Association between the rs2234671 polymorphism and the risk of recurrent urinary tract infections in Iraqi women

Hussein Mahmood Abbas\textsuperscript{a,b}, Harith Jabbar Fahad Al-Mathkhury\textsuperscript{a,*}

\textsuperscript{a} Department of Biology, College of Science, University of Baghdad, Iraq
\textsuperscript{b} Department of Medical Laboratory Techniques, Al-Esraa University College, Iraq

1. Introduction

The recurrent urinary tract infection (rUTI) can be defined as the occurrence of a UTI with symptoms that encountered at least three times in the same year followed by previous symptoms of UTI (Giarenis and Robinson, 2016). rUTI is one of the most common diseases affecting women around the world. Women have 50% higher chance of developing a UTI (Griebling, 2005) in their lives with a 25% chance of recurring infection (Scholes et al., 2000).

Interleukin-8 (IL-8) is the main pro-inflammatory cytokines, which are produced by several immune and non-immune cells (Munoz et al., 2005). IL-8 is also considered as a chemokine, and it is one of the members of a CXC chemokine subfamily, where exerts its biological activity via two G protein-coupled receptors called CXCR1 and CXCR2. CXCR1 receptor mainly binds IL-8, while CXCR2 receptor also binds IL-8, IL-7, IL-5, IL-3 and IL-2 (Yao et al., 2013; Visciano et al., 2015). The IL-8 attached with neutrophils through CXCR1 receptor usually begins with a series of signals that direct and control the movement of neutrophils, inflammatory mediator release, and phagocytic activity (Almontaser et al., 2017; Baggioolini, 2004).

The CXCR1 variant and polymorphisms were associated with IL-8 level in urine and asymptomatic bacteriuria. These findings propose an early in vivo association of genetic factors for the immune response to the bladder in human before the symptomatic UTI development (Hawn et al., 2009). The low levels of mRNA, reduce expression of ccr1 gene, and heterozygous polymorphisms ccr1 gene in patients with UTI are a good example of interindividual variability (Svanborg, 2013). The children who have suffered from acute pyelonephritis may be due to CXCR1 receptor deficiency. Similarly, the reason for the increased UTI susceptibility in premenopausal women may be due to the low level of CXCR1 receptor expression (Smithson et al., 2005). The low expression of the CXCR1 receptor may be due to functional genetic variation in ccr1 gene promoter, and the inability of elimination microorganisms due to defective neutrophil dependent defences (Svanborg et al., 2001).

In the ccr1 gene, one SNP rs2234671(+2607G/C) was detected which encodes for a missense mutation resulting from one polar amino acid change serine (Ser) to threonine (Thr). This mutation in the IL-8RA protein located at position 276 (Renzoni et al., 2000).

A case-control study was designed to explore the association of rs2234671, alongside with ccr1 gene expression and serum level of IL-8 with rUTI risk in a sample of Iraqi women.

2. Materials and methods

2.1. Ethical statement

All participants agreed to provide the investigator with blood specimens. Informed consent according to the Declaration of Helsinki was obtained from all participants.

2.2. Study groups

A total of 80 unrelated Iraqi females (aged 18 to 45 years) were included in the current study and distributed as follows: 40 of them were suffering from rUTI; thereby, considered as the case group. The other 40 women were apparently healthy and never been infected with UTI at all hence they represented the control group.

2.3. Specimen collection

Around 5 ml of venous blood were collected from healthy and rUTI women patients enrolled in this study and divided into two parts. The first part (3 ml) was kept in EDTA tube for DNA and RNA extraction; whereas the other part (2 ml) was submitted for serum isolation and used in estimation of IL-8 level.

2.4. DNA and RNA extraction

The genomic DNA and RNA were extracted using FavorPrep Blood Genomic DNA Extraction Mini Kit (Invitrogen/Taiwan) and Norgen’s Total RNA Purification Mini Kit, respectively, according to the recommended procedures.

\*Corresponding author.
E-mail address: harith.fahad@sc.uobaghdad.edu.iq (H.J.F. Al-Mathkhury).

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2.5. Amplification of target polymorphism in the cxcr1 gene

2.5.1. DNA extraction

The genomic DNA was extracted using FavorPrep Blood Genomic DNA Extraction Mini Kit (Invitrogen/Taiwan) according to the recommended procedures.

