Association of Some NKG2 gene Family and it's Polymorphism with Pulmonary Tuberculosis

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Abstract

The case-control study was aimed to evaluate the effect of NKG2D and NKG2F, gene polymorphism on susceptibility to pulmonary tuberculosis in Babylon province.

The polymorphism of NKG2D gene at (rs1049174) and (rs2255336) were detected for 60 PTB cases and 60 of control. For rs1049174, allele frequency for patients and control were significant (P<0.05) and the C allele represent the risk allele for PTB patients with odd ratio (95% CI) of 2.04 (1.16-3.57) while G allele represent protective allele for control group with odd ratio (95% CI) of 0.49 (0.28-0.86), (P<0.05). The genotype (C/C, G/C, G/G) were (61.7, 30, 8.3)% respectively for PTB patients and (36.7, 50, 13.3)% respectively for control with significant association with disease (P<0.05). On the other hand, the SSCP and sequencing results of rs2255336 revealed that there were no gene variation in rs2255336 among patients and control subjects.

The polymorphism of NKG2F gene at (rs1841958) was also detected for 60 PTB cases and 60 of control. For rs1841958, allele frequency between patients and control were insignificant (P>0.05). The genotypes (C/C, A/C, A/A) were (38.3, 40, 21.7)% respectively for PTB patients and (36.7, 40, 23.3)% respectively for control with insignificant association between these genotype and PTB (P>0.05).

Key Words: *Health; ; gene polymorphism; NKG2F, rs1049174, rs2255336 and rs1841958.*

Introduction

Tuberculosis is an old infectious disease triggered by the bacillus *Mycobacterium tuberculosis*, which is now afflicting humans all over the world. In 2018, 1.2 million deaths and 10 million new TB cases were reported by the World Health Organization [1].

The association of NKG2 gene family with pulmonary tuberculosis was a limited studied. NKG2D and NKG2-F is a transmembrane protein of the NKG2 family of C-type lectin-like receptors. It's encoded by KLRK1and KLRC4 gene respectively, which is located in the NK-gene complex (NKC) situated on chromosome 12 in humans. It is expressed by NK cells, γδ T cells and $CD8^+$ αβ T cells [2].

The NKG2D activating receptor is peculiar in its ability to bind to numerous and highly diversified MHC class I-like self-molecules. These ligands are poorly expressed in normal cells, but can be induced in infected, transformed or damaged cells [3].

NKG2D is a main receptor for the recognition and removal of infected and transformed cells because its ligands are stimulated during cellular stress, either as a result of genomic stress or infection such as cancerous cells and TB infected cells [4]. While the function of NKG2F receptor has not yet been clarified. It may not have a C-type lectin domain and contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs-like motif) in the cytoplasmic tail. However, ITIM-like motif looks to be non-functional, thus NKG2F was considered to be an

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activating receptor [5].

Material and Method

Samples selection and MTB detection

A case-control study involved 60 newly diagnostic pulmonary tuberculosis and 60 samples of apparently healthy persons were taken as a control group.

A blood and sputum samples were collected from all study cases at the center of chest and respiratory disease in AL-Hillah / Babylon province, during the period from November 2018 to July 2019.

The sputum sample was subjected to direct smear examination by Ziehl-Neelsen technique [6], routine culture by use Lowenstein-Jensen medium [7] and use of GeneXpert for detection of MTB [8].

The blood samples were used for genotyping study, as the DNA extraction was achieved according to the method recommended by the manufacturing company (favorgene) in the user manual of Favor Prep Genomic DNA Mini Kit general protocol for fresh and frozen blood samples. The extracted genomic DNA from the blood was checked by using nanodrop spectrophotometer which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm). All the primers that used in this study for detection NKG2D and NKG2F were designed by the aid of NCBI-primer BLAST online software which include NKG2D-rs1049174 (Forward- TGCTGTGTCTCTCTGCTGTG and Revers-TCAGATATCCCCAAGGCTGC), NKG2D-rs2255336 (Forward- TTCTGGACT AATAGCAAAAATGTGA and Revers- AGCCATGGGAATCCGTTTCA), and NKG2F-rs1841958 (Forward-TGTCA TTCCCTTGATGATCCGAAGA and Revers-GTGCAGTTATCATAGAGCACAGTC), at the same time the produced primers were tested for specificity of their target sequences by conducting the BLAST against the human genome, then the primers pair was selected according to the demand criteria such as: product length, the similarity of melting temperature, primers length, specificity, etc. Then the mutations were interred according to the design demands.

