Table 1 Primer sequences used in this study

	Forward or sense	Reverse or antisense
Probe preparation	CAGGAATGCATCTCTGGCTC	CTCCTATCTCTTGCTACATTCC
cDNA library screening		
5′-gene specific linker (GSP) primer	GAGAGAGAGAGAGAGAGAGAACTAGTC-TCGAGCAGTTGATGAAGACAGCACAG	
3′-GSP linker primer	GAGAGAGAGAGAGAGAGAGAACTAGTC-TCGAGCTGTGCTGTCTTCATCAACTG	
Random hexamer	GAGAGAGAGAGAGAGAGAGAACTAGT-CTCGAGNNNNNN	
5′-RACE		
GSP-1	CCACATGGGTGGACAGATGCCTCC	
Nested GSP-1	CTAGTGCAGAGGGCTCTTTGTGCC	
GSP-2	ACGGGGTCATGGTGGCCACATGAAC	
Nested GSP-2	AACCACATGGGTGGACAGATGTCCTCC	
In vitro translation assay	ATCCTCGAGACAAAGAGCCCTCTGCACTAG	ATCGGATCCGAGCAAATG-GAGACAAAGGAC
Luciferase assay		
Intron 1 5,338 C>T FP	ATCACGCGTCCAGAGTCAGGGAAAAAGACC	ATCCTCGAGTTGAATTCTACCATTTTCT-TACATC
Exon 3 8,813 G>A and exon 3 9,186 G>A	ATCACGCGTGTCTTCTCCAGACTGGTGAC	ATCCTCGAGCTCCCTGCTGAAGAA-AGAAGG
Exon 5 11,093 G>A	ATCACGCGTCTTGATTCCTCCTCGCGTTTC	ATCCTCGAGGAGCAAATG-GAGACAAAGGAC
Exon 5 11,741_G	CGCGTCTATTCATCAGCTTGGGCTGC	TCGAGCAGCCCAAGCTGATGAATAGA
Exon 5 11,741_A	CGCGTCTATTCATCAACTTGGGCTGC	TCGAGCAGCCCAAGTTGATGAATAGA
Exon 5 12,311_C	CGCGTTGCAGGGAGGCGGAGGCCCTGC	TCGAGCAGGGCCTCCGCCTCCCTGCAA
Exon 5 12,311_T	CGCGTTGCAGGGAGGTGGAGGCCCTGC	TCGAGCAGGGCCTCCACCTCCCTGCAA
Gel shift assay		
Exon 5 11,741_G	GTGGTGTGTACTATTCATCAGCTTG-GGCTGCCA	GCTGTGGCAGCCCAAGCTGATGA-ATAGTACACA
Exon 5 11,741_A	GTGGTGTGTACTATTCATCAACT-TGGGCTGCCA	GCTGTGGCAGCCCAAGTTGATGA-ATAGTACACA

N represents a mixture of nucleotides A, C, G and T

full-length cDNA, were transcribed and translated using TNT T7 Quick Coupled Transcription/Translation Systems (Promega, Madison, USA) and Transcend Biotin-Lysyl-tRNA (Promega) according to the manufacturer's protocols. For negative control experiments, the antisense plasmids were constructed by inserting each PCR-amplified variant product in the opposite direction to pBluescript SK(+). Primers for constructing of the antisense plasmids were shown in Table 1. After SDS-PAGE and electro-blotting, the biotinylated products were visualized using the Transcend Nonradioactive Translation Detection Systems (Promega).

Luciferase assay

To investigate functions of six SNPs, we cloned genomic fragments into pGL3-promoter vector (Promega) in the 5′–3′ orientation. For intron 1 5,338 C>T and exon 5 11,093 G>A SNPs, each PCR-amplified product was used to be cloned into each of the vectors; for two SNPs in exon 3, one single PCR-amplified product containing both of the SNP loci was used; for exon 5 11,741 G>A and exon 5 12,311 C>T SNPs, double stranded oligonucleotides was cloned into pGL3-promoter vector. Primers and oligonucleotides for cloning of luciferase construct were shown in Table 1. We grew HEK293 cells (RIKEN Cell Bank, Wako, Japan) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. We then performed luciferase assay according to the manufacturer's protocol. After 24 h of the transfection, we lysed the cells in passive lysis buffer (Promega) and then measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility-shift assay (EMSA)

We prepared nuclear extract from HEK293 cells as previously described (Andrews and Faller 1991) and then incubated it with 33-bp oligonucleotides labeled with digoxigenin-11-ddUTP using the Dig Gel Shift Kit, second generation (Roche, Mannheim, Germany). The oligonucleotides used in EMSA experiments were shown in Table 1. The reaction was done with 1/10 volume of Poly [d(I-C)]. For competition studies, we

pre-incubated nuclear extract with unlabeled oligonucleotides (200-fold excess) before adding digoxigenin-labeled oligonucleotide. We separated the protein-DNA complexes on a non-denaturing 6% polyacrylamide gel in 0.5× Tris–Borate-EDTA buffer. We transferred the gel to nitrocellulose membrane and detected the signal with a chemiluminescent detection system (Roche) according to the manufacturer's instructions.

In vitro RNA stability assay

First, we genotyped five SNP loci using DNAs extracted from 20 different human B cell lines (HEV cell lines; RIKEN cell bank). For further RNA stability assay, we used one cell line that was heterozygous for all of these SNPs. Two DNA fragments, corresponding to nt 956–1,827 and nt 1,975–3,400 of *MIAT* variant 4 (GenBank accession number; AB_263417) were amplified by PCR and used as a template. These fragments were cloned into a pBlueScript II SK(+) in the 5′–3′ orientation. We purified the vectors and performed transcription reaction using MEGAscript high yield transcription kit (T3 kit) (Ambion) according to the manufacturer's instruction. Using 3 µl of the transcription products and 3 µl of HEK293 cell extracts (diluted 20-folds with distilled water), we performed RNA stability assay as described previously (Suzuki et al. 2003).

Results

Large-scale SNP association study

We performed a large scale case-control association study using 188 MI patients and 752 general Japanese population by our high-throughput multiplex PCR-Invader assay method (Ohnishi et al. 2001) for 52,608 gene-based SNPs selected from the JSNP database on the basis of the haplotype block structure reported previously (Haga et al. 2002; Tsunoda et al. 2004). Detailed screening strategy and results will be described elsewhere (Ebana et al., in preparation). From this first-stage screening, we identified one SNP (rs2301523) in *FLJ25967* (GenBank accession number, AK098833) on chromosome 22q12.1 to reveal a significant association with MI (P=0.0006). Further investigation of this SNP using a total of 3,464 MI patients and 3,819 general population confirmed the association with MI with a χ^2 value of 22.71 (P=0.0000019; comparison of allele frequency) and odds ratio of 1.36 (95% confidence interval (CI); 1.20–1.55, Table 2).

Table 2 Association analyses between MI and six SNPs in *MIAT*

SNP position	MI (%)				Control (%)				Comparison of allele frequency		
	11	12	22	Sum	11	12	22	Sum	χ^2	P value	Odds ratio (95% CI)
Intron1 5,338 C>T	2,885 (84.0)	528 (15.4)	22 (0.6)	3,435	3,320 (88.0)	443 (11.7)	11 (0.3)	3,774	25.27	0.0000005	1.38 (1.22–1.57)
Exon 3 8,813 G>A	2,926 (85.1)	500 (14.5)	14 (0.4)	3,440	3,308 (88.9)	406 (10.9)	8 (0.2)	3,722	23.22	0.0000014	1.38 (1.21–1.58)
Exon 3 9,186 G>A	2,919 (84.3)	528 (15.2)	17 (0.5)	3,464	3,364 (88.1)	445 (11.7)	10 (0.3)	3,819	22.71	0.0000019	1.36 (1.20–1.55)
Exon 5 11,093 G>A	2,885 (84.3)	523 (15.3)	15 (0.4)	3,423	3,314 (88.1)	438 (11.6)	8 (0.2)	3,760	22.89	0.0000017	1.37 (1.20–1.56)
Exon 5 11,741 G>A	2,888 (84.2)	525 (15.3)	15 (0.4)	3,428	3,308 (88.1)	440 (11.7)	8 (0.2)	3,756	22.47	0.0000021	1.36 (1.20–1.55)
Exon 5 12,311 C>T	2,896 (84.3)	525 (15.3)	15 (0.4)	3,436	3,315 (88.0)	442 (11.7)	8 (0.2)	3,757	21.85	0.0000030	1.36 (1.19–1.56)

CI confidence interval

11, 12 and 22 indicate homozygote of major allele, heterozygote and homozygote of minor allele, respectively

Identification of the full-length gene

Since cDNA sequences of 1,713 bases for FLJ25967 (GenBank Accession code; AK098833) did not contain a long open reading frame (ORF), we first examined expression of *FLJ25967* in 4 fetal and 16 adult human tissues by Northern blot analyses. As shown in Fig. 1, the transcript of approximately 10 kb in length was detected in spleen, peripheral blood leukocyte, lung, liver, thymus, colon and small intestine, although predominant signals were detected in fetal brain and brain. To obtain the full-length cDNA sequence for this gene, we screened the fetal brain cDNA library and subsequently carried out 5′- and 3′-RACE experiments. From the results of the full-length cDNA sequences, this gene was considered to have four splicing variants (GenBank Accession number: AB263414, AB263415, AB263416, and AB263417), each consisting of 10,142, 10,016, 10,068 and 9,942 nucleotides, respectively. However, we still found no long ORF in either of the four cDNA sequences; the longest ORF we identified was 447-base long encoding 149 amino acids (Fig. 2b). Although we compared this ORF with protein sequecnes in the public databases using the BLAST program (Altschul et al. 1990), we found no protein showing a significant similarity. To investigate whether the transcript was really translated into protein, we carried out in vitro translation assay for each of the four variants, but no translated product was detected (Fig. 3), suggesting this gene encodes a functional RNA. We designated this gene *MIAT*, myocardial infarction associated transcript.

