

Neuropeptide Y associated with asthma in young adults

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ABSTRACT

Objective: Neuropeptide Y, a widely circulating neurotransmitter, plays a pivotal role in energy balance, immunomodulation and asthma, and several NPY polymorphisms are promising genetic risk factors for asthma and obesity. We explored the associations of candidate NPY gene polymorphisms with prevalent asthma and its relationship with obesity in young adult asthma patients free of other chronic medical morbidity.

Methods: Five common gene variants of NPY (rs16147 (−399T/C), rs17149106 (−602G/T), rs16140 (+1000C/G), rs5573 (+1201A/G), rs5574 (+5327C/T)) previously validated to account for most of the NPY expression in vitro and in vivo were investigated in 126 physician-diagnosed asthma patients without other chronic medical morbidity and 182 healthy controls (21–35 years). Plasma levels of NPY, adiponectin, and CRP were determined using ELISA, and IL-6 was measured by Luminex in a subgroup of 70 patients and 69 age- and sex-matched healthy controls.

Results: In logistic regression models controlling for gender and obesity, the CT genotype of rs5574 (OR = 0.54, 95%CI: 0.30–0.89) and the GT genotype of rs17149106 (OR = 5.58, 95%CI: 1.09–28.54) were significantly associated with asthma. No significant interaction between NPY SNP polymorphisms and obesity were detected. Plasma NPY level was correlated with adiponectin levels ($p < 0.05$). Compared with the healthy controls, patients with asthma had higher BMI ($p < 0.001$), adiponectin ($p < 0.05$), IL-6 ($p = 0.001$) and CRP ($p < 0.001$), and lower NPY levels ($p < 0.01$).

Conclusions: The CT genotype of rs5574 and the GT genotype of rs17149106 are significantly associated with prevalent asthma.

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1. Introduction

Neuropeptide Y (NPY) is a highly conserved 36 amino acid neurotransmitter involved in a broad range of fundamental physiological activities such as vasoconstriction, regulation of bodyweight and energy balance, immune function, and emotional response (Zhou et al., 2008). A key point of the pivotal regulatory role of NPY is its immunomodulatory function and maintenance of body homeostasis (Thangaratnarajah et al., 2016). In asthma, NPY is suggested to play a bimodal role in active

T cell priming through activating neuropeptide Y receptor Y1 (NPY Y1R) on antigen-presenting cells (APCs), and negative regulation by modulating T cell hyper-responsiveness (Makinde et al., 2013). NPY is also secreted by lung and attenuates the Th1/Th2 imbalance in asthma (Makinde et al., 2013). In addition to immunomodulation, NPY is also important in the regulation of appetite and bodyweight. NPY is found to co-localize with adiponectin receptors in the arcuate nucleus (Guillod-Maximin et al., 2009). Under stressful situations, NPY is released from sympathetic nerve terminals of visceral adipose tissue and accelerates adipocyte growth and fat storage, to increase the supply of energy for dealing with the external threat (Kuo et al., 2007). This may explain why some people gain weight under stress. Stress such as life events could increase the secretion of peripheral NPY from sympathetic nerves and activate related NPY receptors in the abdominal fat,

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stimulating fat angiogenesis, macrophage infiltration, and the proliferation and differentiation of new adipocytes and facilitating food storage and obesity. NPY gene is located near the gene clusters regulating immunological functions. There are limited studies which assessed the role of NPY genotypes in asthma. Aldrich et al. (2009) showed that NPY genetic variation is associated with asthma prevalence (rs5574) and severity (rs5574 and rs16143) in Puerto Ricans, but this effect was not observed in Mexicans.

