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Association between LZTFL1 gene variation and risk infection of COVID-19 in holy Najaf province patients

Zahraa Razaq
Kufa University, , https://orcid.org/ 0000-0002-1482-8672
Email: ZhraarazaqS2022@gmail.com

Suad Gazi Jaafer Alkufi
Nahrain university, https://orcid.org/0000-0003-4156-4446
Email: suad.alkufi@nahrainuniv.edu.iq

***Abstract*---**Coronavirus disease 19 (COVID-19), is acute aspiratory syndrome caused by coronavirus 2 (SARS-CoV-2). Factors such as age, health status (cardiovascular disease, diabetes) and gender are the most important risk factors in terms of their relationship to the host ability to infected with COVID-19. However, the genetic association evidence has been unclear yet in our population. This study, demonstrated genetic predisposition investigation to detect SNPs allele locus 3p21.31at of rs11385942 associations with COVID-19 infection. Statistical analysis and DNA sequences analysis were conducted to the study cases. The results showed male incidences is higher than female number (60 cases- 60% male) corresponding (40 cases40% female). COVID-19 impact significantly on C-reactive protein and the age group 55-65 appeared high level of CRP (130.5 ± 58.93). While, DNA sequence using Sanger sequencing method with further alignments analysis showed the association signal at locus 3p21.31 genes homozygous allele AA/AA frequent exist for the rs11385942 as risk allele in sever patients who have diabetes type 1 or 2 and received oxygen supplementation. However, no heterozygous alleles mutant was detected To conclude, that aged patients and male paralleled the severity of COVID-19. Homozygous gene more common SNPs in the position 3p21.31 LZTFL1 rs 11385942 as others south Asian countries population associated with severe infection with COVID- 19 in Najaf province. Our study is the first step population genome-wide analysis, further analysis need to be done to confirm our result with larger sample and longer time.

***Keywords*---**COVID-19, SARS-CoV suitability, SARS-CoV-2, SNP, LZTFL1 Gene rs11385942.

Introduction:

The coronavirus disease 2019 (COVID-19) is a pandemic caused by new beta-coronavirus SARS-CoV-2. They are wide variation between individuals in symptoms of COVID -19 from severe respiratory asymptomatic syndrome to organ failure which requires hospitalization and need intensive care. Consequently, accurate diagnosis of COVID-19 is more challenging (Li et al., 2020a, Shereen et al., 2020). Since, February 2019 the begging of the pandemic diffusion in the worldwide of COVID-19 most researches focused in clinic and pathogenesis of disease. Recently, most COVID -9 pandemic studied focused on human susceptibility, and genetic different factors impact on disease severity for instance, age, gender, blood group type and the host genetic background (Rashedi et al., 2020, Vannabouathong et al., 2020). The routine clinical diagnosis for this disease is based primarily on epidemiological history, clinical symptoms, a variety of methods of detection, including computed tomography (CT) scan, nucleic acid amplification test (NAAT), and serological techniques (Corman et al., 2020, Wan et al., 2020).

Recently, (Schmiedel et al., 2021) reported that gene variants encoding proteins associated with the sensitivity and severity to COVID -19 infections have been identified. Two independent Genome- Wide Association Studies (GWAS) were carried out among Italian and Spanish test subjects (Ellinghaus et al., 2020) in addition to individuals from the US and the UK (Shelton et al., 2021). Results from these studies showed the presence of an association of *loci* 3p21.31 and 9q34.2 with severity of COVID-19.

Moreover, the associations of *LZTFL1* (HGNC:6741) rs11385942, at locus 3p21.31 with genetic susceptibility to COVID -19 was reported by (Ellinghaus et al., 2020)

Hospitalization risk factors such as various non-genetic conditions and the genetic variants *LZTFL1* rs13078854 with COVID-19, respectively were identified by (Shelton *et al.*,2020). Other study conducted by (Downes *et al.*, 2021) identified that *LZTFL1* encodes the ubiquitously expressed protein leucine zipper transcription factor-like 1, and it is strongly expressed in human lung cells.

However, studies have not been able to explain the role of the *LZTFL1* gene in susceptibility to infection with COVID-19 or its role in the severity of infection with the presence of several genes near 3p21.31 loci that may control the association, including *SLC6A20* (HGNC:30927), *CCR9* (HGNC:1610), *FYCO1* (HG NC:14673), *CXCR6* (HGNC:16647), and *XCR1* (HGNC:1625) (Shelton *et al.*,2020). Finally, we analysis the DNA sequence by using Sanger sequencing method detect the variant and mutant to the single nucleotide polymorphism SNPs as markers to see that certain areas of the genome people who have COVID-19, and that helps us to explain why some people have the ability to be infected with COVID -19 more others.

