RESEARCH ARTICLE

Immune Molecular Characterization of NOD Like Receptors (NOD1, NOD2) in some Immune Cells of Patients with Urinary Tract Infection

Hassan Mazin[1], Ali H. Alwan[2]*

[1,2]*Department of Biology, College of Science, University of Mustansiriyah, Baghdad, Iraq*

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ABSTRACT

Urinary tract infection (UTI) is considered the second bacterial infection globally that causes harmful and bad effects on human health. This study was performed on 62 isolates and blood patients. Definitive diagnosis of positive UTI patients was based on the growth of urine bacteria on blood agar and MacConkey agar. Depending on the characteristic examination, morphological of the colonies phenotypic and microscopic when grown in differential and selective media, as well as biochemical tests, 31 isolates identified as *E.coli* (45.16%), *Klebsiella pneumoniae* (19.35%)*, Pseudomonas aeruginosa* (9.67%), *Enterobacter spp.* (9.67%), *Staphylococcus aureus (*6.45%), *Enterococcus spp*.(3.22%), *Acinetobacter spp.* (3.22%) and *proteus spp.* (3.22%). Genotypic detection was done by using conventional polymerase chain reaction (PCR) for the Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene by using four primers responsible for the inflammatory response in the human body. All four primers designed for the human NOD2 gene were 100% positive. Sequencing for 60 samples of NOD2 gene PCR products was done to detect variations in genes of the Iraqi population which 11 SNPs have been detected and From all the 11 SNPs detected, it showed 121 of 623 samples have heterozygous nucleotides. Serum of 30 patients and 28 healthy controls with age range (14–88) years and for both genders were included to measure the level of IL-18 and TNF-α as an immunological parameter for NOD-like receptor activation against UTIs. The findings of comparison between patients and healthy control for TNF-α level showed there is a significant correlation in group B (40–64) years and C (65–88) years, (P-value 0.003 and 0.01). For IL-18 level, it showed there are significant in the group C (65–88) years, (p-value = 0.04).

Keywords: IL-18, NOD-like receptor, NOD2 gene, TNF-α, UTIs.

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INTRODUCTION

Urinary tract infection (UTI) is an infection that affects part of the urinary tract. About 150 million people get infected by UTIs, and without treatment, it could be fatal.[1] Both gram-negative and gram-positive bacteria can cause urinary tract infection, *E.coli* is the most common cause of UTIs. Females are more susceptible to get urinary tract infections than males because a woman's urethra (the tube from the bladder to where the urine comes out of the body) is shorter than a man's. This makes it easier for bacteria to get into the bladder. UTIs can be more classified into complicated UTIs and uncomplicated UTIs, which the first type can be riskier on some people who had health problems.[2,3] Innate immunity is the first line of defense against infections, it works by complex strategies and different mechanisms. One of these mechanisms is done by specialized cells called pattern recognition receptors (PRR). Pattern recognition receptors are germline-encoded host sensors that detect molecules typical for the pathogens[4] and ATP, nucleic acid, flagellin, etc.

The molecules found on the pathogen called pathogen-associated molecular patterns (PAMPs). Five types of pattern recognition receptors are found[5] toll-like receptors (TLRs), nucleotides binding and oligomerization domain (NOD)-like receptors (NLRs), a retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), C-type lectins (CTLs), and absent-in-melanoma (AIM)-like receptors (ALRs). One of the crucial members of PRR is the Nucleotide-binding and oligomerization domain (NOD) like receptors which is a cytoplasmic receptor that can detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).[6] The activation of NLRs leads to activate multiple signaling pathways, including nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and type I interferon (IFN) response, resulting in processing and induction of pro-inflammatory and anti-microbial responses.[7] The NOD-like receptor contains 22 members found in the human body. Each member has a specific function, but the structure is the same, which contains the N-terminal effector

**Author for Correspondence: dralialameri587@gmail.com*

domain, central NOD (NACHT: NAIP, CIITA, HET-E, and TP-2), and C-terminal leucine-rich repeats (LRRs).[8] The most important members are the NOD1 and NOD2. NOD1 can detect γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and initiates inflammation,[9] while NOD2 can activate the NF-κB protein and start the immune response by recognizing the muramyl dipeptide (MDP).[10,11] The activation of NLRs is divided into four groups; inflammasome formation by activating of caspase-1, which in turn will process pro-inflammatory cytokines the interleukin (IL)-1β and IL-18 as well as pyroptosis.[12] Signaling transduction activation will start by NOD1 and NOD2 by recognizing the γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), leading to the transcription of pro-inflammatory cytokines. The third group which is called Transcription activation can activate by NLRA (CIITA); this protein works as a transactivator on the expression of MHC class II gene.

