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Review

Genome-wide association study to identify SNPs conferring risk of myocardial infarction and their functional analyses

K. Ozaki and T. Tanaka*

Laboratory for Cardiovascular Diseases, SNP Research Center, the Institute of Physical and Chemical Research (RIKEN) 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639 (Japan), Fax: +81 35449 5674,
e-mail: toshitan@ims.u-tokyo.ac.jp

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Abstract. Myocardial infarction might result from the interactions of multiple genetic and environmental factors, none of which can cause disease solely by each of themselves. Although molecular biological studies revealed that a number of proteins are possibly involved in its pathogenesis, little, if any genetic findings have been reported so far. To reveal genetic backgrounds of myocardial infarction, we performed a large-scale, case-control association study using 92,788 gene-based single-nucleotide polymorphism (SNP) markers. We have identified functional SNPs within the lymphotoxin-α gene (*LTA*) located on chromosome 6p21 that conferred susceptibility to myocardial infarction. Furthermore, we could identify galectin-2 protein as a binding partner of LTA protein. The association study further revealed that a functional SNP in *LGALS2* encoding galectin-2, which led to altered secretion of LTA, also indicated a risk of myocardial infarction. A combined strategy of genetic and molecular-cellular biological approaches may be useful in clarifying pathogenesis of common diseases.

Key words. Myocardial infarction; common disease; single-nucleotide polymorphism (SNP); whole-genome association study; lymphotoxin-α (LTA); galectin-2.

Introduction

Despite changes in life-style and the development of new pharmacologic approaches, coronary artery diseases, including myocardial infarction (MI) continue to be the principal cause of death in many countries [1, 2], indicating the importance of identifying genetic and environmental factors in their pathogenesis. Common genetic variants are widely believed to contribute significantly to genetic risks of common diseases [3–5]. Although it is known that coronary risk factors such as diabetes mellitus, hypercholesterolemia and hypertension have genetic components, a positive family history is an independent predictor, which in itself suggests genetic a background [6]. In recent years, a candidate MI susceptibility gene was identified on several chromosome loci using linkage analysis and/or single-nucleotide polymorphism (SNP) case-control association studies [7–10].

For genome-wide association studies to succeed in identifying genes related to common diseases, linkage disequilibrium (LD)-based and haplotype-based mapping must define critical regions. Recent efforts from haplotype analyses of the International HapMap Project [11, 12] which genotyped 600,000 SNPs, will provide more insight into marker-trait association studies.

Plaque rupture with thrombosis is well established as a critical factor in the pathogenesis of MI [13, 14]. Although the detailed mechanisms of plaque rupture are unknown, inflammation plays an important role in its pathogenesis [15]. Inflammatory mediators such as cytokines are involved in atheroma formation and rapid evolution of the

* Corresponding author.

Table 1. Summary of genotyped 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*	6,601
Total	65,671

* The SNPs were not mapped within gene regions.
Reprinted from [16].

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

P value	Number of SNPs	
	Dominant association	Recessive association
>0.01	64,898	64,822
$<1 \times 10^{-2}$	672	715
$<1 \times 10^{-3}$	88	110
$<1 \times 10^{-4}$	12	18
$<1 \times 10^{-5}$	1	5
$<1 \times 10^{-6}$	0	1
Total	65,671	65,671

Reprinted from [16].

atheromatous injury, leading to rupture of the plaque and intraluminal thrombosis [15].

Through the whole genome case-control association study, using 92,788 SNP markers, we previously identified a candidate locus on chromosome 6p21 that confers susceptibility to MI. Subsequent linkage-disequilibrium mapping and analyses of haplotype structure revealed markedly significant associations between MI and functional SNPs present in the *LTA*, encoding lymphotoxin-α [LTA, also known as tumor necrosis factor (TNF)-β] [16]. LTA, a member of the TNF family that also includes TNF-α and lymphotoxin-β (LTB), is expressed as an secreted LTA homotrimer as well as a LTB heterotrimeric complex on the surface of activated lymphocytes [17]. LTA is also known as one of the earliest cytokines produced in vascular inflammatory processes, activating a cytokine cascade by inducing interleukin-1 and other mediators such as adhesion molecules [18]. Furthermore, we have also found that galectin-2, a member of the galactoside-binding lectins, bound directly to LTA, and this interaction may affect the extracellular secretion of LTA [19]. The case-control association study revealed that a transcriptional regulatory SNP in *LGALS2*, encoding galectin-2, was also associated with MI [19]. These findings are clues to new means of prevention, therapy and diagnosis of myocardial infarction.

