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Expression of herpesvirus entry mediator gene as a Potential Biomarker for Disease Severity in Patients with Persistent Asthma.

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ABSTRACT:

Objectives

Herpes virus entry mediator (HVEM) is a costimulatory molecule, and has been proved to play an important role in airway inflammatory and remodeling processes of asthma. We aimed to investigate the expression of HVEM gene in patients with asthma as a means of assessing disease severity.

Methods

This study was carried out on 59 subjects, 16 patients with mild persistent asthma, 11 patients with moderate persistent asthma, 13 patients with severe persistent asthma, and 19 age and gender matched healthy controls. The HVEM mRNA expressions of all subjects were determined by real time PCR. Correlations between HVEM mRNA expression and fractional exhaled nitric oxide (FeNO), pulmonary function test values, total blood white cell count and differential, total immunoglobulin E (IgE) level, and Asthma Control Test (ACT) score were analyzed respectively. The discrimination abilities of HVEM mRNA between different groups were tested using receiver operating characteristics (ROC) curve analyses.

Results

This study showed the expressions of HVEM mRNA were significantly higher in the patients with severe and moderate persistent asthma than in patients with mild persistent asthma and healthy subjects (2.97 ± 1.23 vs. 1.17 ± 0.42 vs. 0.62 ± 0.38 vs. 0.46 ± 0.18 / NAPDH, $P < 0.001$), but there was no significant difference between patients with mild persistent asthma and healthy controls (0.62 ± 0.38 vs. 0.46 ± 0.18 / NAPDH, $P = 0.557$). HVEM mRNA expression at cut off point [1.01 / NAPDH, area under the ROC curve (AUC) = 0.99] is sufficient to discriminate severe patients from mild-to-moderate patients, and at cut off point (0.93 / NAPDH, AUC = 0.91) for discrimination of moderate-to-severe patients from mild ones, while at cut off point (0.76 / NAPDH, AUC = 0.75) for discrimination of asthmatic patients from controls. Furthermore, HVEM mRNA expression was positively correlated with FeNO level ($r = 0.524$, $P = 0.015$), and total lymphocyte count ($r = 0.426$, $P = 0.017$) in patients with persistent asthma.

Conclusions

HVEM gene expressions can be used as a potential biomarker for evaluating the severity of patients with persistent asthma.

Keywords:

Asthma, HVEM, FeNO, Lymphocyte, Severity

Background

Asthma is one of the most common health problems in the world^[1]. Airway inflammation, airway hyper-responsiveness, and airway remodeling are now recognized as the hallmark pathological features of asthma^{[2],[3]}. Large amounts of data suggest that airway remodeling may be attributed to the persistent airway inflammation. However, a growing body of research demonstrates that airway remodeling might develop as a main event that occurs early without any detectable inflammation^[4]. Therefore, the biological behaviors of asthma have not yet been completely elucidated.

The essence of asthma is a complex and chronic inflammatory disorder, whether in its acute phase, chronic phase, or remission phase^[5]. Our widely used markers of airway inflammation include sputum eosinophils, eosinophil cationic protein, interleukin-4 (IL-4) and fractional exhaled nitric oxide (FeNO). Among these markers, FeNO can be of great value in the diagnosis of asthma, identifying types of airway inflammation, and assessing therapeutic sensitivity to steroids^{[6],[9]}. However, single measurement of FeNO is insufficient to evaluate structural remodeling of airway wall, and to assess severity level of asthma.

In recent years, a newly discovered costimulatory molecule, herpesvirus entry mediator (HVEM), broadly expressed on immune cells including T cells, B cells, natural killer (NK) cells, polymorphonuclear cells (PMN), dendritic cells (DCs), and myeloid cells. Its biological ligand is the TNF superfamily member 14 (TNFSF14, also known as LIGHT), and the ligation by LIGHT provides a costimulatory signal for regulating T cell activation. Previous studies have confirmed that HVEM plays an important role in the pathophysiology of asthma. Most of the effects of immunoregulation are mediated through HVEM-LIGHT signaling pathway, such as promoting pulmonary fibrosis^{[10],[11]}^{Error! Reference source not found.}, inducing inflammatory chemokine production, and developing airway remodeling^{[12],[13]}. Based on these results, we suspect that HVEM expression is likely to be a relevant marker of airway inflammation and remodeling, further reflects the symptom severity of asthma. The aim of this work was to evaluate changes of HVEM expressions in patients with mild, moderate, and severe persistent asthma compared to healthy control subjects, and its association with disease severity in these patients.

