Research Article

TLR-8, TNF-α, and ESR-1α Gene Polymorphism Susceptibility in Onset of Arthritis

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Arthritis is a genetic disorder characterized by bones and joint degradation assisted by severe pain and inflammation. It is evident by the studies that 0candidategenes variations play vital role in its development and progression. Therefore, we investigated the genetic variation of TLR-8, TNF, and ESR-1α genes in the Pakistani population. A case-control study comprising 300 RA, 316 OA, and 412 control subjects was conducted. PCR-RFLP and direct sequencing methods were used for determining genetic variations. Analysis was performed by using PLINK and MEGA6.0 software. Allelic and genetic frequencies of polymorphisms identified on rs3764879 (TLR-8), rs3764880 (TLR-8), rs5744080 (TLR-8), rs1800629 (TNF), rs2228480 (ESR-1α), and rs1451501590 (ESR-1α) were significantly varied among RA, OA, and controls. Novel functional mutations SCV000844945 and SCV000844946 on TLR-8 as well as a non-functional SCV000804801 and functional variation SCV000804802 on ESR-1α were also identified and reported for the first time in the studied population. Multiple site analyses indicated that polymorphisms on TLR-8 and ESR-1α genes were significant risk factors in disease onset to the next generation. In conclusion, TLR-08 and ESR-1α were significant in the onset of arthritis whereas the TNF was not found as a significant risk factor in the onset of RA and OA.

1. Introduction

Rheumatoid arthritis (RA) and osteoarthritis (OA) both are bone disorders characterized by the mutual interactions of environment and genetics. However, RA is an autoimmune disorder whereas OA is non-autoimmune but in both diseases bones and joints get affected and degraded one way or another [1]. Innate and adaptive immunities enable the immune system to fight against multiple disorders. Innate immunity detects the presence of antigens through pattern recognition receptors (PRRs) recognized specific pathogenic molecules in microbes along with host endogenous ligands and leads to the pro-inflammatory cytokines, costimulatory molecules, and interferon (IFNs) expression [2,3]. The PRR was further classified into two major classes, i.e., cytosolic and transmembrane. Toll-like receptors (TLRs) are the first known transmembrane PRR. In humans, total of ten TLRs were classified on cell surfaces (TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR-10) and in endosomes (TLR-3, TLR-7, TLR-8, and TLR-9) [4]. TLR-8 functions like TLR-7 as involved in the reorganization of self RNA, within snRNP autoantibodies complexes, viral RNA, and multiple small molecule agonists [5–10]. However, TLR-8 is more likely activated by AU-rich ssRNA and senses it through its secondary structures [6, 11]. TLR-8 expressed itself in myeloid DCs, monocytes, and neutrophils [6, 12, 13]. Human TLR-8 got activated by the endogenous ligands might lead to the onset of many inflammatory diseases such as rheumatoid arthritis RA as well as OA.

Tumor necrosis factor-alpha (TNF-α), a pleiotropic and pro-inflammatory cytokine, is encoded by the TNF-α gene...
which is allocated on chromosome 6:31 [14]. In RA patients, its overproduction primarily by macrophages in joints causes the establishment of rheumatoid synovitis, formation of pannus tissue as well as the destruction of the joint. Its elevated level results in the increase of synoviocyte proliferation and leads to the activation of a secondary mediators’ cascade, playing a role in inflammatory cell recruitment, joint destruction, and neo-angiogenesis [15]. It along with interleukin-1β (IL-1β) increased impaired cartilage function and stiffness of articular chondrocytes [16, 17]. In vitro studies indicated that pro-inflammatory TNF-α upregulates mRNA matrix metalloproteases-1 and -3 chondrocyte which leads to the pathogenic of OA as a potential risk factor [16–18]. TNF-α mRNA expression in knee OA patients was found to be 1.56 times greater as compared to controls [14].

