Evaluating Cytokine of Human of Cytomegalovirus by RT-qPCR

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KEYWORDS
Renal Failure, Cytomegalovirus,qPCR, IL17, IFN

ABSTRACT
Human samples from renal failure patients were collected from 25 July 2023 to 19 October 2023. Renal Failure was included (1 – 80 years). The RT-qPCR, Method was detected The RT-qPCR, Method was used to detect INF and IL17 of all samples. The population groups studied samples subject groups were distributed into 5 groups including (1-16,17-33,34-50,51-67,68-84) years, changed age to gender. The samples were isolated from the hospitals, including (Al-Nasiriyah Teaching Hospital and Dialysis Center, and Al-Hussein Hospital) The first study in Iraq to immunity study cytokine in patients with renal failure by molecular technique RTqPCR.

1. Introduction

Cytomegalovirus is the largest member of the herpes virus family, which also includes herpes simplex types 1 and 2, Epstein-Barr virus (EBV), and varicella-zoster. Cytomegalovirus (CMV) is a common virus with no known seasonal predominance and a prevalence that ranges between 50% and 85% of adults (Zuhair et al.,2019).

Transmission of CMV infection may occur from person to person throughout life, chiefly via close contact with an individual who is excreting the virus. It can be spread through the placenta, blood transfusions, organ transplantation, and breast milk. It can also be spread through sexual transmission (Leruez et al.,2020).

Cytokines (Greek cyto-, cell; and –kinos, movement) are a category of signalling molecules that are used extensively in cellular communication, and they are low-molecular-weight regulatory proteins or glycoproteins 5-50 KD secreted by white blood cells, including macrophages, fibroblasts and lymphocytes and various other cells in the body in response to several stimuli. These proteins assist in regulating the development of immune effector cells, and some cytokines possess direct effector functions of their own (Mehta et al.,2020).

Cysteine knot cytokines are members of the interleukin 17 (IL17) family of cytokines that induce inflammation. In response to IL-23 activation, a subset of T helper cells known as T helper 17 cells produces them. IL17A transcript, which was isolated from a rodent T-cell hybridoma in 1993, allowed for the first Th17 identification. One of the first members of the IL17 family is the protein that IL17A encodes (Moseley et al., 2023).

In humans, the IL-17 family consists of IL17A (often misleadingly referred to as "IL-17"), IL17B, IL17C, IL17D, IL17E, and IL17F. Another name for IL-17E is IL-25. The protein structures of all IL-17 family members are comparable. Four extremely conserved cysteine residues may be found in their protein sequences. The proper three-dimensional structure of the complete protein molecule depends on these conserved cysteine residues (Albeltagy et al., 2021).

Interferons got their name from their ability to prevent viral multiplication (Kang et al., 2022). Interferons are a type of antiviral protein that is released in response to stimuli. Interferons are divided into three categories: type I, type II, and type III. IFN-γ, or interferon-gamma, is a type II protein. This interferon's protein structure differs from type I and type III interferons (Heras et al., 2020).

In vivo, IFN-γ is a pro-inflammatory cytokine that activates effector immune cells and boosts antigen presentation. Natural killer (NK) cells secrete IFN-γ during the innate immune response, whereas CD4+ Th1 cells and CD8+ cytotoxic T lymphocytes secrete IFN-γ after adaptive immunity is triggered.

IFN-γ disrupts cytokine signalling by altering transcription factor expression (Merli et al., 2021).

Renal failure (also kidney failure or renal insufficiency) is a medical condition in which the kidneys fail to adequately filter waste products from the blood (Fadem et al.,2018) .The two main forms are acute kidney injury, which is often reversible with adequate treatment, and chronic kidney disease, which is often not reversible (Alkhaqani, 2022). In both cases, there is usually an underlying cause (Brady et al.,2019).