The whole-genome case-control association study

As the first step of our comprehensive association study, we genotyped 94 MI patients using the high-throughput multiplex polymerase chain reaction (PCR)-Invader assay method [20] and 92,788 gene-based SNPs [21]. We compared the results with the allelic frequencies found in 658 subjects from the general Japanese population. The success rate for this genotyping was 70.8% (65,671 SNPs) and distribution of their allelic frequency was largely even [22]. Characteristics of these 65,671 SNPs are summarized in table 1. The cut-off *P* value of 0.01 for association in either the recessive or dominant models was used for this screening, and as shown in table 2, ~99% of the SNP loci that were successfully genotyped were excluded from the second screening. We estimated the power (β value) for detection of a locus with a genotypic risk ratio of 1.6 by this strategy to be 0.82, assuming the frequency of the disease-associated allele to be 0.4 and the disease prevalence of 0.01 in the general population. Considering the cost-effectiveness of the studies, we thought the value was acceptable. We further genotyped SNPs that showed a *P* value less than 0.01 in a larger replication panel of MI patients and control subjects and found that most of the loci eventually showed a lack of association at this screening phase of the project. However, when one SNP (intron 1; 252A>G) in the *LTA* gene on chromosome 6p21 (HLA region), associated with MI in the screening (χ^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]. Then we looked to see whether the SNP in *LTA* was related to conventional risk factors of MI, including diabetes (DM), hypertension (HT) and hyperlipidemia (HL). We examined the genotype frequencies in the presence and absence of each risk factor in 1133 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 homozygotes 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 from each of these conventional risk factors. We also investigated the possibility that population stratification might be influencing the results. We examined the distribution of χ^2 values for all SNP loci where the χ^2 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 the dominant-inheritance model, respectively, indicating that there is no population stratification. These analyses suggested that a gene conferring susceptibility to MI itself was present within this region.

Table 3. Association between MI and SNPs in LTA, NFKBIL1 and BAT1 [16].

Genotype	MI (n=1133)	Control 1 (n=1006)	χ^2 [P value]	Odds ratio (95% CI)
LTA exon 1 10G>A				
GG	416 (36.7%)	378 (37.6%)	AA vs GG+GA	
GA	504 (44.5%)	512 (50.9%)	21.6	1.78
AA	213 (18.8%)	116 (11.5%)	[0.0000033]	(1.39-–2.27)
LTAintron 1 252A>G				
AA	413 (36.5%)	371 (36.9%)	GG vs AA+AG	
AG	511 (45.1%)	516 (51.3%)	18	1.69
GG	209 (18.4%)	119 (11.8%)	[0.000022]	(1.32–2.15)
LTA exon 3 804C>A,T26N				
CC	414 (36.5%)	376 (37.4%)	AA vs CC+CA	
CA	506 (44.7%)	514 (51.1%)	21.6	1.78
AA	213 (18.8%)	116 (11.5%)	[0.0000033]	(1.39–2.27)
NFKBIL 1 promoter –63T>A				
TT	406 (35.8%)	374 (37.2%)	AA vs TT+TA	
TA	521 (46.0%)	509 (50.6%)	14.5	1.6
AA	206 (18.2%)	123 (12.2%)	[0.00013]	(1.25–2.03)
BAT1 promoter –23G>C				
GG	411 (36.3%)	374 (37.2%)	CC vs GG+GC	
GC	517 (45.6%)	510 (50.7%)	14.7	1.6
CC	205 (18.1%)	122 (12.1%)	[0.00013]	(1.26–2.04)

Nucleotide numbering starts from the first nucleotide of exon 1. Adapted from [16].

Linkage disequilibrium and haplotype structure of MI-related loci

We attempted to construct a high-density SNP map for LD mapping by direct sequencing of DNAs from 16 MI patients and 16 controls. We screened approximately 130 kb of the relevant region on 6p21, which included several other genes encoding molecules related to inflammation such as *TNF, LTA, NFKBIL1* and *BAT1*. We genotyped 94 MI patients and 94 subjects from the general population for those markers, selected on the basis of allele frequencies greater than about 10% (estimated roughly by comparing the peak levels of nucleotide signals on the electropherogram). Finally, 26 SNPs with frequencies high enough to make them relevant to the search for genes predisposing to common diseases (>25% of minor alleles) [5] revealed one extended block of intense LD, with a D′ dropoff near *p5-1* and *AIF-1*. Therefore, we concluded that the MI-susceptibility gene was likely to be located between these two loci, and typed these 26 SNPs by expanding the sample size. Although most of them showed no significant association with the MI phenotype, five of these 26 SNPs revealed a tight association with MI when we compared frequencies of homozygosity for the minor allele between cases and controls (table 3). The distribution of genotypes for each SNP deviated slightly from traditional Hardy-Weinberg equilibrium (HWE); however, considering we had analyzed more than 65,000 SNP loci, we thought the cutoff *P* value for HWE should be less than 1×10^{-6}. These SNPs were in almost complete linkage disequilibrium with each other. Therefore, we further confirmed the association between MI and *LTA* exon 3 804C>A SNP using increased sample sizes (2927 case and 2780 control subjects) and obtained a similar association result (P=0.000013, recessive association model [unpublished data]). At this point, the distribution of genotypes for the SNP was not deviated from HWE (P>0.01). Furthermore, a recent transmission disequilibrium test (TDT) analysis of 447 trio families with coronary artery disease (CAD) in white European (Procardis) demonstrated that the *LTA* 804C allele (26N-LTA) was transmitted in excess to affected offspring (χ^2=8.44, P=0.0018) [23].

