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Studying the estimation levels of inflammatory markers and NF-κB in MDR and DS-TB patients

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***Abstract*— Tuberculosis (TB) is one of the most infectious and deadly diseases, especially after the emergence of drug-resistant bacteria. Therefore, in this study, we developed a method to evaluate the ability of using CXCL10, CXCL9, MMP9, suPAR and the expression gene (NF-κB) and their rolesin the diagnosis and treatment of the disease in patients. Also, we used ELISA and qPCR techniques to compare the serum levels of CXCL10, CXCL9, MMP9, suPAR and NF-κB gene in drug resistant and drug sensitive TB. The serum levels of CXCL10, CXCL9, MMP9, suPAR and the expression of NF-κB gene was compared among drug resistant and drug sensitive TB using ELISA and qPCR Techniques, respectively. Our results showed an increasing in the serum levels of CXCL10, CXCL9 and suPAR, in the drug resistant of TB patients but not in the drug sensitive group, however, all of them did not reach the significant level, it was found that expression of NF-κB in drug sensitive group was higher (3.22-fold) than that in the drug resistant-TB patients (2.70-fold).**

***Keywords*—MDR-Tuberculosis, ELISA, CXCL10, CXCL9, suPAR, MMP9, NF-κB gene**

I. Introduction

Tuberculosis is an infectious disease that considers one of the top leading causes of mortality in the whole world. (Annabel *et al.*, 2019). Itcaused by *Mycobacterium tuberculosis*, which Robert Koch initially identified in 1882 (Marjanovic *et al.*, 2010; Cole, 1999).*Mycobacterium tuberculosis* is a tiny, weakly gram-positive bacillus with a waxy, thick, and strong cell wall that renders it hydrophobic by nature (Grzegorzewicz *et al.*, 2016).

The resistant of tuberculosis to drug considers dangerous globally because of the difficulty to control the disease.. In 2014, MDR-TB (multidrug-resistant tuberculosis) caused 190,000. The majority of second-line medications were found to be ineffective for about 30% of Multi-Drug Resistant-Tuberculosis (MDR-TB) patients. Because of the high mortality rate (40%) and low diagnosis rate, the prevalence rate ofDrug Resistant-Tuberculosis (DR-TB)was only 4.9% of the reported TB patients reportedlow. Only 4.9% of the reported TB patientsreported underwent medication resistance testing in 2009.In 2015, the percentage increased to 30% (Nimmo *etal.*, 2017).In all types ofTuberculosis infections, chemokines and cytokines playimportantof functions in proliferation, migration,mediating innate immunity, angiogenesis,inflammation of cells. (Domingo-Gonzalez *et al.*, 2016; Torraca *et al.*, 2017). Inactual, innatechemokine/cytokine pathways play importantroles in dominant primary infection and in helpingadaptation immune responses (Cooper *et al.*, 2011). However, anti- the balanced activation between pro- and inflammatory cytokines/chemokines is critical for increasing effective host resistance against *Mycobacterium tuberculosis*infection (Cooper *et al.*, 2011; Domingo Gonzalez *et al.*, 2016). Sothat the immune system is amongst the maximum vital inner

danger elements (Ali *et al.*, 2021).Nuclear factor kappa B(NF-κB) activation controls the transcription of the majority of genes encoding pro-inflammatory cytokines (Gilmore, 2006). The stimulation of several inflammatory genes in response to diverse infections and inflammatory cytokines depends on Nuclear factor kappa B (NF-κB), a major mediator of inflammation. One such cytokine is tumor necrosis factor-α (TNF-α), a crucial regulator of human defenses against infection, particularly against the tuberculosis-causing bacteria *Mycobacterium tuberculosis* (MTB). TNF-α influences the immune response to MTB through a variety of mechanisms, such as inducing macrophage activation to effectively kill bacteria (Gutierrez *et al.*, 2008) inducing chemokine and cytokine expression (Algood *et al.*, 2003), and inducing apoptosis (Keane *et al.*, 2002). Because of these activities, which are controlled by the NF-κB signaling pathway, TNF-α is essential for limiting bacterial development in granulomas, lung-based bacterial and immune cell aggregations (Davis & Ramakrishnan, 2008) Therefore, the management of intracellular NF-κB signaling dynamics may be essential for preventing MTB infection. The TNF-induced NF-κB signaling pathway is key to the MTB immune response.

