# Transcriptomic Analysis Identifies Phosphatases as Novel Targets for Adenotonsillar Hypertrophy of Pediatric OSA

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**Online Data Supplement**

## SUPPLEMENTARY METHODS

### Patients

The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained from the legal caregiver of each participant, with assent being obtained from children older than 7 years of age. Consecutive prepubertal non-obese children diagnosed with OSA at the University of Louisville Pediatric Sleep Research Center in Louisville, KY, invited to participate. Inclusion criteria were the presence of OSA according to polysomnographic criteria and age between 6 and 11 years. Exclusion criteria were any children with chronic medical condition, receiving medications, and any genetic or craniofacial syndromes. Age, gender, and ethnicity; use of medications, antihistamines, and presence of co-morbidities were gathered from each participant.

Children with recurrent tonsillar infection (RI) were selected based on a history of at least five tonsillar infections requiring administration of antibiotic courses over a period of less than 6 months, as well as the absence of any symptoms suggestive of OSA using a previously validated questionnaire (1). Patients referred for RI were selected based on a history of at least five tonsillar infections in < 6 months and the absence of any symptoms suggestive of OSA using a previously validated questionnaire (2). However, to further ascertain the absence of sleep-disordered breathing they were evaluated by an overnight sleep study.

### Polysomnographic assessment

Children were studied for up to 12 hours in a quiet, darkened room with an ambient temperature of 24°C in the company of one of their parents. No drugs were used to induce sleep. The following parameters were measured during the overnight sleep recordings: chest and abdominal wall movement by respiratory impedance or inductance plethysmography; heart rate by ECG; and air flow, which was triply monitored with a side-stream end-tidal capnograph that also provided breath-by-breath assessment of end-tidal carbon dioxide levels ($PET_{CO_2}$; BCI SC-300, Menomonee Falls, Wis), a nasal pressure cannula, and an oronasal thermistor. Arterial oxygen saturation ($Sp_{O_2}$) was assessed by pulse oximetry (Nellcor N 100; Nellcor Inc, Hayward, CA), with simultaneous recording of the pulse waveform. The bilateral electro-oculogram, 8 channels of electroencephalogram, chin and anterior tibial electromyograms, and analog output from a body-position sensor (Braebon Medical Corp, Ogdensburg, NY) were also monitored. All measures were digitized with a commercially available polysomnography system (Rembrandt, MedCare Diagnostics, Amsterdam, Netherlands). Tracheal sound was monitored with a microphone sensor (Sleepmate, Midlothian, VA), and a digital time-synchronized video recording was performed.

The proportion of time spent in each sleep stage was expressed as percentage of total sleep time. Obstructive apnea was defined as the absence of airflow with continued chest wall and abdominal movement for duration of at least 2 breaths (2, 3). Hypopneas were defined as a decrease in oronasal flow of ≥50% with a corresponding decrease in $Sp_{O_2}$ of ≥4% and/or arousal (2). The obstructive apnea/hypopnea index was defined as the number of apneas and hypopneas per hour of total sleep time. The obstructive apnea index was defined as the number of apneas per hour of total sleep time. The diagnostic criteria for OSA included an obstructive apnea index >1 per hour of total sleep time and/or an obstructive apnea-hypopnea index >5 per hour of total sleep time with a nadir oxygen saturation value <92% (2). The diagnosis of OSA was established by

overnight polysomnography in the sleep laboratory and required the presence of an apnea-hypopnea index more than five events per hour of sleep (4).

**Body mass index**

Height and weight were obtained using standard techniques from each child. BMI was then calculated (body mass/height$^2$) and was expressed as BMI z-score using an online BMI z score calculator (http://www.cdc.gov/epiinfo/). Children with BMI z-score values exceeding 1.20 were classified as fulfilling the criteria for overweight/obesity (5), and were excluded from this study.

**Statistical analysis**

Results are presented as mean ± SD unless stated otherwise. All analyses were conducted using statistical software (version 11.5; SPPS; Chicago, IL). Comparisons according to group assignment were made with independent *t* tests or analysis of variance followed by *post hoc* comparisons, with p values adjusted for unequal variances when appropriate (Levene test for equality of variances), or $\chi^2$ analyses with Fisher's exact test (dichotomous outcomes). A two-tailed *P*-value < 0.05 was considered statistically significant.

**Tonsillar tissue collection**

Since tonsil cannot be obtained from normal children for obvious ethical reasons, consecutive children undergoing tonsillectomy at Kosair Children's Hospital for either OSA or RI were identified before surgery and recruited to the study. OSA and RI children were also required to have received their last dose of antibiotic therapy at least 6 wk before the day of the surgery. Children with OSA were excluded if they suffered from RI (based on aforementioned criteria). Children with known asthma, allergic rhinitis, history of allergies, and/or having received corticosteroid or leukotriene modifier therapy within 12 months from surgery were excluded (for both groups). Tonsils were removed by a pediatric ENT specialist, and a portion of each tonsil was stored in RNALater (Applied Biosytems/Ambion Woodward St. Austin, TX) as recommended by the manufacturer protocol, and stored at -80°C.

