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Expression of astrocyte elevated gene-1 protein in ameloblastomas, keratocystic odontogenic tumors, and dentigerous cysts

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BACKGROUND: Benign epithelial odontogenic tumors such as ameloblastoma and keratocystic odontogenic tumor (KCOT) may exhibit an aggressive clinical course reminiscent of malignancies. Recent studies have indicated that astrocyte elevated gene-1 (AEG-1) is highly expressed in a variety of malignant neoplasms and its overexpression is associated with tumor invasion, metastasis, and poor survival. However, the role of AEG-1 in odontogenic tumors and cysts is still undiscovered.

METHODS: Immunohistochemical staining of AEG-1 was performed in 42 cases of ameloblastoma, 29 cases of KCOT, and 19 cases of dentigerous cyst. Correlations between AEG-1 expression and clinical parameters of ameloblastomas or KCOTs were statistically analyzed.

RESULTS: AEG-1-positive staining was found in 37 (88%) of 42 ameloblastomas and in 24 (83%) of 29 KCOTs. None of 19 dentigerous cysts were positive for AEG-1 protein. For ameloblastomas, AEG-1 protein expression was significantly higher in ameloblast-like cells than in stellate reticulum-like cells ($P = 0.003$). For KCOTs, AEG-1 protein was diffusely expressed in all lining epithelial cells except the superficial parakeratinized cells. Moreover, the frequency of cortical plate perforation was significantly higher in ameloblastomas with high AEG-1 expression than in ameloblastomas with low or negative AEG-1 expression ($P = 0.043$).

CONCLUSION: Significantly higher expression of AEG-1 protein in ameloblastomas and KCOTs than in dentigerous cysts and significantly greater frequency of cortical plate perforation in high AEG-1-expressed ameloblastomas than in low or negative AEG-1-expressed ameloblastomas may imply the high potential of AEG-1 to serve as a locally invasive biomarker and a target for novel therapy.

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Introduction

Odontogenic tumors are derived from epithelial, ectomesenchymal, and/or mesenchymal elements of the tooth-forming apparatus. The majority of these entities have been regarded as benign lesions. Nevertheless, certain types of epithelial odontogenic neoplasms such as ameloblastomas and keratocystic odontogenic tumors (KCOT) demonstrate locally invasive clinical features including extensive jaw bone resorption and prominent cortical plate expansion and perforation (1). The genes and proteins involved in these aggressive phenotypes have not yet been fully understood but are considered to be due to dysregulation of oncogenes, tumor-suppressor genes, growth factors, cell cycle regulators, apoptosis-related factors, cell adhesion molecules, angiogenic factors, osteolytic cytokines, and matrix-degrading proteinases (2).

Astrocyte elevated gene-1 (AEG-1) was initially cloned from HIV-1-infected primary human fetal astrocytes (3). Structurally, human AEG-1 is a type II transmembrane protein of 582 amino acids. AEG-1 mRNA is detected in heart, skeletal muscle, adrenal gland, and liver (4) and may be induced by human immunodeficiency virus (HIV)-1 (3), tumor necrosis factor (TNF)-α (5), and oncogenic Ha-ras (6). The major downstream signaling cascades activated by AEG-1 are nuclear factor (NF)-κB (5) and phosphoinositide 3-kinase (PI3K) pathways (6). AEG-1 has also been reported to physically interact with p65 subunit of NF-κB, cyclic adenosine monophosphate (AMP)-responsive element-binding protein-binding protein (CBP), and activator protein 1 (AP1) (5, 7). NF-κB and AP1 are transcription

factors binding to the promoters of matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9), which play an important role in tumor invasion and metastasis as well as in extracellular matrix remodeling and degradation. Expression of MMP-2 and MMP-9 in ameloblastomas and KCOTs has been shown to be associated with the local invasiveness of these two specific odontogenic tumors (8–13). Because AEG-1 is the upstream regulator of MMP-2 and MMP-9, it is interesting to know whether there is a higher expression of AEG-1 protein in aggressive odontogenic tumors such as ameloblastomas and KCOTs than in less aggressive odontogenic cysts such as dentigerous cysts.

