The involvement of growth differentiation factor 5 (GDF5) and aggrecan in the epithelial–mesenchymal transition of salivary gland pleomorphic adenoma

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Abstract

Pleomorphic adenoma is the most common salivary gland tumor with annual incidence of 2–3.5/100 000 in population. The histogenesis of salivary gland pleomorphic adenoma is still unclear. One concept sustains the existence of an epithelial–mesenchymal transition (EMT) process in pleomorphic adenomas by which neoplastic epithelial cells transdifferentiate into mesenchymal cells and leading to tissue heterogeneity from this salivary gland neoplasia. Here we investigate by immunohistochemistry the expression of growth differentiation factor 5 (GDF5) and aggrecan in 15 cases of salivary gland pleomorphic adenomas. We found that both markers were present in normal salivary gland, mainly in the cells that line striated and intercalated ducts suggesting their involvement in the morphogenesis of this duct system. A constant positive reactivity for both markers was recorded in transition regions from tubular proliferative units to myxoid areas suggesting the involvement of an EMT process during the tumorigenesis of such salivary gland neoplasia. Also, both markers may be implicated in the transdifferentiation of neoplastic myoepithelial cells from the myxoid zones to lacuna cells of adjacent chondroid areas completing the morphology of this salivary gland tumor.

Keywords: aggrecan, epithelial–mesenchymal transition, growth differentiation factor 5, pleomorphic adenoma, salivary gland.

Introduction

Pleomorphic adenoma is the most common salivary gland tumor, comprising 45% to 75% of all salivary gland neoplasms [1, 2], and average annual age-adjusted incidence rate per 100 000 of 2–3.5 [3]. Usually it has a benign behavior, but can recur after inappropriate treatment [4], especially those developed in parotid glands [5], and 2–8.5% of cases could became malignant [6, 7].

The histogenesis of salivary gland pleomorphic adenoma still remains a controversial topic. Thus, while some authors suggest different-mesenchymal and (myo) epithelial-origins for the two tumor components [8–11], others support the origin from a single cell type: either epithelial [12, 13], modified myoepithelial [12–14], or mesenchymal [9, 15] cells type. In support of the later origin theory, some authors sustain the existence of an epithelial–mesenchymal transitions (EMT) process in pleomorphic adenomas by which neoplastic epithelial cells transdifferentiate into mesenchymal cells leading thus to the tissue heterogeneity from this salivary gland neoplasia [16].

The EMT plays key roles during embryogenesis and also in wound healing and regeneration of fully differentiated tissues [17, 18]. In the last decade, the EMT have been implicated in fibrosis [20, 21] and cancer metastasis [19]. In fact, the process itself means down-regulation of epithelial markers and an upregulation of mesenchymal markers, accompanied by an increase in cell migration and invasion [22]. Such EMT features were noticed in human cancers developed in colon [23], esophagus [24], breast [25] and ovary [26]. EMT is involved in tumor invasion process by: loss of apicobasal polarity [27] and cell–cell contacts [28], cytoskeleton and matrix remodeling [29, 30], together with increasing motility and invasiveness [31–33].

In this paper, we investigated the immunohistochemical expression of growth differentiation factor 5 (GDF5) and aggrecan in 15 cases of salivary gland pleomorphic adenomas, and their involvement in the EMT process.

Materials and Methods

We reviewed medical records from the Laboratory of Pathology, Emergency County Hospital of Craiova, Romania, and identified 15 patients who had been diagnosed with salivary gland pleomorphic adenomas. Twelve specimens included normal salivary gland tissues. Tumor samples were obtained during surgical removal of tumors in the Department of Oral and Maxillofacial Surgery of the same hospital and classified in accordance with the WHO classification of salivary gland tumors [34].
The study was carried out with full local ethics approval.

Surgical specimens were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin wax. Sections of 4-mm thick were then prepared and routinely stained with Hematoxylin–Eosin stain.

