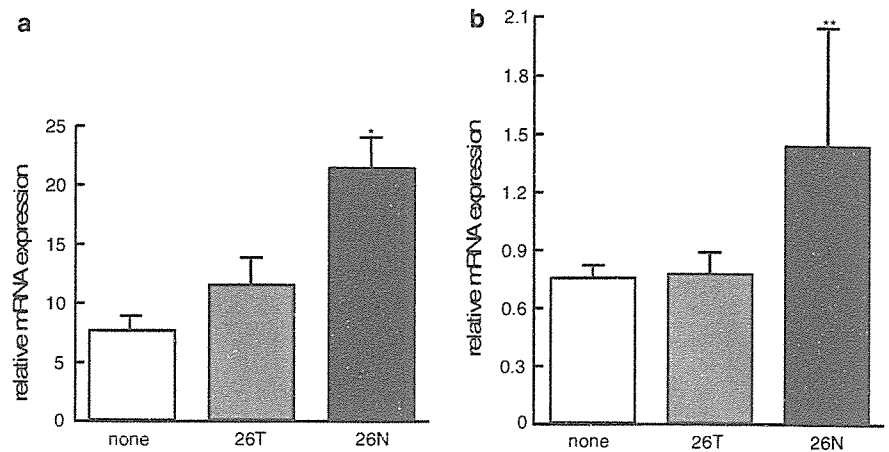**Fig. 2** Differing abilities of 26N- and 26T-lymphotoxin-α (*LTA*) to induce mRNA expression of adhesion molecules (Ozaki et al. 2002). Relative mRNA levels are indicated for **a** VCAM1 and **b** SELE in HCASMC following treatment of each cell with medium only (*white bars*), 26T-LTA (*gray bars*) or 26N-LTA (*black bars*) (20 ng/ml) for 4 h. The figure is taken from Ozaki et al. (2002)

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 the *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 bacterial expression system, and direct binding between galectin-2 and LTA was tested using in vitro binding assay. As shown in Fig. 3a, galectin-2 bound directly to recombinant LTA. We further examined whether the interaction took place in mammalian cells with constructs encoding FLAG-tagged LTA and S-tagged 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).

Association of intron 1 SNP in *LGALS2* with myocardial infarction

Since galectin-2 was shown to bind to LTA, we next examined whether the variation(s) in *LGALS2* (encoding galectin-2) were also associated with susceptibility to myocardial infarction. By re-sequencing of the *LGALS2* genomic region using 32 MI samples, we found 17 SNPs (Table 5). We then compared genotype frequencies of approximately 600 individuals with MI and 600 controls for these SNPs and found one SNP (3279C > T) in intron1 of *LGALS2* showed significant association with MI (Table 5). 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 2,302 for patients with MI and 2,038 for controls. Since minor

allele frequency of the SNP was lower in the group of patients (Table 6), we concluded that the minor variant has a protective role against the risk of MI. To examine
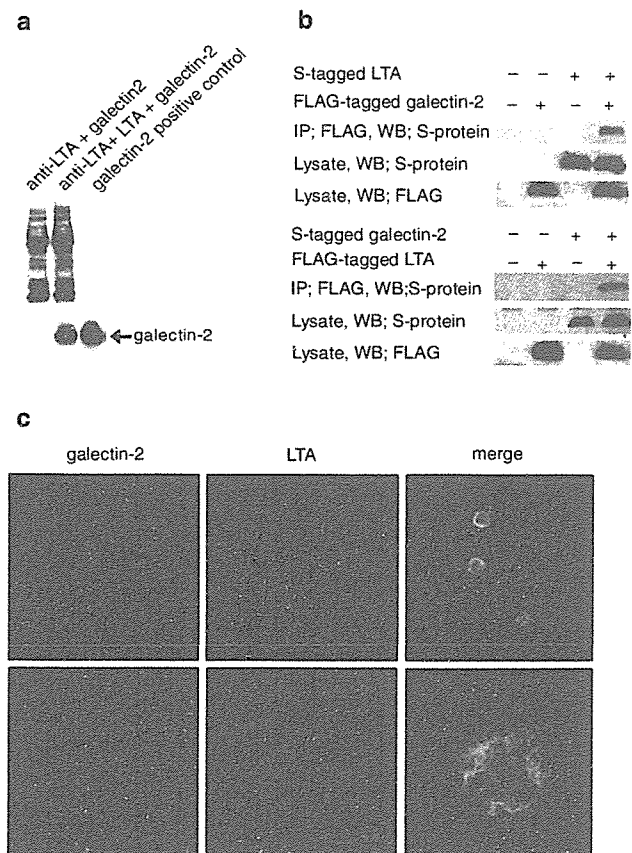

Fig. 3a–c Lymphotoxin-α (*LTA*) binds to galectin-2 (Ozaki et al. 2004). **a** In vitro binding assay. **b** Co-immunoprecipitation of tagged LTA and galectin-2 in COS7 cells. For immunoprecipitation (*IP*) and western blot (*WB*), anti-FLAG tag antibody agarose and S-protein horseradish peroxidase (*S-protein*) were used, respectively. **c** Co-localization of endogenous LTA with galectin-2 in U937 cells. *Bottom panels* are enlarged images of representative cells in the *upper panels*. The figure is taken from Ozaki et al. (2004)

Table 5 Association analyses of 17 single nucleotide polymorphisms (SNPs) in *LGALS2* region with myocardial infarction (MI)

SNP ID	SNP position [a]	dbSNP ID	MI [b]						Control [b]						Allele 1 versus allele 2		
			11	12	22	Sum	MAF	HWE test (P)	11	12	22	Sum	MAF	HWE test (P)	χ^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)

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

[b] 11, 12 and 22 indicate major homozygotes, heterozygotes and minor homozygotes, respectively

A *dash* indicates that the variant was not included in the dbSNP database (build 118)

The table is taken from Ozaki et al. (2004)

Table 6 Association of a single nucleotide polymorphism (SNP) in *LGALS2* with myocardial infarction (MI)

Genotype	MI	Control	Allele C versus allele T	
			χ^2 (*P* value)	Odds ratio (95% CI)
Intron 1 3279C > T[a]				
CC	1,077 (46.8%)	856 (42.0%)	22.1	1.57
CT	1,014 (44.0%)	903 (44.3%)	(0.0000026)	(1.30–1.90)
TT	211 (9.2%)	279 (13.7%)		
Total	2,302 (100%)	2,038 (100%)		

[a]Nucleotide numbering is according to the mutation nomenclature (den Dunnen and Antonarakis 2000)
The table is taken from Ozaki et al. (2004)

whether the intron1 SNP in *LGALS2* would affect its expression level, we constructed reporter plasmids with a genomic fragment containing the SNP in the downstream of a luciferase gene and SV40 enhancer sequence. The clone containing 3279T allele showed two-fold less transcriptional activity than clones containing the 3279C allele and vector only (Fig. 4 a, b), indicating that the substitution repressed the transcriptional level of galectin-2.

Function of galectin-2 protein

Although galectin-2 was thought to be a member of the carbohydrate-binding lectin family based on sequence similarity (Gitt et al. 1992), its function was largely uncharacterized. As several members of the galectin family are known to be secreted (Hughes 1999), we first investigated the possibility of 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 vector. However, we could not detect galectin-2 in culture media after transfection for 24 and 48 h, even by concentrating the FLAG- or Myc-tagged protein using the corresponding anti-tag antibody agaroses (Fig. 4c, d), indicating that galectin-2 predominantly functions as an intracellular protein, although this remains to be clarified using stably transformed cell lines. Since susceptibility to SNP in *LGALS2* was shown to affect expression level of galectin-2, we hypothesized that intracellular amounts of galectin-2 might regulate the secretion level of LTA, thus influencing the degree of inflammation. To clarify this, we examined changes in the secreted level of LTA caused by quantitative alteration of galectin-2, using 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 Fig. 4g, 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 the tandem affinity purification (TAP) system (Rigaut et al.
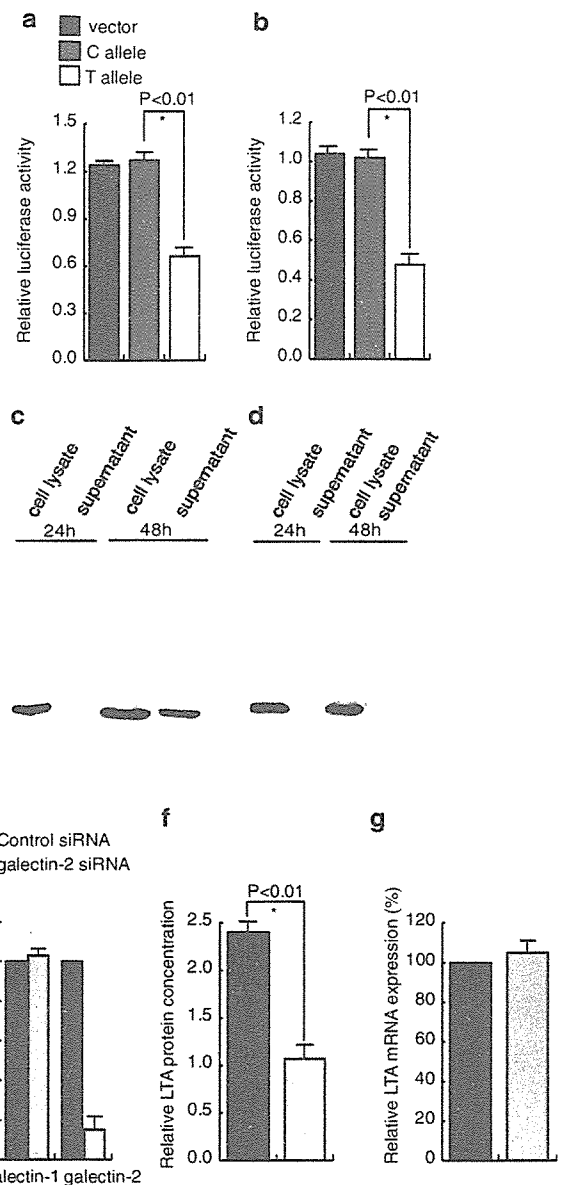

