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Original article

Assessment of IL-12, mRNA expression, vitamin-D level, and their correlation among the *Mycobacterium tuberculosis* cases

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ABSTRACT

Background: Tuberculosis (TB) presents serious health related complications caused by the Mycobacterium tuberculosis pathogen. Interleukin 12 (IL-12), plays a central role in T helper 1 (Th1) cells development that are implicated in chronic inflammatory pathogenesis as well as level of 1,25-dihydroxyvitamin D3 can impact on IL-12 mRNA expression at the transcriptional level.
Methods: The present study included clinically confirmed 100 Mycobacterium tuberculosis cases (TB) for assessment of IL-12 mRNA expression and vitamin-D level as well as equal number of healthy controls were also included.
Results: In TB cases, overall 13.01-fold higher IL-12 mRNA expression and 30.69 ng/ml vitamin-D level were observed. It was observed that higher expression of IL-12 mRNA expression was linked with TB cases had fever (p < 0.0001), night sweat (p = 0.003), sputum with blood (p = 0.03) as well as decreased vitamin-D level was linked with weight loss (p = 0.01), fever (p < 0.0001), night sweat (p = 0.008), sputum with blood (p = 0.005). TB cases with smoking (p < 0.0001) and alcoholism (p = 0.01, p = 0.0001) had significantly higher IL-12 mRNA expression and lower vitamin-D levels compared to its counterpart. It was observed that TB cases with vitamin-D deficiency, insufficiency, sufficiency had 19.51-fold, 14.64-fold, and 10.54-fold IL-12 mRNA expression respectively (deficiency vs insufficiency; p = 0.0003, deficiency vs sufficiency; p < 0.0001). A negative correlation was observed between IL-12 mRNA expression and vitamin-D level among the TB cases (r = −0.68, p < 0.0001).
Conclusions: Higher IL-12 mRNA expression and lower vitamin-D expression among the TB cases may be responsible for the severity and pathogenesis of TB and alterations in IL-12 mRNA expression and vitamin-D may be influenced by the smoking and alcoholism habit of TB cases.

1. Introduction

Mycobacterium tuberculosis (MTB) is an infectious bacterial pathogen that can survive by blocking phagolysosome functions in host macrophages (Vergne et al., 2005; Almeleebia et al., 2021). This bacterium, particularly in developing countries, causes millions of deaths annually worldwide. Tuberculosis (TB) related disease development and outcomes depend on the host's ability to elicit a potent immune response mediated by cells, resulting in activation of the macrophage. Among T cells, macrophages, dendritic cells, and other immune cells, several cytokines are released

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and mediate signaling. The outcome, presentation, and/or severity of the disease can be defined by these interactions (Jo et al., 2003). Recently, In TB cases, cytokine profiles have been studied to develop novel treatments and diagnostic approaches (Mihret et al., 2013). Assessment of cytokines in TB cases and healthy persons help to detect the MTB infection. MTB usually causes a latent form of infection, which affects 23% of the world's population. (Pai et al., 2016). It is believed that progression from latent to active infection occurs through an event of intermediate stages that include early and subclinical stages (Cadena et al., 2017). Although immunological pathways are important in the control of latent TB infection, the mechanisms that drive active disease pathogenesis from the latent stage are not fully understood (O'Garra et al., 2013).

Vitamin D deficiency (VDD) is very widespread globally (Gois et al., 2017) and results in systemic inflammation and immune dysregulation. The mechanism behind the interlink concerning VDD and TB might be as follows. It is recognized that vitamin D is necessary for health and vitamin D receptors on myocytes are involved in cell function regulation., endothelial cells, B and T lymphocytes, macrophages, neutrophils and dendritic cells (Esposito and Lelii, 2015) and modulate innate and adaptive immunity (Esposito and Lelii, 2015). In macrophages treatment with 25 (OH)D, different cytokines are expressed. Additionally, vitamin D exposure stimulates antimicrobial peptides (b-defensin and cathelicidin) that induce autophagy in *M. tuberculosis* (Esposito and Lelii, 2015). Vitamin D is an immunoregulatory hormone, and in the pre-antibiotic era, skin TB was treated effectively with UV light (Martinez et al., 2013). By the 1920s, with regular sun exposure, pulmonary TB was being treated. Vitamin-D has a critical role in innate immunity and boosts the immune response to mycobacterium (Liu et al., 2007). Vitamin D has antibacterial effects and is demonstrated in promoting the environment associated with antibacterial activity and linked to immune against TB disease (Liu et al., 2007). The status of vitamin D was related to NO-mediated mycobacterial killing (Waters et al., 2004). Therefore, present study aimed to evaluate the level of IL-12 mRNA expression, and vitamin-D and their association among the active TB cases as well as association of smoking and alcoholism with IL-12 mRNA expression, and vitamin-D level among the active pulmonary TB cases.

