The importance of BMP6 marker within the process of epithelial–mesenchymal transition in the pleomorphic adenoma of the salivary gland

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Abstract
The pleomorphic adenoma is the most frequently encountered tumor of the salivary glands, representing between 45% and 75% of the total number of the tumors of the salivary glands. According to the literature, there are many studies on the immunohistochemical aspects of the myoepithelial cells, present in the pleomorphic adenoma of the salivary gland. A big diversity of mono and polyclonal antibodies, such as the cytokeratins, muscular proteins and other markers, has been used. In our study, we investigated the immunohistochemical aspect of bone morphogenetic protein 6 (BMP6) marker concerning 15 cases of pleomorphic adenomas of the salivary glands. In the immunohistochemical study, we used the paraffin blocks that served for obtaining the sections necessary for the classical histopathological processing by means of the usual stainings. The immunohistochemical study used the enzymatic detection and the LSAB 2 (Labeled Streptavidin–Biotin 2) System technique as the working method. In order to underline the process of epithelial–mesenchymal transition, we also used double sequential immunohistochemical reactions. By the use of the BMP6 marker, we intended to evaluate the reactivity of the various tumor components in the pleomorphic adenomas of the salivary gland, for this marker, taking into consideration its possible involvement in the process of the epithelial–mesenchymal transition. The maximum reactivity for BMP6 was recorded at the level of the normal, excretory, intratubular units, in the luminal cells of the proliferative ductal units, in the myxoid matrix, the cytoplasm of the myxoid stellate cells and the plasmacytoid matrix and the cytoplasm of the chondroid lacunar cells. Some of the cells belonging to the solid, proliferative areas, some of the abluminal cells, that are part of the proliferative ductal units and certain myxoid stellate or plasmacytoid cells contain the S100 protein, which would indicate the existence of some processes of mesenchymal epithelial/myoepithelial transdifferentiation in the development of this type of salivary tumor. The BMP6 expression is specific to the serous acini salivary cells, which are the most specialized epithelial salivary gland cells. The study demonstrated that the mesenchymal epithelial/myoepithelial potential of transdifferentiation of the luminal cells that make up the proliferative units is certified by the immunohistochemical expression of some BMP6 purely mesenchymal protein cells.

Keywords: pleomorphic adenoma, BMP6 marker, epithelial–mesenchymal transition, immunohistochemistry.

Introduction

The pleomorphic adenoma is the most common tumor of the salivary gland, with an annual incidence rate of 3.5 out of 100 000 citizens and it represents between 45% and 75% of the total number of salivary glands tumors [1]. Usually, this type of tumor is benign, but it can have a relapse after an incomplete excision [2], especially in the parotid area and in a percent of 2–8.5% of cases, it can turn into a malignant tumor [3, 4]. As far as the localization of this kind of tumor is concerned, the specialty literature shows that, although it can derive from any salivary gland, in most of the cases the pleomorphic adenoma, it is localized at the end of the superficial lobe of the parotid gland (70–80%). Rarely, the tumor derives from the submandibular glands (10%) and sublingual glands (1%) [5]. The process of histogenesis specific to the pleomorphic adenoma of the salivary glands continues to remain a controversial subject. Thus, while some researchers suggest the origin of the two tumor components, namely parenchyma and stroma from different sources, that is mesenchymal and myoepithelial [6], others point out the unicellular origin of this tumor, more precisely epithelial [7], myoepithelial or mesenchymal cells [8]. The last theory is also supported, according to some researchers by the process of epithelial mesenchymal transition in the pleomorphic adenoma, by means of which the epithelial neoplastic cells turn into mesenchymal cells, which would lead to the tissue heterogeneity specific to this type of salivary tumor [9]. The process of epithelial mesenchymal transition plays important roles during the embryogenesis, in injury healing and the regeneration of the completely differentiated tissues [10, 11].

In the last decade, researchers emphasized the involvement of this process in fibrosis [12, 13] and within the metastasis of cancer cells [14]. We are talking about the
subexpression of some of the epithelial markers corroborated with the mesenchymal markers, accompanied by a growth of motility and cell invasion [15]. According to certain researches, a squamous calciform or ciliated metaplasia can be noticed at the level of the excretory salivary ducts in the context of the canalicular obstruction and a chronic sialadenitis [16]. The myoepithelial cells are characterized by strong plasticity, having a hybrid epithelial/myoepithelial aspect from a morphological point of view.