2.5.2. Primer selection

Specific primers for rs2234671 were provided in a lyophilized form (Eurofins, Germany) and dissolved in sterile nuclease-free water to give a final concentration of 100 μM and stored at −20 °C until use. At required, 10 μM concentration was prepared and used immediately. The investigated DNA sequences were amplified by the following primers: forward1 5′-CCCAGGTGATCCAGGAGAG-3′, forward2 5′-CTACCTGATAAGGGGTTCAGG-3′ and common reverse primer 5′- TCAGGGGTTGGAAGAGACATT-3′ (Renzoni et al., 2000).

2.5.3. Sequence-specific PCR and amplification conditions

A sequence-specific primer-polymerase chain reaction (SSP-PCR) method, was used to detect the rs2234671 polymorphisms in cxcr1 gene. The amplification conditions for PCR reaction was performed in a final reaction volume of 20 μl, excluding 10 μl of (Quick-Load* Taq 2 × Master Mix), 5 μl of template DNA (25–50 ng), 2 μl of each of forward and reverse primers (10 μM), and nuclease-free water was added to PCR mixture to get a final volume of 20 μl. The Eppendorf tubes were then briefly mixed by the vortex before being placed in the PCR apparatus. The PCR amplifications were applied according to Renzoni et al. (2000) with some modifications. The cycling parameters were applied as follows: 5 min at 96 °C, and 35 repeated cycles of 25 s at 96 °C for denaturation, 45 s at 62 °C for annealing and 30 s at 72 °C for extension and 1 cycle for 72 °C at 3 min. The resultant amplicons (205 bp) were resolved in 1.5% agarose gel.

2.6. Estimation of serum IL-8 levels

Two ml of venous blood was transferred into gel tube and let to clot; thereafter the serum was separated by centrifugation at 5000 × g for 5 min and kept frozen (−20 °C) during the period of sample collection until they were used for estimating the IL-8 level by ELISA technique (Biotech/Korea).

2.7. cxcr1 gene expression

2.7.1. RNA extraction

Total RNA was extracted using Total RNA Purification Mini Kit (Norgen, Canada) following the manufacturer protocol.

2.7.2. cDNA synthesis

cDNA was synthesized by reverse transcriptase polymerase chain reaction using the recommended protocol of Fast HiSenScript™RH (−) RT Premix (Intron/Korea) as follows: 5 μl (300 ng) of total RNA were completed to 20 with nuclease-free water (Biolab, USA). Samples were incubated in thermocycler T100 (Biorad, USA) at 42 °C for 30 min then 95 °C for 3 min., afterward, the reactions were stopped at 4 °C.

2.7.3. Primers selection

Specific primers for 18S (forward primer 5′-AAGTACGACCGCGGTACA-3′ and reverse primer 5′-AGCGCCGTCGAGCATGTATT-3′) and cxcr1 gene (forward primer 5′-TTTGTGTGTTGCGGTGTG-3′ and reverse primer 5′-AGTGTAGCGAGGTGAATCC-3′) were provided in a lyophilized form (Eurofins, Germany) and dissolved in sterile nuclease-free water to give a final concentration of 100 μM and stored at −20 °C until use. At required, 10 μM concentration was prepared and used immediately (Wedel et al., 2008).

2.7.4. qPCR protocol

PCR mixture was composed of 10 μl of Luna Universal qPCR Master Mix (2 ×), template cDNA (3 μl), sense and antisense primers (2 μl for each), and nuclease-free water was added to PCR mixture to reach a final volume of 20 μl. The Eppendorf PCR tubes are mixed briefly by the vortex before being placed in the Open qPCR thermocycler (Chai, USA). One cycle of an initial denaturation step was achieved in the reaction mixture at 96 °C for 5 min. Cycling conditions involved denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 1 min. Over 25 cycles Cauli et al. (1997). Expression levels were quantified using relative quantitation strategy. The results were normalized to 18S RNA (housekeeping gene) expression as shown below.

\[ \Delta C_t = C_t - C_{housekeeping gene} \]

Gene expression = \( 2^{-\Delta C_t} \) (1)

A melting curve with temperatures ranging from 60 °C to 95 °C was obtained, with a temperature increase of 1 °C every second.

2.8. Statistical analysis

The data were examined for normality, homogeneity and normal distribution, mean ± SE of mean by using the IBM SPSS version 25.0. The probability also examined by using student t-test and ANOVA. For non-parametric data, Pearson's chi-square test used to calculate the probability. For genotyping and allele frequencies, the odd ratio, 95% confidence interval and Fisher's exact probability calculated by WinPepi version 11.65. An online Hardy-Weinberg calculator used for genotyping and alleles frequencies calculations.

