International Journal of Pediatric Otorhinolaryngology 110 (2018) 100–104

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: www.elsevier.com/locate/ijporl

Tonsillar antimicrobial peptide (AMP) expression profiles of periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA) patients

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ARTICLE INFO

Keywords:
PFAPA
Tonsil
Beta-defensin-1
AMP
Autoinflammation

ABSTRACT

Introduction: PFAPA (periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis) is the most frequent non-infectious cause of high fever observed among the European child population. While its cause is still not yet fully identified, PFAPA patients were previously shown to have altered tonsillar microbiome composition. Our study hypothesized that this is associated with a change in antimicrobial peptide (AMP) expression levels, as in the case of Crohn's disease which is another autoinflammatory disorder.

Methods and materials: The tonsil specimens were isolated from seven patients with PFAPA syndrome, and six patients with group A beta-hemolytic streptococcal (GAβHS) recurrent tonsillitis. Tonsillar expression levels of human beta-defensin 1-2, cathelicidin, ribonuclease-7, and liver expressed antimicrobial peptide-1 were monitored by immunohistochemistry (IHC). Expression levels were scored using semi-quantitative analysis method and were statistically analyzed by Two-Way Repeated Measures Analysis of Variance test.

Results: Our results showed no significant difference in AMP expression levels between PFAPA and GAβHS patients. Immunolocalization of human beta-defensin 1 was different between the two groups; expressed at higher levels on tonsil surface epithelium (SE) than lymphoid interior (LI) in PFAPA patient group, while this was not evident in GAβHS patients group.

Conclusions: Our results suggest that, PFAPA patients may be associated with altered AMP expression as in other autoinflammatory diseases. Future studies with subjects without any inflammatory condition are required for more precise conclusions.

1. Introduction

Periodic fever, apthous somatitis, pharyngitis and cervical adenitis (PFAPA) is a syndrome named according to its clinical criteria that include fever flares associated with pharyngitis, adenitis, and/or aphthous stomatitis. According to the Eurofever project report, it is the most common non-infectious reason for high fever cases observed in Western European child population [1,2]. PFAPA is thought to be an auto-inflammatory disease (AID) since the fever attacks are associated with elevated levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), leukocyte recruitment and are responsive to cortiosteroid treatment [3–5].

Up till today, the cause of the syndrome is not yet fully identified and there has not been any study showing a link between PFAPA and any known infectious or autoimmune cause [1]. However, tonsillar microbiome was suggested to play a role in the development of syndrome since its composition was shown to differ between control subjects and PFAPA patients [6,7]. Moreover, removal of tonsils by tonsillectomy (TE) is considered as one of the most effective treatment for PFAPA syndrome [8].

Apart from providing protection against infections, antimicrobial peptides (AMPs) produced by epithelial cells are also involved in the regulation of microbial community composition; their altered levels of expression were suggested to cause gut microbiota community changes in patients with Crohn's disease (CD) which is a gastrointestinal AID [9]. Our study aims to evaluate the association between PFAPA and AMP production by monitoring the tonsillar expression levels of human beta-defensin 1-2 (HβD-1-2), cathelicidin (LL-37), ribonuclease-7 (RNase-7), and liver expressed antimicrobial peptide-1 (LEAP-1/hepcidin) which were previously demonstrated in tonsillar tissues [10–13].

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https://doi.org/10.1016/j.ijporl.2018.05.005
Received 19 February 2018; Received in revised form 25 April 2018; Accepted 4 May 2018
Available online 08 May 2018
0165-5876/

2. Materials and method

2.1. Sample collection

The study was performed at Near East University (NEU) Hospital and approved by NEU Scientific Research and Evaluation Ethics Committee (Project No: YDU/2016/42–346). The tonsil specimens used in the study were collected from 13 children who were hospitalized between 2012 and 2016, and were separated into two groups. PFAPA group consisted of seven patients with PFAPA syndrome, and Group A beta-hemolytic streptococcus (GAβHS) group included six patients admitted to the hospital with complaints of recurrent tonsillitis caused by GAβHS. The PFAPA group consisted of 4 female and 3 male subjects, whereas GAβHS group included 2 female and 4 male subjects. The age of patients in the PFAPA group ranged from 3 to 10 years, with an average age of 5.16 years, while the age range of GAβHS was 6–13 years, with an average age of 8.35 years. Tonsil specimens were removed by surgical operation and stored as formalin-fixed paraffin-embedded tissues. All patient databases are obtained using the hospital information system after receiving informed consent from the parents. PFAPA patients were diagnosed according to the Marshall criteria modified by Thomas et al. [14]. Those treated with corticosteroids were excluded from the study.

