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Common *MCL1* polymorphisms associated with risk of tuberculosis

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MCL1 expression has been found to be up-regulated during infection with virulent *Mycobacterium tuberculosis*. We investigated the genetic polymorphisms in *MCL1* as potential candidate gene for a host genetic study of clinical TB infection. We have sequenced exons and their boundaries of *MCL1*, including the 1.5 kb promoter region, to identify polymorphisms, and eight polymorphisms were identified. The genetic associations of polymorphisms in *MCL1* with clinical TB patients (n = 486) and normal controls (n = 370) were analyzed. Using statistical analyses, one common promoter polymorphism (*MCL1-324C>A*) which is absolutely linked with three other SNPs in the promoter and 3'UTR regions, were found to be significantly associated with increased risk of clinical TB disease. The frequency of the A-bearing genotype of *-324C>A* was higher in clinical TB patients than in normal controls (P = 0.0008, OR = 1.68). Our findings suggest that polymorphisms in *MCL1* might be one of genetic factors for the risk of clinical tuberculosis development. [BMB Reports 2008; 41(4): 334-337]

INTRODUCTION

Approximately one-third of the world's population is infected with the bacterium *M. tuberculosis* that causes tuberculosis (TB). This makes TB a significant cause of morbidity/mortality, as it results in approximately 2 million deaths annually (1). However, only 10% of those infected are estimated to progress to active (clinical) TB disease. Host genetic factors are, at least partly, important determinants of susceptibility to TB (2). The doubly high risk of disease in identical twins compared with non-identical twins (3) indicates a host genetic component in susceptibility. Understanding the molecular mechanisms underlying protective immunity is a prerequisite for the development of improved therapies and vaccines for TB.

MCL1 (Myeloid Cell Leukemia 1) is one of anti-apoptotic B-Cell CLL/Lymphoma 2 (BCL2) family which is involved in the control of cell viability (4). MCL1 protein expression has also been found to be up-regulated during infection with virulent *M. tuberculosis*. Thus, the anti-apoptotic effect of the induction of MCL1 expression in infected macrophages promotes the survival of virulent *M. tuberculosis* (5). Moreover, in a previous study, a 6- or 18-bp sequence insertion was found in the *MCL1* promoter region and was associated with rapid disease progression and failure to respond to chemotherapy in chronic lymphocytic leukemia patients (6).

Here we present the genetic polymorphisms in *MCL1* as potential candidate gene for a host genetic study of TB. We performed extensive screening of *MCL1* by direct sequencing, as well as statistical analysis to examine the genetic effects on the risk of TB in Korean male subjects.

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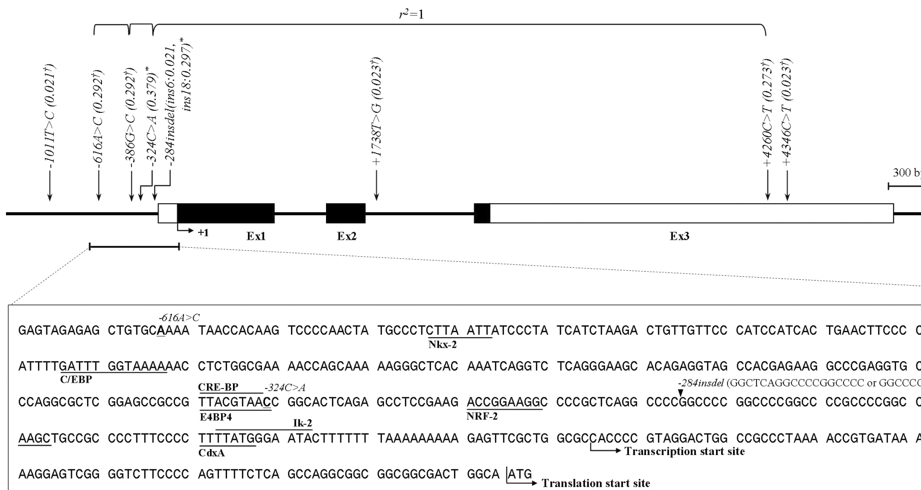
RESULTS