Increase in transcriptional activity brought about by the SNP in intron 1 of *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 figure 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.

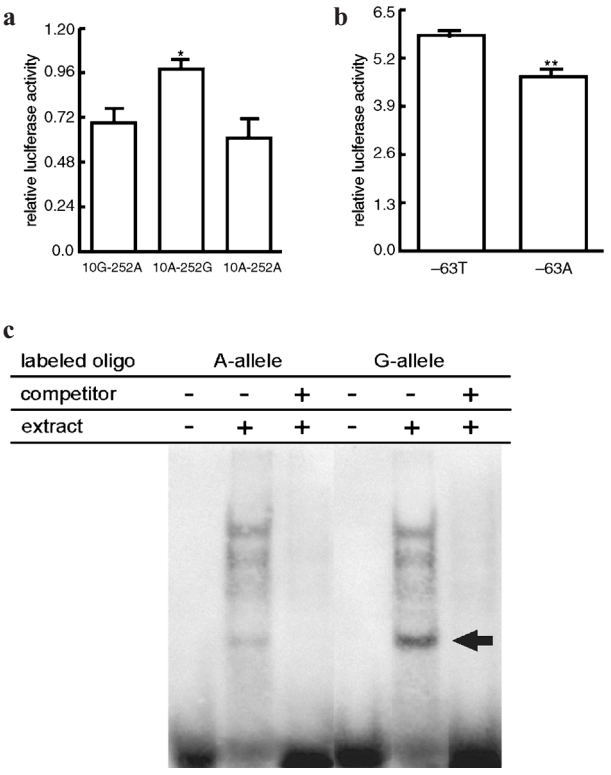


Figure 1. Modification of transcriptional activity by *LTA* SNPs [16]. Transcriptional regulatory activity affected by SNPs in (*a*) intron 1 (252A>G) of *LTA* and (*b*) the promoter region (–63T>A) of *NFKBIL1*. Each experiment was performed independently three times, with each sample measured in triplicate. **P*<0.01 in comparison between 10G-252A and 10A-252G haplotype; ***P*<0.01 in comparison between the -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. Reprinted from [16].

We also used reporter-gene assays to investigate the 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 of transcriptional activity (fig. 1B).

Furthermore, since any known DNA binding proteins could probably bind to parts of the DNA sequence containing the SNP in intron 1 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 figure 1C, the band that appeared when we used the oligonucleotide corresponding to the G allele 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.

Ability of the T26N variation in LTA protein to alter induction of adhesion molecules

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 [4, 5]. 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 smooth-muscle cells (HCASMCs). Although both wild-type (26T) and mutant (26N) LTA protein stimulated messenger RNA (mRNA) expression of vascular cell-adhesion molecule-1 (VCAM-1), intercellular cell-adhesion molecule-1 (ICAM-1), selectin E (SELE), TNF-a, interleukin-1a (IL-1a) and interleukin-1b (IL-1b) within 4 h, 26N-LTA revealed a twofold higher level of transcriptional activity for VCAM-1 than did 26T-LTA (fig. 2a). In addition, 26N-LTA had significantly higher mRNA expression of SELE mRNA in HCASMC, whereas 26T-LTA did not affect its expression (fig. 2b).

Identification of galectin-2 as a binding partner of LTA

After identification of LTA as a novel genetic risk factor for myocardial infarction, we searched for proteins that interact with LTA, to better understand the role of LTA in the pathogenesis of this disease. With both *E. coli* two-hybrid system and phage display method, we could identify a protein, galectin-2, as a binding partner of LTA (data not shown). We purified both recombinant proteins raised by the bacterial expression system, and direct binding between galectin-2 and LTA was tested using an in vitro binding assay. As shown in figure 3a, galectin-2 bound directly to recombinant LTA. We further examined whether the interaction took place in mammalian cells with constructs encoding FLAG- or S-tagged LTA and galectin-2. Western blot analysis showed that galectin-2 was co-immunoprecipitated with LTA (fig. 3b). Using antibodies that specifically recognize each protein, we also investigated subcellular localization of endogenous galectin-2 and LTA proteins in U937 cells and found that these proteins co-localize in the cytoplasm (fig. 3c).

