Estimation of Serum YKL-40 by Real-Time Surface Plasmon Resonance Technology in North-Indian Asthma Patients

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Background: Many studies reported for estimating serum YKL-40 using ELISA or RIA methods. This study introduces the plausible utilization of real-time surface plasmon resonance (SPR) technology in investigating the expression of serum YKL-40 protein levels and ELISA method for serum IgE in bronchial asthma. **Methods:** A commercially available BIAcore 2000 instrument, based on SPR technology, was utilized for assessing serum YKL-40 levels in a control sample size of 45 and active sample size of 97. Antibody immobilization was optimized to obtain the best sensor performance and a sensitive analytic detection. A commercially available ELISA kit was utilized for detecting

serum IgE to estimate allergic condition-associated asthma. **Results:** The results of SPR technology could distinctly classify with highly statistical significance, the asthma severities by estimating the elevated levels of YKL-40 in blood sera of minute quantities (up to 0.33 ng/ml), and thus differentiates superior utility in comparison with ELISA method. No statistically significant correlation of YKL-40 and IgE was observed. **Conclusions:** Serum YKL-40 may be used as a protein marker in classifying asthma severity by applying SPR technology as a reliable, label-free, highly sensitive, and cost-effective tool. J. Clin. Lab. Anal. 31:e22028, 2017. © 2016 Wiley Periodicals, Inc.

Key words: Asthma severity; Enzyme linked immunosorbent assay; FEV1; Surface Plasmon Resonance technology; YKL-40

ABBREVIATIONS

BIAcore = Biomolecule interaction analyzer core

FEV1 = Forced expiratory volume in 1 second

HCgp39 = Human cartilage glycoprotein 39

SPR = Surface plasmon resonance

INTRODUCTION

Chitinases are the members of 18 glycosyl hydrolase family and are widely known for their role as hydrolytic enzymes capable of cleaving environmentally abundant chitin polymer. Earlier studies demonstrated their roles in diseased conditions associated with inflammation in both human epithelial cells (1), and in mouse models of asthma (2, 3). It has been established that both human and bacterial chitinases evoke airway inflammation by type 2 helper T cells and protease-activated receptor 2-dependent mechanisms in epithelial cells (1, 2).

Chitinase-like protein “YKL-40” is also known as a human cartilage glycoprotein 39 (HCgp39). It shares structural homology with chitinase protein family and binds chitin, but unlike chitinases, it is deficient in chitinase activity (4–8). This lack of chitinolytic activity is attributed to an amino acid substitution at the active site (9). The exact function of YKL-40 remains unclear. However, it has been reported that YKL-40 plays a role in preventing the death of inflammatory cells (including eosinophils) and promotes the alternative activation of macrophages resulting in increased inflammation (10). Proinflammatory cytokines such as TNF- α , IL-1 (11), and IL-13 (12) are known to induce YKL-40 production.

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The role of YKL-40 in bronchial remodeling has been recently evoked because its concentration has been found to be increased in the serum of patients with asthma and correlated with subepithelial membrane thickness. In a previous study, circulating YKL-40 levels were observed to be correlated positively with asthma severity and inversely with lung function, suggesting a role of circulating YKL-40 as a potential biomarker for asthma (12). In another study, YKL-40 concentrations were found to be higher at the site of allergen deposition during allergen challenge (13). A recent study on 62 Chinese patients established that at the time of exacerbation attacks, serum YKL-40 level increases as compared to stable asthma period and healthy persons (14). The elevation level of serum YKL-40 in mild and moderate-to-severe asthma is still needed to be investigated among the subjects of different ethnic groups. Till date, no study has been conducted in this context on Indian population.

Surface Plasmon Resonance (SPR) technology involves an optical phenomenon which enables the detection and quantification of protein–protein interactions in real time, without the use of labels. This technique is widely used for characterizing the interactions of proteins with other proteins, peptides, nucleic acids, lipids, and small molecules (15). SPR technique involves passing a fraction of the light energy onto a thin metal film of the instrument at a certain angle resulting in delocalization of electrons in the metal film (plasmon) and hence the reflected light intensity gets reduced upon interaction, thereby changing the local refractive index according to the sample characteristics. The changes in the intensity pattern of the reflected light are detected in a sensorgram and, hence, a quantitative information on the specificity of binding and active concentration of molecules in a sample can be obtained in real time.

This study quantified serum YKL-40 levels by SPR technique in asthmatic individuals with respect to the reference group levels. Furthermore, the relationship among the serum YKL-40 levels, patient's age, gender, allergy, disease duration, total serum IgE, lung function, and length of disease exacerbation was also investigated in this study.

