A functional SNP in *ITIH3* is associated with susceptibility to myocardial infarction

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Abstract Myocardial infarction (MI) results from complex interactions of multiple genetic and environmental factors. To disclose genetic backgrounds of MI, we performed a large-scale, case-control association study using 52,608 gene-based single-nucleotide polymorphism (SNP) markers, and identified a candidate

SNP located on chromosome 3p21.2–p21.1. Subsequent linkage–disequilibrium mapping indicated very significant association between MI and a SNP in exon 2 of the inter-alpha (globulin) inhibitor 3 gene (*ITIH3*; $\chi^2 = 24.88$, $P = 6.1 \times 10^{-7}$, 3,353 affected individuals versus 3,807 controls). In vitro functional analyses showed that this SNP enhanced the transcriptional level of the *ITIH3* gene. Furthermore, we found expression of the *ITIH3* protein in the vascular smooth muscle cells and macrophages in the human atherosclerotic lesions, suggesting *ITIH3* SNP to be a novel genetic risk factor of MI.

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Introduction

Coronary artery diseases, including myocardial infarction (MI), have been one of major leading causes of death in the developed countries (Ross 1999). Multiple lines of evidence have revealed several coronary risk factors contributing to the development of atherosclerosis (Libby 2002). Although each factor by itself seems partly under genetic control, a positive family history is an independent predictor (Shea et al. 1984), which implies genetic contribution to MI. Numerous reports based on family and/or twin studies have provided evidence for a genetic component in the etiology of MI (Marenberg et al. 1996). In recent years, candidate MI susceptibility genes were identified on several chromosome loci using linkage analysis (Wang et al. 2004) and/or single-nucleotide polymorphism (SNP)

case-control association studies (Yamada et al. 2002; Ozaki et al. 2002, 2004, 2006; Helgadottir et al. 2004).

Inflammation is thought to contribute to atherosclerosis and increase the risk of MI (Libby 2002). It is a consequence of complex interactions among modified lipoproteins, monocyte-derived macrophages, T lymphocytes, and vascular smooth muscle cells (SMCs) from the vessel wall. Recent studies have shown the importance of chemoattractants, e.g., adhesion molecules (Cybulsky and Gimbrone 1991), chemokines (Boring et al. 1998), growth factors (Brogi et al. 1993), and proteolytic enzymes and their inhibitors (Galis et al. 1994), in atherosclerotic plaque formation and rupture.

Inter-alpha (globulin) inhibitor 3 (ITIH3), one of the constituents of plasma serine protease inhibitors, has been shown to be related to the proinflammatory process (Fries and Kaczmarczyk 2003). ITIH3 protein is characterized by its unique covalent binding to the chondroitin sulfate chain of bikunin proteoglycan (Zhuo et al. 2004). This complex, named pre-alpha trypsin inhibitor (P α I) is synthesized by hepatocytes and released to the blood vessel upon stimulation of the proinflammatory cytokines (tumor necrosis factor or interleukin-1). Then, ITIH3 makes a complex with the locally synthesized hyaluronan (HA) and interacts with inflammatory cells (Fries and Kaczmarczyk 2003). ITIH3-HA complex has been reported to be involved in inflammatory diseases, including rheumatoid arthritis and inflammatory bowel diseases (Zhuo et al. 2004).

We report here identification of a significant association of a SNP in *ITIH3* with MI through a large-scale association study using approximately 50,000 gene-based SNPs. We also analyzed its functional significance indicating a possible role of this genetic variation in the pathogenesis of MI.

Materials and methods

DNA samples

The study included Japanese individuals with MI who were referred to the Osaka Acute Coronary Insufficiency Study group. The diagnosis of definite MI has been described previously (Ohnishi et al. 2000; Ozaki et al. 2002). The control subjects consisted of general populations recruited through several medical institutes in Japan. Clinical characteristics have been described previously (Ozaki et al. 2006). All subjects were Japanese and provided written informed consent to participation in the study or, if they were under

Table 1 Distribution of *P* values in the first stage screening of 188 cases and 752 controls

<i>P</i> value	Number of SNPs	
	Recessive association	Dominant association
>0.01	48,669	48,818
<0.01	442	322
<0.001	48	23
<0.0001	5	4
<0.00001	3	0

20 years old, their parents gave consent according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama.

