Apurinic/apyrimidinic endonuclease 1 mRNA level in peripheral blood neutrophils is associated with asthma.

Zhou Hai and Qin Jia

Department of Respiration, Shidong Hospital of Yangpu District, No. 999, Shi Guang Rd, Yangpu District, Shanghai 200438, China.

Abstract. Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional key protein. Recent studies suggest APE1 is closely associated with inflammatory response, but its role in asthma remains unknown. We recruited 116 patients with asthma, including 50 with severe asthma (NSA) and 66 with non-severe asthma (SA), and 140 controls. Serum APE1 was detected using the ELISA method. APE1 mRNA in peripheral blood neutrophils and eosinophils were detected using real-time PCR assays. Compared to healthy controls, we observed significant elevations of serum APE1 mRNA levels in peripheral neutrophils (~1.75 folds increase, $p<0.05$) and eosinophils (~2.2 folds increase, $p<0.05$) in patients with asthma. The peripheral blood neutrophil APE1 mRNA can distinguish asthmatic patients from healthy controls with the area under the curve (AUC) 0.893 and a 95% confidence interval (CI) 0.847-0.938 ($p < 0.001$). Also the APE1 mRNA can identify severe asthma from non-severe asthma (AUC 0.759, 95% CI, 0.674-0.846; $p < 0.001$). However, The serum APE1 and eosinophil mRNA levels did not correlate with asthma incidence and severity. Our finding confirms the association between APE1 and asthma and suggests that peripheral blood neutrophil APE1 mRNA may be used as a marker for this condition.

Corresponding author: Qin Jia. Department of Respiration, Shidong Hospital of Yangpu District, No. 999, Shi Guang Rd, Yangpu District, Shanghai 200438, China. Email: qingjia1_sh@hotmail.com

El nivel de ARNm de la endonucleasa 1 apurínica/apirimidínica en los neutrófilos de sangre periférica se asocia con el asma.

Resumen. La endonucleasa apurínica/apirimidínica 1 (APE1) es una proteína clave multifuncional. Estudios recientes sugieren que APE1 está estrechamente asociada con la respuesta inflamatoria, pero hasta el momento se desconoce su papel en el asma. Reclutamos a 116 pacientes con asma, incluidos 50 con asma grave (NSA) y 66 con asma no grave (SA), y 140 controles. Se detectó APE1 en suero usando el método ELISA. El ARNm de APE1 en neutrófilos y eosinófilos de sangre periférica se detectó mediante ensayos de PCR en tiempo real. En comparación con los controles sanos, observamos una elevación significativa de los niveles séricos de ARNm de APE1 en pacientes con asma en neutrófilos periféricos (aumento de ~1,75 veces, p<0,05) y eosinófilos (aumento de ~2,2 veces, p<0,05). El ARNm de APE1 de neutrófilos de sangre periférica puede distinguir a los pacientes asmáticos de los controles sanos con un área bajo la curva (AUC) de 0,893 y un intervalo de confianza (IC) del 95% de 0,847 a 0,938 (p<0,001). Además, el ARNm de APE1 puede identificar el asma grave del asma no grave (AUC 0,759, IC del 95%, 0,674-0,846; p < 0,001). Sin embargo, el nivel sérico de APE1 y ARNm de eosinófilos no mostró correlación con la incidencia y la gravedad del asma. Nuestro hallazgo confirma la asociación entre APE1 y asma y sugiere que el ARNm de APE1 de neutrófilos en sangre periférica puede usarse como marcador para el asma.

INTRODUCTION

Asthma is a major public health problem worldwide. It is a multifactorial disease characterized by chronic airway inflammation, leading to bronchial hyperresponsiveness and airway remodeling [1]. Neutrophils and eosinophils are two major pro-inflammatory cell types that play essential role in the pathogenesis of asthma [1-3]. So far, few biomarkers have been evaluated to reflect the airway inflammation in the asthmatic patients, but with unsatisfactory sensitivity or reliability.

The apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional key protein initially identified to play an important role in the base-excision repair by recognizing the abasic site [4, 5]. Besides its DNA repair function, recent studies showed that APE1 also regulates the expression of different transcription factors, notably, the inflammatory pathway regulator NF-κB, thus contributing to inflammation regulation [6]. It has been proved that APE1 controls IL-6 and IL-8 expression through its redox function [7]. APE1 also regulates inflammatory response in macrophages and keratinocyte [8,9]. Indeed, APE1/Ref-1 has been viewed as an emerging therapeutic target for various inflammatory diseases, including inflammatory pain sensi-

tization, murine myocarditis and spontaneous chronic colitis [10-12].