In Singapore, the prevalence of asthma is as high as 13.2% in young adults (Ministry of Health, 2009). Obesity is well documented to be associated with increased risk and severity of asthma, although the underlying biological mechanism and relationship with NPY is not well understood (McLachlan et al., 2007). In a 10-year longitudinal study of 939 Singaporean asthma patients (Tan et al., 2016), the prevalence of being overweight or obese accounts for more than half of the sample (31% obese (BMI ≥ 25 kg/m²) and 20% overweight (BMI 24–26.9 kg/m²)) and is among the main predictors of subsequent asthma costs. Obesity is associated with increased risks of asthma onset by approximately 2.0 folds in children (Chen et al., 2013) and 2.3 folds in adults (Beuther and Sutherland, 2007) respectively. Body mass index (BMI) increases asthma incidence and severity in a dose-dependent manner (Beuther and Sutherland, 2007; Weiss and Shore, 2004) and decreases patients' response to inhaled corticosteroids (Celedon and Kolls, 2014), and weight loss is reported to improve lung function, asthma symptoms, and asthma control (Maniscalco et al., 2008; Stenius-Aarniala et al., 2000). NPY is a strong candidate for BMI pathway genes. NPY Y1R and Y5R knockout mice have increased food intake, body weight, and adipose deposition (Hsieh et al., 2013; Vahatalo et al., 2016). The Pro7 allele of NPY SNP rs16139 (NPY + 1228), a functional polymorphism which regulates an amino acid change of Leucine7 to Proline7 (Leu7Pro) in the signal peptide of NPY, is reported to be associated with higher cholesterol levels in a population study in Northern Europe (Karvonen et al., 1998). The only other study which investigated the role of NPY gene variation in asthma among Finnish young adults found that rs16147 was associated with an increased risk for self-reported asthma in overweight (BMI ≥ 25 kg/m²) individuals (Jaakkola et al., 2012), suggesting an interaction between NPY gene polymorphism and obesity.

Genetic variation at the NPY loci has been demonstrated to be associated with multiple conditions including cardiovascular disease and high blood pressure (rs16147, rs16139, rs3037354), (Jaakkola et al., 2009; Shah et al., 2009; Zhang et al., 2012) responses to stress and emotional challenge (rs16147), (Zhou et al., 2008) alcohol dependence (rs16139, rs17149106), (Lappalainen et al., 2002; Mottagui-Tabar et al., 2005) obesity (rs3037354), (Bray et al., 2000) ischemic stroke (rs16147), (Yu et al., 2010) schizophrenia (rs16147), (Itokawa et al., 2003) and early-onset atherosclerosis (rs16147) (Shah et al., 2009). In this study, we explored NPY gene polymorphism as a possible genetic predisposing factor for prevalent asthma using a candidate gene approach, and investigated the interaction between NPY gene polymorphism and obesity in predicting the likelihood of prevalent asthma. We investigated all five common mutations (reported in White, Black, Asian, and Hispanic ethnicities) of NPY (rs16147 (−399T/C), and rs17149106 (−602G/T) located in the promoter region; rs16140 (+1000C/G) located in intron-1; rs5573 (+1201A/G) located in exon 2; rs5574 (+5327C/T) located in exon 3) which were previously validated to account for most of the NPY expression in vitro and in vivo (Mickey et al., 2011; Zhang et al., 2012; Zhou et al., 2008).

2. Methods

2.1. Study design and participants

In this clinical study of 308 young adults (aged 21–35 years), 126 physician diagnosed asthma patients and 182 healthy controls aged 21–35 years were recruited from National University Hospital of

Singapore during June 2011 to April 2013. Patients were included in this study only if they were free from oral steroid treatment for at least two weeks before visiting. Individuals were excluded if they had any other medical disease, neurological or psychiatric illness, mental incapacitation or learning disability, or disturbances in the HPA axis. All participants were South-east Asian ethnicity. Twenty milliliter venous blood samples were collected into the BD Vacutainer® CPT™ Cell Preparation tube with Sodium Citrate. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from each blood sample and cryopreserved in −80 °C freezer and liquid nitrogen in 10% Dimethyl Sulfoxide (DMSO)/fetal bovine serum (FBS) respectively until the time the experiments were performed. Plasma was used for the test of levels of NPY, adiponectin, IL-6, and CRP. PBMCs were used for DNA extraction and genotyping of NPY SNPs. All participants signed written informed consent for the study which was approved by the National Healthcare Group Domain Specific Review Board of Singapore.