Autophagy, the last group, works by specialized organelles called Autophagosomes, making the cell autodigest its cytoplasm by lysosomal enzymes.[13]

MATERIALS AND METHODS

Patients and Samples Processing

Thirty-one patients with urinary tract infections were enrolled in this study. The patients were followed up in Ghazi Al-Hariri hospital in the department of the medical city in Baghdad between December 2019 and March 2020. Data regarding age, gender, and treatment medical history were also included. Urine was collected from the catheter and midstream urine and cultured on Blood agar, a general medium for the growth of both gram-negative and gram-positive bacteria, and MacConkey agar, a selective and differential medium for gram-negative bacteria. Growth was seen after incubation at 37°C for 24 hours, and identification was performed using microscopic examination and standard biochemical tests.

Collection of Blood Samples

Five mL of blood samples were collected from patients suffering from UTIs to study serum levels and for DNA extraction for genetic and molecular studies.

Measurement of Serum Cytokines

To explore the interaction between the bacteria and host immune response, serum levels of TNF-α and IL-18 were measured in all patients and control (healthy) group by Enzyme-Linked Immunosorbent Assay (ELISA) sandwich kit. The commercial ELISA kits used for TNF-α and IL-18 were from BTlab - Zhejiang, China. The protocols used followed the instructions from the manufacturer.

Tumor Necrosis Factor Alpha

Serum levels of tumor necrosis factor-alpha (TNF-α) were measured using an Enzyme-Linked Immunosorbent Assay (ELISA) sandwich kit, the plate has been pre-coated with human TNF-α antibody, streptavidin-HRP was also added to sample wells and standard wells. After adding 50μL of stop solution, the yellow color will appear using a microplate reader; each microwell's optical density (OD) was measured at 450 nm.

Interleukin 18

Serum levels of interleukin 18 (IL-18) were determined using an Enzyme-Linked Immunosorbent Assay (ELISA) sandwich kit; the plate has been pre-coated with human IL-18 antibody then streptavidin-HRP was added to sample wells and standard wells. After adding 50 μL of stop solution, the yellow color will appear, the optical density (OD) of each microwell was measured at 450 nm using a microplate reader.

Human DNA Extraction

According to manufacturer instructions using Wisard® genomic DNA purification kit (Promega Corporation – Madison, USA), human DNA was extracted by taking 300 μL of blood samples and adding cell lysis solution to destroy the red blood cells, then adding nuclei lysis and protein precipitation solution, the supernatant which contains the DNA was transferred and mixed with isopropanol in a new tube, after adding DNA rehydration solution the DNA was stored at -20°C for further analysis and PCR amplification.

Protocol of Gene Amplification

Conventional polymerase chain reaction technique was carried out to amplify the NOD2 gene. Each PCR mixture consisted of 12.5 μL of green master mix, 1 μL of forward and reverse primer, 5 μL of extracted DNA and 5.5 μL of nuclease-free water to reach 25 μL as a total volume, and then the PCR mixture was transported to a thermal cycler to start the reaction depending on insertion program. Four primers were used, rs2066842-F (5`-GGCAGGACAGATATCAGAATAC-3`), rs2066842-R (5`-GCAGTGCTCAAAGAGTAG AG-3`), rs2066844-F (5`-CACTTTCCAGTGCT TCTTTG-3`), rs2066844-R (5`-CAGGGTGGTT GTAGAGATTAG-3`), rs2066845-F (5`-TGGA CACCGTCTGGAATA-3`), rs2066845-R (5`-TCCTCCCAAGAGCCTTATAG-3`), rs2066847-F (5`-GCAGGTACTTAACCACTA TCC-3`) and rs2066847-R (5`-CTGGAAACA AGAAGGACAAATC-3`).