3. Results

3.1. rs2234671 polymorphism

Fig. 1 shows the specific band of rs2234671 polymorphism in cxcr1 gene at 205 p. These results represent the two alleles G and C in rs2234671 SNP in both case and control groups.

The results in Table 1 recorded the percentage of genotype and

![Fig. 1](image-url)
allele frequency for cxcr1 SNP (rs2234671) in case and control groups in the current study. The results revealed that GC genotype (62.5%) was more than other genotypes GG and GC in the case group, where the GC and CC genotypes were 30% and 7.5%, respectively. In contrast, the results in the control group reported a high percentage of GG genotype (77.5%) compared with GC and CC genotypes, where the GC and CC scored 17.5% and 5%, respectively. On the other hand, the results in Table 1 reported a high percentage of the G allele (61.3%) and (86.3%) in case and control group, respectively. Concerning C allele, a lower frequency percentage in both case (38.7%) and control (13.7%) groups were seen.

In regard to GG genotype, OR was 0.12 (95% CI = 0.05–0.34); therefore, the risk factor (0.67) is significantly considered protective factor (P = 4 × 10 \(^{-5}\)). Whereas OR of GC genotype was 7.86 (95% CI = 2.82–21.87); hence, risk factor (0.54) is significantly considered etiological factor (P = 7 × 10 \(^{-5}\)). The OR of CC genotype was 1.54 (95% CI = 0.25–9.53), upon that, it is considered insignificantly (P = 1) an etiological factor (0.026). Also, the OR of G allele was 0.25 (95% CI = 0.13–0.51), because of that, risk factor (0.64) is significantly assumed protective factor (P = 9.8 × 10 \(^{-5}\)) and the OR of C allele was 3.93 (95% CI = 1.97–7.83), thus, risk factor (0.29) is deemed to be a significant etiological factor (P = 9.8 × 10 \(^{-5}\)). Depending on these findings, the genotype GC is significantly associated with rUTI.

### 3.2. Estimation of IL-8 level

In the present study, the results of IL-8 levels in sera of the study group were significantly (P = 0.01) higher in cases than in controls (215.52 ± 131.25 vs 141.77 ± 7.21 pg/ml, respectively) as it is illustrated in Fig. 2.

### 3.3. cxcr1 gene expression

The gene expression of cxcr1gene was assayed by RT-PCR technique in case and control groups. Fig. 3 and 4 demonstrates the gene expression for the two genes and single peak for each one.

Insignificant differences (P = .706) were found in cxcr1 expression between the two groups (0.059 ± 0.01 and 0.063 ± 0.016 for control and case groups, respectively). The results in Table 2 pointed out to the insignificant differences between the gene expression level of cxcr1 between cases and controls according to the genotypes of SNP rs2234671.

### 4. Discussion

The results of the current study demonstrated compatibility with those obtained by Javor et al. (2012) who have suggested that the carriers of rs2234671 C allele have a significantly increased risk of developing recurrent APN. Moreover, the results of the current work are in agreement with those submitted by Han et al. (2019) who found that rs2234671 was associated with an increased risk of UTI under allelic comparisons C and G alongside with genotypes GG, GC and CC between cases and controls in children. Otherwise, the results of current study disagreed with the findings of a study carried out by Almontaser et al. (2017) as they have claimed that rs2234671 is not associated with APN in children and not differ significantly in children with lower UTI, APN and renal scar compared with the control group. This differences between these studies may be returned to the functional genetic variants in cxcr1 promoter which also associated with low expression of cxcr1 gene (Yang et al., 2009).

Albeit the significant difference between the IL-8 concentration of case and control groups, findings of the current study demonstrated an existence of interference. Approximately, 75% of case group records are within range of control group. In plain words, only 25% rUTI patients developed high IL-8 level. Thereby, it is hard to say that IL-8 level is a differential marker for rUTI. Furthermore, the data of control group seems more flexible than those of case group; besides, control data skewed down more than those of case group data. More likely, if the sample size was bigger, the results would reveal more interpretations.