2.2. Candidate NPY gene genotyping

“Genomic DNA was isolated from 2 million peripheral blood mononuclear cells (PBMC) using the Qiagen DNeasy Blood & Tissue Kit (Catalogue number: 69504) according to manufacturer's instructions. The DNA samples were prepared at 15 ng/μl concentration for usage in SNP genotyping.” Five candidate NPY SNPs were investigated: rs16147, rs17149106, rs16140, rs5573, and rs5574. Applied Biosystems Assays-on-Demand and custom-made primers/probes for the SNPs of NPY were used to amplify specific transcripts based on the unique promoter and exon structure of each isoform. Genotyping was carried out using 5'-nuclease assays in accordance with the standard Taqman Assay protocol (Applied Biosystems). Gene-specific primers and probes for the target NPY genes and DNase-free water were mixed separately with TaqMan GTXpres Master Mix (Applied Biosystems) and added to the plate. Each 96-well plate contained two control samples (one heterozygote and one homozygote) as well as two negative controls (no template). Genotype data were not available for four participants. PCR conditions were 60 °C for 5 min, 95 °C for 20 min, and 95 °C for 3 min/60 °C for 35 min for 40 cycles. PCR analysis was performed on an ABI 7500 Sequence Detector and the data obtained were analyzed with ABI Sequence Detector System (SDS) Software 3.0.

2.3. Body mass index (BMI)

Height and weight were measured wearing light clothing only. BMI was calculated as weight (kg) divided by the squared height (m²). BMI was missing in four participants at baseline. Obesity was defined according to WHO recommended cutoff for Asians (BMI ≥ 25 kg/m²).

2.4. Elisa and Multiplex analysis

Plasma specimens were prepared for ELISA and Multiplex analysis in a subgroup of 70 patients and 69 age- and sex-matched healthy controls. Neuropeptide Y-like immunoreactivity was measured using a competitive enzyme immunoassay (Peninsula Laboratories, Inc., Bachem, San Carlos, California). The levels of adiponectin and CRP were tested by human adiponectin ELISA (competitive) kit (Biovendor Laboratory Medicine Inc., Brno, Czech Republic) and human CRP ELISA kit (Millipore Corp., Billerica, MA). Plates were run according to the manufacturer's instructions.

Plasma concentration of IL-6 was determined using a custom Milliplex MAP Human Cytokine Magnetic Bead Panel (Millipore Corp., Billerica, MA). Results were generated utilizing the Luminex 200 platform (Millipore), with Bioplex Manager 6.0 software, based on standard curves plotted through a 5-parameter logistic curve setting.

Table 1
Case-control analysis of association of BMI and NPY genotypes with asthma (n = 308).

	Genotype frequencies	OR	95% Confidence Intervals		p
rs5574 Model (<i>Coding synonymous SNP</i>)					
Obese	NA	2.14	0.76	6.03	0.15
rs5574 CT	0.434	0.54	0.30	0.98	0.041
rs5574 TT	0.116	1.02	0.39	2.65	0.97
Interaction (obese * rs5574CT)	NA	2.56	0.58	11.32	0.22
Interaction (obese * rs5574TT)	NA	4.99	0.42	59.33	0.20
rs17149106 Model (<i>Promoter SNP</i>)					
Obese	NA	4.27	2.14	8.50	<0.001
rs17149106 GT	0.036	5.58	1.09	28.54	0.039
rs16140 Model (<i>Intronic SNP</i>)					
Obese	NA	4.33	1.78	10.53	0.001
rs16140 CG	0.431	1.09	0.61	1.96	0.76
rs16140 GG	0.079	1.92	0.73	5.09	0.19
Interaction (obese * rs16140CG)	NA	1.51	0.30	7.56	0.61
Interaction (obese * rs16140GG)	NA	0.23	0.02	2.38	0.22
rs16147 Model (<i>Promoter SNP</i>)					
Obese	NA	1.73	0.52	5.75	0.373
rs16147 TT	0.227	0.83	0.39	1.80	0.640
rs16147 CT	0.467	0.77	0.41	1.47	0.430
Interaction (obese * rs16140TT)	NA	3.35	0.59	19.11	0.174
Interaction (obese * rs16140CT)	NA	3.61	0.67	19.60	0.137

Data are adjusted for gender. Obese is defined as BMI ≥ 25 kg/m².

