Research article
Assessing miR-146a-5P with IL-6 and TLR-4 serum levels in diabetic mellitus patients with or without urinary tract infection

Rabiha Q. Thajel 1, Abdulaumeer M. Ghareeb1, Qays A. M. Al-Khafaji2

1Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Baghdad, Iraq
2Consultant Immunologist, Kamal Al-Samarraee Hospital for Infertility and IVF, Baghdad, Iraq

(Received: August 2023 Revised: September 2023 Accepted: October 2023)

Corresponding author: Rabiha Q. Thajel. Email: rabeha.abode2020@gmail.com

ABSTRACT

Introduction and Aim: Diabetes is a chronic metabolic disease that affects glucose levels. This condition increases blood sugar and weakens immunity, damaging neutrophil function and urine antibacterial activity. IL-6 and TLR-4 are involved in inflammation and immune system control, while miR-146-5p may be a biomarker for diabetes. This study examines IL-6, TLR-4, and miR-146-5p in diabetics with and without UTI infection.

Materials and Methods: In this study, blood samples were collected from 100 diabetic mellitus patients suffering from UTI in addition to 100 individuals apparently healthy as control. The serum levels of IL-6 and TLR-4 were detected using commercial BTab brand ELISA kits. The plasma RNA expression level of the miR-146a-5p was determined by real time-PCR using a specific primer for miR-146a-5p with the U6 gene utilized as a reference gene.

Results: The results showed that diabetic patient’s group had a significantly higher response of IL-6 than controls (75.6 vs. 27.6). TLR-4 levels showed high levels in diabetic patients’ group (1.3) in comparison with the control group (0.441). Both IL-6 and TLR-4 levels showed a significant difference between the diabetic patients with UTI compared to control. The results revealed no-significant differences in the levels among both diabetic patients with UTI and patients without UTI. TLR-4 and IL-6 showed a significant inverse relationship while the relation between TLR-4 level and miR-146a-5p expression level showed a negative relationship.

Conclusion: The levels of IL-6, TLR-4, and miR-146-5p expression were found to be considerably greater in individuals with diabetes having UTIIs compared to patients with diabetes alone. An inverse relationship existed between IL-6 and TLR-4 levels as well as between TLR-4 and miR-146a-5p. The expression levels of IL-6 and miR-146-5p were nearly equal.

Keywords: Diabetic mellitus; UPEC; IL-6; TLR-4; miR-146a-5p.

INTRODUCTION

Diabetes mellitus, commonly known as diabetes, is a chronic metabolic disorder that affects how the body utilizes glucose, the main source of energy for the cells in the body (1,2). Diabetes is characterized by elevated blood glucose levels, either as a result of inadequate insulin production (Type 1 diabetes) or ineffective insulin utilization (Type 2 diabetes). Diabetes mellitus must have an efficient management plan and be continuously monitored if there is any hope of preventing complications and keeping the patient's health at its highest possible level (3).

Urinary tract infections, also known as UTIs, are common bacterial disorders that can affect multiple parts of the urinary system, including the kidneys, ureters, bladder, and urethra (4). The most common bacterial species that causes urinary tract infections (UTIs) are Escherichia coli, and it is responsible for a sizable number of UTI cases, which can range anywhere from fifty percent to ninety percent (5). Other known bacterial types associated with UTIs are Proteus mirabilis and Proteus vulgaris (6). It has been established that diabetes mellitus and UTIs are closely related to one another (7). People who have been diagnosed with diabetes have a greater risk of developing UTIs in comparison to individuals who do not have diabetes. One of the variables that may contribute to this higher susceptibility is the presence of elevated glucose levels in the urine. Presence of glucose in urine is conducive for the proliferation of bacteria, causing UTIs (8). In addition to this, having high amounts of glucose in the blood has the potential to weaken the immune system, which in turn hinders the body's ability to successfully fight against infections. Urinary tract infections in diabetes mellitus patients could potentially lead to more serious complications, such as kidney infections or sepsis, while also making it more difficult to manage diabetes (9).