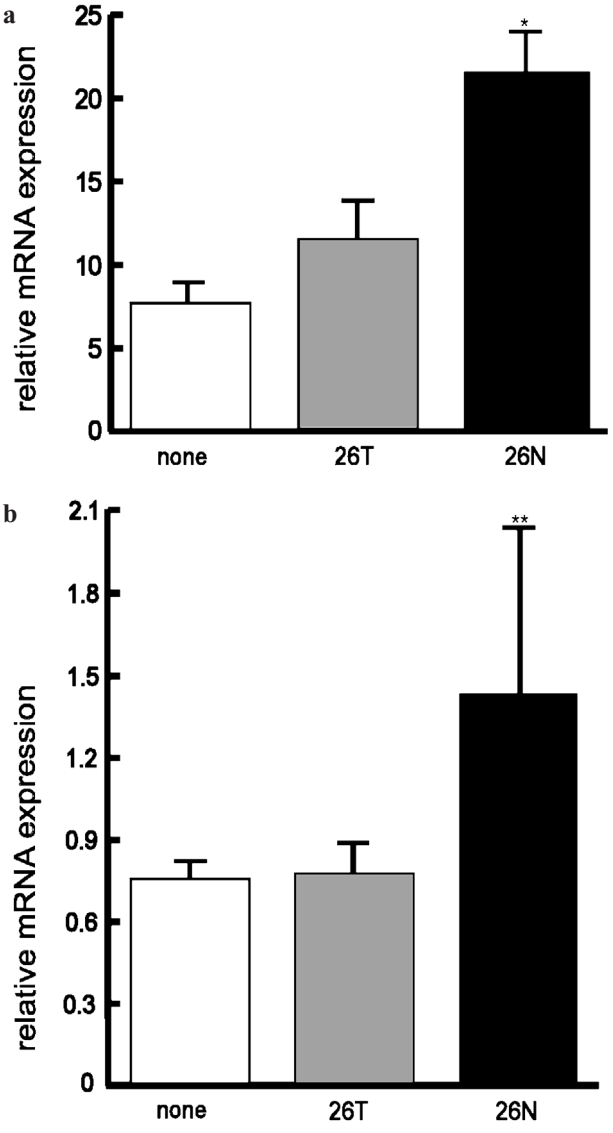


Figure 2. Differing abilities of 26N- and 26T-LTA to induce mRNA expression of adhesion molecules [16]. Relative mRNA levels are indicated for VCAM1 (*a*) and SELE (*b*) in HCASMCs following treatment of each cell with medium only (white bars) 26T-LTA (gray bars), or 26N-LTA (black bars) (20 ng/ml) for 4 h. Reprinted from [16].

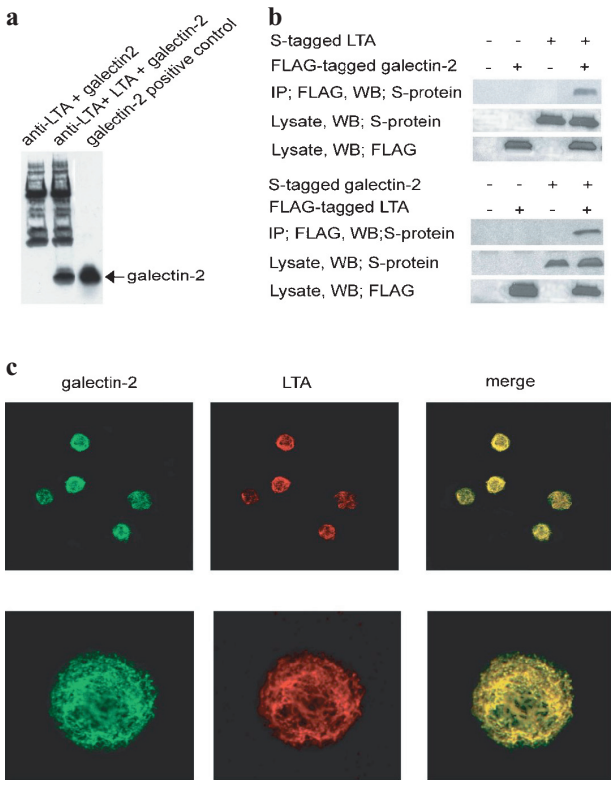


Figure 3. LTA binds to galectin-2 [19]. (*a*) In vitro binding assay. (*b*) Co-immunoprecipitation of tagged LTA and galectin-2 in COS7 cells. For immunoprecipitation (IP) and Western blot (WB), anti-FLAG tag antibody agarose and S-protein horseradish peroxidase (S-protein-HRP) were used, respectively. (*c*) Co-localization of endogenous LTA with galectin-2 in U937 cells. Bottom: Enlarged images of representative cells in the upper panels. Reprinted from [19].

