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Sequencing of exon 14 region and exon 15 of *SPINK5* gene for identification of SNPs in Iraqi asthmatic patients

Forqan Jabbar Taher and Asmaa Mohammed Saud*

Dep. of Biotechnology, College of Science, University of Baghdad, Iraq.
*Corresponding author: asmagenetic2015@gmail.com

Abstract

The serine peptidase inhibitor kazal type 5 (SPINK5) gene is located on chromosome 5q31-32 and it has been implicated in the biological function of asthma. The aim of this study was to correlate between *SPINK5* and asthma. This study was carried out on 140 study samples (100 asthmatic patients and 40 non-asthmatic controls) with the age range of 7-50 of both sexes (71males and 69females). These samples were collected from the Asthma and allergy center, Kadhimiya teaching hospital, the central child hospital in Baghdad, as well as asthma and allergy center in Wasit province, asthma and allergy Center and Marjan Teaching Hospital in Babil province, asthma and allergy center in Misan province during the period of November 2015 to March 2016.Genomic DNA was extracted from blood samples followed by PCR amplification of exon 14 region and exon 15*SPINK5*for subsequent DNA sequencing using Sanger method. Samples of patients with similarities in asthmatic phenotypes were sent for direct sequencing by Macrogen Company, South Korea, along with control samples. Bio Edit results revealed that region contained 11 SNPs along exon 14 and exon 15. All of SNPs are reported for the first time in this study. SNPs found only in asthmatic patients are likely associated with asthma could alter expression of the gene thus contributing to the pathogenicity of asthma.

Keyword: Asthma, SPINK5, SNP.

Introduction

Asthma is a chronic inflammatory disease, which involves a combination of genetic and environmental interactions. There are increasing evidence that contact with antigens and viral infections early in life lowers the risk of developing allergic diseases later in life (Von Mutius*et al*., 1994; Illi*et al*., 2001). Asthma is a wide separated and complicated condition, with considerable heterogeneity both in its phenoty and in the underlying pathophysiology that is characterized by abnormal and inflamed mucosa of the airways, wheezing, and shortness of breath. In some patients, irreversible airway remodeling and intractable airflow limitation may develop. Asthma usually include increasing response of bronchial to multiple of stimuli (Smith *et al*., 1997), also different inflammation cell like the eosinophil will increased In number through the airways, airway destruction, reatraction high secretion of the mucosa through lung wall (Jenna *et al*., 2010).

The disease has a high prevalence and a chronic relapsing course. Although some effective therapies exist for mild asthma, severe asthma remains difficult to treat. The societal cost of the disease is substantial (Smith *et al*., 1997).

Asthma runs strongly in families, and its heritability has been estimated as 60 %(Duffy *et al*., 1990). Genetic studies offer a structured means of understanding the causes of asthma as well as identifying targets that can be used to treat the syndrome (Miriam *et al*., 2010). Asthma is a common and clinically heterogeneous disorder and poses huge costs to society (Barnett and Nurmagambetov, 2011). The risk for asthma is determined in infancy and childhood, it is highly heritable, and the phenotypes are conferred by both genetic susceptibility and environmental exposures (Gary, 2008; Wenzel *et al*., 2012).Asthma heredity doesnot subject to mendelian inheritance modality,since a lot of studies

revelead that asthma has aspecial pattern of substantial familial aggregation .

The prevalence of asthma has rapidly increased over the last few decades to epidemic proportions and there are approximately 300 million people worldwide (Masoli *et al*., 2004). Thus, host genetic susceptibility may play a crucial role in the pathogenesis of asthma.SPINK5 is located in chromosome 5q31-32, Chromosome5q31-33 is one of the main regions implicated in susceptibility to asthma and asthma-related traits (Ober *et al*., 2000; Ryu *et al*., 2006). This locus harbors multiple genes that may influence atopic responses, inflammation and drug responses (Wu *et al*., 2010).The aim of this study was to correlate between *SPINK5* and asthma.

Materials and Methods

One hundred patients (55 males and 45 females) with age range of (7-50) years and diagnosed with asthma were selected for this study. All patients received a comprehensive description of the study, and gave written informed consent for their participation. Control group included 40 non-asthmatic controls. All the study samples were collected from Asthma and allergy center, Kadhimiya teaching hospital, the central child hospital in Baghdad, as well as Asthma and allergy center in Wasit province, Asthma and allergy Center and Marjan Teaching Hospital in Babil province, Asthma and allergy center in Misan province during the period of November 2015 to March 2016.