Genotyping

Designs for polymerase chain reaction (PCR) primers, PCR experiments, DNA extraction, DNA sequencing, SNP discovery, genotyping of SNPs and statistical analysis have been described previously (Ohnishi et al. 2001; Ozaki et al. 2002).

Haplotype block

We used Haploview software for the identification of haplotype. We adopted the solid spine of LD for analysis and extended spine if $D' > 0.90$. We defined strong LD if the one-sided upper 95% confidence boundary on D' was >0.98 and the lower boundary was <0.80 . Conversely, we defined strong recombination pairs for which the upper confidence bound on D' was less than 0.90.

Luciferase assay

DNA fragments corresponding to nt 642 of intron 1 to 54 of intron 2 of *ITIH3* were amplified by PCR using genomic DNA as template, and cloned into pGL3-promotor vector (Promega) in the 5′–3′ orientation. For the SV40-SNP-luciferase construct for *ITIH3* SNP and all constructs for *ITIH1* SNP, double-stranded oligonucleotides (nt 695 of intron 1 to 21 of intron 2 for *ITIH3* and nt 517 to 551 of intron 10 for *ITIH1*) were cloned into pGL3-promoter vector. We grew Jurkat cells and HepG2 cells (obtained from RIKEN Cell Bank; RCB0806, RCB1681, respectively) in RPMI1640 medium and Dulbecco's modified eagle medium (DMEM), respectively, supplemented with 10% fetal bovine serum. We then transfected cells (approximately 1×10^5 and 3×10^6 cells in HepG2 and Jurkat cells, respectively) with 1 μ g of either construct and