Methods

Study subjects

This study was carried out on 59 subjects, 40 asthmatic patients that were admitted in Pulmonary Department, the Second Clinical College, Jinan University, and 19 age and gender matched healthy controls from May 2016 to March 2017. Inclusion criteria were physician-diagnosed asthma according to 2015 Global Initiative for Asthma (GINA) guidelines^[14]. All patients underwent pulmonary function test (PFT) and FeNO test. Subjects were excluded if they had a history of autoimmune or other lung diseases, including but not limited to pneumonia, bronchiectasis, cystic fibrosis, interstitial lung disease, and lung cancer. All subjects enrolled in this study were nonsmokers, and they were divided into four groups: Mild asthma group included 16 patients with mild persistent asthma; Moderate asthma group included 11 patients with moderate persistent asthma; Severe asthma group included 13 patients with severe persistent asthma; and Control group included 19 age and gender matched healthy subjects served as a control group. The impact of therapy of all enrolled patients was evaluated, and asthma severity were assessed from the level of treatment required to achieve good control as defined by 2015 GINA guidelines^[14].

The project was conducted according to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Second Clinical College of Jinan University. Written informed consent has been obtained from all subjects.

All patients and healthy controls were subjected to the following: patient characteristics [including gender, age, body mass index (BMI), history of atopy], treatment modalities, and real time PCR for assessment of HVEM mRNA expressions. Asthma control test (ACT) score, total blood white cell count and differential, total immunoglobulin E (IgE) level, FeNO level, and PFT values were obtained from asthmatic patients. The correlation between HVEM mRNA expression and other clinical indicators were calculated.

FeNO measurements

Before spirometry analyses, all subjects underwent standard measurement of FeNO at an exhalation flow rate of 0.05 L/s, during single exhalation, using the NIOX MINO (Aerocrine AB, Solna, Sweden), according to the manufacturer's instructions. The measurement unit was expressed as ppb. The FeNO measurements were consistent with the American Thoracic Society (ATS)/European Respiratory Society (ERS) 2005 guidelines methods^[11]. All patients refrained from eating and drinking for at least 1 hour prior to the FeNO measurement. Each patient was allowed up to no more than 6 attempts to perform a single-exhalation maneuver, and the FeNO level was calculated as the mean of 3 correct exhalations. Following ATS/ERS 2005 guidelines, the normal exhaled NO value was set as 5–35 part per billion (ppb) for healthy adults.

PFT measurements

Subsequent to the FeNO measurements, patient spirometry testing was performed using a V6200 Series Autobox (SensorMedics, American) spirometer device. PFT parameters like forced expiratory volume in 1 second (FEV1) predicted, FEV1/forced vital capacity (FVC), maximal expiratory flow at 25% of the forced vital capacity (MEF25), and MEF75 were collected according to the methods of the ATS / ERS task force~~Error! Reference source not found.~~. A predicted ratio for each parameter was calculated based on age, sex, height, and race.

Blood white cell differential count and total IgE measurements

Three-milliliter samples of venous blood were collected into tubes with Ethylenediaminetetraacetic acid (EDTA) for laboratory investigations. The total count and percentage for each type of blood white cells (eg, eosinophil, neutrophil, and lymphocyte), and total IgE level were investigated in patients with asthma.

Real time PCR

Total RNA was extracted from mononuclear cells using Trizol reagent (Takara, Dalian, China) following the manufacturer's instruction. The quantity and quality were determined by microplate reader (thermo, Shandong, China): A260/A280=1.8-2.0, indicating perfect purity. Reverse transcription was conducted using 2 µg total RNA with the High-Capacity cDNA reverse transcription kit (Promega, Peking, China) according to the manufacturer's instructions. The real time PCR was performed with GoTaq Green Master Kit (Promega, Peking, China) in accordance with the manufacturer's recommendation. The primers were ordered from Yiji Biological Technology, China, and listed as below:

HVEM Forward Primer: 5'- GGTGCTGTATCTCACCTTCCTG -3'

HVEM Reverse Primer: 5'- TCCTTCACACGATAACCTGGAC -3'

GAPDH Forward Primer: 5'- TCCAAAATCAAGTGGGGCGA -3'

GAPDH Reverse Primer: 5'- TGATGACCCTTTTGGCTCCC -3'.