Five milliliters of blood were collected by vein puncture in EDTA anticoagulant tubes from all patients and control groups then stirred gently for few seconds to avoid blood's clotting. Total genomic DNA extraction from blood collected in EDTA tubes was applied using Geneaid Genomic DNA ® Gsycn DNA Purification kit (GSYN USA) according to the manufacturer's protocol. After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA (Sambrook *et al*., 1989). PCR reaction was used to amplify the two targeted regions: exon 14 using the specific primers (Forward: 5'TTGTTTAAAAGCTGAAAAACTGAG-3'and Reverse: 3'-TCAAACATCTACATTGGCAGAAA-5') and exon 15 using thespecific primers (Forward:5'TTGTTTAAAAGCTGAAAAACTGAG-3'and Reverse: 3'-TCAAACATCTACATTGGCAGAAA-5')(Alpha DNA Company, Canada), these primers were designed by Primer3 plus software program.Components of PCR reaction and mixing amounts for both exon 14 and exon 15regions are shown in Table 1.

Table (1): Initial concentration of components of optimization of amplification reaction of targeted regions.

Components	Concentration	Components of one sample (µl)	Components of 10 sample (µl)
Distilled water	---	1	10
Go Taq® green master mix	1 X	5	50
Forward primer	10 picomols/µl	1	10
Reverse primer	10 picomols/µl	1	10
DNA Sample	100 ng/µl	2	*
Total Volume	---	10	80

PCR programs: Two PCR programs were adopted during this study. First, optimization program shown in table (2) was applied to all primers with gradient temperature in the range of (55-70ºC). After determination of the most perfect annealing temperature for each primer, a new program was set for each of the primers as shown in Tables (3). A successful PCR is achieved when a single sharp band with a specific molecular size appears on the gel.

The PCR products were separated by 2 % agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet light (302 nm) using (Gel Documentation System).

Table (2): PCR program for optimization reaction for two primers

No.		Steps	Temperature (C°)	Time	No. Of cycles
1		Initial denaturation	95	5 min	1
	A	Denaturation	95	1 min	
2	B	Annealing	Gradient temperature	1 min	35
	C	Extension	72	1 min	
3		Final extension	72	10 min	1

Table (3): PCR program for *SPINK5* region (exon 14)

No.		Steps	Temperature (C°)	Time	No. Of cycles
1		Initial denaturation	95	5 min	1
	A	Denaturation	95	1 min	
2	B	Annealing	58	1 min	35
	C	Extension	72	1 min	
3		Final extension	72	10 min	1

Table (4): PCR program for *SPINK5* region (exon 15)

No.		Steps	Temperature (C°)	Time	No. Of cycles
1		Initial denaturation	95	5 min	1
	A	Denaturation	95	1 min	
2	B	Annealing	60	1 min	35
	C	Extension	72	1 min	
3		Final extension	72	10 min	1

Sequencing of PCR Products and Data analysis: After successful amplification of the targeted regions, 25µl of PCR product of several samples (asthmatic and controls), along with primers, were sent abroad the country to Bioneer Company (Korea) for direct sequencing. Results of each group of sequenced samples were obtained within one month.

Sequence Analysis: After receiving of DNA sequence results, Detection of mutations was achieved using the NCBI alignment tool, BioEdit for nucleotide sequence.

Results and Discussion

The results presented in this study were based on analyses of data from a total of 140 cases: 100 asthmatic patient and 40 healthy control. 55 (77.46 %) of males were showed more variation than females 45 (65.21 %) which pointed to the higher risk of asthma in males than in females.

Polymerase chain Reaction (PCR): Successful PCR amplification reaction for the targeted regions was confirmed by (2%) agarose gel electrophoresis when DNA bands of a molecular size related to the targeted regions were visualized under UV light (Gel Documentation System) following 30 minutes staining with ethidium bromide. Molecular sizes for amplified exon 14 and exon 15 were 875bp and 925bp respectively as shown in Figures 1, 2, 3, 4,5,6,7 and 8.

Sequencing results of exon 13 and exon 14 of SPINK5: In exon 14 , five SNPs were found only in asthmatic patients, Bio Edit results showed the presence of three nucleotide transition in sample 1; trans. C>A at 169, trans. C>A at 455 and trans. C>A at 526. Location of transition at (169 and 455) appeared to have SNPs in other asthmatic patients which increases the likelihood of association of these locations with asthma as shown in Figure (9).