MiR-146a is a short noncoding RNA molecule with therapeutic and biomarker potential. Several disorders are associated with abnormal miR-146a expression (10). The microRNA miR-146a-5p has been studied for its potential role in the development and progression of diabetes mellitus and shown to play a role in regulating glucose metabolism and insulin signaling including IRS1, AKT2, and GLUT4. miR-146a-5p has also been implicated in the regulation of
inflammation and oxidative stress, dysregulation of which results in the development of diabetes (11). Thus, miR-146a-5p expression levels have been used as a biomarker in diagnosis and progression of the disorder in diabetes patients

Interleukin-6 (IL-6) is a cytokine protein that regulates immune system function and plays a role in inflammation (12). IL-6 is produced by a variety of cells in the body, including immune cells and adipose tissue, which are involved in a range of activities such as cell proliferation, differentiation, and survival (13). Dysregulation of IL-6 has been implicated in several diseases, including autoimmune disorders, cancer, and metabolic disorders such as obesity and diabetes mellitus (13). Investigations into the relationship between IL-6 and miR-146-5p, particularly in the context of inflammation and immune system regulation have shown that IL-6 can control the immune response by either upregulating or downregulating the miR-146-5p expression levels, while others have suggested that miR-146-5p control the inhibit IL-6 expression (14). A similar study, studying the relationship between miR-146-5ap and IL-6 in rheumatoid arthritis patients showed that miR-146-5p was upregulated in response to IL-6, and that miR-146-5p played a role in regulating the inflammatory response in rheumatoid arthritis (15).

Toll-like receptor 4 (TLR-4) is a receptor protein that is part of the innate immune system. It recognizes and responds to components of bacterial cell walls, and triggers an immune response to fight off infection. Dysregulation of TLR-4 has been implicated in a range of diseases, including autoimmune disorders, cancer, and metabolic disorders such as obesity and diabetes mellitus (16). TLR-4 and miR-146a-5p are molecules that have been studied for their roles in inflammation and immune system regulation (17). While few studies have shown that TLR-4 can upregulate miR-146-5p expression, others have suggested that miR-146a-5p can inhibit TLR-4 expression. The exact relationship between these two molecules is still not fully understood and requires further research. MiR-146a-5p was shown to be increased in response to TLR-4 activation and to have a role in regulating the inflammatory response in sepsis (18).

The purpose of this study was to determine the expression levels of IL-6, TLR-4, and miR-146a-5p in order to determine the link between each of these parameters in patients suffering from UTI and diabetes mellitus in comparison to healthy controls.

MATERIALS AND METHODS

The participants in this study were separated into three groups: those who had diabetes and had UTI infection (n=100), those who had diabetes but did not have an UTI (n=100), and healthy control subjects (n=100) who did not have diabetes or an infection of the urinary tract. The protocol for the study has been approved by the Ethics Committee of the University of Baghdad's Institute of Genetic Engineering and Biotechnology for Postgraduate Studies. The participants were given information regarding the voluntary nature of their participation, and they were required to submit written informed consent forms.

Samples

Blood (3 ml) was collected from each of the participants in the patients and control group. 100µl of the blood drawn was mixed with EDTA, transferred into a new sterile test tube, followed by addition of 200 µl of DNA/RNA shield and kept in the freezer until use. The rest of the blood was subjected to centrifugation for 20 min., and then the upper layer of serum transferred to a tube and used in ELISA. Urine s collected from each participant included in this study was diagnosed for urinary tract infection by culturing on selective media.

Determination of IL-6 and TLR-4 by ELISA

The ELISA kits that are commercially available from the Chinese company CUSABIO were used to test serum concentrations of human Toll-like receptor (TLR-4) and human Interleukin 6 (IL-6), respectively. Briefly, to ELISA plates pre-coated wells with human antibodies the given specimen was added. After that, the streptavidin-HRP conjugate is injected into the system, which makes it easier for it to bind to the antibody that has been biotinylated. After the incubation period has concluded, a subsequent washing step is carried out in order to remove any unbound streptavidin-HRP. After that, the substrate solution is added, which causes the formation of color that is directly proportional to the amount of human IL-6 and TLR4 that is present. After the addition of an acidic stop solution, the reaction is brought to a close, and the absorbance measured at a wavelength of 450 nm.