Association of intron 1 SNP in *LGALS2* with MI

Since galectin-2 was shown to bind to LTA, we next examined whether the variation(s) on *LGALS2* (encoding galectin-2) were also associated with susceptibility to MI. By resequencing the *LGALS2* genomic region using 32 MI samples, we found 17 SNPs (fig. 4a, table 4). We then compared allele frequencies of ~600 individuals with MI and 600 controls for these SNPs and found that one SNP (3279C>T) in intron1 of *LGALS2* showed a significant association with MI (table 4). No particular haplotype showed higher statistical significance for association with MI than the significant SNP alone. We further confirmed this association by increasing the number of samples up to 2302 for patients with MI and 2038 for controls (fig. 4b). HWE of the distribution of genotypes for each SNP was evaluated by χ^2 tests for both patient and control groups, and showed no significant deviation (P>0.01). Since minor allele frequency of the SNP was lower in the patients' group (fig. 4b), we concluded that the minor variant has a protective role against the risk of MI from these genetic studies. To date, we have further confirmed the association with MI and the SNP in *LGALS2* using increased sample sizes (2907 case and 2579 control subjects) and obtained tight association results (P=0.00000012, HWE test P>0.01 [unpublished data]). To examine whether the intron1 SNP in *LGALS2* would affect its expression level, we constructed reporter plasmids with a genomic fragment containing the SNP downstream of a luciferase gene and SV40 enhancer sequence. The clone containing the 3279T allele showed twofold smaller transcriptional activity than clones containing the 3279C allele and vector only (fig. 4c, 4d), indicating that the substitution repressed the transcriptional level of galectin-2.

Table 4. Association analyses of 17 SNPs in the LGALS2 region with MI.

			MI						Control						Allele 1 versus allele 2			
SNP ID	SNP position*	dbSNP ID	11	12	22	Sum	MAF**	HWE test (*P*)	11	12	22	Sum	MAF**	HWE test (*P*)	$\chi 2$	*P*	Odds ratio (95% CI)
SNP1	5′-flanking –1775 G → A	–	270	240	61	571	0.32	0.48	269	233	67	569	0.32	0.13	0.08	0.78	1.03 (0.86–1.22)
SNP2	5′-flanking –1543 G → A	–	402	142	19	563	0.16	0.15	414	138	22	574	0.16	0.02	0.01	0.93	1.01 (0.81–1.26)
SNP3	5′ flanking –1497 A → G	–	563	6	0	569	0.01	0.90	569	5	0	574	0.00	0.92	0.10	0.75	1.21 (0.37–3.98)
SNP4	5′-flanking –1480 A → T	rs140059	434	118	10	562	0.12	0.55	431	131	12	574	0.14	0.58	0.76	0.38	1.12 (0.87–1.43)
SNP5	5′-flanking –1367 C → T	–	373	158	27	558	0.19	0.06	378	175	21	574	0.19	0.89	0.00	0.95	1.01 (0.82–1.24)
SNP6	5′-flanking –1344 C → A	–	514	53	1	568	0.05	0.76	522	51	2	575	0.05	0.53	0.00	0.95	1.01 (0.69–1.49)
SNP7	5′-flanking –1038 C → T	rs1969369	321	205	45	571	0.26	0.13	325	210	32	567	0.24	0.81	0.85	0.36	1.09 (0.90–1.32)
SNP8	5′-flanking –853 G → A	rs5750457	161	275	129	565	0.47	0.58	144	291	125	560	0.48	0.34	0.29	0.59	1.05 (0.89–1.23)
SNP9	intron 1 3279 C → T	rs7291467	285	229	48	562	0.29	0.84	247	241	79	567	0.35	0.11	10.19	0.0014	1.34 (1.12–1.59)
SNP10	intron 1 3469 T → C	rs7291162	207	273	93	573	0.40	0.85	226	261	87	574	0.38	0.42	1.13	0.29	1.10 (0.93–1.30)
SNP11	intron 1 3621 A → G	rs5750452	135	300	135	570	0.50	0.21	164	282	128	574	0.47	0.75	2.25	0.13	1.13 (0.96–1.34)
SNP12	intron 2 451 C → T	–	563	10	0	573	0.01	0.83	556	10	0	566	0.01	0.83	0.00	0.98	1.01 (0.42–2.44)
SNP13	intron 2 562 S → G	–	550	14	0	564	0.01	0.77	538	22	0	560	0.02	0.64	1.87	0.17	1.59 (0.81–3.13)
SNP14	exon 3 146 G → A;R49H	–	540	14	0	554	0.01	0.76	535	9	0	544	0.01	0.85	1.01	0.32	1.53 (0.66–3.56)
SNP15	intron 3 61 G → C	–	513	37	1	551	0.04	0.70	498	44	2	544	0.04	0.34	1.09	0.30	1.26 (0.82–1.94)
SNP16	exon 4 365 G → A;V119I	rs2235339	471	100	3	574	0.09	0.35	474	87	6	567	0.09	0.38	0.18	0.67	1.06 (0.80–1.42)
SNP17	3′-flanking 135 C → T	–	446	94	2	542	0.09	0.20	452	82	6	540	0.09	0.30	0.08	0.78	1.04 (0.78–1.40)

–; the variant was not included in the dbSNP database (build 118).

*; nucleotide numbering is according to the mutation nomenclature [38].