In the tumors of the salivary glands, the neoplastic abluminal cell transdifferentiates at the mesenchymal level, turning into a stellate cell, detached from the myoid areas of the tumors that secrete glycosaminoglycans [9]. Finally, these would turn into chondroblastic cells that by the synthesis and secretion of mucopolysaccharides would make up areas with cartilaginous differentiation at the level of the pleomorphic adenoma. The cuboid cells, present in the hypocellular myoid areas of the pleomorphic adenomas, have a prehondrogenic phenotype, they render cartilage-derived morphogenetic protein-1 (CDMP-1), which plays a major role in the acceleration of transdifferentiation process specific to the neoplastic myoepithelial cuboid cell towards the phenotype of lacunar cells by means of an autoimmune mechanism [17]. Other studies underlined the fact that the abluminal cells, best represented at the numerical level, were those cells with a plasmacytoid morphology [18] that seem to turn into stellate morphology cells [19].

They originate in the luminal cells, rather than in the myoepithelial cells [20]. Clinically speaking, the pleomorphic adenoma appears initially under the form of small nodules, clearly delineated, solitary or multiple, usually elastic, mobile. The diagnosis is based on the relatively big frequency of this tumor, on the form and consistency of the nodule, on the very slow growth and the absence of any kind of local or general change. The evolution of the pleomorphic adenoma is extremely slow, maintaining sometimes under the form of a solitary nodule for years.

In this paper, we analyzed the immunohistochemical expression of the bone morphogenetic protein 6 (BMP6) marker for 15 cases of pleomorphic adenomas of the salivary glands and its role in the process of epithelial–mesenchymal transition.

Materials and Methods

The studied material was represented by 15 cases of pleomorphic adenomas of the salivary gland found in the cases of the Laboratory of Pathological Anatomy within the Emergency County Hospital of Craiova, Romania, more precisely the archived paraffin blocks. The tumor samples were obtained by the tumor surgical resection performed within the oral and maxillary facial surgery in the same Hospital, and the classification was in accordance with the World Health Organization (WHO) classification of salivary gland tumors [1]. The research period was between October 2014–September 2016. The material was used after obtaining the informed consent of the patients and the approval of the Ethics Board of the Hospital.

Of the 15-selected paraffin blocks, there were performed 4 μm-thick serial sections in the microtome, treated with poly-L-lysine. After drying, they were first deparaffinized in three successive xylene baths, followed by a rehydration through four successive alcohol baths with descending concentrations and a distilled water bath, respectively.

The immunohistochemical study was based on the enzymatic detection, using the LSAB 2 (Labeled Streptavidin–Biotin 2) System technique as working method. The kit used was produced by Dako, Redox Romania – code K0675.

At first, there was blocked the endogenous peroxidase (3% oxygenated water), followed by an antigen demasking (in the microwave, at 450 W, for 30 minutes in a pH 6 citrate solution) and, in the end, there was blocked the endogenous Biotin with the Avidin/Biotin blocking kit (Dako, Redox, Romania). After that, the sections were incubated over night, at 37°C, with the primary antibodies S100 (Z0311, Dako Corp., Carpinteria, CA, USA) and BMP6 (MAB1048, Chemicon International, Temecula, CA, USA), having the characteristics written in the table below (Table 1).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Unmasking</th>
<th>Dilution</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100</td>
<td>Polyclonal rabbit</td>
<td>Citrate, pH 6</td>
<td>1:400</td>
<td>Tegment</td>
</tr>
<tr>
<td>BMP6</td>
<td>Monoclonal mouse – Morph 6.1</td>
<td>Citrate, pH 6</td>
<td>1:50</td>
<td>Gastric mucosa</td>
</tr>
</tbody>
</table>

After the washing in a buffering solution Tween–PBS (phosphate-buffered saline), pH 8.4, the sections were incubated with the secondary antibody, following the instructions of the visualization kit K0675.

The result of these immunohistochemical reactions consists in the visualization of the antigens investigated, by means of the 3,3'-diaminobenzidine (DAB) chromogen, by the brown staining. In order to emphasize the process of epithelial–mesenchymal transition, we used double sequential, immunohistochemical reactions. Thus, in the case of the first reaction, we visualized the primary target using the DAB chromogen, whereas in the second part of the protocol, concerned with the process of immunostaining, we used primary antibodies developed in the serum of an animal species, different from the one used for the creation of the primary antibodies used for the first reaction.

In the second part of the protocol concerned with the double immunostaining, we used the ABC–AP (Avidin–Biotin Complex–Alkaline Phosphatase) kit (1:100 dilution, Dako), whereas for the visualization, we used the fast red (Dako) chromogen, that stained the antigenic target in red. The blades were stained with the Hematoxylin–Eosin (HE) and were assembled by means of a watery medium based on glyceryl (Dako).