Interaction (obese * rs17149106 GT) not shown: estimate out of range due to small numbers.

2.5. Statistical analyses

The levels of BMI, adiponectin, NPY, and cytokines (IL-6 and CRP) were evaluated in patients with asthma versus their healthy counterparts using *t* tests. Spearman correlation of NPY with adiponectin was performed. Logistic regression models allow us to assess the main effects of NPY genotype and obesity as well as their interactions while adjusting for the potential confounding of sex-specific effects (Mottagui-Tabar et al., 2005). Data analyses were performed using PASW/SPSS 18.0 and R package with statistical significance set at $p < 0.05$. Figures were prepared using GraphPad Prism version 5.0.

3. Results

Among these 5 candidate SNPs, two SNPs rs5573 and rs16147 had strong linkage, with $r^2 = 0.894$ and $D' = 0.963$ (Table E1). Thus 4 non-linked SNPs which were all in Hardy-Weinberg equilibrium were genotyped and related to asthma. Table E2 shows primer and probe sequences for SNPs rs3037354 and rs17149106.

In logistic regression models controlling for gender and obesity, the CT genotype of rs5574 ($OR = 0.54$, $p = 0.041$) and the GT genotype of rs17149106 ($OR = 5.58$, $p = 0.039$) were significantly associated with asthma Table 1 and Table E3. In the same models, obesity (defined as BMI ≥ 25 kg/m² based on criteria for Asian populations) was independently associated with a higher risk of asthma, supporting previous reports of the association between obesity and asthma. No significant interactions between NPY SNP polymorphisms and obesity were detected. (See Table 1.)

Fig. 1 shows that compared with the healthy controls, patients with asthma had significantly lower level of NPY ($t = -3.087$, $p = 0.003$), suggesting the involvement of neuroendocrine regulation in asthma. As expected, patients with asthma had higher mean BMI ($t = 4.068$, $p < 0.001$), adiponectin ($t = 2.001$, $p = 0.048$) and obesity-related inflammatory cytokines IL-6 ($t = 3.341$, $p = 0.001$) and CRP ($t = 4.270$, $p < 0.001$) than the healthy controls. Furthermore, NPY was significantly correlated positively with the levels of adiponectin ($\rho = 0.283$, $p = 0.021$) in patients with asthma. Table E4.

4. Discussion

In the present study, we measured the peripheral level of NPY in participants and found that patients with asthma had lower level of NPY than their healthy counterparts. Furthermore, participants with GT genotype for the rs17149106 SNP are more susceptible to asthma while those with rs5574 CT are more resistant to asthma. This is consistent with findings from previous studies and suggests that NPY may be involved in the immunomodulation of asthma. Both studies by others and our prior research reported that the presence of asthma is associated with the change of NPY level which is inversely correlated with the level of IL-4, suggesting an effect in attenuating Th2 cytokine production and