The association between APE1 and asthma has not been established yet. Based on the established association between APE1 and inflammatory diseases, we hypothesized that APE1 may play a role in asthmatic inflammation. To test this notion, we detected the serum APE1 protein expression levels, mRAN levels from neutrophils and eosinophils isolated from peripheral blood in adult asthmatic patients and healthy controls.

PATIENTS AND METHODS

Study subjects

The diagnosed asthmatic patients and healthy controls were enrolled at the Department of Respiration, Shidong Hospital of Yangpu District between March August, 2018 and October, 2020. The diagnosis of asthma was made in line with the criteria of the Global Initiative for Asthma (GINA) and described elsewhere [13]. The asthmatic subjects were classified as patients with severe asthma (SA) and patients with non-severe asthma (NSA) by the International European Respiratory Society/American Thoracic Society guidelines [14]. Any patient who had known to have underlying respiratory diseases other than asthma was excluded. We also recruited sex and age matched healthy individuals who had annual checkups at our hospital, but did not have any acute or chronic illness (such as cancer, inflammatory diseases, cardiovascular diseases, etc.), atopic diseases or any symptoms of obstructive airway disease. The body mass index (BMI), smoking status, asthma duration (years), allergic history, blood eosinophils and blood neutrophils counts were obtained from their medical charts.

Ethical statement

The ethical committee of Shidong Hospital of Yangpu District approved the study. This research was conducted in accordance with the principles embodied in the Declaration of Helsinki. All participants were given written informed consent forms to participate in the study.

Pulmonary function

Pulmonary function tests were performed using a SYSTEM 21® device (Minato Medical Science Co., Osaka, Japan), according to the criteria of the American Thoracic Society (ATS)/European Respiratory Society and the Japanese Respiratory Society [15]. The pulmonary function was measured and included the percentage of predicted volume (FEV1% pred).

Serum samples collection and protein quantification

Peripheral blood was drawn in each participant, followed by centrifugation at 3500 rpm for 10 min to isolate serum. Serum samples were collected and APE1 levels were determined using Human APEX1 ELISA kit (Cusabio, Houston, USA). The optical density (OD) was detected with an EnSpire microplate reader (PerkinElmer, Waltham, USA), at a wavelength of 450 nm with a correction set at 540 nm. The concentration of serum APE1 (pg/mL) was calculated using the standard curve. The serum high-sensitivity C-reactive protein (Hs-CRP) was detected using human high sensitivity C-Reactive Protein ELISA kit (Sunlong Biotech, Hangzhou, China) according the manufacturer's protocol. The total IgE level was detected using Human IgE ELISA Kit (Abcam Biotech, Waltham, MA, USA) according the manufacturer's protocol.

RNA isolation and reverse transcription and real-time PCR

Neutrophils and eosinophils were isolated from fresh drawn peripheral blood using the MACSxpress Whole Blood Eosinophil Isolation Kit and MACSxpress Whole Blood Neutrophil Isolation Kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), according to the manufacturer's manual. Total RNA was extracted using the TaKaRa RNA PCR

Kit (Takara, Dalian, China) from neutrophils and eosinophils. The expression of APE1 mRNA was performed by quantitative real-time polymerase chain reaction (rt-qPCR) with the SYBR Premix Ex Taq II (Takara, Dalian, China). All samples were performed in triplicate. β-actin was applied for the internal normalization of RNA. The PCR reaction was performed at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 61°C for 30 s. The comparative Ct method (ΔCt) was exploited to calculate the relative expression levels of miRs. The mean cycle threshold (Ct) values and deviations between the duplicates were calculated for all samples. The primers for the APE1 were as following: miR-30,Forward: CTGCTCTTGGAATGTGGATGGG, Reverse TCCAGGCAGCTCCTGAAGTTCA. β-actin, Forward AGAGCTACGAGCTGCCTGAC and reverse GGATGCCACAGGACTCCA.

Statistical analysis

The data are expressed in terms of mean (± standard deviation). Student's t-test and one-way ANOVA were used to compare two or more groups. Pearson's correlation analysis was conducted and the correlation coefficients (r2) were used to measure correlation. A receiver operating characteristic (ROC) curve was performed to the diagnostic value of serum APE1 and its mRNA in neutrophils and eosinophils in the discrimination between asthmatics from healthy controls. Statistical analysis was performed using SPSS version 19.0.0. P <0.05 was considered significant.