We also performed for the used antibody the external positive and negative control, by means of the same LSAB technique. We performed the external positive control on normal tissues that contain the target-investigated antigen (positive sections). These were processed in the same way as the investigated tumor. This test was interpreted with a view to checking the efficiency of the chemical agents used, as well as the efficiency and correctness of the technique used.
We created some of the positive sections out of the areas with normal tissue, adjacent to the tumor. The tissue provides useful information on the presence and sensitivity of the antigen investigated. The evaluation of the immunohistochemical reactions was made independently by two researchers, whereas in the cases where disharmonies were found, these were reinvestigated until an agreement was reached.

The estimation of the markers’ expression was carried out from the qualitative point of view, the reactions’ intensity being quantified according to the scores: 0 – negative, 1 – poorly positive, 2 – moderately positive, 3 – strong positive. BMP6 dealt with the evaluation of the responsiveness manifested by the various tumor components present in the pleomorphic adenomas of the salivary gland in the case of this marker, with the possible participation in the process of epithelial–mesenchymal transition.

The images were captured by means of the Nikon Eclipse 55i microscope (Nikon, Apidrag, Bucharest, Romania), equipped with a 5 megapixel video camera, provided with a cooling system, whereas the processing and interpretation was made by the use of the imagistic software Image Pro Plus, AM97 (Media Cybernetics Inc., Buckinghamshire, UK). There was used the square chi statistical test in order to see if there are any possible correlations between the immunohistochemical scores and the morphoclinical parameters, and for the correlations between the two immunoreactive scores for the two antibodies, there was used the Student’s t-test.

Results

The pleomorphic adenoma in the researched cases largely developed from the second until the tenth life decade. The youngest age was 17 years old, while the oldest was 84 years old. The maximum case incidence was found in the fifth decade (35.55% of the total number of patients), followed by the sixth decade (20%), seventh (15.55%) and fourth decade (11.11%). The average age of the pleomorphic adenoma onset in the studied cases was 48 years old. The pleomorphic adenomas were developed especially in women (57.77%). The gender ratio was 1.37 for women. The pleomorphic adenoma most frequently developed in the parotid gland (71.11%), being followed by the sub mandibular localization (11.11%) and the palatine one (6.66%), respectively. The main types of stroma found in our study were myxoid, chondromyxoid, hyaline, fibrous, sclerohyaline and sclerous. Most frequently, the stromal component presented a myxoid aspect. The 15 cases of pleomorphic adenoma, according to the relative rate between the stromal component and the epithelial one of tumors, were distributed in the following subtypes: adenomas with predominating tumoral stromal component (42.2%), adenomas with a balanced rate between the stromal component and the parenchyma (40%) and adenomas with predominating epithelial component (17.8%). In the cases of pleomorphic adenoma with predominating stromal component, the epithelial component represented up to 20% of the tumor mass. The neoplastic epithelial proliferations had a trabecular, tubular and insular pattern, with small epithelial islands reduced to several groups of cells, commonly arranged at the margin of the myxoid stromal component or inside it.

In the pleomorphic adenomas with a balanced rate between the stromal component and the parenchyma, the epithelial component was well represented. The dominant tumor pattern was the tubular one and the solid-tissular one. The stromal component was most frequently of the myxoid type, chondromyxoid and chondroid. In the cases of typical pleomorphic adenomas where an epithelial component was dominant, the most frequent epithelial patterns were the tubular and the tissular ones.

In the residual glandular parenchyma, we observed reactivity for BMP6, present both in the secretory units and in the excretory ones. In the acinar structures, the reactivity was poorer than in the excretory ducts, the reaction pattern being a cytoplasmic diffuse one (Figure 1). In the excretory units, the BMP6 reactivity was manifested both in the intralobular ducts and in the interlobular ones. The reactivity was higher in the luminal cells of the intralobular channels, in comparison to the extralobular ones (Figure 2). In the proliferation areas with ductal differentiations, we observed the presence of reactivity for BMP6, especially in the luminal cells (Figure 3). With a focal feature, some of the abluminal cells became positive for BMP6, most of them being positive for the S100 mesenchymal marker. In the solid areas of epithelial proliferation, the reactivity was heterogeneous, some areas presenting a high reactivity for BMP6, especially the ones where the abluminal cells had plasmocytoid morphology (Figure 4), others where the abluminal cells had a fusiform morphology presenting a poor reactivity (Figures 5). In the squamous differentiation areas, the BMP6 reactivity was more intense.