The relative expression of target gene was calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical Evaluation

SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical evaluations. Normal distribution measurement data was expressed as the mean $\pm$ standard deviation. ONE-WAY ANOVA was performed to compare the four groups. Dunnett method was used for the comparison between the two groups. Non-parametric data was expressed as median and interquartile range. The two-sample Wilcoxon Rank Sum test was used to test non-parametric data between 2 independent groups, and the Chi-Square test for categorical data. Linear correlation regression was used to certify whether there was a correlation between HVEM mRNA expression and other clinical parameters. Receiver operating characteristic (ROC) curves were generated, and the area under the curve (AUC) was calculated to identify HVEM for discriminating asthmatic patients from healthy subjects, moderate-to-severe patients from mild patients, severe patients from mild-to-moderate ones, and to provide the best cutoff values. A *P* value of less than 0.05 (*P* < 0.05) was considered as a statistically significant difference.

RESULTS

Baseline characteristics of the study populations

In total, 59 subjects were included, of which 40 subjects were patients with mild, moderate, and severe persistent asthma (19 men and 21 women). The obtained medical data and demographic characteristics of the study group are reflected in Table 1. There was no statistical difference in age, gender, BMI, and atopy status among the four groups. It is interesting to note that all the results of total white cell count differential and IgE level obtained for mild asthma group were similar with those for moderate and severe asthma group (*P* all > 0.05), while the PFT

parameters (including FEV1 of predicted, FEV1/FVC and MEF75) of mild and moderate asthma group were higher than those of severe asthma group (mild vs. moderate vs. severe asthma: FEV1 of predicted, 86.39 ± 3.11 vs. 71.46 ± 10.45 vs. $49.28 \pm 10.46\%$, $P < 0.001$; FEV1/FVC, 73.71 ± 1.95 vs. 60.03 ± 7.80 vs. $51.52 \pm 9.58\%$, $P < 0.001$; and MEF75, 78.31 ± 8.38 vs. 69.69 ± 16.35 vs. 31.60 ± 12.15 L/s, $P < 0.001$). Furthermore, there was also a statistically significant difference in FeNO level among patients with mild, moderate, and severe persistent asthma (mild vs. moderate vs. severe asthma: 33.45 ± 7.51 vs. 90.00 ± 32.74 vs. 106.00 ± 22.19 ppb, $P < 0.001$), and no statistical difference was found in ACT scores among the three asthma groups ($P = 0.350$). Other details of the clinical data in these four groups were showed in Table 1.

Comparison of HVEM mRNA expressions among the study groups

The blood levels of HVEM mRNA in different groups are shown in Table 1 and Figure 1. The HVEM mRNA expression was significantly higher in patients with severe and moderate persistent asthma than in patients with mild persistent asthma and healthy subjects (severe vs. moderate vs. mild asthma vs. healthy subjects: 2.97 ± 1.23 vs. 1.17 ± 0.42 vs. 0.62 ± 0.38 vs. 0.46 ± 0.18 / NADPH, $P < 0.001$). In addition, no significant difference was observed between patients with mild persistent asthma and healthy subjects ($P = 0.557$).

Correlation analysis of HVEM mRNA in the study groups

Through linear correlation analysis, we found HVEM mRNA was positively correlated with FeNO level ($r = 0.524$, $P = 0.015$), and total lymphocyte count ($r = 0.426$, $P = 0.017$), respectively (Table 2 and Figure 2). However, the HVEM mRNA expression has no association with other clinical parameters including ACT score ($r = 0.124$, $P = 0.582$), total neutrophil count ($r = 0.131$, $P = 0.583$), total eosinophil count ($r = 0.248$, $P = 0.179$), neutrophil ratio ($r = -0.111$, $P = 0.551$), eosinophil ratio ($r = 0.009$, $P = 0.963$), lymphocyte ratio ($r = 0.137$, $P = 0.461$), total IgE level ($r = 0.058$, $P = 0.838$), FEV1% predicted ($r = -0.195$, $P = 0.424$), FEV1/FVC ($r = -0.275$, $P = 0.254$), MEF25 ($r = -0.274$, $P = 0.256$), and MEF75 ($r = -0.227$, $P = 0.350$).

