Copy number variation, gene expression and histological localization of human beta-defensin 2 in patients with adeno-tonsillar hypertrophy

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ABSTRACT

Both bacterial infections and innate oral immunity response participate in development of adeno-tonsillar hypertrophy (ATH). ATH can lead to obstructive sleep apnea. We investigated the beta-defensin 2 (hBD-2) encoding gene, *DEFB4*, by analyzing the copy number variations (CNVs) of the defensin gene cluster in patients with ATH and by correlating CNV with *DEFB4* gene expression. We enrolled 79 patients with ATH, 21 of whom presented with only adenoid hypertrophy, while 58 exhibited hypertrophy of both adenoid and tonsil. CNVs of the defensin gene cluster, *DEFB4* mRNA, and hBD-2 protein expression were assessed. Also, beta-defensin 2 was localized histologically using immunohistochemistry. The distribution of defensin gene cluster CNV was similar among the 79 subjects. *DEFB4* expression analysis exhibited considerable inter-individual variability, but with neither specific differences among subjects nor correlation with the CNV number. Immunohistochemistry enabled localization of hBD-2 in the tonsil and adenoid epithelium. No differences in localization between the two ATH presentations were found. Inducible antimicrobial defensin peptides exhibited great inter-individual variability in terms of both CNV and gene expression, but no correlation with presentation of ATH was found.

KEYWORDS

Adeno-tonsillar hypertrophy; copy number variation; defensins; gene expression; human beta-defensin 2

Beta-defensins are a group of cationic antimicrobial peptides that are components of the innate immune system. These act as a first line of defense at sites of infection in mucosa and epidermis; they are produced mainly by epithelial cells (Dale and Krisanaprakornkit 2001). Beta-defensin 2 (hBD-2) is an inducible molecule that is produced in response to inflammation (Dale and Krisanaprakornkit 2001). hBD-2 binds to the outer surface of microorganisms and forms pores in the cell membranes, which kills the microorganism. hBD-2 is more active against Gram-negative than Gram-positive bacteria, and also possesses antifungal properties (Dale and Krisanaprakornkit 2001). hBD-2 is encoded by the *DEFB4* gene, which is located in a cluster with the genes for the other beta-defensins at 8p23.1. The gene varies in copy number among individuals (Hollox 2008).

hBD-2 is expressed in many sites including the oral cavity (Polesello et al. 2017), the adenoids (Choi et al. 2013) and the tonsils (Meyer et al. 2006). Tonsils and adenoids are part of Waldeyer's ring of upper respiratory tract-associated lymphoid tissues (Brandtzaeg 2003). Although tonsils and adenoids produce immune system molecules, recurrent or chronic infections may cause histomorphologic modifications that decrease functionality and cause hypertrophy with concomitant obstruction of the upper airway (Zautner 2012). Consequently, innate immune system molecules could play a role in susceptibility to adeno-tonsillar hypertrophy (ATH). Deficient production of antimicrobial peptide could increase susceptibility to infections (Dale and Krisanaprakornkit 2001) and hypertrophy. It has been suggested that *DEFB4* gene expression can be influenced by the genetic background, specifically by the copy number variation (CNV) of *DEFB4* (Hollox 2008).

We investigated hBD-2 by comparing its expression of mRNA and protein levels in children who present with ATH. We explored whether different levels of hBD-2 production could be related to susceptibility to hypertrophy. We analyzed the genetic background of hBD-2 to determine whether CNVs in *DEFB4* could be correlated to gene and protein expression. Finally, we localized the hBD-2 protein in both tonsil and adenoid specimens using immunohistochemistry.