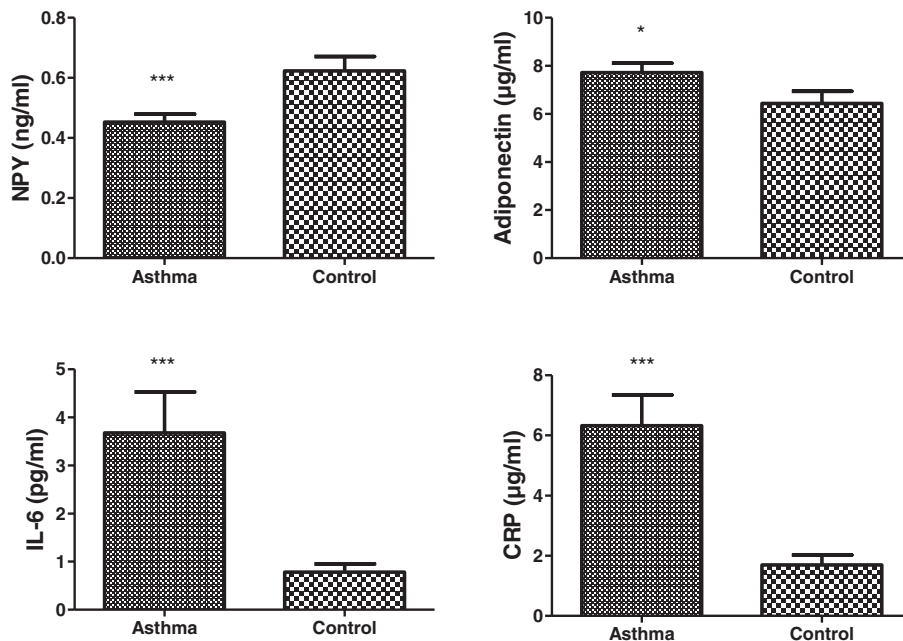


Fig. 1. Plasma levels of NPY, adiponectin, IL-6, and CRP in patients with asthma (n = 70) and the healthy controls (n = 69). Mean values are shown and error bars stand for standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group.

release (Lu et al., 2015; Makinde et al., 2013). Aldrich et al. (2009) showed that NPY genetic variation is associated with asthma prevalence (rs5574) and severity (rs5574 and rs16143) in Puerto Ricans, but this effect was not found in Mexicans. Another study (2012) (Jaakkola et al., 2012) reported that a NPY gene (rs16147/NPY-399) is associated with increased risk for self-reported asthma in overweight individuals. The NPY gene contains 4 exons and is located on chromosomal 7p15.1 in close association with other genes involved in immune regulation. rs17149106 and rs5574 are polymorphisms in the promoter region and third exon of the gene respectively. The mechanism underlying the observed association of rs17149106 and rs5574 with asthma is unclear and may be explained by its pivotal role in immunomodulation. Exonic SNPs present in the coding region of the gene can lead to functional outcomes relevant for diseases. Synonymous SNPs which do not result in the change of the amino acid can potentially influence promoter activity, mRNA stability or rate of protein folding (Capon et al., 2004; Thomas and Kejariwal, 2004). Hence it would be important for future studies to evaluate the role of our exonic SNP in asthma. NPY exerts a major influence on humoral and cellular immune functions, which intimately involve airway inflammatory response in asthma (Groneberg et al., 2004). Studies found that NPY activates NPY Y1R on APCs in active T cell priming, while on the other hand NPY suppresses T cell hyper-responsiveness in asthma (Sood et al., 2006). Further studies are required to understand the molecular mechanisms of this relationship. As expected, this study found that asthma is associated with higher BMI, adiponectin level as well as increased level of obesity-related cytokines IL-6 and CRP (Kattan et al., 2010), suggesting the link between obesity and asthma. This is consistent with previous research showing the link between obesity and asthma incidence (Li et al., 2003; Rastogi et al., 2012; Weiss and Shore, 2004). However, analysis in logistic regression models indicates that the link between NPY and asthma is not via adiposity regulation, although NPY was observed significantly correlated with adiponectin in asthma patients. Our results do not replicate the prior finding reported for Finnish adults that rs16147 was associated with an increased risk for self-reported asthma in overweight (BMI ≥ 25 kg/m²) individuals (Jaakkola et al., 2012). Population difference in genetic susceptibility as was observed by Aldrich et al. may explain this discrepancy, but comorbid atherosclerosis may possibly confounding, whereas participants in this study were free of other chronic disease.

The NPY rs16139 is a functional Leu7Pro (+1128T/C) SNP residing in the signal peptide part of preproNPY which is suggested to affect intracellular processing of preproNPY and release of mature NPY. But the Pro7 allele is absent in Japanese (Makino et al., 2001) and Korean (Ding et al., 2002) population. The polymorphism of rs16139 was, therefore, not examined in the present study of a South-east Asian sample. However, a recent study by Wang et al. (2013) reported that the Pro7 allele is detectable in Chinese Han population, although the frequency of the Pro7 allele relatively low (0.002 in 673 controls, 0.039 in 700 patients with major depressive disorder). The role of the polymorphisms of rs16139 in asthma requires further investigation in future studies of the Asian population.