2. Materials and methods

2.1. Study area and population selection

Present research work included the 100 confirmed untreated newly diagnosed active pulmonary TB cases and 100 healthy controls. The functional pulmonary TB diagnosis was established on clinical presentation, abnormal chest radiograph, and confirmed by positive cultures for *M. tuberculosis* (sputum or BAL fluid). Blood samples from cases and healthy controls were drawn in plain as well as EDTA vials. Blood samples were withdrawn in EDTA vials, and further total RNA extraction for IL-12 mRNA expression was done. The sample was collected in plain vials and centrifuged for 10 min at 1500 rpm and stored at −80 °C for vitamin D measurement.

2.2. Total RNA extraction and complementary DNA synthesis

RNA Isolation kit (Thermo Fisher Scientific, Massachusetts, USA) was carried out for the total RNA extraction from frozen whole blood using the following manufacturer-provided instructions from all the collected samples from cases and healthy controls. RNA quantification was done by using nanodrop by taking OD on 260/280, and further, 100 ng of total RNA was used to synthesize the cDNA using the manufacturer provided kit protocol (Verso, Thermo scientific, USA).

2.3. IL-12 mRNA expression

After the cDNA synthesis, expression of IL-12 was analysed by using SYBR green dye in quantitative real-time PCR machine by forwarding primer sequence 5′-ACCTCCACCTGCCGAGAAT-3′and reverse primer sequence 5′-CATGGTGGATGCCGTTCA-3′, and β-actin were employed as a housekeeping gene. The forward primer sequence 5′-CGACAACGGCTCCGGCATGTGC-3′ and reverse primer sequence 5′-GTCACCGGAGTCCATCACGATGC-3′. It is followed as qRT-PCR for β-actin and IL-12 was performed for 40 cycles; initial denaturation was done at 94 °C for 40 s, annealing temperature was at 60 °C for 40 s, the extension was at 72 °C for 40 s, and 20 μl reaction volume was used. To confirm the specific amplification, an additional step was taken at 72 °C for 5 min to finish the reaction, and the melting curve was studied between the 35 °C to 90 °C ranges. In each experiment, a control without cDNA was comprised, and each reaction was done twice. The relative quantification by the $2^{-[\Delta\Delta CT]}$ process was employed to compute the IL-12 mRNA expression level.

2.4. Vitamin D level assessment

Stored serum from TB cases and controls were thawed for serum vita-D level measurement by electrochemiluminscence based immunoassay method [11493580]. Serum 25(OH) D levels <20 ng/ml was considered as deficient, <30 ng/ml was considered as insufficiency, and >30 ng/ml was deliberated as sufficient.

2.5. Statistical analysis

All the clinical presentations and experimental data were recorded in excel, and analysis was performed by the SPSS 20.0 and Graph Pad Prism 5 statistical software. Depending on the data type nonparametric Mann Whitney U test was done to compare the two groups. A nonparametric spearman correlation analysis was performed between vitamin-D level and IL-12 mRNA expression to see the correlation type (negative correlation). A p value < 0.05 were studied to be statistically significant.

3. Results

3.1. Demographic characteristics

In brief, the present study included 100 cases of TB cases and 100 healthy controls. Mean age of TB cases was 39.89 (SD = 7.65), and healthy controls were 40.79 (SD = 8.64). 63% of cases were males, and 37% were females though in healthy controls, 60% were males and 40% females (Table 1). Several clinical observations were recorded and presented in Table 1.