Material and Methods:

In current study, 100 blood sample were collected (20 control and 80 sample are infected with COVID -19) since October 2021to April 2022. 4 ml of fresh venous

blood samples from COVID-19 infected patients by sterile syringes which divided into (2 ml). Genomic DNA extraction from frozen whole blood by using Geneide Kit according to the manufacturer's protocol (use frozen 200 µl with PBS. 20 µl of Proteinase K was added then mix by pipetting. It was incubated at 60°C for 5-7 minutes, 200 µl of lysis buffer was added then mixed by shaking vigorously, incubated at 60°C then runn5 min., to participate with 200 µl absolute ethanol was added. The, transfer the mixture to 2mL purification DNA column with a specific DNA filter supplied with collection tube as part tools with the kit). 400 µl of wash buffer was added to the column. It was centrifuged at 14-16,000 x g for 30 seconds. Finally, elute the DNA.

Gene Amplification LZTFL1

PCR reaction was used for detection *LZTFL1*genomic DNA COVID -19 blood. Firstly, forward and reverse primers were generated.

Froward primer: - GCTTCTTAAAGCACCACCTTCT
Revers primer: - CTTGGTCAGTATTACCATCAGTCTTTAT.

The reaction was submitted using the Expand High Fidelity PCR System from using amplification reaction kit. (Master mix10 µL**,** forward and revers primer 2µL of each one, 2-4 µL) the final volume was completed with nuclease free water till volume 20 µL and distributed in the PCR tubes & run according to the Thermal cycling conditions in addition, (Initial denaturation 95°C 5 min, denaturation 95°C 30 sec, annealing 58C, extension 72° C).

Analytical agarose gel electrophoresis To separate and detect different DNA fragments agarose gels were prepared and submerged in 0.5 × TBE buffer. Samples were loaded. A DNA size marker was used alongside the samples. A typical gel run was performed at 70 volts for 40 minutes. DNA

PCR DNA fragments sequencing Extract PCR products. Then, nucleic acid solution was diluted in 20 µL ddH2O to a concentration of 100 ng/µL. PCR fragment products sequencing was carried out by the company 'by Macrogen, Korea using Sanger sequencing method.

Statistical Analysis

Data was analyzed using Excel and GraphPad Prism Version 5.00 for window normally distributed data was analyzed using one-way analysis of variance (ANOVA, with Bartlett's test significant difference (HSD) comparison test). A p value of < 0.001 was considered significant from in vivo studies

Results and discussion

The results obtained from the initial analysis showed that number of infected males was higher than female number (60cases- 60% male), while the female were (40 cases - 40 % male) (Figure1A) this result showed the agreement with statistical analysis of the WHO rescores (Jin et al., 2020). Also, this finding is

showing clear similarity with Iraqi researchers in 2021 published by , (Suad G.J. ALkufi et al., 2021)

The cases were distributed in to eight age groups (Figure 1B). The most interesting result, the highest number of patients with coronaviruses was within age group 25-35 cases in male. Female showed the highest percentage infection in the age group 15-25. Several studies confirmed the higher mortality is consistently associated with older age and male sex (Chen and Zheng, 2020, Li et al., 2020b). Although, these results differ from our result in the highly incidence within age group 35-25, but they are mostly consistent with Iraqi statistical analysis (Suad G.J. ALkufi et al., 2021). A possible explanation for these results may be the lack of registration for the aged people in our society in term their ability to reach any COVID -19medical center comparing to the young age groups.