The current study based on the immunological biomarkers

TABLE (1) PCR PRIMERS FOR AMPLIFICATION THE GENE ENCODE NF-KB

Gene	Primer	Sequence (5' to 3')	Reference
NF-kB	Forward	GCAGCACTACTTCTTGACCACC	Ref: (Zhao & Erle,2018)
	Reverse	TCTGCTCCTGAGCATTGACGTC	
GAP-DH	Forward	TTCCAATATGATTCCACCCA	
	Reverse	GATCTCGCTCCTGGAAGATG	

and their levels in(Multidrug resistant) MDR and Drug Sensitive (DS)tuberculosis patients(under treatment patients) andused previous studies that demonstrated the importance of these immune indicators in follow up patient's response for treatment. We debated here some chemokines and immune indicators that tuberculosis patients (under treatment) display in resisting the disease as one of the defenses of the immune system. We also intended to study the control of NF-κB on these biomarkers and their concentrations in the patients' bodies.

II. Materials and Methods

A. Samples Collection

Samples of blood were collected from pulmonary tuberculosis patients in the Advisory Clinic for Chest Disease and Respiratory (ACCDR), the only health center

TABLE (2) LEVELS OF INFLAMMATORY MARKERS IN SERUM INUNDER TREATMENT TB PATIENTS'GROUPS.

Inflammatory markers	Under treatment TB Patients		Healthy people	P. value *
	DS-TB patients N= (16) Median (Mini-Maxi)	MDR-TB patients N= (14) Median (Mini-Maxi)	N= (30) Median (Mini-Maxi)	
CXCL10 (pg/ml)	14.75(11.53 -28.68)	15.14(11.53 -30.12)	19.07(0.96-25.68)	0.755
CXCL9 (ng/ml)	3.15 (1.40–4.65)	3.07(1.92 – 6.51)	2.89(0.68-7.00)	0.547
MMP-9 (ng/ml)	18.97(10.00 -39.60)	21.80(11.65 -37.00)	20.70(0.88-53.95)	0.318
suPAR (pg/ml)	87.94(44.92 – 158.37)	89.66(26.64 – 167.51)	80.71(6.09-226.14)	0.575

* Kruskal Wallis Test.

that deals with TB patients in Basra province, from January 2022 to May 2022.Thirty patientswere recruited in this study. The consent form was obtained from each participant.Treated patients were selected from TB patients who already diagnosed and had taken their treatment for more than three months.

B. Assessment of immunological markers in serum

The levels of four inflammatory markers in serum were evaluated by using ELISA (Enzyme–linked Immunosorbent assay) with specific commercial kits as following: CXCL10, CXCL9, MMP-9 and suPAR (Sunlong Biotech, China) all the ELISA procedures were carried out according to manufacture instructions.

C. RNA Extraction

The ribonucleic acid (RNA) was extracted from blood by using Kit GENE ZO "Tri RNA pure Kit (Geneaid, Taiwan) according to the manufacture's guideline. Nanodrop spectrophotometer was used to determine the concentration and the purity of extracted RNA. The extracted RNA was used as template and subjected to reverse transcription process by the first conversion of RNA that was used as a template to synthesize complementary DNA (cDNA) by transcription enzyme.

The analysis of gene expression of NF-κB was performed by using a real time PCR machine (Applied Biosystem, USA). The reaction tube contained (10µl of GoTaq® qPCR Master Mix with SYBR® Green (Promega, USA), sample cDNA (2µl), forward primer (1µl), revers primer (1µl) and Nuclease-free-water (6µl)) a total of 20µL. Two sets of specific primers were used to amplify the NF-κB and reference gene (GAP-DH). The primers synthesized by OriGene Company (USA) table (1). The program of thermocycle machine was set first at 95°C for 1 minute, then 95°C for 15 seconds with 40 cycles. After that, it was set at 55-63°C for 1 minutes and at 95°C for 15 seconds. The quantity of NF-κB expression in the blood was determined as units relative to the quantity of GAP-DH expression which was calculated using ΔΔCT formula (Livak & Schmittgen, 2001).

D. Statistical analysis

For the purpose of statistical analysis, the Statistical Package for the Social Sciences (SPSS) versionsnumber 24 was used. To describe quantitative data, the mean standard deviation, median and minimum- maximum values were calculated. For the description of qualitative data, absolute numbers with percentage were used. Normality of distribution was tested using appropriate tests including Kruskal Wallis and Mann Whitney tests. Any probability value of < 0.05 was considered significant.

III. RESULTS

A. Comparison of Inflammatory markers levels between MDR and DS TB patients

As shown in table (2), our results showed an increasing in the serum levels of CXCL10, CXCL9 and suPAR in drug resistant TB patients when compared with responding group, but none of them reached the significant level.