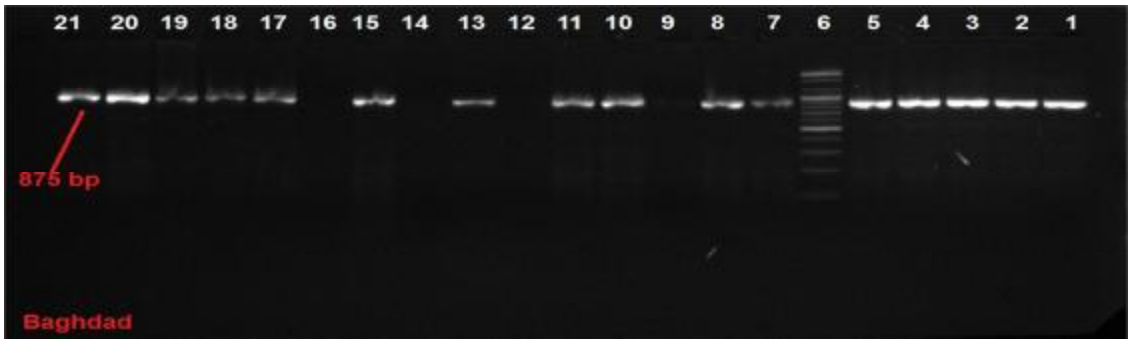

Figure (1): PCR products of SPINK5 gene – exon 14 of the molecular size 875 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 14 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 14 from asthmatic sample.

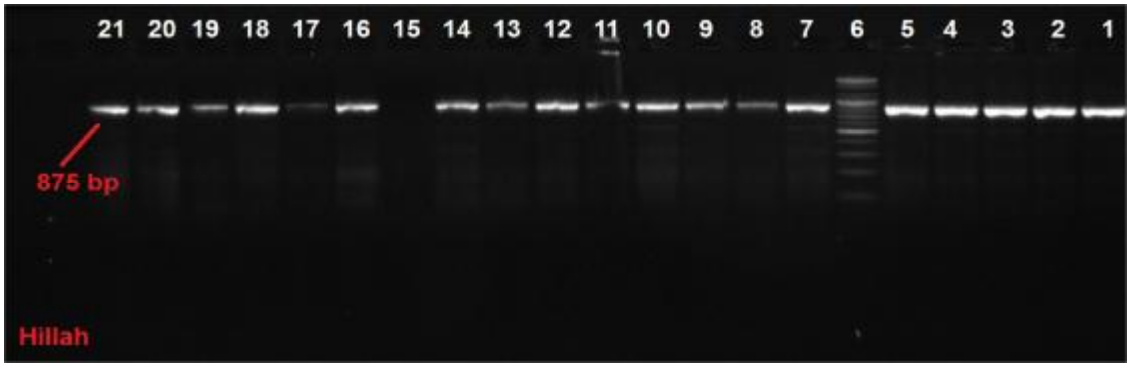

Figure (2): PCR products of SPINK5 gene – exon 14 of the molecular size 875 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 14 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 14 from asthmatic sample.

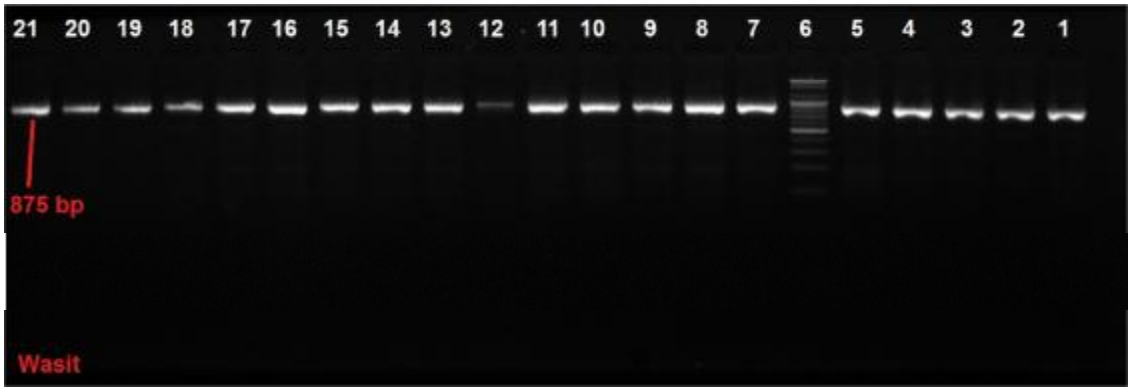

Figure (3): PCR products of *SPINK5* gene – exon 14of the molecular size 875 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 14 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 14 from asthmatic sample.