RESULTS

Demographic and clinical parameters of the study subjects

We enrolled 140 healthy normal controls (NC) and 116 asthmatic patients, among which there were 50 that were assigned into the severe asthma (SA) group, while 66 were patients with non-severe asthma (NSA). There were no differences in age, BMI and sex distribution among the three groups. There was a higher rate of smokes among the asthmatic patients than in healthy controls (32.4 and 45.1 vs. 16.4%, both p<0.05). The SA group had longer asthma duration compared to the NSA group (9.56±4.38 vs. 14.37±8.56 years, p=0.014). The SA group had significantly lower baseline forced expiratory volume in 1 second (FEV1; 77.23±26.45 vs. 58.34±22.25, p=0.012) compared to the NSA group. The asthmatics had dramatically elevated serum total IgE level and serum hs-CRP levels than normal controls. In addition, the SA patients had even more increased total IgE level, serum hs-CRP levels than NSA patients (Table 1).

Association between APE1 and asthma

Compared to the NC group, the serum APE1 level in the NSA and SA group were higher than that in control group. However, no significant difference was noted between the NSA and SA groups, as shown in Fig. 1A. Similarly, we found that APE1 mRNA in peripheral blood eosinophils of NSA and SA patients were significantly increased in comparison to control groups. The SA group had slightly higher eosinophil APE1 mRNA level in contrast to NSA patients, but did not reach statistical significance (Fig. 1B). As for APE1 mRNA in peripheral blood neutrophils, we observed it was significantly up-regulated in NSA and SA groups compared to controls. Noticeably, the SA patients also had a dramatically higher level of APE1 than NSA patients (Fig. 1C).

We also performed the Pearson's correlation analysis and found that APE1 mRNA levels of neutrophils of peripheral blood were significantly correlated with the other clinical indices, such as hs-CRP and Fev1%, as shown in Table 2. The serum APE1 and mRNA level in eosinophils are not correlated to the levels of hs-CRP and FEV1% pred. None of the three was correlated to total Ig E level.

Table 1
Demographic and clinical parameters of the study subjects.

Index	Controls (n=140)	NSA (n=50)	SA (n=66)
Age	42.43±7.23	41.45±10.31	46.63±11.45
Male (%)	51	54	48
BMI(kg/m²)	24.37±3.14	25.52±3.67	25.72±4.43
Smoking rate(%)	16.4	32.4*	45.1*#
asthma duration (years)	-	9.56±4.38	14.37±8.56 #
Allergic history (%)	13	25*	27*#
Blood eosinophils(×10⁹/L)	0.18±0.13	0.25±0.11	0.35±0.14#
Blood neutrophils (×10⁹/L)	4.11±1.04	4.44±1.23	5.67±3.14#
Serum Hs-CRP	0.45±0.01	11.12±6.67*	14.12±6.45 *#
FEV1% pred	-	77.23±26.45	58.34±22.25*
Serum total IgE(IU/mL)	68.16±23.52	199.17±45.87	621.45±133.59*

BMI, Body mass index; Serum Hs-CRP, high-sensitivity C-reactive protein; FEV1% pred, forced expiratory volume in one second % of predicted value. * vs control, p<0.05; #, vs NSA, p<0.05; SA, severe asthma; NSA, Non-severe asthma.

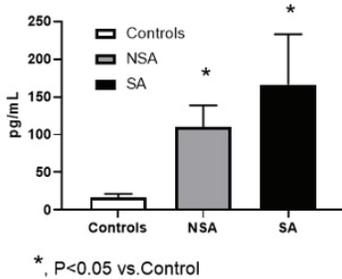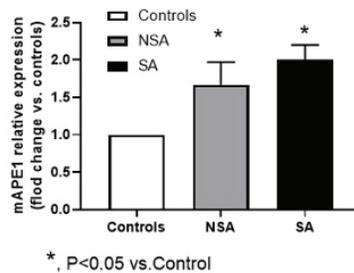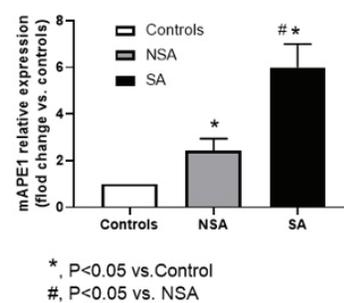**Fig. 1A.** Serum APE1 levels detected using ELISA in control, non-severe asthma (NSA) and severe asthma (SA) groups by using the ANOVA test. **Fig. 1B.** APE1 mRNA in peripheral blood eosinophils using Realtime PCR assays in control, NSA and SA groups. **Fig. 1C.** APE1 mRNA in peripheral blood neutrophils using Realtime PCR assays in control, NSA and SA groups.