11, 12 and 22; major homozygote, heterozygote and minor homozygote, respectively.

**; minor allele frequency=[{(the number of 22) x 2 + the number of 12}/the number of total sample]/2

HWE (Hardy-Weinberg equilibrium) test; an index for random selection of population sample.

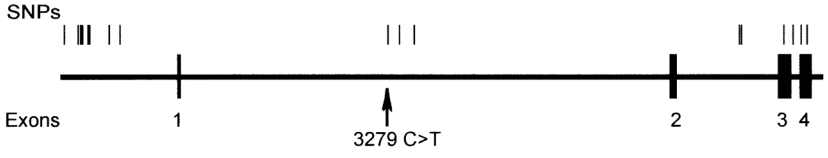
Data are from [19].

Galectin-2 affects secretion of LTA through binding to membrane-trafficking machinery

Although galectin-2 was thought to be a member of the carbohydrate-binding lectin family by sequence similarity [24], its function was largely uncharacterized. As several members of galectin family are known to be secreted [25], we first investigated the possibility whether galectin-2 was also secreted using HeLa or HepG2 cell lines transfected with C-terminal FLAG- or N-terminal Myc-tagged galectin-2 expression vectors. However, we could not detect galectin-2 in culture media after transfection for 24 and 48 h, respectively, even by concentrating the FLAG- or Myc-tagged protein using the corresponding anti-tag antibody agaroses [unpublished data], indicating that galectin-2 functions primarily as an intracellular protein, although it remains to be clarified by stably transformed cell lines. Since the susceptibility SNP in *LGALS2* was shown to affect the expression level of galectin-2, we hypothesized that the intracellular amount of galectin-2 might regulate the secretion level of LTA, thus leading to influencing the degree of inflammation. To clarify this hypothesis, we examined changes in the level of LTA secreted caused by quantitative alteration of galectin-2, using the small interfering RNA (siRNA) technique. One siRNA for galectin-2 was shown to specifically repress ~80% of galectin-2 mRNA (fig. 4e) and to significantly inhibit the secretion of LTA (fig. 4f). As shown in figure 4g, the LTA mRNA level did not change by knockdown of galectin-2.

To investigate the regulatory mechanism of LTA secretion by galectin-2, we searched for intracellular molecules that associate with galectin-2 using a tandem affinity purification (TAP) system [26]. We identified two specific bands that could be seen only when the galectin-2-TAP tag was expressed (fig. 5a). Based on MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry analyses, the two bands were identified as α- and β-tubulins, both constituting microtubules. Using FLAG-tagged galectin-2 transfected HeLa cells, we con-

a



b

Genotype	MI (%)	CO (%)	χ^2 [P value] (Odds ratio) <95%CI>			
			Genotype frequency	Allele frequency	CC vs others	TT vs others
LGALS2 intron1 3279 C>T						
CC	1077 (46.8)	856 (42.0)	25.2	21.1	10.0	22.1
CT	1014 (44.0)	903 (44.3)	[0.0000034]	[0.0000045]	[0.0016]	[0.0000026]
TT	211 (9.2)	279 (13.7)		(1.23)	(1.21)	(1.57)
Total	2302 (100.0)	2038 (100.0)		<1.08-1.37>	<1.12-1.35>	<1.30-1.90>