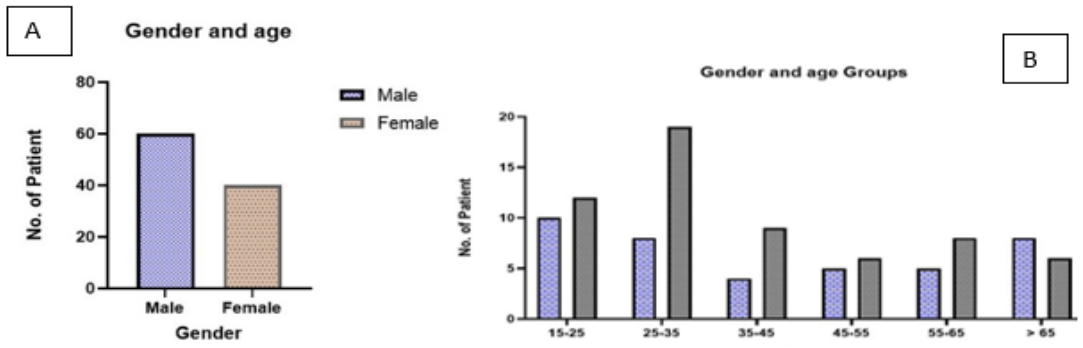

Figure1: Statistical finding of 100 COVID -19cases. A, diagram shows the highest numbers of male 60 while female number are 40. B shows collected cases of 100 patients divided eight age groups the highest COVID -19cases in age group 25-35 male while 15-25 in female.

Genetic Association of COVID-19

According the Human Genome Project was created by all international scientist efforts (Lander et al., 2001) a reference sequence of the human genome was derived. In addition, the Human Genome Project provided the development of technologies for high data sequencing of DNA that lead to improve methods for DNA sequence detection to use them to test infectious disease pathogenesis (Smith, 2017).

Availability of the reference human genome sequence allowed the generation of oligonucleotide probes and short sequence markers (SNPs) mapped on chromosomes positions. In the other hand polymorphic SNP markers play a significant role to analysis of segregation of specific genomic regions in families and analysis of inheritance of specific genomic regions indifferent population (Smith, 2017, Bansal et al., 2010). A variety of software have been developed that can efficiently align a large numbers of sequence even in the presence of multiple errors in the reads (Li et al., 2009, Li and Durbin, 2009). As a result, the current study analyzed single nucleotide polymorphisms and association signal at locus 3p21.31 located on Chromosome 3 short arm p.

Detection of gene variant SNPs LZTFL1 rs11385942 in Patients of COVID -19

Genomic DNA was extracted from collection whole blood of cases that have e infection of corona virus and also 20 control sample (Figure 2). As can see in figure 2 of agarose gel of the successful DNA extraction from frozen blood of patients with corona sever infection with two control sample migrated in one gel. The genomic DNA clearly appears with a strong band in all well successfully.

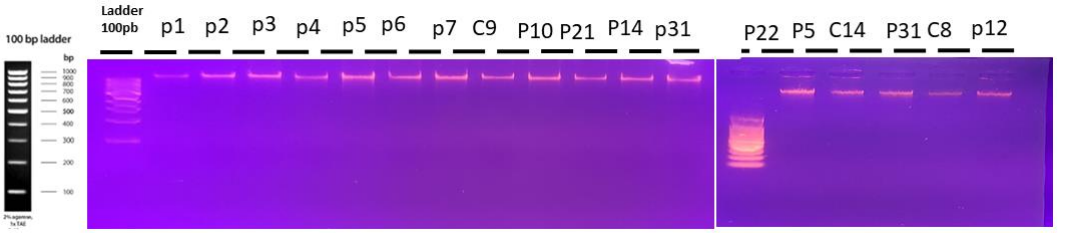

Figure 2. Isolated genomic DNA from control samples who infected and people had Mild infection and sever infection with COVID-19, P symbol C none infected patients sample (Agarose gel 8% ladder 100pb) at 5 v/cm² for 45 min.

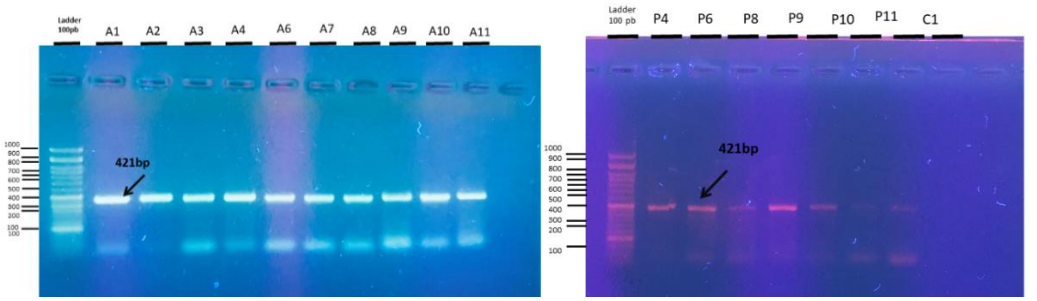

Figure 3: PCR reactions confirmation of genomic on genomic DNA to detect LZTFL1 gene fragment rs11385942 specific band with a perfect product size **421 bp.** Bands ladder 100pb were separated on 1% agarose gel at 5v/cm² for 45 min.