The PCR technique was done on all samples by use (Promega master mix,USA) and then the amplified products were subjected to RFLP or SSCP and Sequencing technique to determine SNPs.

The PCR-RFLP of rs1049174 and rs1841958 was done for all sample by using *BstDE I* and *Alu I* endonucleases respectively and according to instructions of manufactured company (New England Biolabs Inc./ USA) by add 5 µl amount DNA from PCR product to 4 µl restriction enzyme buffer and 1 µl of the selected restriction enzyme *BstDE I* and the reaction mixture then completed to 15 µl by Free nuclease water.

Statistical Analysis

Statistical analysis was done using SPSS version 23; variables were described as mean, standard deviation, number and percentage. Risk was estimated using odds ratio and the level of significance was set at $P < 0.05$.

Results and Discussion

DNA Extraction and PCR Products Detection

Human DNA genome was extracted from whole blood of all the 60 samples of PTB patients and 60 samples of apparently healthy control, the concentration was (50-150 ng) and purity was (1.8-2.0). These DNA was subjected to PCR amplification using specific primers targeting specific regions in the DNA and then enrolled for detection of single nucleotide polymorphisms (SNPs) by RFLP or SSCP-Sequencing techniques. Then the optimized of PCR products of the designed primers pair which would be used in NKG2D (rs1049174 and rs2255336) and NKG2F (rs1841958) genotyping was done by the gradient-PCR at (55-66 C^0) and then the PCR product were electrophoresed to evaluate the most appropriate condition for PCR technique. The most appropriate annealing temperature was $66C^0$ for (rs1049174) and $60C^0$ for (rs2255336 and rs1841958).

Genotypic Characterization of NKG2-D (rs1049174)

The amplification of NKG2D gene at region rs1049174 appeared the presence of gene amplicons on electrophoresis gel for all groups of study.

Genotype association and allele's frequency for patients and control are listed in table (1). The results revealed that there were a significant allele frequency

differences between patients and control group (P<0.05). Allele C represents the risk allele with odd ratio (95% CI) of 2.042(1.166-3.578), while allele G represents the protective allele with odd ratio (95% CI) of 0.49 (0.28-0.86).

The restriction fragment length polymorphism of PCR products of rs1049174 gene revealed three genotypes; C/C, G/C and G/G (figure 1).