The amplification program was run as 95° for 5 minutes, thirty cycles (95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec) and 1 final extension 72°C for 7 minutes and hold phase 10°C for 10 minutes. Agarose gel electrophoresis was used to amplify the products that were run in 1.5% agarose and stained by ethidium bromide.

DNA Sequencing

Sanger sequencing used for PCR products using ABI3730XL, an automated DNA sequencer, by Macrogen Corporation-Korea. The results were received then analyzed using Geneious software.

RESULTS AND DISCUSSION

Identification of Urine Samples

Most samples, when cultured on blood agar and MacConkey agar for 24 hours and 37°C, showed growth of *E. coli* bacteria found in the intestines on the fecal matter, and few amounts can enter and multiply in the bladder by the urethra.

Most UTIs cases are caused by Uropathogenic Escherichia coli (UPEC) (about 80%), which can encode additional genes and virulence factors like adhesins, toxins, iron acquisition systems, and additional metabolic enzymes that enable the UPEC to take advantage and infect the host urinary tract.[14,15]

Detection of Target Gene by Conventional PCR

The Nucleotide-binding Oligomerisation Domain 2 (NOD2) protein is encoded by the Caspase Recruitment Domain 15 (CARD15) gene, which found on chromosome 16.[16] Human DNA was detected by NOD2 gene by using conventional PCR technique. The gene products were confirmed by a run in 1.5% agarose gel electrophoresis stained with ethidium bromide, then electrophoresis in 100 volts for 50 minutes, and finally photographed under UV transilluminator.

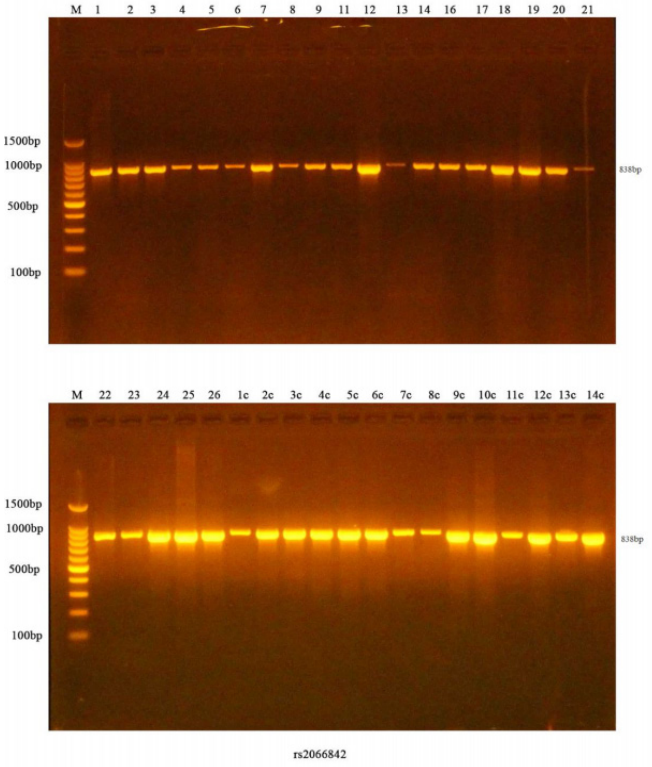

Figure 1: Results of the amplification of rs2066842 primer of Human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1- 14C resemble 838bp PCR products.

Table 1: The percentage of isolates according to the type of bacteria

Bacteria	*No. of isolate*	*Percentage %*
E.coli	14	45.16%
K. pneumoniae	6	19.35%
P. aeruginosa	3	9.67%
Enterobacter spp.	3	9.67%
S. aureus	2	6.45%
Enterococcus spp.	1	3.22%
Acinetobacter spp.	1	3.22%
Proteus spp.	1	3.22%
Total	31	100%

Studies have identified several Single Nucleo-tide Polymorphisms (SNPs) in the NOD2 /CARD15 gene, these SNPs have shown to be related with some pathogens which cause diseases to human. This study used four SNPs primers (rs2066842, rs2066844, rs2066845, and rs2066847) to amplify genomic DNA isolated from patients and control by PCR. All 60 samples showed positive results (Table 1).