Recently, AEG-1 gene, a newly established oncogene, has been found to be overexpressed in human neuroblastomas as well as esophageal and head and neck squamous cell carcinomas (14–16). High expression of AEG-1 protein is associated with tumor progression, invasion, and metastasis. Moreover, AEG-1 has also been reported to upregulate the expression of MMP-2 and MMP-9 in glioma and prostate cancers (17–19). In this study, we evaluated the AEG-1 protein expression statuses and patterns in ameloblastomas, KCOTs, and dentigerous cysts and assessed the correlations between AEG-1 protein expression and clinical parameters of ameloblastomas and KCOTs. We believe that the results of this study may give further insight on the understanding of aggressive clinical behavior of ameloblastomas and KCOTs.

Materials and methods

Sample selection

Formalin-fixed and paraffin-embedded tissue blocks of 42 cases of ameloblastoma, 29 cases of KCOT, and 19 cases of dentigerous cyst were retrieved from the archives of Department of Oral Pathology from 2003 to 2014. Diagnosis of the lesions was based on histological examination of hematoxylin and eosin-stained (H&E) tissue sections by two board-certified oral pathologists (YP Wang and CP Chiang). This study was approved by the Institutional Review Board of the National Taiwan University Hospital (NTUH IRB, No 201405070RIND).

Immunohistochemical staining

Tissue sections were cut 4 μm in thickness, deparaffinized, and rehydrated. Antigen retrieval was performed in the Trilogy buffer system (Cell Marque, Rocklin, CA, USA) in accordance with the manufacturer's instructions. Endogenous peroxidase activity was blocked by immersing the sections in 3% H_2O_2 in methanol for 30 min. After washing in phosphate-buffered saline (PBS), sections were incubated with 1% bovine serum albumin for 30 min to block nonspecific binding and then incubated with the anti-AEG-1 monoclonal antibody Lyric 4–7 with a concentration of 1 μg/ml for one hour at room temperature (20). Tissue sections were washed in PBS containing 0.1% Tween 20 and then treated with the polymer-based super sensitive IHC detection system (Biogenex, San Ramon, CA, USA), including incubation with super enhancer reagent for 20 min at room temperature, rinsing with PBS containing 0.1% Tween 20 for 5 min three times, and reaction with Poly-HRP reagent for 30 min at room temperature. Then, 0.02% diaminobenzidine hydrochloride containing 0.03% H_2O_2 was used as a chromogen to visualize peroxidase activity. The sections were counterstained with hematoxylin, mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA), and examined under an Olympus BX53 microscope (Olympus, Tokyo, Japan).

Evaluation of immunostained sections

Immunostained sections were observed under an Olympus BX53 microscope (Olympus). Tumor or cyst epithelial cells exhibiting predominantly a brown cytoplasmic staining were counted as positive for expression of AEG-1 protein in our samples. The sections were initially scanned at low power. For sections that showed heterogeneous staining, the predominant pattern was taken into account for scoring. At least five high-power fields were chosen randomly, and 1000 cells were counted for each case. The AEG-1 protein-labeling indices (LIs) were counted as a ratio of immunostaining-positive cells to the total number of cells counted. An eyepiece graticule was used to ensure that all cells were evaluated once only. Each of these assessments was independently carried by two investigators (YH Wu and YP Wang). The sections with an interobserver variation of more than 10% were reassessed using a double-headed light microscope to achieve consensus. In this study, the interobserver reproducibility was 93%. The slides with discrepant assessments were reevaluated, and a consensus was reached in all cases. The AEG-1 protein expression was further defined as follows: negative or −, LI < 10%; low expression or +, LI between 10% and 49%; and high expression or ++, LI ≧ 50%.

Statistical analysis

Comparisons of AEG-1 protein expression among different histological subtypes or cell types of ameloblastomas and different disease entities were analyzed by chi-square or Fisher's exact test, where appropriate. A *P*-value of less than 0.05 was considered statistically significant for all tests.