A comparison of genomic DNA sequences in GenBank database with each of the cDNA sequences determined a genomic structure of the *MIAT* gene. This gene consists of five exons (Fig. 2a) and all the splice junctions were considered to conform to the basic GT/AG rule (Mount 1982; Shapiro and Senapathy 1987).

Linkage disequilibrium and haplotype analysis

To search for the possibility that another SNP(s) in this locus confers risk of MI, we investigated SNPs in this region thoroughly, except for the regions corresponding to repetitive sequences. By direct sequencing of genomic DNA from 24 individuals in Japanese population, we identified a total of 60 SNPs, including 14 novel ones which were not registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html; build 126, Table 3). Subsequently, we selected 35 SNPs on the basis of the following two conditions; (1) SNPs should show minor allele frequencies (MAF) >5%, and (2) only one SNP can be selected from a group of SNPs that are in perfect LD among them. Then, we genotyped 96 individuals with MI and investigated a precise haplotype structure in this region using Haploview software (http://

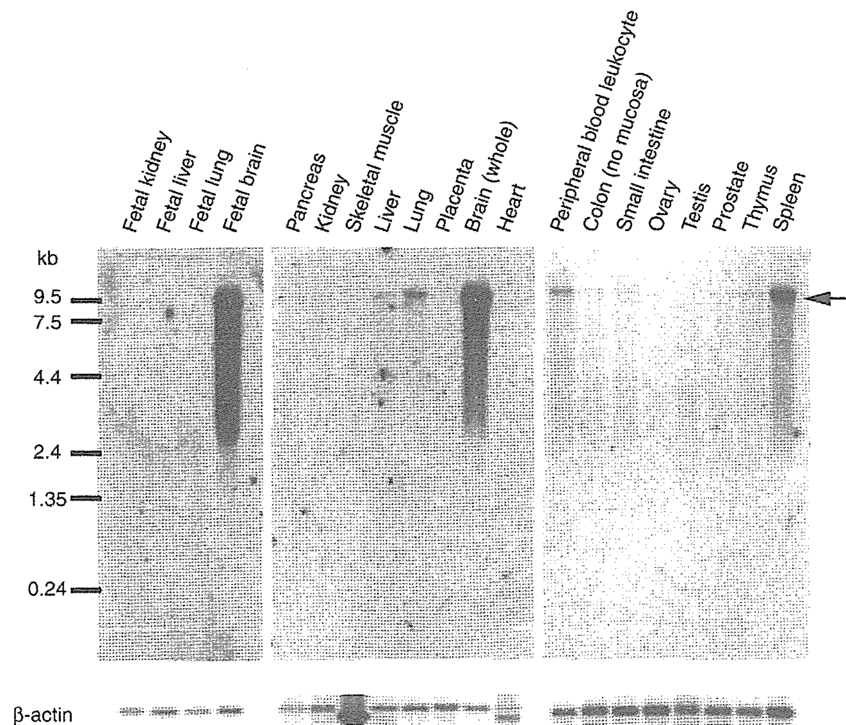
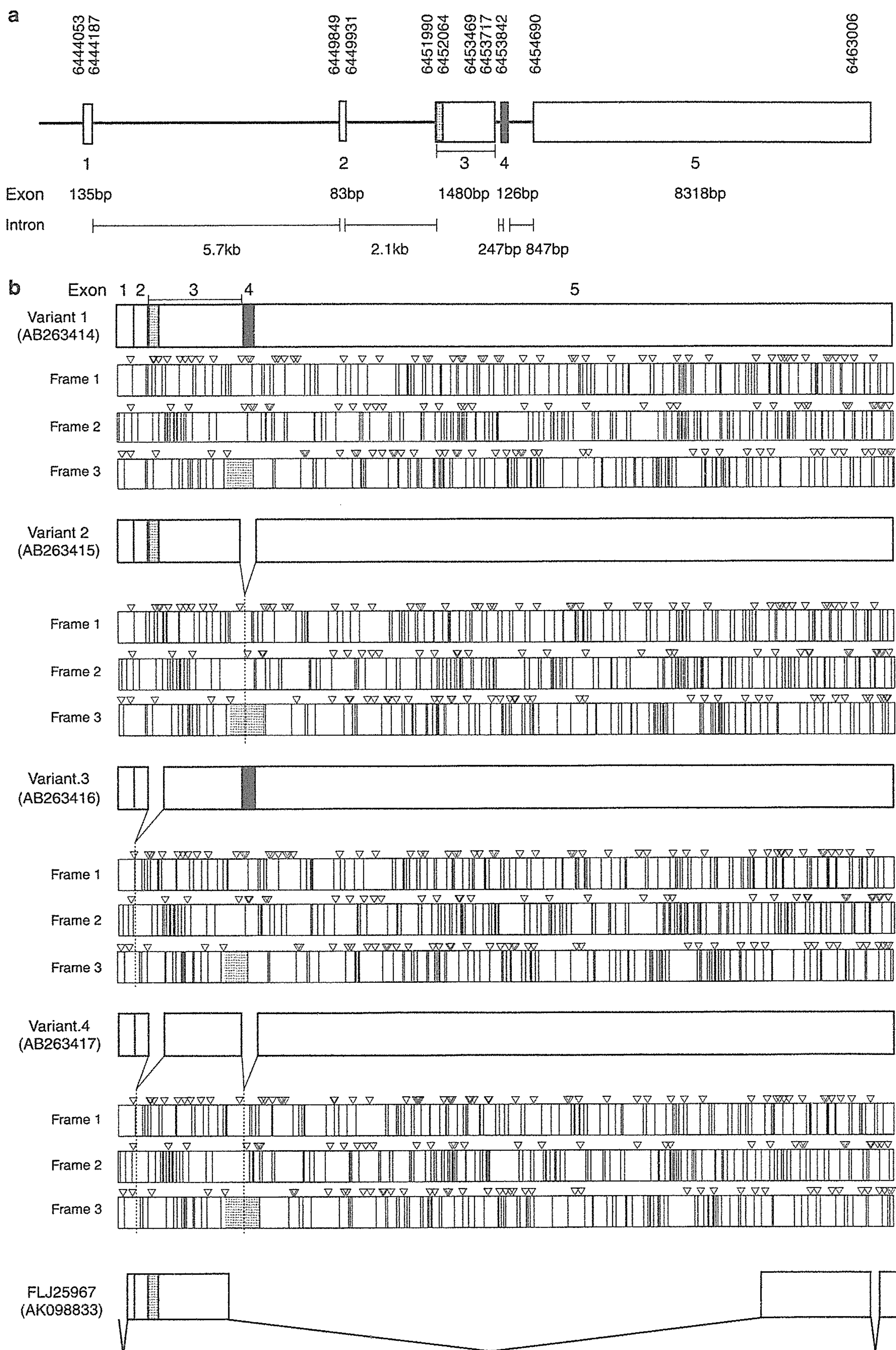


Fig. 1 Expression of *MIAT* in human tissues. *Arrows* indicate the transcript of approximately 10 kb in size. β-actin cDNA was used as the quantity control


a
6444053
6444187
6449849
6449931
6451990
6452064
6453469
6453717
6453842
6454690
6463006
1
2
3
4
5
Exon
135bp
83bp
1480bp
126bp
8318bp
Intron
5.7kb
2.1kb
247bp
847bp
b
Exon 1 2 3 4 5
Variant 1
(AB263414)
Frame 1
Frame 2
Frame 3
Variant 2
(AB263415)
Variant.3
(AB263416)
Variant.4
(AB263417)
FLJ25967
(AK098833)

◀ **Fig. 2** Genomic organization and splicing variants of *MIAT*. **a** Genomic structure of *MIAT*. Base-pair numbering at the top was based on an entry from GenBank DNA database (NT011520.10). **b** Four splicing variants of *MIAT* and their possible open reading frames. The longest ORF was shown in gray. *Inverted triangle* and *vertical line* indicate ATG initiation codon and stop codon, respectively

www.hapmap.org). As shown in Fig. 4, we identified one haplotype block including the marker SNP rs2301523, which was first identified to have an association with MI through our large-scale study. In addition, we found five SNPs (intron 1 5,338 C>T, exon 3 8,813 G>A, exon 5 11,093 G>A, exon 5 11,741 G>A, exon 5 12,311 C>T) in the block in strong linkage disequilibrium (LD) with the marker SNP. Therefore, we examined whether these five SNPs were also tightly associated with susceptibility to MI by genotyping approximately 3,400 individuals with MI and 3,700 control subjects. As shown in Table 2, all of them showed significant associations with MI. In particular, one SNP (intron 1 5,338 C>T; rs2331291) presented the most significant association with MI (χ^2=25.27, P=0.0000005; comparison of allele frequency).

To clarify a possibility that one particular haplotype in the block confers risk of MI, we selected five tag SNPs (exon 3 8,555 T>C, exon 3 9,186 G>A, exon 5 10,804 G>A, exon 5 14,569 A>T, exon 5 15,219 C>A) that could cover 90% of haplotypes in this block. We compared frequency of haplotypes and genotypes of 652 individuals with MI and 620 control subjects using these tag SNPs. Since neither haplotypes nor four other tag SNPs showed statistical significance for the association with MI (Tables 4, 5), we considered that the first marker SNP rs2301523 and additional five SNPs showing the strong LD with it were candidate SNPs associated with MI.

We investigated relationship between patients' genotype information and clinical profiles including diabetes, hypertension, smoking, hyperlipidemia, sex, and age by one-way ANOVA and χ^2 test. Since we could not find positive association for any of the coronary risk factors, age, or sex (data not shown), we concluded our findings are directly related to the pathogenesis of MI.