To discover polymorphism(s) in *MCL1*, we performed direct DNA sequencing in 24 unrelated Korean individuals. We identified 8 polymorphisms; five in the promoter region, one in intron 2, and two in 3'UTR in *MCL1* (Fig. 1A, and Ad. Table 2). Pair-wise comparisons among polymorphisms revealed one set of absolute LD in *MCL1* ($|D'|$ = 1 and r^2 = 1) (*-616A>C* : *-386G>C* : *-324C>A* : *+4260C>T*). The *-284insdel* showed two insertion types: 6-nucleotide (GGCCCC, *ins6*) and 18-nucleotide (GGCTCAGGCCCCGGCCCC, *ins18*) insertion (Fig. 1A). Among identified polymorphisms, two (*-324C>A* and *-284insdel*) in *MCL1* were selected for larger-scale (n = 856) genotyping for a TB genetic association study based on frequencies (> 0.05) and LDs (only one SNP if there is absolute LDs (r^2 = 1)). Three major (freq. > 0.05) haplotypes in *MCL1* were constructed from the Korean male subjects using the algorithm developed by Schaid *et al.* (7) (Haplo.Score) (Fig. 1B).

Logistic regression analyses controlling for age and smoking status (non-smoker = 0, ex-smoker [ex-smokers in TB patients, and current smokers in controls] = 1; no smokers were in patient groups because smoking is not allowed for in-hospital patients) as covariates revealed that one common (freq. = 0.379) promoter SNP, *-324C>A* that is in absolute LD with *-616A>C*, *-386G>C*, and *+4260C>T*, showed strong association



B. Haplotypes in *MCL1*

Hap.	-324C>A	-284insdel	Freq.
ht1	A	del	0.372
ht2	C	del	0.311
ht3	C	ins18	0.290
Others[(1)]	-	-	0.027

Fig. 1. Gene structure, polymorphisms and haplotypes of *MCL1*. Coding exons are marked by black blocks and 5' and 3' UTRs by white blocks. First base of translation sites are denoted as nucleotide +1. Asterisks (*) indicate polymorphisms genotyped in a larger Korean population (n = 856). The minor allele frequency based on 24 sequencing samples, denoted as daggers (†), might be different from those based on genotyping in the larger population. The lines with "r^2 = 1" indicate absolute LD among polymorphisms. (A) Gene structure and polymorphisms in *MCL1* on chromosome 1q21. Putative transcription factor-binding sites around *MCL1-324C>A* are shown. 6-bp insertion and 18-bp insertion alleles of *-284insdel* polymorphism in *MCL1* represented a 6-nucleotide (GGCCCC) insertion and an 18-nucleotide (GGCTCAGGCCCCGGCCCC) insertion, respectively. The putative transcriptional factor-binding sites identified by sequence homology in relation to negative and positive regulatory elements (Heinemeyer *et al.*, 1998) are also shown. (B) Haplotypes in *MCL1*. [(1)]*Others* contain rare haplotypes: C-ins6, A-ins18, A-ins6.

Table 1. Logistic analysis of association of *MCL1* polymorphisms with risk of clinical tuberculosis disease in Korean male subjects

Gene	Loci	Minor allele frequencies		*P*
		Case (n = 486)	Control (n = 370)	
MCL1	*-324C>A*	**0.407**	**0.342**	**0.002**
	*-284insdel**			
	deletion	0.313	0.325	0.33
	6-bp insertion	0.026	0.028	0.92
	18-bp insertion	0.302	0.303	0.53
	ht1[A-del]	**0.397**	**0.341**	**0.004**
	ht2[C-del]	**0.291**	**0.335**	**0.04**
	ht3[C-ins18]	0.284	0.299	0.27

Logistic regression models were used for calculating P-values of polymorphisms, controlling for age and smoking status as covariates. P-values of co-dominant models are given. P-values of haplotype association were calculated using the algorithm developed by Schaid *et al.* (Haplo.Score), while controlling for age and smoking status as covariates.

with the increased risk of clinical TB disease (Table 1). In further haplotype analysis, a susceptible effect of *MCL1 ht1* (A-*del*) and a protective effect of *MCL1 ht2* (C-*del*) were also observed (P = 0.004 and P = 0.04, respectively) (Table 1). The frequency of *-324A*-bearing genotypes (AC or AA of *MCL1-324C>A*) was higher in patients (64.7%) than in normal controls (55.4%) (P = 0.0008, OR = 1.68, Table 2).

