TO MEASURE THE PLASMA LEVELS OF LEUKOTRIENE B4 (LTB4) PROTEIN EXPRESSION IN BOTH CASE AND HEALTHY CONTROL GROUP IN EXTRA-PULMONARY TUBERCULOSIS PATIENTS

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Abstract:

Initial diagnosis and prompt treatment of extra pulmonary tuberculosis continues to be a challenge in all over the world as well as India. First time, this study will prospect the role of LTA4H gene and may be establishing another candidate that influences the susceptibility to extrapulmonary tuberculosis in the North Indian population. This study will be the first report on LTA4H gene various diagnostic markers, expression of gene may validate as a prognostic factor in extrapulmonary tuberculosis. The associations between LTA4H SNPs levels. The diagnosis of extra pulmonary tuberculosis poses a special challenge, as it is often missed or misdiagnosed due to its atypical presentations and difficult to isolate *M.tuberculosis* due to the small number of organisms present at these sites. Subsequently the outcome of present study will reinforce possible use of LTA4H as biomarkers and the therapeutic utility for extra pulmonary tuberculosis. This study will be a step to deflate the diagnostic and therapeutic window to identify another risk factor (LTA4H) for extra pulmonary tuberculosis and will spawn new ways to safeguard and recuperate the health status of individuals and population groups. On RT-PCR, Extra pulmonary patients had lower expression of LTA4H compared to the controls.

Keywords: LTB4GENE, ELISA, EPTB, RT-PCR

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1. Introduction

Tuberculosis (TB), caused by bacillus Mycobacterium tuberculosis, is one of the top 10 causes of mortality globally and the leading introduce of death from a single infectious agent (ranked higher than HIV/AIDS) **(1)**. M. tuberculosis infects almost one-quarter of the global population. However, among the one-quarter of the global population infected by Mycobacterium tuberculosis, only about 10% progress to clinical disease **(2).** Most infected individuals carry the bacteria without expressing overt disease for their entire life, which indicates that host susceptibility is an important risk factor for the progression of active tuberculosis after infection **(3).** A number of genes have been identified in studies of diverse origins to be important in tuberculosis **(4, 5).**

Leukotriene A4 hydrolase (LTA_4H) is a monomeric, cytosolic, zinc metalloenzyme which catalyzes the Leukotriene B4 (LTB4) generation in amino acid metabolic pathway and regulates the balance of LTB4 and LXA4 generation **(6)**. This balance can affect TNF synthesis. TNF is an important factor which influences the manner of macrophage death after M. tuberculosis infection. Macrophage apoptosis can control the diffusion of M. tuberculosis but macrophage necrosis may increase the diffusion of M. tuberculosis. Therefore, there is an inference that the activity of LTA4H can affect the progression of tuberculosis after M. tuberculosis infection.

Recently, as a candidate gene involved in tuberculosis, the polymorphisms in LTA4H gene were investigated in a case control association study in a Vietnamese population **(7)**. Of the six single nucleotide polymorphisms (SNPs) studies, two were found to be significantly associated with tuberculosis. The same 6 SNPs were also studied in a Russian population, but, none of them exhibited significant association with pulmonary tuberculosis **(8)**. Yang et al. suggested that polymorphisms in the LTA4H gene might affect susceptibility to extra-pulmonary tuberculosis and change the risk of developing the disease in the Han nationality in the East China **(9)**.

2. Methods and Materials:

Study design: It is a case control study. The topical investigation was performed on patients suffering from Extra- pulmonary Disease, fulfilling the inclusion & exclusion criteria. Patients were cataloged from the Department of Respiratory Medicine, King George's Medical University (KGMU), Lucknow attending OPD. Authored notified authorization was taken from every subject before inclusion in the study. The study was endorsed by the official institutional ethics committee (Registration no: ECR/262/Inst/up/2013/RR-16)-Ref.code:94th CMIIB-PhD/PI

- **EPTB patients:**

Subject recruitments and collaborative departments for present work-

The investigational work was accomplished in the Departments of Biochemistry and Respiratory Medicine, KGMU. Lucknow.