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Material and methods

Study population

We enrolled 79 Italian children (mean age = 6 ± 2.14 years, range = 1–12; 35 females and 44 males) with surgically removed adenoid or adeno-tonsillar hypertrophic tissue at the Otolaryngology and Audiology Unit Department of the Institute for Maternal and Child Health IRCCS Burlo Garofolo (Trieste, Italy). All subjects presented with documented obstructive sleep disorder symptoms with apnea (Chervin et al. 2000). Twenty-one subjects presented with only adenoid tissue hypertrophy and no clinically appreciable tonsil hypertrophy, i.e. tonsils occupied < 50% of the posterior pharynx, while 58 exhibited hypertrophy of both adenoids and tonsils. Higher risk children with conditions including craniofacial abnormalities, Down syndrome, mucopolysaccharidoses, neuromuscular disorders or obesity were excluded from the study. Adeno-tonsillar specimens from surgery were stored in physiological solution (0.9% NaCl) at 4 °C until they were transported to the laboratory and processed.

Written informed consent was obtained from the children's parents. All experiments and procedures were performed following the ethical standards of the 1975 Declaration of Helsinki (7th revision, 2013). The IRCCS Burlo Garofolo Ethical Committee (RC03/04, L1055, protocol number 118/10) approved the study.

Defensin gene cluster CNV analysis

Genomic DNA was extracted from the tonsil specimens using the standard phenol-chloroform method (Green and Sambrook 2017). Briefly, the samples were digested overnight with proteinase K (1 mg/ml) in reaction buffer (0.01 M Tris-Cl, 0.005 M EDTA, 0.5%SDS) at 56 °C. An equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol then was added to the samples. After centrifugation at 14,000 x g for 5 min at room temperature the aqueous phase was transferred to a new tube and the procedure repeated. The DNA then was precipitated using ethanol, washed, then the DNA pellet was re-suspended in water. All the chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Because the entire region constituting beta-defensin genes exhibits CNVs (Nuytten et al. 2009), we used a CNV Applied Biosystems TaqMan custom assay (Thermo Fisher Scientific, Foster City, CA) whose primers and probe paired within the *DEFB104* gene. To confirm that our custom *DEFB104* assay tagged the 8p23.1 locus correctly, another CNV TaqMan custom assay (Applied Biosystems, Thermo Fisher Scientific) located within the same genetic region in the locus of *DEFB4* gene was used to validate the analysis for 10 randomly chosen samples. The RNAseP (4403326; Applied Biosystems, Thermo Fisher Scientific) assay was used as the reference. Each reaction was performed in a total volume of 15 µl using both CNV TaqMan assays (reference RNAseP and the custom assay) in the same well and the TaqMan Genotyping Master Mix (Applied Biosystems) using an ABI Prism 7900HT instrument (Applied Biosystems) following manufacturer's protocols. Briefly, an initial step for Taq polymerase activation at 95 °C for 10 min was followed by 40 cycles of 15 sec at 95 °C for denaturation and 1 min at 60 °C for extension. In each well, 15 ng of 5 ng/µl genomic DNA were tested, and for each sample, the reactions were performed in quadruplicate. The amplification curves were checked manually using the Safety Data Sheets software v. 2.3 (Applied Biosystems). For gene CNV assignment, absolute raw data were analyzed using CopyCaller software v. 2.1 (Applied Biosystems), which uses a comparative CT (ΔΔCT) relative quantitation analysis of real-time data, assuming "2" is the most frequent CNV for the defensin gene cluster (Ma and Chung 2014).

RNA expression

Specimens, 2 × 2 mm, were cut and homogenized using a T25 basic Ultra-Turrax device (IKA, Wilmington, NC) for 2 min. RNA was extracted with Eurogold Trifast Reagent following the manufacturer's instructions (Euroclone, Milan, Italy) and retro-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). The mRNA expression was evaluated using TaqMan assay for *DEFB4* (Applied Biosystems) and beta-actin as calibrator and reference (ACTB: Hs99999903_m1) using a 7900HT Fast Real-Time PCR System platform (Applied Biosystems). Raw fluorescence data were collected and converted to fold-increase using the Relative Quantification Manager software (Applied Biosystems). As reference for comparison using the ΔΔCt method, a sample with CNV = 1, detected by the CNV analysis described above, was chosen randomly from among the 21 specimens that exhibited CNV = 1, the lowest *DEFB4* CNV detected.