ROC curves to identify subjects with persistent asthma

HVEM expression at cut off point (1.01 / NADPH, AUC = 0.99) is sufficient to discriminate severe patients from mild-to-moderate patients that gives a sensitivity of 81.3% and a specificity of 100% (Table 4, Figure 3A), and at cut off point (0.93 / NADPH, AUC = 0.91) for discrimination of moderate-to-severe patients from mild ones with a sensitivity of 92.9% and a specificity of 76.5% (Table 4, Figure 3B). However, HVEM expression at cut off point (0.76 / NADPH, AUC = 0.75) can discriminate asthmatic patients from healthy subjects with a sensitivity and specificity of 67.7% and 100% (Table 4, Figure 3C).

DISCUSSION

To our knowledge, this study provides the first evidence that the HVEM gene expressions were significantly higher in patients with persistent asthma than in age, sex-matched controls. Furthermore, HVEM gene expression was positively correlated with disease activity in patients with asthma, which suggests that HVEM gene expression can be regarded as a potential, novel biomarker of asthma severity. These findings also implied the potential role of HVEM gene expression in predicting therapeutic effects in asthmatic patients.

Previous investigators have reported that HVEM is an important immune-checkpoint in regulating the course of immune responses, including enhancing CD4⁺T cells expansion, restraining B cells growth, activating NK cells, and participating mucosal immunity (Figure 4). Therefore, it has been considered as a vital mechanism in different pathophysiological conditions such as asthma, virus infection, cancer recognition, dermatitis, and autoimmune diseases^{[11][14]}. **Error! Reference source not found..** Our previous studies in asthma models have shown that LIGHT binding HVEM plays an important role in chronic airway inflammation, and interaction between T cells and airway smooth muscle (ASM) cells in asthma. Blockade of LIGHT can reduce ASM cell proliferation, survival, CD4⁺T cell differentiation, and cytokines release, which may be modulated through HVEM pathways^{**Error! Reference source not found..**[13]}. However, clinical researches on the changes of HVEM expressions in asthmatic patients are absent. Thus, we intended to explore the expression levels of HVEM mRNA in peripheral blood of patients with persistent asthma, and analyse its relationship with clinical parameters.

In the current study, there was a statistically significant increase of HVEM mRNA expression in patients with persistent asthma when compared to the healthy subjects ($P = 0.004$). Moreover, the HVEM mRNA expression was significantly increased in patients with moderate and severe persistent asthma than ones with mild persistent asthma ($P < 0.001$). These observations are in line with the results we previously reported that, the mRNA expressions of HVEM and its ligand LIGHT were shown to be higher in the lung tissue of asthmatic mice as

compared to control mice^{Error! Reference source not found.}. HVEM is widely expressed in multiply immune cells, such as T cells, B cells, NK cells, and proposed as an important receptor in regulating inflammatory response, as well as host defence against pathogens. We found that HVEM mRNA expressions in asthmatic patients were linearly correlated with total lymphocyte counts ($r = 0.426$, $P = 0.017$). It was suggested that HVEM in peripheral blood is mainly expressed in circulating lymphocytes including T cells and B cells, and may play a pathogenetic role in asthma exacerbation through transepithelial migration to airway tissues. It was also implied that peripheral lymphocytes could contribute to airway inflammation and remodeling via increased expression of HVEM in patients with asthma. Future experiments are needed to prove the validity of the hypothesis.

More recently, FeNO has been found to indicate upregulating of eosinophilic airway inflammation, and correlate with allergic sensitization^{[18][19]}. Our study revealed that there was a linear correlation between HVEM expression and FeNO level ($r = 0.524$, $P = 0.015$), suggesting a pathophysiological link between HVEM molecule and eosinophilic inflammation in airway. Additionally, PFT has an important diagnostic value in clinical examination of asthma patients^{Error! Reference source not found. [20][20]}, and also reflects the degree and reversibility of airway obstruction. We found that the FEV1/FVC value of moderate and severe asthma group were lower than that of mild asthma group ($P = 0.006$), which indicate that patients with moderate and severe persistent asthma have a higher level of airway obstruction of than patients with mild persistent asthma. However, no correlation between the PFT parameters and HVEM mRNA expression was found in our study, which may suggest that HVEM mRNA expression cannot directly affect the ventilating function of patients with asthma.

This study revealed a statistically significant association between HVEM mRNA expression and severity of patients with persistent asthma. The ROC curve showed that HVEM mRNA is an excellent parameter to discriminate severe from moderate-to-mild asthma ($AUC = 0.99$), and moderate-to-severe from mild asthma ($AUC = 0.91$), which suggested that HVEM gene over-expression in peripheral blood confers an increased risk of having severe and poorly controlled asthma. However, HVEM mRNA expression is not good at discriminating normal from asthma ($AUC = 0.75$). This finding does not consistent with the results from our previous study^[8], which demonstrated that, the expression of HVEM mRNA was significant higher in the airway of asthmatic model as compared to controls. It may be the results of a limited number of study population and different sources of HVEM mRNA.