Analysis of putative transcription factor-binding sites using the software TFSEARCH (8) was performed with the promoter sequence of *MCL1*. Putative transcription factor-binding sites that had putative score greater than 0.9 based on the TRANSFAC database are shown in Fig. 1A. The DNA sequences surrounding *MCL1-324C>A* was shown to include motifs specific for CRE-BP (cycling AMP response element-binding protein) and E4BP4 (adenovirus E4 promoter-binding protein) binding sites.

DISCUSSION

TB kills about two million people each year, making it one of the world's leading infectious causes of death among young people and adults (9). Each year, more than 8 million people become sick with TB. Due to a combination of factors including economic decline, the breakdown of health systems, insufficient application of TB control measures, the spread of HIV/AIDS, and the emergence of multidrug-resistant TB (MDR- TB), TB is on the rise in many developing and transitional economies (9).

MCL1 is induced by infection of T helper cells with viable and virulent *M. tuberculosis*, but not in response to phagocytosis or ingestion of heat-killed or attenuated *M. tuberculosis*. Induction of this antiapoptotic protein limits the extent of apoptosis in virulent *M. tuberculosis*-infected macrophages as inhibition of MCL1 expression results in increased apoptosis of infected cells. This mechanism appears to contribute to the dif-

Table 2. Association analysis of *MCL1 -324C>A* with risk of clinical tuberculosis disease in Korean male subjects

Subgroup	Genotype			Analyzing model					
				AA vs. AC vs. CC		(AA+AC) vs. CC		AA vs. (AC+CC)	
	CC	*AC*	*AA*	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
TB patients	**165 (35.3%)**	**225 (48.1%)**	**78 (16.7%)**	**1.40 (1.13-1.73)**	**0.002**	**1.68 (1.24-2.27)**	**0.0008**	1.34 (0.88-2.03)	0.17
Normal controls	**164 (44.6%)**	**156 (42.4%)**	**48 (13.0%)**						

Logistic regression model was used for calculating odds ratios (95% confidential interval) and corresponding P-values for SNP sites, controlling for age and smoking status as covariates. P-values of co-dominant, dominant, and recessive models are also given. The significance of association (P = 0.0008 in the dominant model) was retained even after the strictest correction for multiple tests (20 tests in this study).

ferential amounts of apoptosis observed when comparing virulent and avirulent mycobacterial strains and species (5).

Although the mechanisms of MCL1 polymorphisms in manifestation of clinical TB are not fully understood, by screening a large number of TB patients, we are able to suggest, for the first time, that one *MCL1* promoter SNP (*MCL1-324C>A*) and two haplotypes carrying the *del* allele of *-284insdel* (*MCL1 ht1* and *ht2*) are significantly associated with the risk of clinical TB disease. It seems likely that the genetic effects of *MCL1 ht1* (*A-del*) susceptible to clinical TB disease come from the *A* allele of *MCL1-324C>A*, because it was mostly tagged by *MCL1-324C>A* (97.3%, Fig. 1B). Likewise, the opposite direction of effects of *MCL1 ht2* (*C-del*) might come from the *C* allele of *-324C>A*, although the strength was less than that of *MCL1 ht1* (*A-del*).

Using statistical analysis, it would be hard to tell which site(s) is causal for the increased risk of clinical TB because four SNPs (*-324C>A*, *-616A>C*, *-386G>C*, and *+4260C>T*) were in absolute LD. However, when considering that the promoter SNP, *-324C>A*, was shown to include motifs specific for CRE-BP (cycling AMP response element-binding protein) and E4BP4 (adenovirus E4 promoter-binding protein) binding sites, it could be speculated as the causal site for increased risk of clinical TB manifestation. The possibility that the promoter polymorphisms may alter MCL1 protein regulation/transcriptional binding activity will need to be evaluated in future studies.

In summary, one common promoter SNP (*-324C>A*) of *MCL1* was found to have significant association with risk of clinical TB disease through screening of a large number of TB patients and normal controls recruited from Korean male subjects.

MATERIALS AND METHODS

Subjects

A total of 486 patients with clinical pulmonary TB (mean age, 46.7 years; range, 20-86 years, all male) were recruited from the Clinical Research Center for TB, National Masan Tuberculosis Hospital, Korea.