3.2. IL-12 mRNA expression level among TB cases

Relative IL-12 mRNA expression was analysed in TB cases by taking control as a reference to calculate the fold change in expression. Overall, 13.01-fold increased mRNA expression of IL-12 was detected in TB cases in contrast to the healthy control group. TB cases who experience the fever had higher IL-12 mRNA expression level (15.70-fold) compared to TB cases without fever (9.98-fold), and differences among them were established to be statistically significant (p < 0.0001). TB cases who presented the night sweat sputum with blood had higher IL-12 mRNA expression (15.12-fold, 14.49) compared to its contrast (p = 0.003, p = 0.03) respec-

Table 1
Demographic and clinical characteristics of TB cases and healthy controls.

Variables	Active TB cases (%)	Healthy controls (%)
Age (years)	39.89 ± 7.65	40.79 ± 8.64
Gender		
Male	63 (63)	60 (60)
Female	37 (37)	40 (40)
Weight loss		
Yes	65 (65)	
No	35 (35)	
Loss of appetite		
Yes	46 (46)	
No	54 (54)	
Fever		
Yes	53 (53)	
No	47 (47)	
Night sweat		
Yes	36 (36)	
No	64 (64)	
Weakness		
Yes	58 (58)	
No	42 (42)	
Sputum with blood		
Yes	40 (40)	
No	60 (60)	
Smoking		
Yes	37 (37)	
No	63 (63)	
Alcoholism		
Yes	33 (33)	
No	77 (77)	

tively (Table 2). There was no as such significant differences was observed in IL-12 mRNA expression with respect to age, gender, weight loss, loss of appetite, and weakness.

3.3. Level of Vitamin-D among TB cases

Vitamin-D levels were calculated among the TB cases (overall 30.69 ng/ml) and compared with the clinical presentations. It

Table 2
Association of IL-12 mRNA expression with different clinical presentations of TB cases.

Variables	Relative IL-12 mRNA expression (Fold)		P value
	Mean	SD	
Overall expression	13.01	4.72	–
Age (years)			
≤40 years	13.11	5.64	0.73
>40 years	12.89	5.72	
Gender			
Male	12.91	5.55	0.81
Female	13.20	5.87	
Weight loss			
Yes	13.21	5.67	0.71
No	12.66	5.65	
Loss of appetite			
Yes	12.97	6.15	0.83
No	13.06	5.24	
Fever			
Yes	15.70	4.83	<0.0001
No	9.98	4.96	
Night sweat			
Yes	15.12	5.03	0.003
No	11.83	5.67	
Weakness			
Yes	13.84	5.51	0.08
No	11.88	5.70	
Sputum with blood			
Yes	14.49	5.50	0.03
No	12.04	5.57	

was observed that TB cases showed weight loss had decreased vitamin-D level (28.83 ng/ml) while TB cases with no weight loss had vitamin-D level (34.17 ng/ml) comparatively (p = 0.01). TB cases who had fever (24.32 ng/ml), night sweat (26.94 ng/ml), and Sputum with blood (26.99 ng/ml) had lower vitamin-D level compared to TB cases without fever (37.89 ng/ml), night sweat (32.18 ng/ml) and sputum without blood (33.17 ng/ml) (p < 0.0001, p = 0.008, p = 0.005) respectively (Table 3). There was no as such significant differences was observed in vitamin-D level with respect to age, gender, loss of appetite, and weakness.

3.4. Association of smoking and alcoholism with IL-12 mRNA expression among the TB cases

Smoking and alcoholism were analyzed with IL-12 mRNA expression to establish a link to understand the association (Fig. 1). It was observed that TB cases with smoking had 16.41-fold higher IL-12 mRNA expression while non-smokers had 11.2-fold IL-12 mRNA expression (p = 0.0001). TB cases who were alcoholic also showed 14.98-fold higher IL-12 mRNA expression, while non-alcoholic TB cases had 12.5-fold higher IL-12 mRNA expression (p = 0.01).

