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ORIGINAL ARTICLE

The protective role of *Helicobacter pylori* neutrophil-activating protein in childhood asthma

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Abstract
Background: *Helicobacter pylori* quantity and HP-NAP gene expression were evaluated in the faeces of healthy and asthmatic children.
Methods: *H. pylori* DNAs and RNAs were isolated from the stool samples of 92 asthmatic children (AC; 3–8 years) and 88 healthy controls (HC). Quantitative PCR was used to determine the quantity of *H. pylori* and HP-NAP expression relative to the 16S rRNA (reference gene). Gene expression was analysed using the delta delta-Ct method.
Results: *H. pylori* DNA was detected in the stool samples of 18 (20.4%) of the 88 HC ($p < 0.0001$, OR = 0.79) and none of AC. No meaningful statistical differences were found between individuals with positive and negative family histories for asthma in AC and HC ($p > 0.05$). *H. pylori* quantity was higher in seven of 18 *H. pylori*-positive samples, but HP-NAP expression levels were low in four of these seven samples. Based on a multivariate logistic regression analysis of these three variables together, only males displayed a significant difference based on gender differences ($p < 0.02$) and it was determined that, based on the OR value of 0.46 and the 95% CI range of 0.241–0.888, male gender was an independent protective factor in asthma.
Conclusions: HP-NAP levels vary to the relative concentrations of bacteria in the stationary or late logarithmic phases. Different *napA* expression levels may be caused by different endogenous *napA* gene expression or different environmental conditions.

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Introduction

Asthma is a common chronic disease in childhood that is defined by chronic inflammation of the lower airways and the development of wheezing, cough, shortness of breath, and chest tightness.[1] Asthma increased from 235 to 334 million cases between 2011 and 2014, while 14% of the world's children experience asthma symptoms and the burden of asthma is greatest for children aged 10–14 and the elderly aged 75–79.[2] The hygiene hypothesis, an inverse association between infection and atopy, was first proposed by David Strachan in 1989. In this hypothesis, reduced childhood exposure to microorganisms shifts Th1/Th2 immune responses to Th1, reducing the number of allergic disorders. Strachan suggested that fewer family members would result in unhygienic contact, which would be protective and avoid cross-infection from siblings.[3]

Several studies support an inverse association between *Helicobacter pylori* infection and the frequency of allergic asthma.[4,5] *H. pylori* is a Gram-negative, microaerophilic gastric bacterium that colonises much of the world's population and is commonly acquired in childhood. In recent years, *H. pylori* prevalence has decreased substantially in developing countries due to socioeconomic development, effective therapy, and hygiene improvements.[6,7] An inverse correlation was observed between prior acquisition of *H. pylori* and the possibility of being diagnosed with asthma or allergy, and the decreasing incidence of *H. pylori* in the developed world paralleled an increase in the incidence of childhood allergies and autoimmune diseases. This observation came from the third National Health and Nutrition Examination Survey (NHANES).[8] Another suggestion was the relation of reduced risk of childhood-onset ($\leq$15 years) asthma with cagA+ strains of *H. pylori*.[9,10] In a study from Finland, a three-fold increase in the incidence of allergy was reported, with a 30% decrease in *H. pylori* prevalence, between 1973 and 1994.[11] *H. pylori* infections are usually acquired in early childhood, when the immune system is not mature. *H. pylori* infections stimulate a Th1 response in the gastric mucosa and also in peripheral blood. Cytokines of Th1 cells can suppress a Th2 response, which is related to the *H. pylori* neutrophil-activating protein (HP-NAP), an important Th1-promoting virulence factor that inhibits Th2 cytokine release in humans and mice.[5]

We suggested that the dominant factor of the hygiene hypothesis may possibly be the immunomodulation function of *H. pylori* by HP-NAP. In our study, we aimed to determine the relationship between allergic asthma and *H. pylori*'s protective effect by quantifying the number of *H. pylori* bacteria in the stool samples of diagnosed asthmatic children (allergic asthma) and matched healthy control children, and also by quantifying the expression of the HP-NAP gene implicated in the disease.

Materials and methods

Patient and control groups

Our study was conducted as a cross-sectional, case control study conducted from March 2014-January 2015. Ninety-two children diagnosed with clinical asthma, as defined by the international diagnostic criteria,[12] between the ages of three and eight who applied to the Allergy Polyclinic of the Pediatrics Department of Istanbul University Cerrahpasa Medical Faculty were included in the study. Fifty-four (58.6%) of the cases were boys and 38 (41.3%) were girls. The mean age was 5.67 ± 1.24 years. All clinical symptoms and laboratory data (immunological, biochemical, etc.) belonging to the patients that were consistent with allergic asthma were taken from the patients' files. The control group was composed of 88 healthy children with a similar mean age, gender distribution, and standard of living who applied to the healthy children polyclinic of the same medical centre in the same timeframe. Of the control group, 52 (59%) were boys and 36 (40.9%) were girls. The mean age was 5.43 ± 1.61 years ($p > 0.05$). While the control and study groups were being assembled, those who had used antibiotics at any time during the two weeks prior to the beginning of the study, as well as those who had a history of an infectious disease in the past month, were excluded from the study.

Collection of the stool sample

In order to investigate *H. pylori* quantity and gene expression, 20 g stool samples were collected from cases consistent with the study criteria who applied to the Allergy and Healthy Children Polyclinics of Cerrahpasa Medical Faculty. Stool samples were divided into two parts: one part stored at $-20\,^{\circ}C$ for *H. pylori* DNA isolation and the other stored at $-80\,^{\circ}C$ for RNA isolation until molecular investigations began. The location of storage was inside the MagNA PureLC DNA isolation kit in the Medical Microbiology department.

Molecular tests

Bacterial DNA isolation from stool samples was performed using a Stool DNA isolation kit (cat: 27600, Norgen Biotek Corp, Canada); bacterial RNA isolation was performed using a Stool Total RNA isolation kit (cat: 49500, Norgen Biotek Corp, Canada) in accordance with the manufacturer's instructions. The DNA and RNA concentrations in the specimens following all isolations were quantified spectrophotometrically using a Nanodrop instrument (Thermo Scientific, USA). The obtained DNA samples were stored at $-20\,^{\circ}C$ until the *H. pylori* quantitative PCR (qPCR) study began. The RNA samples obtained were reverse-transcribed into cDNA using the Transcriptor First Strand cDNA synthesis kit (cat: 04896866001, Roche Diagnostics GmBH, Germany) in accordance with manufacturer instructions. The obtained cDNAs were stored at $-20\,^{\circ}C$ until the HP-NAP A qPCR study began.

In order for the obtained DNA to be detected by *H. pylori* qPCR, the Genesig *H. pylori* kit (cat: Path-Hpylori, Genesig, PrimerDesign Ltd, UK) and the oasig lyophilised qPCR MasterMix kit (cat: Precision-oasig150, Genesig, PrimerDesign Ltd, UK) were used and the studies were performed on a LightCycler 96 (Roche Diagnostics GmBH, Germany) qPCR instrument in accordance with manufacturer instructions. The PCR protocol of the study was as follows: denaturation for 2 min at $95\,^{\circ}C$, followed by 50 cycles of 10 s at $95\,^{\circ}C$ and 60 s of $60\,^{\circ}C$. The primer and probe series were

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