Diagnostic value determined by ROC analysis

To test the diagnostic value of serum APE1 and its mRNA in eosinophils and neutrophils, we performed the Receiver Operating Characteristic (ROC) curve analysis. As shown in Fig. 2A, the peripheral blood neutrophil APE1 mRNA can distinguish asthmatic patients (NSA+SA) from healthy controls, at a cutoff value of 2.14, with the AUC

of 0.893 (95% CI, 0.847-0.938; p<0.001, with 87.5% sensitivity and 84.6% specificity). We next tested if neutrophil APE1 mRNA is related to the asthma severity. As shown in Fig. 2B, the APE1 mRNA at a cutoff value of 4.24, is adequate to identify SA subject from NSA subjects, with an AUC of 0.759 (95% CI, 0.674-0.846; p<0.001, 83.4% sensitivity and 80.3% specificity). On the other hand, the serum APE1 and eosinophil mRNA level,

Table 2

The correlation between APE1 protein or mRNA levels with the hs-CRP, Total Ig E and FEV1%.

	hs-CRP	Total Ig E	FEV1% pred
Serum APE1	R2=0.562, P=0.032	R2=0.223, P=0.115	R2=-0.307, P=0.055
Eosinophils APE1 mRNA	R2=0.215, P=0.084	R2=0.3632, P=0.064	R2=-0.103, P=0.774
Neutrophils APE1 mRNA	R2=0.775, P=0.003	R2=0.326, P=0.076	R2=-0.708, P=0.001

Serum Hs-CRP, high-sensitivity C-reactive protein; FEV1% pred, forced expiratory volume in one second % of predicted value.
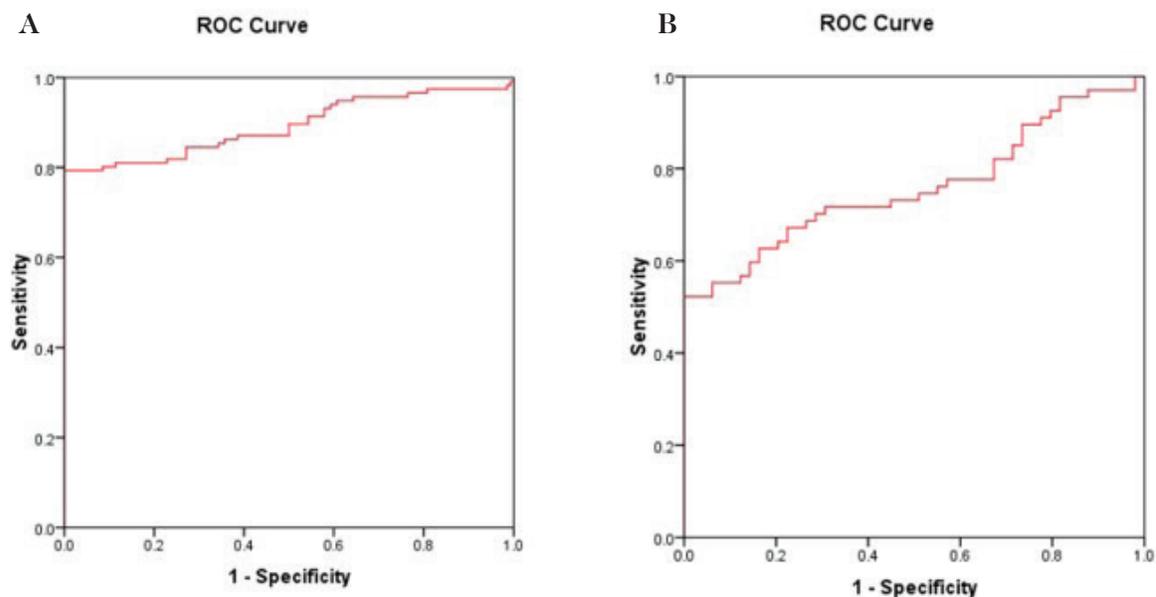