3.5. Association of smoking and alcoholism with Vitamin-D level among the TB cases

The impact of smoking and alcoholism were analysed with vitamin-D levels among the TB cases (Fig. 2). It was observed that the smokers' TB cases had 23.40 ng/ml vitamin-D level while non-smoker TB cases had 34.98 ng/ml of vitamin-D level (p < 0.0001). TB cases who were alcoholic showed lower vitamin-D level (24.60 ng/ml) while non-alcoholic TB cases had comparatively higher (33.70 ng/ml) vitamin-D level (p = 0.0001).

3.6. Association of Vitamin-D level with IL-12 mRNA expression among the TB cases

TB cases were grouped into 3 categories based on vitamin-D level (Vitamin-D deficient, sufficient, and insufficient) and IL-12

Table 3
Association of vitamin-D level with the different clinical presentation of TB cases.

| Variables	Vitamin-D level (ng/ml)		P value
	Mean	SD	
Age (years)			
≤40 years	30.86	10.82	0.62
>40 years	30.46	9.17	
Gender			
Male	30.62	10.06	0.78
Female	30.83	10.40	
Weight loss			
Yes	28.83	10.71	0.01
No	34.17	7.99	
Loss of appetite			
Yes	29.69	11.25	0.63
No	31.56	9.09	
Fever			
Yes	24.32	8.72	<0.0001
No	37.89	5.92	
Night sweat			
Yes	26.94	10.95	0.008
No	32.18	9.06	
Weakness			
Yes	29.10	10.76	0.10
No	32.90	8.85	
Sputum with blood			
Yes	26.99	11.02	0.005
No	33.17	8.74	
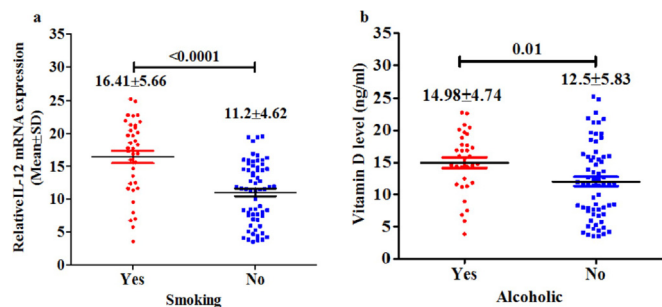
Fig. 1. IL-12 mRNA expression among TB cases (a) Association of smoking with IL-12 mRNA expression. (b) Association of alcoholism with IL-12 mRNA expression.
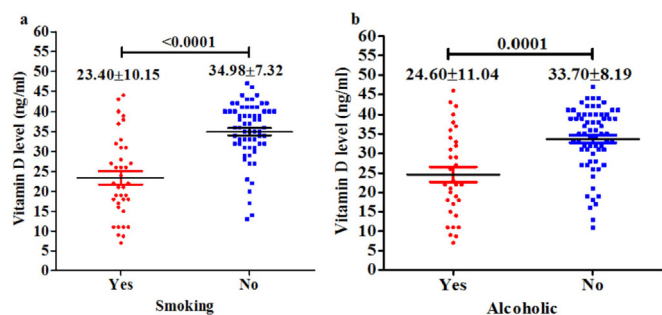

Fig. 2. Vitamin-D level among TB cases (a) Association of smoking with Vitamin-D level, (b) Association of alcoholism with Vitamin-D level.

mRNA level were computed. TB cases with Vitamin-D deficiency was compared with insufficient and sufficient TB cases and was observed that deficient cases had 19.51-fold IL-12 mRNA expression, insufficient had 14.64-fold IL-12 mRNA expression, sufficient had 10.54 fold IL-12 mRNA expression and significant differences among them was found to be statistically significant (p = 0.0003, p < 0.0001)(Fig. 3).

