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Human beta defensin-1 is involved in the susceptibility to adeno-tonsillar hypertrophy

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ABSTRACT

Introduction: Innate immunity molecules are known to play a pivotal role in the homeostasis of the oral mucosa, permitting the presence of commensal microflora and, at the same time, providing a first line of defense against pathogens attempting to invade the oral cavity.

Tonsils represent the local immune tissue in oral cavity, being able to provide a non-specific response to pathogens; however, in the presence of microbes or foreign materials present in the mouth tonsils could became infected and develop chronic inflammation, thus leading to hypertrophy.

The etiology of the disease is multifactorial depending upon environmental and host factors, the latter including molecules of mucosal innate immunity.

Methods: Ninety-five children with adeno-tonsillar hypertrophy subjected to adeno-tonsillectomy were recruited at the pediatric otorhinolaryngology service of the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste (Italy).

The specimen discarded from the surgery were used for genomic DNA extraction and genotyping, for mRNA extraction and gene expression analysis, finally the samples were cut and used to prepare slides to perform immunohistochemistry.

Results: Functional polymorphisms within *DEFB1* gene, encoding the human beta defensin-1 (hBD-1), were analyzed finding association between *DEFB1* rare haplotypes and susceptibility to adeno-tonsillar hypertrophy. *DEFB1* mRNA expression was detected in the tonsils and the hBD-1 protein was localized at the epithelia of tonsils mainly in the proximity of the basal lamina.

Conclusion: Our findings lead us to hypothesize an involvement of hBD-1 mediated innate immunity in the modulation of the susceptibility towards adeno-tonsillar hypertrophy development.

1. Introduction

Adeno-tonsillar hypertrophy (AH) is a common otolaryngologic paediatric disorder due to proliferation of B-cells, helper T-cells and regulatory T-cells that can cause upper respiratory ways partial obstruction [1]. Two forms of the disease are known: the sleep related breathing disorder and recurrent throat infections; common features are nasal congestion, rhinorrhoea, mouth breathing, snoring, hyponasal speech and cough [2]. Recurrent throat infections are diagnosed in the presence of multiple sore throat episodes (at least 7 episodes in the preceding year, or 5 in each of the preceding 2 year, or 3 in each of the preceding 3 years), while sleep related breathing disorder are characterized by abnormality in respiration during sleep, with different degree of severity ranging from primary snoring to the most severe obstructive sleep apnea [3]. In the most severe cases adeno-tonsillectomy is considered the gold standard to improve the symptoms [4], although the intervention is simple and in day hospital some researchers reported complications, such as haemorrhage, pain, infections and in some cases the persistence of symptoms [5]. The causes of AH are not well defined, however, in the AH form linked to recurrent throat infections, bacteria are considered the main etiologic agents leading to the disease [6]. The lymphoid Waldeyer's-Pirogov ring, consists of adenoids, tubal, palatine and lingual tonsils, is an important immunological region in human body, providing an immune system response against pathogens [7], therefore recurrent infections in these sites could produce chronic inflammation and hypertrophy [8].

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Immune response plays a fundamental role in the fighting against pathogens, and innate immunity is the first line of defence in the human body. B-defensins are antimicrobial peptides produced in different region of the human body belonging from innate immunity system. They are particularly expressed in the oral cavity [9] where they exert a strong antimicrobial activity against virus, bacteria and fungi [10] but they also present immuno-modulatory and chemotactive capacity contributing at the interplay between innate and adaptive immune system [11].

Among β-defensins, the β-defensin 1 (hBD-1) is one of the better studied and characterized. It is constitutively expressed at the oral mucosa [12], but it could be also induced by inflammation [13], moreover mRNA expression was reported in epithelium of tonsils surface [14].

hBD-1 production is also under genetic control: three polymorphisms, within the 5′untranslated region (UTR) of *DEFB1* gene, specifically -c.52G > A (rs1799946), -c.44C > G (rs1800972), -c.20G > A (rs11362) have been reported as able to influence the protein level [15].

In our study we investigated the role of *DEFB1* functional polymorphisms, influencing hBD-1 production, in the AH disorder. Specifically, *DEFB1* 5′UTR polymorphisms were analysed in a population of children that underwent adeno-tonsillectomy in order to explore the contribution of genetic background in the risk of AH development. Moreover, the *DEFB1* expression was measured in tonsils specimens and the hBD-1 protein was analyzed in tonsils histologic samples, in order to disclose hBD-1 localization.

2. Materials and methods

2.1. Study population

Ninety-five children with AH and obstructive sleep apnoea were enrolled at the Ear, Nose Throat Unit Department of the Institute for Maternal and Child Health IRCCS Burlo Garofolo (Trieste, Italy). All patients with an history of snoring and sleep apnoea were evaluated by a pediatric sleep questionnaire [16], for children below three years of age or for whom with a discrepancy between symptoms and physical examination were performed poly-somnography and sleep endoscopy before surgery. Patients who presented obstructive sleep apnoea associated with hypertophy of adenoids or tonsils o both were submitted to surgical adeno-tonsillectomy; they are European, mean age 8, range 2–18. The specimens were stored in physiologic solution at 4 °C until transported to the laboratory for the processing.

