BPIFA1 Gene Expression in the Human Middle Ear Mucosa

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INTRODUCTION

The bactericidal/permeability-increasing protein fold-containing family member A1 (BPIFA1) gene has a chromosomal location 20q11.21 and codes the BPIFA1 protein. It is part of a group of seven proteins that are structurally and functionally similar, namely BPIFA1, BPIFA2, BPIFA3, BPIFB1, BPIFB2, BPIFB3, and BPIFB4. They are part of the proteins in the airway surface liquid (ASL) [1]. They protect the respiratory tract via bactericidal and non-bactericidal mechanisms [2]. Their presence was first reported in humans in the year 2000 as “Palate, lung and nasal epithelium clone proteins” (PLUNC), as described by Bingle [3].

The BPIFA1 protein consists of 256 amino acids, has a mass of 26.6 kD, and has distinct hydrophobic properties. It is present in significant amounts in the ASL, as it constitutes approximately 10% of all proteins expressed in ASL of the respiratory tract [4]. It is a secretory protein found in the nasopharyngeal secretion and sputum [5, 6]. It is produced mainly in the mucosal cells of the major and minor salivary glands and in the submucosal glands of the respiratory tract. It is not synthesized in the serous cells of the parotid gland, which confirms the connection between BPIFA1 and the mucosal cells [7]. It is not present in the goblet cells of the respiratory tract and in the alveolar epithelium. Its presence in the mucosa of the nasal cavity, paranasal sinuses, root of the tongue, palatine tonsils, saliva, and nasal secretion has been confirmed. It is also expressed in the columnar epithelial cells of the upper respiratory tract [8]. As for the lower respiratory tract, the BPIFA1 has been found in sputum, tracheal secretion, bronchial secretion, and bronchoalveolar lavage fluid [9]. The BPIFA1 expression is quite clear in the proximal part of the trachea. It gradually decreases in the distal parts of the main bronchi and is completely absent in the peripheral part of the lungs. Besides the respiratory tract, BPIFA1 has a very low expression in the kidneys, colon, as well as in specific granules of the neutrophils [10]. BPIFA1 has antimicrobial properties, antibiofilm effect, and immunomodulatory function and is part of the mucociliary clearance. The BPIFA1 expression has not been examined in the normal human middle ear mucosa and chronic otitis media.

Chronic otitis media with cholesteatoma is very often the cause of hearing loss and the individual’s difficult functioning in the society. In COMC, there is a persistent infection and inflammation of the middle ear cavity and the cells of the processus mastoi-
deus. COMC is a disease that is characterized by permanent hearing loss, intermittent or constant otorrhea, perforation of the tympanic membrane, and presence of a cholesteatoma. The cholesteatoma is formed from desquamated epithelial cells with no nuclei (arranged like onion scales), cholesterol crystals, and fatty acids. This structure is surrounded by a matrix with a histological structure similar to that of the skin but without any hairs and glands. Cholesteatomas develop via a retraction pocket in the pars flaccida or during migration of the epidermis from the outer ear canal through a sidewall perforation in the pars tensa. Pseudomonas aeruginosa (Pa) and Staphylococcus aureus are the most common pathogens isolated from patients with COMC.

MATERIALS and METHODS

In this study, 32 patients were included for the period between March 2016 and September 2016. A detailed anamnesis and clinical status, as well as laboratory tests information were obtained. The anamnesis provided information about time of disease onset, progression, concomitant illnesses, past illnesses, and risk factors.

The participants were divided into two groups—Group I and Group II.

The patients with COMC were assigned to Group I. The inclusion criteria were long-term (over a year), constant, or intermittent otorrhea; permanent hearing loss; perforation of the tympanic membrane; and presence of a cholesteatoma. Only patients with perforation located in the pars flaccida or in the postero-superior quadrant of the pars tensa where the integrity of the annulus fibrosus of the tympanic membrane is always corrupted were included. We routinely performed pure tone audiometry and a computed tomography (CT) scan of the head for each patient. Cholesteatoma was confirmed intraoperatively and histologically in all subjects from Group I. They underwent radical mastoidectomy. During the surgery, part of the pathologically changed mucosa (approximately 2-3 mm³) of the mastoid cells was collected using a micro alligator ear forceps (the jaws are 5 mm long and 1 mm round with cupped tips). The samples were placed in a container with RNAlater and transferred to the Molecule Medicine Center after they were elaborately labeled.

