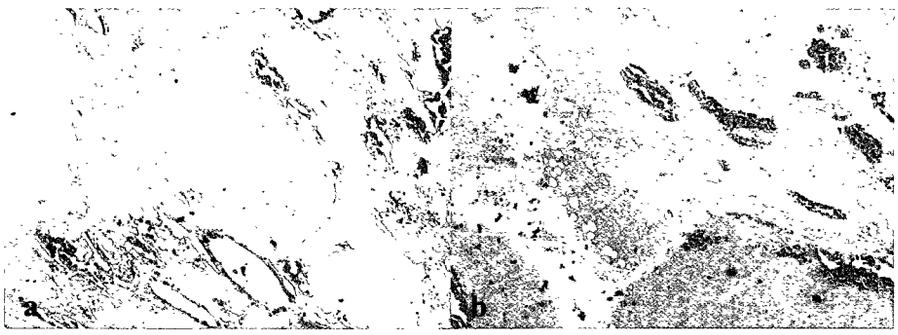


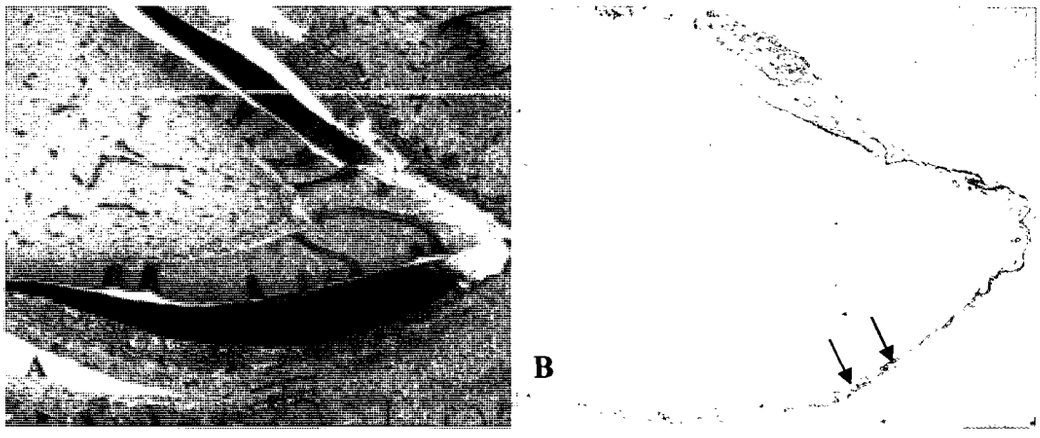
Figure A2.2. Peptide competition procedure performed in the only case of Ameloblastoma that presented AMTN staining, showing that the signal is authentic. **a.** Competition with the human peptide AMTN-1; **b.** Competition with the mouse peptide Kfp60.



Appendix 3. AMTN expression analysis in human tooth samples by immunohistochemistry

In the course of this work, paraffin-embedded human tooth tissue samples (age: 4 months; source anonymous) were supplied by the Department of Oral Pathology of Semmelweis University (Budapest, Hungary), which had been collected following all guidelines of the local ethics review board. These tissues were used as positive controls to test the affinity purified anti-human AMTN antibodies. The procedure was performed as described in Chapter 2. A strong and specific signal was observed in the ameloblast layer and also accumulating in the basal lamina between these cells and the enamel matrix, demonstrating the expected expression pattern of the protein (Figure A3.1).

Figure A3.1. Human tooth sample tested for AMTN-1 epitope, showing specific staining in the ameloblast layer (arrows). **A.** H&E staining; **B.** AMTN signal. Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).



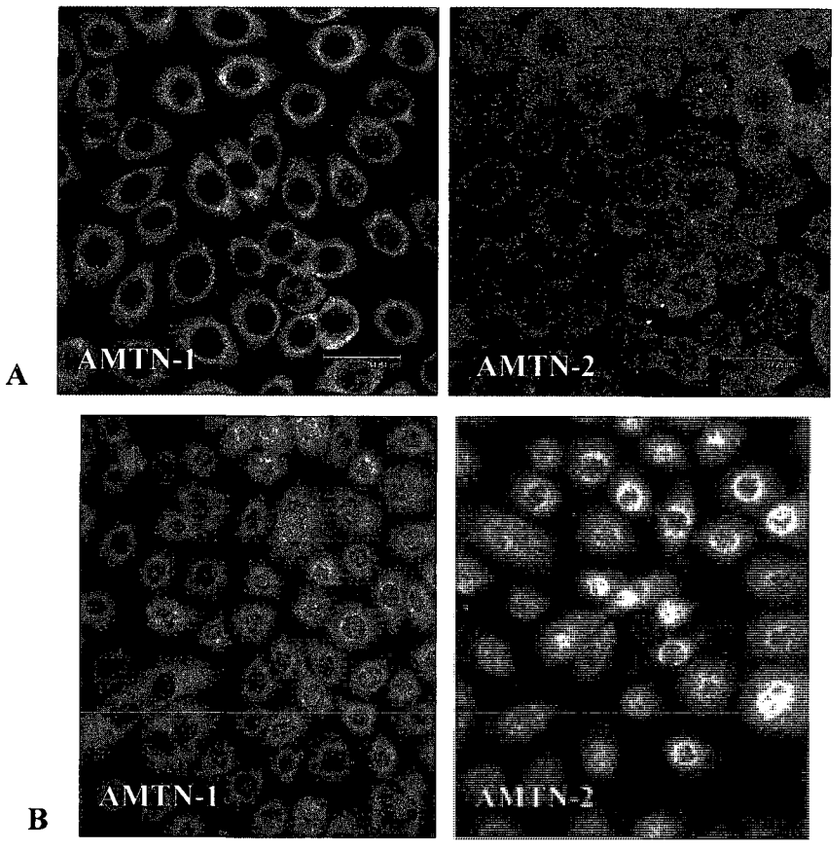
Appendix 4. AMTN expression analysis of AM-1 cell line by immunocytochemistry using crude and partially purified antibodies