Estrogen Receptor 1 (ESR-1) gene encodes for estrogen receptors (ERs), located on chromosome located on 6q25 [19]. The main determinant of bone strength is the cortical bone dimensions, and it is regulated by the estrogen receptor (ER) mediated and mechanical loading (see [20, 21]). It has been evident that estrogens function as a protector against bone matrix loss and are mediated by estrogen receptor-alpha (ER-α) [22, 23, 24]. It stimulates transcription through activation functions (AFs), i.e., AF-1 located on N-terminal and AF-2 located in the ligands binding domain [25]. ER-α activates the genes by the hormone-receptor binding which is then followed by gene activation with estrogen response element (ERE) having promoters. It increased the transcriptional activity from ERE-reporter transfected into osteoblast cell-line [26]. We have recently found that PADI-4 gene polymorphism was not only involved in the onset of RA but was also found to be a significant risk factor in OA onset [1]. Both RA and OA are genetic disorders attributed to the environment and daily lifestyle, but the etiology of both diseases is still a mystery concerning the Pakistani population; therefore, the current study was designed to determine the polymorphism of TLR-8, TNF, and ESR-1α gene and its association with the onset of both RA and OA.

2. Materials and Methods

2.1. Ethical Approval and Sampling. The current study was approved by the ethical committee of the Department of Zoology, University of the Punjab, Lahore, Pakistan, and ethical committees of the government and semi-government hospitals of Punjab province of Pakistan from where samples were collected. All participants were already diagnosed with RA and OA by the physician. Blood samples (3 cc) were collected from each subject in EDTA coated tubes with BD syringes after taking informed written consent from the patient/guardian along with their clinical data. Blood samples were also collected from age and sex-matched healthy control subjects with a negative family history of arthritis. Samples were stored at 4°C before being further processed.

2.2. Genotyping. DNA was extracted from each blood sample of controls as well as patients manually by using a modified organic DNA extraction method [27] and was quantified (conc. ng/μl) and qualified (260/280) by using Nanodrop. Three SNP’s rs3764879, rs3764880, and rs5744080 on TLR-8; two SNP’s rs1800629 and rs361525 on TNF, and four SNP’s rs2234693, rs9340779, rs2228480, and rs1451501590 on ESR-1α were selected on basis of their either direct or indirect involvement in either the pathogenesis or progression of disease. For the amplification of targeted polymorphic sites, specific primers used are presented in Table 1. The polymerase chain reaction (PCR) was conducted in a 25 μl reaction mixture consisting of 10 μl of master mix (Thermo Scientific), 3 μl of forward and reverse primer each, and 12 μl of DNase, RNase-free water. The PCR conditions include initial denaturation at 94°C for 5 mins followed by 35 repeated cycles of denaturation at 94°C for 45 secs, annealing temperature presented in Table 1 for 45 secs, extension at 72°C for 30 secs, and then final extension at 72°C for 10 mins. The products were run on 2% agarose for confirming their product sizes. Genotyping was performed by restriction fragment length polymorphism (RFLP) and direct sequencing. For RFLP, PCR products were digested with their respective enzymes (Table 1) and were run on 2% agarose gel. For the direct sequencing method, PCR products were sequenced by using a sequencer, and sequences were visualized on BioEdit software.

2.3. Statistical Analysis. The genetic data for each subject was tabulated and passed through Hardy Weinberg Equilibrium (HWE) Test. Allelic and Genotypic Test was performed. The significance level was determined by the chi-square test and Fisher exact test. Linkage Disequilibrium and Haplotype analysis were performed fanalysesple site analysis. The change in the amino acid sequence was determined by MEGA 6.0 software.

3. Results

The mean age of RA males was 38.62 years and that of RA females was 38.98 years with a mean age of diagnosis in years was 33.06 and 31.56, respectively. Both RA males and females were in the normal BMI range, i.e., 18.75–25.5 kg/m². The mean age of OA males was 55.27 years and for females was 49.19 years with a mean age of diagnosis in years was 46.97 and 42.73, respectively. In the OA group, obesity, i.e., BMI range of 25.5–30.5 kg/m² was predominating.

