Expression of Basophil Activation Markers in Pediatric Asthma

Masooma Abdullahi[1,2], Reza Ranjbaran[1], Soheaila Alyasin[3], Zeinab Keshavarz[1], Amin Ramezani[4], Abbas Behzad-Behbahani[1], Sedigheh Sharifzadeh[1]*

[1]Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, [2]Student Research Committee, [3]Department of Clinical Allergy and Immunology, [4]School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Asthma is very common in children and its diagnosis is based on clinical manifestations, which can be misdiagnosed as other respiratory diseases with similar signs and symptoms. **Objective:** To analyze the expression of ST2L and CD203c in the diagnosis of pediatric asthma. **Methods:** Basophils were purified from whole blood samples of patients and healthy controls using Ficol-Paque gradient and Basophil Isolation Kit. RNA extraction was done by RNX-Plus solution and after synthesis of cDNA, the gene expression was analyzed by means of real time PCR. **Results:** Patients expressed significantly higher levels of CD203c than healthy controls ($p=0.01$). Although there was an increase in the transcription level of ST2L gene in patients, the results were not statistically significant compared to those obtained from the healthy controls ($p>0.05$). A Specificity of 60% and a sensitivity of 73% were foundusing ROC curve for CD203c expression. Patients with positive family history of asthma exhibited more CD203c and ST2L expression ($p<0.05$). **Conclusion:** It is proposed that determining CD203c expression by real time PCR may be an effective technique for diagnosis of pediatric asthma.

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Keywords: Asthma, Basophil, CD203c, ST2L

*Corresponding author: Dr. Sedigheh Sharifzadeh, Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran, Tel: (+) 98 713 2289113, e-mail: sharifsd@sums.ac.ir

INTRODUCTION

Asthma is the most confounding and the most common disease among chronic respiratory diseases (1). Asthma is characterized by intermittent airway narrowing, air flow obstruction and inflammation, leading to common symptoms including cough, wheeze and shortness of breath (2,3). Health, social and economic disadvantages of this disease is immense, so that world health organization has considered it as 'social health issue'(4). More than 300 million individuals are suffering from asthma, a figure which is expected to rise to 400 million by 2020 (5). Asthma and Allergy Association of Iran has estimated asthma prevalence at 5.9% with about 6.5 million individuals suffering from this disease in Iran (6).

Asthma is the most common chronic disease in children (7). In fact, it is a group of diseases caused by an interplay of environment and genetic factors (8). Common symptoms include airway obstruction, smooth muscle contraction, bronchial wall edema andmucus plugging (9). Asthma in children is most often associated with allergies (atopic asthma) and is one of the most important causes of child mortality (10).

Early diagnosis of asthma among children can facilitate clinical control and result in reduction in morbidity. Delayed diagnosis causes lung dysfunction while proper treatment at the right time can inhibit it (11). On the other hand, incorrect diagnosis of asthma results in incorrect treatment and receiving unnecessary medications (12). Diagnosis is often missed due to similar symptoms of other diseases like Aspirin overdose, human rhinovirus, COPD, cystic fibrosis and emphysema (13-15). It is also important to have a correct and early diagnosis because child's energy and health can be affected by misdiagnosis and mistreatment (11).

Despite the importance of this disease, there is no definite histologic, immunologic or physiologic test for pediatric asthma, the diagnosis is often based on clinical presentation and/or response to treatment model (16). Detection of diagnostic biomarkers in the blood of infants and preschool children has been a challenge for scientists in this field.

Basophils are a small population of granulocytes, making up less than 1% of all leukocytes in the peripheral blood. The role of basophils in allergies and anaphylactic reactions and in asthma due to allergic reactions has been known. These cells play their role through the expression of receptors with high affinity for IgE on the cell surface and alsodegranulation and the release of chemical mediators, chemokine and different cytokines (17).

ST2 is amember of IL-1R family (19) which is important in inflammatory and immunological challenges (18). There are three different transcripts of ST2 gene by alternative splicing. One encodes a soluble protein (sST2), and the other encodes a membrane-bound protein (ST2L) and a less-known variant called ST2V (20). Increased sST2 in inflammatory diseases like asthma has been associated with disease severity (21). ST2 attached to the membrane (ST2L) plays an important role in immune responses such as airway inflammation in asthma.

