NRC Research Press

ARTICLE

A passenger strand variant in miR-196a2 contributes to asthma severity in children and adolescents: A preliminary study

Mohammad H. Hussein, Eman A. Toraih, Nagwa M. Aly, Eman Riad, and Manal S. Fawzy

Abstract: There is emerging evidence to support the role of microRNAs in allergic airway diseases and inflammation. Genetic variants in microRNA genes might affect microRNA-mediated cell regulation. This preliminary study was designed to investigate the association of the microRNA-196a2 rs11614913 (C/T) polymorphism with susceptibility to asthma and clinical outcomes in children and adolescents. Genotyping of rs11614913 polymorphism was determined in 96 patients with bronchial asthma (6–18 years of age) and 96 unrelated controls, using real-time polymerase chain reaction technology. In-silico target prediction and network core analyses were performed. The asthmatics did not show significant differences in genotype distribution ($p = 0.609$) and allele frequencies ($p = 0.428$) compared with the controls. There were also no associations with disease duration, age at onset, asthma phenotype, asthma control, therapeutic level, airway hyper-responsiveness, or biochemical parameters in the blood. However, the CC genotype was associated with a more severe degree of asthma ($p = 0.023$) and higher frequency of nocturnal asthma ($p = 0.002$). Carriers for CC were 17 times more likely to develop nocturnal asthma, and had a more than 2.5-fold increased risk for poor disease outcome compared with CT and TT individuals. In conclusion, microRNA-196a2 rs11614913 polymorphism might be associated with asthma severity in our sample of the Egyptian population. Further investigations in studies with a larger sample size and functional tests are needed to validate our findings and to explore the detailed biological mechanisms.

Key words: asthma, Egyptians, miR-196a2, polymorphism, real-time qPCR.

Résumé : De plus en plus de données ont appuyé le rôle des microARN dans les maladies allergiques respiratoires et l'inflammation. Des variants génétiques des microARN pourraient affecter la régulation cellulaire par les microARN. Cette étude préliminaire a été conçue pour examiner l'association entre le polymorphisme du microARN-196a2 rs11614913 (C/T) et la susceptibilité à l'asthme et le résultat clinique chez les enfants et les adolescents. Le génotypage du polymorphisme re11614913 a été réalisé chez 96 patients souffrant d'asthme bronchique (6–18 ans) et 96 sujets contrôles non reliés, par la technologie de la réaction en chaine par polymérase en temps réel. Une prédiction de la cible « in silico » et une analyse de réseau cœur ont été réalisées. Les patients asthmatiques ne présentaient pas de différences significatives sur le plan de la distribution du génotype ($p = 0{,}609$) et de la fréquence des allèles ($p = 0{,}428$) comparativement aux contrôles. Il n'y avait pas d'association non plus à la durée de la maladie, l'âge à son apparition, le phénotype de l'asthme, le contrôle de l'asthme, le niveau thérapeutique, l'hyperréactivité bronchique ou toute autre donnée de laboratoire. Cependant, le génotype CC était associé à un asthme plus sévère ($p = 0{,}023$) et à une fréquence plus élevée d'asthme nocturne ($p = 0{,}002$). Les porteur de CC étaient 17 fois plus susceptibles de développer un asthme nocturne et plus de 2.5 fois d'être à risque d'une issue défavorable de la maladie comparativement aux individus CT et TT. En conclusion, le polymorphisme du microARN-196a2 rs11614913 pourrait être associé à la sévérité de l'asthme dans leur échantillon de patients égyptiens. Des études supplémentaires comportant un échantillon plus grand et des tests fonctionnels sont nécessaires pour valider les résultats des auteurs et explorer les mécanismes biologiques détaillés. [Traduit par la Rédaction]

Mots-clés : asthme, Égyptiens, miR-196a2, polymorphisme, RT-qPCR.

Introduction

Bronchial asthma is a highly prevalent chronic respiratory disease worldwide, affecting people of all ages. The current Global Initiative for Asthma (GINA) guidelines reported that the prevalence of asthma is estimated to be 1% to 18% (GINA 2014). In Egypt, asthma is a major health problem among school children, with a prevalence of 9.1% (Mansour et al. 2014). Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation with infiltration of mast cells, eosinophils, and T-lymphocytes in the airway wall (Bonsignore et al. 2015). It has two key defining features: a history of variable symptoms of wheeze, shortness of breath, chest tightness; and cough that varies over time and in intensity, together with variable expiratory airflow limitation (GINA 2014). The precise mechanism for asthma remains unclear. Several reports have suggested many environmental risk factors that may predispose to bronchial asthma. However, many people exist in the same environment and do not develop the disease, which highlights the contribution of genetic factors to the pathogenesis (Ober and Yao 2011). Understanding the molecular mechanisms in asthma may shed new light on novel biomarkers for disease prediction and risk stratification (Xu et al. 2014).

MicroRNAs (miRNAs) are small non-coding RNA molecules that target genes by inducing mRNA degradation or inhibiting translation. Single miRNAs may regulate translation of numerous

Received 19 January 2016. Accepted 19 April 2016.

M.H. Hussein. Pulmongist, Ministry of Health, Egypt; Emergency Department, Al Jahra Hospital, Al Jahra, Kuwait.
E.A. Toraih. Department of Histology and Cell Biology (Genetics Unit), Faculty of Medicine, Suez Canal University, PO Box 41522, Ismailia, Egypt.
N.M. Aly and M.S. Fawzy. Department of Medical Biochemistry, Faculty of Medicine, Suez Canal University, PO Box 41522, Ismailia, Egypt.
E. Riad. Department of Chest Diseases and Tuberculosis, Faculty of Medicine, Suez Canal University, PO Box 41522, Ismailia, Egypt.
Corresponding authors: Manal S. Fawzy (email: manal2_khashana@ymail.com) and Mohammed H. Hussein (email: hosny.mh@gmail.com).
Copyright remains with the author(s) or their institution(s). Permission for reuse (free in most cases) can be obtained from RightsLink.

downstream mRNAs (Kishore et al. 2014). Studies have revealed altered expression profiles in various pulmonary disorders, ranging from inflammatory diseases to lung cancer (Tomankova et al. 2010; Rupani et al. 2013; Savarimuthu Francis et al. 2014). Most importantly, inherited single-nucleotide polymorphisms (SNPs) in miRNA genes may have important consequences on their expression, processing, or maturation, and may contribute to susceptibility or progression to various disorders (Su et al. 2011; Kishore et al. 2014). There is emerging evidence to support the role of hsa-microRNA-196a2 (miR-196a2) in a wide range of human diseases (Wang et al. 2013; Yuan et al. 2013). A common variant (rs11614913; C/T) in the passenger strand of the miRNA has been associated with chronic pulmonary disease (COPD) (Li et al. 2011) or its response to the bronchodilators (Fawzy et al. 2016) and lung cancer (Tian et al. 2009; Wang et al. 2014). In addition, this particular miR-196a2 SNP was previously reported to confer risk for other types of cancer (Zhang et al. 2012; Du et al. 2013), inflammatory diseases (Huang et al. 2013; Qi et al. 2013; Kim et al. 2014), and vascular conditions (Jeon et al. 2012, 2013). Thus, this study was conducted to investigate the association between rs11614913 polymorphism and susceptibility to asthma disease in children and adolescents, and to further assess its impact on the clinical outcome and bronchial hyper-responsiveness (BHR) in patients.

Materials and methods

Study population

The study population included 96 patients with bronchial asthma, aged between 6 and 18 years old, and 96 normal, age-matched controls with no history of wheezes, atopy, or chronic upper or lower airway disease. Patients were recruited from the Pediatrics and Chest Diseases and Tuberculosis Departments, Suez Canal University Hospital, Ismailia, and the Chest and Tuberculosis Department, Kasr Al Ainy Hospital, Egypt, between January 2014 and December 2014. Asthma was diagnosed according to the Global Initiative for Asthma (GINA 2014), and the asthmatics underwent a detailed "history taking" via a structured interview and thorough clinical examination. Clinical asthma phenotyping was determined through the patients' clinical, biochemical, and physiological parameters, which were assessed during multiple visits. Levels of asthma symptom control, disease severity, and future risk of exacerbations were assessed based on GINA guidelines. In brief, asthma control in children and adolescents was assessed by the Asthma Control Questonaire (ACQ), based on day and night symptoms, limitation of activities, and rescue medication use for the previous 4 weeks. These 4 variables were used to define the level of asthma control: controlled, partially controlled, and uncontrolled (Matsunaga et al. 2015). The classification of asthma severity was assessed retrospectively from the level of treatment required to control symptoms and exacerbations. As a result, asthma patients were distributed among 3 groups: mild, moderate, or severe asthma (GINA 2015).

Therapeutic level and response, the patient's inhaler technique, and adherence to treatment were checked. Patients were also surveyed for the presence of comorbidities, including rhinitis, sinusitis, gasteroesophageal reflux disease, obesity, obstructive sleep apnea, depression, and anxiety. Chest radiographs were performed to exclude other causes of airflow limitation. Patients with concomitant chronic respiratory disease, non-compliant to treatment, or with manifestations of recent acute respiratory infection were excluded. Body mass index (BMI) was calculated in kilograms per square metre and transformed into z scores, and percentile-adjusted for age and sex (Fawzy et al. 2015). Sexual maturity rating for the patients and controls was assessed according to the Tanner staging by a single specialist (Marshall and Tanner 1969). The study was conducted in accordance with the guidelines in the Declaration of Helsinki and had the approval of the Ethics Committee of Faculty of Medicine, Suez Canal University (Research No. 2512). Written informed consent was obtained from the guardians of the participants.