PATIENTS AND METHODS

The step-by-step methodology followed during the study has been depicted in the flow diagram as shown in Fig. 1 and has been detailed as in the following subsections.

Location of the Study and Study Subjects

This case-controlled study was conducted on 142 adult inhabitants of North India over a period from

December 2009 to January 2012. The active group comprised of 97 patients who were diagnosed to have bronchial asthma based on GINA guidelines (16) and were attending the Outpatient Respiratory Clinic or Emergency Department of All India Institute of Medical Sciences (AIIMS), New Delhi, India. The reference group comprised of 45 healthy individuals without any episode of asthma symptom. This study was approved by Institute's Ethical Committee (RT-24/30.10.2009 dated 04.11.2009) and a written informed consent was obtained from every subject before enrolling them in the study.

Subject Inclusion

In active group, patients with mild-to-severe asthma were included. The visits of patients were either scheduled or more often unscheduled prompted by acute asthma attack characterized by a decreased FEV1 and presence of various symptoms like cough, wheeze, chest tightness, and dyspnea. The active group subjects were subdivided into three groups as follows: active group 1 comprised of mild persistent asthma patients ($n = 25$), active group 2 of subjects with moderate asthma ($n = 32$), and active group 3 consisted of severe asthma patients ($n = 40$). These classifications were based upon the pulmonary function test results and as per the GINA Guidelines (GINA, 2006). The selection criteria included subjects with diagnosed asthma suffering for a period of ≥ 6 months, with or without family history of disease, and reference group involved healthy individuals without any clinical history of asthma. Exclusion criteria for both the study groups were the following: concomitant chronic respiratory diseases [chronic obstructive pulmonary disorder (COPD), interstitial lung disease, etc.], cancer, osteoarthritis, rheumatoid arthritis, schizophrenia, diabetes, inflammatory bowel disease, and any other inflammatory conditions which could affect serum YKL-40 levels.

Collection of Blood Samples and Serum Separation

After clinical assessment and spirometry of active group subjects, blood samples from the exacerbation group (moderate or severe asthma requiring urgent medical attention) were collected prior to administration of treatment and within 2 h from the stable group (mild asthma) and serum were isolated. Blood samples were collected from healthy individuals after taking written informed consent. A quantity of 3 ml of venous blood was collected from every participant in a microcentrifuge tube (Axygen, Inc., Union City, CA, USA). Samples were placed standing for 30 min to

Fig. 1. Flow diagram of methodology adopted.

allow clot formation and then centrifuged at 3500 rpm for 10 min. The obtained serum was aliquoted in three different tubes and stored at -20°C till use.

Biochemical Analysis

This had been carried out following the steps as detailed below.

Binding Studies Using Surface Plasmon Resonance (SPR)

The experiment with SPR measurements was carried out at 25°C using a biosensor-based system for real-time-specific interaction analysis (BIAcore 2000 system; Pharmacia Biosensor AB, Uppsala, Sweden).

Anti-YKL-40 goat antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) was immobilized at a flow rate of $10\ \mu\text{l}/\text{min}$ onto a CM5 sensor chip (dextran matrix covered with carboxy-methyl group in a thin gold layer for covalent protein immobilization) (Pharmacia Biosensor AB, Uppsala, Sweden) using the amine coupling method. The amine coupling kit containing N-hydroxysuccinimide (NHS), ethanolamine hydrochloride and N-ethyl-N'-3 (diethylaminopropyl) carbodiimide (EDC) (Pharmacia Biosensor AB), and surfactant P20 were utilized for antibody immobilization. The HBS-EP buffer of pH 7.4 (10 mM HEPES, 150 mM Sodium Chloride, 3 mM EDTA, and 0.005% Surfactant P20) was used as a running buffer. The dextran on the chip was equilibrated with running buffer and the matrix of the chip was activated with 0.4 M

EDC/0.1M NHS mixture (1:1) at a flow rate of 10 μ l/min. After activation of the chip, 23 μ l of anti-YKL-40 antibody in 10 mM sodium acetate (pH 4.2) was injected and all the unreacted groups were blocked by injecting 1 M ethanolamine hydrochloride (pH 8.5). The antibody-immobilization profile is presented in Fig. 2(a). Eight different known concentrations of

commercially available YKL-40 pure protein (R&D Systems, Inc., Minneapolis, MN, USA), viz., 0.33, 0.57, 2.00, 3.30, 4.60, 6.60, 10.00, and 13.30 ng/ml, were diluted in the ratio of 1:69 using EP buffer out of which 20 μ l was passed over the immobilized antibody at a flow rate of 10 μ l/min. The binding of an analyte to the antibody resulted in an SPR signal [expressed in