Amplification of SNP (rs2066842) shows positive results of all isolates resembling 838bp (Figure 1).

Amplification of (rs2066844) was positive for all DNA samples that resemble 911bp PCR products (Figure 2).

NOD2 (rs2066845), when amplified, was all positive results resembling 952bp PCR products (Figure 3).

Amplification of (rs2066847) was positive for all isolates resembling 944bp PCR products (Figure 4).

NOD2 Gene Sequencing

Direct sequencing was used for 240 samples to detect SNPs within these sequences then compare it with the reference sequence of NOD2 gene in the National Center of Biotechnology Information (NCBI) Gene Bank.

This is the first study to give gene variation of inflammatory NOD2 in Iraqi bacterial infection patients. The most common type of human genetic variation is SNPs, which account for two to three cm of the human genome.[17] In addition, protein expression or structure directly affect by SNPs which exhibit high genetic stability, therefore, underlie numerous genetic

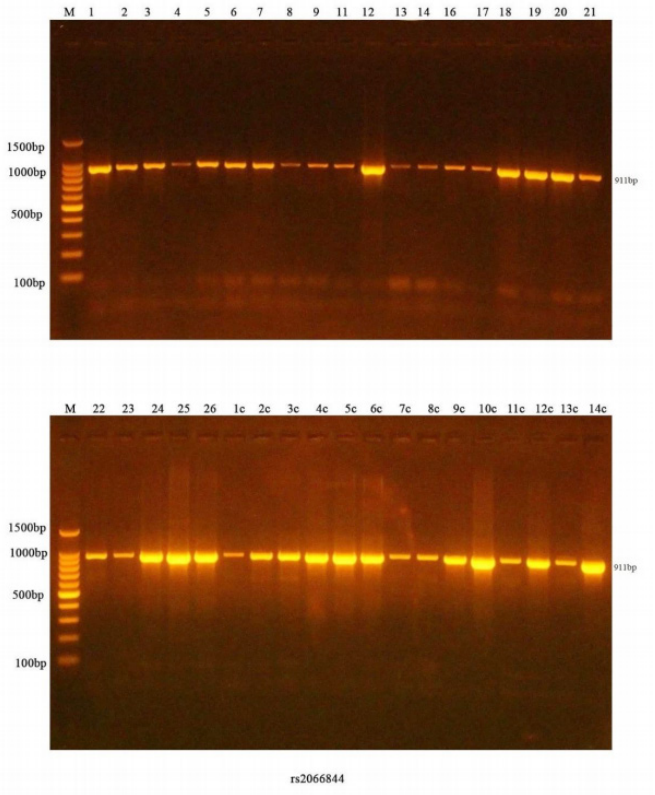

Figure 2: Results of the amplification of rs2066844 primer of Human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1- 14C resemble 911bp PCR products.