Another relatively specific and unique activation marker for basophils is ectonucleotide pyrophosphatase/phosphodiesterase 3 ectoenzyme (3-ENPP) which is called CD203c (22). There is some evidence showing that when basophils are activated in response to allergens, the expression of markers such as ST2L and CD203c on their surface increases. Therefore, in this study our aim was to determine mRNA levels of these markers in order to evaluate their changes in children with asthma compared to healthy controls.

MATERIALS AND METHODS

Patient Population and Sampling. The samples were collected from 2 groups of cases and controls. The case group consisted of 30 children less than 5 years of age who were referred to Imam Reza Clinic, Shiraz, Iran and were clinically diagnosed as being asthmatic. Recurrent wheezing and cough, history of hospitalization due to asthma, wheezing with a family history of asthma or history of atopic disease in patients were inclusion criteria while children with chronic lung disease such as cystic fibrosis or those with foreign bodies in their airways were excluded. The control group consisted of 15 healthy children less than 5 years of age without asthma.

Sample Preparation, Isolation of Basophil Cells, RNA Extraction and cDNA Synthesis. Whole blood samples (5 ml) were obtained from 30 children with asthma and 15 healthy children and collected in K2-EDTA tube. Blood was poured on the equal volume of Ficoll-Paque (Lymphodex, Germany), which slowly reached room temperature in the RNase-Free tubes. The tube containing ficol and blood samples were centrifuged at 430 ×g for 25 min at 25°C to separate the plasma, ficol layer and red blood cells, and then ficol layer was recentrifuged at 3000 rpm for 15 min. The supernatants were then separated and cells were washed with 1 ml PBS 1X (Inoclon, Iran) and counted.

Basophil cells were purified using Basophil Isolation Kit II human according to the manufacturer's instructions (Miltenyi Biotec, USA). The purity of the basophil preparations was 68% as assessed by Wrigth-stian. RNX-Plus Solution was used for RNA extraction according to the manufacturer's instructions (SinaClon, Iran). Spectrophotometer (OD A260/A280 and A260/230) was applied to determine concentration and purity. Integrity was verified by 2.0% agarose gel electrophoresis and distinguishing 28s and 18s rRNAs. cDNA synthesis of Total RNA (5 µg) was performed using random hexamers and oligo-dT primers (RevertAid H Minus First Strand cDNA Synthesis Kit, Fermentase, Litvanya).

Real-Time PCR. Real-time PCR was done by Rotor-Gene Q (Qiagen, Hilden, USA) using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentase, Litvanya). PCR reaction, including 5µl of cDNA and 300nmol L^{-1} primer concentration in every tube with a final volume of 25 µl and the same reaction mixture for all target genes was performed in the same cycling condition: two-step hold temperature: 50 ° C for 2 min, 95° C for 10 min before 45 cycles of 95° C for 20s, 59° C for 30s, and 72° C for 30s.

The Pfaffl method was employed to determine the level of mRNA through normalizing the target genes to the housekeeping gene GAPDH.

The gene sequences of ST2L, CD203c and GAPDH were obtained from the Gen Bank nucleotide databases (accession number of ST2L: 016232, accession number of CD203c: 005021.3 and accession number of GAPDH: :0012567), and then their primers with amplicons of 170, 148 and 128 bp, respectively, were designed by Allele lD6 primer software (Table 1).

Quality Control. We prepared 10 fold serial dilutions to determine test sensitivity. We used No-template controls (NTC) including sterile H2O in every run to check the possible contamination (Figure 1). GAPDH gene sequence was used to assess the quality of the cDNA synthesis and real-time PCR products were run on 2.0% agarose gel (Cinnagen, Iran) to ensure the amplification of the primers. In each run, the test was duplicated for each sample to increase sensitivity.

Table 1. Primer sequences used for real-time PCR.

