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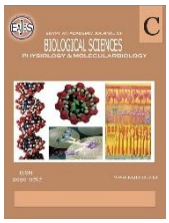

Correlation Between Cytokine Gene CCL3, miRNA146-B and Otitis Media Patients in AL-Najaf Governorate, Iraq

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ABSTRACT

The current study aims to detect the gene expression of genes (CCL3, miRNA-b) in Otitis Media patients and healthy persons. This finding included 50 samples collected from healthy subjects and 100 samples from a patient suffering from otitis media who attended Al-Sadr Medical City (ENT Department) in Al-Najaf Governorate during the period from February 2022 to June 2022. The samples had an average age ranging from 5 to 70 years. In this case-control study, the gene expression of these genes was compared in people with Otitis media and healthy people. PCR technique is used. The patient and control groups' samples were amplified using polymerase chain reactions. The results of the molecular study (gene expression) showed a high significant increase in the level of gene expression in patients for the two genes CCL3, miRNA146-b (9.807 ± 1.14 (6.405 ± 0.7576), respectively, compared to the control group, with a high significant difference at P≤0.05.

INTRODUCTION

Otitis medium, an infection of the upper respiratory tract (URT), is characterized by inflammation of the middle ear and the tympanic membrane (Suzuki *et al*.,2020). It is one of the most prevalent infections in children because the bacteria can enter more easily through the nasopharynx due to the shorter and anatomically horizontal Eustachian tube(Bowatte *et al*., 2018). The most common reason for antibiotic resistance in the developing world is otitis media. The existence of middle ear effusion symptoms and indicators of ear inflammation, such as a bulging tympanic membrane, should be used to make the diagnosis of otitis media (Bergenfelz and Hakansson 2017).

Otitis media comes in three different forms: Acute otitis media (AOM) is defined as the presence of an effusion in the middle ear along with one or more symptoms or indicators of middle ear inflammation (Venekamp *et al*., 2020). It is the most common bacterial illness and the reason why young children need antibiotics(Deniz *et al*.,2018). Tympanostomy tube insertion (TTI) is the preferred course of treatment for acute otitis media because it can prevent the accumulation of chronic fluid in the middle ear (Rosenfeld *et al*.,2016). It is the most widely used surgical operation performed on kids(Pedersen *et al*.,2016). Children are more likely than adults to suffer acute otitis media due to immunological and structural immaturity, while adult ear infections are often persistent (Schilder *et al*.,2016).

Otitis media with effusion (OME), a chronic form of otitis media without the symptoms or physical signs of acute otitis media, may develop as a result of recurrent acute otitis media (AOM). The tympanic membrane is left intact, and the local inflammation causes the liquid to collect in the middle ear cavities and epithelial changes (metaplasia). The persistent effusion following (AOM), which disappears after 2 months in 90% of patients, is distinct from this collection of effusions because it is mucous or sero-mucous but not purulent and lasts for at least three months (Blanc *et al*.,2018). The middle ear and mastoid cavity are chronically inflamed in the third type, known as chronic suppurative otitis media (CSOM), which is identified by persistent otorrhea (ear drainage) through a perforate in the tympanic membrane that lasts for at least two to six weeks (Leach *et al*.,2021).

Cytokines involve chemokines that promote chemotaxis, interferons that regulate innate immunity, interleukins that are responsible for white blood cell communication, lymphokines and tumor necrosis factor which can cause bone resorption and have a pro-inflammatory activity(Araujo-Pires *et al*., 2014). The large subfamily of cytokines are Chemokines which have the ability to coordinate leukocyte recruitment and immune system activation in the pathogenesis of varied human diseases. Chemokines are recognized by their small molecular weight and have the ability to attract macrophages, neutrophils and lymphocytes to the site of inflammation (Ramadan *et al*.,2020).

Chemokines have chemotactic properties and can migrate to the sites of inflammation (chemotaxis) which is the reason that they are called chemokines) (Braz-Silva *et al*.,2019). Chemokines involve two major families: CC and CXC with their receptors CCR and CXCR these receptors are expressed on Th1, and Th2 cells monocytes and neutrophils(Azuma *et al*., 2014).

MicroRNAs, which are small molecules of about 22 nucleotides that can control gene expression by binding to the 3′-(UTR)untranslated region of the target gene, are the next frontier in the regulation of gene expression. They are crucial in inflammatory, immunological, and metabolic disorders(Rasko and Wong,2017). Some significant genes' expression is altered as a result of altered miRNA expression. MiRNA may regulate about 30% of human genes and expression patterns are greatly comparable between healthy and patient persons (Keshavarz, 2015).