Luciferase and Gel-shift assay

Since *MIAT* was considered to be a non-coding functional RNA, we investigated the functions of these SNPs by examining their effect on transcriptional regulation by luciferase assay using HEK293 cell. As shown in Fig. 5d, the clone containing a G allele at position 11,741 in exon 5 had approximately 1.3-fold greater transcriptional activity than an A allele or the vector only. However, the remaining five SNPs did not show statistical difference in their transcriptional activity (P>0.05; Fig. 5a–c, e). Subsequently, to examine whether a nuclear factor(s) might bind to genomic sequence around the exon 5 11,741 SNP, we searched for binding motifs for known transcription factors around this SNP sequence by TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCHJ.html) based on the TRANSFAC database (Heinemeyer et al. 1998). Although no motif for binding of the transcriptional factors was predicted, we attempted to examine binding of some nuclear factor(s) in nuclear extract from HEK293 cells to oligonucleotides corresponding to the 11,741 G and 11,741 A alleles, respectively. As shown in Fig. 5f, the A-allele oligonucleotide bound more tightly to some nuclear factor present in HEK293 cells than the G-allele oligonucleotide.

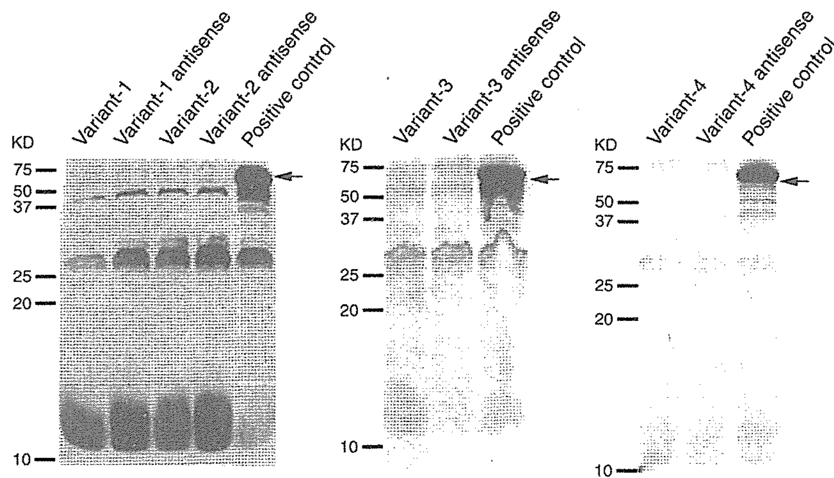


Fig. 3 In vitro translation assay The *arrows* indicate the bands for positive controls

Table 3 SNPs in *MIAT*

db SNP	SNP position	Location in Z99774.1	Allele	MAF
rs5761663	5′-flanking –1,150	22,542	G/C	0.271
–	5′-flanking –1,035	22,657	C/T	0.063
rs5752375	5′-flanking –976	22,716	C/T	0.063
–	5′-flanking –927	22,765	C/T	0.063
rs9608515	5′-flanking –902	22,790	C/T	0.063
rs2157598	5′-flanking –594	23,098	A/T	0.341
rs5761664	5′-flanking –364	23,328	C/G	0.333
rs8142890	5′-flanking –106	23,586	C/T	0.063
rs16982564	Intron 1 696	24,387	G/A	0.271
rs7284198	Intron 1 716	24,407	T/C	0.063
rs5752376	Intron 1 1,349	25,040	G/A	0.063
–	Intron 1 1,506	25,197	G/A	0.333
rs9613239	Intron 1 1,933	25,624	G/A	0.188
–	Intron 1 2,035	25,726	T/G	0.271
rs6005116	Intron 1 2,614	26,305	G/A	0.056
rs9613240	Intron 1 2,851	26,542	A/G	0.065
rs1007476	Intron 1 3,166	26,857	G/T	0.326
rs11912702	Intron 1 3,280	26,971	C/G	0.286
rs1981493	Intron 1 3,923	27,614	G/A	0.5
rs5761665	Intron 1 3,956	27,647	G/T	0.125
rs5752377	Intron 1 3,967	27,658	T/A	0.146
rs6005119	Intron 1 4,504	28,195	A/C	0.435
–	Intron 1 5,042	28,733	C/T	0.271
rs2331291	Intron 1 5,338	29,029	C/T	0.188
rs5761666	Intron 1 5,522	29,213	T/G	0.313
–	Intron 2 5,917	29,608	T/C	0.042
rs5997112	Exon 3 8,308	31,999	G/A	0.478
rs1061541	Exon 3 8,454	32,145	T/C	0.043
rs1061540	Exon 3 8,555	32,246	T/C	0.479
rs12166567	Exon 3 8,639	32,330	G/C	0.313
–	Exon 3 8,813	32,504	G/A	0.188
rs2301523	Exon 3 9,186	32,877	G/A	0.208
rs2301524	Exon 3 9,312	33,003	C/T	0.333
rs4274	Exon 5 10,804	34,495	G/A	0.292
rs3752603	Exon 5 11,011	34,702	C/T	0.417
–	Exon 5 11,093	34,784	G/A	0.188
rs6005121	Exon 5 11,634	35,325	G/A	0.021
–	Exon 5 11,741	35,432	G/A	0.167
–	Exon 5 12,311	36,002	C/T	0.182
–	Exon 5 12,388	36,079	C/T	0.048
rs7985	Exon 5 13,052	36,743	A/G	0.45
rs3180936	Exon 5 13,083	36,774	A/T	0.45
rs4280	Exon 5 13,562	37,253	G/T	0.294
–	Exon 5 14,160	37,851	A/G	0.413
rs8142049	Exon 5 14,309	38,000	C/T	0.435
rs5761670	Exon 5 14,569	38,260	A/T	0.25
–	Exon 5 14,588	38,279	G/A	0.042
rs9625066	Exon 5 15,219	38,910	C/A	0.271
–	Exon 5 15,515	39,206	C/T	0.042
rs9620625	Exon 5 15,771	39,462	G/A	0.022
rs5761672	Exon 5 15,776	39,467	G/A	0.386
rs6005127	Exon 5 16,074	39,765	C/T	0.273
rs5752378	Exon 5 16,352	40,043	G/A	0.176
rs1018834	Exon 5 16,244	40,115	T/A	0.292
rs713720	Exon 5 16,968	40,659	G/C	0.021
rs4822768	Exon 5 17,266	40,957	T/C	0.261
rs7293223	Exon 5 18,211	41,902	G/A	0.262
rs3747138	Exon 5 18,529	42,220	A/G	0.063
rs1045362	Exon 5 18,657	42,348	A/G	0.292
rs1045363	Exon 5 18,663	42,354	C/T	0.25

MAF minor allele frequency

'–' The variant was not registered in dbSNP database (build 126)

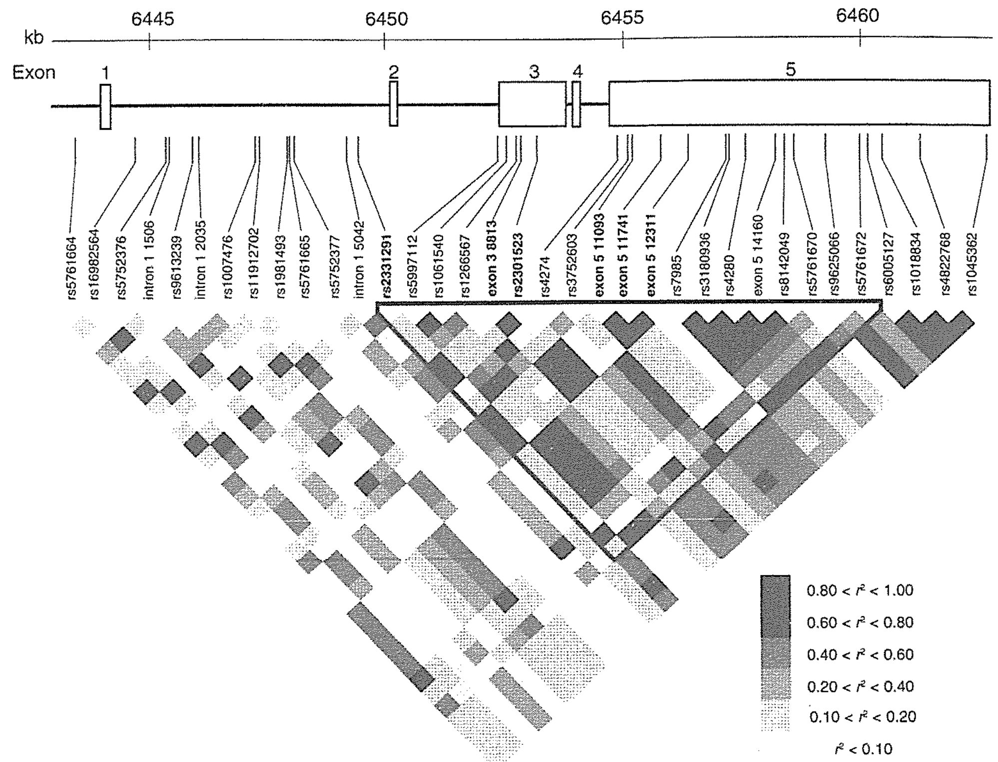


Fig. 4 Linkage disequilibrium structure at the *MIAT* locus SNPs identified by this study are shown. SNPs in *bold* indicate significant association with MI (see also Table 2). Those which were not deposited in dbSNP database (build 126) were labeled with their location in the gene (e.g., intron 1 5,042). Pairwise r^2 values for all combination of SNP pairs are shown in *gray scale*

Table 4 Association analyses of the tag SNPs in the haplotype block

dbSNP	SNP position	MI (%)				Control (%)				Comparison of allele frequency	
		11	12	22	Sum	11	12	22	Sum	χ^2	*P* value
rs1061540	exon 3 8,555 T>C	182 (27.9)	288 (44.2)	182 (27.9)	652	173 (27.9)	291 (46.9)	156 (25.2)	620	0.48	0.49
rs2301523	exon 3 9,186 G>A	534 (81.9)	115 (17.6)	3 (0.5)	652	545 (87.9)	73 (11.8)	2 (0.3)	620	8.89	0.0039
rs4274	exon 5 10,804 G>A	258 (39.6)	268 (41.1)	126 (19.3)	652	237(38.2)	285 (46.0)	98 (15.8)	620	0.31	0.57
rs5761670	exon 5 14,569 A>T	356 (54.6)	237 (36.3)	59 (9.0)	652	326 (52.6)	244 (39.4)	50 (8.1)	620	0.09	0.77
rs9625066	exon 5 15,219 C>A	437 (67.0)	188 (28.8)	27 (4.1)	652	431 (69.5)	169 (27.3)	20 (3.2)	620	1.26	0.26