Table 2 Association analysis of SNPs in the third stage screening

Genotype	MI (%)	CO (%)	Statistical test	Allele frequency	Dominant model
rs2286797					
GG	2,022 (60.3)	2,094 (55.0)	χ^2	24.88	20.49
GA	1,161 (34.6)	1,449 (38.1)	<i>P</i> value	6.1×10^{-7}	6.0×10^{-6}
AA	170 (5.1)	264 (6.9)	Odds ratio	1.216	1.243
Total	3,353 (100)	3,807 (100)	95% CI	1.126–1.313	1.131–1.365
rs2301523 ^a					
GG	2,919 (84.3)	3,364 (88.1)	χ^2	22.71	22.37
GA	528 (15.2)	445 (11.7)	<i>P</i> value	1.9×10^{-6}	2.3×10^{-6}
AA	17 (0.5)	10 (0.3)	Odds ratio	1.362	1.38
Total	3,464 (100)	3,819 (100)	95% CI	1.199–1.547	1.207–1.578
rs2074396					
CC	1,354 (40.3)	1,523 (40.5)	χ^2	0.00	0.00
CA	1,557 (46.4)	1,740 (46.2)	<i>P</i> value	0.95	0.98
AA	446 (13.3)	501 (13.3)	Odds ratio	1.002	1.002
Total	3,357 (100)	3,764 (100)	95% CI	0.936–1.073	0.874–1.149
rs989437					
AA	2,122 (65.8)	2,396 (64.0)	χ^2	1.96	0.07
GA	971 (30.1)	1,188 (31.7)	<i>P</i> value	0.16	0.79
GG	133 (4.1)	159 (4.2)	Odds ratio	1.062	1.032
Total	3,226 (100)	3,743 (100)	95% CI	0.976–1.155	0.976–1.155
rs2240999					
GG	2,544 (75.4)	2,947 (78.0)	χ^2	8.06	4.23
GC	767 (22.7)	786 (20.8)	<i>P</i> value	0.0045	0.04
CC	61 (1.8)	46 (1.2)	Odds ratio	1.155	1.495
Total	3,372 (100)	3,779 (100)	95% CI	1.046–1.276	1.017–2.199
rs2242421					
GG	1,400 (41.6)	1,485 (41.6)	χ^2	0.57	0.42
GA	1,502 (44.6)	1,748 (46.9)	<i>P</i> value	0.45	0.52
AA	462 (13.7)	492 (13.2)	Odds ratio	1.027	1.046
Total	3,364 (100)	3,725 (100)	95% CI	0.959–1.100	0.913–1.199
rs2242442					
GG	1,072 (32.0)	1,143 (30.6)	χ^2	0.26	0.4
GA	1,605 (47.9)	1,868 (50.0)	<i>P</i> value	0.61	0.53
AA	672 (20.1)	728 (19.5)	Odds ratio	1.017	1.038
Total	3,349 (100)	3,739 (100)	95% CI	0.952–1.087	0.924–1.167
rs2292428					
TT	1,631 (48.4)	1,844 (49.1)	χ^2	0.51	0.39
TC	1,425 (42.3)	1,580 (42.1)	<i>P</i> value	0.48	0.53
CC	313 (9.3)	333 (8.9)	Odds ratio	1.026	1.053
Total	3,369 (100)	3,767 (100)	95% CI	0.955–1.103	0.896–1.238
rs2290140					
CC	1,810 (54.9)	2,099 (56.7)	χ^2	2.96	1.23
CT	1,265 (38.3)	1,373 (37.1)	<i>P</i> value	0.085	0.27
TT	224 (6.8)	227 (6.1)	Odds ratio	1.069	1.114
Total	3,299 (100)	3,699 (100)	95% CI	0.991–1.154	0.921–1.348
rs3748037					
CC	1,604 (47.5)	1,796 (47.8)	χ^2	0.28	0.65
CT	1,435 (42.5)	1,610 (42.8)	<i>P</i> value	0.6	0.42
TT	336 (10.0)	353 (9.4)	Odds ratio	1.019	1.067
Total	3,375 (100)	3,759 (100)	95% CI	0.949–1.094	0.912–1.248
rs1043228					
CC	3,205 (95.2)	3,623 (96.0)	χ^2	2.92	0.02
CT	159 (4.7)	147 (3.9)	<i>P</i> value	0.087	0.89
TT	3 (0.1)	3 (0.1)	Odds ratio	1.214	1.121
Total	3,367 (100)	3,773 (100)	95% CI	0.972–1.516	0.226–5.556
rs220079					
TT	1,046 (30.9)	1,240 (32.9)	χ^2	2.04	0.21
TC	1,703 (50.4)	1,841 (48.8)	<i>P</i> value	0.15	0.65
CC	632 (18.7)	689 (18.3)	Odds ratio	1.049	1.028
Total	3,381 (100)	3,770 (100)	95% CI	0.982–1.121	0.912–1.159
rs2808707					
GG	921 (27.3)	1,058 (28.3)	χ^2	1.99	2.04

Table 2 Continued

Genotype	MI (%)	CO (%)	Statistical test	Allele frequency	Dominant model
GT	1,660 (49.3)	1,861 (49.7)	<i>P</i> value	0.16	0.15
TT	789 (23.4)	823 (22.0)	Odds ratio	1.049	1.084
Total	3,370 (100)	3,742 (100)	95% CI	0.982–1.120	0.970–1.212
rs3793136					
TT	3,113 (92.5)	3,510 (92.7)	χ^2	0.18	0.93
TC	249 (7.4)	275 (7.3)	<i>P</i> value	0.67	0.34
CC	4 (0.1)	2 (0.1)	Odds ratio	1.038	2.252
Total	3,366 (100)	3,790 (100)	95% CI	0.873–1.233	0.412–12.301

^a Reported by Ishii et al. (21)

Table 3 Power of screening strategy

	First	Second	Third	Total
Additive model	0.25	0.45	0.90	0.10
Dominant model	0.39	0.76	0.99	0.29
Recessive model	0.41	0.57	0.97	0.23

0.1 µg of pRL-TK vector, an internal control for transfection efficiency, using FuGene transfection reagent (Roche). After 24 h, we collected cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega; Ozaki et al. 2002).