Immunohistochemistry was performed on 4 μm sections from one selected block for each case. The sections were deparaffinized in xylene, dehydrated in ethanol, and immersed in distilled water containing 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Then, we performed an antigen-unmasking step by 20 minutes heat induced epitope retrieval in DakoCytomation Target Retrieval solution, code S1700. Subsequently, the unspecific binding sites were blocked with a 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for one hour. Briefly, the primary antibodies were used at a dilution of 1:100 for GDF5 (rabbit polyclonal, Sigma-Aldrich, Cheminpress, Romania, code HPA015648) and 1:100 for Aggrecan (mouse monoclonal, 4F4, Santa Cruz Biotechnology Redox, Romania, code sc-33695). The primary antibodies were amplified with biotinylated species-specific secondaries and a LSAB2 (Dako, Redox, Romania, code K0675) system. Visualization was done with 3,3′-diaminobenzidine (DAB) (Dako, Redox, Romania, code K3468). For counterstaining, we used Mayer’s Hematoxylin.

Negative-control stainings were done by omitting the primary antibodies.

To establish the myoepithelial origin of some neoplastic cells that were positive to the above-specified primary antibodies, we used double sequentially immunostaining reactions. After DAB staining for GDF5 and respective Aggrecan, the anti-p63 (1:50, mouse monoclonal, 4A4, Thermo Fisher Scientific, Cheminpress, Romania, code MA1-21871) antibody and respective anti-Smooth Muscle Actin (1:100, rabbit polyclonal, Thermo Fisher Scientific, Cheminpress, Romania, code PA1-37024) antibody was added, detected with a goat anti-rabbit biotinylated secondary antibody (1:400, 30 minutes at room temperature, Dako), and amplified with ABC-AP (alkaline phosphatase) (1:100, Dako). The new signal was detected with fast red (Dako), and the slides were coverslipped in a glycerol-based mounting medium (Dako). In order to eliminate the secondary biotinylated antibodies cross-reactivity after the first reaction an unmasking blocking step was made by incubating the slides with Avidin/Biotin kit for 30 minutes (Dako).

The reactions were assessed only qualitative, the intensity of marker expression being quantified using the following scores: 0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive.

The images were acquired by utilizing a Nikon Eclipse 55i microscope (Nikon, Apidrag, Bucharest, Romania) equipped with a 5-megapixel cooled CCD camera and the Image ProPlus AMS7 software (Media Cybernetics Inc., Buckinghamshire, UK).

 résultats

Clinicopathological data

According to the medical records, the median age for the investigated salivary pleomorphic adenomas was around 43 years, with the parotid (80%) as the most frequent involved salivary gland. The majority of pleomorphic adenomas were with well-balanced stromal/parenchyma components (eight cases), in four cases prevailed the parenchyma and in the remainder the stroma was much better represented. The epithelial component consisted mainly of tubulo-glandular structures (45%), followed by solid areas (25%), fascicle/trabecular proliferation (5%), nests of cells (5%) and other type of cellular patterns. The main encountered stromal type was the myxoid (90%), followed by the chondroid and chondro-myxoid (60%), fibrous (30%) and hyaline (40%).

GDF5 immunoreactivity

Immunoreactions for GDF5 were observed through the entire salivary gland parenchyma both in secretory and excretory units. The most intense reaction was noticed in the cytoplasm of both intra- and interlobular ducts (Figure 1, A and B). In the secretory units, the reaction had a lower intensity with some areas lacking reactivity (Figure 1C). Also, a weak immunoreaction was present in endothelial and inflammatory cells (especial plasma cells) (Figure 1D).

The GDF5 reactivity in salivary pleomorphic adenomas was variable with immunostaining intensity varying between tumor areas and from one case to another. In the tumoral parenchyma, the most intense reaction was observed in neoplastic tubular structures (Figure 1, E and F). The luminal neoplastic cells of these structures were more reactive than abluminal cells. In the solid proliferative areas, the reaction was less intense and more obvious in the neoplastic cells that were negative to the p63 myoepithelial marker (Figure 2, A and B).