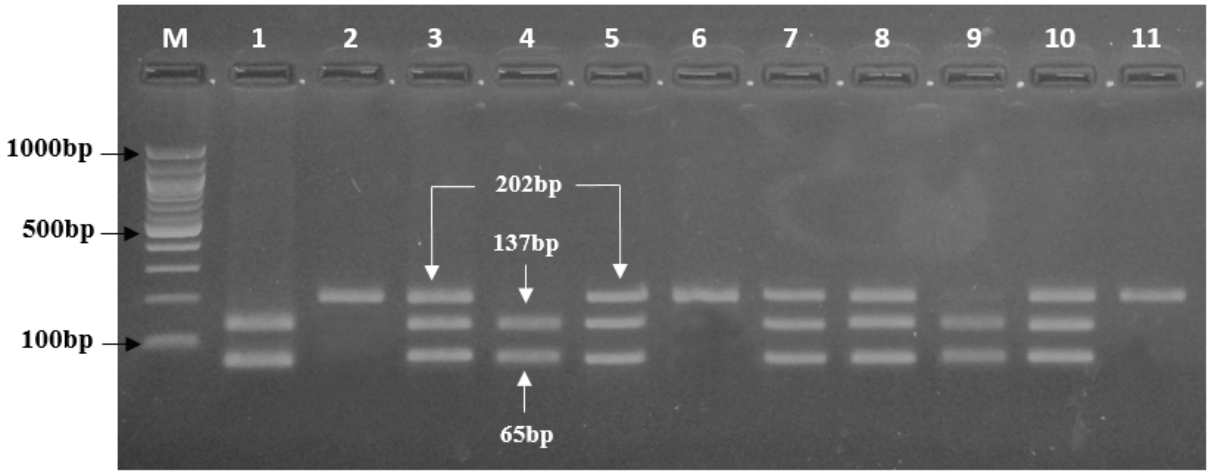

Figure (1): Gel electrophoresis carried on (agarose gel (2%), 75% V, 20 mA for 1hr.) for NKG2D gene (rs1049174). PCR products visualized under U.V. light after staining with ethidum promaide. M: 100 bp DNA marker; the size of product is 202bp and 137+65 bp. The G/G(1,4,9), G/C(3,5,7,8,10) and C/C(2,5,11).

Association of rs1049174 genotypes with PTB was tested, and the results revealed that CC genotype was more associated with pulmonary tuberculosis, while G/C genotype conferring lesser pulmonary tuberculosis susceptibility for the carrier individuals with odd ratio (95% CI) of 0.36 (0.16-0.78), P=0.04.

Table (1): Genotype association and allele's frequency of rs1049174 with PTB.

Genotype	Control	Case	OR (95% CI)	P-value
C/C	22 (36.7%)	37 (61.7%)	1.00	0.046
G/C	30 (50%)	18 (30%)	0.36 (0.16-0.78)	
G/G	8 (13.3%)	5 (8.3%)	0.37 (0.11-1.28)	
Allele Frequency				
C	74 (62%)	92 (77%)	2.042 (1.166-3.578)	0.017
G	46 (38%)	28 (23%)	0.49 (0.28-0.86)	
*(P<0.05), OR: odd ratio, CI: confidence interval				

Genotypic Characterization of NKG2-D (rs2255336) with PTB

The rs2255336 (which partially cover the exon 9 of NKG2-D) were also screened in the present study, and these fragments consist of 201bp.

The PCR products of rs2255336 were subject to single strand conformation polymorphism (SSCP) to detect any small alteration in PCR-amplified product (SNPs). A composition of 12% nondenaturing PAGE of polyacrylamide gels was used for electrophoresis of PCR-amplified product with specialized buffer systems for 20-24hrs and then it is subjected to silver staining to visualize bands.

The DNA polymorphism appears as same band pattern on polyacrylamide gels, the results revealed that rs2255336 gene did not have any polymorphism, and to confirm the results, some samples from patients and control were sent to the macrogen company in South Korea to read the DNA sequencing of amplicons for SNPs detection and After alignment results of sequencing of the 201bp of rs2255336 gene showed 100% sequences similarities between the sequenced samples and the intended reference target sequences.

The results of the current study agree with many studies that reported the implication of rs1049174, produced by a substitution of the C by G nucleotide base in the NKG2D gene with a vast number of diseases [9-11].

In this study, the rs1049174 C/C genotype was significantly associated with susceptibility to PTB, while, the difference in the rates of G/C and G/G genotypes between patients and control, may be associated with decrease the risk of pulmonary tuberculosis (P=0.04) as shown in table (1).

The results of this study were incompatible with results obtained by weiwei *et al.,* [10] who found that the patients who carry the rs1049174 GG genotype have high chance to get infection and more susceptible to develop cancer and have a multivariate odds ratio (OR) of 1.85 (95%CI): 1.02–3.38.

On the other hand, the study finding of rs2255336 genotype revealed unnoticed of any mutation and disassociation of this SNP with PTB.

Ana Paula *et al*, [12] mentioned in study about NKG2D expression from NK-cell in *M. tuberculosis* infection, that there is no change in the expression of these receptors in response to *M. tuberculosis* infection and suggested that although NKG2D receptors are known to distinguish stressed and viral infected cells, but the expression of these specific receptors might not be required for responsiveness to *M. tuberculosis*. This cannot be firmly concluded, since cells other than NK cells can express these receptors.