Electrophoretic mobility-shift assay

We prepared nuclear extracts from Jurkat cells, HCASMCs and HCAECs (Cambrex) as previously described, and then incubated them with three tandem copies of 16 oligonucleotides (8 of exon 2 to 2 of intron 3 of *ITIH3*) labeled with digoxigenin–11-ddUTP using the digoxigenin gel-shift kit (Roche). For competition studies, we pre-incubated nuclear extract with unlabeled oligonucleotide (100-fold excess) before adding digoxigenin-labeled oligonucleotide. We separated the protein–DNA complexes on a non-denaturing 7% 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 (Ozaki et al. 2002).

Immunohistochemistry

Tissue samples were obtained from patients with MI by elective directional coronary atherectomy after obtaining informed consent. Immunohistochemical protocols were carried out as described previously using goat polyclonal anti-human *ITIH3* IgG (Santa Cruz) (Minami et al. 2001; Ozaki et al. 2004).

Statistical analysis

We carried out statistical analyses for the association study, haplotype frequencies and Hardy–Weinberg equilibrium and calculation of r^2 as described elsewhere (Yamada et al. 2001). We calculated the total number of independent SNPs in this study to be 41,362 (the SNPs in LD, $r^2 > 0.80$, were considered as one SNP) and used this to correct the obtained *P* value by the Bonferroni's correction (P_c). The result of luciferase assay were tested using Fisher's exact test (Statistica software, StatSoft), and mRNA stability data were tested using Student *t* test.

Results

Association study

We first genotyped randomly-selected 188 MI patients and 752 general Japanese population, using a high-throughput multiplex PCR-Invader assay method (Ohnishi et al. 2001) using 52,608 gene-based tag SNPs selected from the JSNP database (Haga et al. 2002; Tsunoda et al. 2004). We successfully obtained the genotype information at 49,167 SNP loci and the distribution of *P* values for these SNPs is summarized in Table 1. We further genotyped SNPs that showed *P* value of 0.01 or smaller with a larger panel of individuals (664 MI and 1,473 controls) as the second-stage screening. Through this screening, we found 14 loci revealing *P* values of 0.001 or smaller, and further verified their associations with MI by genotyping 3,353 affected and 3,807 control individuals. Of two SNPs that we considered to have the statistically-significant association (Table 2), one SNP was located within *MIAT* (Ishii et al. 2006) and the other was present in exon 2 (rs2286797) of the *ITIH3* gene on chromosome 3p21.2–p21.1 ($P = 6.1 \times 10^{-7}$; comparison of allele