Pulmonary function test

Spirometry was performed using an electronic spirometer (BTL-08 Spiro Pro system; BTL) with a valve-spacer device, in accordance with the guidelines of the American Thoracic Society/European Respiratory Society (ATS/ERS) (Soriano et al. 2013). Basal lung function, including measurements of forced expiratory volume in first second (FEV_1), forced vital capacity (FVC), and peaked expiratory flow rate (PEFR) were assessed. A bronchial provocation via methacholine challenge test was done to assess airway hyperresponsiveness (AHR). Saline and methacholine solutions were aerosolized using a nebulizer attached to an air compressor with the following gradient doses (0.06, 0.125, 0.25, 1.0, 2.0, 4.0, 8.0, 16.0 mg/mL) at 5 min intervals. The concentration of methacholine that caused a 20% decline in FEV_1 (in mg/mL) was measured for each subject. AHR was categorized according to the American Thoracic Society guidelines, with positive cutoff value defined as a $PC_{20} < 8$ mg/mL (Crapo et al. 2000; Pellegrino et al. 2005).

Biochemical analysis

A blood sample was drawn in EDTA vacutainers at the same time daily (1000–1300 h) to avoid diurnal variation in the measurements. The absolute eosinophilic count (AEC) was calculated as the product of its percentage and total leukocyte count, using an automated cell counter (Sysmex). In addition, serum total IgE was measured using an enzyme-linked immunosorbant assay (C29-800, Immunospec Corporation, Canoga Park, California, USA). The minimum detectable concentration of IgE using this assay is estimated to be 5.0 IU/mL. Samples below this limit were considered negative.

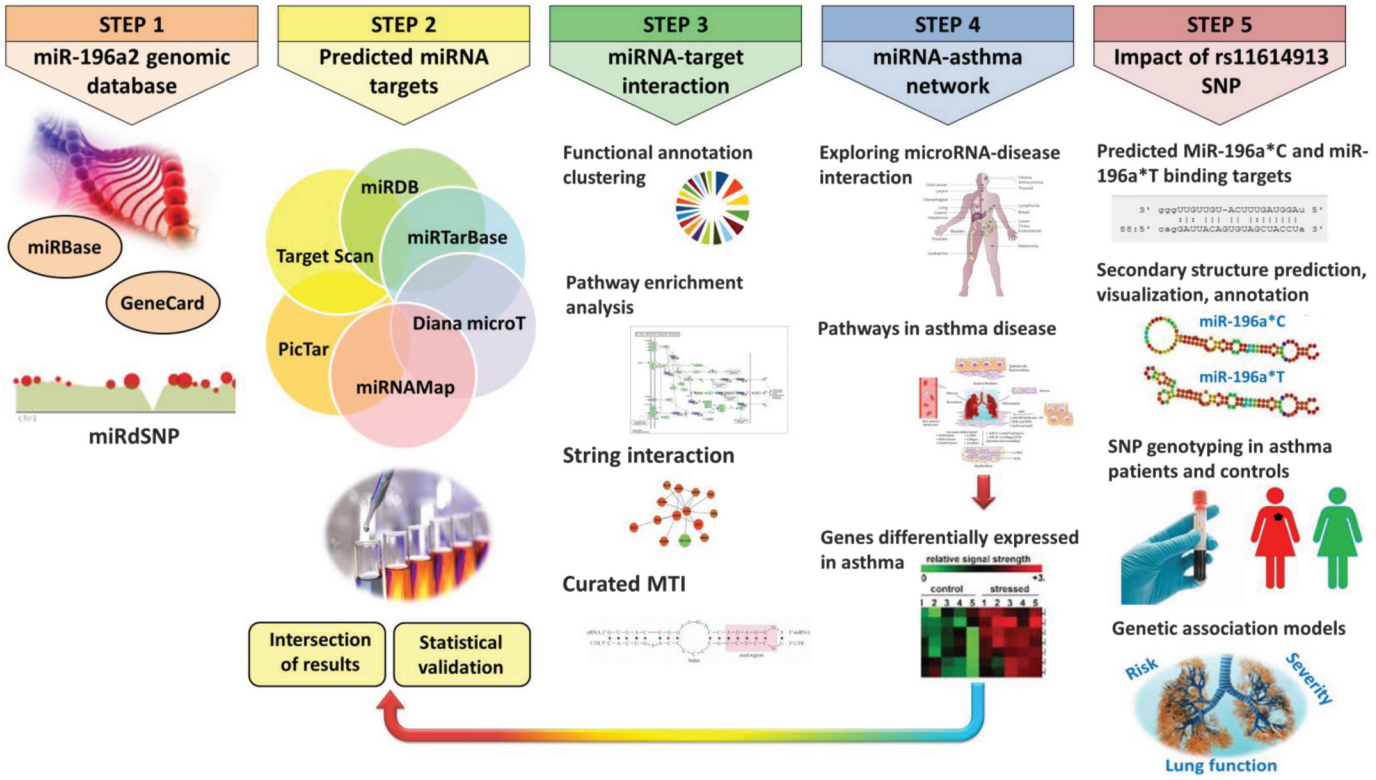
In-silico target gene prediction

The workflow for in-silico data analysis is illustrated in Fig. 1. Multiple computational prediction tools were employed to identify miR-196a2-3p and 5p target genes (in 3′URT, 5′UTR, and CDS of the genes). They included miRBase (http://microrna.sanger.ac.uk/targets/v3/), miRDB (http://mirdb.org/miRDB/), TargetScanHuman version 6.2 (http://www.targetscan.org/), DIANA-microT-CDS version 5.0 (http://diana.cslab.ece.ntua.gr/pathways/), miRTarBase version 18 (http://mirtarbase.mbc.nctu.edu.tw/), PicTar (http://pictar.mdc-berlin.de/), DIANA-TarBase version 7.0 algorithm (http://diana.cslab.ece.ntua.gr/tarbase/), miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/), and miRNAMap version 2.0 (http://mirnamap.mbc.nctu.edu.tw/) databases. Result intersection, statistical validation, and filtration, were applied to reduce the false positive prediction rate. The following criteria were used for filtration of the putative miRNA targets: target sites predicted by at least 2 tools; genes containing multiple target sites; genes with miRNA target sites located in accessible regions; and experimentally validated manually curated miRNA: gene interactions based on high throughput methods (Hsu et al. 2008).

Functional annotation clustering and pathway enrichment analysis

The predicted miRNA target genes were analyzed for gene ontology (GO) terms and Kyoto encyclopedia of genes and genomes (KEGG) enrichment pathway analysis (Kanehisa et al. 2004) using DIANA-miRPath version 2.0 web-server (Vlachos et al. 2012) and miRTar.Human tool (http://miRTar.mbc.nctu.edu.tw/). MiRNA-196a2-asthma association was explored using miRPub server (http://www.microrna.gr/mirpub/), which is a database for searching microRNA publications and their correlation to diseases, tissues, and genes (Vergoulis et al. 2015). Genes and networks involved in the asthma pathogenesis were collected from the Ingenuity Pathway Analysis platform (Ingenuity Systems, Redwood City, Calif.) (Kaneko et al. 2013) and literature review (Xu 2014).

Fig. 1. A flow chart illustrating in-silico data analysis for the miRNA target prediction, enrichment pathway analysis, and miRNA-disease interaction. Genomic databases were retrieved from miRBase release 21 and GeneCard Human gene Database. Gene and microRNA sequence were analyzed. Step 1. All polymorphisms of the *miR-196a2* gene were obtained from miRdSNP database. Step 2. A complete set of miR-196a2-3p tk;1and 5p predicted target genes was downloaded from multiple databases, including identification of new experimentally validated targets. Results are intersected and statistically validated, with functional annotation clustering of target genes and enrichment analysis into a biological pathway (KEGG pathways and REACTOME). Step 3. Selection of the curated microRNA-target interactions (MTI) and analysis of the expression profiles of miR-196a2 and its target genes. Step 4. Disease–miRNA association networking was determined using PhenmiR, miR2Disease, miRPub, and Human microRNA Disease Databases. Pathways involved in pathogenesis of asthma disease were retrieved from KEGG and Reactome pathways, followed by intersection of asthma genes with the miR-196a2 target gene set. Step 5. Prediction of functional impact of rs11614913 SNP on target genes and secondary structure, visualization and annotations of precursor microRNA with C and T alleles using miRmut2Go, RNAfold webserver, and miRNAMap 2.0, based on minimum free energy (MFE). Final experimental SNP genotyping to determine the association of the variant with disease risk and outcome. [Colour online.]



Predicted functional effect of rs11614913 SNP

Distribution of SNPs in miR-196a2 were analyzed via the miRdSNP database (http://mirdsnp.ccr.buffalo.edu/). The predicted functional impact of miR-196* rs11614913 polymorphism (5′-CGGCAACA-AGAAACUGC[C/U]UGAG-3′) at the passenger strand was performed using miRmut2Go (http://compbio.uthsc.edu/miR2GO) for comparative functional analysis of miRNA mutations based on the enriched functional annotations of predicted target gene sets of wild and mutant types (Bhattacharya and Cui 2015). Selected parameter settings were as follows: combining both miRanda and TargetScan methods for miRNA target prediction, *p* value threshold <0.01, and medium hierarchical filtering level. In addition, miRNAMap 2.0 and RNAfold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) were used to predict the minimum free energy (MFE) structures and base pair probabilities from single RNA sequences (Martin-Guerrero et al. 2015).