In the myxoid areas, the GDF5 reactivity was seen at the level of modified myoepithelial cells that had stellate or plasmacytoid morphology (Figure 2, C and D), peculiar around proliferative neoplastic tubular structures. The majority of these cells were negative to the p63 myoepithelial marker. In the chondroid stromal areas, the GDF5 immunostaining was noticed around the lacunae and in the cytoplasm of lacuna cells (Figure 2, E and F).

Aggrecan immunoreactivity

In the normal salivary glands, we saw positive immunoreactions at the level of both intra- and interlobular ducts. The reaction pattern was cytoplasmic and was present in the cells that line striated and intercalated ducts (Figure 3A) and also in cells from the bistratified interlobular ducts (Figure 3B).

In the pleomorphic adenomas specimens, the reactivity was constant present through the entire tumor in both epithelial proliferative areas and stromal regions, but with different intensities from one tumor area to another. In the epithelial proliferative areas, the immunostaining was seeing both in solid areas and in those with ductal pattern differentiation. In the solid areas, the reaction was inconstant with some areas being entirely negative (Figure 3C), and other with focal cytoplasmic reaction at the level of few cuboidal neoplastic myoepithelial that were also positive to α-SMA (Figure 3D). In the
epithelial tumors areas with ductal differentiation the reaction was seen especially at the level of luminal cells and less frequently in the abluminal cells (Figure 3, E and F).

At the level of “tubulo-myxoid” proliferative units, we noticed a gradual increase of aggrecan reactivity from the tubular structures to the surrounding myxoid areas (Figure 4A). In the myxoid areas, the aggrecan deposition was seen both in the intercellular spaces and cytoplasm of spindle-shaped or plasmacytoid cells (Figure 4, B and C). Some of these cells were myoepithelial at the origin being positive to α-SMA. In the stromal chondroid areas the reactivity was present both in the matrix and lacuna cells (Figure 4, D and E). The reaction was more intense in the matrix and at the interface of proliferative epithelium and myxoid/chondroid stroma (Figure 4F). Very few lacuna cells were both positive to aggrecan and α-SMA.

Figure 1 – GDF5 immunoreactivity (DAB-brown) in: (A and B) Normal salivary gland at the level of both intra- and interlobular ducts, ×100; (C) A weak or negative reaction in the secretory units of normal salivary gland, ×200; (D) Weak reactivity in endothelial and inflammatory cells (especially plasma cells), ×200; (E and F) Intense positive reaction in the neoplastic tubular structures, ×200.
Figure 2 – GDF5 immunoreactivity (DAB-brown) in: (A and B) Weak reactivity in the solid proliferative areas with some neoplastic cells negative to the p63 myoepithelial marker (Fast Red – red), ×200; (C and D) Intense positive reaction in the modified myoepithelial cells that had stellate or plasmacytoid morphology from myxoid areas, ×200; (E and F) Positive reaction around the lacunae and in the cytoplasm of lacuna cells from the chondroid stromal areas, ×200.

Figure 3 – Aggrecan immunoreactivity (DAB-brown) in: (A and B) Positive reaction at the level of both intra- and interlobular ducts, ×200.
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Figure 3 (continued) – Aggrecan immunoreactivity (DAB-brown) in: (C) Weak or negative expression in the solid proliferative areas, ×200; (D) Some of the neoplastic cells of these areas were also positive to α-SMA; (E and F) Intense positive reaction in the neoplastic tubular structures, ×200.