RNA extraction and cDNA synthesis

All blood samples have been subjected to extraction of RNA by Direct-zol RNA Miniprep (Zymo, USA, CAT# R2062). The TRI Reagent® prepared sample can be directly applied onto the Zymo-Spin IIC Column, followed by spinning, washing, and eluting the RNA. There is no requirement for phase separation, precipitation, or subsequent purification processes. The concentration and purity of miRNA were assessed in a random selection of five samples using the Quantus Fluorometer for calibration purposes. Following the extraction process, the samples were promptly subjected to conversion from RNA to cDNA using the PrimeScript™ RT reagent Kit. This kit is specifically developed to facilitate
reverse transcription, which is optimized for real-time RT-PCR. The tubes were subsequently inserted into a thermal cycler equipment manufactured by Applied Biosystems, located in the United States. The reaction mixture was subjected to incubation at a temperature of 37°C for a duration of 15 minutes, repeated three times for reverse transcription. Following this, the mixture was exposed to a temperature of 85 °C for a period of 5 seconds to inactivate the reverse transcriptase using heat treatment. Finally, the mixture was cooled to 4°C.

**Measurement of miR-146a-5p gene expression**

The quantification of miR-146a-5p gene expression was performed using the real-time polymerase chain reaction (PCR) method. The KAPA SYBR FAST qPCR Master Mix (2X) has been specifically formulated to facilitate high-performance real-time PCR. The kit includes a newly developed DNA polymerase that has been created through the process of molecular evolution. This has led to the production of a distinct enzyme that is specifically tailored for the purpose of conducting real-time quantitative PCR (qPCR) utilizing SYBR Green I dye chemistry. In this investigation, the RNU43 gene was employed as a reference gene alongside a specific primer for miR146a-5p, as indicated in Table 1. A total of 3 µl of the produced cDNA was used, and the volume was adjusted to 20 µl using nuclease-free water. The tubes were subsequently inserted into a thermal cycler apparatus and configured to undergo 40 cycles, each consisting of a 15-second incubation at 95°C followed by a 30-second incubation at 60°C. Subsequently, a temperature of 95 °C should be maintained for a duration of 5 minutes.

**RESULTS**

Table 2 summarizes the expression levels of IL-6, TLR-4 and MIR146-5p among the studied groups. The samples of UT infected patients that showed high presence of *E. coli* were chosen for further study. The results of IL-6 level showed a significant difference between the diabetic patients with UTI and without UTI and control group with a higher level of IL-6 in diabetic patients with UTI then followed by diabetic patients without UTI compared to control (78.34 and 69.11 vs. 40.92, respectively. p-value=0.001).

The findings of the TLR-4 analysis revealed a notably elevated level in individuals diagnosed with UTI compared to the control group (40.92 vs. 0.69, respectively). The p-value of 0.001 indicates that there is a statistically significant difference between the levels of TLR-4 in diabetic patients with UTI and those without UTI. However, the level of TLR-4 in diabetic patients without UTI (69.11) did not exhibit any significant differences when compared to both diabetic patients with UTI and patients without UTI. The findings of the study indicate that there was no statistically significant difference observed in the levels of miR146a-5p between diabetic patients with UTIs and those without UTIs (81.86 vs 48.42, respectively). However, it is worth noting that the control group exhibited a substantial difference in miR146a-5p levels compared to both diabetes patients with UTIs, with a 3.56-fold increase.