In summary, the present study indicates that NPY gene polymorphisms in rs5574 and rs17149106 are potential genetic predisposing factors for prevalent asthma. Further studies are needed to delineate their precise roles in increasing individual susceptibility to develop incident asthma.

Conflict of interest

None of the authors has any potential conflict of interest including any financial, personal or other relationships that could influence their work and contribution to this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.npep.2016.07.003>.

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Table E1. Linkage for NPY SNPs - 1000 genome project

SNP	Proxy	Distance	RSquared	DPrime	Arrays	Chromosome	Coordinate_HG18
rs16147	rs5573	1599	0.894	0.963	None	chr7	24291534
rs5573	rs16147	1599	0.894	0.963	IM,OQ,OE,O24,O28,O54,O5E,OEE	chr7	24289935
rs16140	rs5573	201	0.655	1	None	chr7	24291534
rs5573	rs16140	201	0.655	1	None	chr7	24291333
rs16147	rs16140	1398	0.542	0.893	None	chr7	24291333
rs16140	rs16147	1398	0.542	0.893	IM,OQ,OE,O24,O28,O54,O5E,OEE	chr7	24289935
rs5573	rs5574	4124	0.289	1	A6,I3,I5,I6,I6Q,IM,IMD,IC,ICQ,IBC,OQ,AxM,IWQ,O E,O24,O28,O54,O5E,OEE	chr7	24295658
rs5574	rs5573	4124	0.289	1	None	chr7	24291534

rs16147	rs5574	5723	0.279	1	A6,I3,I5,I6,I6Q,IM,IMD,IC,ICQ,IBC,OQ,AxM,IWQ,O E,O24,O28,O54,O5E,OEE	chr7	24295658
rs5574	rs16147	5723	0.279	1	IM,OQ,OE,O24,O28,O54,O5E,OEE	chr7	24289935
rs16140	rs5574	4325	0.19	1	A6,I3,I5,I6,I6Q,IM,IMD,IC,ICQ,IBC,OQ,AxM,IWQ,O E,O24,O28,O54,O5E,OEE	chr7	24295658
rs5574	rs16140	4325	0.19	1	None	chr7	24291333

Table E2. Primer and probe sequences for NPY differential allelic expression

Oligonucleotide	Sequence
rs3037354	
Forward primer	5'-TGTTTCATCTTAATCAGAACCCACAT-3'
Reverse primer	5'-GGTCCAAAGTGACGGTGGTTA-3'
Probe for - allele	5'-FAM-AGACAGAAACTTGTTTCATT-3'
Probe for TG allele	5'-VIC-CAGAAACTGTTGTTTCATT-3'
rs17149106	
Forward primer	5'-GCAGAAGCCCCTGAAACCA-3'
Reverse primer	5'-GGCTAGAGGGTCCCAAAGC-3'
Probe for G allele	5'-FAM-CTCCCCACCCACCC-3'
Probe for T allele	5'-VIC-TCCCCACACCACCC-3'

Table E3. Baseline demographic characteristics and frequencies of NPY SNPs of young adults aged 21-35 years by asthma and healthy control groups (n=308)

	Asthma (n=126)	Control (n=182)
Gender		
Male	68 (54.8)	93 (54.7)
Female	56 (45.2)	77 (45.3)
Age	24.56 ± 4.91	22.79 ± 3.31
BMI (kg/m ²)	23.85 ± 5.40	21.51 ± 3.49
rs5574		
CC	57 (47.5)	79 (43.4)
TT	19 (15.8)	16 (8.8)
CT	44 (36.7)	87 (47.8)
rs17149106		
GG	114 (93.4)	179 (98.4)
GT	8 (6.6)	3 (1.6)
rs16140		
CC	58 (47.5)	91 (50.0)
GG	12 (9.8)	12 (6.6)
CG	52 (42.6)	79 (43.4)
Rs16147		
CC	36	57
TT	32	37
CT	54	88

Data are presented as mean ± SD or n (%).

Table E4. Correlation ρ (p) of NPY and adiponectin

	ρ	p
Patient	0.283	0.021
Control	0.211	0.082
All	0.173	0.045

NPY = Neuropeptide Y.