A decrease in glomerular filtration rate mainly determines renal failure, the rate at which blood is filtered in the glomeruli of the kidney. This is detected by a decrease or absence of urine production or determination of waste products (creatinine or urea) in the blood. Depending on the cause, hematuria (blood in the urine) and proteinuria (protein loss in the urine) may be noted (Rodríguez et al.,2019). Increased bodily fluid (causing edema), elevated acid and potassium levels, decreased calcium and increased phosphate levels, and eventually anemia are all possible complications of renal failure. Bone health may also be impacted. Chronic kidney issues have been linked to a higher risk of cardiovascular disease (Richards et al.,2019).

The aim of this study regarding Dhi-Qar province, Iraq, was that there had been no previous studies dealing with the molecular detection of human Cytomegalovirus in patients with Renal Failure. Thus, this work aims to detect cytomegalovirus in renal failure patients of different ages and genders. The main objectives of this study included:

1-Diagnosis of human Cytomegalovirus from renal failure patients by RT-PCR

2-Evaluation of pro and anti-inflammatory

3- Correlation between MiR-UL112-3p and Cytokine Levels in Renal Failure Patients Positive for HCMV

2. Methodology

Design Of Study

In the case sectional study, the healthy control sample was patients suffering from renal failure without viral infections.

Patients

Serum samples used in this study were obtained from one hundred patients with renal failure of age ranging from one year to eighty-one years from both genders who were admitted to Al-Nasiriyah Teaching Hospital and dialysis center, Al-Hussein Hospital during the interval between July 2023until October 2023Serum sample collection.

The serum samples were collected from patients with renal failure. The samples (1 ml) of serum were put into suitable containers, labelled and kept in an icy box. It were then transferred to the laboratory within two hours of collection. In the laboratory, the collected serum samples were stored in the freezer at -20 °C until use for molecular study and detection of viruses by (RT PCR and Sanger sequencing).

Real-time quantitative polymerase chain reaction (RT- qPCR) Technique

This technique was used to amplify the gene of the virus for detection of *Cytomegalo virus* and gene expression and immunological study (INF γ and IL17A) in all tested samples (Total Blood and Serum) by using real-time kits (GoTaq® qPCR and RT-qPCR Systems, Appendix.5 GoTaq®1-step RNA master mix, Appendix.6; Promega, USA. In addition to GoTaq® 2-Step RT-qPCR System, Appendix.7-A and B, Promega, USA), The mixture was prepared with a final volume 20 µl by mixing all contents mentioned intables (3.6,3.7,) This technique was performed Alamin center for advanced research and biotechnology, in Alnajaf province, by using (Analytik Jena\Qtower3G) and in the public health laboratory by using (BIO-RAD/CFX96) devices.

Gene expression

These results explain the use of MicroRNA and cytokines gene expression, including pro-inflammatory(INF γ) and anti-inflammatory(IL17A) biomarkers of one hundred patients infected with renal failure according to the type of human (Cytomegalovirus) positive. RNA, microRNA extraction, and cDNA synthesis were performed according to manufacturing protocols. Expression level(the equation $2^{-\Delta\Delta Ct}$) evaluated and compared with one hundred samples of healthy control by RT-qPCR.House Keeping gene (GAPDH) used for comparative project with patients and healthy control

Extraction of Viral DNA using FavorPrep™ 96-Well Viral DNA/RNA Kit

Materials:

1. 1.5 ml microcentrifuge tubes
2. Proteinase K
3. Serum sample
4. GSB Buffer
5. Absolute ethanol
6. GS Columns
7. 2 ml Collection Tubes
8. W1 Buffer
9. Wash Buffer (ensure absolute ethanol is added)
10. TE Buffer

According to the instructions provided by the manufacturer, the following steps:

Procedure:

1. Add 20 µl of Proteinase K to the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl of serum sample to the tube.
3. Add 200 µl of GSB Buffer to the tube and mix vigorously.
4. Incubate the mixture at 60ºC for 5 minutes, inverting the tube every 2 minutes to facilitate Proteinase K digestion and cell lysis.
5. Add 200 µl of absolute ethanol to the lysate and mix immediately by shaking vigorously for 10 seconds. If precipitate forms, break it up as much as possible with a pipette.
6. Place a GS Column in a 2 ml Collection Tube. Transfer the entire mixture (including any insoluble precipitate) to the GS Column. Centrifuge at 14,000-16,000 x g for 1 minute.
7. If the mixture does not flow through the GS Column membrane, increase the centrifuge time until it passes completely.
8. Discard the flow-through and place the GS Column into a new 2 ml Collection Tube.
9. Add 400 µl of W1 Buffer to the GS Column. Centrifuge at 14,000-16,000 x g for 30 seconds and discard the flow-through.
10. Place the GS Column back into the 2 ml Collection Tube. Add 600 µl of Wash Buffer (ensure absolute ethanol is added) to the GS Column. Centrifuge at 14,000-16,000 x g for 30 seconds and discard the flow-through.
11. Place the GS Column back into the 2 ml Collection Tube. Centrifuge for 3 minutes at 14,000-16,000 x g to dry the column matrix.
12. Transfer the dried GS Column to a clean 1.5 ml microcentrifuge tube. Add 100 µl of TE Buffer to

the center of the column matrix. Let it stand for at least 3 minutes to allow complete absorption. Centrifuge at 14,000-16,000 x g for 30 seconds to elute the purified DNA.

Design and preparation of the primers

The primers used in this study were prepared according to the manufacturer's recommendations. The lyophilized sequences were dissolved in an appropriate volume of nuclease-free water to yield a stock solution with a 100 pmol/µl concentration. A working solution was then prepared by diluting the stock solution to a final concentration of 10 pmol/µl. The primers for Human Cytomegalovirus and immunity genes were designed based on NCBI database sequences, while U6 primers were obtained from Macrogen (OLIGO). Table 3-4 shows the primers' sequences and volumes.

Table (1) the primer design of Human *Cytomegalovirus*

Name	Gene	Sequence	Bases	PCR Products Size
Human Cytomegalovirus Primer F	capsid	TCAAAACCACCGTGACAAGC	20	
Human Cytomegalovirus Primer R	capsid	ACAACGTGCTACGAAAGTGC	20	

Statistical analysis

The statistical analysis involved a one-way ANOVA followed by a two-stage linear step-up procedure by Benjamini, Krieger, and Yekutieli, which was used to compare more than two groups. Further, the Mann-Whitney U test was used to compare the two groups. The results are shown as mean ± SD. This analysis used GraphPad Prism version 10.0.0 for Windows, developed by GraphPad Software in Boston, Massachusetts, USA.

3. Results and discussion

Molecular diagnosis method for detecting pro-inflammatory and anti-inflammatory cytokines by Real-time qPCR.

In this study, two types of immune parameters were detected by using RT-qPCR, selected one pro-inflammatory cytokine INFγ and anti-inflammatory cytokine IL17A for patients of Cytomegalovirus virus in this study; the detection was with a comparative study using Housekeeping gene (GAPDH) and negative control (patients with Renal Failure) Alterations in the levels of cytokines in the infected patients with renal failure.

This section aimed to examine whether the infected patients with renal failure according to type of human (Cytomegalovirus) had statistically different cytokine profiles (IL-17A and INF-γ) relative to those non-infected patients with renal failure.

The RNA was extracted, and the cDNA one-step kit was, after analysing the RT-qPCR data, the fold change was calculated using the equation 2^ (-ΔΔCt). Mann Whitney test found that IL-17A and INF-γ levels in the infected patients with Cytomegalovirus were identified to be significantly increased in comparison to those non-infected patients