In the myxoid areas, we noticed the presence of a responsiveness to BMP6 at the level of the stromal cells with plasmocytoid or stellate morphology that seem to derive from the differentiation of the abluminal cells, of the neoplastic ductal proliferations and from the cuboidal cells, that are to be found at the periphery of the solid neoplastic proliferations (Figure 6). At the level of the chondroid and chondromyxoid areas, most of the lacunar cells have been characterized by BMP6. Most of these stromal cells that are to be found in the myxoid and chondroid areas were also reactive to the S100 mesenchymal marker (Figure 7).

Discussion

The pleomorphic adenoma is characterized by a big morphological plasticity, which is especially due to the myoepithelial component, belonging to this type of salivary neoplasia [21].

These cells were first attributed a contractile function, but it was recently discovered that these are part of the embryonic development, of the synthesis concerned with the modeling of extracellular matrix and paracrine signaling [22]. Furthermore, it was also noticed that in the case of mammary cancer, the myoepithelial cells inhibit angiogenesis, proliferation and invasion of tumor cells [23, 24].

In the course of the neoplastic transformation, the myoepithelial cells change their immunophenotype, losing the reactivity for some of the markers and acquire the features of other types of markers [22], which lie at the basis of the various morphological and cytological aspects, specific to this type of tumor of the salivary gland.
Figure 1 – Pleomorphic adenoma – glandular residual parenchyma. Cytoplasmic reactivity for BMP6 (brown) at the level of the serous acini and of the excretory channels. BMP6 IHC staining, ×200.

Figure 2 – Pleomorphic adenoma – glandular residual parenchyma. Cytoplasmic reactivity for BMP6 (brown) at the level of the excretory intralobular channels. BMP6/S100 (red) IHC staining, ×200.

Figure 3 – Pleomorphic adenoma – parenchyma – solid tubes. Reactivity for BMP6 (brown), manifested especially by the luminal cells, but also by the abluminal ones. BMP6 IHC staining, ×100.

Figure 4 – Pleomorphic adenoma with the prevalence of the parenchyma; reactivity for BMP6 (brown) manifested by the neoplastic cells with a plasmacytoid morphology, present within the solid proliferative areas. BMP6 IHC staining, ×200.

Figure 5 – Pleomorphic adenoma with the prevalence of the parenchyma; faint reactivity for BMP6 (brown) of the neoplastic cells with fusiform and cuboidal morphology, present in the solid proliferative areas, most of them being positive for the S100 (red) protein. BMP6/S100 IHC staining, ×200.

Figure 6 – Pleomorphic adenoma with a balanced relation parenchyma/stroma; reactivity for BMP6 (brown) of the stromal cells present in the myxoid areas. BMP6 IHC staining, ×100.
In the literature, there are numerous studies that deal with the immunohistochemical aspects of the myoepithelial cells present in the pleomorphic adenoma of the salivary gland. The studies resorted to a large diversity of mono and polyclonal antibodies, such as the cytokeratins, the specific muscular proteins (S100 protein, vimentin and other markers) [20, 25–33]. All these studies showed a variable immunoreactivity of the myoepithelial cells present in the structure of the pleomorphic adenoma of the salivary gland. This is the reason why the existence of a marker necessary for their identification is out of the question.

In this matter, we proposed to investigate the expression in various cellular compartments of the pleomorphic adenoma of some markers like BMP6 and S100, which are mainly expressed by the mesenchymal cells and, hence, to observe their involvement degree in the epithelial–mesenchymal transition that take place in this type of tumor. BMP6 is a protein that belongs to the superfamily of transforming growth factor-beta (TGF-β) growth factors involved in the growth of cartilages and bones. This protein is capable of inducing the expression of the osteogenic markers in the mesenchymal stem cells [34]. S100A protein belongs to the superfamily of S100 calcium-binding proteins, comprising at least 25 distinct members [35]. Through calcium binding, these proteins are involved in various intra and extracellular processes, recently being proved their involvement in the tumorigenesis and cancer progression [36]. Moreover, it was also proved the involvement of some of these proteins in inducing the process of epithelial–mesenchymal process, and, therefore, in the promotion of the tumor cells migration and in the tumor invasion, respectively, via Wnt/β-catenin signaling pathways [37].