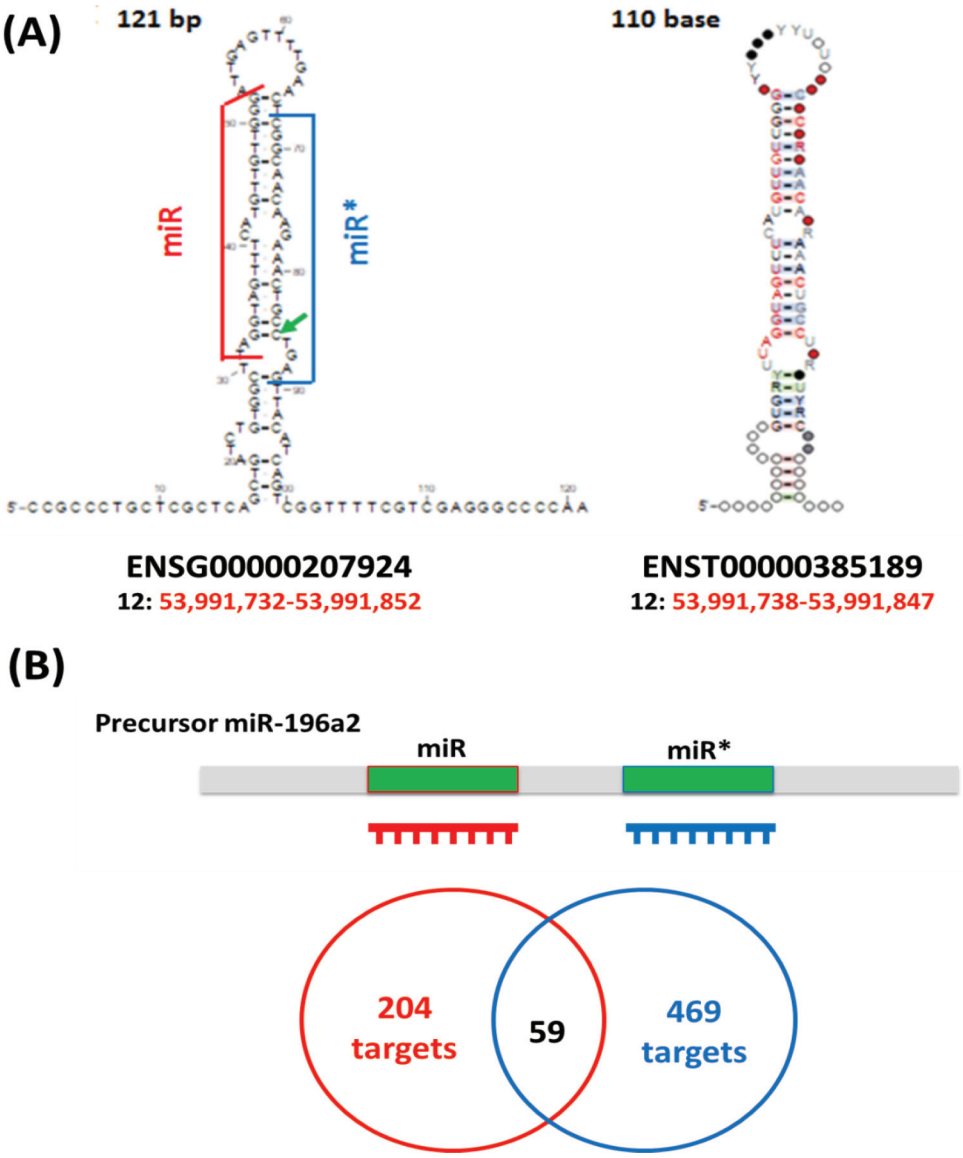
SNP identification in patients and controls

Genomic DNA was extracted from whole blood of asthma patients and controls using QIAamp DNA Blood Mini kit (cat. No. 51104; QIAGEN) following the manufacturer's protocol. DNA concentration and purity was assessed by NanoDrop ND-1000 (NanoDrop). Samples were genotyped, following a blind protocol, for the hsa-miR-196a2 polymorphism (rs11614913) using real-time polymerase chain reaction. PCR was performed in a 25 μL reaction volume containing 12.5 μL TaqMan Universal PCR Master Mix (2×), 1.25 μL 20× TaqMan SNP genotyping assay mix (cat. No. 4351379; Applied Biosystems), and 20 ng genomic DNA diluted to 11.25 μL with Nuclease-free water. Appropriate negative and positive controls were used. Real-time PCR amplification was performed on StepOne Real-Time PCR System (Applied Biosystems, Foster City, Calif.) using the following conditions: 2 initial holds (50 °C for 2 min and 95 °C for 10 min) followed by a 40-cycle 2-step PCR (95 °C denaturation for 15 s and annealing/extension 60 °C for 1 min). Allelic discrimination was called by the SDS software version 1.3.1 (Applied Biosystems). Ten percent of the samples were randomly selected for the repeated assays and the results were 100% concordance (Malkki and Petersdorf 2012).

Statistical analysis

Statistical analysis was carried out using the "Statistical Package for the Social Sciences (SPSS) for windows," version 20. Hardy–Weinberg equilibrium was tested using the Online Encyclopedia for Genetic Epidemiology (OEGE) software (http://www.oege.org/software/hwe-mr-calc.shtml) with a goodness of fit χ^2 test to compare the observed genotype frequencies among the subjects with the expected genotype frequencies. The allele frequency within each group was determined as the number of occurrences of an individual allele divided by the total number of alleles. Genotype and allele frequencies were compared between asthmatic patients

Fig. 2. MicroRNA-196a2 gene and predicted targets of miR-196a2-5p and miR-196a2-3p. (A) MiR-196a2 is expressed from an intergenic region in HOX gene clusters, at chromosome 12q13.13, coordinates: 53991738–53991847 [+] based on the human genome assembly GRCh38. It is 110 nucleotides long. Letters represent the nucleotide sequence. Mature miR-196 (*MIMAT0000226*) is shown in red, and miR-196* (the opposed sequence of mature miRNA at the stem arm of the secondary structure: *MIMAT0004562*) is shown in blue. The studied single nucleotide polymorphism rs11614913 C>T is shown (green arrow). (Data source: miRBase database version 21, http://www.ensembl.org, and http://ferrolab.dmi.unict.it/). (B) Human miR-196a2 is composed of two different mature miRNAs at the 5p arm (miR) and the 3p arm (miR*), which are processed from the same stem-loop. The Venn diagram shows the number of target genes predicted by the miRDB database, illustrating the 59 common genes targeted by both miR-196a2 mature forms. [Colour online.]



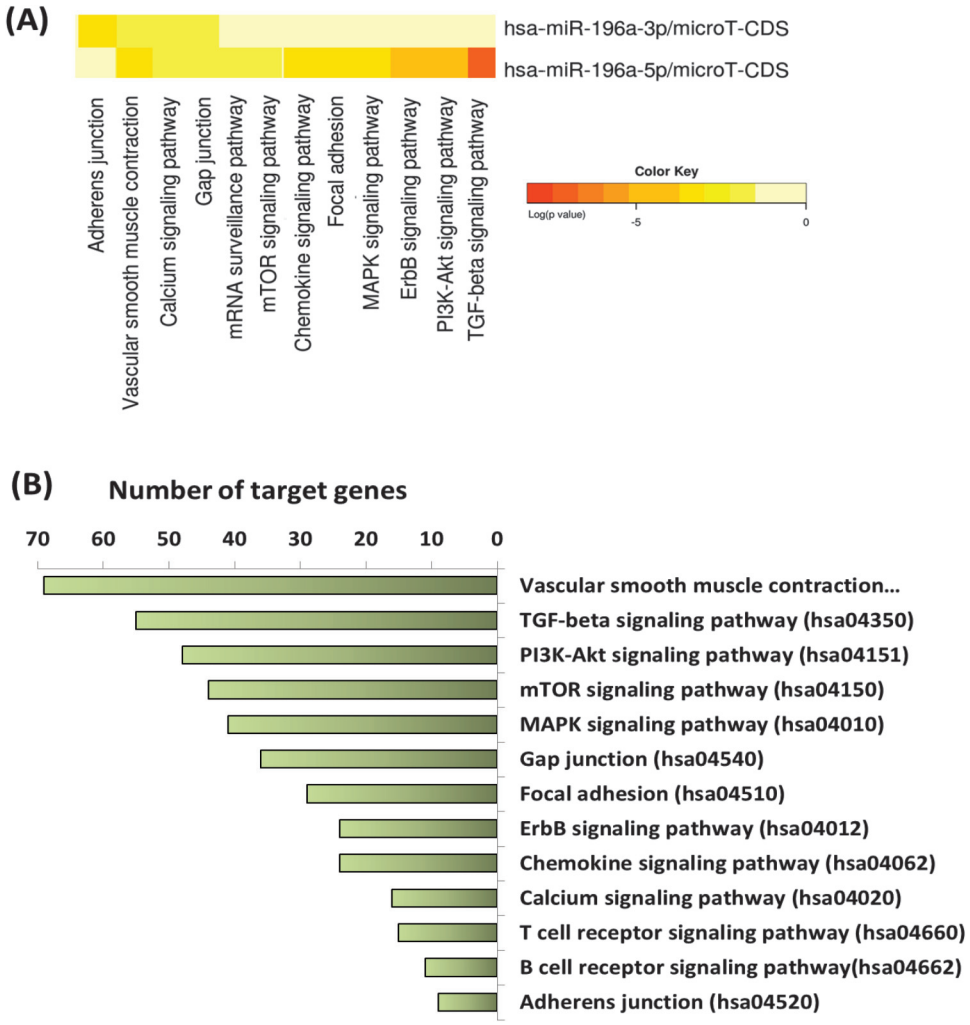
and control subjects using the χ^2 for trend test, and odds ratios (ORs) and 95% confidence intervals (CIs) for multiple genetic association models were used to assess the relative risk conferred by a particular allele or genotype. Binary logistic regression analysis was performed to adjust potential confounders (including age, gender, obesity, puberty, residence, family history of asthma). The BMI *z* score was calculated using an online pediatric calculator (http://www.quesgen.com/BMIPedsCalc.php). Data distribution was checked with the Kolmogorov–Smirnov test. Data presented are the mean ± SD. BDRBASE (change in FEV1 as a percent of baseline FEV1) was calculated with the following equation: {BDRBASE = [(post-BD FEV1 − pre-BD FEV1)/pre-BD FEV1] × 100}. The clinical data and pulmonary function of the groups were compared using the χ^2, Student *t*, and Kruskal–Wallis tests, followed by Newman–Keuls multiple comparison tests. A 2-tailed *p*-value of 0.05 was considered statistically significant.