MATERIALS AND METHODS

Study Design and Patients:

This case-control study used 150 clinical samples in total, 90 males and 60 females, with ages ranging from 5 to 70 years old. It was conducted between February 2022 and June 2022. The first group was patients with discharge Otitis media (100), males (68) and females (32), First, patients were personally questioned by a researcher using an anonymous questionnaire form that included (age and gender). The second control group was 50 randomly selected healthy people (5–70)years old, (30 males, and 20 females). This study agrees with the same ethics as patients admitted to the Al-Sader Teaching Hospital in Iraq's Al-Najaf Governorate. Primers sequence has been mentoied in Table 1.

Table 1: Primers sequence.

Genes	Primer sequence(5′-3′)	Expected size(pb)	Reference
Ccl3	Forward: 5′ATGCAGGTCTCCACTGCTGCCCTT- 3′ Revers: 5′GCACTCAGCTCCAGGTCGCTGACA T-3′	274 bp	(Chui and Dorovini,2010)
miRNA -146B	Forward : 5′-TGAGAACTGAATTCCATAGGCTGT-3′ Revers : 5′-GCTGTCAACGATACGCTACG-3′	85bp	(Ge *et al.*,2016)

Samples Collection:

Each subject's venous blood was collected for five milliliters. Before the blood was taken, a tourniquet was placed immediately on the skin around the arm, and the skin around the vein was sterilized with 70% ethyl alcohol from the patients and control group. The blood is directly taken in sterile tubes containing EDTA for RNA extraction, which is followed by the use of the (Real-time PCR) technology. These samples should be immediately frozen at -20C**.**

Statistical Analysis:

The well-known statistical program (Graph Pad Prism version 7) was employed, and the one-way anova analysis of variance test (by Tukey's multiple comparisons test) was performed to compare the measured parameters (Ramadan *et al*.,2020)**.**

RESULTS AND DISCUSSION

Ccl3 and miRNA146-b Gene Expression:

The melting and temperature curves of the genes Ccl3, TLR-4, NOD-2 and miRNA146-b showed that the RT-qPCR products were clear and homogeneous as shown in **Figures (1 - 6).** Data of gene expression were given as Mean± Standard Error (SE).

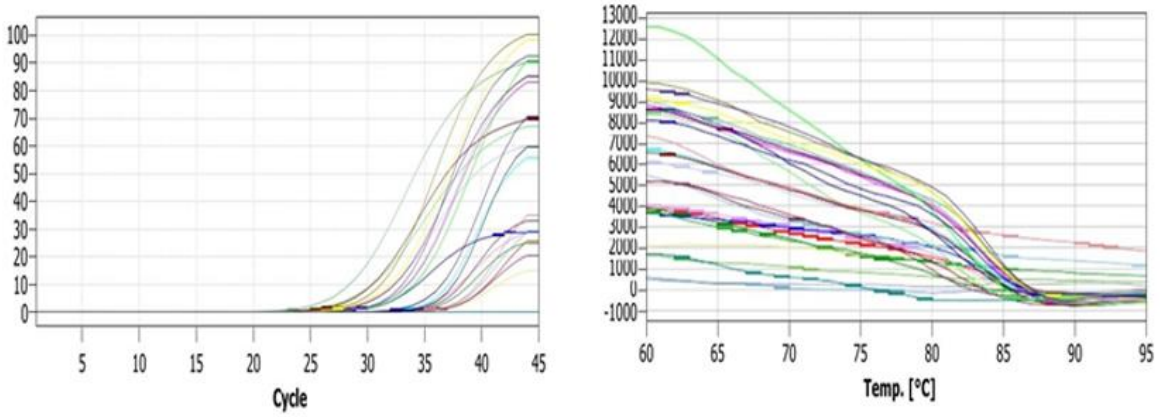

Fig.1: Cycling and melting curve of qPCR amplification for Ccl3 gene.