Fig. 4a–g Function of the single nucleotide polymorphism (SNP) in *LGALS2* associated with myocardial infarction (*MI*) (Ozaki et al. 2004). a, b Transcriptional regulatory activity of intron 1 SNP of *LGALS2* in HeLa (a) and HepG2 (b) cells. c, d Unlike galectin-1 (c), galectin-2 (d) was not a secreted protein. e–g Inhibition of galectin-2 expression. Levels of galectin-1 or galectin-2 mRNA (e), supernatant lymphotoxin-α(*LTA*) protein (f), and LTA mRNA (g) after 48 h of transfection with siRNA vectors. The figure was partly cited from Ozaki et al. (2004)

1999). We identified two specific bands that could be seen only when the galectin-2-TAP tag was expressed (Fig. 5a). Based on a MALDI/ToF mass spectrometry analysis, the two bands were identified as α- and β-tubulins, both constituting microtubules. Using FLAG-tagged galectin-2 transfected HeLa cells, we confirmed co-immunoprecipitation of endogenous tubulins (Fig. 5b). Interestingly, we also found that the
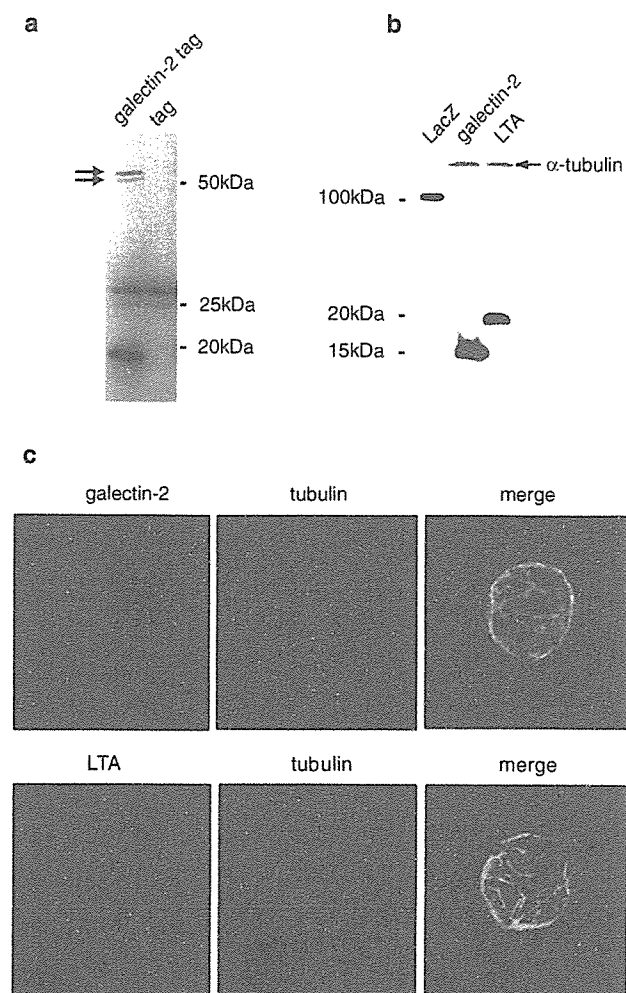
Fig. 5a–c Galectin-2 interacts with microtubules (Ozaki et al. 2004). **a** Isolation of tandem affinity purification (*TAP*)-tagged galectin-2 and interacting proteins. *Arrowheads* indicate α- and β-tubulins. revealed by MALDI/ToF mass spectrometry analysis. **b** Co-immunoprecipitation of endogenous α-tubulin with FLAG-tagged galectin-2 or lymphotoxin-α (*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 lymphotoxin-α (*LTA*) in U937 cells. The figure is taken from Ozaki et al. (2004)

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 α-tubulin co-localized as reticular filamentous networks developed in the cytoplasm (Fig. 5c).

Recently, microtubule cytoskeleton networks have been implicated in the subcellular movements of some proteins including glucose transporter isoform (GLUT4) or thiamine transporter (THTR1) (Liu et al. 2003; Subramanian et al. 2003). 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. although
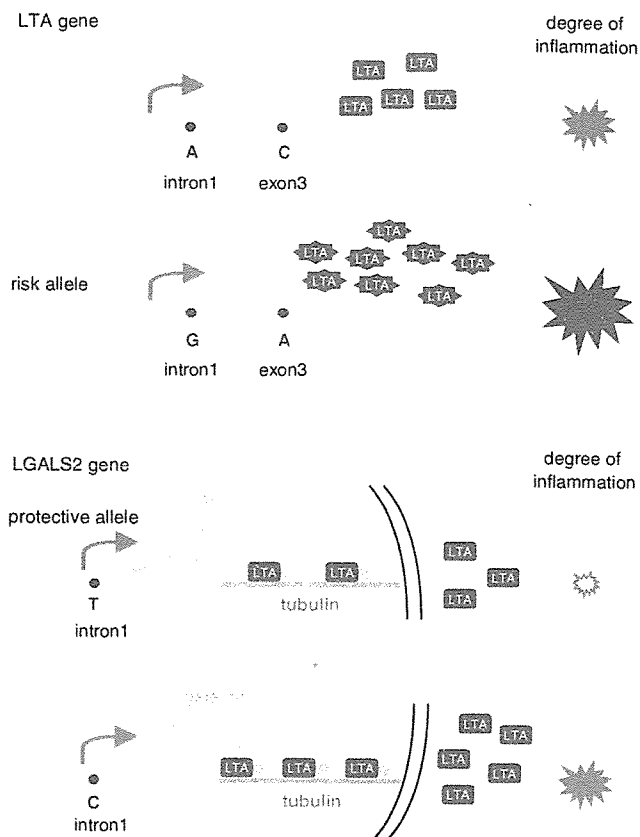

Fig. 6 Hypothetical roles of SNPs in *LTA* (*upper panel*) and *LGALS2* (*lower panel*) in inflammatory process in the pathogenesis of myocardial infarction (MI)

the precise role of galectin-2 in this trafficking machinery complex has yet to be elucidated (Fig. 6).

Other genetic association studies on MI

By searching the OMIM (Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = OMIM) database with the keyword "myocardial infarction," one can find 132 distinct entries related to this disease. Most of the loci were examined using a small-scale patient group, thus indicating the fragility of their association results. However, even with those that examined a large number of patients, there is scarcely a follow-up study to confirm the association with this disorder. It might be conceivable that the next step should be a prospective confirmatory study and/or meta-analysis.

Molecular analyses on MI regarding inflammation

There have been studies that reported the importance of inflammation in the pathogenesis of this disorder. For example. rupture of atherosclerotic plaques occurs pre-

dominantly at the edges of the fibrous cap, an area of accumulated inflammatory cells in close proximity to vascular smooth-muscle cells and endothelial cells (Galis et al. 1994; Jonasson et al. 1986; Kaartinen et al. 1994, 1996; van der Wal et al. 1994). Activated 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 (Galis et al. 1994; Kaartinen et al. 1996). Also, 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 (Schreyer et al. 2002). These implied that LTA, 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.

Conclusions

Through a large-scale case-control association study using SNPs, we found two SNPs in *LTA* confer risk of MI; one in *LGALS2*, encoding a binding protein of LTA protein, was also associated with MI. With these two association results as well as recent molecular studies on MI, we believe inflammation, and especially the LTA cascade, may play a pivotal role in the pathogenesis of MI. It is worth noting that these findings initially came from a hypothesis-free, large-scale SNP association study. This indicates the potent power of this kind of study to identify an anchoring point for pathogenesis, and that for further understanding, combination of a search for a binding partner of the encoded protein and a subsequent genetic analysis might also be powerful. It is also possible that a common final pathway that emerges as inflammation is present in the pathogenesis of MI, and due to these characteristics, identification of genetic risk factors of this clinically heterogeneous disorder was relatively easy. Needless to say, this is a speculation and needs to be clarified.

CAD attributable to atherosclerosis is a leading cause of death for both men and women in many countries (Breslow 1997; Braunwald 1997). We believe that knowledge of genetic factors contributing to the pathogenesis of MI will lead to improved diagnosis, treatment and prevention for the patients.

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ELSEVIER

Impact of atherosclerosis-related gene polymorphisms on mortality and recurrent events after myocardial infarction

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Abstract

Although previous epidemiologic studies have suggested an association between the onset of myocardial infarction (MI) and some genetic variations, the impact of these variants on recurrent cardiovascular events after MI has not been fully elucidated. We genotyped 87 polymorphisms of 73 atherosclerosis-related genes in consecutive acute MI patients registered in the Osaka Acute Coronary Insufficiency Study and compared the incidence of death and major adverse cardiac events (MACE) among the polymorphisms of each gene. After initial screening in 507 patients, we selected nine polymorphisms for screening in all 1586 patients. Multivariate Cox regression analysis revealed that G allele carriers at the position 252 of the lymphotoxin alpha (LTA) gene were independently associated with an increased risk of death (hazard ratio [HR]: 2.46; 95% CI: 1.24–4.86). In conclusion, a 252G allele of LTA is associated with an increased risk of death after AMI and may be a useful genetic predictor.
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Keywords: Myocardial infarction; Polymorphism; Lymphotoxin alpha; Prognosis

1. Introduction

Myocardial infarction (MI) has become one of the leading causes of death around the world. Previous epidemiologic studies have identified certain risk factors related to the onset and prognosis of MI, such as diabetes mellitus, hypertension, hyperlipidemia and smoking. Today, attention is being focused on genetic susceptibility to MI and identification of some genomic markers may provide additional information to the standard risk factor profile as well as some insights into

the underlying pathology. We have reported an association between the functional variant of lymphotoxin alpha [1] or galectin-2 [2] and susceptibility to MI in a case-control study as well as others [3,4]. Although these polymorphisms may be useful genomic markers for distinguishing high-risk subjects from an entire cohort, it does not necessarily mean that these polymorphisms have the same utility to predict recurrent events after MI because there may be different mechanisms for the initial and recurrent cardiovascular events. Accordingly, to determine genetic predictors by performing a prospective observational study on a cohort of MI survivors may provide useful information for the genetic identification of high-risk patients after MI, leading to an improved prognosis.