Results

In-silico data analysis

Human miR-196a2 gene (ENSG00000207924) exists within the region of homeobox (HOX) gene clusters at chromosome 12 from position 53991732 to 53991852 (according to the human genome assembly: GRCh38, release 107). It is 121 nucleotides (nt) long. During biogenesis, it undergoes two cleavage processes, one in the nucleus forming a precursor hairpin transcript (ENST00000385189) of 110 bases long, and another in the cytoplasm forming miRNA duplex. Hsa-miR-196a2 forms two different mature miRNAs: miR-196a

Fig. 3. Functional enrichment pathway analysis of miR-196a2. (A) Enrichment pathway analysis. Heat map showing targeted pathways for both hsa-miR-196a-5p and hsa-miR-196a-3p with *p*-values <0.05 and microT-CDS threshold of 0.4. A total of 12 pathways have target genes (in CDS or 3′-UTR regions) for miR-196a2, with 3 intersecting pathways targeted by both mature miRNAs. The enrichment results of validated target genes showed that most pathways were related to multiple inflammatory and immunological processes, including the chemokine signaling pathway, T and B cell signaling pathways, vascular smooth muscle contraction, and calcium signaling pathway. The degree of color is based on the significant *p* values of the predicted algorithm by DIANA tools; the red has the highest score of statistical significance (data source: DIANA-miRPath version 2.0). (B) Number of target genes in enriched KEGG pathways. The following options were used: microT threshold = 0.4, $p < 0.05$, and significantly clustered target genes in KEGG pathways. [Colour online.]



and miR196a* at the 5p and 3p arms, respectively, processed from the same stem-loop precursor (Fig. 2A). Hundreds of genes were predicted to be targeted by miR-196a2-3p and miR-196a2-5p using multiple microRNA databases; some were experimentally validated by high-throughput techniques. According to the miRDB database, miR-196a2-3p and 5p have 469 and 204 putative gene targets, respectively, with 59 targets in common (Fig. 2B).

Our in-silico analysis determined the presence of a common variant (rs11614913: C/T) in nucleotide 78 (GRCh38 coordinates 12:53991815) at the 3′ end of miR-196a2*. Thus, CC and TT homozygotes produce two mature molecules; one from the guide strand miR-196a2 (uagguaguuucauguuguugg), and the other either miR-196a2*C (cggcaacaagaaacugcCugag), or miR-196a2*T (cggcaacaagaaacugcUugag), respectively from the passenger strand. Whereas, CT heterozygotes produce three mature miRNAs (miR-196a2, miRa2*C, and miR-196a2*T). Predicted functional impact of the SNP using miRmut2Go web-server revealed similar predicted target gene sets and functional annotations. Predicted secondary structure of pre-miR-196a2 with rs11614913 SNP (either C or T alleles) via RNAfold webserver and miRNAMap 2.0 showed no dramatic effect on the folding pattern of the hairpin loop.

Functional annotation clustering of curated targeted genes and enriched pathway analysis revealed that miR-196a2 target multiple molecular and signaling pathways involved in inflammatory and immunological biological processes, including chemokine signaling pathway, T and B cell signaling pathways, vascular smooth muscle contraction, and calcium signaling pathway (Fig. 3). By surveying the literature, multiple genes involved in asthma pathogenesis were collected, the putative predicted genes of miR-196a2 in the asthma disease pathway (KEGG: hsa05310) are shown in Fig. 4.

rs11614913 polymorphism in asthmatic patients

Baseline characteristics of the asthmatic patients and the controls are summarized in Table 1. The distribution of genotypes in both study groups was found to be in accordance with those expected by the Hardy–Weinberg equilibrium ($p > 0.05$). Genotyping of the miR-196a2 rs11614913 polymorphism revealed no significant

Fig. 4. Predicted target genes in the asthma pathway (hsa05310). Genes involved in the asthma pathway are shown in green. MiR-196a2 target IL-5 and IL-13 genes in KEGG pathway; colored in purple and coded below according to the mRNA target regions: 3′UTR in yellow, 5′UTR in green and coding sequence in orange. In the pathway, recognition of the antigen and activation by antigen presenting cells (APC) lining the airway causes differentiation of naive T cells to TH2 cells. Activated TH2 stimulates the formation of IgE by B cells. These IgE molecules bind to their receptors located on mast cells. The cross linking of mast-cell-bound IgE by allergens leads to the release of biologically active mediators (histamine, leukotrienes) by degranulation causing the immediate symptoms of allergy. Mast cells also release chemotactic factors that contribute to the recruitment of inflammatory cells, particularly eosinophils, whose proliferation and differentiation from bone marrow progenitors is promoted by IL-5. The activation of eosinophils leads to release of toxic granules and oxygen free radicals that lead to tissue damage and promote the development of chronic airway inflammation. [Colour online].

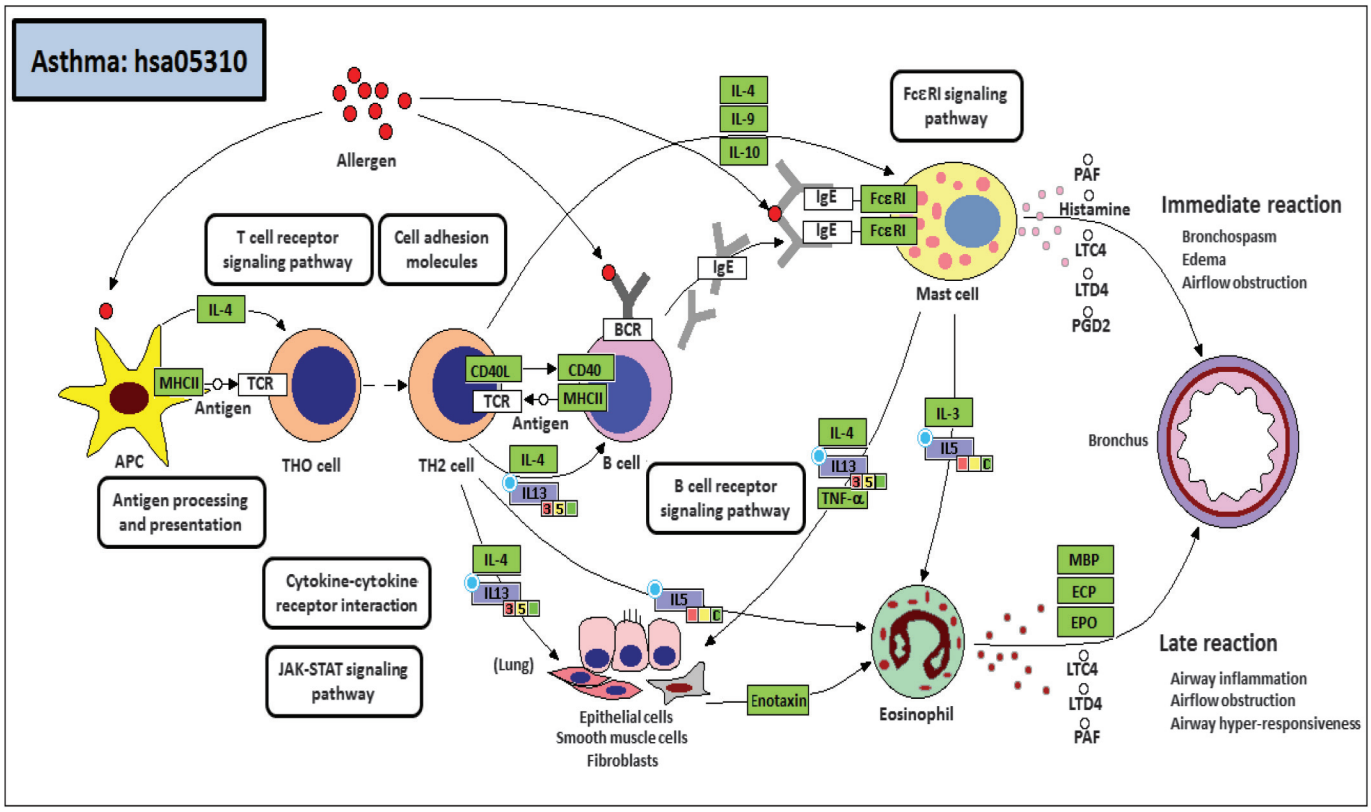


Table 1. Baseline characteristics of the study groups.

Demographic data	Asthma	Controls	*P* values	OR (95% CI)
Total number	96 (100)	96 (100)		
Mean age (years)	9.7±3.0	9.6±3.1	0.761	
Age categories (%)				
6–11	72 (75.0)	73 (76.0)	0.867	Reference
12–18	24 (25.0)	23 (24.0)		1.05 (0.54–2.04)
Gender				
Male	44 (54.2)	40 (41.7)	0.561	Reference
Female	52 (45.8)	56 (58.3)		1.18 (0.66–2.09)
Residence				
Urban	32 (33.3)	45 (46.9)	0.056	Reference
Rural	64 (66.7)	51 (53.1)		0.56 (0.31–1.01)
BMI percentile				
<85th	53 (55.2)	51 (53.1)	0.952	Reference
<95th	32 (33.3)	34 (35.4)		0.90 (0.48–1.67)
≥95th	11 (11.5)	11 (11.5)		0.96 (0.38–2.41)
Pubertal status				
Males	25 (26.0)	19 (19.8)	0.243	Reference
Females	20 (20.8)	25 (26.0)		0.60 (0.26–1.40)
FH of asthma				
Negative	67 (69.8)	89 (92.7)	<0.001*	Reference
Positive	29 (30.2)	7 (7.3)		5.50 (2.27–13.3)

Note: Values are the number (percentage) and mean ± SD. OR, odds ratios; BMI, body mass index; FH, family history. Pubertal status, sexual maturation, and secondary sexual characters were assessed using the Sexual Maturity Rating stages of Marshall and Tanner (1969); *, $p < 0.05$ (χ^2 and Student *t* tests).

Table 2. Genotype and allele frequencies of *hsa-miR-196a2* polymorphism in asthma patients and controls.