Results

Ameloblastomas

Of the 42 ameloblastomas, 28 (67%) were conventional solid ameloblastomas and 14 (33%) were unicystic ameloblastomas. Mural ameloblastomas were found in 12 (86%) of 14 unicystic ameloblastomas (Table 1). There were 25 male and 17 female patients. The mean age of the patients with ameloblastomas was 31.5 (range, 20–71) years. A half of 42 ameloblastomas were ≧5 cm and the other half were <5 cm in the greatest diameter. Five ameloblastomas were located in the maxilla and 37 in the mandible. Cortical plate perforation of the jaw bone by the tumor was present in 10 (24%) of 42 ameloblastomas (Table 2). The AEG-1-positive reaction product was predominantly found in the cytoplasm, especially in the perinuclear region (Fig. 1A–E). In addition, the AEG-1-positive staining was generally lost in stellate reticulum-like cells undergoing acanthomatous change (Fig. 1C). Focal nuclear stain was also observed. Of the 42 ameloblastomas, 8 showed high AEG-1 expression, 29 low AEG-1 expression, and 5 negative AEG-1 expression (Table 1). For

Table 1 Expression of AEG-1 protein in 28 conventional solid ameloblastomas, 14 mural ameloblastomas, 29 keratocystic odontogenic tumors, and 19 dentigerous cysts

		AEG-1 protein expression[a]		
	Case number	−	+	++
Conventional solid ameloblastoma	28			
Follicular	5	1	2	2
Plexiform	7	1	5	1
Acanthomatous	3	0	2	1
Desmoplastic	2	1	1	0
Mixed	11	0	9	2
Unicystic ameloblastoma	14			
Mural	12	2	8	2
Non-mural	2	0	2	0
Keratocystic odontogenic tumor	29	5	0	24
Dentigerous cyst	19	19	0	0

[a]Negative or −, labeling index (LI) < 10%; low expression or +, LI between 10% and 49%; and high expression or ++, LI ≧ 50%.

Table 2 Correlations between AEG-1 protein expression and clinical parameters of 42 ameloblastomas

		AEG-1 protein expression		
Clinical parameter		*High*[a] *Case number (%)*	*Low or negative*[a] *Case number (%)*	P-*value (Fisher's exact test)*
Age (years)	≧30	6 (86%)	12 (34%)	0.031
	<30	1 (14%)	23 (66%)	
Sex	Male	3 (43%)	22 (63%)	0.413
	Female	4 (57%)	13 (37%)	
Greatest diameter (cm)	≧5	3 (43%)	18 (51%)	1.000
	<5	4 (57%)	17 (49%)	
Location	Maxilla	0 (0%)	5 (14%)	0.569
	Mandible	7 (100%)	30 (86%)	
Cortical plate perforation	Yes	4 (57%)	6 (17%)	0.043
	No	3 (43%)	29 (83%)	
Total		7 (100%)	35 (100%)	

[a]High expression, labeling index (LI) ≧ 50%; low expression, LI between 10% and 49%; and negative, LI < 10%.

AEG-1 protein expression in different cell types, AEG-1 protein expression was significantly higher in ameloblast-like cells (−, 6 cases; +, 26 cases; ++, 10 cases) than in stellate reticulum-like cells (−, 11 cases; +, 31 cases; ++, 0 case; $P = 0.003$). The frequency of cortical plate perforation was significantly higher in ameloblastomas with high AEG-1 expression than in ameloblastomas with low or negative AEG-1 expression ($P = 0.043$) (Table 2). Moreover, there was a significant association of high AEG-1 expression with patients of relatively elder age (≧30 years, $P = 0.031$) (Table 2). However, no significant difference in AEG-1 protein expression was observed among different histological subtypes of ameloblastoma.