11, 12 and 22 indicate homozygote of major allele, heterozygote and homozygote of minor allele, respectively

Table 5 Haplotype analyses

Haplotype ID	Tag SNPs					Haplotype frequency		Comparison of haplotype frequency	
	rs1061540	rs2301523	rs4174	rs5761670	rs9625066	MI	Control	χ^2	*P* value
Haplotype 1	T	C	A	A	C	0.361	0.383	1.30	0.25
Haplotype 2	C	C	G	T	C	0.259	0.272	0.65	0.42
Haplotype 3	C	C	G	A	C	0.154	0.160	0.19	0.66
Haplotype 4	T	T	G	A	A	0.052	0.053	0.01	0.90
Haplotype 5	T	C	G	A	A	0.049	0.062	2.06	0.15
Haplotype 6	C	C	G	A	A	0.042	0.047	0.32	0.57

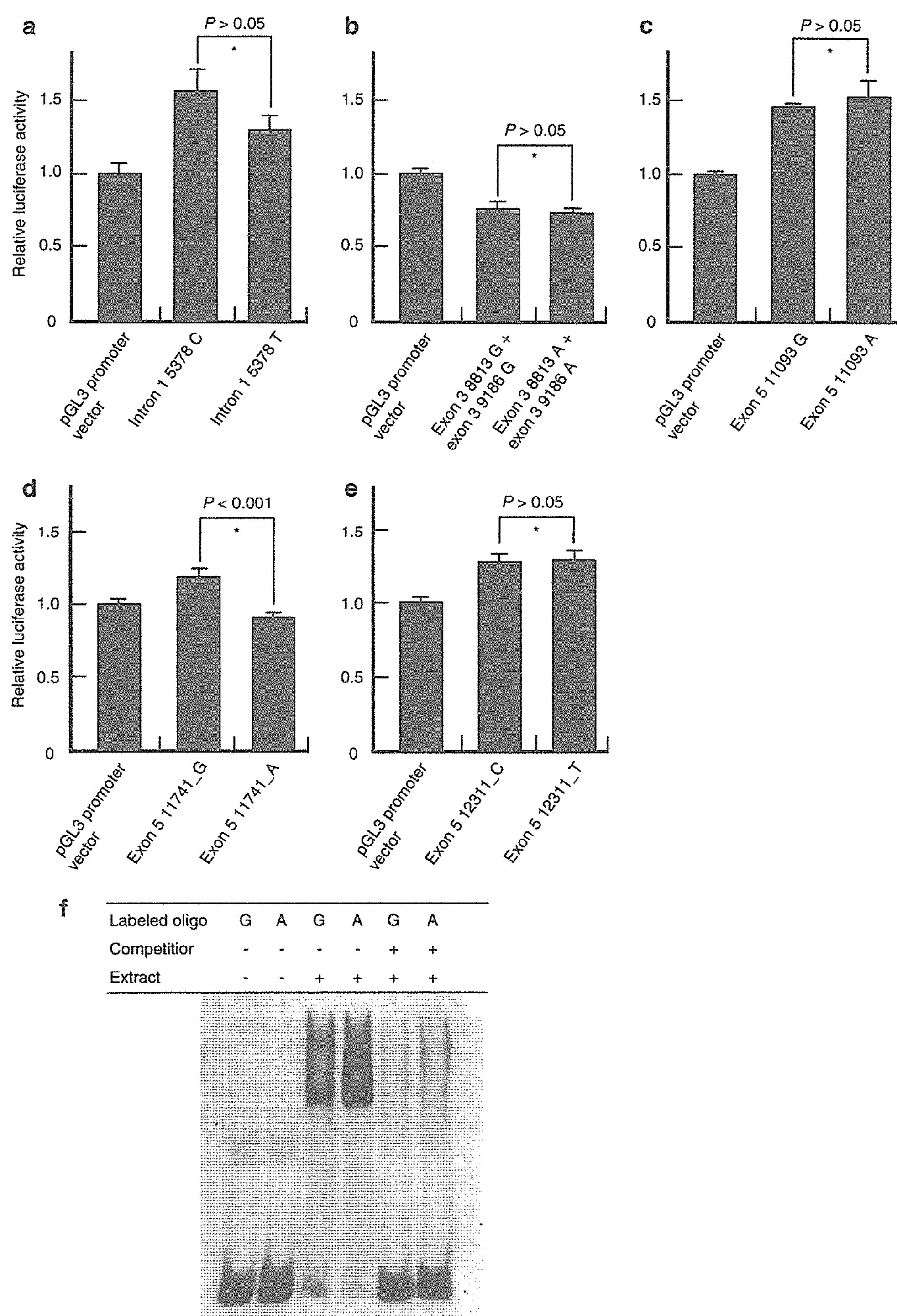


Fig. 5 Transcriptional effect of the six SNPs in *MIAT*. **a–e** Luciferase assays in HEK293 cells. Only exon 5 11,741 G>A SNP affected transcriptional activity (**d**). *Student's *t* test. We repeated each experiment three times and studied each sample in triplicate or duplicate. **f** Binding of an unknown nuclear factor(s) to exon 5 of *MIAT*. The experiments were repeated three times with similar results

Effect of SNP on RNA stability

In two previous studies, SNPs and haplotypes in exons have been implicated in having some roles in the stability of mRNA (Suzuki et al. 2003; Yang et al. 2003). To examine a possibility that the five exonic SNPs showing the significant association with MI (exon 3 8,813 G>A, exon 3 9,186 G>A, exon 5 11,093 G>A, exon 5 11,741 G>A and exon 5 112,311 C>T) might influence the stability of mRNA, we carried out RNA stability assay using HEK293 cells (Suzuki et al. 2003). Although the mRNA corresponding to the minor haplotype tended to

be degraded more rapidly as compared with that corresponding to the major one, the difference was not statistically significant (data not shown).

Discussion

Through a large scale case-control association study using gene-based genome-wide tag SNPs, we found that six SNPs in *MIAT*, a novel gene encoding a possible non-coding functional RNA, might confer the genetic risk of MI. Since the function of this gene was not known, it was not possible to identify this gene as associated with MI by means of candidate gene approach. An advantage of the genome-wide association without any hypothesis is to find genetic variations, even in genes encoding functional RNAs, associated with various diseases. Hence, we are confident that a comprehensive association study using genome-wide tag SNPs is a powerful tool to fully understand genetic backgrounds of common diseases.

Our findings indicated that *MIAT* is a non-coding functional RNA. Biological functions of several non-coding functional RNAs have been investigated intensively; for example, H19 was shown to be involved in imprinting (Pfeifer et al. 1996). Xist was the first example of the functional RNA and was proven to regulate inactivation of the X chromosome (Brown et al. 1991). Hoxa11s is an antisense RNA for Hoxa11 and regulates transcription of Hoxa11 (Hsieh-Li et al. 1995). Recently, Willingham et al. (2005) identified a functional long non-coding RNA (termed NRON), which acts as a repressor of the nuclear factor of activated T cell (NFAT), and showed that specific ncRNAs as NRON may play a role in regulating the complexity of intracellular trafficking. Carninci et al. (2005) found over 23,000 non-coding RNA species through comparison between full-length cDNA sequences and genome sequences, indicating the complexity of mammalian transcriptional landscape. Thus, a large number of non-coding functional RNAs seem to play important roles in a variety of biological functions. Another aspect of functional RNA is a micro RNA (miRNA), one of the sequence-specific post-transcriptional regulators of gene expression (Tang 2005). It is generated by Dicer, a multidomain enzyme of the RNase III family. Dicer cuts precursor miRNAs with hairpin structure into miRNAs. However, sequence comparison of sense strand with antisense one using BLASTN program (Altschul et al. 1990) did not reveal complementary segments within *MIAT*, suggesting no possible hairpin structure that might give rise to double stranded RNA by Dicer. This indicates that *MIAT* is unlikely to contain an miRNA precursor. Although it is very difficult to reveal the function of *MIAT* with the present knowledge, we think the increasing attention to non-coding RNA and subsequent progress will help to solve this problem.

In the present study, we identified SNPs in *MIAT* conferring susceptibility to MI through a large-scale case-control association study. Although function of *MIAT* remains unclear, we believe that knowledge of genetic factors contributing to the pathogenesis of MI as presented here, will lead to improved diagnosis, treatment and prevention.

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nature genetics

A functional SNP in *PSMA6* confers risk of myocardial infarction in the Japanese population

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Inflammation is now considered critical in the pathogenesis of myocardial infarction. One of the mechanisms regulating the inflammatory process is the ubiquitin-proteasome system. We investigated whether variants of the 20S proteasome are associated with susceptibility to myocardial infarction and found a common SNP (minor allele frequency of 0.35) in the proteasome subunit α type 6 gene (*PSMA6*) conferring risk of myocardial infarction in the Japanese population ($\chi^2 = 21.1$, $P = 0.0000044$, 2,592 affected individuals versus 2,851 controls). We replicated this association in another panel of myocardial infarction and control subjects, although its relevance to other ethnic groups remains to be clarified. The SNP, located in the 5′ untranslated region of exon 1 in this gene, enhanced the transcription of *PSMA6*. Moreover, suppression of *PSMA6* expression using short interfering RNA in cultured cells reduced activation of the transcription factor NF-κB by stabilizing phosphorylated IκB. Our results implicate this *PSMA6* SNP as a previously unknown genetic risk factor for myocardial infarction.