In Group II, we included patients with bilateral sensorineural hearing loss (BSHL) established through brain stem evoked response audiometry. Also, each subject underwent a micro-otoscopy, tympanometry, and CT scan. The presence of a normal tympanic membrane was confirmed. We have excluded patients from this group with otoscopic, CT, and intraoperative data confirming the presence of inflammatory process in the middle ear. All subjects were diagnosed with bilateral hearing loss of the cochlear type (deafness) and underwent cochlear implantation. We collected part of the normal mucosa of the antrum (approximately 2-3 mm³) using a micro alligator ear forceps during the mastoidectomy. The samples were placed in a container with RNAlater and transferred to the Molecule Medicine Center after they were elaborately labeled.

The total RNA from tissue samples stored in RNAlater was isolated using RNeasy Plus Micro kit (Qiagen) as per the manufacturer’s instructions. The concentration and quality of the RNA samples were evaluated spectrophotometrically using NanoDrop. For a reverse transcription reaction with high-capacity cDNA—subsequently, quantitative real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7900HT (Applied Biosystems) with QuantiTect SYBR Green PCR Kit and QuantiTect Primer Assay. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference control for normalization. Relative changes in gene expression levels of BPIFA1 were calculated using the 2-ΔΔCt method. The expression levels of BPIFA1 were compared between Groups I and II, with Group II as calibrators. Real-time experiments were performed in triplicates and the mean Ct values were calculated.

Statistical Analysis

The statistical analysis was performed using the Statistical Package for Social Sciences software version 20 (IBM Corp.; Armonk, NY, USA). A receiver operating curve (ROC) analysis was performed for evaluating the specificity and sensitivity of BPIFA1 expression levels for distinguishing the COMC patients from control group.

Our study was performed in accordance with the ethical standards of the Helsinki Declaration and approved by the local ethics committee. Each patient provided informed consent for participating in the study. The study was performed in accordance with the guidelines for human experimentation required by our institute.

RESULTS

RNA was isolated from all tissue samples (n=32). For the first time, BPIFA1 gene expression was examined in both the normal mucosa and mucosa in COMC in the human middle ear and a comparison was drawn for BPIFA1 expression between COMC patients and the control group. We proved that the expression exists in all participating subjects (n=32) regardless of the condition of the mucosa, i.e., both in patients with pathologically changed mucosa in COMC (Group I) and normal mucosa of the middle ear in BSHL (Group II; Table 1).

Table 1. Comparison between COMC and BSHL groups

<table>
<thead>
<tr>
<th></th>
<th>Group I (COMC)</th>
<th>Group II (BSHL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients (n)</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>No of patients with BPIFA1 expression, n (%)</td>
<td>17 (53%)</td>
<td>15 (47%)</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>7 (41%)</td>
<td>8 (53%)</td>
</tr>
<tr>
<td>Females, n (%)</td>
<td>10 (59%)</td>
<td>7 (47%)</td>
</tr>
<tr>
<td>Average age, years (range)</td>
<td>44 (range 27-73)</td>
<td>18 (range 1-50)</td>
</tr>
<tr>
<td>No of patients in the age range 0-18 years, n (%)</td>
<td>0 (0%)</td>
<td>9 (60%)</td>
</tr>
<tr>
<td>Bulgarian, n (%)</td>
<td>15 (88%)</td>
<td>8 (53%)</td>
</tr>
<tr>
<td>Gipsy, n (%)</td>
<td>1 (6%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>Turkish, n (%)</td>
<td>1 (6%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Smoker, n (%)</td>
<td>10 (59%)</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>Non-smoker, n (%)</td>
<td>7 (41%)</td>
<td>12 (80%)</td>
</tr>
<tr>
<td>No of patients with radical mastoidectomy</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>No of patients with cochlear implantation</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>No of patients with BPIFA1 expression &lt;6 ng/µL, n (%)</td>
<td>5 (29%)</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>No of patients with BPIFA1 expression &gt;6 ng/µL, n (%)</td>
<td>12 (71%)</td>
<td>10 (67%)</td>
</tr>
</tbody>
</table>

COMC: chronic otitis media with cholesteatoma; BLS: bilateral sensorineural hearing loss
The average age of the subjects in Group I was 44 years, whereas it was significantly lower in Group II (18 years), since 60% (n=9) of the subjects in Group II were aged ≤18 years. The Bulgarian ethnicity was confirmed in 88% (n=15) of Group I and 53% (n=8) in Group II. Also, 40% (n=6) of the subjects from Group II were Gypsies compared to 6% (n=1) in Group I. The number of smokers in Group I was 59% (n=10). Considering that 60% of the subjects in Group II were under 18 years of age, the number of smokers in this group is also lower (20%; n=3). All the patients Group I underwent radical mastoidectomy with the removal of the cholesteatoma, and all the patients in Group II underwent cochlear implantation.