Protein expression

hBD-2 levels were measured using western blot analysis. Briefly, tissues were homogenized with a T25 basic Ultra-Turrax device (IKA, Wilmington, NC) for 2 min in 200 µl radio-immunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40

detergent (1% sodium deoxycholate, 0.1% sodium dodecylsulfate; Sigma Aldrich). Lysates were quantified using a Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were loaded onto Tris-glycine gels prepared according to the protocol reported by Schägger (2006), then transferred to polyvinylidene difluoride membrane using the Transblot Turbo Transfer system (Bio-Rad).

The membranes were incubated with primary antibody for hBD-2 (ab63982; Abcam, Cambridge UK) and beta-actin (sc-130656; Santa Cruz Biotechnologies, Dallas, TX) overnight at 4 °C, then developed with horseradish peroxidase-conjugated secondary antibody (ab97051; Cell Signaling Technologies, Leiden, The Netherlands) and Clarity substrate (Bio Rad). Staining intensity of hBD-2 was normalized to actin optical density using Image J software.

IHC

Specimens, 3 × 3 mm, were embedded in Polyfreeze (P0091; Sigma Aldrich) and snap frozen in liquid nitrogen as directed by manufacturer; specimens were stored at −80 °C. Sections were cut at 10 µm using a CM3000 cryostat (Leica, Wetzlar, Germany), then mounted on glass slides. Vectastain ABC HRP kit (PK-4000; Vector Laboratories, Burlingame, CA) was used for IHC using beta-defensin 2 primary antibody diluted 1:200 (Abcam, Cambridge, UK) in Tris buffer saline (TBS) with 2.5% normal goat serum and 0.1% Tween (Sigma Aldrich). Sections were counterstained using hematoxylin and eosin.

Statistical analysis

Defensin gene cluster CNVs frequencies were determined by direct counting, and analyzed using the Fisher exact test on R software v. 3.4.3. Values for $p \leq 0.05$ were considered statistically significant.

Results

Defensin gene cluster CNV analysis

The mean CNV is 2 (range 1–5) among the patients; the detailed CNVs frequencies are given in Table 1. The CNVs frequency was compared between patients with adenoid hypertrophy and those with ATH; no difference was found between these two groups. A confirmation analysis using a second CNV assay using 10 random samples showed identical results for CNVs.

Table 1. Comparisons of defensin gene cluster CNV frequencies between children with adenoid hypertrophy and those with ATH.

Defensin gene cluster CNV	Adenoid hypertrophy	Adeno-tonsil hypertrophy	Adenoid hypertrophy vs. adeno-tonsil hypertrophy
	n = 21	n = 58	
2	10 (0.48)	29 (0.50)	Reference
1	4 (0.19)	17 (0.29)	p = 0.75, OR = 0.69, 95% CI = 0.14–2.87
3	6 (0.28)	8 (0.14)	p = 0.31, OR = 2.14, 95% CI = 0.48–9.25
4	1 (0.05)	2 (0.03)	p = 1.00, OR = 1.44, 95% CI = 0.02–30.48
5	0 (0.00)	2 (0.03)	p = 1.00, OR = 0.00, 95% CI = 0.00–16.92

OR, odds ratio; CI, 95% confidence interval.

RNA expression

DEFB4 RNA expression varied among individuals. Figure 1 shows an example of relative quantification of gene expression using the ΔΔCt method; the T40 sample from the adeno-tonsillar group was used as an internal calibrator (selected randomly from patients with CNV = 1, the lowest *DEFB4* CNV). No correlation was found between CNV and the level of gene expression (Figure 1).

Evaluation of protein level

The level of hBD-2 reflected the RNA expression and there was no correlation between CNV and protein concentration (Figure 2). Protein levels varied between adenoids and tonsils and among individuals, e.g. T40 and T69, which possessed the same *DEFB4* CNV (1), exhibited different protein expressions.