Gene	Gene Bank Accession Number	Primer Sequences	Position	Product Size(bp)
ST2L	NM_016232	F:TGGGAGAGATATGCTACCTGGAG R:CCTTGGCGTCGTTCTGGATG	F:1531 R:1700	170
CD203c	NM_005021	F:AGGGAATATGTCAGTGGATTTGG R:GGCTCTCAGAAGGAGGAACC	F:1965 R:2112	148
GAPDH	NM_001256799	F:TTGACCTCAACTACATGGTTTACA R:GCTCCTGGAAGATGGTGATG	F:311 R:436	128

Statistical Analysis. P values were determined by nonparametric Mann-Whitney test using SPSS 16 software.

Ethical Considerations. All methods used in this study were approved by the Ethics Committee of Shiraz University of Medical Sciences (Shiraz, Iran). Informed consent form was filled out by children's parents.

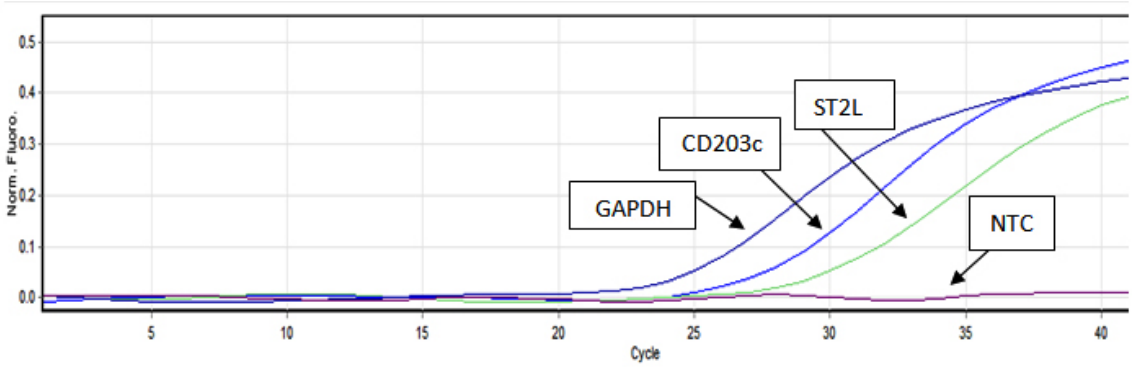

Figure 1. Real-time quantitative PCR. Amplification plots using real-time quantitative PCR for GAPDH, ST2L and CD203c gene for one of the samples. NTC: non-template control.

RESULTS

The test was carried out for the two groups of patients and healthy controls. The patients were under 5 years old with clinical symptoms like recurrent wheezing and cough. The healthy control group was under 5 years of age without clinical allergic asthma. The expression ratio for each gene was calculated based on the pfaff 1 method. Finally, the results obtained from patient and control groups by real time PCR were compared. Efficiency rates were 0.92, 1.1 and 1.1 for GAPDH, ST2L and CD203c, respectively.

The gene expression levels of ST2L and CD203c were determined in patients and healthy controls (Table 2).

Table 2. Mean and SD of Ct values of tested genes for healthy control and patient groups.

		ST2L	CD203c	GAPDH
Healthy Control (N=15)	Mean ± SD	31.662 ± 2	33.2 ± 2.40	24.43 ± 2.42
Patient (N=30)	Mean ± SD	32.33 ± 2.49	34.02 ± 2.45	25.55 ± 2.31

According to our data, there was a significant increase in the expression level of CD203c gene in patients compared to the control group (p=0.01). Although there was an increase in the expression of ST2L gene, the results were not statistically significant (p >0.05) (Figures 2 and 3).