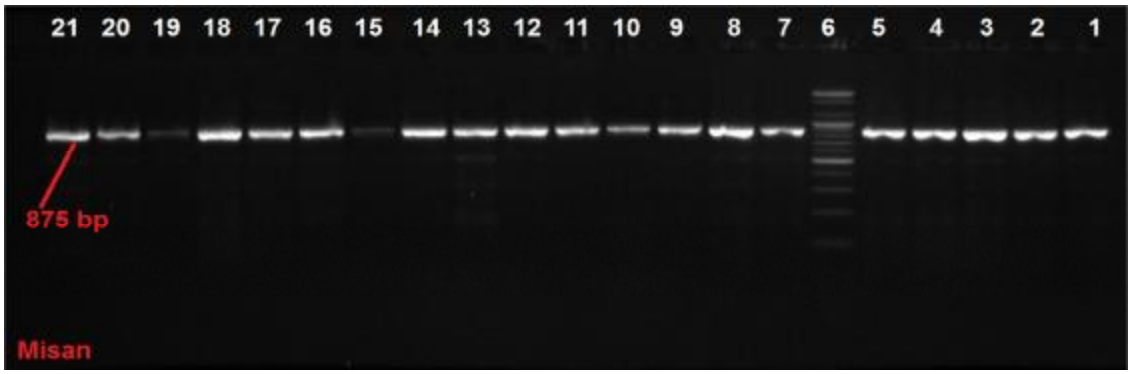

Figure (4): PCR products of *SPINK5* gene – exon 14of the molecular size 875 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 14 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 14 from asthmatic sample.

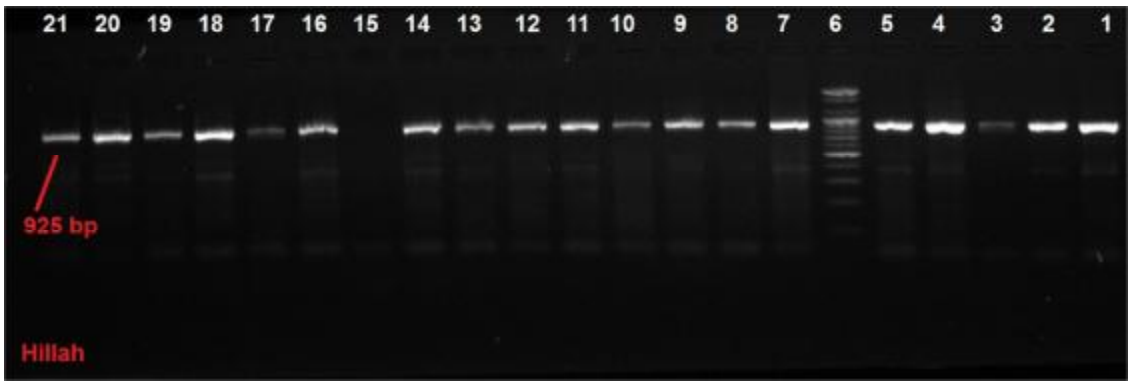

Figure (5): PCR products of *SPINK5* gene – exon 15of the molecular size 925 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 15 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 15 from asthmatic sample.

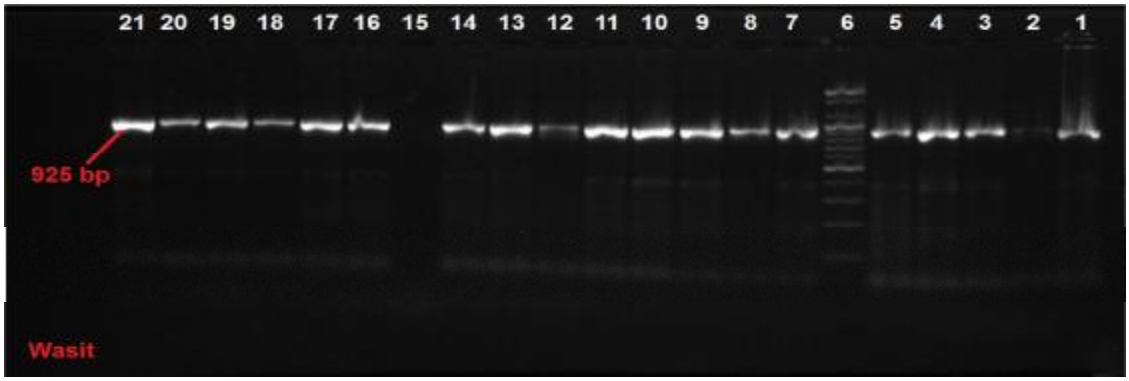

Figure (6): PCR products of *SPINK5* gene – exon 15of the molecular size 925 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 15 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 15 from asthmatic sample.