**Table 1:** Primers name and sequences used in this study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’-3’</th>
<th>Annealing Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR146a-5P-RT</td>
<td>GTTGGCTCTGTGCAAGGTTGGTCCAGGTAATTCG</td>
<td>55</td>
</tr>
<tr>
<td>miR146a-5P-F1</td>
<td>GTTGGTGAAGAATCTGAATTC</td>
<td></td>
</tr>
<tr>
<td>RNU43-RT</td>
<td>GTTGGCTCTGTGCAAGGTTGGTCCAGGTAATTCG</td>
<td></td>
</tr>
<tr>
<td>RNU43 F</td>
<td>GTGAACCTATTAGCGGCG</td>
<td></td>
</tr>
<tr>
<td>Universal Reverse</td>
<td>GTGCAGGGTCCAGG</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** IL-6, TLR-4 and miR-146a-5p expression levels in diabetic patient and control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Test</th>
<th>IL-6</th>
<th>TLR-4</th>
<th>miR146-5p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean</td>
<td>40.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.56&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>22.02</td>
<td>0.37</td>
<td>5.42</td>
</tr>
<tr>
<td></td>
<td>Std. Error of Mean</td>
<td>7.78</td>
<td>0.13</td>
<td>1.91</td>
</tr>
<tr>
<td>Diabetic and UTI</td>
<td>Mean</td>
<td>78.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.86&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>19.74</td>
<td>0.51</td>
<td>69.37</td>
</tr>
<tr>
<td></td>
<td>Std. Error of Mean</td>
<td>3.60</td>
<td>0.09</td>
<td>23.12</td>
</tr>
<tr>
<td>Diabetic without UTI</td>
<td>Mean</td>
<td>69.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>48.42&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation</td>
<td>7.11</td>
<td>0.33</td>
<td>74.22</td>
</tr>
<tr>
<td></td>
<td>Std. Error of Mean</td>
<td>2.68</td>
<td>0.12</td>
<td>28.05</td>
</tr>
</tbody>
</table>

The letters a, b, ab denote Duncun test; differences in letters indicate a significant difference and there is no significant change if the letters are not altered.

DOI: https://doi.org/10.51248/v43i5.3258
**Thajel et al: Assessing miR-146a-5P with IL-6 and TLR-4 serum levels in …… without urinary tract infection**

The findings of the study demonstrated a statistically significant negative correlation between the levels of TLR-4 and IL-6 ($r=0.780$, $p=0.001$). The relation between IL-6 and miR-146a-5p are represented in Fig. 2. The results showed an equilibrium plot ($r=0.041$, $p=0.984$). The relation between TLR-4 level and miR-146a-5p expression level are shown in Fig. 3. The results showed a significant negative relation between the levels of the parameters ($r= -0.100$, $p=0.633$).

**DISCUSSION**

In the urinary tract infected diabetes patients, the most prevalent bacterial species responsible for UTI was found to be *Escherichia coli* (data not shown). This is in agreement with a prior study in which uropathogenic *E. coli* was shown to be responsible for the high prevalence of UTIs in the Iraqi population.
The purpose of this research was to evaluate the levels of expression of IL-6, TLR-4, and miR146-5pa in diabetic patients, comparing those patients who had UTIs to those who did not have UTIs. The findings of the study showed that the levels of expression for IL-6, TLR-4, and miR-146-5p were significantly higher in diabetes patients who had urinary tract infections (UTIs) compared to diabetic patients who did not have UTIs and the control group. IL-6, a cytokine known for its function in starting immunological responses and inflammation as well as its pro-inflammatory qualities, has been related to the development of T2DM as well as UTIs. Studies have revealed that people who have type 2 diabetes and urinary tract infections have higher levels of the inflammatory cytokine IL-6 than healthy people do (20). As per Patil et al., (21) IL-6 levels in the serum of patients who had been diagnosed with UTIs were examined and compared to IL-6 levels in the serum of healthy people who served as controls. According to the findings of the research, patients who were diagnosed with UTIs had considerably higher mean serum IL-6 levels when compared to the control group, which was composed of healthy persons (21). Similarly, the levels of IL-6 were assessed in persons diagnosed with type 2 diabetes and in a control group of healthy individuals. It was noted that the levels of IL-6 were significantly higher in patients with type 2 diabetes compared to the healthy control group. The collective evidence from these findings and our own observations suggests that IL-6 may be implicated in the inflammatory response and serves as a significant factor in the development of both type 2 diabetes and urinary tract infections (22).