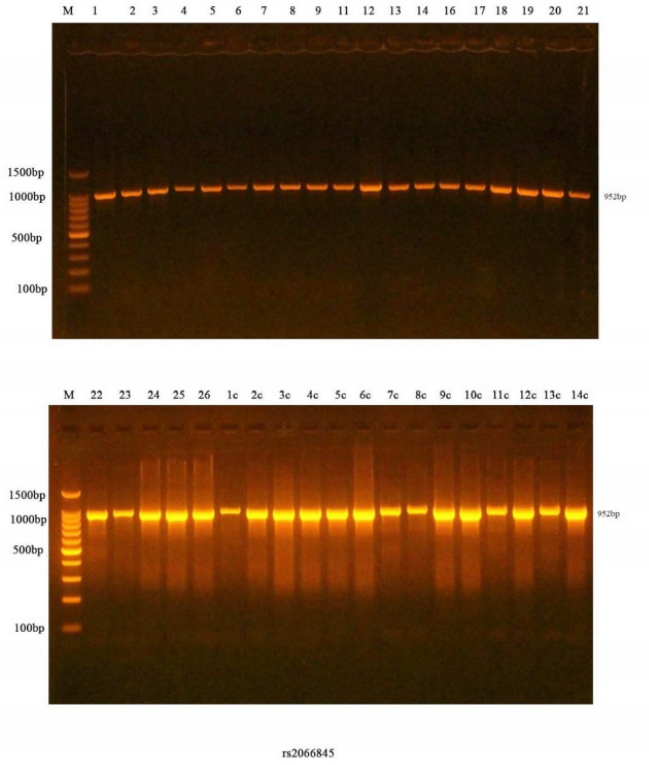

Figure 3: Results of the amplification of rs2066845 primer of Human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1- 14C resemble 952bp PCR products.

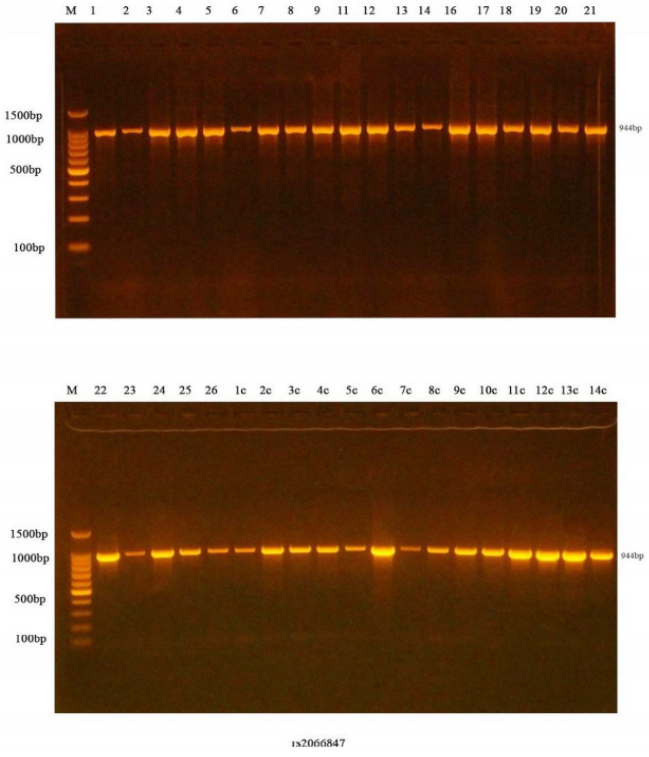

Figure 4: Results of the amplification of rs2066847 primer of Human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1- 14C resemble 944bp PCR products.

disorders.[18] Multiple SNPs has been detected when it compared to the database like rs773749264, rs5743278, rs2067085, rs211-1234, rs2111235, rs34939799, rs20-66847, rs73575774 and rs73575775.

The SNP (rs2066842) involves a C to T change, 57 patients and controls enrolled, 33/57 CC (57.89%), 20/57 CT (35.08%) and 4/57 TT (7.01%). The SNP (rs773749264) enroll 57 samples, 44/57 GG (77.19%), 12/57 GA (21.05%) and 1/57 GT (1.75%). For SNP (rs2066844), 56 samples enrolled, all 56 showed single "C" peak indicative of a C homozygous allele (100%). The SNP (rs5743278) involves "C" to "G" change, 56 samples enrolled, 54/56 CC (96.42%) and 2/56 CG (3.57). For SNP (rs206-7085), 55 samples enrolled, 25/55 CG (45.45%), 9/55 GG (16.36%), 20/55 CC (36.36%) and 1/55 CA (1.81%). For SNP (rs2111234), 55 samples enrolled, 23/55 GA (41.81%), 27/55 AA (49.09%) and 5/55 GG (9.09%). For SNP (rs21-11235), 55 samples enrolled, 23/55 GA (41.81%), 27/55 AA (49.09%) and 5/55 GG (9.09%). For SNP (rs2111235), 55 samples enrolled, 23/55 GA (41.81%), 27/55 AA (49.09%) and 5/55 GG (9.09%). SNP (rs349-39799), 55 samples enrolled, 53/55 CC (96.36%) and 2/55 CT (3.63%). The SNP (rs2066847) showed 59 samples with homo-zygous allele (100%). SNP (rs73575774) shows 53 of 59 with homozygous allele GG (89.83%) and 6 of 59 with heterozygous allele GA (10.16%). The SNP (rs73575775) shows 53 of 59 with homozygous allele AA (89.83%) and 6 of 59 with heterozygous allele GA (10.16%). From all the 11 SNPs detected for NOD2 gene during sequencing to detect variations in Iraqi population showed 121 of 623 samples have heterozygous nucleotides.