**Tonsil Immunohistochemistry**

Tonsils were placed overnight in a fixative containing 1% paraformaldehyde in PBS and 30% sucrose at 4$^o$C. Post-fixed tissues were sectioned on a freezing microtome. Coronal sections (30 microns) of tonsils were initially incubated in 0.3% $H_2O_2$ for 30 minutes, washed several times in PBS, and blocked with a PBS/0.4% Triton X-100/0.5%TSA (Tyramide Signal Amplification, Perkin Elmer Life Sciences, Boston, MA) blocking reagent/10% normal goat serum (Vector Laboratories, Burlingame CA) for 1 hour. Sections were then incubated with primary PSPH antisera (Abcam cta# ab58125; 1:1000) at 4 $^o$C for 24 hours, and then washed in PBS 6 times for 5 minutes each wash. Sections were then incubated at room temperature for 1 hour in biotinylated anti-rabbit antibody (Vectastain Elite ABC kit, Burlingame CA; 1:600) in a PBS/0.5% TSA blocking reagent /10% goat serum solution. After 3 5-min washes, sections were incubated at room temperature with streptavidin-horseradish peroxidase diluted 1:100 in PBS/0.5% TSA blocking reagent. Subsequently, the sections were incubated with tetramethyl rhodamine tyramide (red) diluted 1:50 in amplification diluent (Perkin Elmer Life Sciences , Boston, MA) for 2 minutes. Sections were then washed in PBS, and mounted onto glass slides. Negative controls were prepared by either omitting the primary or the secondary antibody. Sections were prepared from 5 sets of tonsils and of adenoids from either OSA or RI groups, and

were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

**Mixed adenotonsillar primary cell culture system**

Surgically removed tonsils and adenoids were placed in ice cold phosphate buffered saline (PBS) plus antibiotics and processing was started within 30 minutes after surgical excision under aseptic conditions. Briefly, tonsils were washed thoroughly with PBS, manually dissected into Petri dishes, and gently grounded with a syringe plunger through a 70 μm mesh screen to obtain a mixed cell suspension through mechanical dissociation. Red blood cells were removed by lysis buffer. Cells viability of all specimens was determined by trypan blue exclusion. Specimens with a viability of less than 70% were discarded. Cells cultures were established in standard medium RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics, which included streptomycin, fungisone, gentamycin, and penicillin to prevent bacterial and fungal contamination. Mixed cell suspensions were transferred onto 24-well or 96-round bottom-well plates at a concentration of $1x10^6$ cells/well. Cells were cultured in a 5 % $CO_2$ incubator at 37°C for 48 hours. Cells were incubated to evaluate basal proliferation or treated with PSPH inhibitors such as okadaic acid, Calyculin A, and PPI2 at $10^{-6}$M to a$10^{-9}$ concentrations.

**BrdU cell proliferation and apoptosis annexin V assays with flow cytometry**

To detect global cell, T-cell and B-cell specific proliferation, we employed bromodeoxyuridine (BrdU) pulsed proliferation analysis using flow cytometry. All procedures were measured using the APC BrdU flow kit (BD Biosciences, San Diego, CA) as previously described [13, 18] and as recommended by the manufacturer. In brief, at the end of 48 hours of cell culture in 24-well plates, cells were pulse-labeled with 1 mM BrdU for 4 hours. The cells were then washed with PBS, and BrdU labeled cells were stained with a 3-color antibody combination consisting of mouse anti-human CD45/PerCP Cy7, CD3/PE, and CD19/APC-Cy7 antibodies (BD Biosciences, San Diego, CA) in 50 μl staining buffer for 15 min on ice. Following binding, the cell-surface antibodies, cells were fixed and permeabilized with cytofix/cytoperm buffer. After this procedure, cells were suspended with DNase (300 μg/ml) for 1 hour at 37°C. The anti-BrdU APC antibody was added in perm/wash buffer and incubated for 20 min at room temperature. Isotype controls relevant for each of the antibodies were used to establish background fluorescence. Negative control was used as a sample that was untreated with BrdU and was not stained with specific fluorescence antibodies. Data were acquired on a FACS Aria flow cytometer using the FACS Diva 5.5 software (BD Biosciences, San Diego, CA). After gating of lymphocytes based on CD45+ cells, T-cell and B-cell numbers were calculated as CD3+/CD19- and CD3-/CD19+ cell populations, respectively. Proliferation of T-cells and B-cells was identified by counting CD3+/BrdU+ and CD19+/BrdU+ cell populations. A similar approach was undertaken using Annexin V mouse anti-human antibodies to quantify global, T-cell or B-cell specific apoptosis. The results were displayed as two color dot-plots and analyzed by FlowJo software (Tree Star, San Carlos, CA). All data are expressed as the percentage of positive cell from the total cell population.