It was observed that allele G on rs3764879 (TLR-8) was prevalent in patients whereas on rs3764880 (TLR-8) allele G got replaced by allele A in patients. On the rs5744080 (TLR-8) polymorphic site, mutant allele T was prevalent among RA and OA individuals as compared to controls. Two intrinsic novel mutations were also identified of about 50bp G>C and 39bp T>A before the rs3764879 (TLR-8) polymorphic site. The novel SNP’s G>C and T>A identified through sequencing were submitted to ClinVar NCBI for obtaining accession numbers SCV000844945 and SCV000844946, respectively. Allele C replaced allele Ton rs1800629 (TNF) polymorphic site in both cases whereas on rs361525 (TNF) polymorphic site allele C was found to be prevalent among patients as well as controls. No mutation
| Gene     | SNP ID | F.P      | R.P         | A.T (°C) | Product size (bp) | RFLP Enzyme | I.T (°C) | T.D.T (°C) | Fragments size (bp) |
|----------|--------|----------|-------------|----------|------------------|-------------|----------|------------|---------------------|
| TLR-8    | rs3764879 | GTGTGTGTCTGATTTGGTG | TTTCTAGGCTCACACCATTGG | 58       | 390              | HpyCh4IV    | 65       | —          | CC: 390, CG: 390, 228, 162, GG: 228, 162 |
|          |        |          |             |          |                  |             |          |            | AA: 39, AG: 390, 291, 990, GG: 291, 99, CC: 416, CT: 416, 234, 182, TT: 234, 182 |
|          | rs3764880 |          |             |          |                  | NlaIII      | 37       | 65         | CC: 390, CG: 300, 228, 162, GG: 228, 162 |
|          | rs5744080 | CCCCCAATACCCTCTGGTTTT | ATGGAAGCACCACCATCACA | 58       | 416              | HpyCh4IV    | 65       | —          | AA: 226, AG: 226, 202, GG: 202, GG: 226 |
| TNF      | rs1800629 | AGGCAATAGGTTTGGAGGGCAT | ATCTGGAGGAAGCGGTAGTG | 59.5     | 226              | NcoI        | 37       | 65         | CC: 636, CC: 636 |
|          | rs361525 |          |             |          |                  | MspI        | 37       | 65         | AA: 246, CC: 636, GG: 636 |
| ESR-1a   | rs2234693 | CTGCCCATTTCCTATTCCTCC | TCCTTCTGCCACCCCTGGCGATTATTATCTGA | 59       | 636              | PvuII       | 37       | 80         | CC: 636, CC: 636 |
|          | rsS9340779 |          |             |          |                  | XbaI        | 37       | 65         | AA: 246, CC: 636, GG: 636 |
|          | rs2228480 | GTGGAGGAGACCCGACACAA | TGGCCACTCATCTAGAAAGCC | 60.5     | 246              | BglI        | 37       | 80         | AA: 246, 145, 101, GG: 145, 101 |
|          | rs1451501590 |          |             |          |                  |             |          |            | —                   |

F.P: forward primer; R.P: reverse primer; A.T: annealing temperature; RFLP: restriction fragment length polymorphism; I.T: incubation temperature; T.D.T: thermal deactivation temperature.
exists on rs2234693 (ESR-1α) and rs9340779 (ESR-1α), i.e.,
allele G was found to be significant among patients as well as
in controls whereas on rs2228480 (ESR-1α) allele G was
more prevalent in patients as compared to controls. Similarly,
on the rs1451501590 (ESR-1α) site, allele G replaced
allele C in patients. Two novel mutations were also
identified, i.e., SCV000804801 (23G>A) and SCV000804802
(241T>A).