Study Participants: Selection Criteria

A clinically EPTB subject was kept in case group and healthy subjects for control group. Inclusion and exclusion standards of both groups was as follows-

We enrolled a total of 100 EPTB patients completing the inclusion and exclusion criteria from outpatient and indoor patient Department of Respiratory Medicine, Lucknow. Data were collected on a standardized questionnaire for each subject. This included demographic characteristics age and Gender, Clinical history (Case definitions), Past and family history, Anthropometric data. Posted consent was taken from 100 patients or guardian.

Healthy Control: Age and Gender -matched had been selected. An only a healthy person was enrolled with no history of respiratory disease.

Collection of Blood samples: Blood will be transferred in Plasma (for ELISA), Plasma will be separated from first vial and store at -20^{0}C

Diagnostic category:

LTB4 measured by ELISA to see the correlation between LTB4 in both case and healthy group. Blood sample will be aliquot in first part of blood will be transferred in Plasma separating vial (for ELISA), ELISA separated plasma of blood will be use to measurement of LTB4 level for both case and control group by commercially available ELISA kit and follow as per manufacturer protocol.CEA562Ge 96 Tests Enzyme-linked Immunosorbent Assay Kit For Leukotriene B4 (LTB4) Organism Species: Pan-species (General) The kit is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of LBT4 in serum, plasma, tissue homogenates, cell culture supernates and other biological fluids.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1,000xg at 2-8°C within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles. This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to LTB4 has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled LTB4 and unlabeled LTB4 (Standards or samples) with the pre-coated antibody specific to LTB4. After incubation the unbound conjugate is washed off. Next, avid in conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of LTB4 in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of LTB4 in the sample. Matrices listed below were spiked with certain level of LTB4 and the recovery rates were calculated by comparing the measured value to the expected amount of LTB4 in

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of LTB4 and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

3. Statistical Analysis

Continuous data were surmised as mean ±SD while categorical data in number and percentage. One way to analyse covariance (ANOVA) was done for the analysis the correlation between Demographic and *LTA4H* and *LTB4* Gene. Statistical Package for the Social Sciences (SPSS) software version 20.0 was used to analyze the data. Statistical significance was set as p<0.05.

4. Result:

Hundred patients with Extra pulmonary were included. Group 1 related to Gender Male 65 (65%), Female 35 (35%), Group two (Gender) control Male 60 (60%) Female 40 (40%). P value **0. 046.**Group 1 related Religions Hindu 66 (66.0%)Muslim 26 (26.0%) other 8 (8.0%). Group 2 Control (Religions) Hindu 94 (94.0%) Muslim 4(4.0%) other nil (0.0%) and **p value <0.001**. Group 1 of Socioeconomic status lower 66 (66.0%) middle 10 (10.0%) lower middle 8(8.0%) upper middle 12 (12.0%) rich 4(4.0%), Group 2 Control (Socioeconomic status) lower 20(20%), middle 10(10%), lower middle (28%). upper middle 36(36%), Rich 6(6%) **P value 0. 001. Group** 1 related to Occupation student 16(16.0%). Labour 40(40.0%) housewife 36 (36.0%) other 8(8.0%) group 2 (Occupation) Control student 24(24%), labor 26(26%), housewife 20 (20%) others 30(30%) **P value <0. 001.** Group 1 related to the Marital status Married 62(62.0%) Unmarried 34 (34%) Widow 4 (4.0%) Group 2 Control (Marital status) **P value is 0.001.** Group 2 about the habits smoker 26(26.0%) Non-smoker 74 (74.0%0) Group2 control (Habits) smoker 34(34%) and non-smoker 66(66%) P value 0.004 Group 1 about type of smoking Bidi 9(34.6%), cigarette 5(19.2%) others 12(46.2%) and Group 2 Control (type of smoking) Bidi 12(35.3%), cigarette 16(47.1%) others 6(17.6%) **P value 0.027.**