	Asthma ($n = 96$)	Controls ($n = 96$)	*P* value	Crude OR (95% CI)	Adjusted OR (95% CI)[a]
Genotype frequency					
Dominant model					
CC	52 (54.2)	49 (51.0)	0.609	1.0	1.0
CT	37 (38.5)	36 (37.5)		0.96 (0.53–1.76)	2.53 (0.77–8.26)
TT	7 (7.3)	11 (11.5)		0.59 (0.21–1.67)	2.72 (0.81–9.13)
Codominant model					
CC	52 (54.2)	49 (51.0)	0.664	1.0	1.0
CT+TT	44 (45.8)	47 (49.0)		0.88 (0.50–1.55)	0.89 (0.48–1.66)
P-HWE	0.905	0.278			
Allele frequency					
Allelic model					
C	141 (73.4)	134 (69.8)	0.428	1.0	0.36 (0.1–1.23)
T	51 (27.6)	58 (30.2)		0.83 (0.53–1.30)	0.93 (0.48–1.79)

Note: Values presented are a percentage. OR, odds ratios; P-HWE, *p* values for Hardy–Weinberg equilibrium.

[a]Adjusted for confounding variables (age, gender, residence, family history, BMI percentile, puberty). χ^2 test was used.

Table 3. Clinical and laboratory data of asthmatic patients, according to *hsa-miR-196a2* polymorphism.

		MIRN196A2 genotypes				Adjusted OR (95% CI)
Characteristics	No.	CC	CT	TT	*P* value	CC versus CT/TT
Total number (%)	96	52 (54.2)	37 (38.5)	7 (7.3)		
Mean age (years)	96	10.1±3.4	9.5±2.5	9.1±2.5	0.918	
Children (6–11)	72	38 (52.8)	29 (40.3)	5 (6.9)	0.829	1.0
Adolescents (12–18)	24	14 (58.3)	8 (33.3)	2 (8.3)		1.25 (0.49–3.18)
Gender						
Female	52	28 (53.8)	17 (32.7)	7 (13.5)	0.031*	1.0
Male	44	24 (54.5)	20 (45.5)	0 (0.0)		1.02 (0.45–2.30)
FH of asthma	28	15 (51.7)	28 (41.8)	2 (3.0)	0.043*	0.25 (0.10–0.63)*
Pubertal status	45	20 (44.4)	21 (46.7)	4 (8.9)	0.199	0.47 (0.20–1.07)
Obesity	11	4 (36.4)	6 (54.5)	1 (9.1)	0.448	0.86 (0.36–2.08)
Mean age at onset (years)	96	3.7±1.9	3.2±1.7	3.0±1.8	0.470	
Early onset (≤3 years)	45	24 (53.3)	18 (40.0)	3 (6.7)	0.950	1.0
Late onset (>3 years)	51	28 (54.9)	19 (37.3)	4 (7.8)		1.06 (0.47–2.38)
Asthma duration (years)	96	6.4±2.9	6.2±2.4	6.1±1.9	0.938	
Asthma phenotypes						
Atopic asthma	76	38 (50.0)	31 (40.8)	7 (9.2)	0.252	1.0
Non-atopic asthma	7	7 (100)	0 (0.0)	0 (0.0)		15 (0.80–271)
Aspirin-sensitive asthma	11	6 (54.5)	5 (45.5)	0 (0.0)		1.2 (0.33–4.26)
Exercise-induced asthma	2	1 (50.0)	1 (50.0)	0 (0.0)		1.0 (0.06–16.5)
Symptoms						
Daytime symptoms >2/week	48	29 (60.4)	18 (37.5)	1 (2.1)	0.117	1.65 (0.73–3.72)
Nocturnal symptoms	16	15 (93.8)	1 (6.2)	0 (0.0)	0.002*	17.4 (2.19–138)*
Reliever use >2/week	33	21 (63.6)	11 (33.3)	1 (3.0)	0.295	1.29 (0.53–3.16)
Activity limitations	29	16 (55.2)	11 (37.9)	2 (6.9)	0.990	1.05 (0.44–2.54)
Comorbidities	48	30 (62.5)	16 (33.3)	2 (4.2)	0.203	1.96 (0.87–4.44)
Risk for poor outcome	67	41 (61.2)	23 (34.3)	3 (4.5)	0.065	2.58 (1.05–6.32)*
Lab tests						
Total IgE (IU/ml)	96	76±89.1	87±81.2	186±182$	0.128	
AEC (×106/L)	96	170±245	197±266	361±432	0.433	

Note: Values are the mean ± SD or a percentage. OR, odds ratios; AEC, absolute eosinophilic count; FH, family history. Assessment and classifications of asthma were performed based on GINA guidelines. Analyses were made using the χ^2 and Kruskal–Wallis tests, followed by the Newman–Keuls multiple comparison test; *, $p < 0.05$; $, significant difference from homozygote wild type and heterozygote groups.

difference between patients and controls ($p = 0.609$) in the overall and stratified analysis (Table 2). The frequency of the C allele was more common in our population, representing (0.73 and 0.70 in patients and controls, respectively, $p = 0.428$).

The disease characteristics of patients according to rs11614913 genotypes are illustrated in Table 3 and Fig. 5. No observed significant association of miR-196a2 genotypes was found with disease duration, age at onset, asthma phenotype, asthma control, therapeutic level, airway hyper-responsiveness, or any biochemical parameters. However, the CC genotype was associated with a more severe degree of asthma ($p = 0.023$) and higher frequency of nocturnal asthma ($p = 0.002$). Carriers for CC were 17 times more likely to develop nocturnal asthma and had a >2.5-fold increased risk for poor outcome disease compared with the CT and TT individuals. On the other hand, patients with the TT genotype had the best PEFR, pre-FEV1, and post-FEV1 (all $p < 0.05$), and hence, were the least responsive to bronchodilators (Fig. 6).

In the stratified analysis, adjusted ORs showed higher susceptibility to develop severe asthma in CC carriers for all variables (Supplementary data, Table S1[1]). This risk increased significantly in patients with the CC genotype with positive family history of asthma, delayed age at onset (>3 years old), puberty, and uncontrolled symptoms compared with their corresponding genotypes ($p < 0.05$).

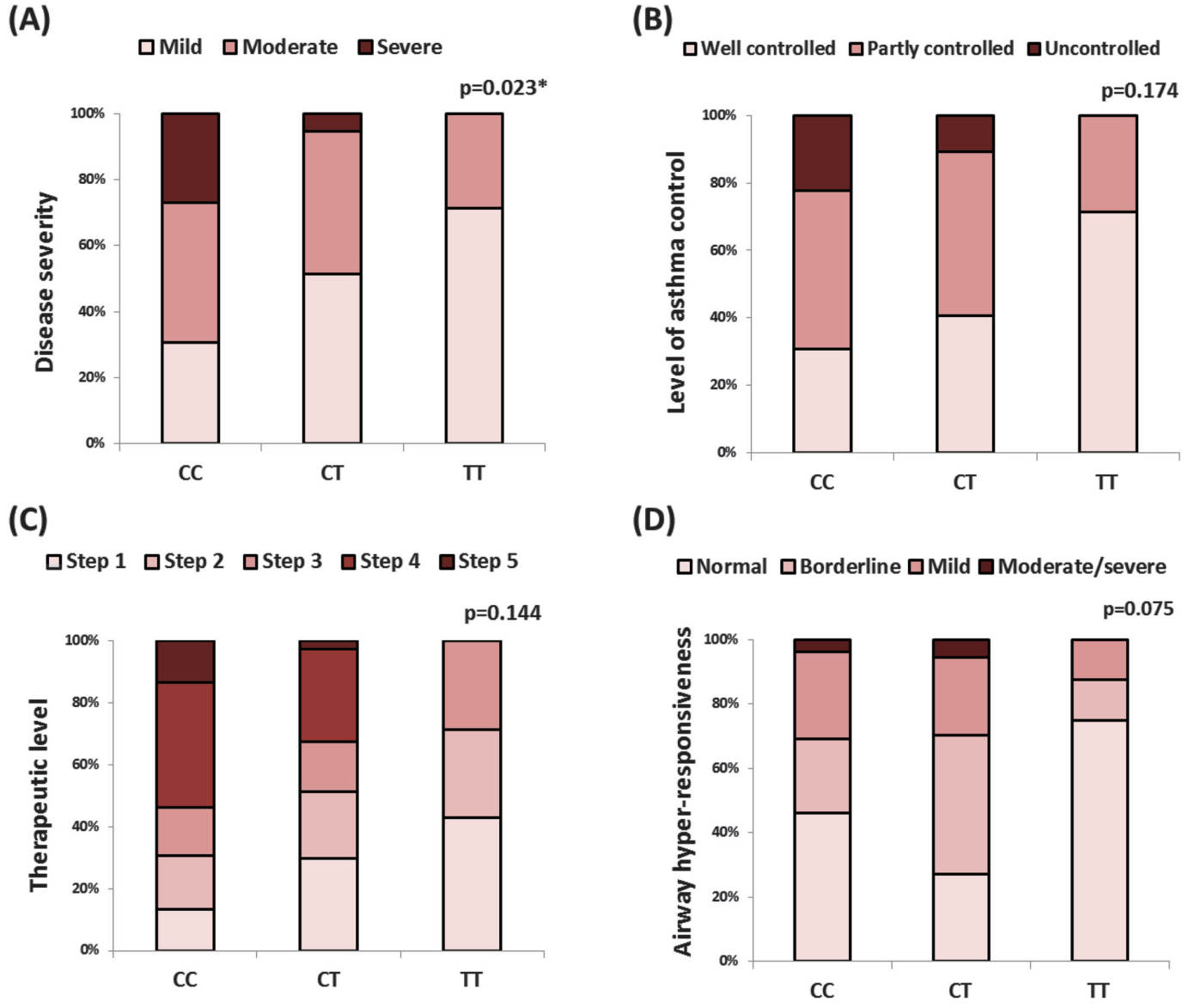
Discussion

In the last few years, several studies have focused on the association between miRNA and increased risk of respiratory system diseases (Li et al. 2011; Omran et al. 2013; Wang et al. 2014). There is evidence that the lung has a very specific miRNA expression profile that undergoes changes during lung development (Tomankova et al. 2010). Numerous studies have provided evidence on the potential role of miRNAs in molecular pathways involved in asthma pathogenesis and progression. To date, there are many miRNAs that have been found to be differentially expressed in the bronchial epithelial cells and circulation of asthmatic patients (Kai et al. 2015), where they act as key regulators in various inflammatory and immunological pathways involved in asthma pathogenesis and in the neuronal control of airway smooth muscles (Omran et al. 2013). Genetic variants within human miRNA regulome have been shown to influence miRNA expression, target interaction, and to affect the phenotype (Obsteter et al. 2015). Many variants in specific miRNA genes and (or) their target genes were previously linked to changes in the susceptibility to develop asthma (Omran et al. 2013). Mutations in miRNA transcripts in the pre-miRNA region of miR-146a (rs2910164*C: C/G) and miR-149 (rs2292832*T: C/T) were associated with lower risk of asthma. A variant in the 3′UTR of the HLA-G gene (rs1063320*G), which is an asthma susceptibility gene, was found to be targeted by miR-148a, miR-148b, and miR-152, and was shown to be protective against asthma, but only in the children of asthmatic mothers (Kishore et al. 2014).