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In this study, we therefore evaluated the influence of 73 candidate genes, which were selected on the basis of an overview of vascular biology, inflammation, and coagulation and fibrinolysis, as well as lipid and glucose metabolism and other metabolic factors, on recurrent events after MI.

2. Methods

2.1. Patients

Among 3788 consecutive Japanese patients with acute myocardial infarction who were registered in the Osaka Acute Coronary Insufficiency Study (OACIS) from April 1998 to April 2004, 1586 survivors who gave written informed consent for data collection, blood sampling and genotyping were included in this study. Details of the Osaka Acute Coronary Insufficiency Study have been reported [5,6]. Briefly, all patients were prospectively registered immediately after the diagnosis of acute MI, based on their symptoms, electrocardiographic findings and release of cardiac enzymes. The study protocol complied with the Guidelines for Genome/Genetic Research issued by the Japanese government and was approved by the institutional ethical committee.

2.2. Selection of polymorphisms

Using public databases such as PubMed, we selected 87 polymorphisms of 73 candidate genes that have been reported to be potentially associated with atherosclerosis, vascular inflammation, coagulation and fibrinolysis and standard risk factors such as diabetes mellitus, hypertension and hyperlipidemia (Table 1).

2.3. Genotyping of polymorphisms

Venous blood was collected from each patient into tubes containing 50 nmol/L of EDTA and genomic DNA was extracted with a kit (Qiagen Hilden, Germany). The genotypes of the 87 polymorphisms were determined with a fluorescence- or colorimetry-based allele-specific DNA primer probe assay (Toyobo Gene Analysis, Tsuruga, Japan). The regions showing polymorphism were amplified by the polymerase chain reaction (PCR) with primers previously described elsewhere [1,7–15]. The reaction mixture (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 1–4 mmol/L of magnesium chloride and 1 U of DNA polymerase (rTaq or KIO-plus, Toyobo) in the corresponding DNA polymerase buffer. The amplification protocol comprised initial denaturation, 35–45 cycles of denaturation at 95 °C for 30 s, annealing at 55–65 °C for 30 s and extension at 72 °C for 30 s, followed by final extension at 72 °C for 2 min.

To detect the genotype by means of fluorescence, amplified DNA samples were incubated with streptavidin-conjugated magnetic beads in 96-well plates at room temperature. The plates were placed on a magnetic stand and the supernatants were harvested and transferred to the wells of a 96-well plate containing 10 mmol/L of sodium hydroxide. Then, fluorescence was assessed at excitation and emission wavelengths of 485 and 538 nm, respectively, for fluorescein isothiocyanate, or 584 and 612 nm, respectively, for Texas red. To determine the genotype by colorimetry, amplified DNA samples were denatured with 0.3 mmol/L of sodium hydroxide and subjected to hybridization at 37 °C for 30 min in hybridization buffer containing 30–45% formamide with each of two allele-specific capture probes fixed to the bottoms of the wells of a 96-well plate. After thorough washing of the wells, alkaline phosphatase-conjugated streptavidin was added to each well and the plate was incubated at 37 °C for 15 min with agitation. The wells were again washed and after the addition of a solution containing 0.8 mmol/L of 2-(4-iodophemyl)-3-(4-nitrophnyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (monosodium salt) and 0.4 mmol/L of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt, the absorbance of the samples was assessed at a wavelength of 450 nm.

The accuracy of genotyping with this method was confirmed by restriction fragment length polymorphism analysis or by twice performing direct DNA sequencing of the PCR products of 50 randomly selected DNA samples. In each instance, the genotype determined by the allele-specific DNA primer probe assay system was identical to that determined by the other methods.

2.4. Data collection, follow-up and association studies

Research cardiologists and trained research nurses recorded data concerning sociodemographic variables, medical history, therapeutic procedures and clinical events during the patient's hospital stay. Information was obtained from the hospital medical records and by direct interview with the patients, their families and the treating physicians. After written informed consent to enter the OACIS was obtained from each patient, all in-hospital data were transmitted to the data collection center for processing and analysis. For patients who were discharged alive, follow-up clinical data concerning subsequent cardiac events were obtained at 3, 6 and 12 months after the onset of AMI and annually thereafter. The data were obtained from the research outpatient clinic or, in a few instances, by verbal or written contact with the patient's physician, the patient, or family members. The incidence of death, the incidence of death plus non-fatal MI and the incidence of major cardiac events (MACE) were compared among the different polymorphisms. MACE was defined as death from any cause, myocardial infarction, unstable angina and revascularization (including target vessel revascularization, percutaneous coronary intervention for vessels other than the primary target vessel and coronary artery bypass grafting).

To evaluate the association between the various polymorphisms and the outcome after MI, we first performed a screening study using 87 polymorphisms of 73 candidate

Table 1
The 87 polymorphisms examined in the screening study

Gene	Polymorphism	Gene	Polymorphism
Adenosine monophosphate deaminase 1	C34T	Interleukin-13	G4166A (Arg110Gln)
Adiponectin	G276T	Interleukin-18	C−607A
Adiponetcin	C383T (Arg112Cys)	Interleukin-18	G−137C
Alpha estrogen receptor	T397C	Interleukin-4 receptor alpha	A398G (Ile50Val)
Angiotensin II receptor type I	A1166C	Interleukin-6	G−174C
Angiotensinogen	T704C (Met235Thr)	Interleukin-6	C−634G
Apolipoprotein E	T3932C (Cys112Arg)	Leptin	C−1887A
Apolipoprotein E	C4070T (Arg158Cys)	Lymphotoxin alpha	A252G
ATP-binding cassette transporter ABCC6	C3421T (Arg1141 stop)	Lymphotoxin alpha	C804A (Thr26Asn)
Atrial natriuretic peptide	C708T	Matrix Gla Protein	G−7A
Beta2 adrenergic receptor	A46G (Arg16Gly)	Metalloproteinase-12 (macrophage elastase)	A−82G
Beta2 adrenergic receptor	C79G (Gln27Glu)	Metalloproteinase-7 (matrilysin) promoter	A−181G
Beta2 adrenergic receptor	C491T (Thr164Ile)	Metalloproteinase-7 (matrilysin) promoter	C−153T
Beta3 adrenergic receptor	T190C (Trp64Arg)	Metalloproteinase-9 (gelatinase B)	C−1562T
CC chemokine receptor	G190A (Val64Ile)	Methylenetetrahydrofolate reductase	C677T (Ala222Val)
CD18	C1323T	Microsomal triglyceride transfer protein	G−493T
Cholesterol ester transfer protein	G1200A (Arg451Gln)	Monocyte chemoattractant protein 1	G−2518A
Coagulation factor V	G1691A (Arg506Gln)	Myeloperoxydase	G−463A
Coagulation factor XII	C46T	Neuropeptide Y	T1128C (Leu7Pro)
C-reactive protein	G1059C	p22phox	C242T (His72Tyr)
Dopamine D2 receptor	C1097G (Ser311Cys)	Paraoxonase	A220T (Met55Leu)
Early growth response protein-1	C−151T	Paraoxonase	A632G (Gln192Arg)
Endothelial nitric oxide synthase	T−786C	Peroxisome-proliferator—activated receptor-alpha	C256G (Leu162Val)
Endotheline-1	G5665T (Lys198Asn)	Peroxisome-proliferator—activated receptor-gamma	C34G (Pro12Ala)
E-selectin	A561C (Ser128Arg)	Plasminogen-activator inhibitor type 1	4G−668/5G
E-selectin	G98T	Prothrombin	G20210A
Flactalkine receptor	G84635A (Val249Ile)	P-selectin	A76666C (Thr715Pro)
Ghrelin	C247A (Leu72Met)	Receptor for advanced glycation end products	G557A (Gly82Ser)
Glutamate-cysteine ligase modifier subunit	C−588T	Receptor for advanced glycation end products	T−429C
Glycogen synthase	A1426G (Met416Val)	Resistin	ATG repeat
Glycoprotein Ia	A1648G (Lys505Glu)	Scavenger receptor BI	G4A (Gly2Ser)
Glycoprotein Ia	C807T	Scavenger receptor BI	G403A (Val135Ile)
Glycoprotein Ia	G873A	Serotonin receptor 2A	T102C
Glycoprotein IIIa	C1565T (Leu33Pro)	Soluble epoxide hydrolase	G860A
Glycoprotein VI	T13254C (Ser219Pro)	Soluble epoxide hydrolase	Arg402–403ins in exon13
Heme oxigenase-1	GT repeats in promoter	Thrombomodulin	G−33A
Hepatic lipase	C−480T	Thrombopoietin	A5713G
Human platelet antigen-2	C1018T (Thr145Met)	Thrombospondin 1	A2210G (Asn700Ser)
Intercellular adhesion molecule-1	G1462A (Glu469Lys)	Thrombospondin 4	G1186C (Ala387Pro)
Interleukin-1 alpha	C−889T	Toll-Like Receptor 2	C2029T (Arg677Trp)
Interleukin-1 receptor antagonist	Tandem repeat in intron2	Transforming growth factor beta 1	T29C (Leu10Pro)
Interleukin-10	G−1082A	Tumor necrosis factor alpha	G−308A
Interleukin-10	T−819C	Vascular endothelial growth factor	C−634G
		von Willebrand factor	G−1051A

Minus signs indicate the number of nucleosides upstream from the transcription-initiation site. For non-synonymous polymorphisms, the resulting amino acid change is shown in parentheses.

genes in 507 patients who were randomly selected from the total study population. This revealed nine polymorphisms that were related to recurrent events. Then, a large-scale study was performed to assess the association between these nine polymorphisms and recurrent events after MI in all 1586 patients.