**RNA isolation and microarray hybridization**

Total RNA from tonsils of 18 children with RI and 18 children with OSA was isolated using RNeasy Lipid Tissue Mini Kit with DNase treatment (Qiagen, Valencia, CA). Tissues were homogenized in 1 ml of QIAzol Lysis Reagent (Qiagen, Valencia, CA) using a PolyTron homogenizer. RNA integrity was assessed for each sample using the Agilent 2100 Bioanalyzer

(Agilent Technologies, Palo Alto, CA), cRNA was generated, fluorescently labeled with Cyanine 3-dCTP (Perkin Elmer, Boston, MA), and hybridized to the Agilent human whole-genome arrays containing 44,000 transcripts. Microarrays were scanned (SureScan, Agilent Technologies) followed by image processing and filtering using Agilent Feature Extraction software.

**Quantitative RT-PCR**

qRT-PCR analysis was performed using ABI PRISM 7500 System (Applied Biosystems, Foster City, CA). The same total RNA was used for both microarray and RT-PCR experiments. cDNA synthesis was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Ribosomal 18S rRNA was used as a reference gene to normalize the expression ratios for the gene of interest. The primer sequences of the genes of interest were designed to be within the same region of the microarray sequence probes. One microgram of total RNA from OSA and RI samples was used to generate cDNA templates and TaqMan® Master Mix Reagent Kit (Applied Biosystems, Foster City, CA) was used to amplify and quantify each transcript of interest in 25 μl reactions. Triplicate PCR reactions were performed in 96-well plates for each gene in parallel with the 18S rRNA. The steps involved in the reaction program included: the initial step of 2 minutes at 50$^{o}$C; denaturation at 95$^{o}$C for 10 min, followed by 45 thermal cycles of denaturation (15 seconds at 95$^{o}$C) and elongation (1 min at 60$^{o}$C). The expression values will be obtained from the cycle number (Ct value) using the Biosystems analysis software. All the genes of interest and 18S rRNA will be performed in triplicates to determine the Ct-diff. These Ct values will be averaged and the difference between the 18S Ct (Avg) and the gene of interest Ct (Avg) will be calculated (Ct-diff). The relative expression of the gene of interest will be analyzed using the $2^{-\Delta\Delta CT}$ method. Quantitative results were expressed as the mean ±standard deviation (SD). Statistical significance was evaluated by the Student's t-test

**Identification of enriched pathways in tonsillar tissue**

Initially, the filtered gene expression intensities of all 36 subjects (OSA and RI) were log-transformed and normalized using the quantile method. Intensities of multiple probes mapping to the same gene were averaged, resulting in ~31,000 unique gene expression values. Enriched pathways in tonsillar tissue from children with OSA were identified using gene set enrichment analysis (6) (GSEA). Approximately 1800 gene sets were computationally sampled and a random permutation analysis of the subjects (n = 1000) was performed to determine enrichment of gene sets in each group at a false discovery rate cutoff ≤ 10%.

**Gene interaction network analysis**

Genes mapped to gene sets enriched in OSA subjects and involved in proliferative pathways were combined, and an interaction network was created using Ingenuity's knowledge base (7) and several publicly available gene product relationship databases (8-10). The interaction network, or interactome, was built around genes with the highest connectivity using an iterative algorithm that systematically connects additional nodes to the initial seed. The topological characteristics of the interactome, i.e., the number of nodes and connectivity, were extracted.

**Ranking of network-associated genes based on their Significance Score**

A score based on the topologic properties of the interactome and the differential expression levels of its nodes was developed to identify the genes most likely to be important in tonsillar

hypertrophy. There is mounting evidence that the functional integrity of genetic networks is highly dependent on hubs of high connectivity (11, 12). Using the interactome's topology, we rank-ordered its members based on their connectivity. Since this method does not provide information on the relative differential expression of a given gene in the tonsillar tissue of children with OSA compared to RI, an integrated Significance Score was defined as:

$$\text{Significance Score} = \text{Ln}[\text{Connectivity}] - \text{Ln}[P\text{-value}]$$

Where the *P*-value was based on a gene-by-gene inter-group comparison using the parametric *t*-test (two-sided, unequal variance) on log-transformed gene expression values. In general, the greater a gene's connectivity and the more significant its differential expression, the higher its score. However, genes with highly significant expression, even if sparsely connected will receive relatively high scores, as will genes with a modest degree of differential expression but high connectivity within the network. Statistical cutoff values were determined by performing a random permutation analysis on the subjects (n = 10,000) and obtaining a null frequency distribution for the Significance Score. Genes with scores above the $95^{th}$ percentile of the null distribution were deemed significant (Supplementary Fig. E3).

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