In this study, a common variant in the passenger strand of miR-196a2 gene (rs11614913: C/T) was investigated, with the aim of analyzing the association between miR-196a2 SNP and clinical asthma phenotype, and to test its potency as a predictive or prog-

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/bcb-2016-0010.

Fig. 5. Frequency of miR-196a2 genotypes in asthma patients, according to disease characteristics. Disease severity, asthma control, and therapeutic levels were assessed according to the GINA (2014) guidelines. Using the methacholine (20%) challenge test, airway hyper-responsiveness PC20 was categorized according to American Thoracic Society guidelines (Crapo et al. 2000). The χ^2 test was used; values for $p < 0.05$ were considered statistically significant.



nostic marker in asthmatic children and adolescents. Genotype and allele frequencies of the SNPs did not deviate from Hardy–Weinberg equilibrium in patients and controls. The C allele was the most prominent allele, representing 73.4% and 69.8% in patients and controls, respectively, with no significant difference between the studied groups in overall and stratified analysis. These frequencies were similar to previous studies conducted in different ethnic populations with overall minor allele frequency (MAF) of 0.33 (1000 Genome project, imported from dbSNP release 144). The HapMap database (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap27_B36/) shows that the variant allele is swapped between different ethnic populations. The CC is identified as the variant form in East Asians, but is the predominant form in European, African, Mexican, and other races (Fig. 7). These differences might reflect the fact that different ethnic (genetic background) and environmental exposures may modify the risk of the disease (Yuan et al. 2013). The absence of association with asthma risk in our population was consistent with another previous study conducted in asthmatic Chinese patients (Su et al. 2011). Also, these findings were in agreement with our in-silico analysis, which revealed no structural impact of the variant on the 3D RNA folding pattern of the hairpin loop, nor functional effect on the type and number of target genes. The location of the studied SNP, outside the 5′ seed region that is essential for mRNA targeting, may explain the lack of association with disease susceptibility. Similarly, no association of rs11614913 was observed with leprosy (Cezar-de-Mello et al. 2014). However, rs11614913*T was significantly associated with lower risk for COPD (Kishore et al. 2014), and the rs11614913*C variant in miR-196a2 precursor was associated with increased risk of multiple types of cancer, including lung cancer and congenital heart disease (Tian et al. 2009). In addition, miR-196a2 SNP was associated with risk for hepatitis B infection in Asian populations (Zhou et al. 2014), inflamed Crohn's disease (Brest et al. 2011), and viral defense (Sonkoly et al. 2008).

One finding in our study is the significant increase in frequency of the C allele in patients with nocturnal asthma. The mecha-

Fig. 6. Bronchodilator response in asthmatic patients, according to miR-196a2 genotype. Pre- and post-FEV1, pre-bronchodilator, and post-bronchodilator forced expiratory volume in the first second; FVC, forced vital capacity; PEF, peak expiratory flow; BDR, bronchodilator response; BDRBASE, change in FEV1 as a percent of baseline FEV1 {BDRBASE = [(post-BD FEV1 – pre-BD FEV1)/pre-BD FEV1] × 100}. The Kruskal–Wallis test was used, followed by Newman–Keuls multiple comparison tests. a, Compared with the homozygote wild-type; b, compared with the heterozygote.

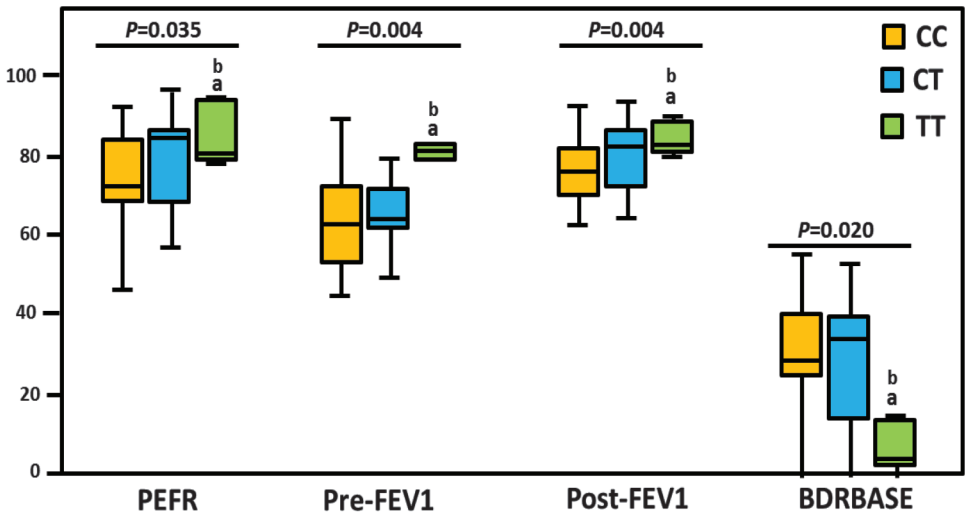
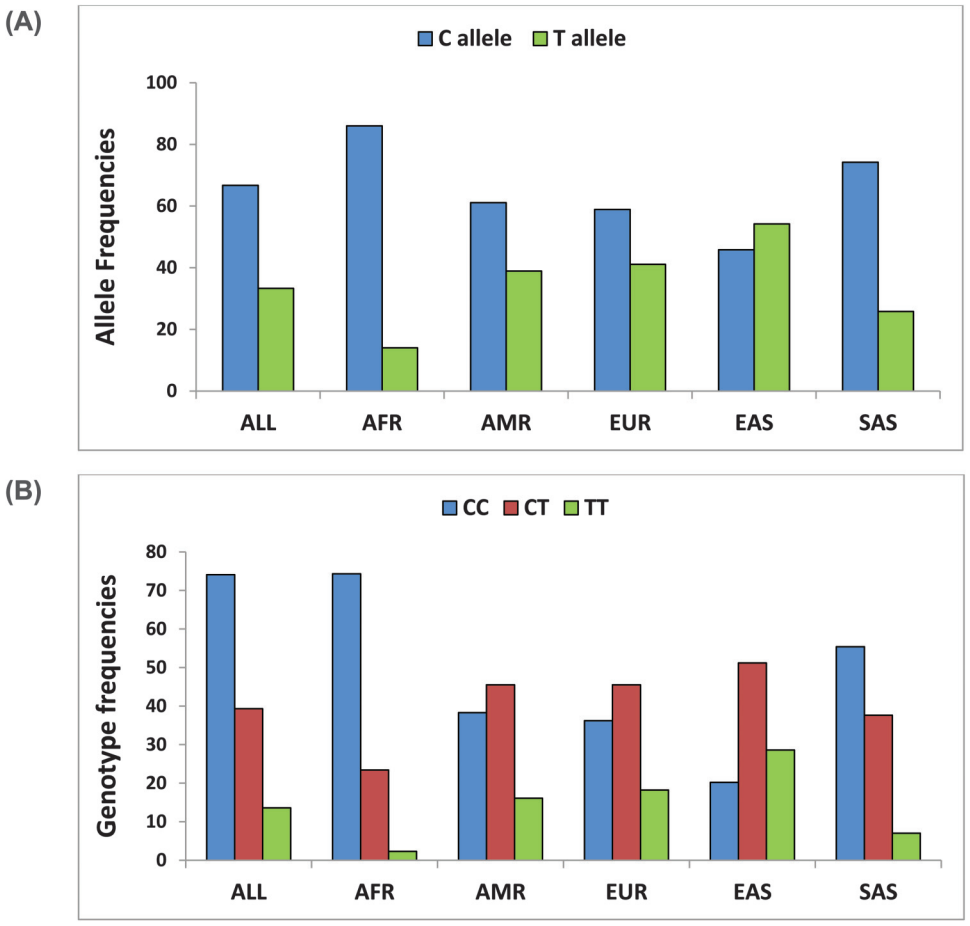


Fig. 7. Allele and genotype frequencies in different ethnic populations (data source: 1000 Genomes Project Phase 3). ALL, all individuals from that release are being considered; AFR, African; AMR, mixed American; EUR, European; EAS, East Asian; SAS, South Asian.



nisms of nocturnal asthma are intimately related to circadian rhythms, which influence inflammatory cells and mediators, hormone levels, and cholinergic tone. Patients with nocturnal asthma symptoms may have greater nighttime activation of inflammatory cells and mediators, lower levels of epinephrine, and increased vagal tone (Skloot, 2002). To the best of our knowledge, our study is the first to reveal the link between rs11614913 (C>T) and nocturnal asthma, but the mechanism needs to be further

elucidated in future studies. Another key finding in this study is the association of rs11614913 SNP with disease severity. The rs11614913*C variant was significantly associated with a higher degree of asthma disease as assessed by the GINA guidelines. In addition, CC genotype carriers were more likely to develop nocturnal asthma and had higher risk of a poor outcome. Individuals with the C allele had significantly lower baseline pulmonary function (PEFR and FEV_1 values) and eventually higher bronchodilator response than T allele. In an in-vitro study, Hoffman et al. (2009) reported an increased expression of mature miR-196a2 in breast cancer cells transfected with pre-miR-196a-C vector, compared with cells transfected with pre-miR-196a-T, but did not observe the differential expression of the precursor miR-196a2 transcript, suggesting that miR-196a2 genotypes may result in altered processing of the precursor hairpin loop into mature forms. This difference in expression levels might provide a plausible explanation for the tendency of having severe disease in C carriers as found in our study. Moreover, in severe asthma, circulating CD8+ T cells were activated, but not CD4+ T cells (Kai et al. 2015). Interestingly, miR-196a2 was predicted to target *HSPA1A* and *TAP1* genes, which are involved in CD8+ T cell function, and not the CD4+ T cell cascade in antigen processing and presentation pathway (hsa04612), thus supporting the hypothesis that severe asthma might show different genetic signatures during progression. Research efforts directed towards a greater understanding of the mechanisms and functional significance of the aberrant expression of miRNAs in bronchial airways will assist in the development of less toxic therapies and provide better markers for disease classification.