Detection C-reactive protein(CRP) **titer in COVID -19 patients.**

The present study used CRP as an important blood parameter of infection pathogenicity and also to evaluate the COVID -19 cases severity. As illustrated in Table (1) the distribution of C -reactive protein in COVID-19 patients sample. One-way (ANOVA) of variance was used with Bartlett's test analysis to determine the significant difference between the diverse group parameters. P values of less than 0.001 were considered as statistically significant.

Table 1
Statistical analysis among age groups and the average standers and difference of CRP level in COVID-19 patient measured from their blood serum.

Age Group	Mean ± SD of CRP titer in different age group
15-25	34.8 ±11.1
25-35	38.1 ±13.5
35-45	55.9 ± 40.7
45-55	130.5 ± 58.93
55-65	108 ± 63.5
65<	112.9 ± 82.9
Total Mean	73.2
Normal value	10mg/dl
P. value 0.001	P. value 0.001

As Table 1 shows, determination of serum mean and SD concentration of CRP in patients with COVID-19 corresponding to the age groups in which, the age group 45-55 appeared to have high level of CRP (130 ±58) followed by 56-65 age group in (108. ± 63.3). Elevated levels of CRP were observed with levels on average **80.09667mg/dl** in severe COVID-19 patients of the study cases.

A possible explanation for this might be that aged people have more susceptibility to get the severe infection comparing to other age group. That mean these people have a low immune response due their age against the COVID-19 infection. Consequently, CRP level may rise from a normal level 10mg/L due low ability of their body to defense against the corona SARs disease. According to (CRP) is synthesized by the liver in response to interleukin-6 and well known as one of the classical acute-phase reactants and as a marker of inflammation. Consequently, the C-reactive protein scored highly percentage comparing to other group. This finding confirms the association between severity and increasing CRP titer. These results are consistent with other study (Zhou et al., 2020).

The available studies that have determined serum concentration of CRP in patients with COVID-19 significant increased on average 20 to 50 mg/L (Chen et al., 2020a). Elevated levels of CRP were observed up to 86% in severe COVID-19 patients(Chen et al., 2020b, Guan et al., 2020). Patients with severe disease progressions had ability to raised titer of CRP than mild or non-severe patients. For instance, a study reported that patients with more severe clinical features had on average CRP concentration of 39.4 mg/L and patients with mild symptoms CRP concentration of 18.8 mg/L (Gao et al., 2020). The patients who died from COVID-19 had about 10 fold higher levels of CRP than the recovered patients (median 100 vs 9.6 mg/L) (Tan et al., 2020). For all these previous reasons this study used C-reactive protein as indicator the severity COVID-19 which support the reason to select high risk patients as a candidate sample for further genetic analysis and gene sequence analysis by using single nucleotide allele detection in further work study.

Detection SNPs LZTFL1 gene Sequencing results

In the present study, 10 PCR reaction fragments products were selected for further DNA sequence analysis. The selection cases depend on the patients' severity infection with COVID -19which related to their C-reactive protein titer table 4-6. In addition, these patients have diabetes type one or two and have in particular respiratory problems. Initially, amplification the target region of LZTFL1 gene of selected patients and for control was submitted. Polymerase chain Reaction to confirm SNPs LZTFL1 rs11385942 in Patients infected with COVID-19. The novel forward and reverse oligonucleotide were generated in this study (Table 2) the expecting size of PCR product 421 pb.

Froward GCTTCTTAAAGCACCACCTTCT

Revers CTTGGTCAGTATTACCATCAGTCTTTAT

Secondly, agarose gel electrophoresis was used to confirm this amplification for this region, a (Figure 6). Then, the DNA fragments were migrated on a gel that separates them by size. It is apparent from figure 6 the PCR DNA products for genomic DNA of frozen blood with correct fragment size as expecting 421pb by using 1% agarose gel and stained with safety stain all samples were amplified successfully with a single band.

Subsequently, DNA fragments were isolated by DNA extraction kit from the gel. And send them to sequence company Macrogen, Korea using Sanger sequencing method.