2.5. Statistical analysis

Discrete variables were expressed as counts or percentages and were compared with the χ^2-test. Continuous variables were expressed as the mean ± S.D. and compared by the unpaired two-sided t-test. In the screening study, we used the χ^2-test with a cut-off p-value of less than 0.1 to avoid false negative associations. In the large-scale study, polymorphisms selected in the screening study were included as covariables as well as age, sex, body mass index, coronary risk factors (including diabetes mellitus, hypertension, hyperlipidemia, smoking and history of prior MI), anteroseptal MI and reperfusion therapy in Cox's proportional hazards regression model. Survival curves were constructed by Kaplan–Meier method and differences in the event-free survival rate were compared among the different genotypes using the log-rank test. Association were considered signif-

Table 2
Patient characteristics

Age (years)	64.0 ± 11.0
Male sex (%)	1225/1586 (77.2)
BMI (kg/m^2)	23.4 ± 4.0
Diabates mellitus (%)	544/1550 (35.1)
Hypertension (%)	811/1546 (52.5)
Hyperlipidemia (%)	718/1529 (47.0)
Smoking (%)	1048/1581 (66.3)
Past history of MI (%)	176/1533 (11.5)
Preangina (%)	364/1540 (23.6)
Anteroseptal MI (%)	766/1586 (48.3)
Killip class > II (%)	191/1524 (12.5)
Reperfusion therapy within 24 h (%)	1382/1582 (87.4)
PCI (%)	1362/1582 (86.1)
PTCR (%)	159/1582 (10.1)
CABG (%)	34/1555 (2.2)
Peak value of creatinine kinase (IU/1)	2628 ± 2371
Medication at discharge	
Aspirin (%)	1452/1586 (91.6)
Angiotensin-converting enzyme inhibitor (%)	1019/1566 (65.1)
Angiotensin-receptor blocker (%)	150/1560 (9.6)
Beta blocker (%)	615/1563 (39.3)
Calcium blocker (%)	375/1562 (24.0)
Statin (%)	384/1586 (24.2)

icant at a p-value of less than 0.05. All statistical analyses were performed using SPSS software (SPSS, Inc.).

3. Results

The baseline characteristics of the patients are listed in Table 2. Long-term follow-up was completed in 1585 patients (99.9%), while contact was lost with one patient. During the mean follow-up period of 831 days, there were 76 deaths, 158 deaths or MIs and 522 MACE.

The screening study in 507 patients identified nine polymorphisms for further study on the basis of a univariate x^2-test with a p-value of less than 0.1. These were C−480T polymorphism of the hepatic lipase gene, A398G polymorphism of the interleukin-4 receptor alpha gene, G−137C polymorphism of the interleukin-18 receptor antagonist gene, A252G polymorphism of the lymphotoxin alpha gene, 4G/5G poly-
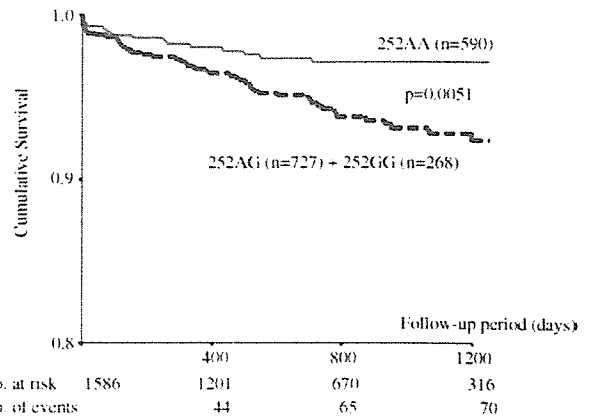

Fig. 1. Cumulative survival rate during follow-up according to the LTA. Solid line indicates LTA-252AA genotype and dashed line indicates LTA-252AG + GG genotype, respectively.

morphism of the platelet activator inhibitor-I gene, G860A polymorphism of the soluble epoxide hydrolase gene, T29C polymorphism of the transforming growth factor beta gene, A5713G polymorphism of the thrombopoietin gene and G−1051A polymorphism of the von Willebrand factor gene (Table 3).

In the entire study in 1586 patients, multivariate Cox regression analysis revealed that A252G polymorphism in the LTA gene was significantly associated with an increased risk of death (Table 4). The hazard ratio for the occurrence of death in LTA G allele carriers (AG or GG genotypes) versus non-carriers (AA genotype) was 2.46 (95% CI: 1.24–4.86). Fig. 1 shows Kaplan–Meier survival curves for patients with each genotype. There was no association between each of nine polymorphisms and MACE.

4. Discussion

In this prospective observational study, we investigated the prognostic significance of 87 polymorphisms of 73 genes that have been suggested to be related to the pathophysiology of MI, and found that an LTA polymorphism were associated

Table 3
The nine polymorphisms selected by the screening study

Gene	Polymorphism	Genetic model	Event	Event rate	p-Value
Hepatic lipase	C−480T	CC + CT vs. TT	MACE	158/382 vs. 30/123	0.0002
Interleukin-4 receptor alpha	A398G (Ile50Val)	AA + AG vs. GG	Death + MI	31/314 vs. 29/192	0.0773
Interleukin-18	G−137C	CC + CG vs. GG	Death	10/116 vs. 18/389	0.0990
Lymphotoxin alpha	A252G	AA vs. AG + GG	Death	6/183 vs. 22/323	0.0949
Plasminogen activator inhibitor-1	4G/5G	4G/4G + 4G/5G vs. 5G/5G	Death	21/436 vs. 7/70	0.0900
Soluble epoxide hydrolase	G860A	AA + AG vs. GG	MACE	63/194 vs. 126/312	0.0737
Transforming growth factor-beta 1	T29C (Leu10Pro)	CC vs. CT + TT	Death	4/146 vs. 24/360	0.0800
Thrombopoietin	A5713G	AA vs. AG + GG	Death	12/107 vs. 16/399	0.0038
Von Willebrand factor	G−1051A	AA vs. AG + GG	Death	10/101 vs. 18/405	0.0319

Minus signs indicate the number of nucleosides upstream from the transcription-initiation site. For non-synonymous polymorphisms, the resulting amino acid change is shown in parentheses. MACE is determined as a combination of death, MI, unstable angina and revascularization.

Table 4
Multivariate Cox regression analysis of association between nine polymorphisms and mortality after myocardial infarction in the entire study

Gene	Polymorphism	Model	Unadjusted HR[a] 95% CI	p-Value	Adjusted[b] HR 95% CI	p-Value
Hepatic lipase	C−480T	CC + CT vs. TT	1.06 (0.61–1.85)	0.829	1.36 (0.72–2.58)	0.342
Interleukin-4 receptor alpha	A398G (Ile50Val)	AA + AG vs. GG	0.76 (0.46–1.28)	0.306	0.69 (0.37–1.28)	0.237
Interleukin-18	G−137C	CC + CG vs. GG	0.88 (0.51–1.53)	0.654	0.75 (0.39–1.44)	0.385
Lymphotoxin alpha	A252G	AA vs. AG + GG	2.33 (1.29–4.20)	0.005	2.46 (1.24–4.86)	0.010
Plasminogen activator inhibitor-1	4G/5G	4G/4G + 4G/5G vs. 5G/5G	0.85 (0.45–1.63)	0.628	1.37 (0.67–2.81)	0.387
Soluble epoxide hydrolase	G860A	AA + AG vs. GG	1.13 (0.68–1.88)	0.636	1.22 (0.67–2.24)	0.516
Transforming growth factor-beta 1	T29C (Leu10Pro)	CC vs. CT + TT	1.13 (0.66–1.94)	0.662	0.77 (0.42–1.42)	0.405
Thrombopoietin	A5713G	AA vs. AG + GG	0.99 (0.55–1.79)	0.981	0.86 (0.44–1.68)	0.664
Von Willebrand factor	G−1051A	AA vs. AG + GG	0.73 (0.42–1.28)	0.266	0.78 (0.40–1.53)	0.475

[a] Hazard ratio of latter genotypes (e.g. TT in hepatic lipase) compared with former ones (e.g. CC + CT in hepatic lipase) are indicated.

[b] Age, sex, body mass index, diabetes mellitus, hypertension, hyperlipidemia, current smoker, past history of MI, anteroseptal MI, Killip class ≥ II and reperfusion therapy are included as covariables.

with an increased risk of death. Although several genes have already been shown to be associated with the onset of MI, few polymorphisms have been reported to show an association with the prognosis of MI. Accordingly, our data might provide some insights for research concerning the genetic risk of recurrent events after MI.

Lymphotoxin alpha (LTA, formerly named TNF-β) is a member of the TNF family that plays a critical role in inflammation and is located within the human leukocyte antigen class III gene cluster on human chromosome 6p21. Many studies have focused on TNF-α as a key cytokine involved in heart failure and atherosclerosis [16,17]. LTA is structurally similar to TNF-α and also has an important role in the inflammatory response by inducing monocyte migration, as well as by promotion of lymphocyte activation and proliferation [18,19]. Although this cytokine has been widely investigated to clarify its association with systemic inflammation, the role of LTA in the pathology of coronary heart disease has not been fully elucidated. Schreyer et al. reported a reduction of atherosclerosis lesion in LTA knockout mice, but not in TNF-α knockout mice, suggesting that LTA may be more important in the proatherogenic response [20]. In the LTA gene, there are some genetic variants with functional significance. An A–G substitution at nucleotide position 252 (A252G) is reported to be associated with increased transcriptional activities [1] or elevated C-reactive protein [21]. Moreover, the 252G allele links to an amino-acid coding polymorphism (804A) which increases mRNA expression of VCAM1 and selectin E [1]. Furthermore, Ozaki et al. [1] and others [22,23] found some variants of the LTA gene were associated with the onset of myocardial infarction or coronary heart disease. Our finding that the LTA polymorphism is associated with recurrent cardiovascular events confirms the important role of this cytokine, as is shown in these previous reports and suggests the need for adjunctive treatment for patients with the polymorphism. The mechanism of the LTA polymorphisms for increased cardiovascular events is still unclear. Several environmental predictors of the prognosis of MI, such as high age, diabetes mellitus, hypertension,

time to revascularization, severity of MI (i.e. Killip class or left ventricular function) and therapeutic interventions, have been established on the basis of clinical data. In the present study, Cox multivariate analysis revealed that the 252G allele of LTA was a predictor of death independent from these known predictors. Detailed in vivo and in vitro studies of the mechanisms by which the genetic polymorphism influences cardiovascular events will be needed to solve this issue and clarify the position of this polymorphism within the constellation of the established predictors.