Fig. 2. (a) Immobilization profile of anti YKL-40 antibody on CM5 sensor chip. RU, resonance unit; EDC, N-ethyl-N'-(3-dimethyl amino-propyl) carbodiimide; NHS, N-hydroxysuccinimide. (b) SPR response to the binding of serum YKL-40 on immobilized antibody

response unit (RU)] which was captured in real time. Figure 2(b) represents a binding characteristics of serum YKL-40 to immobilized antibody. As the concentration of serum protein was found to be increased, a subsequent increment was observed in RU resulting in shifting the binding curve toward higher value of RU. The relation between the known concentrations of pure protein and the RU values was found to be linear and the standard linear graph thus obtained was used for determining the concentration of the unknown ones. The lower limit of detection was 0.33 ng/ml below which no binding was observed with YKL-40 pure protein. The result in this respect is presented in Fig. 3. A quantity of 20 μ l of each diluted serum sample (1:69) was then passed over the immobilized YKL-40 antibody at a flow rate of 10 μ l/min. This procedure was followed for all the active and reference group samples and the respective RU values were recorded in real time. After each binding step, regeneration was performed with 10 μ l of 1 mM NaOH. The unknown concentration of YKL-40 in the serum samples was calculated from the standard plot.

Enzyme Linked Immunosorbent Assay (ELISA)

A sample size of 40 active group and 20 healthy controls were randomly chosen for detecting the serum IgE levels using ELISA method. The active group was constituted of mild ($n = 11$), moderate ($n = 10$), and severe ($n = 19$) asthma categories (GINA, 2006). Total serum IgE was detected using goat anti-IgE antibody (DRG International Inc., Springfield, NJ, USA). A 96 well-plate IgE kit (procured from DRG International Inc.) was utilized for IgE estimation. All the reagents

and samples were brought at room temperature and a dilution of 1:100 was done for each sample. Six different concentrations of IgE standard protein (provided in the kit), viz., 800, 400, 100, 50, 10, and 0.0 IU/ml, were used as a standard. Standard and samples were added in duplication (20 ml each) in appropriate wells. Afterward, zero buffer (100 μ l) was added to each well and left for incubation for 30 min at room temperature. After incubation washing (5 times) was done with distilled water and 150 ml of enzyme conjugate reagent was added to each well. Incubation and washing were repeated and then TMB Substrate (100 ml) was added followed by incubation (20 min) at room temperature. A 100 ml of stop solution was then added and immediately Optical density (OD) was recorded at 450 nm (as specified by the manufacturer).

Statistical Analysis

The data obtained were statistically analyzed using STATA 11.1 software (STATA Corporation, college station, TX, USA). Results are presented as median (range) and categorical variables as proportions, n (n %). A comparison between groups for serum YKL-40 and IgE levels was done by Kruskal–Wallis H test and then multiple comparisons were done by Wilcoxon rank-sum (Mann–Whitney) test with bonferroni correction. The relationship between serum YKL-40 and different variables such as age, gender, allergy, disease duration, length of exacerbation, FEV1, and IgE in active group subjects was assessed by non-parametric Mann–Whitney U -test and Spearman's rank correlation analysis. A P -value ≤ 0.05 was considered as statistically significant.

Fig. 3. Standard calibration curve of YKL-40 pure protein. Each point represents the mean value of triplicate measurements.

RESULTS

Table 1 presents the demographic data of both the groups. The median concentration of YKL-40 protein in the serum of active group was higher than that of reference group. When individual group was compared with reference group, only moderate and severe asthma groups showed significantly increased levels of YKL-40, whereas in mild asthma group, the levels of YKL-40 were not significantly different from that of reference group. We observed a significant difference in serum YKL-40 levels on inter-group comparisons between moderate and severe as well as mild and severe asthma groups. The difference in YKL-40 levels in mild and moderate groups ($P = 0.020$) was not found to be statistically significant after bonferroni correction. The elevation level of YKL-40 protein in active group subjects with respect to that of reference group was calculated using equation 1 which is stated as follows:

$$\% \text{ elevation of serum YKL} - 40 = \frac{\text{Active group level} - \text{Reference group level}}{\text{Reference group level}} \times 100 \quad (1)$$

In equation 1, “active group level” stands for the median value of serum YKL-40 level in the individual category of asthma patients, such as mild, moderate,

and severe. “Reference group level” stands for the median value of serum YKL-40 level of control group subjects. A positive sign indicates elevation in protein levels in the active group and vice versa.