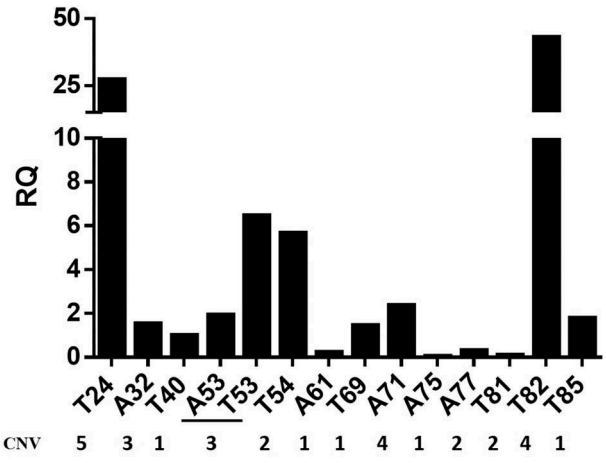

Figure 1. Example of DEFB4 RNA expression analysis using patient T40 as an internal calibrator. Below DEFB4 CNVs are indicated for each sample.

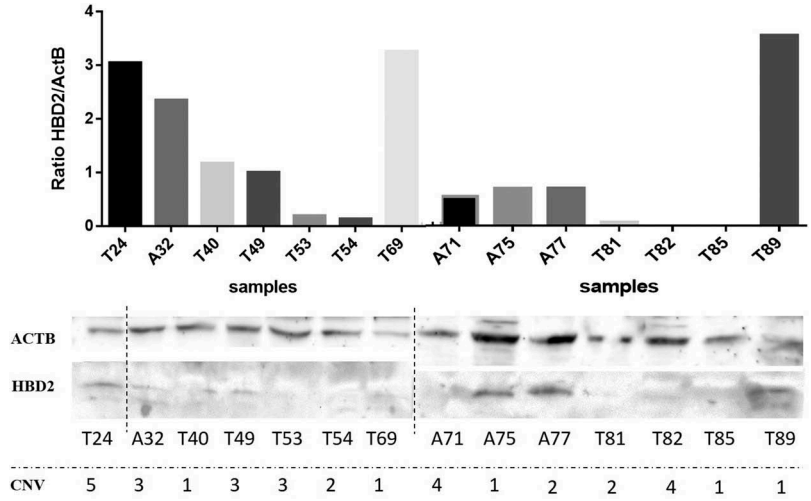

Figure 2. Upper panel: relative quantification of optical density signal. Integrated intensity from β-actin was used to normalize hBD-2 signal and to equalize minimal protein loading differences. Lower panel: western blot membrane showing β-actin (ACTB) and hBD-2 signals in different samples. CNV = *DEFB4* gene CNVs.

Immunohistochemistry

In tonsils, hBD-2 was localized on the external margin of the epithelium and in the lymphoid tissue (Figure 3). In adenoids, however, distribution of hBD-2 staining was diffuse with no clear distinction among different areas (Figure 4a, b).