The present study is only a monocentre explorative analysis of possible biomarker of asthma, like many others have been proposed over time. Even though the size of our study was limited, this is the first study to report that HVEM gene expression is over expressed in patients with persistent asthma, and associated with the disease severity. HVEM is an important checkpoint in the occurrence and development of airway inflammation and remodeling in asthma. Peripheral blood HVEM gene expression provides a potential clinical value for the severity evaluation and personalized therapy of asthmatic patients. Further research is warranted to uncover the correlation between HVEM gene expression and asthma control measures or responses to therapy.

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conflict of interest

We declare that we have no conflict of interest.

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Figure Legends

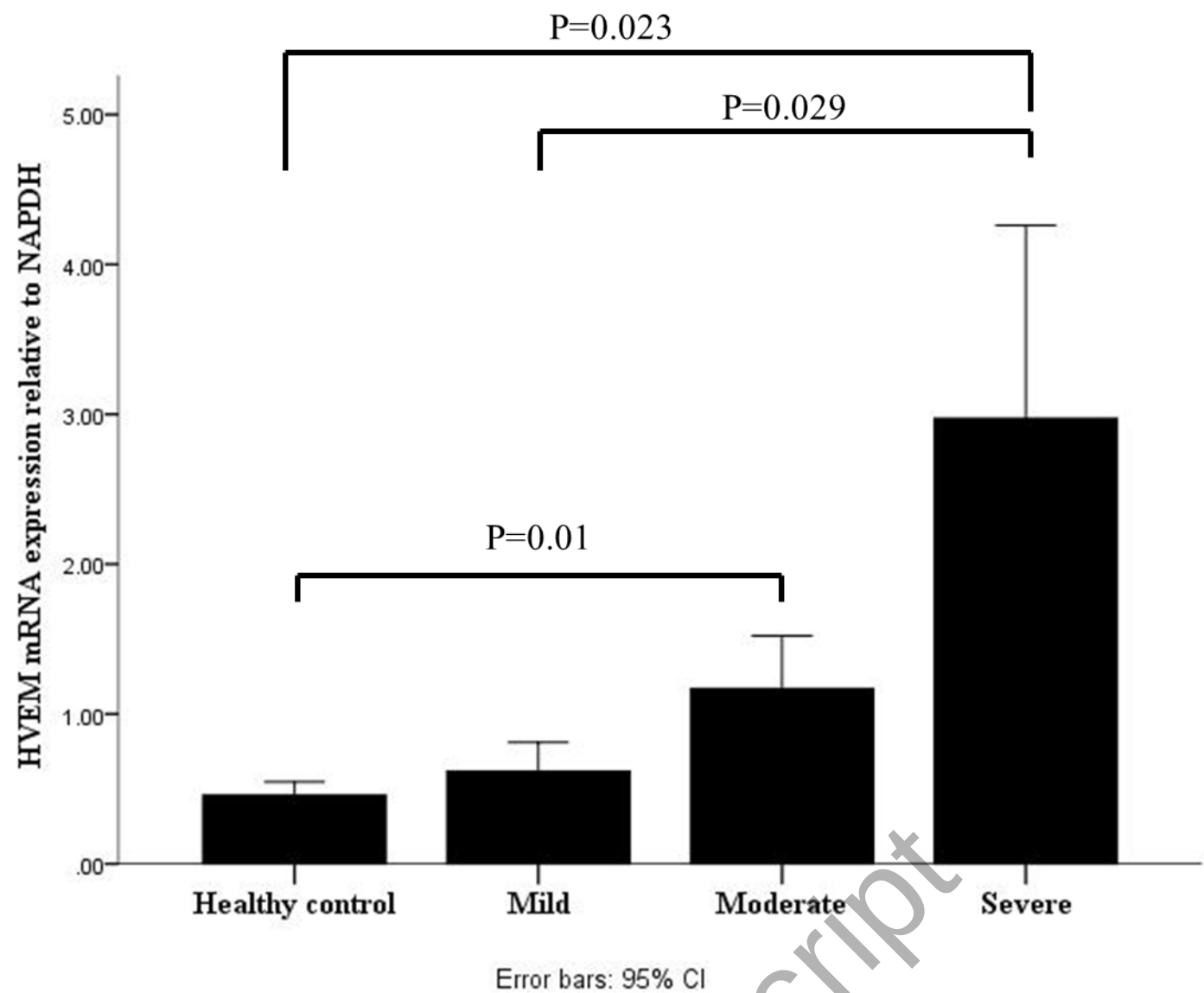
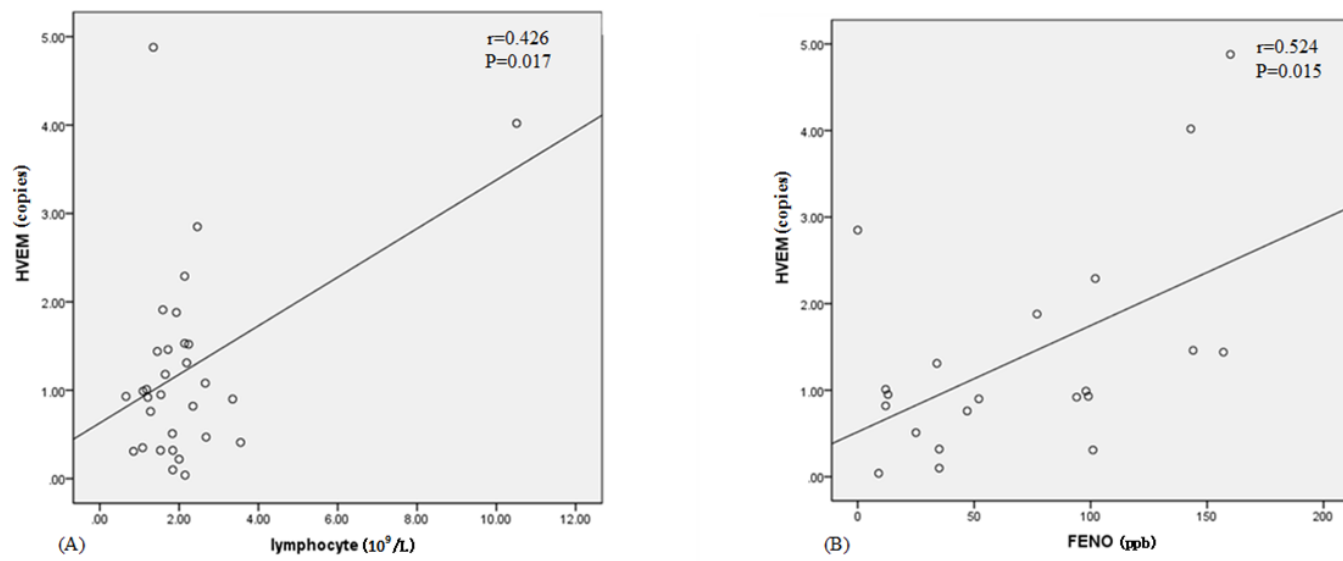