Table 4 Identified polymorphisms in *ITIH1* and *ITIH3*

Polymorphism position ^a	rs number	MAF ^b
<i>ITIH1</i>		
Intron 2 188G > A	–	0.021
Intron 5 646A > T	rs2302417	0.490
Intron 6 1327G > A	–	0.021
Intron 6 1507T > C	rs2710323	0.500
Intron 8 304–305 GTGCCGGGAGTAA/ins	rs3836493	0.486
Exon 10 1197C > G, I399M	rs12638839	0.021
Intron 10 238C > T	rs2710322	0.259
Intron 10 320G > A	rs3774354	0.472
Intron 10 423G > A	rs3774355	0.471
Intron 10 536A > C	rs3774356	0.261
Intron 11 87G > A	rs2239551	0.492
Intron 12 82T > A	rs2268023	0.492
Exon 14 1754A > T, E585V	rs678	0.489
Exon 14 1784A > G, E595R	rs1042779	0.492
Intron 14 36A > G	rs2286799	0.041
Intron 14 62A > C	rs2286798	0.487
Intron 15 148C > G	–	0.023
Intron 15 299G > C	–	0.022
Intron 18 428T > C	rs2284351	0.492
Intron 18 1069T > G	rs4687550	0.483
Intron 18 1087T > C	rs4687551	0.483
Exon 19 2196G > A, R732R	–	0.041
Exon 19 2311C > T, R771W	–	0.041
Intron 19 224A > C	rs2270197	0.492
Intron 20 347–348 G/ins	rs3216516	0.483
Intron 20 525T > C	rs1076425	0.492
Intron 20 591T > C	rs1075653	0.500
Exon 21 2547T > C, S849S	rs9324	0.497
Intron 21 121T > C	–	0.005
<i>ITIH3</i>		
Promoter –1254G > A	rs4687654	0.489
Promoter –905C > T	rs2239699	0.487
Promoter –217C > T	–	0.063
Promoter –192C > T	rs9881468	0.457
Exon 2 108G > A, P36P	rs2286797	0.249
Exon 4 291C > T, D291D	rs2240921	0.001
Intron 4 150C > T	rs2240920	0.481
Intron 4 418C > G	rs2240919	0.482
Intron 7 112G > A	rs2535629	0.474
Exon 9 943C > A, K943Q	rs3617	0.495
Intron 11 24C > T	–	0.027
Intron 11 192C > T	rs736408	0.456
Intron 13 851T > C	rs4481150	0.478
Intron 13 1033T > C	rs2710331	0.459
Intron 14 377T > C	rs4687552	0.458
Intron 19 39G > C	rs2276813	0.006
Intron 19 99G > A	rs13059141	0.024
Intron 21 327C > T	rs3815421	0.004

– The variant was not included in the dbSNP database (build 126)

^a Nucleotide numbering is according to the mutation nomenclature (den Dunnen and Antonarakis 2000)

^b Minor allele frequency

frequency, Table 2). Even after Bonferroni's correction, this result was considered to be statistically significant ($P_c < 0.05$). Through the calculation by FDR (Storey 2002) and the association was unlikely to be false positive (q value = 8.86×10^{-4}). Detection power

of this strategy for association under the condition of odds ratio of 1.5 and MAF of 0.40 is shown in Table 3.

Since another SNP(s) in this locus might confer a genetic risk of MI, we searched the HapMap database (<http://www.hapmap.org>; The international HapMap Consortium 2003) and found that *ITIH3* as well as *ITIH1*, both of which belong to the ITIH family, were contained in the same haplotype block. Hence, we subsequently screened SNPs in the genomic sequences covering *ITIH1* and *ITIH3*, except for those corresponding to repetitive sequences, by re-sequencing of genomic DNAs from 24 Japanese individuals. We identified a total of 31 SNPs and two insertion/deletion polymorphisms in *ITIH1* and 19 SNPs in *ITIH3* (Table 4). A comparison of these SNPs in the dbSNP database (The National Center for Biotechnology Information, USA) identified 9 of them (intron 2 188G > A, intron 5 646A > T, intron 15 148C > G, 299G > C, exon 19 2196G > A; R732R, 2311C > T; R771W, intron 21 327C > T in *ITIH1*; 5' flanking region –205G > A, intron 11 24C > T in *ITIH3*) to be novel as at the end of May 2006. Among the 50 SNPs we identified, we genotyped 38 SNPs with the minor allele frequencies of 10% or greater for 94 MI patients and 94 controls to construct a detailed haplotype map in this region (Fig. 1a). Using Haploview software (<http://www.hapmap.org>), we identified one haplotype block with four common haplotypes that covered more than 90% of the Japanese population. We selected three SNPs, rs2710322, rs2239699, and rs2286797 as tag SNPs that represented this locus (arrows, Fig. 1b) and further examined the association of each of the two additional tag SNPs (rs2710322 and rs2239699) with MI. Comparison of genotypes of 3,464 individuals with MI and 3,819 controls at these SNP loci revealed no significant association of either of the two additional SNP with MI (Table 5).

Subsequent analysis of each of the four common haplotypes against all others indicated the significant association of haplotype 4 with MI. However, since this haplotype contained the A allele for rs2286797 and the remaining haplotypes contained the G allele for it (Table 6), we considered that the haplotype association simply reflected the association of the single SNP (rs2286797).