It has been hypothesized that a transmembrane receptor known as TLR-4 has a role in the progression of multiple diseases, including UTIs and T2DM. In the current study, it was found that diabetic patients who did not have urinary tract infections (UTIs) displayed significantly lower levels of TLR-4 than those who did have UTIs. On the other hand, researchers found that individuals who suffered from both diabetes and UTI had elevated levels of TLR-4 expression. Compared to people who are regarded to be in good health, those who are diagnosed with UTIs (23) and T2DM (24) have been shown to have lower levels of TLR-4 in previous studies. Additional research is required since there is a lack of data about TLR-4 levels in people who have diabetes and UTI at the same time. This spike in TLR-4 levels was noticed in this particular study, and it needs to be investigated further. In the process of regulating inflammatory pathways, the microRNA that is known as miR-146a-5p is an extremely important player. It was discovered that the expression of miR-146a-5p is altered in the target organs that are negatively impacted by the problems of diabetes. In addition to this, it has been hypothesized that the lack of the microRNA miR-146a-5p may have contributed to the development of these issues (25). This study is the first to investigate whether or not there is a link between UTIs and diabetes mellitus and miR-146a. The results of this study indicated an increased level of miR-146a in the blood of persons who were diagnosed with diabetes. In addition, a greater amount of miR-146a was found in patients who were diagnosed with both diabetes and UTI. The findings of this investigation are consistent with those of previous studies which showed that diabetic individuals had significantly higher levels of serum miR-146a when compared to those who had pre-diabetes (26). Based on this discovery, we can deduce that measuring miR-146a levels could be useful as a candidate biomarker for predicting the clinical consequences of diabetes. A strong connection between IL-6 and miR-146a-5p was found to exist as a result of the outcomes of this inquiry. These findings were in line with those of an earlier investigation that had been carried out by Rahman and colleagues (27). That study had compared the blood levels of TLR-4 and IL-6 in people who had T2DM to a control group of individuals who were healthy. A significant and favorable correlation was found in the research examination between the levels of blood TLR-4 and IL-6 in those who had been diagnosed with T2DM (r = 0.50, p 0.05). Zhang et al., (28) carried out a study with the purpose of determining the levels of TLR-4 and IL-6 that were present in blood and urine samples taken from patients diagnosed with UTIs as well as from a control group consisting of healthy persons. The results of this study were compared to the findings of a previous study that investigated the levels of these two proteins. The research conducted revealed a noteworthy and favorable association between the levels of serum TLR-4 and IL-6 in individuals diagnosed with urinary tract infections (r=0.502, p<0.001). In a study conducted by Kim et al., (29), the researchers assessed the concentrations of TLR-4 and IL-6 in both the serum and urine samples obtained from individuals diagnosed with acute pyelonephritis, as well as from a group of healthy individuals serving as controls. The research conducted revealed a noteworthy and favorable association between the levels of urine TLR-4 and urine IL-6 in individuals diagnosed with acute pyelonephritis (r=0.596, p<0.001). The results of this study provide evidence that TLR-4 and IL-6 play a role in a feedback mechanism that contributes to the development of UTIs and T2DM. Furthermore, it suggests that an imbalance in this mechanism may be responsible for the persistent low-level inflammation observed in individuals with these conditions.

CONCLUSION

IL-6, TLR-4 and miR-146-5p expression levels were significantly elevated in patients suffering from
diabetes and UTI in comparison to patients with only diabetes. The results of correlation between TLR-4 and IL-6 showed a significant inverse relationship. While the relationship between IL-6 and miR-146a-5p was in near equilibrium, the relation between TLR-4 level and miR-146a-5p expression level showed a negative relationship.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES


17. Taganov, K., Boldin, M., Chang, K., Baltimore, D. NF-B-dependent induction of microRNA miR-146a, an inhibitor targeted to signaling proteins of innate immune responses. PNAS. 2006;103(33):12481-12486.