All SNPs followed HWE (p = 1.00), and it was observed
that in RA individuals except for rs3764879 (TLR-8) allelic
frequency of rs3764880 (TLR-8), rs5744080 (TLR-8),
SCV000844995 (TLR-8), and SCV000844996 (TLR-8),
polymorphic sites were not significantly varied in com-
parison to controls. However, in OA cases, except for
SCV00084495 (TLR-8), rest of the SNPs were not signifi-
cantly associated with the onset of disease at the allelic
level. As a result of the genotypic analysis, it was observed
that except for rs3764879 (TLR-8), all other SNPs were signifi-
cantly associated with the onset of RA and except for
rs5744080 (TLR-8) all other SNPs were significant risk
factors for OA onset. It was observed that rs1800629 (TNF)
was not significantly associated with the onset of disease
at an allelic level; however, it is significantly associated
at the genotypic level. In RA, subjects rs2228480 (ESR-1α),
SCV000804801 (ESR-1α), and SCV000804802 (ESR-1α)
were significantly associated with the onset of disease at an
allelic and genotypic level whereas rs1451501590 (ESR-1α)
was not found to be a significant risk factor in disease onset.
In OA, subjects rs2228480 (ESR-1α), rs1451501590 (ESR-
1α), SCV000804801 (ESR-1α), and SCV000804802 (ESR-1α)
were significantly associated with the onset of disease at both
allelic and genotypic levels (Table 2).

The linkage disequilibrium (L.D) for RA and OA is
presented in Figures 1(a), 1(b), 2(a), and 2(b). As a result of
L.D, it was observed that in both RA and OA individuals, all
SNPs together were significant risk factors with the onset of
disease as D' = 1.000; r2 = 0.405 and D' = 0.660; r2 = 0.350. It
was observed that SCV000844945 and SCV000844946 were
also significant risk factors for the next generation in the
onset of disease. As no mutation was observed at rs2234693
and rs9340779 polymorphic sites, therefore, they were ex-
cluded from multiple site analyses. As a result of L.D for RA
subjects, it was observed that rs2228480, rs1451501590,
SCV000804801, and SCV000804802 together were 100%
disease onset risk factors in the next generation as D' = 1.000
and r2 = 0.360 whereas it also increased the risk of OA onset
up to 71% in next-generation D' = 0.717; r2 = 0.423. The L.D
for ESR-1 is presented in Figures 3(a) and 3(b) for RA and
Figures 4(a) and 4(b) for OA, respectively.

On the TLR-8 gene, it was shown that all haplotypes were
significant (p < 0.01) for the start of disease because their
frequency was higher in patients compared to control par-
ticipants. This was true for both RA and OA. However,
the frequency of CACGT and CATGT was higher in con-
trols; therefore, they act as protectants in the onset of disease.
ESR-1α haplotype analysis indicated that the frequency of
AAGT was higher in controls as compared to patients;
therefore, it acts as a protectant in RA and OA onset. The
significant haplotype in disease onset is presented in Table 3.

Change in amino acid sequences with respective polymor-
phic sites is presented in Table 4.

4. Discussion

RA and OA are the two major types of arthritis characterized
as multifactorial disorders such as age, BMI, autoimmunity,
hormonal, environmental, and genetics which play role in
their development and progression. Essential information
has been developed concerning the genome-wide causative
mutations and their inheritance through multiple tech-
niques including genome-wide association studies
(GWASs), candidate gene association studies Twin, linkage
analysis, segregation analysis, and twin studies [28]. Genetic
association studies in different populations evidenced that
disease development and progression risk are complex
processes that might interact with innate as well as acquired
host responses to inflammatory, biomechanical, hormonal,
environmental, and immunological stimuli [29]. Therefore,
the current study targeted for the first time to determine the
association of TLR-8, TNF, and ESR-1α gene polymorphism
with the onset of not only RA but also OA in the Pakistani
population.

Currently, the study demonstrated that rs3764879 of
TLR-8 was associated with RA onset at an allelic level
whereas rs3764880 and rs5744080 were associated at the
genotypic level. In OA development, rs3764879 and
rs3764880 were significant risk factors genotypically. Two
novel functional mutations never reported before, i.e.,
rs3764879 and rs3764880 leads to a decrease in inflammatory cytokines
[30]. Polymorphism on rs3764880 leads to a decrease in inflammatory cytokines
levels. This polymorphism in the start codon causes TLR-8’s
first three amino acid deletions giving rise to the TLR8v2
isoform [31]. A study conducted on OA Chinese individuals
reported rs5744080 polymorphisms as a significant risk
factor in the onset of disease in male subjects [32]. It is well
documented that variants on the TLR-8 genes were associ-
ated with autoimmune and infectious diseases like sys-
temic lupus erythematosus, type 2 diabetes, and tuberculosis
[33–35]. However, more studies should be conducted to
determine its association with RA and OA development.