Inflammation is important in the pathogenesis of coronary artery disease through its contribution to atheroma formation and rapid evolution of the atheromatous injury, leading to rupture of the plaque and intraluminal thrombosis[1]. In recent years, several candidate genes and loci associated with susceptibility to myocardial infarction have been identified through case-control association studies[2–7] or linkage analysis (1p34–36, 2q21–22, 2q36–q37.3, 3q13, 14q32 and Xq23–26)[8–13]. Notably, most of the gene products are implicated their involvement in inflammation.

The 26S ubiquitin-proteasome system is a principal proteolytic pathway that is important in the regulation of the abundance of protein involved in apoptosis, cell cycle, cell proliferation or differentiation, and inflammation[14–17]. One of the most important functions of the proteasome associated with the inflammation pathway is the degradation of IκB protein, which inhibits the activation of nuclear factor κB (NF-κB), a central transcription factor that regulates the expression of genes related to inflammation such as cytokines and adhesion molecules involved in atherogenesis[14].

Through a whole-genome case-control association study using 92,788 SNP markers, we had previously found that functional SNPs in the gene (*LTA*) encoding lymphotoxin-α (LTA; ref. 3), one of the cytokines produced in an earlier stage of the vascular inflammatory processes[18], conferred susceptibility to myocardial infarction (ref. 3). Because binding of LTA to its receptor strongly activates NF-κB by proteasomal degradation of its inhibitory partner, IκB[19], we hypothesized that variation(s) in the genes encoding proteasomal proteins could confer risk of myocardial infarction. The 20S proteasome, which is composed of seven α and ten β subunits, is the core particle for the 26S proteasome system[20]. On the basis of the information in the International HapMap Project database (http://hapmap.org)[21] and the JSNP database (http://snp.ims.u-tokyo.ac.jp)[22], we selected SNPs with minor allele frequencies of >10% that covered most of haplotypes in the genomic region of genes encoding these subunits. There were in total 169 common SNPs in these genes, and the selected tag SNPs covered more than 90% of the alleles in each region with an r^2 value of 0.8 or greater. Subsequently, we compared genotype frequencies of about 450 individuals with myocardial infarction and 450 control individuals at these SNP loci (**Table 1** and **Supplementary Table 1** online) and found that one SNP (exon 1 –8C/G, listed in the dbSNP database of the US National Center for Biotechnology Information as rs1048990) in the 5′ untranslated region of exon 1 of *PSMA6* was significantly associated with myocardial infarction (**Table 1**). We have not found any published reports of linkage of the chromosomal region containing *PSMA6* to myocardial infarction. We further analyzed the haplotype structure across the *PSMA6* region using these SNPs, and we found that the associated SNP was not in linkage disequilibrium (LD) with any of the other seven SNPs in or around the *PSMA6* region (**Supplementary Table 2** online). No particular

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Table 1 Association analyses between myocardial infarction and tag SNPs in a haplotype block containing *PSMA6*

	Individuals with MI				Control individuals				Allele 1 versus allele 2		
dbSNP ID	1/1	1/2	2/2	Sum	1/1	1/2	2/2	Sum	χ^2	*P*	Odds ratio (95% CI)
rs8011465	304	151	13	468	263	160	12	435	1.41	0.23	1.15 (0.91–1.45)
rs8008319	330	131	11	472	284	143	14	441	3.16	0.08	1.24 (0.98–1.58)
rs7157492	326	130	11	467	283	145	15	443	3.73	0.05	1.27 (1.00–1.61)
rs4982254	177	225	69	471	127	225	69	421	3.86	0.05	1.21 (1.00–1.46)
rs1048990	185	222	66	473	216	194	41	451	9.56	0.002	1.36 (1.12–1.65)
rs12878391	320	130	11	461	279	144	17	440	4.21	0.04	1.28 (1.01–1.63)
rs4981283	255	192	22	469	222	187	31	440	2.28	0.13	1.17 (0.95–1.45)
rs1957107	351	111	11	473	303	133	8	444	2.60	0.11	1.23 (0.96–1.59)

Genotype is given in the column heading, with 1/1 indicating homozygosity for the major allele, 1/2 heterozygosity and 2/2 homozygosity for the minor allele. CI, confidence interval; MI, myocardial infarction; ID, database identifier.

haplotype of the *PSMA6* region showed statistical significance for association with myocardial infarction ($P > 0.01$, **Supplementary Table 3** online).

Next, to examine the possibility that other unidentified SNP(s) in this gene confers risk of myocardial infarction, we searched for SNPs in a 29-kb region containing *PSMA6*, except for those corresponding to repetitive sequences, by resequencing genomic DNA from 48 Japanese individuals. We identified a total of 13 SNPs (**Fig. 1**). Comparing our results with the dbSNP database showed that 7 of the 13 SNPs (–18T/C and –1C/T in the 5′-flanking region and 1233A/T, 1246A/G, 7239A/G, 7294T/A and 7693G/A in intron 1) were unknown as of the end of January, 2006. Genotyping of about 100 individuals with myocardial infarction and 100 control individuals with respect to these 13 SNPs showed only three SNPs—exon 1 –8C/G (rs1048990), intron 1 1233A/T and intron 1 10820A/G (rs12878391)—to have minor allele frequencies of more than 5% (**Fig. 1**). As the results for the 10820A/G SNP indicated that it might have a slight association with myocardial infarction (**Table 1**), we increased the number of samples (to 1,025 individuals with myocardial infarction and 980 without) and eventually found no association ($P = 0.28$; 95% confidence interval, 0.93–1.28). As for the remaining two SNPs, which were in perfect LD ($r^2 = 1$), we examined the –8C/G SNP in exon 1 by genotyping 2,592 individuals with myocardial infarction and 2,851 control individuals. We found a strong association with myocardial infarction ($\chi^2 = 21.10$, $P = 0.0000044$; comparison of allele frequency, **Table 2**).

We examined the possibility of confounding effects by age, sex and classical risk factors in the myocardial infarction group and found no relation between genotype and these factors, indicating that the SNP rs1048890 in *PSMA6* is an independent risk factor of myocardial infarction (**Supplementary Tables 4** and **5** online). By using genomic control methods[23], we also assessed the stratification of the population used in this study by genotyping 17 randomly selected SNPs. The significance of stratification (*P*) and the inflation factor ($\lambda_{3500,4000}$) were 0.71 and 1.00 (95th percentile upper bound 2.28), respectively. Because the maximum factor (χ^2 statistic for recessive model/χ^2 statistic for threshold) in this study is 2.32, it is unlikely that population stratification is the cause of our case-control association result. To confirm the association, we examined it with another panel of individuals with myocardial infarction and control individuals (867 myocardial infarction and 1,104 controls, respectively) who had been recruited more recently. We again found an association between this SNP (rs1048990) and myocardial infarction ($\chi^2 = 9.02$, $P = 0.0027$ for the recessive association model and $\chi^2 = 8.74$, $P = 0.0031$ for the additive association model; **Supplementary Table 6** online).

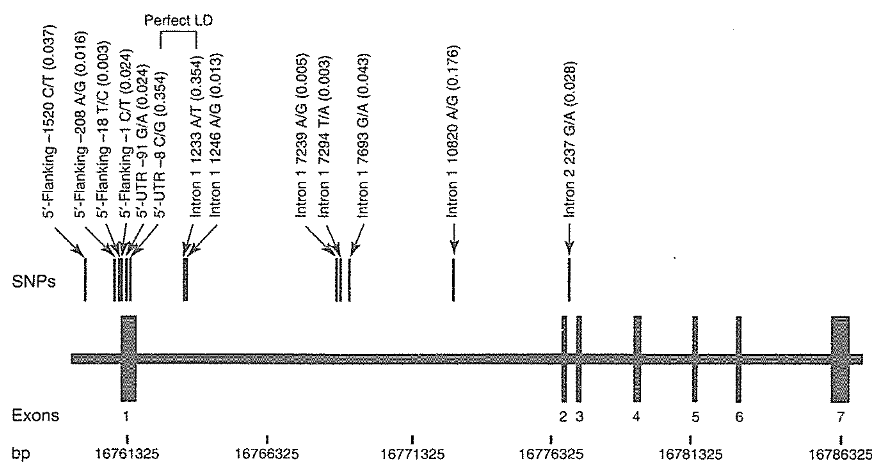

Figure 1 Map of SNPs in the *PSMA6* gene region identified by this study. Minor allele frequencies of each SNP in about 200 Japanese individuals are indicated in parentheses. The base-pair numbering at the bottom is based on the DNA sequence deposited in GenBank database. Nucleotide numbering for the SNPs is according to the mutation nomenclature defined in ref. 30.

To clarify whether either or both of the two SNPs in *PSMA6* associated with myocardial infarction, –8C/G in exon 1 and 1233A/T in intron 1, would affect the gene's expression, we constructed plasmid clones containing genomic DNA fragments corresponding to these SNPs. Each construct included one of the four possible combinations of the exon-1 and intron-1 SNPs (–8C-1233A, –8G-1233A, –8C-1233T and –8G-1233T haplotypes, respectively) and a luciferase gene transcriptional unit in the 5′-to-3′ direction. The clones containing the –8G-1233A and –8G-1233T haplotypes showed approximately 1.5- to 1.7-fold greater transcriptional activity than those containing the other two haplotypes (**Fig. 2**), indicating that the substitution in exon 1, but not the one in intron 1, affected the transcription of *PSMA6*.

We subsequently looked for nuclear factor(s) that might bind to oligonucleotides corresponding to genomic sequences of the

Table 2 Association between myocardial infarction and the exon-1 –8C/G[a] (rs1048990) SNP in *PSMA6*

	Individuals with MI		Control individuals	
Genotype	Number	%	Number	%
CC	1,134	43.8	1,382	48.5
CG	1,137	43.9	1,216	42.7
GG	321	12.4	253	8.9
Total	2,592	100	2,851	100
	χ^2	*P*	Odds ratio	95% CI
Allele frequency	21.1	0.0000044	1.21	1.11–1.31
Recessive model	17.7	0.000025	1.45	1.22–1.73
Additive model	19.2	0.000012	1.48	1.24–1.77

CI, confidence interval; MI, myocardial infarction.
[a]Nucleotide numbering is according to the mutation nomenclature defined in ref. 30.