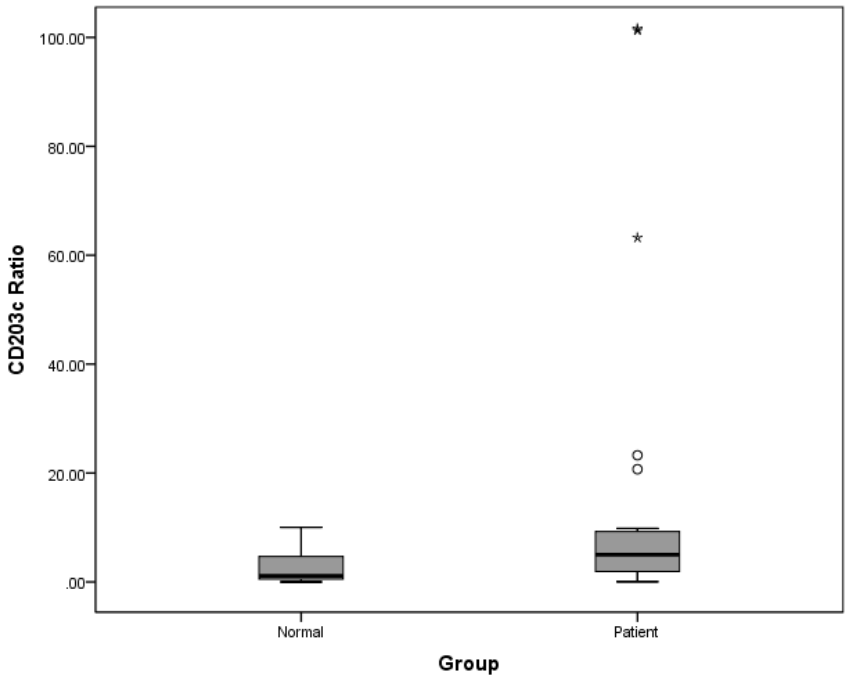

Figure 2. Messenger RNA expression ratio of CD203c in the control and test groups. Increased expression ratio of CD203c in patient group compared with healthy controls was significant (p=0.01).

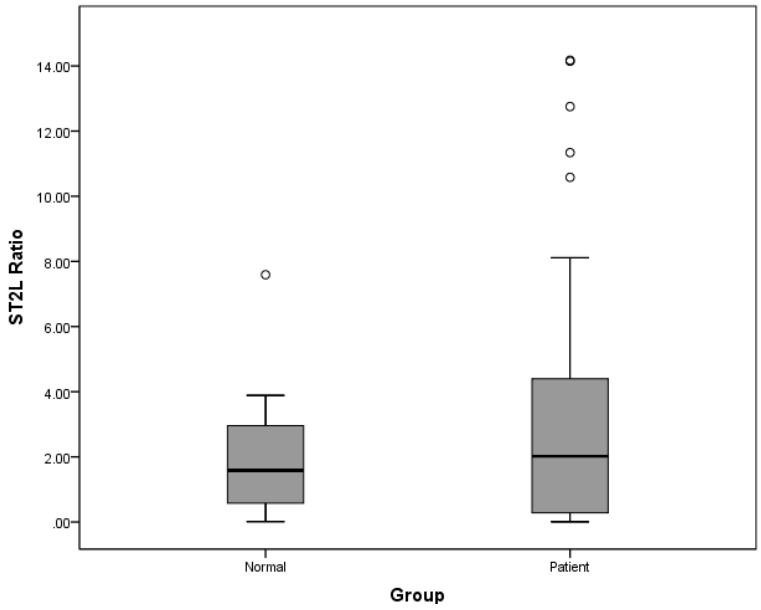

Figure 3. Messenger RNA expression ratio of ST2L in the control and test groups. Increased expression ratio of ST2L in patients compared with healthy control was not significant (P>0.05)**.**

Based on these data, Receiver Operating Characteristic (ROC) curve showed AUC value of 0.72 (95% CI, 0.561-0.879; P=0. 01) (Figure 4).