The current study revealed a strong association of
Rs1800629 polymorphic site variation with both RA and OA
development. In the pipeline of current findings, the same
functional polymorphism rs1800629 with RA as well as OA
onset was also reported in the north Indian population,
Chinese population, and Egyptian population [36–38]. In
contrast, no association between rs1800629 and RA was
reported in the Brazilian population [39]. On the other hand,
the current study reported no association between rs361525
and disease development. However, contrary to current
findings, another study conducted on the Pakistani
Table 2: Single site analysis of all targeted polymorphic sites in RA as well as OA subjects.

| Gene          | Allele/genotype | RA frequency (P/C) | $X^2$ value | $p$ value | OA frequency (P/C) | $X^2$ value | $p$ value |
|---------------|-----------------|-------------------|-------------|-----------|-------------------|-------------|-----------|
| Rs3764879     |                 |                   |             |           |                   |             |           |
| C             | 0.057/1.000     | 1290.075          | 0.002*      |           | 0.361/1.000       | 729.016     | >0.01     |
| G             | 0.943/0.000     |                   |             |           | 0.639/0.000       |             |           |
| GG            | 0.887/0.000     |                   |             |           | 0.278/0.000       |             |           |
| CG            | 0.113/0.000     | 711.999           | >0.01       |           | 0.722/0.000       | 727.999     | 0.002*    |
| CC            | 0.000/1.000     |                   |             |           | 0.000/1.000       |             |           |
| Rs3764880     |                 |                   |             |           |                   |             |           |
| A             | 0.088/1.000     | 1219.788          | >0.01       |           | 0.196/1.000       | 1017.99     | >0.01     |
| G             | 0.912/0.000     |                   |             |           | 0.804/0.000       |             |           |
| GG            | 0.823/0.000     |                   |             |           | 0.608/0.000       |             |           |
| AG            | 0.177/0.000     | 712.000           | 0.001*      |           | 0.392/0.000       | 728.000     | 0.001*    |
| AA            | 0.000/1.000     |                   |             |           | 0.000/1.000       |             |           |
| Rs5744080     |                 |                   |             |           |                   |             |           |
| C             | 0.170/0.981     | 988.959           | >0.01       |           | 0.038/0.981       | 1297.66     | >0.001    |
| G             | 0.830/0.019     |                   |             |           | 0.962/0.019       |             |           |
| TT            | 0.660/0.000     |                   |             |           | 0.924/0.000       |             |           |
| CT            | 0.340/0.039     | 655.964           | 0.002*      |           | 0.076/0.039       | 688.920     | >0.01     |
| CC            | 0.000/0.961     |                   |             |           | 0.000/0.961       |             |           |
| SCV00844945   |                 |                   |             |           |                   |             |           |
| C             | 0.500/0.000     | 521.964           | >0.01       |           | 0.524/0.000       | 558.531     | 0.001*    |
| T             | 0.500/1.000     |                   |             |           | 0.476/1.000       |             |           |
| CC            | 0.000/0.000     |                   |             |           | 0.047/0.000       |             |           |
| CG            | 1.000/0.000     | 712.000           | 0.001*      |           | 0.953/0.000       | 728.000     | 0.001*    |
| GG            | 0.000/1.000     |                   |             |           | 0.000/1.000       |             |           |
| SCV00844946   |                 |                   |             |           |                   |             |           |
| A             | 0.500/0.000     | 521.964           | 0.000       |           | 0.741/0.000       | 899.208     | >0.01     |
| T             | 0.500/1.000     |                   |             |           | 0.259/1.000       |             |           |
| AA            | 0.000/0.000     |                   |             |           | 0.481/0.000       |             |           |
| AT            | 1.000/0.000     | 712.000           | 0.001*      |           | 0.519/0.000       | 727.999     | 0.002*    |