Figure. 1 Comparison of HVEM mRNA expressions among healthy subjects, patients with mild, moderate, and severe persistent asthma (0.46 ± 0.18 vs. 0.62 ± 0.38 vs. 1.17 ± 0.42 vs. 2.97 ± 1.23 / NADPH, $P < 0.001$). Data was shown as mean $\pm$ standard deviation. Healthy control, healthy subjects without asthma; Mild, patients with mild persistent asthma; Moderate, patients with moderate persistent asthma; Severe, patients with severe persistent asthma.



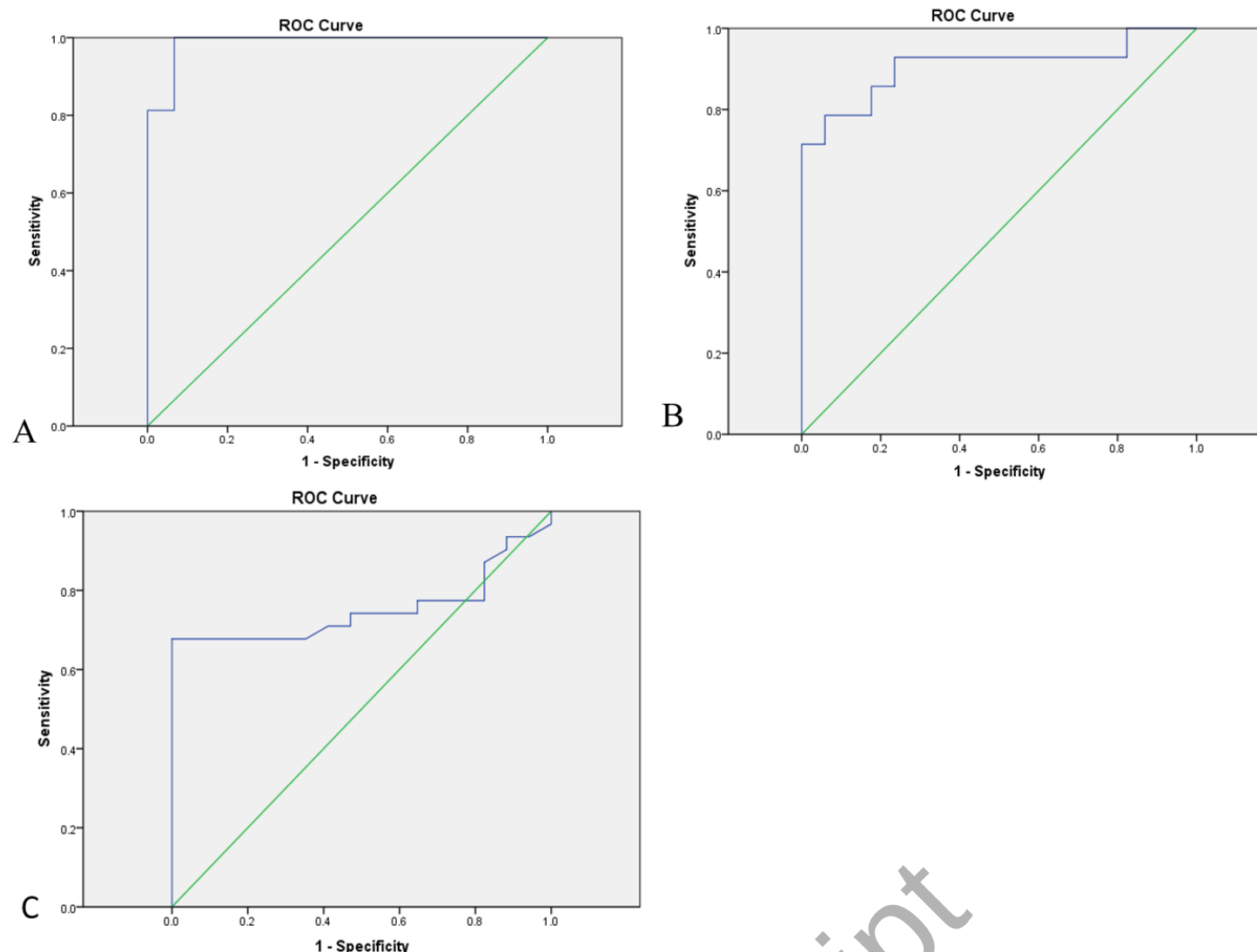


Figure.3 Receiver operating characteristic (ROC) curve analysis of HVEM mRNA expression. **(A)** ROC curve of HVEM mRNA expression at cut off point (1.01 / NADPH) for discrimination of severe from mild-to-moderate asthmatic patients, Area under the curve (AUC) = 0.99, Confidence interval (CI) = 0.96-1.00. **(B)** ROC curve of HVEM mRNA expression at cut off point (0.93 / NADPH) for discrimination of moderate-to-severe from mild asthmatic patients, AUC=0.91, CI=0.79-1.00. **(C)** ROC curve of HVEM mRNA expression at cut off point (0.76 / NADPH) for discrimination of patients from healthy subjects, AUC = 0.75, CI =0.61–0.89.

Table 1. Clinical characteristics of the study groups.