Owing to the method used for examining the comparative expressional analysis of BPIFA1 between patients with COMC and BSHL, only samples of RNA with a concentration above 6 ng/µL were used. Thus, 71% (n=12) samples of Group I and 67% (n=10) samples of Group II were used. The mucosa from the COMC patients was pathologically changed and hence was associated with the chronic inflammatory process. The BSHL patients (Group II) were utilized as a control group because their samples were normal, healthy mucosa of the middle ear (Table 2).

Table 2 demonstrates that there is no significant difference in the BPIFA1 expression in 16.67% (n=12) of the patients with COMC (Group I) compared to the control group (BSHL, Group II). One-half of the patients with COMC show an increased expression and 33.33% (n=4) showed a decreased expression.

In Figure 1, the Box plot graph shows the expression levels of BPIFA1 in both groups. The ROC curve analysis showed that there is no statistically significant difference in the levels of BPIFA1 expression between the patients with COMC and the controls (p= 0.947; Figure 2). This could also be a result of the small number of patients and controls. The examined cohort will have to be increased to confirm the results.

AUC=0.492, p=0.947, sensitivity 58.3%, and specificity 50%.

DISCUSSION

To date, BPIFA1 gene expression has not been examined in the mucosa of the human middle ear. The BPIFA1 expression in the mucosa of the upper respiratory tract in a normal condition of the organism and in chronic rhinosinusitis has already been proven [16]. Due to the morphologic and functional similarity in the mucosa of the middle ear and the mucosa of the upper respiratory tract, we examined the BPIFA1 expression in the mucosa of the middle ear as well. We proved the presence of BPIFA1 expression in the middle ear in all participating subjects (n=32). It has already been established that BPIFA1 protein connects the lipopolysaccharides of different pathogenic bacteria and discontinues the following pro-inflammatory processes in the respiratory tract [17]. In this way, BPIFA1 will have a protective effect in the middle ear where inflammatory diseases of the mucosa are concerned. It participates in the pathogenesis of the acute and chronic otitis media, which requires further research in the future.

The inhibition of BPIFA1 reduces the resistance of the respiratory tract to bacterial infections with *Mycoplasma pneumoniae* (Mp), *Pa*, *Klebsiella pneumoniae*, and non-typable *Haemophilus influenzae* [18,19]. It is possible that the inhibition of BPIFA1 in the middle ear would
increase the risk for developing an otitis media. Pa is one of the main bacteria isolated in COMC patients [12-15]. In vitro studies have proven that BPIFA1 has a direct bacteriostatic effect in Mp and Pa [20]. Therefore, BPIFA1 will have a bacteriostatic effect on some of the most common pathogenic bacteria in COMC.

BPIFA1 is a multifunctional protein that regulates epithelial Na+ channel (ENaC). The ENaC plays a significant role in the regulation of the amount of liquid that covers the upper respiratory tract and in the realization of an adequate mucociliary clearance. BPIFA1 inhibits ENaC in the cellular lines of human bronchial cells. Hence, it blocks the activation of the ENaC, which reduces the absorption of sodium ions and water. The result is a decrease in the viscosity of ASL and improvement in the mucociliary clearance. BPIFA1 protein is a negative regulator of ENaC [21]. The increased expression of BPIFA1 also leads to a better clearance of pathogens from the mucosa. It is also probable that the increase of BPIFA1 expression is the organism's response against the bacterial infection, aiming to improve the mucociliary clearance by liquefying ASL. In 50% (n=6) of our patients with COMC, we could prove increased BPIFA1 expression compared to the control group. It is possible that this increase is a result of the chronic inflammation of the mucosa of the middle ear. A research proved that the lungs of genetically modified mice, which over-express BPIFA1, display less pronounced fibrosis after an inflammatory process. This suggests that BPIFA1 protein has a protective effect against fibrosis [22]. The more evident the BPIFA1 expression is, the less noticeable the fibrotic changes will be. These less noticeable fibrotic changes are proof of the mucosa's better condition. Patients with COMC and increased BPIFA1 expression will probably have less scared mucosa and thus, better surgery results. In patients with decreased BPIFA1 expression, the reverse will be valid.

In 33.33% (n=4) of the patients with COMC, we found a decreased expression. We are guessing that the lowered expression of BPIFA1 is a result of the tissue scarring in the middle ear along with a decrease of the secretory elements.