3.7. Correlation of vitamin D level with IL-12 mRNA expression

A non-parametric spearman correlation analysis was performed to understand the association between vitamin D level and IL-12 mRNA expression. After the analysis a negative correlation was observed between vitamin-D level and IL-12 mRNA expression among the TB cases (r = −0.68, p < 0.0001, Fig. 4). It suggested that increase in IL-12 mRNA expression would decrease the level of vitamin-D and vice versa.
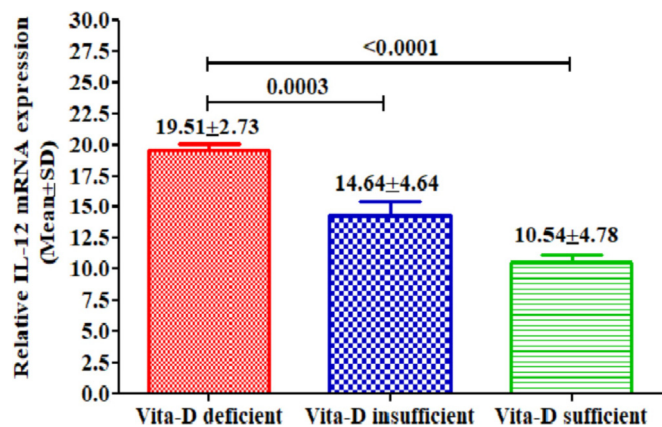

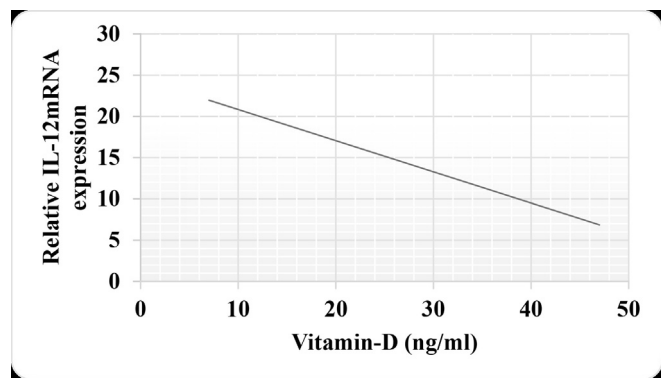**Fig. 3.** Association of Vitamin-D level with IL-12 mRNA expression among TB cases.

Fig. 4. Correlation of Vitamin-D level with IL-12 mRNA expression among TB cases.

4. Discussion

TB causes death worldwide and the foremost cause of HIV/AIDS from a single contagious agent (Organization, 2020). MTB affects the lungs and is a leading cause of death (Organization 2008). An estimated 10 million individuals worldwide fell ill with TB in 2018. In the WHO regions, most of the TB cases occurred in South-East Asia (44%), and Africa (24%) (Organization, 2020).

Concerning this, the present research evaluated the mRNA expression level of IL-12 and vitamin-D level in active TB cases. It was observed that the TB cases had 13.01-fold increased IL-12 mRNA expression compared to healthy controls. TB cases with different clinical presentations were also analyzed and observed that TB cases with fever, night sweat, weakness, sputum with blood had higher expression than its counterpart. TB cases with fever, night sweat, weakness, sputum with blood had 5.72-fold, 3.29 fold, 1.96 fold, 2.47 fold higher mRNA expression compared to TB cases without fever, night sweat, weakness, sputum with blood. *M tuberculosis* infection in TB cases has been reported to alter the IL-12 level, and the IL-12 level was higher in cases with pulmonary TB (Dlugovitzky et al., 2000). It was also found that the TB cases had cells with higher cytokine expression in lymph nodes or BAL (Munk et al., 1996). IL-12 knockout mice are known to be more vulnerable to TB infection in a gene knockout mice study (Cadena et al., 2017). Mycobacterial protective immunity is linked with antigen presentation by antigen-presenting cells (Flynn and Chan, 2001), and APC was able to incite Type 1T helper cells (Th1) and cytokine secretions, such as interleukin (IL)-12 and interferon (IFN)-g, respectively (Giacomini et al., 2001). Protective immunity against MTB is central to the collaboration of T cells with infected macrophages (Munk and Emoto, 1995) and IL-12 is one of the major cytokines in the MTB immune response (Boom et al., 2003).