Identification of putative mRNA targets is important to understand the specific functions of miRNAs. By using miRNA target prediction software, we found that miRNA-196a2 can modulate the expression of multiple cytokines and mediators that are key players in the asthma pathway (KEGG: hsa05310), such as interleukin (IL)-13 and IL-5. The former induces epithelial cell hyperplasia, goblet cell metaplasia, deposition of various ECM proteins in subepithelial regions, and increased airway smooth muscle cell contractility (Schroeder et al. 2010), whereas IL-5 is produced by T helper-2 (Th2) cells, and activates mast cells, contributes to the recruitment of inflammatory cells to the airways, and promotes the proliferation and differentiation of eosinophils from bone marrow progenitors. The activation of eosinophils, in turn, leads to the release of toxic granules and oxygen free radicals that lead to tissue damage, airway dysfunction of cholinergic nerve receptors, airway hyper-responsiveness, mucus hypersecretion, and airway remodeling characterized by fibrosis and collagen deposition (Possa et al. 2013). Molecular events in the asthma process also involve complex cell signaling pathways; miR-196a2 was predicted by miRTar.Human tool (http://miRTar.mbc.nctu.edu.tw/) to target a network of several key molecules in asthma-related pathways, (Fig. 4; Supplementary data, Table S2). Specifically, miR-196a2 was predicted to target 15 genes in the T-cell receptor signaling pathways. T lymphocytes are considered the central effector cells in asthma regulation. Skewing the balance between Th1 and Th2 responses toward Th2 aggravates the allergic immune response (Liu et al. 2010). In addition, miR-196a2 was predicted to mediate the expression of 11 genes in the B-cell receptor signaling pathway, and 15 genes in the Fc epsilon RI signaling pathway (FcεRI). B-cells synthesize and release immunoglobulin E (IgE) antibodies specific for the allergen. Mast cells constitutively bind to free IgE antibodies via their FcεRI receptors. IgE cross-linking on mast cells leads to mast-cell activation. Activated mast cells undergo degranulation and release histamine and other inflammatory factors into the extracellular space. These inflammatory factors aid in the recruitment of Th2 cells, eosinophils, and other leukocytes to the lungs (Janeway et al. 2005).

To the best of our knowledge, this is the first study to indicate that miR-196a2 rs11614913 plays a role in asthma severity. However, there are several limitations that need to be considered. First, the relatively small sample size in this study might limit the statistical power, and could underestimate the weak and potential associations. Second, the miR-196a2 expression profile in the airways and lung tissues did not correlate with the genotype. Third, functional validation of Mir-196a2 target genes involved in asthma pathogenesis and outcome was not covered in this study, and will be explored in future work.

Conclusions

In conclusion, our study suggested that the rs11614913 (C>T) polymorphism in the precursor sequence of human microRNA-196a2 contributes to the severity of bronchial asthma, but does not affect the susceptibility to the disease. Additional studies with a larger sample size, ethnically diverse populations, and functional tests are warranted to validate our epidemiological findings and to explore the detailed biological mechanisms.

Acknowledgements

The authors would like to thank the Oncology Diagnostic Unit, Suez Canal University, Egypt, for providing the facilities for pursuing the research work in the unit. Declaration of interest: The authors report that there are no conflicts of interest associated with this work. The authors alone are responsible for the content and writing of the paper.

References

Bhattacharya, A., and Cui, Y. 2015. miR2GO: comparative functional analysis for microRNAs. Bioinformatics, **31**(14): 2403–2405. doi:10.1093/bioinformatics/btv140. PMID:25762653.

Bonsignore, M.R., Profita, M., Gagliardo, R., Riccobono, L., Chiappara, G., Pace, E., and Gjomarkaj, M. 2015. Advances in asthma pathophysiology: stepping forward from the Maurizio Vignola experience. Eur. Respir. Rev. **24**(135): 30–39. doi:10.1183/09059180.10011114. PMID:25726552.

Brest, P., Lapaquette, P., Souidi, M., Lebrigand, K., Cesaro, A., Vouret-Craviari, V., et al. 2011. A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. Nat. Genet. **43**(3): 242–245. doi:10.1038/ng.762. PMID:21278745.

Cezar-de-Mello, P.F., Toledo-Pinto, T.G., Marques, C.S., Arnez, L.E., Cardoso, C.C., Guerreiro, L.T., et al. 2014. Pre-miR-146a (rs2910164 G>C) single nucleotide polymorphism is genetically and functionally associated with leprosy. PLoS Negl. Trop. Dis. **8**(9): e3099. doi:10.1371/journal.pntd.0003099. PMID:25187983.

Crapo, R.O., Casaburi, R., Coates, A.L., Enright, P.L., Hankinson, J.L., Irvin, C.G., et al. 2000. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. Am. J. Respir. Crit. Care Med. **161**: 309–329. PMID:10619836.

Du, W., Ma, X., Kong, W., Liu, T., Wei, B., Yu,J., et al., 2013. Association between rs11614913 polymorphism in miR-196a2 and colorectal cancer risk: a meta-analysis. Cancer Biomark. **13**(6): 457–464. doi:10.3233/CBM-140389. PMID: 24595083.

Fawzy, M.S., Alhadramy, O., Hussein, M.H., Ismail, H.M., Ismail, N.M., Biomy, N.M., and Toraih, E.A. 2015. Functional and structural impact of atp-binding cassette transporter A1 R219K and I883M gene polymorphisms in obese children and adolescents. Mol. Diagn. Ther. **19**(4): 221–234. doi:10.1007/s40291-015-0150-7. PMID:26243156.

Fawzy, M.S., Hussein, M.H., Abdelaziz, E.Z., Yamany, H.A., Ismail, H.M., and Toraih, E.A. 2016. Association of microRNA-196a2 variant with response to short-acting β2-agonist in COPD: An Egyptian pilot study. PLoS One, **11**(4): e0152834. doi:10.1371/journal.pone.0152834. PMID:27043015.

Global Initiative for Asthma (GINA). 2014. Global strategy for asthma management and prevention 2014. Available from http://www.ginasthma.org/. [Accessed 16 December 2014.]

Global Initiative for Asthma (GINA). 2015. Global strategy for asthma management and prevention 2015. Available from http://www.ginasthma.org/. [Accessed 3 March 2016.]

Hoffman, A.E., Zheng, T., Yi, C., Leaderer, D., Weidhaas, J., Slack, F., et al. 2009. MicroRNA miR-196a-2 and breast cancer: a genetic and epigenetic association study and functional analysis. Cancer Res. **69**(14): 5970–5977. doi:10.1158/0008-5472.CAN-09-0236. PMID:19567675.

Hsu, S.D., Chu, C.H., Tsou, A.P., Chen, S.J., Chen, H.C., Hsu, P.W., Wong, Y.H., et al. 2008. miRNAMap 2.0: genomic maps of microRNAs in metazoan genomes. Nucleic Acids Res. **36**: D165–D169. doi:10.1093/nar/gkm1012. PMID: 18029362.

Huang, Y., Yi, X., Jian, Z., Wei, C., Li, S., Cai, C., et al. 2013. A single-nucleotide polymorphism of miR-196a-2 and vitiligo: an association study and functional analysis in a Han Chinese population. Pigment Cell Melanoma Res. **26**(3): 338–347. doi:10.1111/pcmr.12081. PMID:23433405.

Janeway, C.A., Jr., Travers, P., Walport, M., and Schlomick, M.J. 2005. Immunobiology: The immune system in health and disease, 6th ed. Garland Science, New York.

Jeon, Y.J., Choi, Y.S., Rah, H., Kim, S.Y., Choi, D.H., Cha, S.H., et al. 2012. Association study of microRNA polymorphisms with risk of idiopathic recurrent spontaneous abortion in Korean women. Gene, **494**(2): 168–173. doi:10.1016/j.gene.2011.12.026. PMID:22222140.

Jeon, Y.J., Kim, O.J., Kim, S.Y., Oh, S.H., Oh, D., Kim, O.J., et al. 2013. Association of the miR-146a, miR-149, miR-196a2, and miR-499 polymorphisms with ischemic stroke and silent brain infarction risk. Arterioscler. Thromb. Vasc. Biol. **33**(2): 420–430. doi:10.1161/ATVBAHA.112.300251. PMID:23202363.

Kai, W., Qian, X.U., and Qun, W.U. 2015. MicroRNAs and Asthma Regulation. Iran. J. Allergy Asthma Immunol. **14**(2): 120–125. PMID:25780877.

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. 2004. The KEGG resource for deciphering the genome. Nucleic Acids Res. **32**(Database issue): D277–D280. doi:10.1093/nar/gkh063. PMID:14681412.