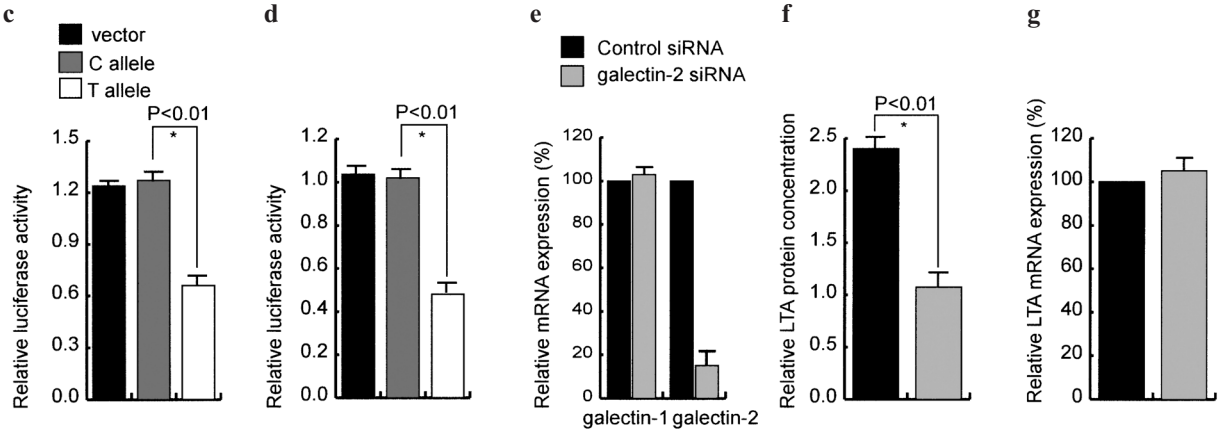


Figure 4. Association of a SNP in *LGALS2* with MI and its functionality [19]. (*a*) Map of SNPs in the *LGALS2* locus. (*b*) Association between MI and intron 1 SNP in *LGALS2*. Nucleotide numbering is according to the mutation nomenclature [15].(*c* and *d*) Transcriptional regulatory activity of intron 1 SNP of *LGALS2* in HeLa (*c*) and HepG2 (*d*) cells. (*e–g*) Inhibition of galectin-2 expression. Levels of galectin-1 or galectin-2 mRNA (*e*), supernatant LTA protein (*f*), and LTA mRNA (*g*) after 48-h transfection with siRNA vectors. Reprinted from [19].

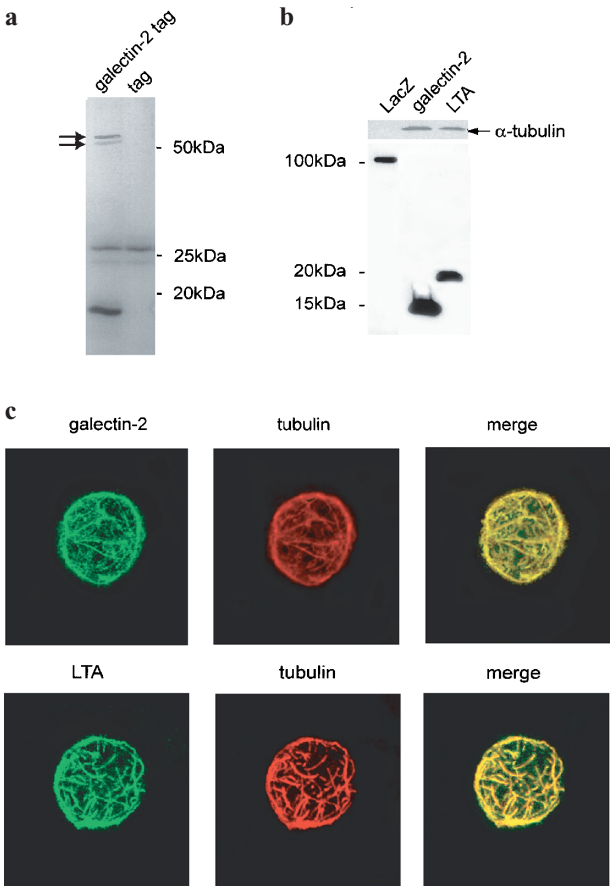


Figure 5. Galectin-2 interacts with microtubules [19]. (*a*) Isolation of TAP-tagged galectin-2 and interacting proteins. Arrowheads indicate α- and β-tubulins, revealed by MALDI/ToF mass spectrometry analyses. (*b*) Co-immunoprecipitation of endogenous α-tubulin with FLAG-tagged galectin-2 or LTA. Immunoprecipitations were done using anti-FLAG M2 agarose, and immunoprecipitates were detected using anti α-tubulin antibody (upper panel) or anti-FLAG antibody (lower panel). FLAG-tagged LacZ encoding β-galactosidase was used as a negative control. (*c*) Co-localization of endogenous α-tubulin with endogenous galectin-2 or LTA in U937 cells. Reprinted from [19].

firmed co-immunoprecipitation of endogenous tubulins (fig. 5b). Interestingly, we found that the endogenous tubulins were also co-immunoprecipitated with LTA (fig. 5b). Images from serial confocal sections of double-immunostained U937 cells revealed that galectin-2 and a-tubulin co-localized as reticular filamentous networks developed in the cytoplasm (fig. 5c).

Discussion

By means of a genome-wide SNP analysis and subsequent functional analyses we have identified candidate genes for susceptibility to MI. We think our screening system offers at least two advantages. First, our SNP database [21] (http://snp.ims.u-tokyo.ac.jp) is an extensive catalogue of SNPs located within exons, introns and promoter regions. Since we are screening selected regions using these gene-based SNPs, we can efficiently examine SNPs that may be associated with susceptibility to common diseases. Second, on the basis of a combination of multiplex PCR and Invader assay [20], our method allows high-throughput, low-cost screening that requires as little as 0.1 ng of genomic DNA per single SNP.

The two SNPs (intron 1 252 A→G and exon 3 804 C→A, T26N) in the *LTA* that showed a strong association with MI were proven to affect *LTA* expression level and also the biological function of the respective gene products. The 252A>G substitution in intron 1 influenced transcriptional activity, as measured by luciferase assay. In addition, by means of a gel-shift assay we found an allelic difference in the protein's affinity for binding to unidentified transcription factor(s). That result suggested that one or more unidentified molecules in nuclear extracts might be related to MI. The variant protein, 26N-LTA, induced greater expression of VCAM-1 and SELE mRNA in vascular smooth-muscle cells than did 26T-LTA. Since these two functional SNPs were in complete linkage disequiliburium, variant protein was estimated to be increased 1.5-fold over wild-type. A recent TDT analysis in white European CAD families confirmed association of the *LTA* 804C allele with an increased risk of CAD, with γ^2 of 1.96 (1.25–3.13) [23]. Our association results of *LTA* SNPs and subsequently its functional roles, together with the white European TDT study, strongly suggest that LTA has an important functional role in the pathogenesis of MI/CAD.