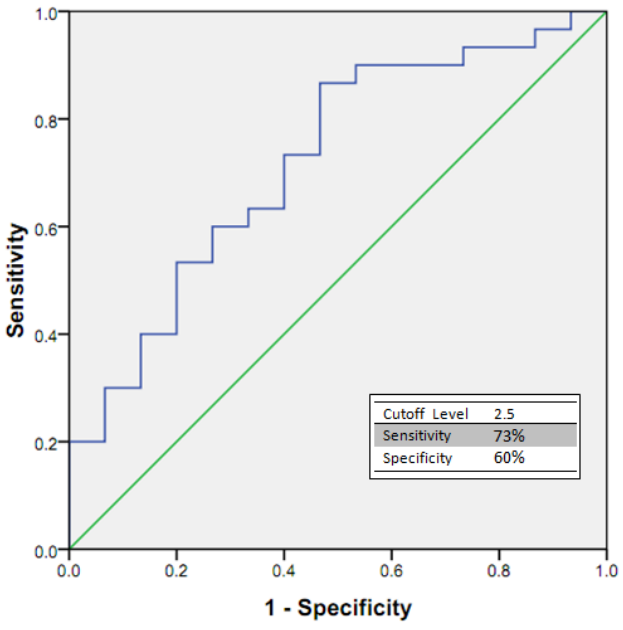

Figure 4. Receiver-operating characteristic (ROC) curve analysis yielded 2.5 CD203c expressing basophils as the optimal cut-off for discriminating asthma from healthy control. The sensitivity and specificity were 73% and 60%, respectively, with an AUC value of 0.72 (95% CI, 0.561-0.879; P=0.01). The sensitivity and specificity were calculated according to the identified optimal cut offs.

There was a significant p value for increased expression of CD203c and ST2L in patients with a positive family history of asthma (p=0. 01 for CD203c, p=0. 048 for ST2L) (Figure 5 and 6).

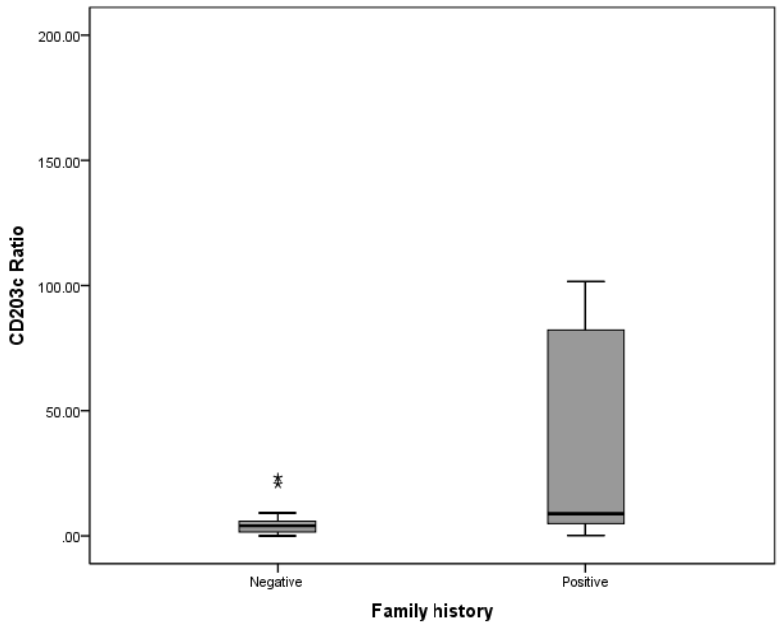

Figure 5. Increased expression ratio of CD203c in patients who had parents or brothers or sisters with asthma than others. There is a significant P value (0.01).