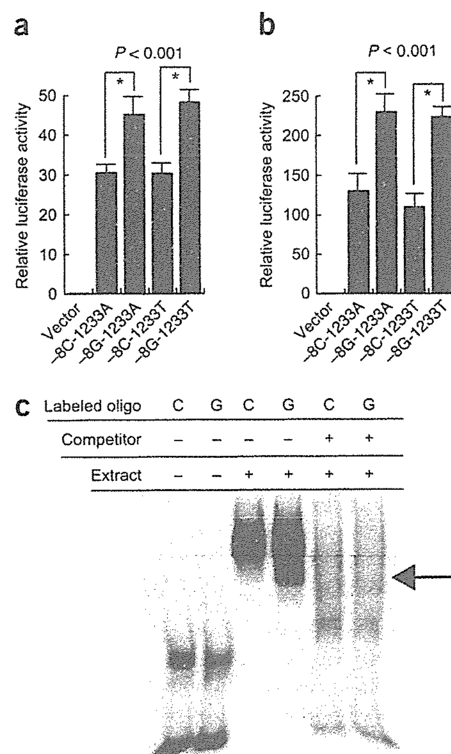

Figure 2 Function of the exon-1–8 C/G (rs1048990) SNP of *PSMA6*. **(a,b)** Transcriptional regulatory activity of the exon-1 SNP of *PSMA6* in **(a)** HeLa **(a)** and HepG2 cells **(b)**. We repeated each experiment three times and studied each sample in triplicate. *Student's *t*-test. **(c)** Gel shift experiment showing binding of unknown nuclear factor(s) to exon 1 of *PSMA6*. An arrow indicates the band showing specific binding of nuclear factor(s) to the G allele. Oligo, oligonucleotide.

–8C or –8G alleles, although no known protein was predicted to bind to this DNA segment. Using nuclear extracts from the HepG2 human liver cell line, we observed one band, indicating binding of nuclear protein(s) to the oligonucleotide, in the G allele lane but not in the C allele lane (**Fig. 2c**). This result raises the possibility that an unidentified nuclear factor(s) interacting with this region might regulate transcription of *PSMA6* and also influence myocardial infarction susceptibility.

To further confirm the effect of the SNP on transcription, we performed allele-specific quantitative PCR using a TaqMan probe[24] on seven independent Epstein-Barr virus–transformed human B cell lines (HEV cell lines) obtained from individuals who were heterozygous at the –8C/G SNP locus. In these cell lines, the G allele showed 1.7- to 1.8-fold higher expression of *PSMA6* than the C allele (**Table 3**). From these observations, we concluded that the exon-1 SNP in *PSMA6* affected its transcription *in vitro* and *in vivo*.

Degradation of the IκB protein is an essential step for activation of NF-κB, a key transcription factor that regulates expression of inflammatory genes[14]. Because the proteasome complex participates in this degradation process, we used the short interfering RNA (siRNA) technique to examine whether IκB degradation and subsequent NF-κB activation would be influenced by the cellular concentration of PSMA6 protein. We targeted five distinct sequences, and one siRNA against *PSMA6* significantly suppressed expression of its mRNA in both Jurkat cells and human coronary artery endothelial cells (HCAECs; **Fig. 3a,b**, respectively) and resulted in inhibition of NF-κB activation in both Jurkat cells and HCAECs (**Fig. 3c,d**, respectively). In addition, we examined whether reduced expression of PSMA6 might have some effect on the IκB pathway, which is stimulated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent activator of NF-κB. Without knockdown of PSMA6 expression, IκB in HCAECs was phosphorylated by TPA stimulation in 5 min and degraded in 15 min (**Fig. 3e**, right). However, degradation of phosphorylated IκB was significantly delayed when the cells were treated with siRNA specific to *PSMA6* (**Fig. 3e**, left). These results imply that the altered expression of PSMA6 could impair physiological ubiquitin-proteasome functions and could influence the expression of genes involved in the NF-κB–dependent inflammatory pathway. Thus, it is conceivable that the higher expression of *PSMA6* seen with the minor (G) allele might enhance the degree of inflammation through activation of NF-κB protein and might thereby be important in the pathogenesis of myocardial infarction.

Ubiquitin is colocalized with α smooth muscle cell actin in neointimal regions of atherosclerotic coronary arteries, and the ubiquitin-proteasome system has been implicated in the initiation, progression and end stage of atherogenesis[25]. Furthermore, pharmacologic inhibitors of the ubiquitin-proteasome pathway significantly attenuate myocardial reperfusion injury, ischemic stroke and atherosclerosis in several experimental animal models by inhibiting activation of NF-κB (refs. 26–28). These results, together with the genetic association results for the *PSMA6* SNP and its functional roles described here, make it seem likely that the ubiquitin-proteasome pathway is important in the pathogenesis of myocardial infarction. We are confident that knowledge of genetic factors and their molecular cascades can contribute to a better understanding of the pathogenesis of myocardial infarction and will lead to improved diagnosis, treatment and prevention.

Table 3 The allelic gene expression ratio for *PSMA6* in seven human B cell lines

	HEV32	HEV36	HEV38	HEV40	HEV43	HEV51	HEV52
G/C ratio	1.68 ± 0.02	1.72 ± 0.02	1.77 ± 0.01	1.79 ± 0.04	1.70 ± 0.02	1.69 ± 0.04	1.71 ± 0.06

The mean ± s.d. is provided for each cell line. We studied each sample in triplicate in one assay and each assay was independently repeated three times.

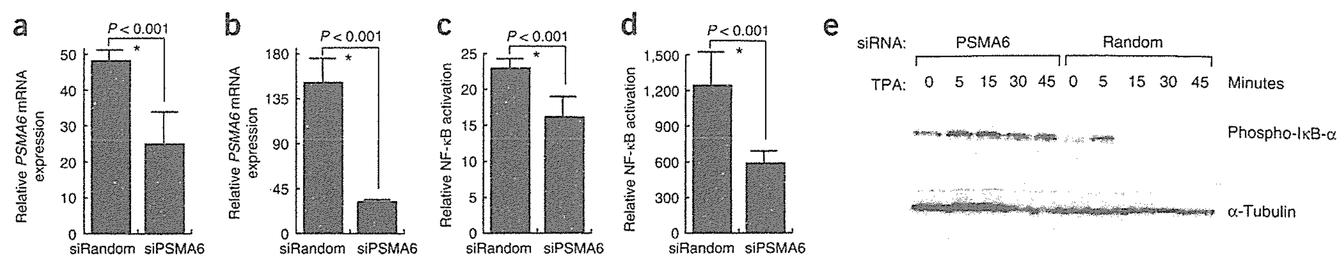

Figure 3 Expression levels of *PSMA6* affect NF-κB activation and IκB degradation. (**a**,**b**) Levels of *PSMA6* mRNA in random (siRandom) or *PSMA6* (siPSMA6) siRNA–treated (**a**) Jurkat cells and (**b**) HCAECs. (**c**,**d**) Relative NF-κB activation in (**c**) Jurkat cells and (**d**) HCAECs. We repeated each experiment three times and studied each sample in triplicate. (**e**) Inhibition of phospho–IκB-α degradation in HCAECs by knockdown of *PSMA6*. We used TPA as a potent activator of NF-κB.

METHODS

DNA samples. The study included 3,459 Japanese individuals with myocardial infarction referred to the Osaka Acute Coronary Insufficiency Study (OACIS) group. The **Supplementary Note** online describes the diagnosis of definite myocardial infarction and clinical characteristics of two cohorts. The control subjects consisted of 3,955 members of the general population who were recruited through several medical institutes in Japan. All subjects were Japanese and provided written informed consent to participate in the study, or their parents gave consent if they were under 20 years old, according to the procedure approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama.

SNP analysis. Designs for PCR primers, PCR experiments, DNA extraction, DNA sequencing, SNP discovery, genotyping of SNPs and statistical analysis were described previously[3,6]. Detection of significance of stratification (P) and estimation of quantitative assessment of population stratification (λ) were done according to previous reports[23,29]. We assessed the relationship between the clinical profiles and the genotype information of individuals with myocardial infarction by one-way ANOVA and χ^2 testing.

Luciferase assay. We cloned DNA fragments from *PSMA6* corresponding to nucleotides (nt) 600 of the 5′ flanking region to nt 10 of exon 1 and nt 1133–1343 of intron 1 into the pGL3-Basic vector (Promega) upstream of the luciferase gene in the 5′-to-3′ orientation. After 48 h of transfection, we lysed the cells in passive lysis buffer (Promega) and then measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

Gel-shift assay. We incubated nuclear extract prepared from HepG2 cells as previously described[3] in the presence of $MgCl_2$ and $CaCl_2$ with three tandem copies of a 16-nt oligonucleotide (positions –15 to 1 of exon 1 of *PSMA6*) that had been labeled with digoxigenin DIG-11-ddUTP using the DIG gel-shift kit (Roche). The reaction was carried out without poly[d(I-C)], reagent at room temperature (22–25 °C) . For competition studies, we preincubated a 125-fold excess of unlabeled oligonucleotide with the nuclear extract before adding DIG-labeled oligonucleotide. The protein-DNA complexes were separated on a nondenaturing 6% polyacrylamide gel (Invitrogen) in 0.5× Tris-borate-EDTA (TBE) buffer (45 mM Tris borate, pH 8.4, 1 mM EDTA) and transferred onto a nitrocellulose membrane. We detected the signal with a chemiluminescent detection system (Roche) according to the manufacturer's instructions.