2.2. Immunohistochemistry (IHC) staining

Following deparaffinization in xyelene, and rehydration by serial dilutions of ethanol, 4-μm paraffin-embedded tissues were loaded into Ventana automated slide stainer (Ventana Medical Systems Inc.) and left for incubation for 90 min (mins) with following primary antibodies (all from Abcam): mouse monoclonal anti-β-defensin-1 antibody (1:500, clone M11-14b-D10); rabbit polyclonal anti-β-defensin-2 antibody (1:500); rabbit polyclonal anti-cathelicidin antibody (1:200); mouse monoclonal ribunuclease-7 antibody (1:100, clone 4C4); and mouse monoclonal anti-hepcidin (LEAP-1) antibody (1:100). To assure specific binding, tissue sections treated with rabbit polyclonal isotype control antibody (Abcam, 1:200) or those left untreated were used side by side. Then the slides were incubated with the ready-to-use horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit secondary antibodies (ScyTek Laboratories) for 20 min, which was then followed by 5 min of incubation with 3,3′-Diaminobenzidine (DAB) chromogen mixture, and counterstaining with hematoxylin and bluing reagent.

Immunohistochemical assessments were performed blindly by two independent researchers without any information regarding the status of the patients, as described previously [15]. Briefly, after the immunohistochemistry staining procedure, 10 fields were chosen at random, and expression pattern was evaluated in 1000 cells (100 cells/field) using a high-powered (200×) microscopic magnification. The mean value of each investigator's scores was rated as follows; the samples with 0%, 1–10%, 11–50%, 51–80% and 81–100% positive cells were scored as 0, 1, 2, 3 and 4, respectively. The intensity of the stained cells was rated on a scale of 0–3; negative, weak, moderate and strong stained samples were rated as 0, 1, 2 and 3 respectively. Final AMP expression level score was obtained by multiplying the positive cell distribution (PCD) score with the staining intensity (SI). Average expression level score for each AMP obtained from each investigator was later used for further statistical analysis.

2.3. Statistical analysis

Semiquantitative immunohistochemical data were analyzed statistically with GraphPad Prism (Version 7.00) software package. Expression score levels from both groups were compared by using Two-Way Repeated Measures Analysis of Variance test. Within group comparisons were adjusted for errors by using Holm-Sidak's multiple comparisons test while for between groups comparisons Tukey post hoc test was applied. Level of significance was accepted to be 0.05.

3. Results

3.1. Comparison of AMP expression levels between PFAPA and GAβHS patient groups

AMPs can be expressed constitutively as well as upon stimulation with microbe-associated molecular patterns. They are involved in the regulation of microbial community such that gut microbiota community changes observed in CD patients were associated with altered AMP production [9]. Besides of being expressed on surface epithelium (SE) which has a direct contact with surface microbiota, AMP expression can also be detected in lymphoid interiors (LI) of tonsils. Apart from exerting direct anti-microbial effects, AMPs were also suggested to facilitate lymphocyte recruitment as well as stimulation [16].

Due to unavailability of tonsil samples isolated from patients without any inflammatory condition, GAβHS patients were used for comparison purposes in our IHC and statistical analysis. IHC results showed high expression levels of all AMPs included in the study on tonsil SE and LI except for HβD-1 which displayed low/moderate level of expression (Fig. 1).

The statistical analysis revealed no significant difference in SE and LI AMP expression levels between the two groups. AMP expression pattern was significantly different; HβD-1 was predominantly expressed on SE of PFAPA tonsils, while there was not any significant difference in expression levels between SE and LI of tonsil samples isolated from GAβHS patients. In contrast, expression pattern for the other AMPs did not differ between the study groups; while LEAP-1 expression level was higher in tonsil LI, expressions of other AMPs were evenly distributed between tonsil SE and LI (Fig. 1).

4. Discussion

According to the Eurofever project report, PFAPA syndrome is the most common reason for non-infectious high fever episodes in Western European children, with an incidence rate of 2/10,000 in pediatric populations [1,7,17]. Even though its etiology is not yet clear, PFAPA is thought to be an AID since the fever attacks are associated with elevated CRP, ESR, leukocyte levels and can respond to single-dose corticosteroids [3–5].

Crohn's disease, which is another AID, is associated with disruption of AMP production that subsequently lead to gut microbiota community changes and chronic inflammation [9,18]. Our study hypothesized that similar association may exist for PFAPA syndrome since altered tonsillar microbiome structure was previously observed in PFAPA patients and TE is considered as one of the most effective treatment for the syndrome [6,7]. For this purpose, tonsillar expression levels of HβD-1-2, LL-37, RNase-7, and LEAP-1 were compared between PFAPA and GAβHS patients. Among those, while HβD-1-2, RNase-7 and LL-37 expressions were previously demonstrated on tonsil tissues by IHC, and tonsillar LEAP-1 expression was only studied by PCR [10–13]. In accord with literature, HβD-1-2, RNase-7 and LL-37 expressions were detected by IHC on tonsil SE, in our study. Our study also demonstrated for the first time the LEAP-1 expression, on tonsil SE, at protein level.

Statistical analysis did not show any significant difference in tonsillar SE expression levels of HβD-1-2, LL-37, RNase-7, and LEAP-1 between PFAPA and GAβHS group patients. In a previous study, recurrent tonsillitis patients were reported to display altered expression levels of HβD-1 and LL-37 on tonsil SE, in comparison to control subjects with no history of recurrent tonsillitis [10,12]. Therefore, our results suggest that, like recurrent tonsillitis patients, PFAPA patients may also have altered AMP expression pattern on the tonsil SE, when compared with uninflamed tonsils. Accordingly, tonsils from PFAPA and recurrent tonsillitis patients were demonstrated to have features of