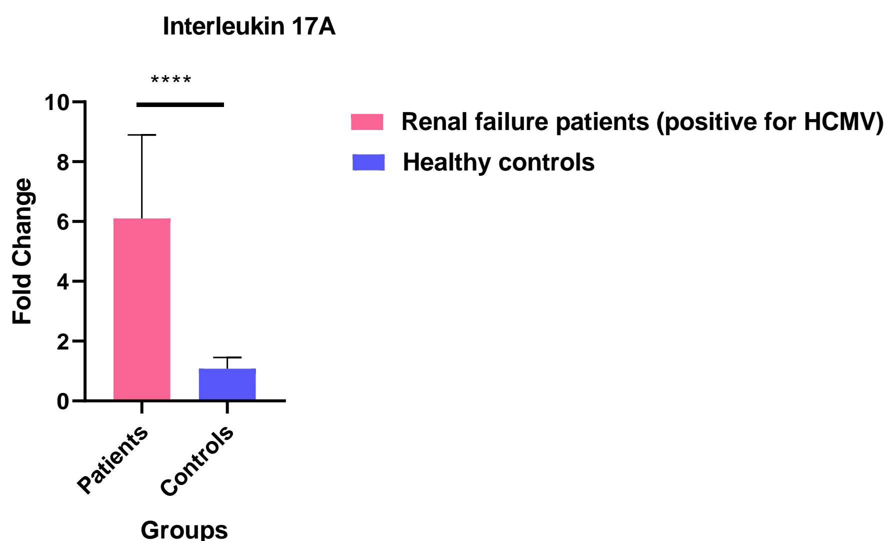

Figure (1) Comparing level of IL17A and gene GAPDH of Renal failure patients and control with RT-qPCR

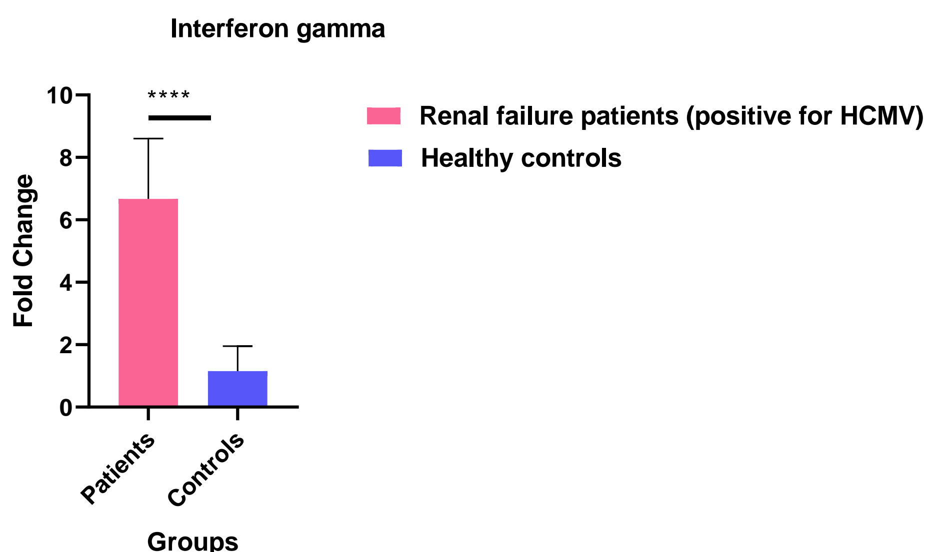

Figure 2. Comparing level of INF γ and gene GAPDH of Renal failure patients and control with RT-qPCR

Table (2) Alterations in the levels of cytokines across groups

Cytokine	Renal failure (positive for HCMV) mean (SD) (pg/ml)	Control mean (SD) (pg/ml)	P value
IL-17A	6.1 (2.8)	1 (0.3)	< 0.0001
INF-gamma	6.6 (1.9)	1.1 (0.8)	< 0.0001

The significance of differences has been tested by a Mann–Whitney U test, where **** p<0.0001 is significant. The Data are Mean (SD) of IL17A and INF-gamma gene expression. This study is recent as this topic links IL17A and INF gama with Cytomegalovirus in renal failure.

As a researcher, the higher levels of interferon-gamma (INF-γ) and IL17Ain individuals with human cytomegalovirus (CMV) infection have been linked to renal failure for several reasons.

The INF-γ is a key cytokine in the immune response against viral infections, including CMV. Elevated levels of IFN-γ indicate a strong immune response, which can inadvertently damage the transplanted kidney through heightened inflammation.