There were some limitations to our study. One limitation was the possibility of survivor bias. Patients dying before or soon after admission were excluded from our study, so it is possible that they had a particular polymorphism and their exclusion biased our results. However, the frequencies of the LTA polymorphism in the study population were in Hardy–Weinberg equilibrium, so it is unlikely that acute mortality is dominated by specific polymorphisms of LTA. Another limitation is ethnic differences. However, the frequency of the 252G allele of LTA in this study population was similar to that in Iwanaga et al. [22] and Keso et al. [24] studies, so the influence of ethnic differences should be minimal.

In conclusion, A252G polymorphism of the LTA gene was significantly associated with an increased risk of death after MI and may be one of the most important genetic determinants that have been detected so far. To confirm our findings, further large-scale epidemiologic studies of various ethnic groups are needed, as well as experimental studies to clarify the precise mechanisms involved. Determination of the genes related to an increased risk of recurrent events after MI could be of clinical significance to identify patients with a high genetic risk, and thus provide better treatment after MI.

Acknowledgements

Appendix A

The following persons and institutions participated in the OACIS. Sakurabashi Watanabe Hospital, Osaka: Fujii K; Osaka Police Hospital, Osaka: Kodama K; Kansai Rosai Hospital, Amagasaki: Nagata S, Nanto S, Morozumi T; Osaka General Medical Center, Osaka: Fukunami M; Ishinkai Yao General Hospital, Yao: Matsu-ura Y; Osaka National Hospital, Osaka: Kusuoka H, Yasumura Y, Koretsune Y; Higashi-Osaka City General Hospital, Higashi-Osaka: Kijima Y; Osaka Rosai Hospital, Sakai: Yamada Y, Tanouchi J, Nishino M; Kawachi General Hospital, Higashi-Osaka: Mishima M, Lim YJ; Osaka Minami National Hospital, Kawachinagano: Kinoshita N, Imai K; Osaka Kosei Nenkin Hospital, Osaka: Sasaki T; Osaka Railway Hospital of West Japan Railway Company, Osaka: Esumi A; Yao Municipal Hospital, Yao: Hoshida S, Umemoto K; Kaizuka City Hospital, Kaizuka: Morita H, Lee JM; Kita-Osaka Hospital, Osaka: Ogitani N, Ikeda S; Setsu Iseikai Hospital, Setsu: Akehi N; Kobe Ekisaikai Hospital, Kobe: Shimazu T, Fuji H; Kashiwara City Hospital, Kashiwara: Naka M, Akashi T; Teramoto Memorial Hospital, Kawachinagano: Hishida E; Shinsenri Hospital, Suita: Hayashi T, Nakatsuchi Y; Meiwa Hospital, Nishinomiya: Sugii M; Osaka Seamens Insurance Hospital, Osaka: Kohama A; Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita: Yamamoto K, Hasegawa S, Minamino T; Department of Medical Information Science, Osaka University Graduate School of Medicine, Suita: Takeda H, Matsumura Y.

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4. 国際ハップマップ計画

田中敏博

国際ハップマップ計画は，効率的な疾患遺伝子解析を行うための基盤づくりを目標に6カ国が共同で行ったプロジェクトである．約3年間でヒトゲノム上に存在するSNPを580万カ所以上調べ，ゲノムのそれぞれの領域における詳細なハプロタイプ構造を明らかにした．

はじめに

国際ハップマップ計画は，日本，米国，英国，カナダ，中国，ナイジェリアの6カ国の国際協力により，2002年10月末に開始された．このプロジェクトの目標はヒトのDNA多型のパターンを把握し，全ゲノムにわたってハプロタイプ地図を作成することである．ハップマップ（HapMap）のハップ（Hap）はハプロタイプ（Haplotype）に由来する．この地図は，高血圧や糖尿病といったcommon disease関連遺伝子や薬剤感受性遺伝子の単離を非常に効率よく進めるための基盤となる．本稿では，ハプロタイプについて説明した後，この国際プロジェクトについて概説する[1]．

1 ハプロタイプ

ハプロタイプとは，染色体上のある領域におけるヒトの多型の組み合わせである．例を挙げて説明する．図1に示すように，4カ所のbiallelic SNP〔対立遺伝子[※1]が2種類の一塩基多型（single nucleotide poly-

morphism）〕が存在する場合，同一染色体上に存在するSNPの組み合わせ，すなわちハプロタイプは理論上2×2×2×2＝16種類考えうる．ところが，この4カ所の多型の距離が比較的近いと，16種類のすべてのハプロタイプが見られることはほとんどない．この理由は，減数分裂の際の組換えで説明される（図2）．すなわち，それぞれのSNPの間で組換えが起きてしまうと，母由来の染色体（GCAT）と父由来の染色体（CGGA）とが混ざり合った形で（キメラ），子に受け継がれていく（図2）．そうすると，今まで存在しなかったGCGAというハプロタイプが誕生する．組換えは1回の減数分裂で約30回起きるとされているが，この現象がある領域で起こる確率はおおよそ領域の大きさに比例する（物理的距離と遺伝的距離）．2つのSNPを考えてみると，その物理的距離が大きければ，その間で組換えの起きる確率は高いが，小さければ組換えは起きにくい．そのため，比較的小さな領域内に存在するSNP群についてみていけば，世代を経てもハプロタイプの種類は増えにくい．

[キーワード＆略語]
SNP，ハプロタイプ，ハプロタイプブロック，タグSNP，基盤研究，国際ハップマップ計画
SNP：single nucleotide polymorphism

> **※1　対立遺伝子（アレル）**
> ある遺伝子座を占める遺伝子の種類が複数ある場合，これら1つ1つを対立遺伝子（アレル）と呼ぶ．現在では，遺伝子に限らず，SNPのような1つの塩基配列に複数の種類が存在する場合にも用いられる．

International HapMap Project
Toshihiro Tanaka：SNP Research Center, RIKEN（理化学研究所遺伝子多型研究センター）

```
        SNP1  SNP2  SNP3  SNP4
       -G/C - C/G - A/G - T/A-

①     - G  -  C  -  A  -  T  -
②     - G  -  C  -  A  -  A  -
③     - G  -  C  -  G  -  T  -
④     - G  -  C  -  G  -  A  -
⑤     - G  -  G  -  A  -  T  -
⑥     - G  -  G  -  A  -  A  -
⑦     - G  -  G  -  G  -  T  -
⑧     - C  -  G  -  G  -  A  -
⑨     - C  -  C  -  A  -  T  -
⑩     - C  -  C  -  A  -  A  -
⑪     - C  -  C  -  G  -  T  -
⑫     - C  -  C  -  G  -  A  -
⑬     - C  -  G  -  A  -  T  -
⑭     - C  -  G  -  A  -  A  -
⑮     - C  -  G  -  G  -  T  -
⑯     - C  -  G  -  G  -  A  -
```

図1　ハプロタイプの種類

図2　減数分裂の際の染色体交叉

ディプロイドゲノム（大半の体の細胞）

ハプロイドゲノム（卵や精子）

2 ハプロタイプブロック

ゲノム上には組換えの起こりやすい箇所（recombination hot spot）が多数存在している．ある領域内にこのhot spotが存在すると，その領域を表すハプロタイプの種類が増える可能性は格段に増す．逆の言い方をすると，あるhot spotと隣のhot spotの間の領域に限定して着目すれば，ハプロタイプの種類は少なくとどまる可能性が高い（最近の知見では3〜5種類）．この，ハプロタイプの種類が比較的少ない領域をハプロタイプブロックとよぶ．

3 タグSNP

ハプロタイプブロックを構成するSNP群については，すべてをタイピングしなくても情報は得られる．その理由を以下に説明する．1つのbiallelic SNPではハプロタイプを2つのサブグループに分けることが可能であるから，n種類のハプロタイプが存在する場合，1つ1つのハプロタイプを識別するためにタイピングすべきSNPの数は，うまく選ぶことができれば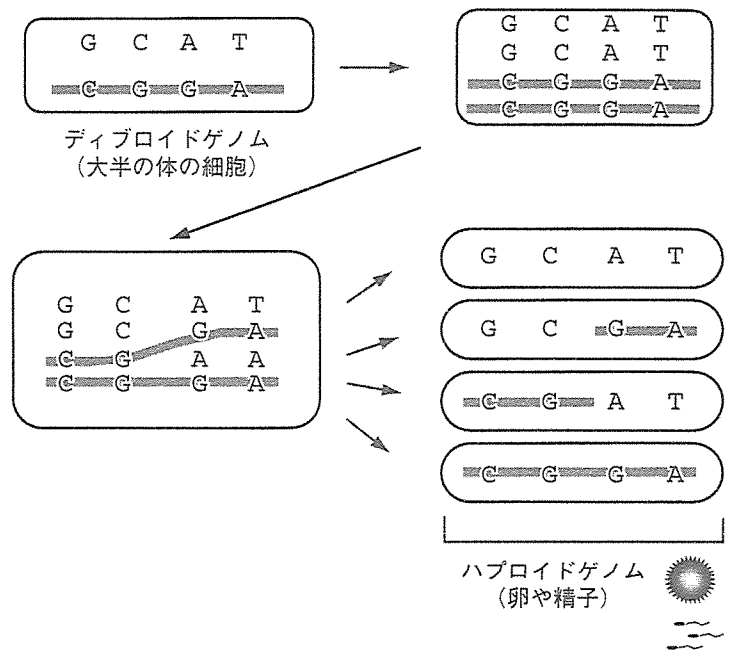理論上 $\log_2 n$ 以上の一番小さな整数となる．ところが，ハプロタイプブロック内ではその定義上，存在するハプロタイプの種類は多くない．よってハプロタイプブロックを構成するSNPの数 $\gg \log_2 n$ となる．これらの，ハプロタイプを代表するように選ばれるSNP（群）をタグSNPという．