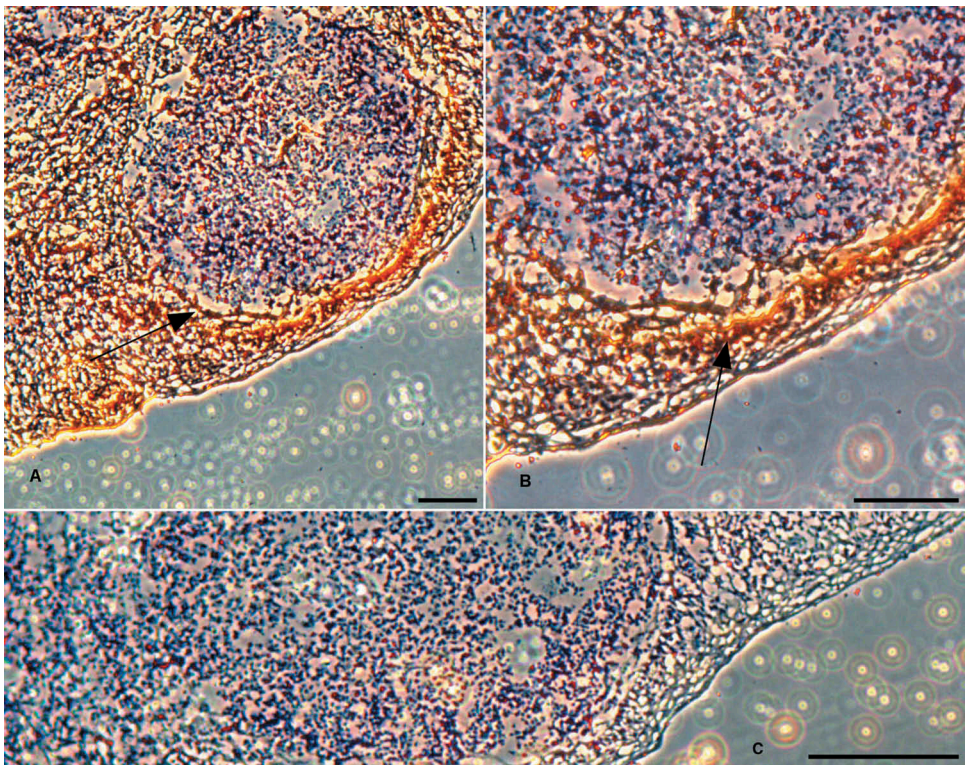

Figure 3. a) Immunohistochemically stained tonsillar section showing hBD-2 localization. A thin layer of hBD-2 immunopositivity can be seen on the epithelial side of the section; hBD-2 is localized in cells (arrow) surrounding a germinative center. Scale bar = 100 μm. b) Higher magnification of the same region as in (a). Arrow, hBD-2 positive cells around a germinative center. Scale bar = 100 μm. c) Higher magnification of the same region as in (a) in which primary antibody was omitted during staining as a negative control. No hBD-2 signal is apparent. Scale bar = 100 μm.