The PCR fragments products DNA sequencing

Ten sever patients were selected as high risk group patients. Table 2 provides titers of C-reactive protein levels for the selected COVID -19 patient measured from their blood serum the type of chronic disease they have. The average of C reactive protein of the cases is higher than normal value more nine folds 90.645 mg/L while the normal value is 10 mg/L. In the other hand the all have diabetic type 1 mostly only two have diabetic 2. These results showed agreement with previous study published by Tan and other colleagues researchers in 2022 they found that patients who died from COVID-19 had about 10 fold higher levels of CRP than the recovered patients (median 100 vs 9.6 mg/L) (Tan et al., 2020).

Firstly, sequence samples were analyzed Aligned using NCBI nucleotide alignment tool (Nucleotide BLAST the Basic Local Alignment Search Tool) (Figure 4). Sequence alignment was compered the result sequence of the cases with COVID-19 corresponding to the NCBI data base of COVID -19 (normal healthy human sequence for the LZTF1 Ref Seq Gene that is deposited in the NCBI (Accession number: NG_033917.1) to detect associated SNP detection (Kerstens, 2010). That analysis shown identity 100% as seen in figure 4 which reflect the correct sequence target gene of the samples that sent to sequence.

Further multiple-sequence alignment in order to compare between PCR fragments chromatogram nucleotide sequence of the high risk patients COVID-2019 (figure 5).

Initially, figure 5 showed that eight samples provided clean sequencing results and can be compared (A1, A4, A5, A6, A7, A8, and A9) table 2 for the whole target area. While samples A2 and A3 showed unclean sequencing results cannot use in alignment analysis.

The results obtained from the primary analysis as shown in figure 5. The alignment of the eight samples showed that the frequency of the risk allele of the main at 3p21.31 (rs11385942) was homozygous allele AA/AA. These patients have been severing COVID -19 patient's diabetes type one or two and have in particular respiratory problems (Figure 5). Figure 6 presents the chromatogram sequence analysis and should clearly the AA/AA allele and confirmed former detection.

In addition, they were received mechanical ventilation and also received oxygen supplementation. We can conclude homozygous are common in Iraqi cases. Overall, these results indicate that no heterozygous associated with severe infection in Iraqi patients otherwise our suggestion increase the number of cases in further work to confirm this finding.

Globally, the available variant database entries in main page of NCBI suggest that the frequency of this risk allele varies among populations worldwide (Table 3). What the researcher identified that the south Asian populations are more likely have more frequency to have the heterozygous allele AA/AAA comparing with other populations (Balanovsky et al., 2021, Downes et al., 2021). While the east Asian showed homogenous allele AA/AA.

Significantly, the results of this study compared to the findings of previous work on different population sequence alignment of gene rs11385942 risk allele. These results match those observations in earlier studies which confirmed that the east Asian population more likely have homogenous allele as can see in table 3. The frequency of the risk allele rs11385942 is high in South Asia, marginally lower in Europe and West Asia, and low in other world counties (Balanovsky et al., 2021). Little is known about LZTFL1, but earlier research had shown a little about the protein.

Recently the researcher found that *LZTFL1*which comprised six genes (SLC6A20, LZTFL1, CCR9, FYCO1, CXCR6, and XCR1). As demonstrated by (Seo et al., 2011) LZTFL1 gene encode a specific type of protein it codes a kinesin like protein for, which called Leucine zipper transcription factor-like protein (Seo et al., 2011). The risk allele of rs11385942 is associated increased expression of SLC6A20, and LZTFL1 is strongly expressed in human lung cells (Singh et al., 2021, Valenti et al., 2021).

It has been suggested that LZTFL1 protein function mechanism is to regulate ciliary formation in ciliated human tissue for example trachea intestinal tissue in villi. This gene interacts with BB some protein to create complex. Together with

the BB Some complex, controls ciliary trafficking. In addition, suggest the LZTFL1 contributes in c hedgehog (SHH) pathway regulation may play a role in neurite outgrowth is involved in a signaling pathways cells response in different tissue. Finally, LZTFL1 may have tumor suppressor (Seo et al., 2011, Wei et al., 2010).

In the context of an infection and inflammation, low levels of LZTFL1 promote the transition of certain specialized lung cells into a less specialized state. Higher levels of LZTFL1 slow this transition (Wei et al., 2016).