| TT            | 0.000/1.000     |                   |             |           | 0.000/1.000       |             |           |
| Rs1800629     |                 |                   |             |           |                   |             |           |
| G             | 0.722/0.000     | 753.867           | >0.01       |           | 0.910/0.000       | 1023.640    | >0.01     |
| A             | 0.228/1.000     |                   |             |           | 0.090/1.000       |             |           |
| GG            | 0.543/0.000     |                   |             |           | 0.820/0.000       |             |           |
| AG            | 0.457/0.000     | 600.000           | 0.001*      |           | 0.180/0.000       | 616.000     | 0.002*    |
| AA            | 0.000/1.000     |                   |             |           | 0.000/1.000       |             |           |
| TNF           |                 |                   |             |           |                   |             |           |
| Rs361525      |                 |                   |             |           |                   |             |           |
| G             | 1.000/1.000     | —                 | —           |           | 1.000/1.000       | —           | —         |
| GG            | 1.000/1.000     | —                 | —           |           | 1.000/1.000       | —           | —         |
| Gene       | Allele/genotype | RA frequency (P/C) | $X^2$ value | $p$ value | OA frequency (P/C) | $X^2$ value | $p$ value |
|------------|-----------------|-------------------|-------------|-----------|-------------------|-------------|-----------|
| Rs2234693  | C               | 1.000/1.000       | —           | —         | 1.000/1.000       | —           | —         |
|            | CC              | 1.000/1.000       | —           | —         | 1.000/1.000       | —           | —         |
| Rs93407779 | C               | 1.000/1.000       | —           | —         | 1.000/1.000       | —           | —         |
|            | CC              | 1.000/1.000       | —           | —         | 1.000/1.000       | —           | —         |
| Rs2228480  | A               | 0.127/1.000       | 930.177     | 0.002*    | 0.286/1.000       | 675.414     | 0.002*    |
|            | G               | 0.873/0.000       | 0.714/0.000 | 0.073/1.000 | 0.427/0.000       | 530.493     | 0.002*    |
|            | AA              | 0.000/1.000       | 0.000/1.000 | 0.000/1.000 | 0.000/1.000       | 0.000/1.000 | 0.000/1.000 |
|            | AG              | 0.253/0.000       | 600.000     | 0.002*    | 0.500/0.000       | 0.500/0.000 | 0.500/0.000 |
|            | GG              | 0.747/0.000       | 0.500/0.000 | 0.500/0.000 | 0.500/0.000       | 0.500/0.000 | 0.500/0.000 |
| Rs1451500  | A               | 0.342/1.000       | 588.819     | >0.01     | 0.509/1.000       | 393.256     | 0.001*    |
|            | G               | 0.658/0.000       | 0.491/0.000 | 0.041/1.000 | 0.041/1.000       | 0.041/1.000 | 0.041/1.000 |
|            | AA              | 0.057/1.000       | 0.057/1.000 | 0.057/1.000 | 0.057/1.000       | 0.057/1.000 | 0.057/1.000 |
|            | AG              | 0.570/0.000       | 535.645     | >0.01     | 0.937/0.000       | 566.126     | 0.001*    |
|            | GG              | 0.373/0.000       | 0.022/0.000 | 0.022/0.000 | 0.022/0.000       | 0.022/0.000 | 0.022/0.000 |
| ESR-1α     | A               | 0.455/0.000       | 353.398     | 0.001*    | 0.562/0.000       | 473.449     | 0.001*    |
|            | G               | 0.545/1.000       | 0.438/1.000 | 0.123/0.000 | 0.877/0.000       | 616.000     | 0.002*    |
|            | AA              | 0.545/1.000       | 0.438/1.000 | 0.123/0.000 | 0.877/0.000       | 616.000     | 0.002*    |
|            | AG              | 0.910/0.000       | 500.917     | 0.001*    | 0.877/0.000       | 616.000     | 0.002*    |
|            | GG              | 0.090/1.000       | 0.000/1.000 | 0.000/1.000 | 0.000/1.000       | 0.000/1.000 | 0.000/1.000 |
| SCV000804801 | A            | 0.437/1.000       | 335.181     | 0.002*    | 0.628/0.000       | 564.014     | 0.001*    |
|            | T               | 0.563/1.000       | 0.372/1.000 | 0.256/0.000 | 0.744/0.000       | 616.000     | 0.002*    |
|            | AA              | 0.563/1.000       | 0.372/1.000 | 0.256/0.000 | 0.744/0.000       | 616.000     | 0.002*    |
|            | AT              | 0.613/0.000       | 354.907     | 0.001*    | 0.744/0.000       | 616.000     | 0.002*    |
|            | TT              | 0.257/1.000       | 0.000/1.000 | 0.000/1.000 | 0.000/1.000       | 0.000/1.000 | 0.000/1.000 |
Figure 1: (a, b) Linkage disequilibrium chart of TLR-8 gene polymorphic sites in RA patients and controls.