Our study pointed out the reactivity of the BMP6 protein at the level of both the normal residual parenchyma of the salivary gland and of the types of the investigated pleomorphic adenoma. The maximum reactivity of BMP6 was recorded in the excretory intralobular normal cells, in the myxoid cells of the proliferative ductal units, in the myxoid matrix, in the cytoplasm of the myxoid stellate and plasmacytoid cells, in the chondroid matrix and the cytoplasm of the chondroid lacunar cells. Some of the cells belonging to the solid proliferative areas, some of the abluminal cells that are part of the proliferative ductal units and certain myxoid stellate or plasmacytoid cells contain the S100 protein, which suggests the existence of some processes of epithelial–myoepithelial/mesenchymal transdifferentiation within the development of this type of salivary tumor.

Contrary to us, other authors have noticed a much more active reaction at the level of the serous acini and reduced in the case of several isolated cells present in the excretory ducts of the normal salivary glands [38]. The authors concluded that the BMP6 expression is specific to the salivary serous acinar cells, the most specialized salivary epithelial cells. Furthermore, some authors suggested that BMP6 present in the saliva is necessary for the regeneration of the oral mucosa, showing the role of this protein in the process of differentiation related to the keratinocytes [39]. The investigations concerned with the responsiveness to BMP6 in the benign tumors of the salivary gland have revealed a decrease in the reactivity to the salivary normal parenchyma, a maximum of expressiveness being noticed within the areas with squamous differentiation [38].

The authors also pointed out a focal reactivity for BMP6, in the case of some of the abluminal cells that are constituent parts of the proliferative ductal-like units, as well as the isolated presence of the BMP6 deposits in some stromal tumor areas. The stromal areas with chondroid differentiation did not record any kind of reactivity to BMP6. The authors suggested that the expression of this protein in the pleomorphic adenoma would be rather associated with epithelial differentiations than with mesenchymal cells [39]. By the use of the reverse transcription-polymerase chain reaction (RT-PCR) technique, the researchers highlighted the RNA supraexpression in the pleomorphic adenoma, a messenger of the BMP2 protein, whereas at immunohistochemical level, this protein was detected in the modified myoepithelial cells, present around the chondroid areas and the basal membranes [40].

The authors decided that the BMP2 protein produced by the modified myoepithelial cells generates the formation of the chondroid areas by the paracrine method. One year later, these authors immunohistochemically highlighted the reactivity for BMP6 of the luminal cells present at the level of the proliferative ductal like units, of the myoepithelial neoplastic cells from the myxoid areas and of the lacunar chondroid cells that have also indicated the type II collagen expression [41]. The authors concluded that the BMP6 protein is part of the chondrogenesis and it intervenes by the autocrine method in the preservation of the chondroid tissue. The protein also contributes to the formation of the proliferative ductal-like units present in the pleomorphic adenoma of the salivary gland.

At the level of the tubular areas found in the pleomorphic adenoma, some authors observed the type IV collagen expression and of the lamina, as well as the absence of the mesenchymal genes, which suggests the purely mesenchymal character of the cells present in these areas [6]. The cells contained by the tubular-myxoid areas seem to have a mixed epithelial and mesenchymal phenotype [42]. Consequently, a progressive process of
epithelial–mesenchymal transdifferentiation of the pleomorphic adenoma takes place. The extracellular abundant accumulation of proteoglycans will finally lead to the disappearance of the tubular-epithelial structures, which are replaced by the isolated neoplastic cells surrounded by the myxoid matrix. The next stage in the process of epithelial–mesenchymal transdifferentiation is represented by the type II collagen expression, with the subsequent formation of the chondroid matrix and of lacunae with typical chondrocytes [9]. In our study, we observed the fact that, some of these myxoid stromal areas give birth to chondroid areas. Quite rarely, we observed the presence of some chondroid existence found in direct contact with the neoplastic proliferative units, a fact that might suggest the genesis of these stromal areas after an epithelial–mesenchymal transition. We also observed some similar aspects in the case of ossified matrix areas. Moreover, the fibrous/sclerous stromal areas give birth to chondroid areas. Quite rarely, we observed the presence of some chondroid existence found in direct contact with the neoplastic proliferative units, a fact that might suggest the genesis of these stromal areas after an epithelial–mesenchymal transition. We also observed some similar aspects in the case of ossified matrix areas. Moreover, the fibrous/sclerous stromal areas seem to also develop directly in the neoplastic epithelial proliferative areas.

Conclusions

The study performed by us shows the involvement of the two proteins in the epithelial–mesenchymal and epithelial–myoepithelial transdifferentiation processes from pleomorphic salivary gland adenomas. This fact would somehow explain the tissueular feature of this type of salivary gland tumor.

Conflict of interests

The authors declare that they have no conflict of interests.

References


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