Our results indicated a statistically significant ($P < 0.05$) difference between moderate and severe groups as compared to the median value of reference individuals. The elevations in YKL-40 protein levels in moderate and severe categories of asthma compared with the reference group were found to be distinct and are stated as follows: moderate (44.79%) and severe (98.84%), respectively. Mild group showed statistically insignificant increase ($P = 0.90$) in serum YKL-40 levels with a value of 5.01% with respect to that of reference group. The estimated YKL-40 levels and P -values for all the group are presented in Table 2 and Fig. 4.

There was a statistically significant difference between reference group and each of the sub-active

groups in serum IgE levels (Fig. 5). When individual active group was compared with reference group, sera IgE levels were found to be statistically significantly

TABLE 1. Clinical Characteristics of Subjects

Variables	Reference group	Active group subjects			
		All	Mild	Moderate	Severe
Sample size	45	97	25	32	40
Age (in years)					
Median (range)	27 (21–43)	38 (18–85)	40 (19–70)	32.5 (18–71)	36.5 (20–85)
Gender: n (%)					
Male		42 (43.3)	12 (48)	16 (50)	13 (32.5)
Female		55 (56.7)	13 (52)	16 (50)	27 (67.5)
Smokers		15 (15.4)			
Never smokers		82 (84.5)			
Allergy					
With		62 (63.9)			
Without		35 (36.0)			
Family history					
Yes		42 (43.3)			
No		55 (56.7)			
Disease duration (years)					
Median (range)		8 (1–35)			
Length of exacerbation (h)					
Median (range)		22 (2–48)			
Sample frequency n (n %) Medications					
β 2 agonist					
Short acting		82 (84.5)			
Long acting		35 (36.0)			
Inhaled steroids		36 (37.1)			
Oral steroids		03 (3.1)			

TABLE 2. Serum YKL-40 Levels in Study Group

Group	Serum YKL-40 level Median (range)	<i>P</i> -values (active group compared to reference one)
Reference	2.6 (1.1–5.5)	
Active		
Mild	2.72 (0.6–7.5)	0.902
Moderate	3.75 (1.8–9.0)	<0.001
Severe	5.15 (2.7–10.5)	<0.001

Fig. 4. Serum YKL-40 levels in asthmatic patients and controls. The levels of YKL-40 were higher in moderate and severe asthma group than those in reference group. Data are presented as median (horizontal line in each box), with 25th and 75th percentiles (top and bottom of each box) and 10th and 90th percentiles (top and bottom of each bar).

Fig. 5. Variation in serum IgE levels in asthma patients and controls.

elevated in moderate and severe subgroups with *P*-values of 0.0004 and <0.001, respectively. The difference in IgE levels between reference group and mild

Fig. 6. Correlation of serum YKL-40 levels with FEV1/%FEV1 predicted. The correlation coefficient is from Spearman's rank-correlation analysis.

group ($P = 0.818$) was not found to be statistically significant. No correlation was observed between YKL-40 and serum IgE levels ($r = -0.124$, $P = 0.447$) when compared between the reference group and active group as whole. A negative association of the serum concentrations of YKL-40 with lung function measured in terms of FEV1 was observed with a correlation value of $r = -0.577$, $P < 0.0001$, and the observed variation in this respect is shown in Fig. 6. No statistical association of YKL-40 levels was observed with age ($P = 0.166$), gender ($P = 0.633$), disease duration ($P = 0.213$), the length of exacerbation ($P = 0.180$), smoking ($P = 0.388$), and allergy ($P = 0.934$) in asthma.

DISCUSSION

It has been reported that human cartilage glycoprotein 39 or YKL-40 acts as a sensor and binds to chitin. As a part of innate immune response against chitin-containing pathogens, YKL-40 attempts to damage the chitin coat of infecting organisms, such as fungus and nematodes (17). YKL-40 is an acute phase protein synthesized by monocytes, macrophages, neutrophils, synoviocytes, epithelial cells, etc. (7, 18), and is expressed in many diseases associated with inflammation (19–21).

