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Genetic Analysis of Gene Responsive to Tuberculosis In Under Developing Areas of Pakistan

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Tuberculosis is a global infectious disease caused by the bacteria *Mycobacterium tuberculosis* (*Mtb*). One third of the world population is estimated to be victim of this bacterial disease with approximately 1.7 million deaths yearly. In this study the presence of *Mtb* was analyzed in suspected tuberculosis (TB) patients and the percentage in the gender of rural population was calculated. TB was determined by sputum analysis in acid fast structures such as *Mycobacterium tuberculosis*. The TB positive patients were further investigated for the detection single nucleotide polymorphisms (SNPs) of chemokine ligands C-C motif (CCL-1). CCL1 has been associated with pulmonary TB. Specific primers for SNP (rs12600717 and rs12603965) were designed for CCL1 and amplified by PCR. The amplified gene was run on agarose gel for determining the size of the gene and run with the known molecular weight size markers. In order to verify mutations in CCL1, the amplified gene was sequenced. Four novel mutations were detected, which gave various protein products. These results may lead to development of new diagnostic markers for TB pathogenesis

Key words: Pulmonary tuberculosis, sputum analysis, CCL1, SNP, novel mutations.

In accordance to the WHO 2013 Global Tuberculosis report, Pakistan is ranked seven globally and the incidence and percentage of the spread of the disease in Pakistan is 0.329 per annum. Approximately 90% of people infected with Mtb show no symptoms; around 10% develop symptoms manifested as pulmonary disease; 1% develops TB meningitis, which is the most severe form and have a mortality rate of 20–25%. Currently immunological methods are used to diagnose tuberculosis infection including the tuberculin skin test or interferon release assay, but microbiological tests such as sputum analysis are mainly used to diagnose active TB[1]. Both TB and chronic obstructive pulmonary disease (COPD) primarily affect the lungs and share common risk factors such as smoking, low socioeconomic status and dysregulation of host defense functions. Furthermore COPD has a high prevalence in elderly with TB[2].

Sputum analysis and culture is the most common and specific method of specific diagnosis of lower respiratory tract diseases usually caused by bacteria[3]. Culture of a well screened, expectorated sputum sample will identify the pathogen which is a bacterium in most cases of bacterial pneumonia[4]. Expectorated sputa, as well as those secretions obtained by aspiration from

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the upper airway or respiratory system, are thought a part of oropharyngeal bacterial contamination. Other techniques in which transtracheal aspiration is included are more "sterile," but have a much higher rate of incidence of morbidity[5]. In the sputum of the positive patient for the tuberculosis acid fast structures are found[6].

In response to infectious TB, inflammatory or immune cells usually migrate to the lungs, where they make granulomas that controls and contains infection. The infection leads to production of specific chemokines, which act as chemotactic and activating factors for leukocytes[7]. In mammals around 27 chemokine ligands C-C motif (where the first 2 Cys residues are immediately adjacent) induced genes (CCL1-28) are known, and with their receptors they are key regulators in recruitment of immune cells to sites of infection and inflammation. The CC chemokines are classified according to their function in inflammation, hemostatis or both. The chemokine (C-C motif) ligand 1 (CCL1) function both in inflammation and homeostatis[8]. CCL1 responsiveness increases with the increase in the age meaning that a direct proportional relationship exists between age of suspected person and the CCL1 gene.

The CCL1 (also called TCA-3 or I-309) protein takes actively part in the signaling of cells and has a role on the defense mechanism. In monocytes (but not neutrophils) the CCL1 chemokine displays chemotactic activity and acts as a ligand for the chemokine (C-C motif) receptor 8 (CCR8) receptor, which is expressed when Th2 cells and T cells are polarized[9]. The host defence against TB is induced by Th1, but sometimes Th1 is inhibited by cytokines secreted from Th2 cells. Hence the expression of CCL1 genes is an important mediator of TB susceptibility and progression of the disease[10]. Polymorphism of TB related genes have been widely investigated. These include genes encoding, tumor necrosis factors (TNFs) and their receptors[11], major histocompatibility complex/human leukocyte antigen (MHC/HLA)[12], Toll-like receptors (TLRs)[13], interleukins and cytokines[14]. In this work single nucleotide polymorphism (SNP) was investigated in CCL1 related to TB, which will be helpful in understanding and developing novel vaccines against TB.

MATERIALS AND METHODS

Study sample

All the participants (n=256) involved were suspected TB patients from the rural population of Burewala, Pakistan. The participants belonged to different health units of Burewala: Tehsil head quarter of Burewala (n=99), Rural health center of Gaggoo (n=73), Basic health unit 177/E.B. (n=52) and Basic health unit 197/E.B. (n=32). All patients' sputum was collected and analyzed by acid fast staining[15]. Positive samples were further investigated.