TB cases who were smokers and alcoholics were also evaluated for IL-12 mRNA expression level. It was observed that the TB cases who were smokers and alcoholics had 5.21-fold and 2.48-fold higher IL-12 mRNA expression compared to TB cases who were non-smoker and non-alcoholic. The excessive rates of TB disease and infection are seen in smokers have affected several structural and host determinants (Edge et al., 2016). E-cigs and cigarette smoke on macrophages were observed to alter the production of cytokines, stimulating both inflammatory and anti-inflammatory responses (Hodge et al., 2011). Active smoking has also been linked with TB infection and mortality (Jha et al., 2008).

Cigarette smoke raises the risk of MTB infection in a variety of ways., decreased the activity of alveolar macrophages, mucociliary clearance impairment, decreased pulmonary lymphocyte immune response, Modified activity of pulmonary dendritic cells, and reduction of the cytotoxic action of NK cells (Underner and

Perriot, 2012). Continuous smoking has contributed to TB relapse, and passive smoking has also increased the risk of both TB infections and disease (Leung, 2010). Evidence showed that cigarette smoke is a risk factor contributing to infection, and progression and severity of the active TB disease (Sitas, 2004). A Chinese study described the mortality rate among tuberculosis cases who smoked as nine times higher (Wen, 2010). Studies from India and South Africa concluded that smokers have a higher risk of death (Gupta et al., 20052005). It has also been shown that smoking tobacco is a predictor and risk factor significantly connected with more excellent default rates in TB care sites (Lavigne, 2006).

We also observed that TB cases showed an overall 30.69 ng/ml vitamin-D level, and their association was evaluated with different clinical presentations. TB cases who had weight loss, fever, night sweat, sputum with blood had 5.34 ng/ml, 13.57 ng/ml, 5.24 ng/ml, 6.18 ng/ml lower vitamin-D levels compared to its counterpart, respectively. TB cases of smokers and alcoholics also showed 11.58 ng/ml, 9.1 ng/ml lower vitamin-D levels than a non-smoker and non-alcoholic TB cases, respectively. TB cases that were VDD, insufficient and sufficient, had 19.51-fold, 14.64- and 10.54-fold IL-12 mRNA expression. It suggested that vitamin-D deficient and insufficient TB cases had higher IL-12 mRNA expression than vitamin-D sufficient TB cases.

A growing body of evidence (Gois et al., 2017; Upala et al., 2015) suggested that Vitamin-D deficiency has an impact on immunity and low vitamin D level involved in MTB infection by higher production of chemokine, dendritic cells activation, and alteration of T cell activation (Zeng, 2015).

A correlation analysis was also done between IL-12 mRNA expression and vitamin-D level among the TB cases and a negative correlation was observed. It suggested that a decrease in vitamin-D level would increase the IL-12 mRNA among the TB cases and vice versa. It has been revealed that vitamin D3 inhibit IL-12 production (Lemire, 1995), and 1,25(OH)2D3 inhibits the development of the NFATp/AP-1 complex, which suppresses IL-2 gene transcription. (Alroy et al., 1995). The inflammatory course may lower 25(OH) D3 levels as a source for activated vitamin D. (Tsiaras and Weinstock, 2011).

It has been demonstrated that children with VDD, because of Nutritional VDD, low exposure to sunlight may be linked with reduced vitamin D levels, had a potential risk of emerging TB in developing countries (Gois et al., 2017; Nwosu et al., 2014). Seasonality incidence of TB disease leads to the assumption that cyclic differences in vitamin D levels may have a role in TB infection or disease progression (Santos et al., 2012). A study on prisoners reported that prisoners' low or poor vitamin D levels were connected with active TB (Kim, 2014; Hong et al., 2014).

5. Conclusions

The present study demonstrated that higher IL-12 mRNA expression and lower vitamin-D expression were linked with Tuberculosis infection. TB cases who was smoker and alcoholic were correlated with higher IL-12 mRNA expression and lower vitamin-D level. An increase in IL-12 mRNA expression was also associated with lower levels of vitamin-D, which suggested that lower vitamin-D levels may be the reason for increased IL-12 mRNA expression.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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