After the first experiments with the AM-1 cell line had confirmed that these cells express AMTN mRNA (i.e. RT-PCR, shown in Chapter 3), immunocytochemistry analysis was carried out with the purpose of assessing the cellular distribution pattern of the protein. The results shown in this section were obtained with the IgG purified antibody.

Cells were grown in chamber slides and fixed with ice-cold Methanol for 10 minutes. The standard immunocytochemistry procedure described in Chapter 3 was utilized. The secondary antibody used was the fluorescein (FITC)-conjugated goat anti-rabbit IgG, fragment specific (Jackson Immuno111-096-047), at different concentrations (1:10, 1:30, 1:100). DAPI stain was applied for 5 minutes for nuclear blue color development according to manufacturer's instructions (Invitrogen, 2006). Confocal microscopic pictures revealed a mainly cytoplasmic signal with less background for samples stained with the antibody for AMTN-1, although a similar localization was observed for the epitope AMTN-2. These results are similar to those shown in Chapter 3 using the affinity purified antibodies, although a more specific signal for AMTN-2 was seen there. Figure A4.1 shows both results for comparison.

Figure A4.1. Immunolocalization of AMTN-1 and AMTN-2 proteins in the AM-1 cell line with IgG (A) and affinity purified (B) antibodies.

Negative control samples were stained with pre-immune serum at the same dilution used for primary antibodies (not shown).



Appendix 5. Mineralization assay performed with AM-1, cementoblast and osteoblast-like cell lines

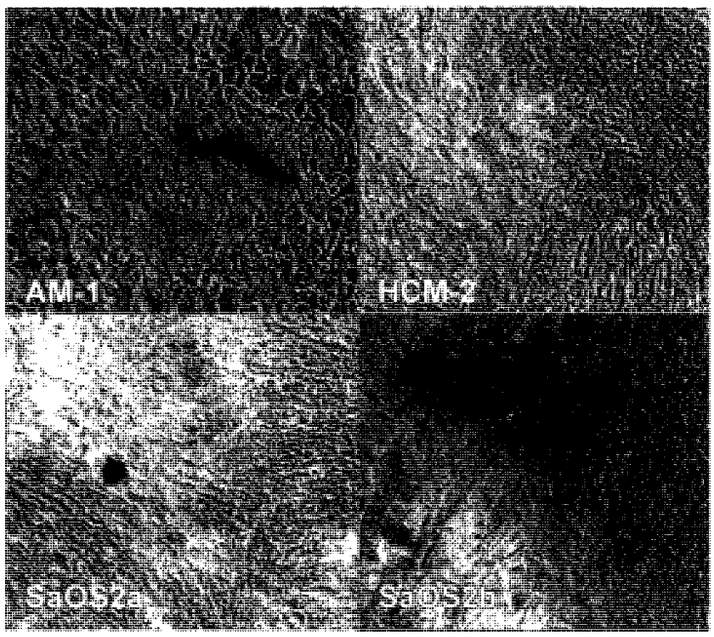
Initial mineralization assays were performed to investigate whether the AM-1 cell line would mineralize upon stimulation. Two additional cell lines of human origin were selected for comparison: the HCM-2 (cementoblast-like cell line) and SaOS2 (osteoblast-like cell line). These cell lines were obtained from Dr. Takashi Takata, Hiroshima University, Japan (Kitagawa et al. 2005) and the Department of Nutritional Sciences, University of Toronto, Canada (Platt et al. 2007), respectively. For the latter, two different FBS sources (utilized in the culture medium) were tested in separated groups: one obtained from Calsera (Etobicoke, Canada), and the other from Invitrogen (Carlsbad, CA).

Fifty µg/ml ascorbic acid and 10^{-8}M dexamethasone were freshly added to the regular culture medium. When cells reached confluency, 10 mM ß-glycerophosphate was also supplemented. Media were changed initially every second day, or every day as the cells became confluent. At the 28th day in culture, cells were fixed with 4% paraformaldehyde overnight, washed and left overnight in distilled water. Following the Von Kossa method, a solution of 2.5% silver nitrate in H2O was applied for 30 minutes in the dark. After several washes with distilled water, FC Buffer containing 25% formaldehyde and 5% sodium carbonate was added for 3 minutes, cells were washed extensively with tap water and dried (modified from Dhore et al. 2008). The results can be seen in Figure A5.1.