Keratocystic odontogenic tumors

There were 12 male and 17 female KCOT patients. The mean age of 29 KCOT patients was 35.6 (range 20–75) years. AEG-1 protein was diffusely expressed in all lining epithelial cells except the superficial parakeratinized cells (Fig. 1E). The AEG-1 reaction product was located mainly in the cytoplasm and focally in the nuclei of the positive epithelial cells. Furthermore, there was a relatively stronger AEG-1 staining in the basal cells than in the suprabasal cells (Fig. 1E). The AEG-1-positive rate was 83% (24/29) for KCOTs and 88% (37/42) for ameloblastomas; there was no significant difference in the AEG-1-positive rate between these two odontogenic tumors (Table 1). In addition, there was also no significant association of AEG-1 protein expression with any one of 5 clinical parameters (age, sex, size, location, and cortical plate perforation) of KCOTs (data not shown).

Dentigerous cysts

There were 10 male and 9 female dentigerous cyst patients. The mean age of 19 dentigerous cyst patients was 22.7 (range 20-51) years. All the 19 dentigerous cysts were negative for AEG-1 (Table 1 and Fig. 1F). Moreover, the AEG-1-positive rate was significantly higher in ameloblastomas (88%) and KCOTs (83%) than in dentigerous cysts (0%, $P < 0.001$).

Discussion

This study showed high AEG-1 expression in 24 of 29 KCOTs and negative AEG-1 expression in only 5 of 29 KCOTs. This finding suggests that the AEG-1 protein has high potential to act as a biomarker for KCOTs. Furthermore, this study demonstrated a significantly higher expression of AEG-1 protein in locally invasive odontogenic tumors such as ameloblastomas and KCOTs than in less aggressive odontogenic cysts such as dentigerous cysts. The frequency of cortical plate perforation was also significantly higher in ameloblastomas with high AEG-1 expression than in ameloblastomas with low or negative AEG-1 expression. These results indicate that the high AEG-1 protein expression is related to the locally invasive clinical behavior of ameloblastomas and KCOTs. A previous study has shown the expression of activated MMPs 1, 2, and 9 in ameloblastomas. It is also found that inhibition of MMP-2 activity may subsequently suppress the local invasiveness of ameloblastoma cells (8). Moreover, the expression of MMP-2 and MMP-9 is detected on the cell surfaces and in the cytoplasm, respectively, of peripheral columnar cells of the tumor nests in ameloblastomas (9). Because MMP2 and MMP-9 can degrade bone matrix and in turn release mitogenic factors to enhance tumor cell proliferation, MMP2 and MMP-9 may play an important role in the local invasiveness of ameloblastomas (10). For KCOTs, the MMP-2 expression is found in all examined samples and in the basal cells and basement membrane zone of the detached lining epithelium (11). Furthermore, the active form of MMP-9 is frequently detected in KCOTs (12). In addition, both NF-κB and MMP-9 are more highly expressed in the lining epithelia of KCOTs than in those of dentigerous cysts and radicular cysts (13). In the present study, the AEG-1-positive reaction products were mainly located in the cytoplasm of ameloblast-like cells of ameloblastomas and in nearly all lining epithelial cells of KCOTs. Because high expression of MMP2 and MMP9 is