Figure 2: (a, b) Linkage disequilibrium chart of TLR-8 gene polymorphic sites in OA patients and controls.

Figure 3: (a, b) Linkage disequilibrium chart of ESR-1α gene polymorphic sites in RA patients and controls.
### Table 3: Haplotype analysis of TLR-8 and ESR-1α gene.

| Haplotype: rs3764879, rs3764880, rs5744080, SCV000844945, SCV000844946 | TLR-8 |  | O A frequency (P/C) | p value |  | Haplotype: rs2228480, rs145105190, SCV000804801, SCV000804802 | ESR-1α |  | OA frequency (P/C) | p value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CACGT | 0.000/0.981 | 0.006* | CACGT | 0.000/0.981 | 0.001* | AAGT | 0.025/1.000 | 0.002 | AAGT | 0.000/1.000 | 0.002 |
| CATGT | 0.000/0.019 | 0.0005* | CATGT | 0.000/0.019 | 0.0004* | AAAT | 0.003/0.000 | — | AAAA | 0.218/0.000 | 0.002 |
| CGCCA | 0.014/0.000 | 0.005* | CATCA | 0.075/0.000 | 0.001* | AGGT | 0.098/0.000 | 0.001 | AGAA | 0.005/0.000 | — |
| CGCCCT | 0.014/0.000 | 0.005* | CATCT | 0.040/0.000 | 0.009* | GAGA | 0.311/0.000 | 0.001 | AGGA | 0.064/0.000 | 0.001 |
| CGCGA | 0.014/0.000 | 0.005* | CGTCA | 0.176/0.000 | 0.001* | GAGTCA | 0.013/0.000 | 0.001* | GATCA | 0.038/0.000 | 0.008* |
| CGCGT | 0.014/0.000 | 0.0005* | CGTCT | 0.070/0.000 | 0.001* | GATCT | 0.018/0.000 | 0.001* | GATCT | 0.068/0.000 | 0.001* |
| GATCA | 0.013/0.000 | 0.001* | GATCA | 0.013/0.000 | 0.009* | GATGA | 0.018/0.000 | 0.001* | GGCCT | 0.038/0.000 | 0.008* |
| GATCT | 0.018/0.000 | 0.001* | GATCT | 0.068/0.000 | 0.001* | GGTA | 0.130/0.000 | 0.001* | GGTA | 0.068/0.000 | 0.001* |
| GATGA | 0.024/0.000 | 0.006* | GGTGC | 0.476/0.000 | 0.002* | GGCGA | 0.024/0.000 | 0.006* | GGTGC | 0.476/0.000 | 0.002* |
| GGCGA | 0.024/0.000 | 0.006* | GGTGC | 0.476/0.000 | 0.002* | GCGA | 0.024/0.000 | 0.006* | GGTGC | 0.476/0.000 | 0.002* |
| GGCCT | 0.024/0.000 | 0.006* | GGTGC | 0.476/0.000 | 0.002* | GCGT | 0.024/0.000 | 0.006* | GGTGC | 0.476/0.000 | 0.002* |