During this culture period, no substantial mineralized nodules evidenced by black staining were seen in any of the three cell lines; however, the level of deep yellow staining was elevated above background levels and, despite its variations in intensity, demonstrated that the cells had the potential to mineralize. Thus, the procedure was repeated for the AM-1 cell line only, utilizing the same method described above, except for two variations: no FBS was utilized in the media and the staining was performed after 35 days in culture. The successful results are shown in Chapter 3 (control samples of the mineralization assay described).

Culture media are usually supplemented with varying amounts of FBS. It has been demonstrated that at least a small amount of FBS is typically required for calcification (Hamlin and Price 2004; Platt et al. 2007). However, since AM-1 cells are grown under serum-free conditions, the presence of FBS may have affected the behavior of the cells in the first experiment, and this would possibly explain the lack of mineralization. Additionally, the timing of culture could also have had a role in the process; in the second experiment, the cells were maintained in culture for 7 additional days and produced substantial calcified nodules.

Figure A5.1. Von Kossa staining for ameloblastoma cells (AM-1), cementoblast-like cells (HCM-2), and osteoblast-like cells (SaOS2) treated with 2 different sources of FBS: SaOS2a, selected source as indicated in Platt et al., 2007 (Calsera (Etobicoke, Canada)); SaOSb, Invitrogen (Carlsbad, CA) source.



Appendix 6. Ethics Approval

The following institutional ethics approvals were obtained for the accomplishment of this study.

MOUNT SINAI HOSPITAL

Research Ethics Board

600 University Avenue, Room 1003A
Toronto, Ontario, Canada, M5G 1X5
t: (416) 586-4875 f: (416) 586-4715
www.mtsinai.on.ca

Notification of REB Initial Approval (Expedited)

Date: October 29, 2008

To: Dr. George Sandor
Department of Dentistry
Division of Dentistry
Mount Sinai Hospital
600 University Avenue
Room 412 Toronto, Ontario

Re: 08-0203-E
Evaluation of Amelotin Expression in Ameloblastomas

Sponsor:	
REB Review Type:	Expedited
REB Initial Approval Date:	29 October, 2008
REB Expiry Date:	29 October, 2009
Documents Approved:	Protocol: received 2008/09/02
	Consent Form: - 2008/10/20
Documents Acknowledged:	Material Transfer Agreement - 2008/09/03

The above named study has been reviewed and approved by the Mount Sinai Hospital Research Ethics Board. If, during the course of the research, there are any serious adverse events, any confidentiality concerns, changes in the approved protocol or consent form, or any new information that must be considered with respect to the project, these should be brought to the immediate attention of the REB. In the event of a privacy breach, you are responsible for reporting the breach to the MSH REB and the MSH Corporate Privacy Office (in accordance with Ontario health privacy legislation – Personal Health Information Protection Act, 2004). Additionally, the MSH REB requires reports of inappropriate/unauthorized use of the information.

If the study is expected to continue beyond the expiry date, you are responsible for ensuring the study receives re-approval. The REB must be notified of the completion or termination of this study and a final report provided. As the Principal Investigator, you are responsible for the ethical conduct of this study.

The MSH Research Ethics Board operates in compliance with the Tri-Council Policy Statement, ICH/GCP Guidelines and Part C, Division 5 of the Food and Drug Regulations of Health Canada.

Sincerely,

Tatiana Santini BHSc., RRCP/RRT
Research Ethicist

For: Ronald Heslegrave, Ph.D.
Chair, Mount Sinai Hospital Research Ethics Board

University of Toronto
Office of the Vice-President, Research

Office of Research Ethics

PROTOCOL REFERENCE #23906

March 16, 2009

Dr. Bernhard Ganss
CIHR Group Dynamics
Faculty of Dentistry
150 College Street
Fritzgerald Building, Room 234
Toronto, ON M5S 3E2

Ms. Daiana P. Stolf
CIHR Group in Matrix Dynamics
Faculty of Dentistry
150 College Street
Fritzgerald Building, Room 234
Toronto, ON M5S 3E2

Dear Dr. Ganss and Ms. Stolf:

Re: Administrative Approval of your research protocol entitled, "Evaluation of Amelotin Expression in Odontogenic Tumors"

We are writing to advise you that the Office of Research Ethics has granted administrative approval to the above-named research study. The level of approval is based on the following role(s) of the University, as you have identified with your submission:

- Graduate Student research – hospital-based only
- Storage or analysis of De-identified Personal Information (data)

This approval does not substitute for ethics approval, which has been obtained from your hospital Research Ethics Board. Please note that you do not need to submit Annual Renewals, Study Completion Reports or Amendments to the ORE unless the involvement of the University changes so that ethics review is required. Please contact the ORE to determine whether a particular change to the University's involvement requires ethics review.

Best wishes for the successful completion of your project.

Yours sincerely,

Daniel Gyewu
Research Ethics Coordinator