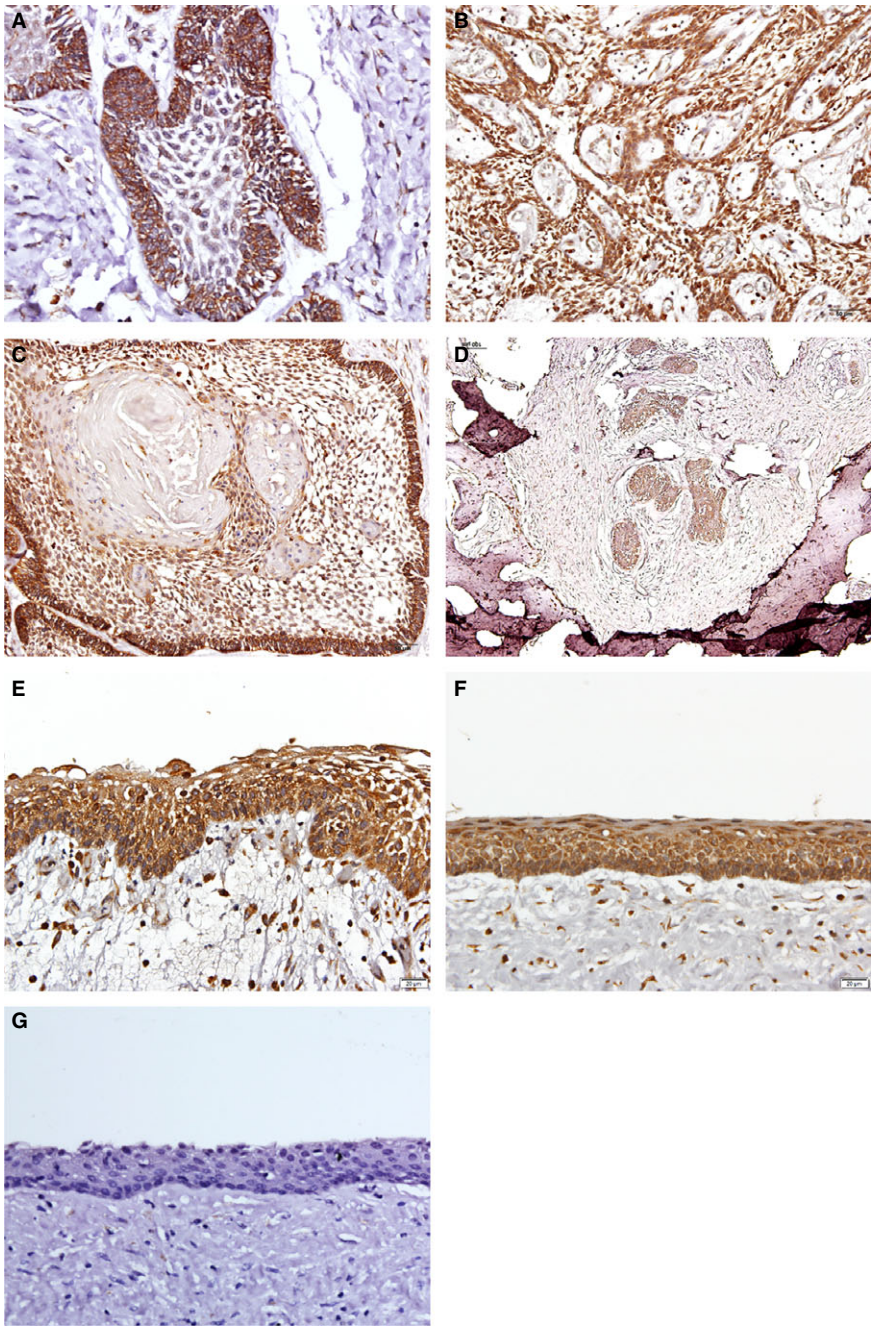

Figure 1 Immunohistochemical staining for AEG-1 protein in ameloblastomas, keratocystic odontogenic tumors, and dentigerous cysts. (A) A follicular ameloblastoma showing strong AEG-1 staining in peripheral ameloblast-like cells and weak AEG-1 staining in stellate reticulum-like cells of the tumor nest. (B) A plexiform ameloblastoma exhibiting strong AEG-1 staining in both peripheral ameloblast-like cells and central stellate reticulum-like cells. (C) An acanthomatous ameloblastoma demonstrating strong AEG-1 staining in peripheral ameloblast-like cells, moderate AEG-1 staining in stellate reticulum-like cells, and negative AEG-1 staining in central acanthomatous cells of the tumor nest. (D) An ameloblastoma showing high AEG-1-expressed tumor nests that invade into bone marrow tissue. (E) A unicystic ameloblastoma showing strong AEG-1 staining in all ameloblastomatous lining epithelial cells. (F) A keratocystic odontogenic tumor exhibiting strong AEG-1 staining in all stratified squamous lining epithelial cells except the superficial parakeratinized cells. (G) A dentigerous cyst showing a negative AEG-1 staining in the stratified squamous lining epithelium. (original magnification, A, E, F, and G, 40 $\times$; B, C, and D, 20 $\times$).

significantly associated with the local invasiveness of ameloblastomas and KCOTs and AEG-1 is the upstream regulator of MMP-2 and MMP-9, it is easy to understand why significantly higher expression of AEG-1 in ameloblastomas and KCOTs than in dentigerous cysts can explain the local invasive clinical feature of these two odontogenic tumors. However, further studies such as MMP2/9 promoter-driven luciferase assays in cell lines of ameloblastoma are required to confirm our findings.