4 タグSNPの選別方法

具体的に説明するため，図3のように6つのSNPで構成されるハプロタイプブロックが存在し，このブロックは5種類のハプロタイプで説明できると仮定する．5種類のハプロタイプブロックを識別するための最小限のSNP数は，先ほどの式より，3カ所である．まず，SNP1を1つ目のタグSNPとして選択したとする．SNP1がAであれば，ハプロタイプは②であることが，一意に決まる．Gであった場合は①，③，④，⑤のいずれかとなる．これらの4つを識別するために2つ目のSNPとしてSNP3を選ぶとする．Gであれば，ハプロタイプは①か④となり，Aであればハプロタイプは③か⑤となる．さらに，この2種類のハプロタイプをいずれも識別できるSNPを探してみると，SNP4となる．すなわち，SNP4がGならハプロタイプは①か③，Cなら④か⑤となる．SNP3の情報をあわせて考えると，SNP3 - SNP4がG - Gなら①，G -

```
            SNP1      SNP2      SNP3      SNP4      SNP5      SNP6
       —    G/A   —   C/T   —   G/A   —   G/C   —   C/A   —   A/T   —
  ①   —    G    —    C    —    G    —    G    —    C    —    A    —
  ②   —    A    —    T    —    A    —    C    —    A    —    T    —
  ③   —    G    —    C    —    A    —    G    —    C    —    A    —
  ④   —    G    —    C    —    G    —    C    —    A    —    T    —
  ⑤   —    G    —    C    —    A    —    C    —    C    —    A    —
```

図3　ハプロタイプを識別するためのSNP（タグSNP）

Cなら④，A－Gなら③，A－Cなら⑤となる．留意すべきは，SNP1とSNP2は同じ情報しかもっておらず，ハプロタイプを識別するためには同時に選んではいけないということである．

ゲノムワイドSNP解析においては，タイピングすべきSNPの数をできる限り少なくすることが，効率よい研究につながる．前述のように，ゲノムにおけるハプロタイプブロックの分布があらかじめわかっていれば，あらかじめ選ばれたタグSNPのみをタイピングすればよいことになり，疾患解析研究の効率化を図るための基盤となるわけである．

⑤ 疾患研究におけるハプロタイプの有用性

ハプロタイプは，疾患解析研究において前記以外にも有用性をもっていると考えられている．この研究の最初の目標は，疾患関連遺伝子（もしくは疾患関連SNP）を同定することにあるが，単独のSNPのみで疾患感受性を規定するのではない場合も考えられる．すなわち，複数のSNPが組み合わせとして，つまりハプロタイプとして疾患に関連することも考えられ，その場合，あらかじめその座位のハプロタイプ地図が得られていれば，当然のことながら効率よく研究が進められる．

もうひとつはhidden SNPの問題である．いくら全ゲノム的に地図を構築するといっても，存在するSNP数の多さを考えると100％穴のない地図を作ることは不可能である．高精度の地図ができ，タグSNPがわかったからといって，この地図から漏れているSNPも当然存在し，それが疾患の感受性に関わっている場合もあると考えられる（hidden SNP）．しかしながら，そういったSNPは代表ハプロタイプのいずれかに載っ

ている可能性が高いことが最近の研究から示唆されており，ハプロタイプの相関解析にてhidden SNPの存在する可能性が判断できると考えられている．

⑥ 国際ハップマップ計画

国際協力のもと，3年間で60万カ所のSNPについて，欧米人，アジア人，アフリカ人をそれぞれ90名ずつタイピングするというのが当面の目標であった．ヒトゲノムは3,000 Mbにわたるので，5 kbに1つのSNPをタイピングすれば，概略は明らかになるという考えである．表1に組織を示すが，サンプル収集は日本，米国，中国，ナイジェリアが，タイピングは日本，英国，米国，カナダ，中国がそれぞれ担当した（表2）．タイピングにおいては，染色体別に各国の担当を決めたが，日本の分担は7本の染色体，ゲノム全体の約4分の1と決まった．これは米国の32.4％に次いで2番目に大きく，単独のタイピングセンターとしては世界最大の貢献をしていることを示している．

1）サンプル収集

アフリカからは，両親に加えて1人の子供（トリオと呼ぶ）を30組集め，ゲノムDNAサンプルを提供いただいた．日本人と中国人はアジアの2つの地域からの代表という意味合いをもつため，それぞれ45人のサンプルを解析する．アジアとして合わせて90サンプルを解析することとなった．この，アジアからの2つのグループに関しては，お互いに血縁関係にない人々に参加いただいた．米国からのサンプルは，以前から解析されてきたサンプル（CEPH：Centre d'Etude du Polymorphisme Humain）について，提供者から再同意をいただく形式を取った．また，CEPHサンプルについては，アフリカからのサンプル同様に30組の

表1 国際ハップマップ計画の組織 (http://hapmap.org/groups.htmlより)

参加国	研究グループ			ダイピングしたゲノムの割合／(役割)	染色体
日 本	中村裕輔 理化学研究所，東京大学			24.3%	5, 11, 14, 15, 16, 17, 19
	松田一郎 北海道医療大学，ユウバイオス倫理研究会			(サンプル収集)	
英 国	David Bentley Wellcome Trust Sanger Institute			23.7%	1, 6, 10, 13, 20
	Peter Donnelly University of Oxford			(解析)	
	Lon Cardon University of Oxford Wellcome Trust Centre for Human Genetics			(解析)	
カナダ	Thomas Hudson McGill University and Génome Québec Innovation Centre			10.1%	2, 4p
中国	Huanming Yang The Chinese HapMap Consortium	Huanming Yang Beijing Genomics Institute		5.9%	3q, 8p, 20 Mb of 3p
		Yan Shen Chinese National Human Genome Center at Beijing			
		Lap-Chee Tsui Hong Kong HapMap Group	Paul Kwong-Hang Tam & William Wai-Nam Mak The University of Hong Kong	2.5% (9.5%)	70 Mb of 3p
			Jeffrey Tze-Fei Wong Hong Kong University of Science and Technology		
			Mary Miu Yee Waye The Chinese University of Hong Kong		
		Wei Huang Chinese National Human Genome Center at Shanghai		1.1%	21
	Houcan Zhang Beijing Normal University			(地域社会に対する取り組み)	
	Changqing Zeng Beijing Genomics Institute			(サンプル収集)	
米 国	Arnold Oliphant Illumina			16.1%	8q, 9, 18q, 22, X
	David Altshuler Broad Institute of Harvard and MIT			9.7%	4q, 7q, 18p, Y
	David Altshuler Broad Institute of Harvard and MIT			(解析) 32.4%	
	Richard Gibbs Baylor College of Medicine with ParAllele Bioscience			4.6%	12
	Pui-Yan Kwok University of California, San Francisco with Washington University in St. Louis			2.0%	7p
	Kelly Frazer Perlegen Sciences			ゲノムワイド，225万カ所のSNP	全染色体
	Aravinda Chakravarti Johns Hopkins School of Medicine			(解析)	
	Mark Leppert University of Utah			(地域社会に対する取り組み&サンプル収集)	
ナイジェリア	Charles Rotimi Howard University with University of Ibadan			(同上)	
	Lincoln Stein Cold Spring Harbor Laboratory, New York			(データコーディネーションセンター)	

表2　タイピングセンター

センター	染色体の分担
理化学研究所	5, 11, 14, 15, 16, 17, 19
ウエルカムトラスト・サンガー研究所	1, 6, 10, 13, 20
マッギル大学とゲノム・ケベック・イノベーションセンター	2, 4p
中国ハップマップコンソーシアム※	3, 8p, 21
イルミナ社	8q, 9, 18q, 22, X
ハーバードおよびMITのブロード研究所	4q, 7q, 18p, Y, mtDNA
ベイラー医科大学とParAllele BioScience社	12
カルフォルニア大学サンフランシスコ校とワシントン大学（セントルイス）	7p
パーレジェンサイエンス社	第2, 4, 7染色体のENCODE領域（5 Mb）， 第8, 9, 12, 18 染色体(CEUのみ)， および第2期プロジェクト

※中国ハップマップコンソーシアムは，北京ゲノム研究所，北京の中国国立ヒトゲノムセンター，香港大学，香港科技大学，
香港中文大学，上海の中国国立ヒトゲノムセンターから成る

表3　国際ハップマップ計画でタイピングされたSNPの総数

グループ	CEU	CHB	JPT	YRI
タイピングされたSNPの総数	5,894,684	5,812,990	5,812,990	5,857,466

CEU：CEPH（北ヨーロッパや西ヨーロッパから来た祖先をもつ米国ユタ州の住民）
CHB：中国，北京の漢民族系中国人
JPT：日本，東京の日本人
YRI：ナイジェリア，イバダンのヨルバ族