### Table 4: Change in amino sequence by MEGA 6.0 software.

| Gene | SNP ID | Wild amino acid | Mutant amino acid |
|---|---|---|---|
| TLR-8 | Rs3764880 | Valine | Methionine |
|  | Rs5744080 | Histamine | Histamine |
|  | SCV000844945 | Arginine | Threonine |
|  | SCV000844946 | Phenylalanine | Isoleucine |
| TNF | Rs1800629 | Upstream transcript variant | Phenylalanine |
|  | Rs361525 | Upstream transcript variant | Pheny alanine |
|  | Rs2228480 | — | 3 prime UTR variant |
| ESR-1α | Rs2228480 | Threonine | Threonine |
|  | SCV000804802 | Phenylalanine | Isoleucine |
|  | Rs145150190 | — | — |
population reported a positive association between rs361525 and OA development in the Pakistani population [40]. Like the findings of the current study, no positive association has been reported in the Chinese, Mexican, and Iraqi populations [40–42].

It was declared from the present study that no association exists between rs2234693 and rs9340779 with the onset of both RA as well as OA in the studied population. The current study reported that polymorphism on rs2228480 polymorphic site acts as a significant risk factor in the onset of both RA and OA. It was also revealed that rs1451501590 polymorphism was associated with OA development only. Although the vital role of estrogen in the pathogenesis of RA was indicated by many studies, no significant effects were reported by estrogen receptor agonists on RA symptoms [43]. Van Vollenhoven [44] reported a lack of clinical advantages of selective ESR-1α agonist treatment in RA patients. Like current findings, no association was reported between RA and rs2234693 and rs9340779 polymorphic sites in the Polish and Japanese populations [43]. Studies conducted in Caucasian, European, and American populations reported a non-significant association between rs2234693 and rs9340779 and RA [45, 46]. In contrast to current findings, strong association of rs2234693 and rs9340779 with OA was reported in Japan, Korean, and Mexican populations [47, 48]. A meta-analysis conducted by Hu et al [49] reported that polymorphism on rs2234693 might reduce the risk of OA risk and the rs9340779 may not be associated with OA risk in the Chinese population. A weak association relationship in rs9340779 and OA was reported in Europeans but not Asians whereas rs2234693 was not significantly associated with OA in both populations [50]. Another study conducted by Ma et al. [51] reported that the risk of OA incidence decreased with rs9340779 and rs2228480 whereas rs224693 was a strong factor in disease onset. Jin et al. [52] reported in their case-control study that the minor allele of rs2228480 was an increased risk of knee OA in his Meta-analysis, indicated the same results. The current study also reported the identification of two significant risk SNPs, i.e., SCV000804801 and SCV000804802. SCV000804802 was a functional novel SNP and caused the replacement of phenylalanine with isoleucine.

ESR-1α polymorphisms altered the estrogen receptor expression in the ESR-1, and ESR-2 genes could affect the expression that leads to immunological consequences. The effect of estrogen was mediated by estrogen receptors located in the multiple immune cells (T cells, B cells, monocytes, and macrophages) as well as in the thymus [53]. This effect can be achieved by directly manipulating the profile of T-helper cytokine from interleukin (IL)-2, TNF-α, and interferon (IFN)-γ (pro-inflammatory) to IL-4, IL-6, and IL-10, transforming growth factor (TGF)-β (anti-inflammatory) direction [54].

The current study concluded that TLR-8 and ESR-1α gene polymorphism are the significant risk factors in the onset of both RA as well as OA individuals in the Pakistani population. However, larger scale studies in other populations should be conducted to determine novel mutation susceptibility.

**Data Availability**

The data used to support the findings of this study are available from the corresponding author upon request.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest.

**Authors’ Contributions**

All authors contributed equally to this study.

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