We also investigated one SNP in *ITIH1* (intron 10 536A > C; rs3774356) that revealed strong LD with rs2286797 SNP ($r^2 = 0.93$, Fig. 1a) in the initial LD mapping. However, genotyping of the maximum number of samples at this SNP locus revealed an association with MI at a much weaker level ($\chi^2 = 19.19$, $P = 1.2 \times 10^{-5}$, comparison of allele frequency, Table 7), indicating that *ITIH3* is a gene susceptible to MI.

Fig. 1 Polymorphism map and haplotype structure of *ITIH1* and *ITIH3* locus. **a** Map of SNPs (MAF > 10%) in the *ITIH1* and *ITIH3* region, and pairwise LD coefficients (r^2) among them. The number of base-pair is based on genomic sequence (NC_000003.10) deposited in GenBank database. **b** Haplotype and tag SNPs in this region

Table 5 Association analysis of tag SNPs in the haplotype block

Genotype	MI (%)	CO (%)	Statistical test	Dominant model
rs2710322				
GG	1,889 (55.7)	2,107 (57.1)		
GA	1,286 (37.9)	1,349 (36.5)	χ^2	1.33
AA	216 (6.4)	236 (6.4)	<i>P</i> value	0.25
Total	3,391 (100)	3,692 (100)		
rs2239699				
CC	947 (27.9)	971 (25.7)		
CT	1,697 (50.0)	1,870 (49.5)	χ^2	7.23
TT	752 (22.1)	939 (24.8)	<i>P</i> value	0.0072
Total	3,312 (100)	3,749 (100)		

Functional analyses of the exon 2 synonymous SNP in *ITIH3*

Since the most statistically-significant SNP did not substitute an amino acid of *ITIH3* protein, we

hypothesized that this synonymous SNP affected the transcriptional regulation, because several papers reported that some transcriptional factors bound to the exonic coding sequences of some genes and regulated their transcriptional level (Mori et al. 2002; Zhang

Table 6 Haplotype association analysis using tag SNPs in the block

Haplotype ID	Case	Control	rs2710322	rs2239699	rs2286797	χ^2	P value
Haplotype 1	0.275	0.259	G	C	G	4.84	0.028
Haplotype 2	0.252	0.244	G	T	G	1.33	0.248
Haplotype 3	0.25	0.24	A	C	G	1.95	0.163
Haplotype 4	0.221	0.256	G	T	A	24.8	6.36×10^{-7}

Table 7 Association analysis of the SNP in *ITIH1*

Genotype	MI (%)	CO (%)	Statistical test	Allele frequency	Dominant model
rs3774356					
AA	2,035 (59.9)	2,093 (55.7)	χ^2	19.19	12.99
AC	1,194 (35.2)	1,402 (37.3)	P value	1.2×10^{-5}	3.1×10^{-4}
CC	166 (4.9)	261 (6.9)	Odds ratio	1.188	1.189
Total	3,395 (100)	3,756 (100)	95% CI	1.100–1.283	1.082–1.306

et al. 2004; Ikeda et al. 2005). We constructed six different reporter plasmids (Fig. 2), each of which corresponded to a genomic fragment including the SNP, and performed a luciferase assay using Jurkat and HepG2 cells. The clones containing the DNA fragment corresponding to the G allele showed greater transcriptional activity in both HepG2 and Jurkat cells than those containing the A allele (Fig. 2a, b), indicating this SNP affects transcriptional level of *ITIH3*.

Using nuclear extracts from human coronary artery smooth muscle cells (HCASMCs), human coronary artery endothelial cells (HCAECs), and Jurkat cells originated from human T lymphocyte, all of which were considered to be important players in the pathogenesis of MI, we subsequently examined the presence of some nuclear factor(s) that might bind to oligonucleotides corresponding to genomic sequences of the 108G or 108A alleles, although no known protein was predicted to bind to this DNA segment. When we used nuclear extracts from Jurkat cells (Fig. 2c), we observed two bands of different molecular weights in the lane corresponding to the G allele or the A allele, suggesting that two different nuclear proteins in Jurkat cells bound to each of the allele. However, this kind of difference was not observed when we used HCASMCs or HCAECs (data not shown).