Figure 4 – Aggrecan immunoreactivity (DAB-brown) in: (A) A gradual increase of reactivity from the tubular structures to the surrounding myxoid areas, ×200; (B and C) Aggrecan deposition both in the intercellular spaces and cytoplasm of spindle-shaped or plasmacytoid cells, ×200; (D) Positive reaction both in the matrix and lacuna cells, ×200.
earliest stages of endochondral ossification, including chondrocytes (hypertrophy and calcification) and at the CDMP-2 is involved in the terminal differentiation of ossifying long-bone centers [46]. Most probable the expression was restricted to the hypertrophic chondrocytes of mesenchymal condensation and throughout the cartilaginous [44, 45]. Studies on human embryos proved that CDMP-1 is expressed in the developing central nervous system [43], and has a role in skeletal and joint development, including angiogenesis and osteoblast differentiation [47].

CDMP-1 and CDMP-2 was not related to maintenance of myoepithelial cells to lacuna cells in an autocrine manner. Both CDMP-1 could play a role in the acceleration of the progenitor phenotype [49]. The authors suggest that CDMP-1 could play a role in the acceleration of the transdifferentiation from cuboidal neoplastic myoepithelial cells to lacuna cells in an autocrine manner. Both CDMP-1 and CDMP-2 was not expressed in the lacuna cells of the tumor chondroid areas and, thus was concluded that CDMP-1 and -2 were not related to maintenance of the chondroid phenotype of the lacuna cells. Also, the

Discussion

Pleomorphic adenoma is characterized by morphological plasticity, which seems to be mainly due to the myoepithelial cells component of this salivary gland neoplasm [35]. Initially, these cells are attributed solely with a contractile function, but more recently, it was proved their involvement in the embryonic development, extracellular matrix synthesis and remodeling and paracrine signaling [36]. Moreover, in breast cancer was proved that myoepithelial cell inhibit proliferation, angiogenesis and tumor cell invasion [37, 38]. During neoplastic transformation the myoepithelial cell change its immunophenotype by losing immunoreactivity for some markers and acquire the expression of other markers [36], which could explain different morphological and cytological features peculiar to such salivary gland neoplasm.

Other studies proved the existence of an EMT process during pleomorphic adenoma tumorigenesis with a complete transition pathway from a pure epithelial to a mixed and finally a true mesenchymal cell phenotype within pleomorphic adenomas [10, 12, 15, 16, 39–41]. In this process, ductal epithelial cells express some mesenchymal genes [16], some of which are involved in bidirectional mesenchymal–epithelial transition [42].

To elucidate the contribution of the epithelial and or the myoepithelial compartments to the histogenesis of this salivary gland neoplasm we immunohistochemically investigated the expression of GDF5 and aggrecan in 15 specimens of pleomorphic adenomas.

Growth differentiation factor 5 (also named Cartilage-Derived Morphogenetic Protein 1) is a member of the bone morphogenetic protein (BMP) family and the transforming growth factor (TGF)-beta superfamily that is expressed in the developing central nervous system [43], and has a role in skeletal and joint development [44, 45]. Studies on human embryos proved that CDMP-1 was found predominantly at the stage of precartilaginous mesenchymal condensation and throughout the cartilaginous cores of the developing long bones; whereas CDMP-2 expression was restricted to the hypertrophic chondrocytes of ossifying long-bone centers [46]. Most probable the CDMP-2 is involved in the terminal differentiation of chondrocytes (hypertrophy and calcification) and at the earliest stages of endochondral ossification, including angiogenesis and osteoblast differentiation [47].

Aggrecan also known as chondroitin sulfate proteoglycan 1 is a critical component for cartilage structure and the function of joints, mediating chondrocyte–chondrocyte and chondrocyte–matrix interactions through its ability to bind hyaluronan [48].

In our study, GDF5 immunoreactivity was present in normal salivary gland, both in secretory and excretory units with the most obvious reaction in the cytoplasm of luminal cells of intra- and interlobular ducts. Kusafuka K et al. proved that CDMP-1 was expressed in the striated and intercalated ducts of the normal salivary glands, suggesting its involvement in branching morphogenesis of this duct system rather than in maintenance of myoepithelial cells in the normal salivary glands [49]. The expression of CDMP-2 was not observed in normal salivary glands.