The parents of the children gave their informed written consent for participating at the study. All the study experiments and procedures followed the ethical standard of the 1975 Declaration of Helsinki (7th revision, 2013) and the IRCCS Burlo Garofolo Ethical Committee (RC03/04, L1055, protocol number 118/10) approved the study.

Genomic DNA of patients was extracted from the tonsils specimens using the Phenol-Chloroform method, following standard protocol [17].

The genomic DNA extracted from hypertrophic tonsils should not be different with respect to healthy tissue. Children hypertrophic tonsils are not neoplastic tissue so we did not expect any neoplastic tissue specific mutation or DNA genomic profile different form other healthy tissues of the same patients. However to triple-check this statement we extracted genomic DNA from buccal swabs of 30 randomly chosen children with AH (blood sampling has been avoided since more invasive), using the Norgen Bioteck DNA Isolation Kit (Thorold, Ontario, Canada): *DEFB1* SNPs genotyping revealed the same SNPs profile of the DNA extracted from hypertrophic tonsils.

Genomic DNAs belonging from 178 Italian healthy children (85 males, 93 females, mean age 8, range 6–12 enrolled in a previous screening program for celiac disease in schools “Good as Rice”), without chronic tonsillitis and tonsillectomy, have been employed as control group for the genetic analysis.

2.2. DEFB1 genotyping

Genomic DNAs of patients have been genotyped for the three 5′UTR *DEFB1* polymorphisms using Taq-man genotyping assays, C_11636795_20 (c.52- G > A), C_11636794_10 (c.-44C > G) and C_11636793_20 (c.-20G > A), on the ABI 7900 SDS real-time PCR platform (Thermo Fisher Scientific, Foster City, California, USA).

2.3. RNA expression

The specimens were submitted to mechanical homogenization using T25 basic Ultra-Turrax (Ika - Werke, Wilmington, North Carolina, U.S.A.) for 2 minutes. Then RNA was extracted using Trizol Reagent (Euroclone, Pero, Milan, Italy) and retro-transcribed using High-Capacity cDNA Reverse transcription kit (Thermo Fisher Scientific). *DEFB1* mRNA expression was evaluated using a specific Taq-man™ probe (Hs00608345_m1), β-Actin (as calibrator and reference, ACTB: Hs99999903_m1) was used as control. Experiments were run on Applied Biosystems 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific) platform. Raw fluorescent data were collected and converted in fold-increase with the Relative Quantification manager software (Thermo Fisher Scientific). The most high fold increase was used as reference for the comparison using the ΔΔCt method [18].

2.4. Immunohistochemistry

The specimens were embedded in Tissue-Tek O.C.T (Miles, Elkhart, Indiana, U.S.A.) and rapidly frozen on liquid nitrogen. Sections were cut (10 μm) with a CM3000 cryostat (Leica, Wetzlar, Germany) and then mounted on glass slides.

Immunohistochemistry was performed with Vectastain ABC HRP kit (PK-4000, Vector Laboratories, Burlingame, California, U.S.A.) following manufacturer's instruction. The sections were incubated with the primary antibody β-defensin 1 (FL-68, SC-20797, Santa-Cruz) at dilution of 1:200. Ematossilin and Eosin was used to counterstain the samples.

2.5. Statistical analysis

DEFB1 gene polymorphisms, allele and genotype frequencies were calculated by direct counting, while haplotype frequencies were computed using the Arlequin software, version 3.1.0 [19]. The Fisher's exact test was used for pairwise comparison of allele and genotype frequencies, and only p-values < 0.01 were considered significant. All statistical analyses were carried out using the open-source R version 3.1.3 [20]. Post hoc power calculations were performed through G*Power software version 3.1.9.2 (χ^2 tests - Goodness-of-fit tests: α error probability = 0.05, degrees of freedom = 3; exact distribution, Fisher's Exact test: tails = two; α error probability = 0.05) [21].

P-value for linkage disequilibrium analysis was calculated using the permutation test with the EM algorithm, Arlequin [19,22] whereas D′ and r^2 measures were computed with SNPstats [22].

3. Results

3.1. DEFB1 genotyping

The three *DEFB1* polymorphisms were in Hardy-Weinberg equilibrium in patients and controls, except for –c.44C > G among the patients (Table 1).

The *DEFB1* 5′UTR polymorphisms allele and genotype frequency distributions were not different between patients and controls, although a trend was observed for the –c.44G/G genotype more frequent among patients (0.14) respect to controls (0.05), but the statistically significance was not achieved (Table 1).

The three *DEFB1* polymorphisms were in linkage disequilibrium