Expression of ITIH3 protein in atherosclerotic lesion

To investigate whether ITIH3 protein was expressed in atherosclerotic plaque, we carried out immunohistochemical staining of human coronary atherectomy specimens with anti-ITIH3 antibody. As shown in Fig. 3, ITIH3 was detected in the macrophages, foam cells, and SMCs. Almost all the macrophages and foam cells were

stained, some of which were aggregated around cholesterol crystals in intima. The SMCs in a deep layer of the vessel wall were stained more intensively.

Discussion

Through a large-scale, case-control association study and subsequent LD mapping, we identified *ITIH3* as a strong candidate gene conferring one of the risk factors for MI. We have demonstrated that the SNP showing the strong association with MI is likely to influence the transcriptional activity of this gene. In addition, by means of electrophoretic mobility-shift assay, we found the difference in the binding affinity to the unidentified transcription factor(s) in lymphocyte-derived cell line (Jurkat cell) in an allele-specific manner, although this difference was not observed in HCASMCs and HCAECs. Through the allele-specific difference in the ITIH3 protein level by some stress responses, T lymphocytes could contribute to the process in atherosclerotic changes through its inflammatory functions and increase the risk of MI (Libby 2002).

In addition to the in vitro experimental data, we have shown that ITIH3 protein was abundantly accumulated in SMCs and macrophages in the intima of human atherosclerotic plaques while they were absent in quiescent or normal medial SMCs, implying that ITIH3 might play a critical role in the pathogenesis of atherosclerosis and subsequent myocardial infarction.

Although functional correlation of ITIH3 with the development, progression or rupture of atherosclerotic plaques remains to be clarified, several papers reported that the members and constituents of the IxI family were detected in synovial fluid of patients with rheumatoid arthritis (Yingsung et al. 2003), in colo-

Fig. 2 Functional analyses of the SNP in exon 2 of *ITIH3* (rs2286797). Transcriptional effect by the SNP using Jurkat cells (**a**) and HepG2 cells (**b**). * $P < 0.01$ and ** $P < 0.05$, by student's t test. The experiment was done in duplicate and repeated three

times independently. **c** Binding of unknown nuclear factors to exon 2 of *ITIH3* in Jurkat cells. The experiment was repeated three times with similar results

rectal mucosal SMCs of patients with inflammatory bowel disease (de la Motte et al. 2003), and also in plasma of patients with sepsis (Balduyck et al. 2000), all of which suggested that they are likely to be involved in both local and systemic inflammatory response (Fries and Kaczmarczyk 2003). Furthermore, in vitro experiments revealed ITIH3 protein incorporated into the cumulus matrix through covalent linkages to HA (Zhao et al. 1995; de la Motte et al. 1999), an

attachment ligand for macrophages (Ross 1999) and lymphocytes (Jean et al. 2001). Since several recent reports also suggested that genes related to inflammation conferred risk of MI (Ozaki et al. 2002, 2004, 2006; Helgadóttir et al. 2004), it is conceivable that this molecule plays an important role in the pathogenesis of MI through inflammatory response.

Although the detailed biological role of ITIH3 protein in the pathogenesis of atherosclerosis is still to

Fig. 3 Expression of ITIH3 protein in coronary atherectomy specimen. **a** Most vascular SMCs in intimal layer and macrophages in deep layer are stained. Magnification $\times 37$. **b** Activated SMCs are migrated and proliferated in shallow layer in the vessel. Magnification $\times 70$. **c** Foam cells aggregate around cholesterol crystals. Magnification $\times 70$. **d** SMCs migrated from medial layer to intimal layer are stained, while those remained in medial layer are not. Magnification $\times 50$

be clarified, we believe our findings would be an anchoring point for functional clarification of this gene and also provide a useful clue for development of novel diagnostic method, treatments and prevention for this common but serious disorder.

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