DNA extraction

Blood samples from all TB positive participants were selected and DNA was isolated for investigation of CCL1 SNP's. 5ml of blood + 45ml of Lysis buffer (2X) were added to 50ml capped centrifuge tube and mixed for 100 min at 3000 rpm. Supernatant was discarded and 3ml of EDTA salt buffer + 0.3 ml of 10% SDS + 0.1ml of proteinase K were added to the pellet. All tubes were incubated over night at 37ºC in shaking water bath. Next day 3ml of liquid phenol was added, mixed thoroughly for 10 min and centrifuged for 10 min at 2000 rpm. 3 ml of chloroform Isoamyl alcohol was added to the upper aqueous phase and centrifuged for 5 min at 2000rpm. 6 ml ethanol was added to precipitate the DNA. For completely dryness of the precipitated DNA completely the tubes were put up side down. After drying 0.5 ml of 10mM EDTA buffer was added to re-dissolve the DNA overnight. Next day 25µl of DNA is mixed with 2ml of distilled water in quartz cuvette and the absorbance at 260 and 280nm was recorded. The absorbance ratio calculated was 260/280nm. The extracted DNA was stored at -20 ºC for further processing.

Amplification of CCL1 gene

CCL1 was amplified using PCR. Two pairs of CCL1 primer were designed using NCBI Genbank and primer3plus. Two SNPs were selected rs12600717 and rs12603965. The primers used to amplify rs12600717 was 5'-TCCCTCCCAGCAAGAAACAC-3' and 3'-TCAACCTCAATGGGGCTGAC-5', and for rs12603965 was 5'-CCTGTGCTCCTCCTCTCTCT-3' and 3'-TTCAGATGCCTGGAAGCCTG-5'.

PCR was performed in a volume of 50 µl containing 2.5 µl cDNA (conc. 2-20 ng), 0.05 µl primer pair, 1.25 mM DNTPs, 1.5 mM $MgCl_2$, 0.2 µl

Tag polymerase (Roche). The PCR protocol was as following:
Step 1: 94 ºC for 3 min; Step 2: 94 ºC for 30 s, 53 ºC for 30 s and 72 ºC for 50 s. This was repeated for 20 cycles; Step 3: 72 ºC for 2 min.

The amplified PCR product was run on 1% agarose gel electrophoresis

After running the segment of DNA on the gel and after finding its exact molecular weight next step was to determine the exact sequence of that gene and find where the change exactly occurred. All the materials (template DNA 5 µl, radioactive labeled primer 0.05 µl, DNA 0.2 µl, dNTPs 2 µl and any one of the four dideoxynucloetide 0.375 µl) were added in automatic DNA sequencer.

Statistical analyses were carried out with SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). The results are presented as the mean ± SEM. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

TB is the severe disease which spreads through air and is a severe infection to the respiratory system of the infectious persons and can infect the inner lining of the cells in the pulmonary system. The epithelial cells in the respiratory system provide a best environment to these bacterial cells for increasing in number[16]. These bacteria are recognized well as compared to other bacteria because they are acid fast stains and they all well capable of living in the body of the infectious person for a long time. Throughout the world the death rate because of this disease is 2 million per annum and a serious reason of decline in life[17]. Tb is transmitted through air from one person to the other causing a serious change in the genetic material of the host cell and some malfunctioning of the codon responses[18].

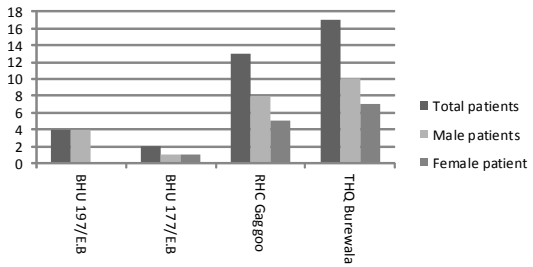

Fig. 1. Graphical view of the ratio of patient in all survey areas

In this study the correlation between TB and gender in rural population of Pakistan was investigated (See Figure 1). Total number of patients were 256, and 36 patients (23 male and 13 female) were found positive for *Mycobacterium tuberculosis*. Statistical test was applied on the total population versus total affected person, with male and female patients and female versus male gave the following results (Table 1). This gave the clear view about the probability of occurring the disease in male is more than that of female ($p \leq 0.05$). This is in agreement with WHO recent surveillance report (2015) which showed that the TB is more prevalent in males as compared to females, though some cases have shown females may be more susceptible to TB due to vitamin D deficiency[19].

TB infected patients blood samples were collected and DNA was isolated to see any genetic change. Furthermore genetic investigations during infection show genetic changes in exons[20]. This is important as changes may lead to expression of aberrant gene products. CCL1 gene was chosen, as it has already been associated with pulmonary TB[21]. Two different SNP's were selected (rs12600717 and rs12603965) from the NCBI database. Primers were designed and the CCL1 gene was amplified. Four changes in the sequence of CCL1 were observed in TB positive patients

Table 1. Statistical test of patient number and gender in TB patients

Sr No.	Statistic Program	Total patient & male	Total patient & female	Male & female patients
1	P-value	0.3206	0.1660	0.0222
2	95% confidence interval	-4.57 to 8.57	-4.39 to 13.06	0.75 to 4.75
3	t	1.3093	2.1372	4.3710
4	df	2	2	3
5	SED	1.528	2.028	0.629