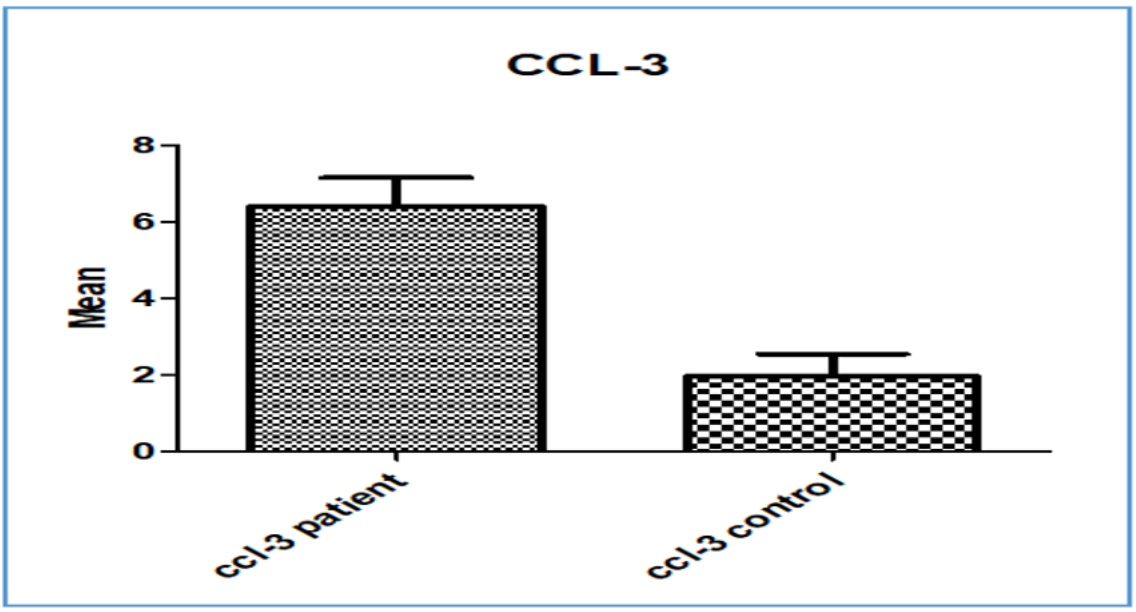

Fig.2: Fold change of CCL3.

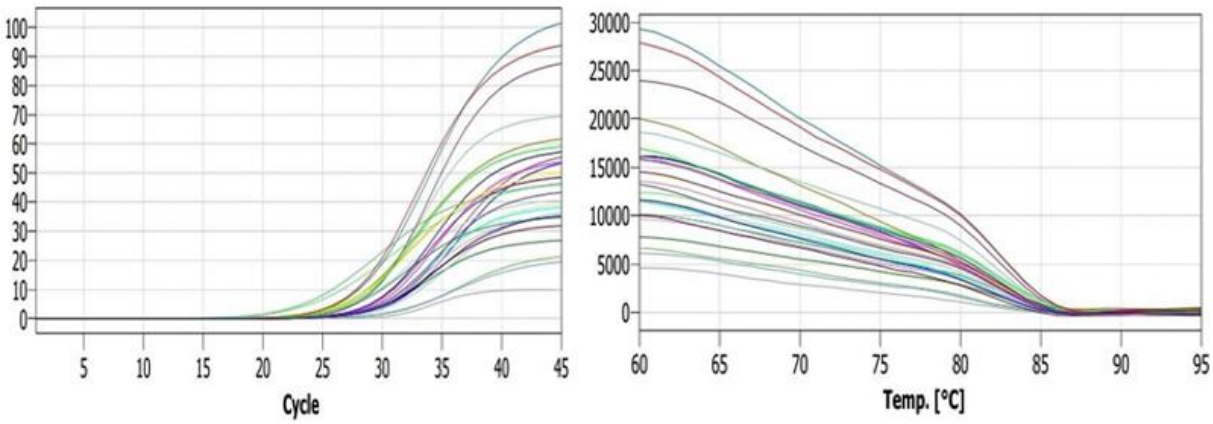

Fig. 3: Cycling and melting curve of qPCR amplification for B-Actin Housekeeping gene.