The SNP variation in could be cause human predisposition to get infected with COVID -19in people more than other. That potentially any defect or mutation in gene sequence could increase clinical symptoms of the new coronavirus SARS-CoV-2 which associated with high severity and bad prognosis in these specific patients. The association of 3p21.31 variations with susceptibility to SARS-CoV-2 infection, along with disease severity, considered as high spot the importance of the respiratory epithelium for this locus. LZTFL1 encodes a cytosolic leucine zipper protein, which associates with the epithelial marker in the trafficking of numerous signaling molecules (Niemi et al., 2021, Seo et al., 2011).

To sum up, it seems reasonable to conclude that the chromosome 3p21.31 loci are involved in COVID -19 susceptibility persons with severe disease. Although the sample in our study was reasonably small, the data used in the study were obtained from multiple medical centers. Moreover, our findings found, that aged patients and male paralleled the severity of COVID-19. Homozygous gene more common SNPs in the position 3p21.31 LZTFL1 rs 11385942 as others south Asian countries population associated with severe infection with COVID- 19 in Najaf province. Additional large-scale researches are required to confirm current study.

Table 2

Summaries of the high risk group patients C-reactive protein levels of 10 selected COVID -19patients (high risk group) measured from their blood serum.

t	Patients	C-Reactive protein mg/L	
1	**A1**	**300.00**	**Diabetic type 1**
2	**A2**	**40.84**	**Diabetic type 1**
3	**A3**	**40.68**	**Diabetic type 1**
4	**A4**	**91.67**	**Diabetic type 1**
5	**A6**	**120.26**	**Diabetic type 1**
6	**A7**	**34.19**	**Diabetic type 1**
7	**A8**	**53.32**	**Diabetic type 1**

8	**A9**	**102.88**	**Diabetic type 1**
9	**A10**	**30.52**	**Diabetic type 2**
10	**A11**	**92.09**	**Diabetic type 2**
Average	**90.645 mg/L**		
Normal value	**10< mg/L**		

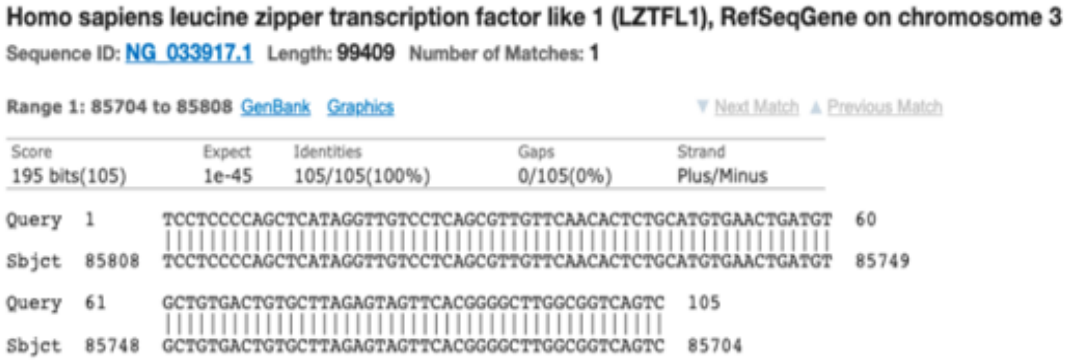

Figure 4: Sequence DNA alignment of LZTFL1 gene between aligned with the RefSeqGene sequence that is deposited in the NCBI (Accession number: NG_033917.1