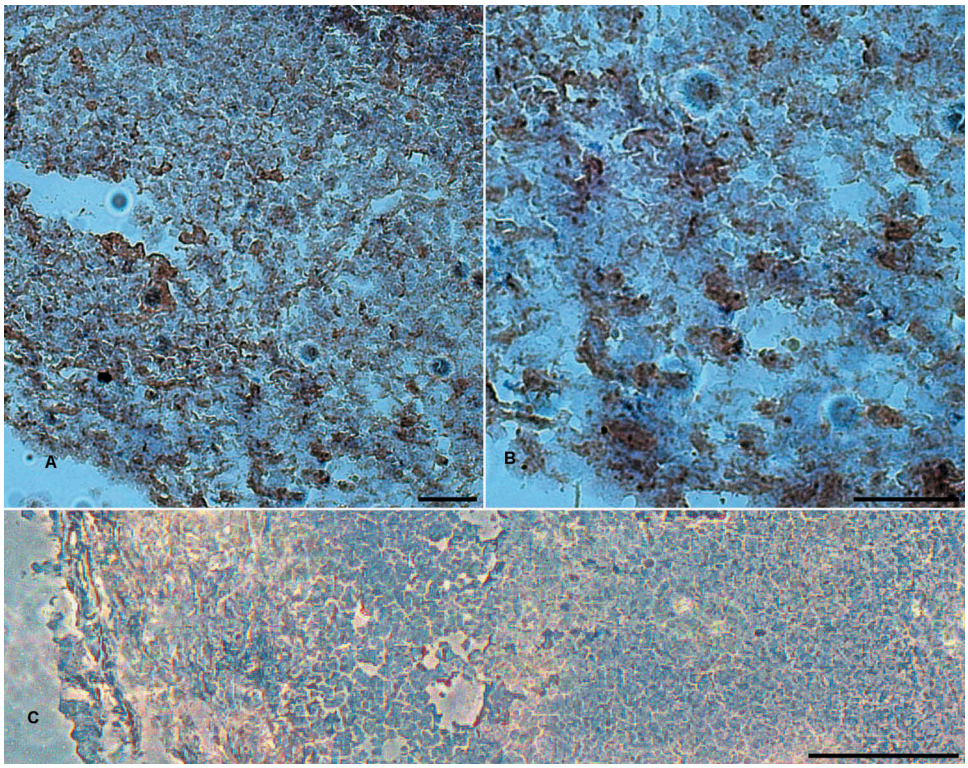

Figure 4. a) Immunohistochemically stained adenoid section showing hBD-2 localization. By contrast to tonsil, staining is diffuse with no specific localization. Scale bar = 100 µm. b) Higher magnification image taken from same region as in (a), confirming that hBD-2 staining is diffuse. Scale bar = 100 µm. c) Higher magnification image of the same region as in (a) in which primary antibody was omitted during staining as a negative control. No hBD-2 signal is apparent. Scale bar = 100 µm.

Discussion

The etiology of ATH is not fully understood (Ingram and Friedman 2015); however, bacteria appear to trigger immune activation with subsequent chronic inflammation and hypertrophy, which suggests that the immune response and its components, e.g. hBDs, participate in the pathology (Zautner 2012). We reported earlier the potential involvement of hBD-1 in adeno-tonsillar disease (Zupin et al. 2016, 2018). We report here our assessment of the role of another member of hBD family, hBD-2.

Our IHC investigation of the tonsils demonstrated that hBD-2 was expressed mainly in the epithelial lining with scattered staining in the lymphoid tissue. Our observations are partially consistent those of Claeys et al. (2003) who reported the presence of hBD-2 peptide in most tonsils, both on the surface and in crypt epithelia. These investigators did not report staining in the lymphoid tissue that we saw. Our findings also were consistent with the report by Choi et al. (2013) that immunoreactive cells were distributed in the surface epithelium and submucosal area of the tonsils. In adenoids, we observed hBD-2 staining of both the surface epithelia and the submucosal area, although the reactivity was weaker than that for the tonsils.

We have found few reports concerning the effect of defensin CNVs on gene and protein expression, and none concerning adenoids and tonsils. Low *DEFB4* CNV was correlated with low *DEFB4* mRNA expression in inflamed colon biopsies from patients with inflammatory bowel disease (Fellermann et al. 2006). Also, low *DEFB4* CNV was correlated with low serum hBD-2 concentration within generalized chronic periodontitis patients (Jaradat et al. 2013) and among healthy individuals (Jansen et al. 2009).

We found no significant association between hBD-2 CNV, mRNA expression and protein levels. Also, no differences in CNVs were observed between children affected by adenoid hypertrophy and children with ATH. The surprising lack of correlation could be due to the state of inflammation and hyperplasia or to other variables that could affect hBD-2 production. For example, hBD-2 is induced by various stimuli, including Gram-negative and Gram-positive bacteria, and *Candida albicans* as well as cytokines such as tumor necrosis factor-α and interleukin-1β (Schibli et al. 2002). Singh et al. (1998) suggested that airway epithelia may require both microbial and immune system stimuli before responses to hBD-2 secretion

occur. On the other hand, it could be that the bacteria are the triggers, but by the time adenoids and tonsils are excised, the infections had resolved and only the established hyperplasia remained (Bell et al. 2012). In the latter case, molecular markers of innate immune system activity could have returned to normal levels.

Our findings could be influenced by the small number of subjects enrolled and the multifactorial etiology of the disease, not yet clearly defined, could have an impact on the laboratory outcomes. Because other reports described similar results (Claeys et al. 2003; Schwaab et al. 2010; Bell et al. 2012), we believe that hBD-2 does not affect adeno-tonsillar disease. Claeys et al. (2003) suggested earlier that the original natural co-existence between microorganisms and human body could not activate and exhaust the innate defenses until the mucosal barrier is broken.

Disclosure statement

The authors declare no conflict of interest

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