In this study we utilized SPR technology for the first time in asthma for serum YKL-40 detection because SPR is a natural phenomenon which delivers high-quality data in real time with a very low sample volume. It is a label-free technique with an advantage of reusability of the sensor surface immobilized with primary antibody unlike other traditional methods such as ELISA, in which the primary antibody can be used only once. Furthermore, SPR technique does not

require any secondary antibody for serum biomolecule detection, unlike ELISA. Also, detection of both low- and high-affinity antibodies is possible with SPR unlike ELISA where low-affinity antibody gets washed away during washing step. Therefore, it is a highly sensitive technique by which molecules in picogram quantity can be detected with appreciable resolution. Hence, SPR (BIAcore 2000 system, Pharmacia Biosensor AB) is a cost-effective technology by which 60–80 samples can be evaluated with one-time immobilized antibody on a sensor chip and the binding response can be obtained in real time unlike ELISA.

Serum IgE levels were found to be elevated in asthma as compared with that of reference group. This is in accordance with the classically recognized fact that IgE-mediated responses contribute to allergy and asthma. No statistically significant correlation was observed between serum IgE and YKL-40 levels when compared between the reference group and active group as a whole. This observation may be due to a small sample size used in this experiment. However, this finding is in contrast with the previous studies (12, 14).

We did not observe any statistically significant dependence of YKL-40 levels on age, gender, disease duration, the length of exacerbation, smoking, allergy, and in asthma. These findings on the non-dependence of serum YKL-40 level on age and gender are in corroboration with the previous studies (22, 23). However, our results are in contradiction to a recently published study on Iranian population where a positive correlation of YKL-40 was observed with age and disease duration (24). It is, therefore, revealed that serum YKL-40 level elevations are independent of the factors: age, gender, disease duration, the length of exacerbation, smoking, and allergy in our study population. Hence, it may be concluded that serum YKL-40 has a potential to be used as a biomolecule in detecting different grades of asthma severity in North-Indian asthma patients.

As the primary outcome measure of this study, significantly increased serum YKL-40 levels were observed in patients with asthma than in reference group. On inter-group comparisons between mild, moderate, and severe asthma conditions, a significant progressive elevation in serum YKL-40 levels was observed with most elevated levels in moderate (44.79%) and severe asthma (98.84%) group. Overlapping in the levels was observed in mild asthma and reference group. Also, a negative correlation was observed between YKL-40 levels and FEV1 which is in accordance with the previous studies (12, 14, 25). This may be due to the increased expression of YKL-40 by bronchial smooth muscle cells,

neutrophils, and macrophages at the site of inflammation which results in excessive mucous secretion and congestion in airways resulting in an obstruction to the airflow (7, 18). These observations support the hypothesis that YKL-40 level elevates with asthma exacerbation (12, 14).

Exploring the expression pattern of YKL-40 in our study subjects using SPR technology, we found distinct difference in the elevated levels as compared with the reference ones in serum concentration of YKL-40 asthma group. It is to be mentioned that this finding resembles with that among the European and Chinese populations reported recently by ELISA method (12, 14, 25). The main contribution using SPR technology in our study, in contradistinction to the results of the reported ELISA method, is that each discrete elevated level of YKL-40 could classify asthma severity (moderate and severe) with high statistical significance even in the presence of the biomolecule in minute quantity (~0.33 ng/ml).

In our previous pilot study, we observed a significant decrease in serum YKL-40 levels in post-treatment asthma subjects as compared to untreated ones. Our findings strongly suggest a need for investigating post-treatment serum YKL-40 levels in larger sample size for its probable role in asthma prognosis (26).

CONCLUSION

Our study showed that serum YKL-40 levels were significantly elevated in patients with moderate and severe asthma compared with reference group. Although there was a positive correlation with disease severity, the study did not suggest serum YKL-40 to be a diagnostic marker of mild asthma as the difference in levels of serum YKL-40 between reference and mild asthma group was not found to be statistically significant (P -value >0.05). However, the serum level of YKL-40 may be used as a protein marker to determine asthma severities in moderate and severe cases. And SPR technology may be recommended as a suitable and sensitive method for detecting serum biomolecules present in minute traces (0.33 ng/ml). To establish YKL-40 as a prognostic marker in asthma, prospective studies on larger sample size with pre- and post-treatment YKL-40 values are warranted.

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AUTHORS' CONTRIBUTIONS

SN proposed to carry out this work, framed the background of the study, performed the experiment, was actively engaged in sample collection, data collection, analysis, interpretation, and drafting manuscript; PA contributed greatly in screening the study subjects, diagnosis, and guided clinical data interpretation; SD was involved in data acquisition through SPR technology (BIAcore 2000 machine); and KD designed the study protocol, organized experimental works, was responsible for data interpretation and comparing the outcome measures, editing the manuscript, guiding the revised manuscript preparation, and final approval of manuscript.

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