Regarding GDF5 immunoreactivity in salivary pleomorphic adenoma, our study proved a strong reaction in neoplastic tubular structures, peculiar in the cytoplasm of luminal cells. A weak reactivity was also seen in the abluminal cells and in some neoplastic cells from the solid proliferative areas. In the stroma, GDF5 was positive in the cytoplasm of modified myoepithelial cells with stellate or plasmacytoid morphology that surround the proliferative neoplastic tubular structures. Such reactivity may suggest a transition of epithelial luminal cells to myoepithelial/mesenchymal cell phenotype. Moreover, Ellis GL and Auclair PL have suspected that plasmacytoid and spindle cells to be in transition from one form to the other [1]. In addition, Ogawa Y et al. hypothesized that plasmacytoid cells could originate from luminal rather than myoepithelial cells [50].

We also found GDF5 immunoreactivity in the cytoplasm of lacuna cells and in the chondroid matrix around these lacunae. In the study of Kusafuka K et al., CDMP-1 was expressed in the cuboidal neoplastic myoepithelial cells in the hypocellular areas, which were considered to have morphologically a pre-chondroprogenitor phenotype [49]. The authors suggest that CDMP-1 could play a role in the acceleration of the transdifferentiation from cuboidal neoplastic myoepithelial cells to lacuna cells in an autocrine manner. Both CDMP-1 and CDMP-2 was not expressed in the lacuna cells of the tumor chondroid areas and, thus was concluded that CDMP-1 and -2 were not related to maintenance of the chondroid phenotype of the lacuna cells. Also, the
authors found that these cells had a similar phenotype (CDMP-2 negative) with the mature to upper hypertrophic chondrocytes [49].

Regarding aggrecan reactivity, our study proved a positive reaction at the level of both intra- and interlobular ducts from normal salivary glands with the highest intensity in the cells that line striated and intercalated ducts. Contrary, Kusafuka K et al. reported no aggrecan expression in the normal salivary gland tissue [51].

In the pleomorphic adenomas specimens, the reactivity was constantly present through the entire tumor in both epithelial proliferative areas and stromal regions. In the tumor parenchyma, the highest reactivity was recorded at the level of luminal cells from neoplastic structures with ductal differentiation. In addition, at the level of “tubulo-myxoid” proliferative units, we noticed a gradual increase of aggrecan reactivity from the tubular structures to the surrounding myxoid areas. In the myxoid areas, the aggrecan deposition was seen both in the intercellular spaces and cytoplasm of spindle-shaped or plasmacytoid cells. Some of these cells had a-α-SMA reactivity proving a myoepithelial origin while other were negative to this marker suggesting an epithelial luminal cells origin. Previous studies proved that aggrecan which is a marker suggesting an epithelial luminal cells origin. Some of these cells had a-α-SMA reactivity proving a myoepithelial origin while other were negative to this marker suggesting an epithelial luminal cells origin. Also, both markers may be implicated in the trans-differentiation of neoplastic myoepithelial cells from the myxoid zones to lacuna cells of adjacent chondroid areas completing the morphology of this salivary gland tumor.

**References**


**Conclusions**

Our study proves that both GDF5 and aggrecan were present in normal salivary gland, mainly in the cells that line striated and intercalated ducts suggesting their involvement in the morphogenesis of this duct system.

A constant positive reactivity for both markers was recorded in transition from tubular proliferative units to myxoid areas suggesting the involvement of an EMT process during the tumorigenesis of such salivary gland neoplasia. Also, both markers may be implicated in the trans-differentiation of neoplastic myoepithelial cells from the myxoid zones to lacuna cells of adjacent chondroid areas completing the morphology of this salivary gland tumor.


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