(see Table 2). The codon AAG gave 3 different changes. When nucleotide A at position 577 was mutated to T, it results in a stop codon, suggesting expression of a truncated protein. When nucleotide G at position by 579 was mutated to C, the amino acid Lys is converted to Asn, and when both nucleotides A and G were mutated then Lys was converted to Tyr. Lys is a very important amino acid, which contain a basic side-chain that can be acetylated and/or methylated. Acetylation is an important modification, and has been shown to play an important role in cellular processes, metabolism, cell signaling, cytoskeleton dynamics and circadian rhythm[22] When the sequence of CCL1 was submitted to the PAIL Lys acetylation prediction server[23], Lys was predicted as a positive acetylation site (results not shown). This means that Lys has a potential for acetylation, and when this amino acid is mutated to either Asn or Tyr, its function abolishes. The same sequence was submitted to the phosphorylation prediction server Netphos 2.0[24] and NetNGlyc 1.0[25], where Lys was replaced with Tyr and Asn, respectively. Tyr was also predicted as a positive predicted phosphorylation site, but Asn did not show any potential for glycosylation. These results suggest that when Lys is mutated to Tyr, the function of CCL1 changes. Phosphorylated Tyr also modulates the function of the protein, and is known to play a role in transmembrane and intracellular signaling by serving as a docking site for proteins that contain phosphorylated Tyr-binding domain, such as an SH2 or PTB domains[26]. When the nucleotide A at position 580 is mutated to T, the amino acid Ser is converted to Cys. Ser phosphorylation potential was predicted by Netphos 2.0 as positive,

Table 2. DNA sequence of CCL1 in normal and TB infected patients

Sr no.	CCL 1 translational view in control sample			CCL1 translational view in affected person		
	Codon	Position	A.A coding	Codon	Position	A.A coding
1	ATG	508,509,510	M	ATG	508,509,510	M
2	CAG	511,512,513	Q	CAG	511,512,513	Q
3	ATC	514,515,516	I	ATC	514,515,516	I
4	ATC	517,518,519	I	ATC	517,518,519	I
5	ACC	520,521,522	T	ACC	520,521,522	T
6	ACA	523,524,525	T	ACA	523,524,525	T
7	GCC	526,527,528	A	GCC	526,527,528	A
8	CTG	529,530,531	L	CTG	529,530,531	L
9	GTG	532,533,534	V	GTG	532,533,534	V
10	TGC	535,536,537	C	TGC	535,536,537	C
11	TTG	538,539,540	L	TTG	538,539,540	L
12	CTG	541,542,543	L	CTG	541,542,543	L
13	CTA	544,545,546	L	CTA	544,545,546	L
14	GCT	547,548,549	A	GCT	547,548,549	A
15	GGG	550,551,552	G	GGG	550,551,552	G
16	ATG	553,554,555	M	ATG	553,554,555	M
17	TGG	556,557,558	W	TGG	556,557,558	W
18	CCG	559,560,561	P	CCG	559,560,561	P
19	GAA	562,563,564	E	GAA	562,563,564	E
20	GAT	565,566,567	D	GAT	565,566,567	D
21	GTG	568,569,570	V	GTG	568,569,570	V
22	GAC	571,572,573	D	GAC	571,572,573	D
23	AGC	574,575,576	S	AGC	574,575,576	S
24	AAG	577,578,579	K	AAC	577,578,579	N
24(1)				**TAG**	**577,578,579**	**Stop codon**
24(2)				**TAC**	**577,578,579**	**Y**
25	**AGC**	**580,581,582**	**S**	**TGC**	**580,581,582**	**C**
26	**ATG**	**583,1773,1774**	**M**	**CTG**	**583,1773,1774**	**L**

The bold underlined nucleotides are mutations of the CCL1 gene in infected patients

which suggest that if Ser is converted to Cys, CCL1 function may be lost. Finally when the nucleotide A is converted to C at position 583, the amino acid Met is changed to Leu. Met is necessary for donation of its methyl group for different function. Overall the different SNPs produce different aberrant protein products, which may prevents CCL1 from normal functioning.

CCL1 is produced mostly by monocytes and binds to its receptor CCR8, present on lymphocytes and monocytes, where it regulates migration of dendritic cells to lymph nodes. CCR8 is highly enriched on Th2 and regulatory T cells, and influence the progression of Th2 type T cell. Expression of the receptor CCR8 is up-regulated by CCL1 stimulation of peritoneal macrophages, and lead to cell aggregation. Genetic variation or alteration of CCL1 function influence the migration of T cells, monocytes and dendritic cells to the site of infection. This may result in unconstrained bacterial growth leading to pulmonary disease.

CONCLUSION

Diagnosis and determination of the genetic bases of the infection provides a good basis for management and control of TB infection. In this work novel SNPs has been detected in TB positive patients, which may lead to novel protein products. Further studies in this area could lead to understanding and treatment with more accurate prognostic information. In addition, this work may lead to novel molecular and development of new diagnostic markers for TB pathogenesis.

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