Immunological Tests

For comparison, 28 healthy controls were included in this study, which were negative for UTIs. According to their ages, the patients have been divided into 3 groups; group A (14–39) years, group B (40–65), and group C (65–88). The comparison was between patients and healthy controls to find a significant correlation in cytokine production (Tumor Necrosis Factor-alpha and interleukin 18). For TNF-alpha, it showed there is a significant correlation in group B (40–64 years) (P-value 0.003) and group C (65–88 years) (P-value 0.01).

For IL-18 it showed there is a significant correlation in group C (65–88 years) (P value 0.04). When it's made statistical analysis for TNF-α, it found the highest significance when compared the patient to the control in the age (40–64) years (P-value 0.003).

Statistical analysis for IL-18 found the highest significance when it compared the patient to the control in the age (65-88) years (P-value 0.04).

During urinary tract infection, some immune cells like uroepithelial cells and macrophages provide pro-inflammatory cytokines and chemokines, including TNF-α and other cytokines. So it increases to enhance the antibacterial defenses.[19,20]

IL-18 is a pleiotropic cytokine required to manage both innate and acquired immune responses.[21] IL-18 increases in number -besides IL-1β- and trigger classic components of the

inflammatory response due to activation of the inflammasome, as eight members of NLRs can activate the inflammasome, especially the NLRP3, which play an essential role in urologic pathologies.[22]

In 2018, Zheng *et al.*[23] studied the expression of pro-inflammatory cytokines in mice during pneumococcal meningitis infection. He saw that the level of TNF-α was elevated in the mouse brain, which explains that the NOD2-RIP2 pathway is required to trigger inflammatory cytokine and chemokine production during the infection.

Jürgen *et al.*[24] studied the cytokine level of IL-18 in patients who have Crohn's disease, and he noticed that IL-18 was highly expressed in the intestinal epithelial cells; he noticed the NOD2 was also overexpressed in intestinal mononuclear and epithelial cells in Crohn's disease patients,[25] so he observed that there is an interaction between IL-18 and NOD2 genotype in patients with Crohn' disease.

Some studies approved an association between NOD2 polymorphisms and susceptibility to Crohn's disease.[26,27] Wang *et al.*[28] performed a study on the Dutch population cohort in 2014 for rs2066844, rs2066845 and rs2076756, and he found no interaction between patients infected in the urinary tract NOD2 protein.

Other studies suggest that immunity does not depend on NOD2 to defend against the pathogen, like Jeong *et al.,*[29] who showed that there is no need for NOD2 to start the innate immunity and prevent the pathogenicity of *Yersinia enterocolitica* as bacterial clearance and serum cytokine production levels upon systemic infection are indistinguishable between NOD2 mice and control mice.

CONCLUSION

The most common bacterial species found in patients with urinary tract infection was *E.coli* in 14 total 31samples (45.16%). All four primers designed for the human NOD2 gene were 100% positive. All the 11 SNPs detected for the NOD2 gene during sequencing to detect variations in the Iraqi population showed that 121 of 623 samples have heterozygous nucleotides. When compared to the patient to the control for TNF-alpha, the highest significance was in the age (40–64) years (P-value 0.003). The highest significance compared the patient to the control for IL-18 was in the age (65–88) years (P-value 0.004). The isolates possessed NOD2 gene responsible for activating the inflammatory response, which in turn synthesized the TNF-α and IL-18 at high levels in patients, and these results were confirmed by sequencing and compared with the reference sequence of NOD2 approved by NCBI.

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