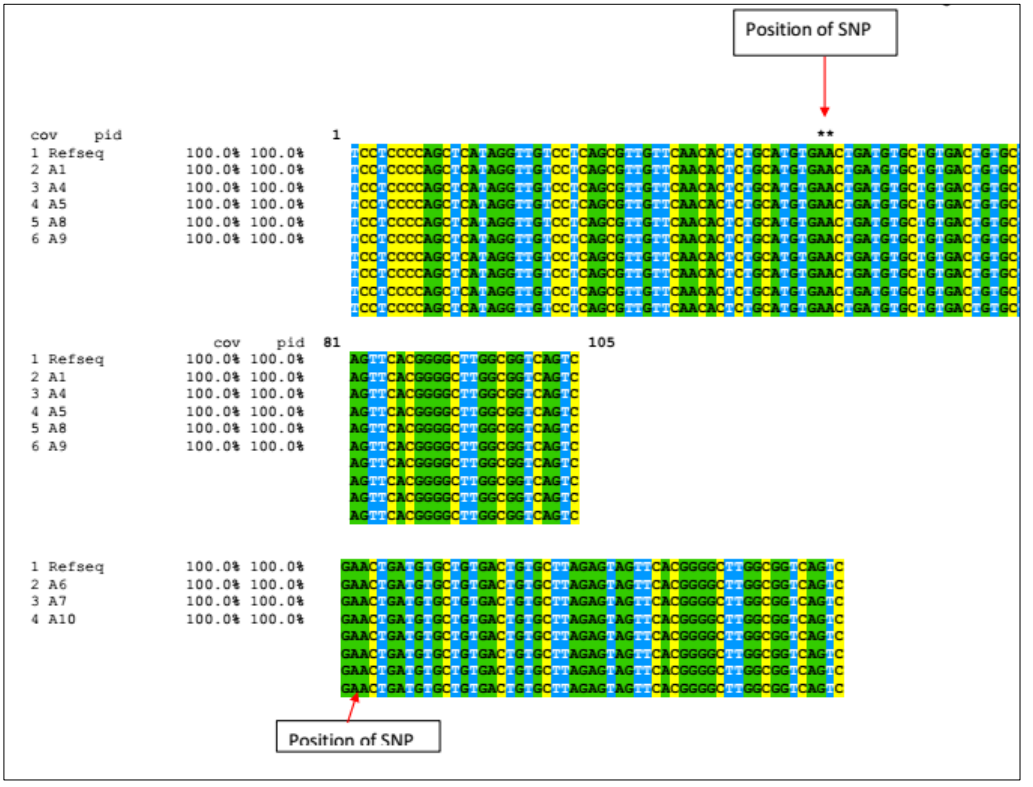

Figure 5: Alignment of partial LZTFL1 gene sequences of three selected samples against a reference LZTFL1 gene sequence of Homo sapiens in the NCBI (Accession number: NG_033917.1) for detection of SNP rs11385942 AA/AAA (highlighted using **).

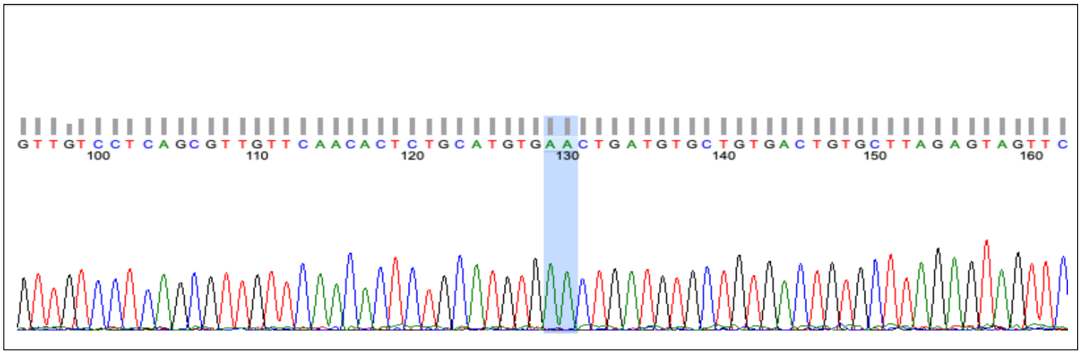

Figure6 : Chromatogram of the sequence of AAA alleles in the DNA sequences of samples A1 (Highlighted in a box). This figure is provided by Macrogen, Korea using Sanger sequencing method. BioEdit Sequence Alignment Editor software.

Table 3 Distribution of SNPs Allele frequent in alignment sequence in different populations

Population	Group	Sample Size	Ref Allele	Alt Allele
Total	Global	21204	AA=0.92388	AAA=0.07612
European	Sub	14152	AA=0.91697	AAA=0.08303
African	Sub	5530	AA=0.9410	AAA=0.0590
African Others	Sub	198	AA=0.965	AAA=0.035
African American	Sub	5332	AA=0.9402	AAA=0.0598
Asian	Sub	112	AA=1.000	AAA=0.000
East Asian	Sub	86	AA=1.00	AAA=0.00
Other Asian	Sub	26	AA=1.00	AAA=0.00
Latin American 1	Sub	146	AA=0.932	AAA=0.068
Latin American 2	Sub	610	AA=0.961	AAA=0.039
South Asian	Sub	98	AA=0.63	AAA=0.37
Asian	Sub	112	AA=1.000	AAA=0.077

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