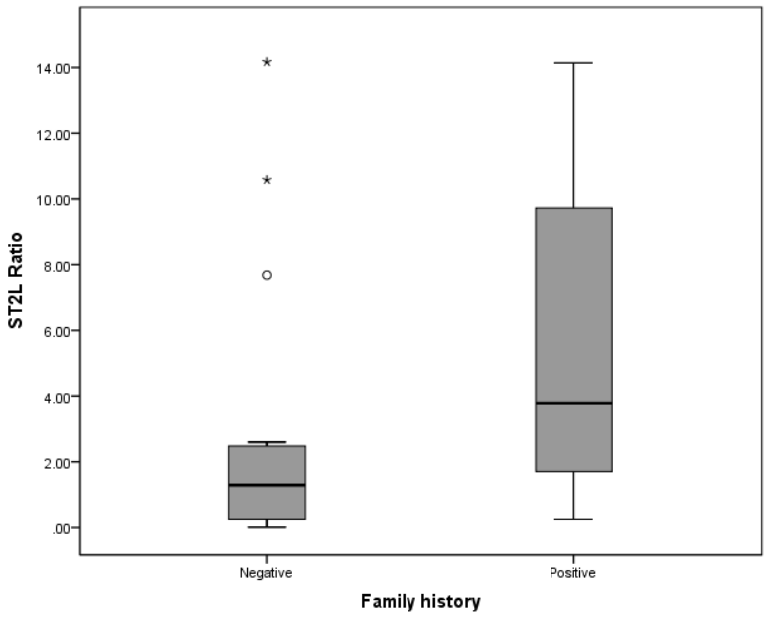

Figure 6. Increased expression ratio of ST2L in patients who had parents, brothers or sisters with asthma than others. There is a significant P value (0.04).

Coefficient of Correlation for the two genes was found to be 0.511, which is statistically significant (p=0.004). Therefore, these two genes have a significant correlation (Table 3).

Table 3. Correlations of ST2L and CD203c genes expressions in patients.

			CD203c Ratio	ST2L Ratio
Spearman's rho	CD203c Ratio	Correlation Coefficient	1.000	0.511**
		Sig. (2-tailed)	.	0.004
		N	30	30
	ST2L Ratio	Correlation Coefficient	0.511**	1.000
		Sig. (2-tailed)	0.004	.
		N	30	30

**Correlation is significant at the 0.01 level (2-tailed)

DISCUSSION

Studies have shown that basophils and their markers like ST2L and CD203c have an important role in asthma (17,23) and that detecting basophil markers, ST2L and CD203c are useful in the diagnosis of asthma. This study was conducted to analyze the expression of basophil activation markers in the diagnosis of pediatric allergic asthma using real time PCR. Based on our results, expression of CD203c is enhanced in children suffering from asthma, which can be detected by real time PCR method in basophils of blood sample. We could not detect an increased expression of ST2L in in basophils of blood samples of children suffering from asthma by this method.

The purity of enriched basophils by Basophil Isolation Kit II human was 68% as assessed by Wrigthstian. Ocmant *et al*. also isolated 60% basophil by this method (24).

Our findings showed an increased ST2L expression in patients, but the result was not statistically significant. Recent studies showed an increased ST2L expression level using stimulation of basophils by IL-3 or IL-33 *in vitro*. Pecaric-Petkovic, *et al*. showed an increased ST2L expression after stimulating basophils by IL-3 using real time PCR (10). Molly *et al*. showed the same results (25). In another *in vitro* study, it was reported that ST2L expression increased after stimulating epithelial cells by IL-33 (26). It was also reported by Maho Suzukawa *et al.* that ST2L expression increased after stimulating mast cells by IL-33 (27). We did not stimulate cells because we intended to check the mentioned markers in blood samples of patients directly without any intervention.

We found a significant increase in the expression of CD203c ratio in patients. Hauswirth *et al.* showed increased expression of CD203c in mast cells by RT-PCR and flowcytometery (28). Potapinska *et al*. showed this increase in basophils of patients with asthma (29). Another important finding was the significant relationship between increased expression of CD203c and ST2L and having first degree family history of asthma. As London *et al.* showed, parents with asthma increase the risk of asthma in their children (30).

In conclusion, our experiments revealed that increased CD203c expression detected by real time PCR could be used for the diagnosis of asthma through acquiring a cut-off point especially in cases where clinical diagnosis can be misled by other diseases. Invasiveness of other methods like tissue biopsy limit their use in clinical practices. The significant relationship between family history and increased expression of CD203c and ST2L can help better diagnosis. Further studies with larger sample sizes

are recommended to corroborate these results. Therefore, it will be a good idea to perform flowcytometery to confirm CD203c expression simultaneously. Sharifzadeh *et al.* conducted a similar study and showed there was not a significant increase in the expression of CD203c (Unpublished article) by flowcytometry. This difference between our results may be as a result of higher sensitivity of real time PCR in quantitative expression assay. Also, evaluating sST2 in serum can be done for confirming ST2L expression simultaneously. We can do such studies for adults and evaluate the expression of other genes which play a role in asthma.

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