Quantification of allelic variation in expression. We obtained EBV-transfected B cell lines from the BioResource Center, RIKEN. We prepared mRNA from seven cell lines that were heterozygous with respect to the –8C/G SNP in exon 1 and synthesized cDNA from the mRNA. We performed an allelic gene expression experiment by TaqMan assay essentially as described previously[24], using the published primer pair and allele-specific probes. (All of the primers and probes for the TaqMan assay were synthesized by Applied Biosystems.) **Supplementary Table 7** online shows the primer sequences.

We first made a linear regression line for the log of fluorescent intensity ratio (VIC / FAM) versus the log of allele ratio (standard line $y = a + bx$, where y is the log of (VIC intensity / FAM intensity) at a given mixing ratio, x is the log of the mixing ratio, a is the intercept and b is the slope) by measuring genomic DNA from CC and GG homozygous individuals mixed at different ratios (8:2, 7:3, 6:4, 5:5, 4:6, 3:7 and 2:8). VIC and FAM signals represent amounts of mRNA expressed from each of the G and C alleles, respectively. Then we measured allele-specific gene expression for each cDNA and genomic DNA (as an internal control) from each individual by using real-time quantitative PCR. We calculated the allele ratio on cDNA and genomic DNA amplicons by intercepting log of (VIC intensity / FAM intensity) on the standard line. We calculated the expression ratio of G allele to C allele by comparing those of the cDNAs to those of the genomic DNAs from the same individual. The PCR reactions were performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems) under the following conditions: 1 cycle of 10 min at 90 °C, then 40 cycles of 0.25 min at 92 °C and 1 min at 60 °C.

siRNA experiments. We cloned the target sequences for *PSMA6* into the pSilencer 2.0-U6 siRNA vector (Ambion). **Supplementary Table 7** shows the target sequences. We used the pSilencer negative control vector (Ambion) as negative control. After using the Nucleofector system (Amaxa) to cotransfect Jurkat cells with the pNiFty plasmid vector (InvivoGen)—a luciferase reporter vector carrying the NF-κB–specific E-selectin promoter—and the pRL-TK vector (Promega) as an internal control, we stimulated them with TPA (20 ng ml^{-1}) for 2 h, and then we collected the cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System. For the experiment using HCAECs (CAMBREX Corp.), we used the pSilencer 5.1-U6 Retro system (Ambion) to establish a stable pT67 cell line constitutively expressing retroviral *PSMA6* siRNA. We infected HCAECs for 72 h using supernatant from the stable pT67 cell line and then transfected them with the pNiFty vector. After 24 h of transfection, we measured luciferase activity using the Dual-Luciferase Reporter Assay System and normalized it by comparison with total cellular protein concentration. The mRNA quantification procedure was described previously[3].

Protein blot analysis. We transfected the *PSMA6* and control siRNAs into HCAECs as described in the siRNA experiment section. We stimulated the cells using 20 ng ml^{-1} TPA (Sigma) for 0, 5, 10, 15 and 45 min and then collected and lysed them in standard SDS sample buffer. After SDS-PAGE and blotting, we visualized immune complexes using a rabbit polyclonal antibody to phospho–IκB-α (Cell Signaling) and a horseradish peroxidase–conjugated secondary antibody to rabbit IgG (Amersham), or a mouse antibody to human α-tubulin (Santa Cruz) and a horseradish peroxidase–conjugated antibody to mouse IgG (Amersham).

Accession codes. GenBank: nucleotide sequence for proteasome (prosome, macropain) subunit, α type, 6 (*PSMA6*): NM_002791.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

K.O. performed most of the experiments and wrote the manuscript; H.S., H.M., Y.M., S.I. and M.H. managed DNA samples and clinical information; A.I. contributed to SNP discovery; T.N., A.T., T. Tsunoda and N.K. performed the data analyses; Y.N. contributed to SNP discovery and preparation of the manuscript; T. Tanaka supervised this study.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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MINIREVIEW

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Inflammation as a risk factor for myocardial infarction

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Abstract Myocardial infarction (MI) is one of the common diseases whose pathogenesis includes genetic factors. To reveal genetic backgrounds of this clinically heterogeneous disorder, we started our case-control association study by examining large-scale gene-based single nucleotide polymorphism (SNP) sets for ~1,000 patients and controls. As a core genotyping method, a combination of multiplex PCR and Invader method was used, and after genotyping ~65,000 SNPs, we found two SNPs located within lymphotoxin-α (*LTA*) gene showing significant association with MI. LTA is one of the cytokines produced in the early stages of vascular inflammatory processes. These SNPs seem to be involved in inflammation by both qualitatively and quantitatively modifying the function of LTA protein, thereby conferring a risk of MI. The genetic association was further confirmed by other researchers using white European trios. To further understand the roles of LTA protein in the pathogenesis of MI, we searched for proteins that interact directly with LTA protein and identified galectin-2 protein as a binding partner of LTA protein. It is a member of galactose-binding lectin family whose function has not been well characterized. Genetic association study again revealed that an SNP in *LGALS2* encoding galectin-2 was also associated with susceptibility to MI. This genetic substitution seemed to affect the transcriptional level of galectin-2, which led to altered secretion of LTA, thereby affecting the degree of inflammation. Thus, our findings indicate the importance of inflammation, especially the LTA cascade, in the pathogenesis of MI. Also, combined strategy of genetic and molecular-cellular biological approaches may be useful for clarification of the pathogenesis of common diseases in general.

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Keywords SNP (single nucleotide polymorphism) · Association study · Myocardial infarction · Inflammation

Introduction

Coronary artery diseases including myocardial infarction (MI) continue to be the principal cause of death in many countries despite changes in lifestyle and the development of new pharmacologic approaches (Breslow 1997; Braunwald 1997), indicating the importance of identifying genetic and environmental factors in the pathogenesis of these diseases to provide better treatment to patients.

MI is a disease of the vessel that feeds the cardiac muscle, called the coronary artery. Abrupt occlusion of the coronary artery results in irreversible damage to cardiac muscle. Plaque rupture with thrombosis is a well-established critical factor in the pathogenesis of MI (Falk et al. 1995; Libby 1995). Although detailed mechanisms of plaque rupture are unknown, inflammation is thought to play an important role in its pathogenesis (Ross 1999). Inflammatory mediators like cytokines are involved in atheroma formation; rapid evolution of the atheromatous injury, leading to rupture of the plaque; and intraluminal thrombosis (Ross 1999).

Epidemiological studies have revealed several risk factors for MI, including diabetes mellitus, hypercholesterolemia, and hypertension. Although each of the risk factors should have genetic components, a positive family history of MI is an independent predictor, which suggests it has a genetic background of its own (Marenberg et al. 1994). To reveal genetic backgrounds of MI, investigation through common genetic variants should be of great use since they are widely thought to contribute significantly to genetic risks of common diseases (Collins et al. 1997; Lander 1996; Risch and Merikangas 1996). Naturally, comprehensive analyses, rather than the candidate gene approach, have much

more power to detect genetic factors in that, in theory, they can examine a whole set of genetic variations. Among the variations in the human genome, single nucleotide polymorphisms (SNP) have recently attracted attention. SNP is the most common variations and is usually biallelic. These characteristics provide a greater probability of identifying the associated variations directly if comprehensive analysis is possible, and their binary nature is also of great help in constructing a high-throughput genotyping system.

In this mini-review, we focus on our genetic association results and show that our initial hypothesis-free strategy unexpectedly revealed the importance of inflammation in the pathogenesis of MI.

Identification of susceptibility locus for MI through large-scale gene-based SNP association study

As a first step in our comprehensive association study, we genotyped 94 MI patients with our high-throughput multiplex PCR-Invader assay method (Ohnishi et al. 2001) using 92,788 gene-based SNPs (Haga et al. 2002) and compared the results with the allelic frequencies found in 658 members of the general Japanese population. We think our screening system is superior to others in two major ways. First, our SNP database (Haga et al. 2002, http://snp.ims.u-tokyo.ac.jp) was an extensive catalogue of SNPs located within exons, introns and promoter regions. Since we screened selected regions using these gene-based SNPs, we could efficiently examine SNPs that might be associated with susceptibility to common diseases. Second, based on a combination of multiplex PCR and Invader assay, our method allows high-throughput, low-cost screening that requires as little as 0.1 ng of genomic DNA per single SNP (Ohnishi et al. 2001).

The success rate for this genotyping was 70.8% (65,671 SNPs), and distribution of the allelic frequency was largely even (Ozaki et al. 2002). Characteristics of these 65,671 SNPs are summarized in Table 1. The cut-off P value of 0.01 for association in either recessive or dominant models was used for this screening, and as shown in Table 2, approximately 99% of the SNP loci that were successfully genotyped were excluded from the second screening. We further genotyped SNPs that showed P value less than 0.01 in a larger replication panel of MI patient and control subjects and found that most of the loci eventually showed the lack of association at this screening phase of the project. From these observations, we think one should carefully handle the association results using fewer (~100) samples for common diseases. However, when one SNP (intron 1; 252A > G) in the lymphotoxin-α (*LTA*) gene on chromosome 6p21 (HLA region), which was associated with MI in the screening ($\chi^2=9.4$, $P=0.0022$), was verified, the association became much more significant, with a χ^2 value of 18.0 ($P=0.000022$; homozygotes for the minor allele versus others) and an odds ratio of 1.69 [95% confidence interval (CI): 1.32–2.15; Table 3].

Table 1 Summary of genotyped single nucleotide polymorphisms (SNPs)

Genes screened	13,738
Exon	11,694
Synonymous	1,493
Non-synonymous	1,491
Non-coding region	8,710
Intron	43,910
5' flanking region	2,038
3' flanking region	1,428
Other regions[a]	6,601
Total	65,671

[a]Not mapped within gene regions
The table is taken from Ozaki et al. (2002)

Then we examined whether the SNP in *LTA* was related to conventional risk factors of MI, including diabetes (DM), hypertension (HT) and hyperlipidaemia (HL). We examined the genotype frequencies in the presence or absence of each risk factor in 1,133 MI samples, and found that an association between each of the three risk factors and SNP at *LTA* was not statistically significant in a comparison of homozygote for the minor allele versus others ($P=0.14$, 0.96 and 0.68 for DM, HT and HL, respectively). Thus, the association of *LTA* was shown to be independent of each of these conventional risk factors.