PCR has been routinely tested in all sputum AFB culture-positive samples to distinguish Mycobacterium Tuberculosis (MTB) from non-tuberculous mycobacterial (NTM). The diagnosis of pulmonary TB was confirmed by the isolation of *M. tuberculosis* from sputum or bronchoalveolar lavage fluid. The patients with NTM infection were excluded from this study. TB patients who had a family history of the disease were excluded to eliminate the additional risk factors of exposure to TB. A total of 370 healthy controls (mean age, 54.9 years; range, 40-69 years, all male; Ad Table 1) were simultaneously recruited from an unselected population who had come in for routine health checkups in the same regional area. Only subjects whose ages were greater than 40 were included in normal controls to exclude the possibility of TB infection among young individuals (TB may subsequently develop in a proportion of the controls). Individuals with other apparent diseases such as HIV infection (no HIV-positive patient have been reported in our hospital so far), hepatitis (mainly chronic hepatitis B infection), diabetes, alcoholism, autoimmune diseases, and cancers were also excluded (in both cases and controls). The ethnicity of all patients and controls was Korean. Informed consents were obtained from all subjects before drawing blood. The study protocol was approved by the Institutional Review Board of National Masan Hospital. Written informed consent was obtained from each subject.

Sequencing analysis of the *MCL1* gene

Genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (Wizard Genomic DNA purification kit, Promega, Madison, WI, USA). We sequenced exons and their boundaries in *MCL1*, including the promoter region (~1.5 kb), to discover genetic variants in 24 Korean DNA samples using a DNA analyzer (ABI PRISM 3730, Applied Biosystems). Twenty-four primer sets of the *MCL1* gene for the amplification and sequencing analysis were designed based on GenBank sequences (Ref. Genome sequence for *MCL1*; NT_086596 released in Aug. 2004). The primer information is available as Ad Table 3 (http://www.snp-genetics.com/reference/SupplementaryInformationToMCL.doc) and sequence variants were verified by chromatograms.

Genotyping

For genotyping of polymorphic sites, amplifying primers and probes were designed for TaqMan. Primer Express (Applied Biosystems) was used to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labeled with the FAM dye and the other with the fluorescent VIC dye. PCRs were run in the TaqMan Universal Master mix without UNG (Applied Biosystems), with PCR primer concentrations of 900 nM and TaqMan MGB-probe concentrations of 200 nM. Reactions were performed in a 384-well format in a total reaction volume of 5 μl using 20 ng of genomic DNA. The plates then were placed in a thermal cycler (PE 9700, Applied Biosystems) and heated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The TaqMan assay plates were transferred to a Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity in each well of the plate was read. Fluorescence data files from each plate were analyzed using automated software (SDS 2.1). The *-284insdel* in the promoter of *MCL1* was genotyped using an ABI3100 DNA sequencer in conjugation with GENESCAN and GENOTYPER software (Applied Biosystems). Oligonucleotides used in *-284insdel* genotyping include the fluorescent labeled forward primer FAM-CGAGGTGCTCATG-GAAAGAG and reverse primer TTCCCATAAAAGGGGAAAGG. PCR fragments of 212, 218, and 230 bp were obtained, corresponding to deletion, 6 bp (GGCCCC, 6 bp insertion) and 18 bp (GGCTCAGGCCCCGGCCCC, 18 bp insertion) insertion types. The primer information is available as Ad Table 4 (http://www.snp-genetics.com/reference/SupplementaryInformationToMCL.doc).

Statistics

We examined Lewontin's D' (|D'|) and the linkage disequilibrium coefficient r^2 between all pairs of biallelic loci (5). Haplotype frequencies were inferred using the algorithm developed by Schaid *et al.* (7) (Haplo.Score). Logistic regression models were used for calculating odds ratios (95% confidential interval) and corresponding P-values, controlling for age (continuous value) and smoking status (non-smoker = 0, ex-smoker/smoker = 1) as covariates. P-values of haplotype associations were also calculated by the algorithm developed by Schaid *et al.* (9) (Haplo.Score). Putative transcription factor- binding sites were examined using the software TFSEARCH: Searching Transcription Factor Binding Sites (version 1.3, http://www.cbrc.jp/research/db/TFSEARCH.html) (putative score > 0.9) based on the TRANSFAC database (6).

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