Genotypic Characterization of NKG2-F (rs1841958)

The amplification of NKG2F gene at region rs1841958 was also investigated and the results of genotype association and allele's frequency for patients and control are listed in table (2). The results revealed that there were insignificant allele frequency differences between patients and control group (P >0.05).

The RFLP studies of PCR products of rs1841958 gene revealed three genotypes; C/C, A/C and A/A as shown in figure (2).

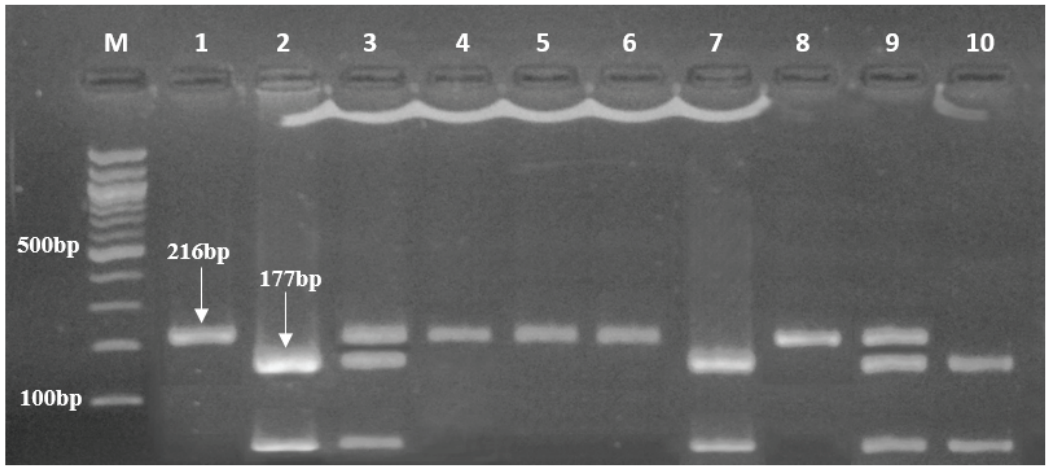

Figure (2): Gel electrophoresis carried on (agarose gel (2%), 75% V, 20 Am for 1hr.) for NKG2F gene (rs1841958). PCR products visualized under U.V. light after staining with ethidum promaide. M: 100 bp DNA marker; the size of product is 216bp, 177+39bp. The C/C(1,4,5,6,8), A/C(3,9) and A/A(2,7,10).

Association of rs1841958 genotypes with PTB was further tested. The result displays that there was insignificant association of any genotypes with pulmonary tuberculosis.

Table (2): Genotype association and Allele's frequency of rs1841958 with PTB.

Model	Control	Case	OR (95% CI)	P-value
C/C	22 (36.7%)	23 (38.3%)	1.00	0.98
A/C	24 (40%)	24 (40%)	0.96 (0.35-2.60)	
A/A	14 (23.3%)	13 (21.7%)	0.89 (0.28-2.85)	
Allele Frequency				
C	68 (56.7%)	70 (58.3%)	1.071 (0.572-2.003)	0.83
A	52 (43.3%)	50 (41.7%)	0.934 (0.499-1.748)	
*(P<0.05), OR: odd ratio, CI: confidence interval				

Interestingly, the rs1841958 SNP in NKG2F is missense mutation, and to validate the result of RFLP and to detect the type and the exact location of this SNP in NKG2F gene, some DNA samples were sent to Macrogen Company to read the DNA sequencing of rs1841958 amplicons. The sequencing analyzed and alignment results of rs1841958 gene 216bp revealed the presence of one nucleic acid substitution SNP which is G133T and was detected in this study in the investigated P1-P5samples as shown in figure (3).