The CMV infection or reactivation triggers the production of INF-γ by T cells and natural killer cells. Persistent or reactivated CMV can lead to sustained high levels of INF-γ, contributing to chronic inflammation and graft injury.

The INF-γ plays a role in activating and differentiating immune cells involved in the alloimmune response, such as T-helper 1 (Th1) cells. This can increase the likelihood of acute and chronic kidney graft rejection.

The IL-17A is a pro-inflammatory cytokine. Its elevated levels indicate a heightened inflammatory response, which can damage kidney tissues and contribute to chronic rejection.CMV infection or reactivation can trigger a robust immune response, including the production of IL-17 by Th17 cells. This response can exacerbate inflammation in renal failure; renal failure often receives immunosuppressive drugs to prevent rejection. These drugs can also affect the immune system's control of CMV, leading to reactivation and higher IL-17A levels.

The IL-17A can enhance the alloimmune response against renal failure by promoting the activation and infiltration of immune cells that attack the graft. High IL-17A levels can lead to tissue damage and fibrosis, further compromising kidney function and potentially leading to graft failure.

Discussion

A total (of 100) different clinical cases collected were arbitrarily (79) positive cases infected with Human Cytomegalovirus, while (21) were negative cases.

This study, included serum samples of patients who were diagnosed with renal failure in hospitals during sample collection, and this corresponds to the study of (AL-Fayyadh and Mezher,2020) and (Karalos and Al-azzawi et al 2020), who also used the serum sample from renal failure, results in figures (4-1) shows the CT value that means the concentration of virus in each sample.

The Study of (Bottino et al 2024) used the same technique to detect CMV by RT-qPCR .These are the percentages we got Because the appearance of the number of positive samples depends on the collection of patients with renal failure according to the region and cause of the infection. We noticed that patients with the acute condition develop into chronic conditions. If we collect the samples in another place, the percentage may differ according to the location and type of samples.

The results show that among (41) females out of a hundred samples, the results show higher positive cases of *Cytomegalovirus,* which includes (38) cases of males of samples.

A study by (Perry et al 2022) showed that females were infected more than females with Cytomegalovirus , occurring in 30% of females versus 16% of males (Mohsin et al 2022). This study also agrees with our study, which found that HCMV DNA was detected in males at 1(16.66) %. while in females was 5(83.34) %. As a researcher, the number of females collecting samples from the number of visitors is greater than the number of males, and the second possibility is that the large number of infections is due to the weakness of the immune system in women due to hormonal changes, as they are more susceptible to infection.

The results of this study show that Cytomegalovirus infection is higher in all age groups, especially (51-67) age group, with (23) cases more than other groups and the lower infection in age group (1-16) with (10) cases, that illustrated.

According to(Perry et al 2022), Females 50 years or older were at particular risk compared with males under 50 years and another study showed high precent differences among both sexes. These results

showed that the age group 60-70 had the highest infection rate among other groups(Mohsin et al. 2020). according to (Siddiqu et al 2018), CMV PCR was detected in one hundred and two kidney transplant recipients. Of those, 79 were live unrelated (commercial) kidney transplants, and 23 patients had live related kidney transplants. Donor age modifies the association between donor sex and graft survival. Older female donors were associated with similar or lower hazards of graft failure than older male donors in both male and female recipients, suggesting a better functional reserve of older female donor kidneys(Melk et al 2024).

As a researcher, For this age group, immunity is weak, and they are vulnerable to infection due to chronic diseases of the age, high blood pressure and diabetes, and also due to the use of medications with incorrect or excessive diagnosis.

This study indicates the MicroRNA-UL112-3p gene expression level as a pro-inflammatory biomarker of patients infected with renal failure. MicroRNA-UL112-3p expression levels in patients were elevated compared to healthy control, Figure 4.2. The miR-UL112-3p was significantly elevated in Renal Failure (< 0.0001) and compared to healthy controls,

These results agree with(Afshar et al in 2022) examination, which used blood samples to measure the expression of MicroRNA UL112-3p by real-time qPCR; the role of miRNAs is detected in both lytic and latent phases of CMV infection. Consequently, in the present study, an extremely high expression level of miR-UL112-3p, -UL112-5p, -UL22A-3p, -UL22A-5p, -US25-1-5p, -US25-2-5p, -UL36-3p, -UL36-5p and -UL70-3p detected in the active CMV infected KTRs in comparison to latent ones, which prove their possible critical and central biological role in the CMV pathogenesis.