Table 1: Demographic correlation of patients of EPTB

			Group				p-value
s. no.	Demographic parameters		Cases		Controls		
			N	%	N	%	
1	Gender	Male	65	65.0%	64	64.0%	**0.046**
		Female	35	35.0%	36	36.0%	
2	Religion	Hindu	66	66.0%	96	96.0%	**<0.001**
		Muslim	26	26.0%	4	4.0%	
		Other	8	8.0%	0	.0%	
3	Socioeconomic status*Kuppuswammy 2021. ***Indian J Forensic Community Med*** 2021;8(1):1-3	Lower	66	66.0%	20	20.0%	**<0.001**
		Middle	10	10.0%	10	10.0%	
		lower middle	8	8.0%	28	28.0%	
		upper middle	12	12.0%	36	36.0%	
		Rich	4	4.0%	6	6.0%	
4	Occupation	Student	16	16.0%	24	24.0%	**0.001**
		Labour	40	40.0%	26	26.0%	
		Housewife	36	36.0%	20	20.0%	
		Other	8	8.0%	30	30.0%	
5	Marital status	Married	64	64%			**0.001**
		Unmarried	34	34%			
		Widow	4	4%			
6	Habits smoker	Smoker	26	26.0%	34	34.0%	**0.004**
		Non-smoker	74	74.0%	66	66.0%	

7	Type of smoking	Bidi	9	34.6%	12	35.3%	**0.027**
		Cigarette	5	19.2%	16	47.1%	
		Other	12	46.2%	6	17.6%	

s.no.	**Radiological findings**	N	Percent
1.	ULTRASOUND	23	23.0%
2.	CT	20	20.0%
3.	FNAC	21	21,0%
4.	CULTURE	36	36.0% Total-100

Confirmation of EPTB Patients

Medical history presents 100 (100.0%), absent 0 (0.0%)**.** History presents 12 (12.0%) and absent 88(88.0%). Family history Present 16 (16.0%) and absent 84 (84.0%). Afb smear Positive 10 (10.0%) and negative 90 (90.0%) 0.81 Confirmation of EPTB Patients had definite Extra pulmonary patients. ultrasound 23(23.0%) CT 20(20.0%) FNAC 21(21.0%) CULTURE 36(36.0%) Plasma *LTB4* levels were measured in 100 subjects of each case and controls by *LTB4* Immunoassay. ELISA kit and follow as per manufacturer protocol. CEA562Ge 96 Tests Enzyme-linked Immunosorbent Assay Kit for Leukotriene B4 (*LTB4* Plasma *LTB4* levels were lower in cases than in controls. *LTB4* measured by ELISA and *LTA4H* mRNA expression evaluated by real time PCR using SYBR green method. The expression levels of *LTA4H*gene, were lower in patients than control. *LTA4H* gene were considerably correlated with Age Sex, Medical history, Socioeconomic status, occupation and other clinical parameter lymph node tuberculosis, bone tuberculosis, pleural tuberculosis and other extra-pulmonary tuberculosis. These findings were suggested that Expression in the *LTA4H* gene might be influence the sensitivity to extra-pulmonary tuberculosis and a new potential genetic risk factor of extra-pulmonary tuberculosis. This study will be a step to reduce the diagnostic and therapeutic window to identify another risk factor for extra pulmonary tuberculosis and will spawn new ways to safeguard and improve the health status of individuals and population groups. On ELISA Method for, Extra pulmonary patients had lower expression of compared to the controls. Correlation of biomarkers: *LTA4H* level correlated with age, Sex Smoking, , Other Clinical Parameter On ELISA, Extra pulmonary patients(2304.52pg/ml) had lower expression of *LTB4* compared to the controls (3096.142pg/mls) (P value = 0.0012).

5. Measurement of protein level of LTB4

On ELISA, Extra pulmonary patients (2304.52pg/ml) had lower expression of LTB4 compared to the controls (3096.142pg/mls) (P value = 0.0012).

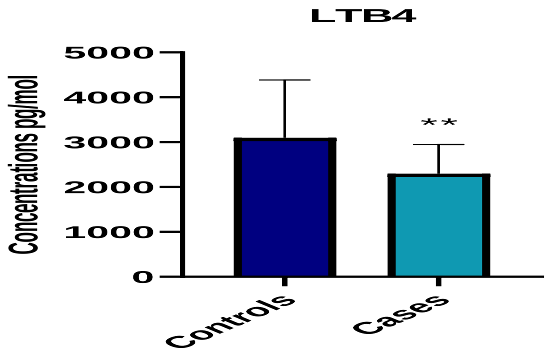

Fig no 01: Error Bar diagram Protein Level of LTB4 lower Expression of the patients with extra pulmonary (case) compared to controls.