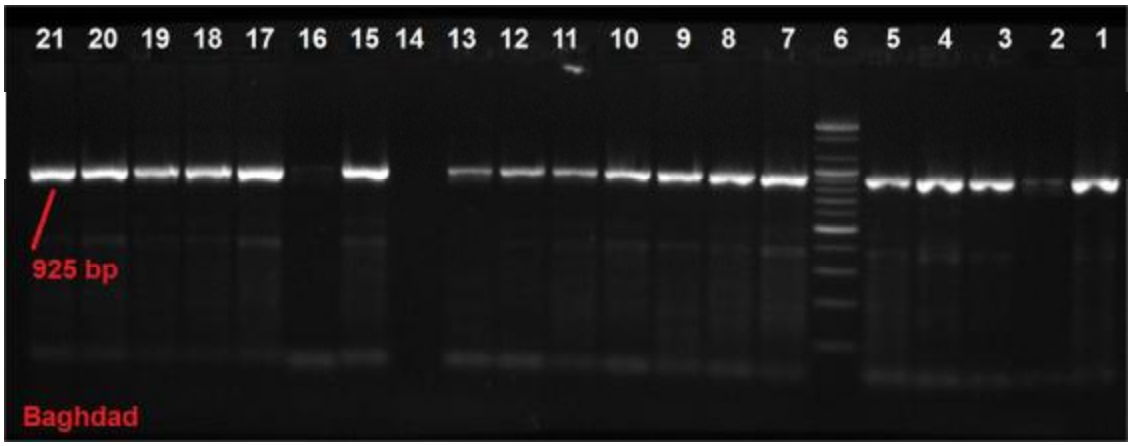

Figure (7): PCR products of *SPINK5* gene – exon 15of the molecular size 925 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 15 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 15 from asthmatic sample.

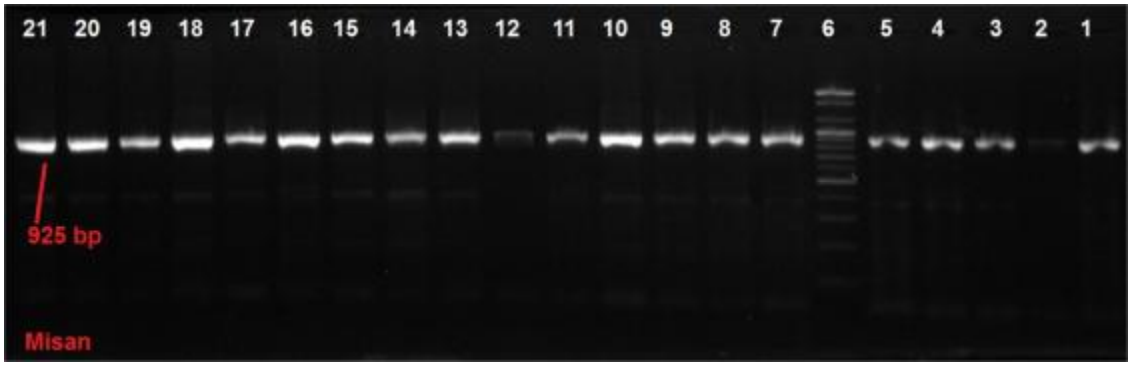

Figure (8): PCR products of *SPINK5* gene – exon 15of the molecular size 925 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr . Lane (1-5): products for exon 15 from healthy sample. Lane 6: DNA ladder (100-1500 bp). Lane (7-21): products for exon 15 from asthmatic sample.