	Mild asthma group (n=16)	Moderate asthma group (n=11)	Severe asthma group (n=13)	Control Group (n=19)	P value
Age (year), mean (SD)	44.06 (10.20)	49.88 (11.64)	50.17 (16.08)	45.59 (2.97)	0.390
Sex, male, n (%)	8 (50.0)	6 (54.5)	5 (38.5)	6 (31.6)	0.550
BMI (kg/m ²), mean (SD)	24.45 (2.24)	25.84 (2.16)	23.78(3.12)	24.40 (2.17)	0.766
History of atopy, n (%)	5 (31.3)	4 (36.4)	7 (53.8)	7 (36.8)	0.840
ACT, mean (SD)	15.85 (3.65)	13.80 (6.50)	18.00 (1.63)	NA	0.350
Total neutrophil count, (/uL), mean (SD)	5.73 (2.52)	5.94 (3.65)	5.45 (2.99)	ND	0.629
Total eosinophil count, (/uL), mean (SD)	0.29 (0.25)	0.22 (0.37)	0.40 (0.44)	ND	0.601
Total lymphocyte count (/uL), mean (SD)	2.02 (0.73)	1.48 (0.57)	3.33 (3.53)	ND	0.114
Neutrophil ratio, (%), mean (SD)	59.58 (15.75)	68.46 (16.25)	56.85 (14.64)	ND	0.323
Eosinophil ratio, (%), mean (SD)	4.44 (3.80)	1.30 (2.13)	3.95 (4.22)	ND	0.132
Lymphocyte ratio, (%), mean (SD)	28.22 (10.61)	20.68 (9.87)	29.55(10.47)	ND	0.194
Total IgE, (IU), mean (SD)	229.69 (270.37)	314.24 (388.73)	200.82 (94.85)	ND	0.882
FeNO, (ppb), mean (SD)	33.45 (7.51)	90.00(32.74)	106.00(22.19)	ND	0.000*
FEV1 of predicted, (%), mean (SD)	86.39 (3.11)	71.46 (10.45)	49.28 (10.46)	ND	0.000*
FEV1/FVC, (%), mean (SD)	73.71 (1.95)	60.03 (7.80)	51.52 (9.58)	ND	0.000*
MEF25, (L/s), mean (SD)	41.33 (22.38)	25.58(8.75)	28.53(9.97)	ND	0.237
MEF75, (L/s), mean (SD)	78.31 (8.38)	69.69(16.35)	31.60 (12.15)	ND	0.000*

HVEM mRNA, (/NADPH), mean (SD)	0.62 (0.38)	1.17 (0.42)	2.97(1.23)	0.46 (0.18)	0.000*
Treatment					
Budesonide, dose, (mg/day)	3.0	6.0	8.0	NA	0.000*
Methylprednisolone, dose, (mg/day), median (IQR)	NA	NA	40 (40-80)	NA	NA
Long-acting β -agonist, n (%)	0 (0)	6 (54.5)	8 (61.5)	NA	0.001*
Leukotriene receptor antagonist, n (%)	13 (81.3)	11 (100)	13 (100)	NA	0.088

* Indicates a significant value, $P < 0.05$.

Abbreviations: SD, standard deviations; BMI, body mass index; ACT, asthma control test; NA, not applicable; FeNO, fractional exhaled nitric oxide; ppb, part per billion; ND, not done; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MEF25, maximal expiratory flow at 25% of FVC; MEF75, maximal expiratory flow at 75% of FVC; IgE, immunoglobulin E; HVEM, herpesvirus entry mediator; IQR, interquartile range.

Table 2. Correlation between HVEM mRNA expression and other clinical indicators in the patients with persistent asthma.

clinical indicators	R value	P value
Total neutrophil count	0.131	0.483
Total eosinophil count	0.248	0.179
Total lymphocyte count	0.426	0.017*
Neutrophil ratio	-0.111	0.551
Eosinophil ratio	0.009	0.963
Lymphocyte ratio	0.137	0.461
Total IgE	0.058	0.838
FEV1% predicted	-0.195	0.424
FEV1/FVC	-0.275	0.254
MEF75	-0.227	0.350
MEF25	-0.274	0.256
FeNO	0.524	0.015*
ACT	0.124	0.582

* Indicates a significant value, $P < 0.05$.

Abbreviations: FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MEF25, maximal expiratory flow at 25% of FVC; MEF75, maximal expiratory flow at 75% of FVC; IgE, immunoglobulin E.

Table 3. Validity of HVEM mRNA expression for discrimination of asthmatic patients from healthy subjects, moderate-to-severe patients from mild patients, and severe patients from mild-to-moderate ones.

Discrimination ability of HVEM mRNA	AUC	95% CI	Cut-off	Sensitivity	Specificity	P-value
Discriminating asthmatic patients from healthy subjects	0.752	0.61~0.89	0.76	67.7%	100%	0.004*
Discriminating moderate-to-severe patients from mild patients	0.91	0.79~1.00	0.93	92.9%	76.5%	0.000*
Discriminating severe patients from mild-to-moderate patients	0.99	0.96~1.00	1.01	81.3%	100%	0.000*

* Indicates a significant value, $P < 0.05$.

Abbreviations: HVEM, herpesvirus entry mediator; AUC, area under the curve; CI, Confidence interval.