We also investigated the possibility that population stratification might be influencing the results. We examined the distribution of chi-square values for all SNP loci where chi-square test was appropriate. The mean and 95% upper bound for the values were 1.05 and 4.03 for recessive-inheritance model, respectively, and 1.04 and 3.96 for dominant-inheritance model, respectively, indicating that there was no population stratification. These analyses indicated that a gene conferring susceptibility to MI itself was present within this region.

Linkage disequilibrium and haplotype structure at *LTA* locus

We constructed a high-density SNP map for LD mapping by direct sequencing of DNA by screening approximately 130 kb of the relevant region on 6p21, which included several other genes encoding molecules

Table 2 Distribution of P value in the screening of 94 cases and 658 controls

P value	Number of single nucleotide polymorphisms	
	Dominant association	Recessive association
>0.01	64,898	64,822
$<1\times10^{-2}$	672	715
$<1\times10^{-3}$	88	110
$<1\times10^{-4}$	12	18
$<1\times10^{-5}$	1	5
$<1\times10^{-6}$	0	1
Total	65,671	65,671

The table is taken from Ozaki et al. (2002)

Table 3 Association between myocardial infarction (MI) and single nucleotide polymorphisms in *LTA*

Genotype	MI (n = 1.133)	Control (n = 1.006)	χ^2 (P value)	Odds ratio (95% CI)
LTA exon 1 10G > A				
GG	416 (36.7%)	378 (37.6%)	AA vs GG + GA	1.78 (1.39–2.27)
GA	504 (44.5%)	512 (50.9%)	21.6	
AA	213 (18.8%)	116 (11.5%)	(0.0000033)	
LTA intron 1 252A > G				
AA	413 (36.5%)	371 (36.9%)	GG vs AA + AG	1.69 (1.32–2.15)
AG	511 (45.1%)	516 (51.3%)	18	
GG	209 (18.4%)	119 (11.8%)	(0.000022)	
LTA exon 3 804C > A.T26N				
CC	414 (36.5%)	376 (37.4%)	AA vs CC + CA	1.78 (1.39–2.27)
CA	506 (44.7%)	514 (51.1%)	21.6	
AA	213 (18.8%)	116 (11.5%)	(0.0000033)	

Nucleotide numbering starts from the first nucleotide of exon 1
The table is taken from Ozaki et al. (2002)

related to inflammation, such as *TNF, LTA, NFKBIL1*, and *BAT1*. We found 26 SNPs with frequencies high enough to make them relevant to the search for genes predisposing to common diseases (>25% of minor alleles), and consequently found one extended block of intense LD. We further genotyped these 26 SNPs by expanding the sample size. Although most of them showed no significant association with the MI phenotype, three of these SNPs revealed a tight association with MI when we compared frequencies of homozygosity for the minor allele between cases and controls (Table 3). We further confirmed association with MI and *LTA* exon3 804C > A SNP using increased sample sizes (2,833 cases and 3,399 control subjects) and obtained similar association results (P=0.0000014, recessive association model; Table 4). Furthermore, a recent transmission disequilibrium test (TDT) analysis of 447 trio families with coronary artery disease (CAD) in white Europeans (PROCARDIS) demonstrated that the *LTA* 804C allele (26N-LTA) was excessively transmitted to affected offspring (χ^2=8.44, P=0.0018, γ^2=1.96, PROCARDIS Consortium 2004), indicating the generality of this variation in the pathogenesis of MI. Recently, other Japanese researchers have claimed the lack of association (Yamada et al. 2004); we think there is one flaw in their paper. The genotype frequencies of our MI group and their MI group were almost the same, and the difference was in control groups. Their control group included only those who had at least one coronary risk factor, which created a risk of sampling biases. Also, several other studies have examined this locus, and their genotype frequencies were similar to our control groups (Nishimura et al. 2000; Quasney et al. 2001; Tokushige et al. 2003; Iwanaga et al. 2004). Thus, it might be natural to consider that the different association results were due to the unique characteristics of their control group, not ours.

Function of intron1 SNP in *LTA*

To determine whether the two SNPs in the *LTA* gene, 10G > A in exon 1 and 252A > G in intron 1, would affect its expression level, we constructed three kinds of plasmids with a genomic fragment containing both SNPs (10G-252A, 10A-252G, and 10A-252A haplotypes, respectively) upstream of a luciferase gene transcriptional unit. We did not investigate a construct containing 10G-252G because this haplotype was not present in the individuals we examined. As shown in Fig. 1a, the clone containing the 10A-252G haplotype showed 1.5-fold greater transcriptional activity than clones containing the other two haplotypes, indicating that the substitution in intron 1, but not the one in exon 1, affected transcription of the *LTA* gene. We also used reporter-gene assays to investigate potential effects of SNPs in the promoter regions of the *IKBL* and *BAT1* genes, and found that a -63A allele of *IKBL* might cause a moderate reduction in transcriptional activity (Fig. 1b).

Furthermore, since any known DNA-binding proteins could probably bind to parts of the DNA sequence containing the SNP in intron1 of *LTA*, we examined whether a nuclear extract from Jurkat cells was able to bind to oligonucleotides corresponding to genomic sequences that included 252A or 252G alleles. As shown in Fig. 1c, the band that appeared when we used the oligonucleotide corresponding to the G allele

Table 4 Confirmation of association between myocardial infarction (MI) and lymphotoxin-α (*LTA*) gene single nucleotide polymorphisms

Genotype	MI (%)	Control (%)	χ^2 (P value)	HWE-χ^2 test (P)	
				MI	Control
Exon 3 804 C > A, T26N					
CC	1.028 (36.3)	1.333 (39.2)	AA vs CC + CA	3.34 (0.068)	3.25 (0.072)
CA	1.318 (46.5)	1.630 (48.0)	23.31		
AA	487 (17.2)	436 (12.8)	(0.0000014)		
Total	2.833	3.399			

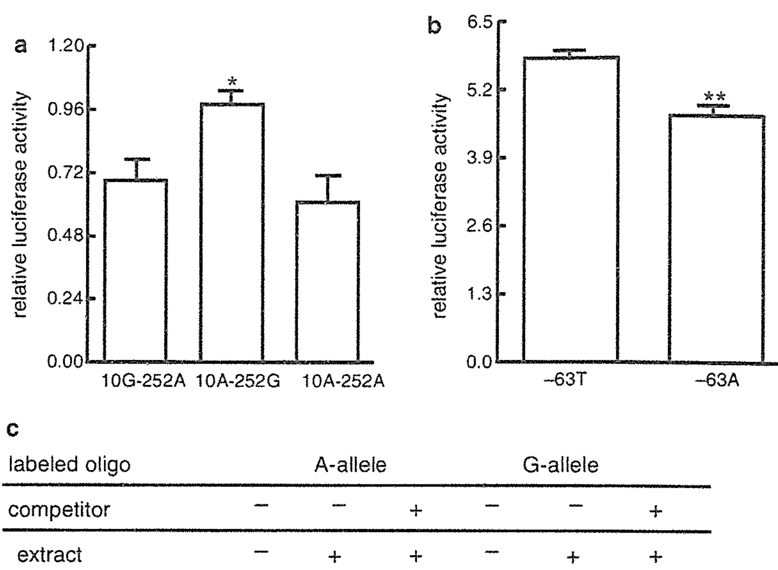

Fig. 1a–c Modification of transcriptional activity by lymphotoxin-α (*LTA*) gene single nucleotide polyphormisms (SNPs) (Ozaki et al. 2002). Transcriptional regulatory activity affected by SNPs in **a** intron 1 (252A > G) of *LTA* and **b** the promoter region (-63T > A) of *NFKBIL1*. Each experiment was performed independently three times, with each sample measured in triplicate. $^{*}P < 0.01$ in comparison between 10G-252A and 10A-252G haplotypes; $^{**}P < 0.01$ in comparison between -63A allele and -63T allele (Student's *t*-test). **c** Binding of unknown nuclear factor(s) to intron 1 of *LTA*. An *arrow* indicates the band showing tighter binding of nuclear factor(s) to the G-allele. The figure is taken from Ozaki et al. (2002)

was more intense than the band corresponding to the A allele, indicating that some nuclear factor present in Jurkat cells was binding more tightly to the G allele than to the A allele. This result raised the possibility that unidentified nuclear factor(s) regulating transcription of *LTA* by binding to this region may represent novel molecular entities related to MI susceptibility.

T26N variation in LTA protein

In addition to SNPs in the putative promoter or transcriptional-regulator sequences of the *LTA* gene, we found one SNP that would substitute asparagine for threonine at codon 26 (T26N). The LTA product can induce adhesion molecules and cytokines from vascular endothelial cells, vascular smooth-muscle cells, and several kinds of leukocytes, as its contribution to the inflammatory process (Libby 1995; Ross 1999). To address whether these biological activities could be influenced by the amino-acid substitution in the gene product, we constructed expression vectors containing each allele, and purified mature recombinant LTA proteins in *Escherichia coli*. The ability of each allele to induce expression of adhesion molecules and cytokines was examined in cultured human coronary artery endothelial cells (HCAEC) and in cultured human coronary artery smooth-muscle cells (HCASMC). Both types of LTA protein stimulated mRNA expression of vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1), E-selectin, TNF-α, interleukin-1α (IL-1α), and interleukin-1β (IL-1β) within 4 h, and we observed no difference in transcriptional activity when we used HCAEC. However, in HCASMC cells, 26N-LTA revealed a two-fold higher level of transcriptional activity for VCAM-1 and E-selectin than 26T-LTA (Fig. 2).

Thus, it seems that increased level of functionally modified LTA protein is associated with increased degree of inflammation, thereby conferring risk of MI (Fig. 6).