B. Comparison the expression of NF-κB in two groups (MDR and DS-TB patients)

The current resultsthat the expression of NF-κB in responding group was higher (3.22-fold change) in than the drug resistant-TB patients (2.70-fold change) (table 3).

TABLE (3) NF- KB GENE EXPRESSION IN UNDER TREATMENT TB PATIENTS ACCORDING TO THEIR DRUG RESISTANCE.

Under treatment TB Patients	**NF-κB gene expression (fold)** **Median (Mini-Maxi)**	**P. value***
MDR	2.70 (0.25 – 41.61)	1.000
DS-TB	3.22 (0.009 – 50.52)	

*Kruskal Wallis Test

IV. DISCUSSION

Monitoring TB treatment response is very valuable and important in making clinical decision. It wasreported that the host immune markers play significant roles indevelopment or reduce and control TB infection, so they may be important in monitoring the extent of disease development or recovery(de Martino *et al.,* 2019). The available methods were not fully adequate in detection and monitoring TB treatment. GeneXpert, for example, can detects *Mycobacterium tuberculosis* genome regardless dead or live bacilli found in clinical sample. Additionally, it is not available low- income countries (Li *et al.*,2017).Immunological markers base techniques are capable to provide more reliable method to follow up the prognosis and monitoring the treatment of TB. Based on ~~may~~ the previous studies, which showed the roles of CXCL10, CXCL9, MMP-9, suPAR in the pathology of TB disease, we selected these immune markers to study their roles in the prognosis of TB in drug-resistant-Pulmonary Tuberculosis (DR-PTB) and drug sensitive-Pulmonary Tuberculosis (DS-PTB) (Brahmbhatt *et al.,* 2006; Djoba Siawaya *et al.,* 2009; Adekambi *et al.,* 2015). According to the results shown by the current study regarding the two participatedgroups of patients, the DR-PTB and DS-PTB, the levels of the tested immune markers were relatively higher in drug-resistant patients than in the drug sensitive group.

However, none of these differences reached the significant level. This could be due to several reasons. One of these reasons might be the condition of the pathological process in the treated patients. in general, due to the granulomas and sputum from big cavities both contain latent foci where bacteria may be able to persist (Ojha *et al.,* 2008).

For example, suPAR level is relatively steadily maintained in the serum of relapse TB patients, according to the previous study's findings (Mardining Raras *et al.,* 2012). A tiny percentage of *Mycobacterium tuberculosis* in the macrophages of tuberculosis relapse patients survives first-line anti-tuberculosis therapy, survives for an undetermined period of time, then reemerges following immunosuppression of host, according to one hypothesis (Wayne & Sohaskey, 2001). Furthermore, this could be due to the small sample size wasused in this study. Despite of the non-significant results obtained in this study, but the increasingin the serum levels of the investigated immunomarkers in MDR TB patients in comparing with DS TB patients suggests further study using more sensitive method and large samples size to verify the importance of these immunomarkers in differentiating Multidrug resistant tuberculosis patients.

In an attempt to know the response of DR patients in tuberculosis, the NF-κB gene expression level was evaluated and compared with the rest of the DS patients, and its level was lower (2.70-fold change) in DR patients than DS patients (3.22-fold change).

The reason of decreasing NF-κB gene expression in DR patientsmay be due to the intensive treatment in MDR patients, especially with the presence of new drugs that have shown their high efficacy in reducing inflammation and a noticeable immune response. Where some drugs have proven effective in inhibiting NF-κB, as was observed in a previous study (Shree *et al.,* 2016) where they proved that Clofazimine, a medication used to treat TB cases who have developed extensive drug resistance and multidrug resistance. It was believed to successfully restore the antimycobacterial cellular response, reinforcing the theory that *M. tuberculosis* directly inhibits NF-κB activation to suppress host resistance.

Both the NLRP3 inflammasome and the NF-κB pathway may serve as crucial targets for the development of innovative treatments to treat *Mycobacterium tuberculosis*infection when combined with the processes involved in inflammatory signaling pathways. The inhibition of NLRP3 inflammasome and NF-κB signaling pathway by MCL may participate to its anti-inflammatory role during *Mycobacterium tuberculosis* infection (Zhang, Jiang, *et al.,* 2017). Bai et al., (Bai, *et al.*,2013) found that the NFκB inhibition involved in decreasing the viability of intracellular MTB in human macrophages through prompting the apoptosis and autophagy.

However, decreasing the expression of NF-κB in MDR patients' needs for further immunological and molecular studiesto clarified and understood.

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