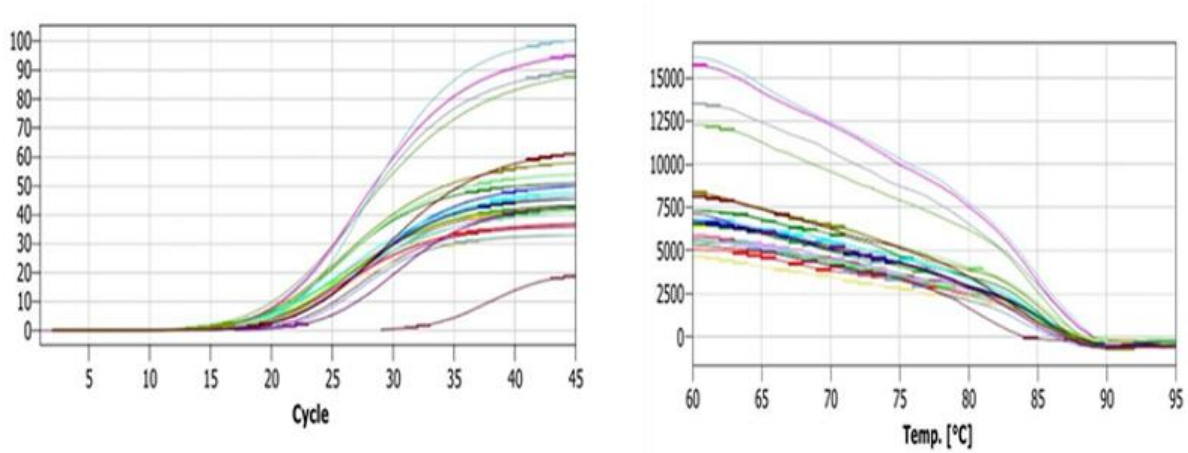

Fig.4: Cycling and melting curve of qPCR amplification for miRNA146-b gene.

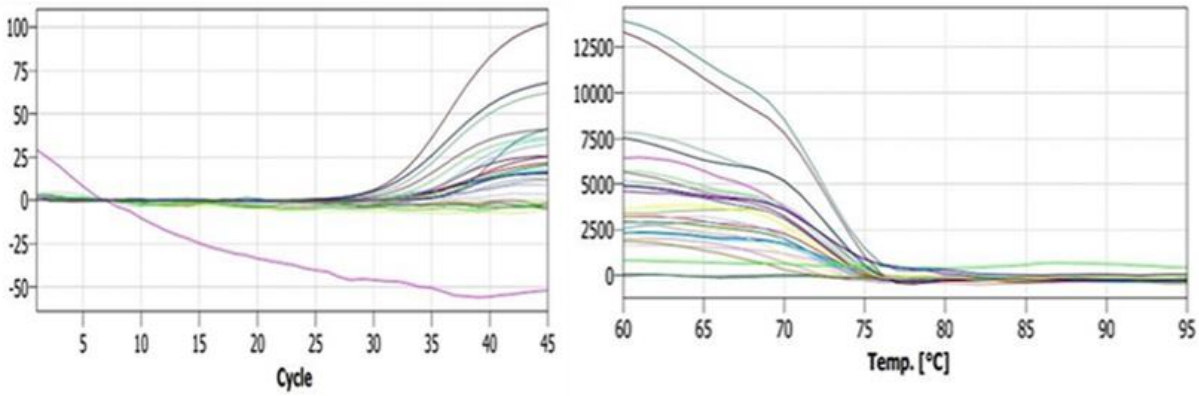

Fig. 5: Cycling and melting curve of qPCR amplification for U6 Housekeeping gene.

Table (2), shows the CCL3 gene expression level in studied groups, the Mean±SE of CCL3 gene expression level for OM patients was increased (6.405±0.7576) as compared to control(1.950 ± 0.6030). The results showed a higher significance of CCL3 gene expression level in patients as compared to control (p=0.0006).

Table 2: Expression fold of CCL3 gene in OM patients and controls.

Parameter ccl-3	Mean ±SE	P-value
Patient	6.405 ± 0.7576	0.0006***
Control	1.950 ± 0.6030	