In murine embryonic development, AEG-1 is found to be expressed in the first pharyngeal arches. Moreover, co-localization of AEG-1 and Ki-67 has been found in the

epidermis compartment of the skin between E12.5 and E18.5 in murine model (21). Previous studies have demonstrated the expression of Ki-67 in all basal lining epithelial cells of KCOT (22) but in only few basal lining epithelial cells of dentigerous cysts (23). Furthermore, Ki-67 expression is also found predominantly in ameloblast-like cells in solid ameloblastoma and unicystic ameloblastomas and there is a higher Ki-67 expression in solid ameloblastoma than in unicystic ameloblastomas (24, 25). Our study also showed diffuse and strong AEG-1-positive stain in nearly all lining epithelial cells of KCOTs and ameloblast-like cells of ameloblastomas but no AEG-1-positive stain in lining epithelial cells of dentigerous cysts. Expression of AEG-1 in murine embryonic cells and co-localization of AEG-1 and Ki-67 in the same tumor epithelial cells in ameloblastomas and KCOTs suggest that AEG-1 may be involved in murine embryonic development and proliferation of tumor cells of ameloblastomas and lining epithelial cells of KCOTs.

In addition to augmentation of cellular proliferation, AEG-1 is also involved in resistance of apoptosis, as exemplified in retinoblastoma and renal cell carcinoma cells (26, 27). Knockdown of AEG-1 in Caki renal cell carcinoma cells results in the appearance of apoptotic bodies and remarkable elevation of the ratio of apoptotic cells. Expression of the anti-apoptotic factor Bcl-2 is also significantly reduced, whereas the pro-apoptotic factors Bax, caspase-3, and poly (ADP-ribose) polymerase (PARP) are significantly activated (27). Similar results have also been confirmed in human retinoblastomas, that is, significantly reduced apoptosis rate as well as increased Bax/Bcl-2 ratio and cleaved-caspase-3 protein level are demonstrated in AEG-1-siRNA lentivirus-transfected cells when compared with the control cells (28). It is also interest to find that the Bcl-2-positive rate (85%) of ameloblastomas or KCOTs is comparable to AEG-1-positive rate of ameloblastomas (88%) or KCOTs (83%) (29). Furthermore, most of the Bcl-2-positive signals are found in the peripheral ameloblast-like cells, while most of the central stellate reticulum-like cells are positive for Bax and Bak (28). The upregulation of sonic hedgehog (SHH) signaling axis, including Bcl-2, is also noted in KCOTs with a strong resemblance to ameloblastomas rather than odontogenic cysts (30). These results suggest AEG-1 may play important roles in proliferation and anti-apoptosis of tumor cells and augmentation of matrix-degradation ability in ameloblastomas and KCOTs, which in turn bolster the locally aggressive behavior of these two odontogenic tumors and finally leading to considerable bony destruction of the primary tumor sites.

In this study, the ameloblastomas with high AEG-1 protein expression were more frequently found in patients ≧30 years of age than in patients <30 years of age. In neuroblastomas and gliomas, patients with high AEG-1 protein expression also have significantly elder age than patients with low AEG-1 protein expression (14, 19). Although there is a significant association of high AEG-1 protein expression with elder age of the patients, the underlying mechanisms that lead to this significant association need further studies.

In conclusion, significantly higher AEG-1 protein expression in ameloblastomas and KCOTs than in dentigerous cysts and significantly greater frequency of cortical plate perforation in ameloblastomas with high AEG-1 expression than in ameloblastomas with low or negative AEG-1 expression indicate that the AEG-1 protein has a high potential to act as a biomarker for aggressive clinical behavior and a target for novel therapy.

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Conflicts of interest

The authors have no conflict of interests relevant to this article.