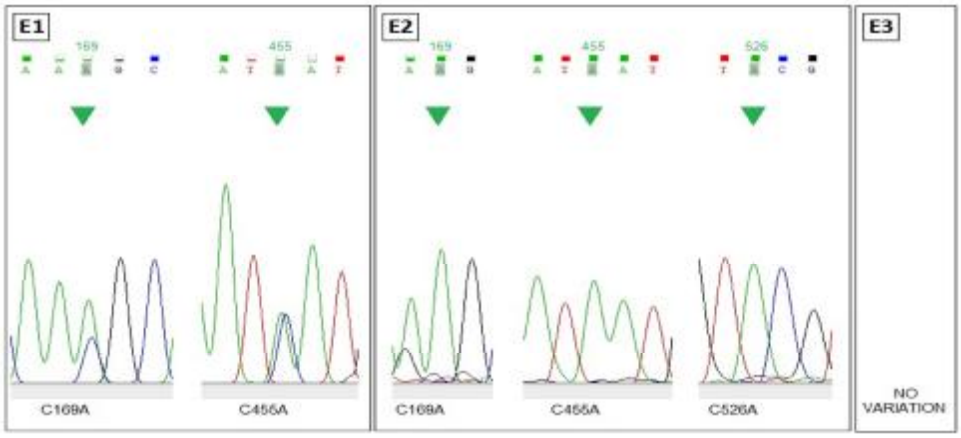

Figure (9): Distribution of SNPs identified in this study along *SPINK5* gene (exon 14).

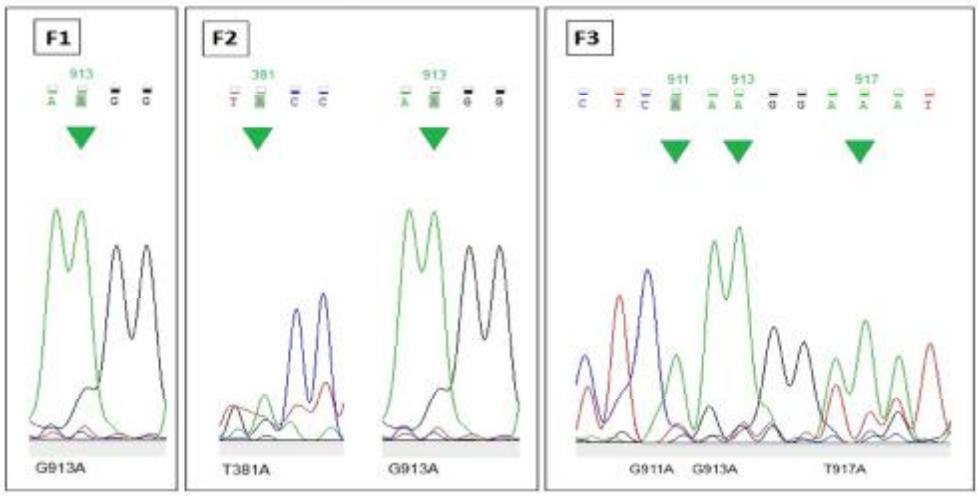

Figure (10): Distribution of SNPs identified in this study along *SPINK5* gene (exon 15).

Coding polymorphisms in SPINK5 exons 13 and 14 have been reported to be associated with atopy, asthma and atopic dermatitis (AD)(Kato *et al*., 2004). Alternatively, Zhao *et al.,* (2012) demonsetrated coding polymorphisms in SPINK5 exons 13, 14 and 26 have been reported to be associated with atopic dermatitis (AD), asthma and high level of IgE the SPINK5 gene polymorphisms was found not to be associated with AD in regard to either serum IgE levels, concurrent allergic asthma or early onset of AD.

For exon 15 region of SPINK5, BioEdit alignment for all 3 samples of DNA sequence showed 6 new ones located along this region, one was mutual (found both in asthmatic and control samples), one was found only in asthmatic patients and two In control patient. There are some scientists did not find any relationship between this gene and asthma, Folster *et al*. (2005) genotyped four nonsynonymous SNPs (Asp106Asn, Asn368Ser, Asp386Asn, and Glu420Lys), and detected no association between *SPINK5* and atopic dermatitis in populations of Northern German origin. Jongepier *et al.* (2005). Failed to detect any association between *SPINK5* and asthma, atopic phenotypes and atopic dermatitis in a Dutch population. These discordant findings probably reflect different genetic and environmental backgrounds in various populations.

Figure (9) and Figure (10) shows the location of SNPs in exon 14 of the gene and the SNP detected in exon 15. To our knowledge, this is the first time to report these SNPs in Iraq.

The results obtained in this study suggest the possible role of *SPINK5* activity in the pathogenicity of asthma. The two SNPs found in control are possibly not associated with asthma while the SNPs found only in asthmatic patients are likely associated with asthma and SNPs could alter expression of the gene thus contributing to the pathogenicity of asthma. It is highly recommended for further studies to include the remaining regions of this gene in sequencing analysis as well as gene expression assays using real time PCR to confirm the association of this gene with asthma (Zhao *et al.,* 2012).

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