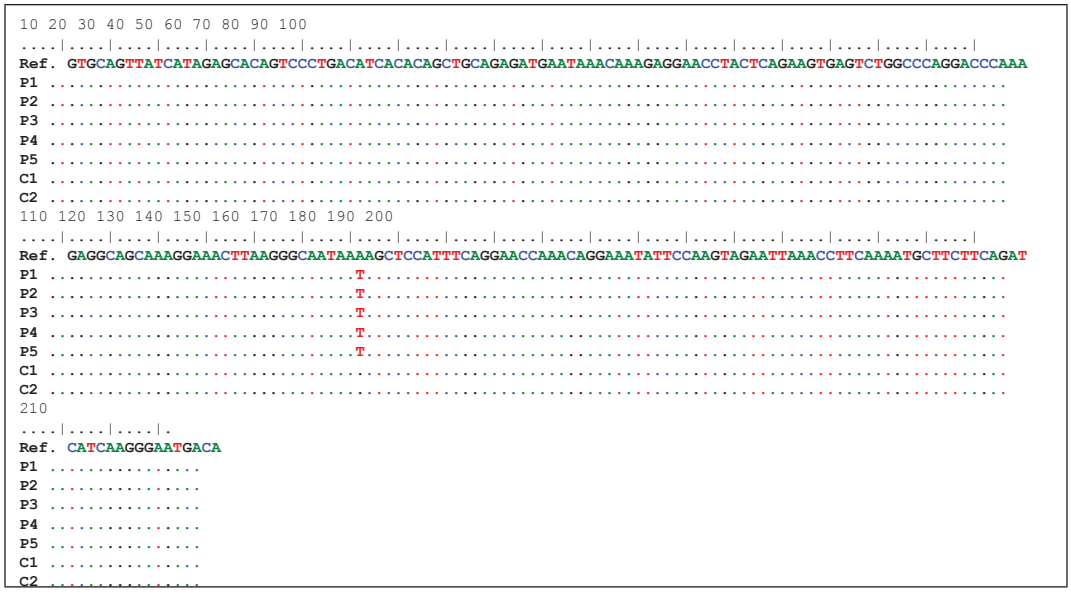

Figure (3): DNA sequences alignment of DNA samples with their corresponding reference sequences of the 216 bp amplicons of rs1841958. The symbol "ref" refers to the NCBI referring sequence; "P1-P5" refers to the samples; "C1-C2" refers to control.

However, this SNP leads to amino acid substitution represented by the conversion of Isoleulcine with Valine at 29th position (I29V) of the mature NKG2-F type II integral membrane protein and also this SNP was not reported in the ClinVar database too. The sequencing chromatogram of the identified variation region revealed that this SNP was found in a heterozygous status in both P1 and P2, and in a homozygous status in P3,P4 and P5.. Whereas C1 and C2 had exhibited a normal homozygous (A/A) status for this specified locus.

Hayashi *et al,* [13] reported the implication of NKG2F (rs1841958) SNPs produced by a substitution of the C by A nucleotide base with reduced peripheral blood leukocyte cytotoxicity and increased incidence of microbial infection and/or cancer.

The strongest linkage peak in Turkish familial with some disease was reported in persons have rs1841958 SNP in NKG2-F [14]. The NKG2F gene, encodes the C-type lectin receptor, the function of which is largely unknown. A possible hint to its function might be found in a related family member, NKG2D, encoded by KLRK1 and also located within the same disease-associated haplotype block [15].

The results of the association of rs1841958 in NKG2F gene with pulmonary tuberculosis revealed insignificant association of any genotypes and any allele variant in these SNPs with pulmonary tuberculosis.

This study may consider the first study that investigates the role of NKG2D and NKG2F with PTB in Iraq and also among few studies that investigate the role of these genes with PTB in the word.

Conclusion

The polymorphism of NKG2D gene at (rs1049174) was appear significant allele frequency differences between patients and control ($P<0.05$). The genotype C/C revealed significant association with disease ($P<0.05$). On the other hand, the NKG2D (rs2255336) revealed no gene variation among patients and control subjects.

The polymorphism of NKG2F gene at (rs1841958) showed insignificant allele and/or genotypic association with pulmonary tuberculosis ($P>0.05$).

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

Funding: Self-funding

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