We further demonstrated that LTA protein binds directly to galectin-2. The intron 1 SNP of *LGALS2*, which down-regulates the expression of galectin-2 mRNA, was also strongly associated with MI, and the variant has a protective role against the MI phenotype based on its genotype. The intracellular amount of galectin-2, determined by the intron 1 SNP in *LGALS2*, was shown to alter secretion of LTA through binding to microtubules. Recently, microtubule cytoskeleton networks have been implicated in the subcellular movements of some proteins, including glucose transporter isoform (GLUT4) and thiamine transporter (THTR1) [27, 28]. It is likely that LTA is another molecule that uses the microtubule cytoskeleton network for translocation, and galectin-2 mediates LTA trafficking through binding to microtubules (fig. 6), although the precise role of galectin-2 in this trafficking machinery complex remains to be elucidated. Since LTA is an inflammatory mediator, it is likely that functional variations in the genes encoding these proteins confer a risk of MI by affecting the degree of inflammation at the lesion (fig. 6). Rupture of atherosclerotic plaques occurs predominantly at the edges of the fibrous cap, an area of accumulated inflammatory cells in close proximity to vascular smooth-muscle cells and endothelial cells [29–34]. Activated

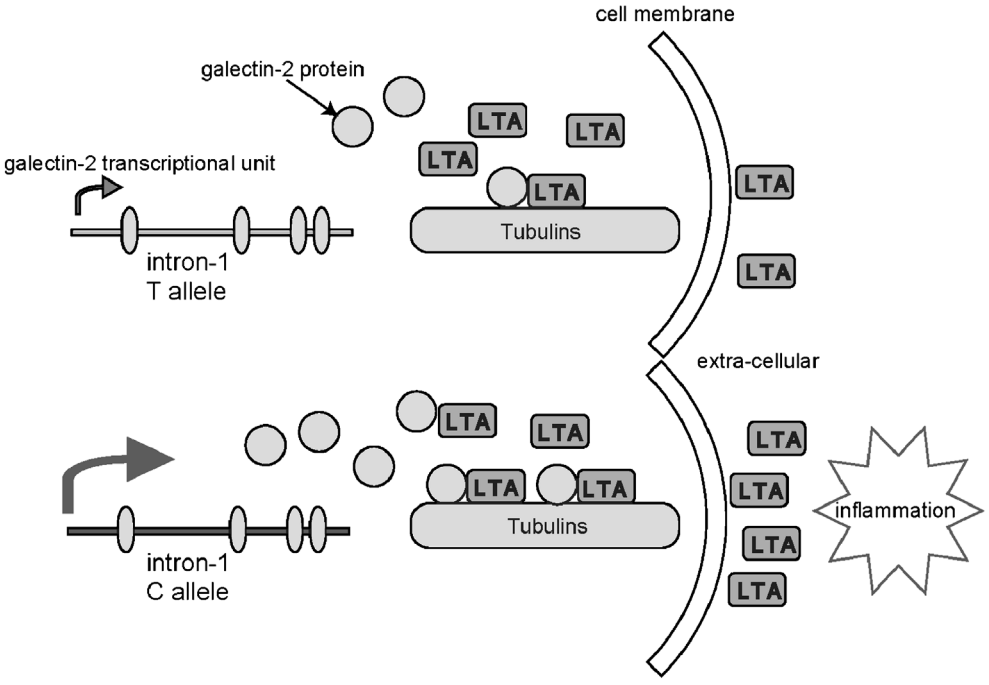


Figure 6. The possible mechanism of LTA, galectin-2 and tubulins in the inflammatory process.

inflammatory cells stimulate their neighboring cells to erode the extracellular matrix through the release of inflammatory cytokines, and decay of the framework forming the plaque cap leads to plaque rupture [29, 35, 36]. A recent report demonstrated that LTA was expressed in atherosclerotic lesions in mice and that loss of LTA reduced the size of those lesions; on the other hand, loss of TNF did not alter development of lesions in mice fed an atherogenic diet [37]. These implied that LTA findings imply, as one of the mediators of inflammation, along with galectin-2 as a regulator of LTA secretion, might play an important role in the pathogenesis of MI.

Coronary artery disease attributable to atherosclerosis is a leading cause of death for both men and women in many countries [1,2]. We believe that knowledge of the genetic factors contributing to the pathogenesis of MI will lead to improved diagnosis, treatment and prevention.

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