トリオとなるように選択した．各地から集められたサンプルは，収集地にて連結不可能匿名化を受けCorriell研究所に送られ，そこでセルライン化された．この研究所では研究計画の審査の後に，このプロジェクトに関わらない研究者にもDNAサンプルを提供している．データの連続性が確保されるため，将来，本プロジェクトにより得た知見をさらに深く追求することが可能となっている．

2）データの公開

各国で分担して得られたタイピング情報はData Cordination Centerに転送され，速やかに公開されている．データはhttp://hapmap.org/downloads/index.html#releasesから閲覧できる．

3）プロジェクト第2期

計画の途中から，さらに詳細に地図を作成することとなり，さらに460万カ所のSNPを米国のベンチャー企業であるPerlegen Sciences社が請け負うこととなった．このステップは非常に早く進み，2005年9月ごろ終了した．全体としては，表3のように1つの民族集団について580万を超えるSNPをタイピングした結果が，すべてダウンロード可能である．

文献

1）The International HapMap Consortium：Nature, 437：1299-1320, 2005

＜著者プロフィール＞
田中敏博：鹿児島県出身．1996年東京大学大学院医学系研究科修了．2004年日本人類遺伝学会奨励賞，2005年文部科学大臣表彰若手科学者賞．日本人類遺伝学会臨床遺伝専門医，日本内科学会認定内科医，日本循環器学会認定循環器専門医．これまで循環器病の遺伝子解析，ゲノム解析のための基盤整備などに携わる．臨床の現場に還元できる成果を目指している．

TRANSLATIONAL PHYSIOLOGY

WTC deafness Kyoto (*dfk*): a rat model for extensive investigations of *Kcnq1* functions

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Kyoto (*dfk*): a rat model for extensive investigations of *Kcnq1* func-
tions. *Physiol Genomics* 24: 198–206, 2006. First published Decem-
ber 20, 2005; doi:10.1152/physiolgenomics.00221.2005.—KCNQ1
forms K channels by assembly with regulatory subunit KCNE
proteins and plays a key role in the K homeostasis in a variety of
tissues. In the heart, KCNQ1 is coassembled with KCNE1 to produce
a cardiac delayed rectifier K current. In the inner ear, the KCNQ1/
KCNE1 complex maintains the high concentration of K in the
endolymph. In the stomach, KCNQ1 is coassembled with KCNE2 to
form the K efflux channel that is essential for gastric acid secretion.
In the colon and small intestine, KCNQ1 is coassembled with KCNE3
to play an important role in transepithelial cAMP-stimulated Cl
secretion. For further understanding of *Kcnq1* function in vivo, an
animal model has been required. Here we reported the identification of
a coisogenic *Kcnq1* mutant rat, named deafness Kyoto (*dfk*), and the
characterization of its phenotypes. WTC-*dfk* rats carried intragenic
deletion at the *Kcnq1* gene and showed impaired gain of weight,
deafness, and imbalance resulting from the marked reduction of
endolymph, prolonged QT interval in the electrocardiogram (ECG),
and gastric achlorhydria associated with hypertrophic gastric mucosa.
Surprisingly, WTC-*dfk* rats showed hypertension, which suggested
that *Kcnq1* might be involved in the regulation of blood pressure.
These findings suggest that WTC-*dfk* rats could represent a powerful
tool for studying the physiological functions of KCNQ1 and for the
establishment of new therapeutic procedures for *Kcnq1*-related dis-
eases.

voltage-dependent potassium channel; deafness; long-QT syndrome;
achlorhydria; hypertension

Kcnq1 ENCODES A PORE-FORMING (α) subunit of the voltage-gated
K channel. It encodes six membrane-spanning domains (S1–
S6), including the voltage sensor (S4) domain, and a K-
selective pore between S5 and S6 (2, 24). To form native
channels, KCNQ1 coassembles with small β-subunits, so-

called KCNE proteins. Although the stoichiometry of coassem-
bly is not yet known, it is likely that four α-subunits assemble
with four β-subunits to form the channels.

In the heart, KCNQ1 is coassembled with KCNE1. The
KCNQ1/KCNE1 complex produces a slowly activating de-
layed rectifier K current (I_{Ks}) that contributes to the later
phase of action potential repolarization, returning to the resting
potential (2, 24). Mutations of the human *KCNQ1* gene are
associated with the congenital long-QT syndrome, an inherited
disorder that is characterized by abnormal ventricular repolar-
ization and increases the risk of sudden death from cardiac
arrhythmias. There are at least two familial forms of long-QT
syndrome. One is the Jervell and Lange-Nielsen syndrome,
which is believed to be inherited as an autosomal recessive trait
and associated with congenital deafness (12). A second, more
common familial form is inherited as an autosomal dominant
trait with no other phenotypic abnormalities. This form, which
is sometimes referred to as the Romano-Ward syndrome (34),
is usually associated with a lower arrhythmia risk than the
autosomal recessive form.

In the inner ear, both KCNQ1 and KCNE1 are expressed. In
this tissue, the KCNQ1/KCNE1 complex produces a K-rich
fluid known as endolymph that bathes the organ of Corti, the
cochlear organ responsible for hearing, and the utricle, saccule,
and semicircular canal, which are responsible for balance and
equilibrium. Functional loss of KCNQ1 provokes congenital
deafness in the individuals with Jervell and Lange-Nielsen
syndrome. The mechanism of deafness is that the lack of I_{Ks}
leads to inadequate endolymph production and deterioration of
the organ of Corti (31).

In the stomach, KCNQ1 and KCNE2 are both expressed in
the luminal membrane of the acid-secreting parietal cells (6,
11), where H/K-ATPase, a pump responsible for the trans-
fer of H ions into the stomach, is coexpressed (9). The
KCNQ1/KCNE2 complex yields K currents that are activated
by acidic pH stimulation at resting membrane potential. Thus
KCNQ1/KCNE2 forms acid-activated luminal K channels
whose function is to supply K to the luminal surface to allow
H-for-K exchange by the pump (11). Disruption of *Kcnq1* in
mice causes a large increase in stomach pH that is accompa-
nied by gastric hyperplasia (16).

In the small intestine and colon, KCNQ1 colocalizes with KCNE3 in the basolateral membranes of crypt cells (6). The KCNQ1/KCNE3 complex is constitutively opened at the more negative membrane potential of intestinal epithelial cells and can be further activated by the action of cAMP (25). This complex is thought to be important for maintenance of transepithelial transport in the colon and the small intestine, by recycling K^+ that is transported into the cell by basolateral Na^+-K^+-2Cl⁻ (NaK2Cl) cotransporters and Na^+/K^+-ATPases. Thus the KCNQ1/KCNE3 channel is required for transepithelial cAMP-stimulated Cl⁻ secretion.

In the airway epithelia, KCNQ1 mediates a basolateral K^+ conductance that plays an important role in maintaining cAMP-dependent Cl⁻ secretion (10, 17). The β-subunit coassembled with KCNQ1 in the airway epithelia has been a matter of debate. In the mouse, KCNE3 is thought to be coupled with KCNQ1 (10). In the pancreas, KCNQ1 is expressed in the insulin-secreting cells. Inhibitors for the KCNQ1 channels increase the insulin secretion, which suggested that KCNQ1 would be involved in insulin secretion by the regulation of membrane potentials in the insulin-secreting cells (29).

Kcnq1 expression is not only confined to the tissues described above but also occurs in many epithelial tissues, such as placenta, kidney, liver, and thymus (7, 33, 38). However, no clear functional role of Kcnq1 in these tissues has been found as yet. For a further understanding of Kcnq1 functions in these tissues as well as the establishment of novel therapeutic procedures for diseases associated with Kcnq1 dysfunction, such as the long-QT syndrome, deafness, and achlorhydria, an animal model that is easy to investigate and manipulate is required. To address this issue, a rat model would be suitable, since the rat has an ~10 times larger size than the mouse and offers several unique advantages in various fields of research. In particular, the rat has been used as a variety of disease models because of its easier clinical examination and sampling as well as therapeutic administration and manipulation.

In this report, we describe the characteristics of a novel Kcnq1 mutation, deafness Kyoto (dfk), in the rat. Positional candidate cloning revealed that the dfk allele is an intragenic deletion including exon 7 of the Kcnq1 gene. WTC-dfk rats suffer from deafness and imbalance resulting from profound morphological abnormalities of the inner ear. These rats exhibit prolonged QT intervals and T-wave abnormalities on electrocardiogram (ECG) measurements and elevation of pH to almost neutral in the gastric secretion. Additionally, WTC-dfk rats display hypertension.

MATERIALS AND METHODS

Animals. Rats showing abnormal behaviors characterized by head tossing, drawing back, stepping back, and circling were found in the $N_{12}F_{60}$ generation of a WTC/I-Atrn' congenic strain (15) at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, in 1999. Even after elimination of the Atrn' mutation on chromosome (Chr) 3 (14), these phenotypes were observed and were inherited in an autosomal recessive manner. These mutant phenotypes resembled behavioral features of some types of deafness mutants. Therefore, we called the causative gene "deafness Kyoto" (gene symbol, "dfk"). Because the backcross generation in which the dfk mutation was found was sufficient to replace the genetic background with WTC, dfk was thought to be synonymous with a mutation arising on the genetic background of an inbred WTC strain. Therefore, WTC-dfk and WTC are coisogenic; their genetic backgrounds were

identical except for the dfk mutation. ACI/NKyo and WTC rat strains were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. Animal care was conducted according to the Guideline for Animal Experiments of Kyoto University. All experimental procedures were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

The ACI/NKyo (NBRP no. 0001), WTC (NBRP no. 0020), and WTC-dfk (NBRP no. 0289) strains have been deposited in the National BioResource Project for the Rat in Japan and can be obtained from the Project (http://www.anim.med.kyoto-u.ac.jp/nbr/).