The current results are in accord with Sheu et al. (2006) explaining a positive significant correlation between acute pyelonephritis and IL-8. Similarly, Mohkam et al. (2008) confirmed the increase in urinary IL-8 level in children with febrile UTI. Besides, Rodhe et al. (2009) compared the level of IL-8 in sera of patients with acute cystitis, ASB, and negative controls and they found significantly higher levels of urine IL-8, and IL-6 in subjects with ASB than in negative controls, and even

### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (n = 40)</th>
<th>Controls (n = 40)</th>
<th>OR (95% CI)</th>
<th>RF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>Observed</td>
<td>Expected</td>
<td>Observed</td>
<td>Expected</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>G</td>
<td>12</td>
<td>30</td>
<td>15.01</td>
<td>37.52</td>
<td>31</td>
</tr>
<tr>
<td>GC</td>
<td>25</td>
<td>62.5</td>
<td>18.99</td>
<td>47.47</td>
<td>7</td>
</tr>
<tr>
<td>CC</td>
<td>3</td>
<td>7.5</td>
<td>6.01</td>
<td>15.02</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>100</td>
<td>40</td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>

Fig. 2. Box plot of IL-8 concentration in case and control groups. Boxes range from the 25th to 75th percentile and are intersected by the median line. Asterisks denotes to the mean. Whiskers extending below and above the box range represent the maximum and minimum values, respectively. Outliers are indicated as individual data points.
higher levels in acute cystitis. Drage et al. (2019) noticed highly significant differences ($P = .0001$) in patients with rUTI compared with controls. On the other hand, Mahyar et al. (2013) observed an insignificant correlation between acute pyelonephritis and IL-8. Likewise, the study of Krzemien et al. (2004) showed that IL-6 and IL-8 are not suitable markers to distinguish the differentiation between APN and lower UTI in children up to 2 years old.

This controversy between these studies could be due to variables such as time of sampling, geographical area, the health aspect and perhaps a healthy diet that affects, in general, all the vital systems of the body, age of patients, some of the determinants belonging to the individuals themselves, and perhaps the most significant, the variability of strains. Knowing that during the infection process and in response to the tumour necrosis factor-$\alpha$ and IL-1, IL-8 as an inflammatory cytokine is released from a variety of cells like monocytes, neutrophils, and endothelial cells (Diepold et al., 2008). Drage et al. (2019) pointed out that IL-8 has a significant part in the pathophysiology of uropathogens colonisation and persistence. IL-8 is hormonal mediators which produced in response to infectious and inflammatory conditions in various tissues of the body (Hopkins, 2003). The elevated level of IL-8 in the tissue is very important for the rescue of bacterial infections (Brauner et al., 2001) due to increased trans-epithelial neutrophil migration, also the higher level of IL-8 contributes to the release of cytotoxic neutrophil products such as lysozymes, myeloperoxidase, elastase and eventually leads to tissue destruction (Leitch et al., 2008).

Obviously, the insignificant differences between controls and cases, in terms of ccr1 gene expression, would refer to the irrelevant role of ccr1 in the pathophysiology of rUTI. In that manner, other authors have pointed out similar findings. For instance, Frendeus et al. (2000) and Lundstedt et al. (2007) have found decreased surface expression and mRNA levels of ccr1 gene with APN-prone children and their family members. Moreover, Wedel et al. (2008) recorded that the expression levels of ccr1 was similar in tumour and normal prostate

<table>
<thead>
<tr>
<th>Genotyping</th>
<th>Gene expression level (mean ± SE)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>0.055 ± 0.010</td>
<td>0.074 ± 0.024</td>
</tr>
<tr>
<td>GC</td>
<td>0.070 ± 0.04</td>
<td>0.058 ± 0.022</td>
</tr>
<tr>
<td>CC</td>
<td>0.021 ± 0.002</td>
<td>0.068</td>
</tr>
</tbody>
</table>

The elevated level of IL-8 in the tissue is very important for the rescue of bacterial infections (Brauner et al., 2001) due to increased trans-epithelial neutrophil migration, also the higher level of IL-8 contributes to the release of cytotoxic neutrophil products such as lysozymes, myeloperoxidase, elastase and eventually leads to tissue destruction (Leitch et al., 2008).

Fig. 3. Amplification curves of ccr1, ccr2 gene.

Fig. 4. Amplification curves of 18S gene.
tissue. The low expression of cxcr1 receptor gene may be back to the highly specific for IL-8 while cxcr2 receptor gene is indiscriminate and binds to IL-8 as well as to other CXC chemokine’s that containing a common amino terminal Glu-Leu-Arg (Murphy, 1997). Additionally, the polymorphisms (Godaly et al., 2001) and functional genetic variants (Svanborg et al., 2001) in the promoter region of the cxcr1 gene could be related to the low level of expression of cxcr1 gene. The polymorphisms reducing cxcr1 gene expression were associated with acute pyelonephritis and an increased risk for renal scarring (Svanborg, 2013).

Disclosure

None to report.

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Declaration of Competing Interest

None.

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