The goals are to study the miRNAs encoded by viruses and their roles in viral replication, expression, and infection, including but not limited to developing rapid and accurate detection methods for HCMV infection, to develop the planning of effective antiviral therapies, and providing new molecular targets for the development of antiviral drugs. Recently, investigators have proposed a bioassay based on microgels with optical fluorescent oligonucleotide probes to detect circulating endogenous miR-US4-5p; this has improved the accuracy and reduced the cost of detecting HCMV infection.The antagomir (also known as anti-miRs or blockmirs) miR-122-3p is now used to treat hepatitis C infection, and other antagomirs of virus-encoded miRNAs may be used to treat many viral diseases in the future (Zhang et al in 2020).

Higher levels of miR-UL112-3p, a microRNA encoded by human cytomegalovirus (CMV), have been implicated in Renal Failure through several mechanisms.

The first reason: miR-UL112-3p helps CMV evade the host immune response by targeting and downregulating key components of the immune system. This immune evasion allows CMV to persist and reactivate, leading to continuous low-grade inflammation and damage to the renal failure and

miR-UL112-3p can modulate the expression of cytokines and other immune molecules. By interfering with the immune response, this microRNA can reduce the immune system's effectiveness in controlling CMV infection, leading to prolonged inflammation and increased risk of graft rejection; mir-ul112-3p can inhibit apoptosis (programmed cell death) in infected cells. By preventing the death of CMV-infected cells, the virus can maintain a reservoir within the kidney, contributing to ongoing inflammation and tissue damage, Chronic CMV infection and the persistent presence of viral components like miR-UL112-3p can promote fibrosis in the transplanted kidney. Fibrosis is characterized by the accumulation of extracellular matrix proteins, leading to scarring and functional decline of the graft; mir-ul112-3p can interfere with various cellular signaling pathways involved in immune responses, cell proliferation, and inflammation. This interference can exacerbate graft injury and contribute to chronic allograft dysfunction. miR-UL112-3p targets and downregulates the expression of specific host genes involved in antiviral responses and immune regulation. This downregulation impairs the host's ability to mount an effective defense against CMV, allowing the virus to persist and cause ongoing damage to the graft.

In summary, the higher levels of miR-UL112-3p in CMV-infected renal failure contribute to immune evasion, chronic infection, and inflammation, all of which can lead to graft dysfunction and eventual failure. Managing CMV infection and its associated microRNAs is crucial to improving outcomes in renal failure.

The role of miR-UL112-3p in the context of human cytomegalovirus (HCMV) infection, particularly in patients with renal failure, is multifaceted and significant due to its impact on the immune response and viral persistence. Here's a detailed look at the role of miR-UL112-3p in this specific context:

Immune Evasion: miR-UL112-3p downregulates the expression of immune response genes in host cells. This includes inhibiting major histocompatibility complex class I (MHC-I) molecules, which are essential for presenting viral antigens to cytotoxic T cells.

Inhibition of Cytokines: It can also inhibit the expression of cytokines and chemokines, such as interferon-gamma (IFN-γ), which are crucial for an effective antiviral response

Latency and Reactivation: miR-UL112-3p helps HCMV establish and maintain latency within host cells. By modulating the host's immune response, the virus can persist without being detected and cleared by the immune system. This is particularly important in immunocompromised individuals where viral reactivation is common.

4. Conclusion and future scope

In the light of the current study, it is concluded that:

The findings highlight the significant P <0.0001 of miR-UL112-3p, IFN- γ and IL17A in the pathogenesis of these conditions, influenced by human CMV infection status compared to healthy control.

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