Auditory brainstem response measurement. Auditory brainstem response (ABR) measurements were performed in three individuals each for WTC and WTC-dfk rats at 26 wk of age. The following experiments were done on animals that were anesthetized with ketamine hydrochloride (60 mg/kg, im) and pentobarbital sodium (21 mg/kg, ip) and kept at 38°C. Stainless steel needle electrodes were inserted subcutaneously into the vertex (indifferent) and one side (active) and the other side (ground) of the retroauricular region, respectively. The ABR was obtained by averaging 1,000 evoked responses to click stimuli at intensities of 60, 65, 70, 75, 80, 100, 120, and 135 dB peak equivalent sound pressure level (peSPL), with 50-ms intervals generated by an acoustic stimulator (MEB-5504: Nihon Koden, Tokyo, Japan). Clicks were delivered through an inner ear-type earphone facing the meatus acusticus externus.

Genetic mapping. (WTC-dfk × ACI)F₁ rats were intercrossed to obtain F₂ rats. Homozygous WTC-dfk animals were identified on the basis of appearance of head-tossing and/or circling behaviors and inability to swim at 3–4 wk of age. Two hundred and forty WTC-dfk animals were obtained from 1,000 F₂ progeny and used for genetic mapping. For the initial mapping of dfk, we employed pooled simple sequence-length polymorphism (SSLP) analysis (27). DNAs from 45 randomly selected rats were standardized to 20 ng/μl, and equal amounts of individual DNAs were pooled. The dfk DNA pool was genotyped for 75 microsatellite markers distributed among all autosomes. For the fine mapping of dfk, all dfk/dfk animals were genotyped.

Sequencing. PCR products were treated with ExoSAP-IT (Amersham Biosciences, Piscataway, NJ) to digest single-strand DNAs and excess primers. Cycle sequencing was performed with the BigDye Terminator Ready Reaction Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). PCR samples were purified with CENTRI-SEP spin columns and were then loaded into an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Northern blotting. Poly(A) RNA was purified from total RNA by using Oligotex-dT30 Super (Roche Diagnostics). Two micrograms of poly-A RNA were electrophoresed on formaldehyde-denaturing agarose gels and blotted onto Hybond N+ membrane (Amersham Biosciences). Bands on the autoradiograph were detected by using an imaging plate and a computerized image display system (BAS2000: Fuji Film, Tokyo, Japan). A DNA fragment containing exons 9–14 of rat Kcnq1 was used as a probe.

Electrocardiograms. ECG recordings were obtained from adult (12–18 wk) wild-type (n = 6) and WTC-dfk (n = 6) rats using implantable Physiotel TA10EA-F20 radio frequency transmitters and receivers (Data Sciences International, Arden Hills, MN). After an animal was anesthetized with chloral hydrate (600 mg/kg, ip), the transmitter was placed within the peritoneal cavity. The electrodes were placed at the right axilla and at the left side of the xiphoid. Each electrode was sutured subcutaneously. After implantation of the transmitters, the animals were allowed to recover for at least 72 h. Twenty consecutive beats were recorded from individual animals under resting conditions. Unfiltered data were analyzed offline, and QRS, QT, PQ, and RR intervals were measured. Rate-corrected QT values (QTc) were derived using the formula $QTc = QT/SQRT(RR/100)$ (19).

Histopathological analyses. To evaluate the histological phenotype of dfk rats, WTC-dfk rats were killed under anesthesia at 34 wk of age (n = 3). Age-matched WTC rats (n = 3) were examined as controls

Perfusion fixation through the left ventricle was performed with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains, spinal cords, inner ears, stomach, and representative visceral organs were removed and fixed with the same fixative solution. Before processing for paraffin embedding, the ear tissues were decalcified with 5% EDTA-dipotassium salt. The samples were embedded in paraffin, and sections (4 μm) were stained with hematoxylin and eosin stain (HE). The cochlea was also embedded in epoxy resin, and then semithin sections (1 μm) were stained with toluidine blue.

Stomach pH and acid output measurements. The WTC-*dfk* rats (11 wk old, $n = 5$) and the age-matched, wild-type, control WTC rats ($n = 5$) were fasted overnight before the experiment with free access to water. Each rat was anesthetized, and the abdominal cavity was opened and the pylorus ligated. Four hours after treatment, the rat was killed and the stomach was removed. The intraluminal contents were drained into a centrifuge tube after a small nick was made in the stomach wall along the greater curvature adjacent to the pyloric ligature. The solutions with the stomach contents were centrifuged to pellet the insoluble material. The pH of the supernatants was measured, and then acid was titrated using an automatic titrator COM-TITE-500 (Hiranuma Sangyo, Ibaraki, Japan). The results were expressed as microequivalents of acid per liter of gastric volume.

Blood pressure measurement. Systolic blood pressure was measured by the tail-cuff method using a nonpreheated, noninvasive blood pressure monometer (MK-2000; Muromachi Kikai, Tokyo, Japan). The average values of three measurements were determined for individual animals. All measurements were performed on 11 male rats at 9 or 10 wk of age (18).

RESULTS

Clinical features of WTC-dfk rats. The *dfk* rats were recognized at 10 days after birth by their twisting of their necks toward the back when lifted by the tail. After weaning, they exhibited hyperactivity and usually showed rapid head bobbing and occasionally a head tilt. Mature WTC-*dfk* rats displayed bidirectional circling behavior. In addition, they were unable to swim. When WTC-*dfk* rats were placed into a deep tank filled with warm water, WTC-*dfk* rats immediately began rotating along their long axis and sank underwater. While underwater, the rats were still rotating along their body length. The rats seldom resurfaced until they were rescued. These findings suggested that WTC-*dfk* rats might have lost their balance and have defects in the inner ear, which is responsible for linear and angular acceleration.

The adult WTC-*dfk* rats seemed to be smaller than the normal littermates, so we measured the body weights of the WTC-*dfk* rats (male, $n = 6$) at 5, 6, and 10 wk of age. At 5 or 6 wk of age, body weights were not significantly different between WTC and WTC-*dfk* rats. At 10 wk of age, WTC-*dfk* rats showed significantly lower body weight than WTC rats (246.3 ± 3.2 vs. 190.6 ± 8.8 g, $P < 0.01$) (Fig. 1A).

ABR. In addition to the imbalance, WTC-*dfk* rats showed no response to sounds such as rapping and clapping. To test the
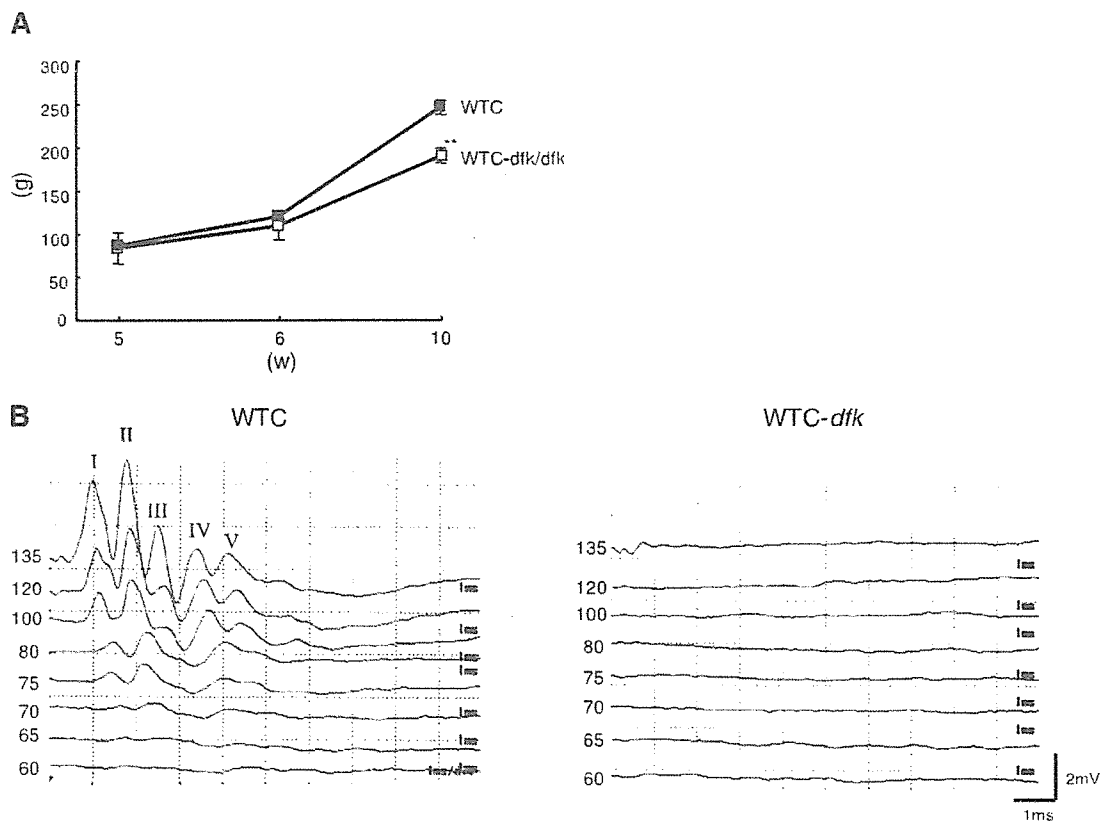

Fig. 1. Body weight and auditory phenotypes of the WTC-*dfk* rat. *A*: body weights of WTC and WTC-*dfk* rats. Body weights were measured in male WTC ($n = 6$) and male WTC-*dfk* ($n = 6$) rats at 5, 6, and 10 wk (w) of age. Impaired gain of body weight was observed in WTC-*dfk* rats at 10 wk of age. *B*: representative auditory brainstem response (ABR) of the WTC and WTC-*dfk* rats. The 5 major peaks (labeled *I, II, III, IV,* and *V*) were clearly detectable with >100 dB in the WTC rat (*left*), whereas no waveform was recorded at the highest stimulus level of 135 dB in the WTC-*dfk* rat (*right*). ABR measurements were performed in WTC ($n = 3$) and WTC-*dfk* ($n = 3$) rats at 26 wk of age.