6. Discussion:

Tobin &co-workers in the year (2012) were again did an experiment on animal model zebrafish and found susceptibility for *Mycobacterium marinum*, can result from either inadequate or excessive acute inflammation. They also revealed the two distinct molecular routes to *Mycobacterial* susceptibility converging on dysregulated TNF levels: inadequate inflammation caused by excess lipoxins and hyper inflammation driven by excess LTB4. In humans, recognize a single nucleotide polymorphism (SNP) in the LTA4H promoter region that regulates its transcriptional activity. In tubercles meningitis, polymorphism was associated with inflammatory cell recruitment along with patient survival and response to adjunctive anti-inflammatory therapy. A study by **(10)** Some other studies have shown a similar effect in different diseases, single nucleotide polymorphisms in 5-lipoxygenase activating protein (ALOX5AP) and LTA4H genes were associated with LTB4 over production in myocardial infarction susceptibility. A single study of India **(11)** has evaluated the genetic contribution of Leukotriene variants with coronary artery disease (CAD) and also observed the association of these variants with plasma LTB4 levels. Plasma LTB4 levels were measured in 150 subjects of each cases and controls by LTB4 Immunoassay. Plasma LTB4 levels were higher in cases than in controls LTB4 measured by ELISA and LTA4H mRNA expression evaluated by real time PCR using SYBR green method. The expression levels of LTA4H gene, TNF, IL-8 were higher in patients than control. Finally they concluded that LTA4H exhibit close association in subject with cardiovascular disease and may provide incremental values for predicting cardiovascular risks. Elisa Method to Extra pulmonary patients had lower expression of compared to the controls in zebrafish embryo and discovered a class of mutants hyper susceptible to M. marinum infection. Mutations in the lta4h gene of zebrafish were displayed increased bacterial growth and reduced TNF signalling **(12).** Mutations of the lta4h gene reduced its expression and redirected LTB4 synthesis along with production of the anti inflammatory LXA4. These results suggest that common polymorphisms in the LTA4H gene do not play any major role in susceptibility to clinical pulmonary; zebrafish and found susceptibility for Mycobacterium marinum, can result from either inadequate or excessive acute inflammation **(13).** They also revealed the two distinct molecular routes to Mycobacterial susceptibility converging on dysregulated TNF levels: inadequate inflammation caused by excess lipoxins and hyperinflammation driven by excess LTB4 **(14).** In humans, recognize a single nucleotide polymorphism (SNP) in the LTA4H promoter region that regulates its transcriptional activity. In tuberculous meningitis, polymorphism was associated with inflammatory cell recruitment along with patient survival and response to adjunctive antiinflammatory therapy. ELISA, Extra pulmonary patients (2304.52pg/ml) had lower expression of LTB4 compared to the controls (3096.142pg/mls) (P value = 0.0012). Extra pulmonary patients. Plasma *LTB4* levels were measured in 100 subjects of each case and controls by *LTB4* Immunoassay. ELISA kit and follow as per manufacturer protocol. CEA562Ge 96 Tests Enzyme-linked Immunosorbent Assay Kit for Leukotriene B4 (*LTB4* Plasma *LTB4* levels were lower in cases than in controls. *LTB4* measured by ELISA

Indirectly LTA4H gene coordinates to the balance of pro and anti-inflammatory eicosanoids but polymorphisms in this gene may govern the susceptibility to *Mycobacterial* infections. Impairment of the LTA4H gene by genetic polymorphism can lead to inadequate immune response by affecting the levels of TNF which in turn results in a high or low inflammatory **(15,16).** In zebrafish larvae infected with *Mycobacterium marinum*, LTA4H deficiency and excess both produce hyper-susceptibility with increased bacterial burdens. Over-expression of LTA4H produces TNF excess during infection and can be rescued by genetic knockdown or pharmacological modulation of TNF. The human LTA4H gene is a single-copy gene with a length greater than 35 kb and localized on chromosome 12q22 **(17,18).** The protein encoded by LTA4H gene contains 610amino acid residues with a molecular weight of 69,140 Da. LTA4H may affect TNF levels via LTB4 generation. Hence the single nucleotide polymorphisms (SNPs) of the LTA4H gene lead to a selective loss of the ability of the enzyme to produce LTB4 and may affect the susceptibility of the host to tuberculosis through participating in immune responses **(19, 20).** Commonly susceptibility to tuberculosis is described as insufficient immune response that fails to control infecting *Mycobacteria*. Ultimately this is very fascinating to know the associations of SNPs and mRNA expression of LTA4H gene and plasma LTB4 level with EPTB patients.

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