***(p<0.05)higher significant, SE Standard Error

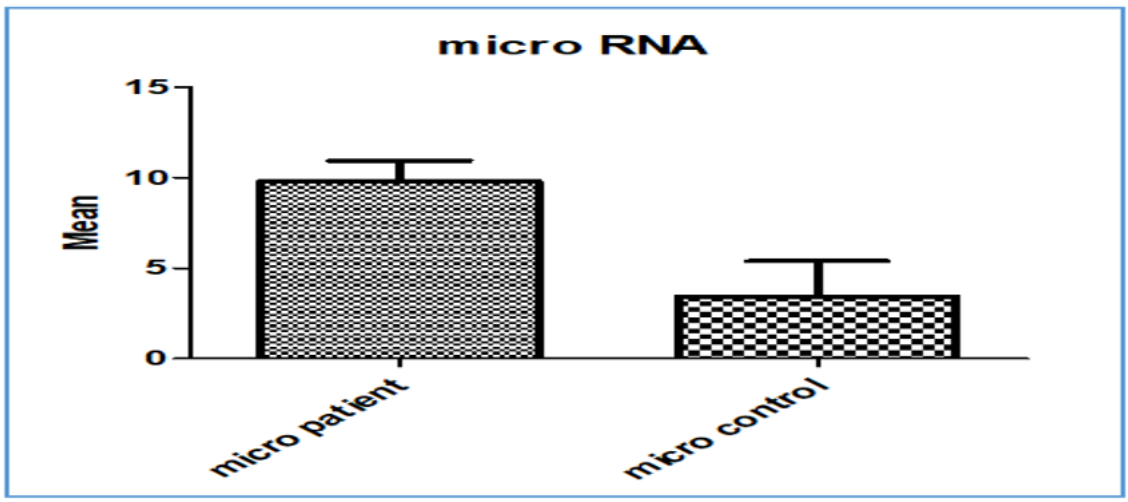

Fig.6: Fold change of microRNA146-b

Table (3), shows the miRNA146-b gene expression level in studied groups, the Mean±SE of miRNA146-b gene expression level for OM patients was increased (9.807 ± 1.140) as compared to control (1.962±3.438).

Table 3: Expression fold of miRNA146-b gene in OM patients and controls.

Parameter micro RNA 146-b	Mean ±SE	P-value
Patient	9.807 ± 1.140	**0.0067
Control	1.962 ± 3.438	

** (p<0.05) higher significant

Table (2), finding agreed with research conducted by Kauer et al.,(2015) which demonstrated that Children with AOM who have a bacterial Otopathogen have higher expression levels of proinflammatory, cytokines/chemokines(ccl3) and TLRs, and this depending on the number of bacterial species found. Few studies have been conducted on the local immune mediator response to colonization in connection to otitis media. There has only been one cross-sectional investigation on the nasal wash of 98 infants aged 7 to 26 months, and it found no correlation between otitis susceptibility and expression of (IL-6, IL-8, CCL3, CCL5, and TNF) during health, URI, or AOM (Morris and Pichichero, 2017).

Pro-inflammatory substances such as TNF-α, IL-1β, and C-C motif chemokine ligand 3 (CCL3) are essential for attracting inflammatory cells to the ME and activating them to eliminate bacteria from the body, decreasing pro-inflammatory substances, including TNF-α and CCL3, showed decreased but extended leukocyte recruitment, poor macrophage function, and failure to remove bacteria from the ME cavity (Leichtle *et al.*, 2010; Deniffel *et al.*, 2017). Exogenous CCL3 can also totally restore OM recovery and phagocytosis, indicating that it functions downstream of TNF-α (Leichtle *et al.*, 2010).

The results in Table (3) show that miRNA146-b expression was more

significant and this agreed with Samuels et al.,(2016) studies that have shown associations between miRNAs and OM, and miR-146b expression was elevated in ME of OM patients and in vitro cultured ME epithelial cells activated with pro-inflammatory cytokines, therefore, identifying miRNA target genes and their downstream pathways may offer fresh insights into OM. They also examined the expression of miR-146 in middle ear biopsies from otitis media patients. In particular, only the recurrent group's miR-146b expression was considerably higher than that of the controls.

MiRNAs may play a role in the pathophysiology of OM, according to several investigations. Human ME epithelial cells (HMEECs) treated with lipopolysaccharides (LPS), a gram-negative bacterium cell wall component, revealed 15 differently expressed miRNAs, according to Song *et al*.,(2011), and the expected targets of these miRNAs include genes that control cell proliferation, innate immunity, acute inflammatory responses, the IkB kinase/NFkB cascade, cell communication, complement activation, and cell differentiation.

Val *et al*.,(2018) discovered five miRNAs that are known to target innate immunity genes, including miR-378a-3p + miR-378i, miR-200a3p, miR-378g, miR30d-5p, and miR-222-3p. These miRNAs were detected in chronic OM ME effusions. Samuels *et al*.,*(*2016) also discovered an inverse relationship between TNF receptor-associated factor (TRAF6), a component of the Toll-like receptor signaling cascade, and miR-146a and miR-146b expression. As a result of targeting TRAF6, that study showed that miR-146a and miR-146b may be crucial in the pathogenesis of otitis media.

Conclusion

CCL3 and miRNA 146- B gene expression levels in OM patients were significantly increased compared to the control group.

Ethical Approval:

This study was conducted under approval by the medical ethics committee at the University of Kufa (2017).

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