There is also a structural similarity between BPIFA1 and a certain horse protein, latherin. It has been isolated from horse sweat and saliva. Latherin has a very high surfactant effect on the air-water surface [23]. The biological role of the surfactant on the respiratory tract has not been defined clearly yet but it could be different from that of the alveolar surfactant. Probably the surfactant of the upper respiratory tract destroys biofilms and prevents their development. Biofilms are bacterial communities attached to the surface of the respiratory tract and included in a matrix that provide protection against the factors of the environment and the immune system. Possibly, part of the strategy of the upper respiratory tract for fighting against bacterial colonization is forming a surfactant. In vitro experiments confirm that the presence of BPIFA1 significantly diminishes the development of a biofilm from Pa [24]. BPIFA1 lowers the surface tension of the extracellular matrix/bacteria. Thus, BPIFA1 corrupts the early stages of the development of biofilm-bacteria aggregation and the formation of a microcolony.

We believe that in the middle ear mucosa, BPIFA1 will limit the formation of bacterial biofilms, which are exceptionally resistant to pharmaceutical therapy. This will make the treatment of the chronic inflammatory process fundamentally easier and time saving. The tissue remodeling process in the middle ear mucosa will progress slower. Our opinion is that the better the BPIFA1 expression is, the more positive the prognosis of COMC will be. This will probably be true for the early stages of COMC because following the progression of the disease, the BPIFA1 expression will decrease.

According to McGillivary and Bakaletz, BPIFA1 as a surfactant plays an important role in the homeostasis and protection of the middle ear in chinchillas [2]. Using tensiometry, they compared the surface tension of a 72 mN/m drop of water and a drop of BPIFA1 (64 mN/m) at a concentration of 20 µg BPIFA1/mL in water (lowest possible concentration). They found statistically significant difference (p<0.0001) in the decrease of the surface tension of BPIFA1 compared to clear water. They also established that the surface tension of a drop of BPIFA1 (29 mN/m) at a concentration of 500 µg BPIFA1/mL of water is approximately equal to the surface tension of a drop of the detergent sodium dodecyl sulphate. These results demonstrate the important function of BPIFA1 as a surfactant of the respiratory tract Eustachian tube and its possible role in the pathogenesis of the inflammatory diseases of the respiratory tract and the middle ear. The animals that have their BPIFA1 expression blocked experience negative tension in the cavum tympani along with retraction of the tympanic membrane compared to the healthy controls.

The results from the experiment with chinchillas and our research on the BPIFA1 expression in the mucosa of the middle ear in humans suggest that the BPIFA1 protein plays an analogous role in humans. The BPIFA1 protein is definitely part of the innate immune system of the middle ear and is highly likely involved in the pathogenesis of the inflammatory diseases of the middle ear. It is possible that BPIFA1 affects the function of the normal commensal microflora of the middle ear.

The levels of BPIFA1 may vary depending on the different paths of the non-specific immunity. This fluctuation in the concentration of BPIFA1 is probably part of the initiation of the immune response and the activation of other protective mechanisms that are more effective in eliminating bacterial infections. BPIFA1 changes during an infection. In our patients with COMC, we observed lowered BPIFA1 expression in 33.33% (n=4), increased in 50% (n=6), and no significant difference in 16.67% (n=2), indicating that a difference in the BPIFA1 expression in 80.33% of all patients with COMC was not ed compared to the control group. The difference between the two groups did not reach a statistically significant difference (p=0.947), which is probably due to the small number of subjects. We believe that future research in this area will show significant difference between patients with COMC and controls as well as indicate the role of BPIFA1 protein in the pathogenesis of inflammatory diseases of the middle ear.

CONCLUSION

In conclusion, we believe that the BPIFA1 protein has an undeniable presence in the mucosa of the middle ear in humans. It protects the mucosa via different mechanisms, including inhibition of bacterial growth, decrease in ASL viscosity, improvement in the clearance of the mucosa, prevention of bacterial colonization, and development of the bacterial biofilms on the surface of the mucosa. There is a defi-
nite difference in the BPIFA1 expression in healthy mucosa and the mucosa of COMC patients. In the experiment models, BPIFA1 affects the function of the Eustachian tube. Therefore, it will also play a role in the inflammation of the middle ear in humans, which is still unclear at this point. New studies on the functions of the BPIFA1 protein will prove its significance for the mucosa of the middle ear in norms and pathology.

Ethics Committee Approval: Ethics committee approval was received from the ethics committee of COESRMUS-19/08.05.2015.

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - Y.H.; Design - Y.H.; Supervision - D.P.; Resource - Y.H.; Materials - S.Y.; Data Collection and/or Processing - D.K.; Analysis and/or Interpretation - D.P.; Literature Search - S.Y.; Writing - Y.H.; Critical Reviews - D.P.

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