Fig. 2A. The Receiver-Operating Characteristic (ROC) analysis of APE1 mRNA in eosinophils of peripheral blood in distinguishing asthmatic patients (NSA+SA) from healthy controls, with an area under the curve value of 0.893 (95% Confidence Interval, 0.847-0.938; p < 0.001, with 87.5% sensitivity and 84.6%). **Fig. 2B.** The Receiver-Operating Characteristic (ROC) analyses of APE1 mRNA in eosinophils of peripheral blood distinguishing asthmatic patients NSA from SA groups, with an area under the curve value of 0.759 (95% Confidence Interval, 0.674-0.846; p < 0.001, 83.4% sensitivity and 80.3% specificity.

however, did not show a diagnostic difference in separating asthmatic patients from controls, nor are they related to the asthma severity (data not shown).

DISCUSSION

In this study, we detected the serum APE1, the peripheral blood eosinophil and neutrophil APE1 mRNA in adult asthmatic patients and healthy controls. We found although all of these markers were increased in asthmatic patients, only neutrophil APE1 mRNA has diagnostic significance in distinguishing asthmatics from controls, and also in separating severe patients from nonsevere patients. This finding establishes, for the first time the association between APE1 and asthma, and also provides an easily accessible biomarker to evaluate the asthma development and severity in a clinical setting. To the best of our knowledge, we are

the first to confirm the association between APE1 and asthma.

APE1 has been increasingly viewed as a potent inflammatory regulator in a variety of inflammatory processes. In psoriatic skin, APE1 was markedly up-regulated in epidermal layers. APE1 the transcriptionally activated hypoxia-inducible factor-1α and NF-κB, two crucial transcription factors responsible for inflammation in keratinocytes. APE1 is essential for the expression of inflammatory cytokines and chemokines in HaCaT cells and primary keratinocytes [16, 17]. In ApoE-/- mouse model of atherosclerosis, plasma APE1 correlates with Atherosclerotic Inflammation levels and APE1/Ref-1 expression was upregulated in aortic tissues [18]. In macrophages, pharmacological inhibition of APE1 with its redox function inhibitor suppresses inflammatory response in activated macrophages [19]. Elevation of Serum APE1 was reported in experimental murine model for myocarditis [11].

APE1 has been used as a prediction marker of Environmental Carcinogenesis Risk, including smoking [20]. Smoking can in induce a various types of DNA damage and prompts cancers. Several previous studies reported the association between genetic variability of APE1 with lung cancer. Some researchers reported that APE1 genotypes were correlated with the risk of lung cancer among smokers [21], while the others reported that APE1 polymorphisms of −656T > G located in the promoter region and D148E are closely associated with lung cancer risk under cigarette smoking exposure [22, 23]. Smoking has been shown to exacerbate asthma severity by aggravating inflammation [24, 25]. Consistent with this, in our study, we observed that the severe asthma patients have a higher smoking status than non-severe asthma and healthy controls. The smoking amount is positively associated with the asthma severity (data not shown). However, the role of APE1 in asthma has not been elucidated so far.

Our study, for the first time, confirms the diagnostic significance of APE1 mRNA in peripheral blood neutrophils. Airway inflammation in bronchial asthma is characterized by infiltration with eosinophils and neutrophils [26, 27]. That was the reason we detected APE1 mRNA levels from eosinophils and neutrophils. Compared to the sputum, human peripheral blood is a stable source of eosinophils and neutrophils. Our data suggests APE1 mRNA in peripheral blood neutrophils, rather than that in eosinophils, can be used as a biomarker. Since we enrolled adult asthmatics, we cannot exclude the role of eosinophils APE1 m RNA in asthmatic children. To our surprise, our data did not reveal the clinical significance of serum APE1 for asthma diagnosis and classify its severity. In conclusion, our study discovered an easily accessible biomarker for asthma evaluation. Of course, further validation of our finding with a larger scale of sample size is needed.

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Conflict of interest

None.

Author's ORCID numbers

- Qin Jia: 0000-0001-5576-9843
- Zhou Hai: 0000-0003-2815-2746

Contributions of authors

QJ conceived the study. ZH and QJ enrolled patients, collected patient's information and performed the lab assays. QJ analyzed the data and drafted manuscript.

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