Kaneko, Y., Yatagai, Y., Yamada, H., Hiroki, I., Hironori, M., Tohru, S., and Hizawa, N. 2013. The search for common pathways underlying asthma and COPD. Int. J. Chron. Obstruct. Pulmon. Dis. **8**: 65–78. doi:10.2147/COPD.S39617. PMID:23378757.

Kim, H.Y., Yoon, J.H., Lee, H.S., Cheong, J.Y., Cho, S.W., Shin, H.D., and Kim, Y.J. 2014. MicroRNA-196A-2 polymorphisms and hepatocellular carcinoma in patients with chronic hepatitis B. J. Med. Virol. **86**: 446–453. doi:10.1002/jmv.23848. PMID:24248733.

Kishore, A., Borucka, J., Petrkova, J., and Petrek, M. 2014. Novel insights into miRNA in lung and heart inflammatory diseases. Mediators Inflamm. **2014**: 259131. doi:10.1155/2014/259131. PMID:24991086.

Li, L.J., Gao, L.B., Lv, M.L., Dong, W., Su, X.W., Liang, W.B., and Zhang, L. 2011. Association between SNPs in pre-miRNA and risk of chronic obstructive pulmonary disease. Clin. Biochem. **44**: 813–816. doi:10.1016/j.clinbiochem.2011.04.021. PMID:21565178.

Liu, G., Friggeri, A., Yang, Y., Milosevic, J., Ding, Q., Thannickal, V.J., et al. 2010. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J Exp. Med. **207**: 1589–1597. doi:10.1084/jem.20100035. PMID:20643828.

Malkki, M., and Petersdorf, E.W. 2012. Genotyping of single nucleotide polymorphisms by 5′ nuclease allelic discrimination. Methods Mol. Biol. **882**: 173–182. doi:10.1007/978-1-61779-842-9_10. PMID:22665234.

Mansour, A.E., Yasein, Y.A., Ghandour, A., Zaidan, O., and Abo El-Abaas, M.M. 2014. Prevalence of bronchial asthma and its impact on the cognitive functions and academic achievement among preparatory school children in Damietta governorate, Egypt. J. Am. Sci. **10**(7): 119–127.

Marshall, W.A., and Tanner, J.M. 1969. Variations in pattern of pubertal changes in boys and girls. Arch. Dis. Child. **44**(235): 291–303. doi:10.1136/adc.44.235.291. PMID:5785179.

Martin-Guerrero, I., Gutierrez-Camino, A., Lopez-Lopez, E., Bilbao-Aldaiturriaga, N., Pombar-Gomez, M., Ardanaz, M., and Garcia-Orad, A. 2015. Genetic variants in miRNA processing genes and pre-miRNAs are associated with the risk of chronic lymphocytic leukemia. PLoS One, **10**: e0118905. doi:10.1371/journal.pone.0118905. PMID:25793711.

Matsunaga, N.Y., Ribeiro, M.A., Saad, I.A., Morcillo, A.M., Ribeiro, J.D., and Toro, A.A. 2015. Evaluation of quality of life according to asthma control and asthma severity in children and adolescents. J. Bras. Pneumol. **41**(6): 502–508. doi:10.1590/S1806-37562015000000186. PMID:26785958.

Ober, C., and Yao, T.-C. . 2011. The genetics of asthma and allergic disease: a 21st century perspective. Immunol. Rev. **242**(1): 10–30. doi:10.1111/j.1600-065X.2011.01029.x. PMID:21682736.

Obsteter, J., Dovc, P., and Kunej, T. 2015. Genetic variability of microRNA regulome in human. Mol. Genet. Genomic Med. **3**(1): 30–39. doi:10.1002/mgg3.110. PMID:25629077.

Omran, A., Elimam, D., and Yin, F. 2013. MicroRNAs: new insights into chronic childhood diseases. Biomed. Res. Int. **2013**: 291826. doi:10.1155/2013/291826. PMID:23878802.

Pellegrino, R., Viegi, G., Brusasco, V., Crapo, R.O., Burgos, F., Casaburi, R., et al. 2005. Interpretative strategies for lung function tests. Eur. Respir. J. **26**(5): 948–968. doi:10.1183/09031936.05.00035205. PMID:16264058.

Possa, S.S., Leick, E.A., Prado, C.M., Martins, M.A., and Tibério, I.F. 2013. Eosinophilic inflammation in allergic asthma. Front. Pharmacol. **4**: 46. doi:10.3389/fphar.2013.00046. PMID:23616768.

Qi, J., Hou, S., Zhang, Q., Liao, D., Wei, L., Fang, J., et al. 2013. A functional variant of pre-miRNA-196a2 confers risk for Behcet's disease but not for Vogt–Koyanagi–Harada syndrome or AAU in ankylosing spondylitis. Hum. Genet. **132**(12): 1395–1404. doi:10.1007/s00439-013-1346-8. PMID:23928854.

Rupani, H., Sanchez-Elsner, T., and Howarth, P. 2013. MicroRNAs and respiratory diseases. Eur. Respir. J. **41**(3): 695–705. doi:10.1183/09031936.00212011. PMID:22790917.

Savarimuthu Francis, S.M., Davidson, M.R., Tan, M.E., Wright, C.M., Clarke, B.E., Duhig, E.E., et al. 2014. MicroRNA-34c is associated with emphysema severity and modulates SERPINE1 expression. BMC Genomics, **15**: 88. doi:10.1186/1471-2164-15-88. PMID:24479666.

Schroeder, J.T., Bieneman, A.P., Chichester, K.L., Breslin, L., Xiao, H., and Liu, M.C. 2010. Pulmonary allergic responses augment interleukin-13 secretion by circulating basophils yet suppress interferon-α from plasmacytoid dendritic cells. Clin. Exp. Allergy, **40**: 745–754. doi:10.1111/j.1365-2222.2010.03456.x. PMID:20184608.

Skloot, G.S. 2002. Nocturnal asthma: mechanisms and management. Mt. Sinai J. Med. **69**(3): 140–147. PMID:12035073.

Sonkoly, E., Stahle, M., and Pivarcsi, A. 2008. MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. Semin. Cancer Biol. **18**(2): 131–140. doi:10.1016/j.semcancer.2008.01.005. PMID:18291670.

Soriano, J.B., Alfageme, I., Almagro, P., Casanova, C., Esteban, C., Soler-Cataluña, J.J., et al. 2013. Distribution and prognostic validity of the new Global Initiative for Chronic Obstructive Lung Disease grading classification. Chest, **143**(3): 694–702. doi:10.1378/chest.12-1053. PMID:23187891.

Su, X.W., Yang, Y., Lv, M.L., Li, L.J., Dong, W., Miao-Liao, Gao, L.B., et al. 2011. Association between single-nucleotide polymorphisms in pre-miRNAs and the risk of asthma in a Chinese population. DNA Cell Biol. **30**(11): 919–923. doi:10.1089/dna.2010.1164. PMID:21663520.

Tian, T., Shu, Y., Chen, J., Hu, Z., Xu, L., Jin, G., et al. 2009. A functional genetic variant in microRNA-196a2 is associated with increased susceptibility of lung cancer in Chinese. Cancer Epidemiol. Biomarkers Prev. **18**(4): 1183–1187. doi:10.1158/1055-9965.EPI-08-0814. PMID:19293314.

Tomankova, T., Petrek, M., and Kriegova, E. 2010. Involvement of microRNAs in physiological and pathological processes in the lung. Respir. Res. **11**: 159. doi:10.1186/1465-9921-11-159. PMID:21092244.

Vergoulis, T., Kanellos, I., Kostoulas, N., Georgakilas, G., Sellis, T., Hatzigeorgiou, A., and Dalamagas, T. 2015. mirPub: a database for searching microRNA publications. Bioinformatics, **31**(9): 1502–1504. doi:10.1093/bioinformatics/btu819. PMID:25527833.

Vlachos, I.S., Kostoulas, N., Vergoulis, T., Georgakilas, G., Reczko, M., Maragkakis, M., et al. 2012. DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. Nucleic Acids Res. **40**(online): W498–W504. doi:10.1093/nar/gks494. PMID:22649059.

Wang, G., Wang, W., Gao, W., Lv, J., and Fang, J. 2014. Two functional polymorphisms in microRNAs and lung cancer risk: a meta-analysis. Tumour Biol. **35**(3): 2693–2699. doi:10.1007/s13277-013-1355-1. PMID:24254303.

Wang, N., Li, Y., Zhu, L.J., Zhou, R.M., Jin, W., Guo, X.Q., et al. 2013. A functional polymorphism rs11614913 in microRNA-196a2 is associated with an increased risk of colorectal cancer, although not with tumor stage and grade. Biomed. Rep. **1**(5): 737–742. PMID:24649020.

Xu, W. 2014. Expression data analysis to identify biomarkers associated with asthma in children. Int. J. Genomics, **2014**: 165175. doi:10.1155/2014/165175. PMID:24790987.

Yuan, Z., Zeng, X., Yang, D., Wang, W., and Liu, Z. 2013. Effects of common polymorphism rs11614913 in Hsa-miR-196a2 on lung cancer risk. PLoS One, **8**(4): e61047. doi:10.1371/journal.pone.0061047. PMID:23593385.

Zhang, H., Su, Y.-L., Yu, H., and Qian, B. 2012. Meta-Analysis of the association between Mir-196a-2 polymorphism and cancer susceptibility. Cancer Biol. Med. **9**(1): 63–72. doi:10.3969/j.issn.2095-3941.2012.01.012. PMID:23691458.

Zhou, J.J., Chen, R.F., Deng, X.G., Zhou, Y., Ye, X., Yu, M., et al. 2014. Hepatitis C virus core protein regulates NANOG expression via the stat3 pathway. FEBS Lett. **588**: 566–573. doi:10.1016/j.febslet.2013.11.041. PMID:24462277.

Copyright of Biochemistry & Cell Biology is the property of Canadian Science Publishing and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.