Circ J 2005; 69: 1029–1034

Validation of the Association Between the Gene Encoding 5-Lipoxygenase-Activating Protein and Myocardial Infarction in a Japanese Population

Kazuaki Kajimoto, PhD; Keisuke Shioji, MD; Chisaki Ishida, PhD; Yoshitaka Iwanaga, MD; Yoshihiro Kokubo, MD; Hitonobu Tomoike, MD; Shun-ichi Miyazaki, MD; Hiroshi Nonogi, MD; Yoichi Goto, MD; Naoharu Iwai, MD

Background Recently, the 5-lipoxygenase activating protein gene (*ALOX5AP*) was reported to confer a risk of myocardial infarction (MI) and stroke, independent of conventional risk factors. The purpose of the present study was to validate those findings in a Japanese population.

Methods and Results The study population consisted of 1,875 subjects (males 871, females 1,004) recruited from the Suita study (control group) and 353 subjects (males 306, females 47) with MI. The promoter, all of the exons, and 3'UTR regions of *ALOX5AP* were sequenced in 96 subjects, and 8 polymorphisms were found. There were significant differences in the frequencies of the haplotypes constructed from the 2 SNPs (A162C and T8733A) between the control and MI groups. Multiple logistic analysis indicated that the homozygous genotype of the (CA) haplotype was significantly associated with a reduced risk for MI.

Conclusion The hypothesis that *ALOX5AP* contributes to susceptibility for MI was validated in a Japanese population. (*Circ J* 2005; 69: 1029–1034)

Key Words: *ALOX5AP*; Haplotype; Myocardial infarction

Myocardial infarction (MI) is a multifactorial disease caused by environmental and genetic factors. There are an increasing number of studies that identify genes contributing to MI[1–5] for personalized prevention from the disease. Recently, the 5-lipoxygenase activating protein gene (*ALOX5AP*) was reported to confer a risk of MI and stroke, independent of conventional risk factors[6] This possibility was based on findings in genome-wide scans and subsequent case–control studies. A haplotype, HapA, defined by 4 single nucleotide polymorphisms (SNPs) and which spanned *ALOX5AP*, was shown to be associated with MI in an Icelandic population[6] and subsequently, another SNP-based haplotype within *ALOX5AP*, HapB, showed a significant association with MI in British cohorts from Leicester and Sheffield[6]

The *ALOX5AP* gene encodes the membrane-associated 5-lipoxygenase (LO)-activating protein, an important mediator of the activity of 5-lipoxygenase, a key enzyme in the biosynthesis of leukotrienes[7] Leukotrienes are not only smooth muscle constrictors but also proinflammatory mediators that are produced predominantly by inflammatory cells[8,9] Studies have indicated that inflammatory processes play an important role in the progression of atherosclerotic disease[10,11] The 5-LO pathway could be an important contributor to the pathophysiology of atherosclerosis through the formation of leukotriene (LT) B4 via an increase in vascular permeability[12] Antagonists of LTB4 have been reported to attenuate the development of atherosclerosis in apoE-deficient and LDLR-deficient mice[13] Furthermore, 5-LO has been localized to macrophages, dendritic cells, foam cells, mast cells, and neutrophilic granulocytes, and the number of cells that expressed 5-LO was markedly greater in advanced lesions[14] Leukocytes that were positive for 5-LO accumulated at distinct sites that are most prone to rupture[15] Taken together, these findings suggest that upregulation of the leukotriene pathway may contribute to the progression of atherosclerotic progression and plaque stability. However, the precise role of leukotrienes in the pathogenesis of atherothrombotic diseases awaits further investigation.

Thus, it is likely that *ALOX5AP* contributes to MI. However, we are now recognizing that the contribution of common alleles is less than expected, and any single study that considers a few thousand subjects may not be large enough to support concrete conclusions and should be viewed as providing only tentative results. The purpose of the present study was to validate the findings of DeCode genetics[6] in a Japanese population and to evaluate the possible importance of *ALOX5AP* in the pathogenesis of MI.

Methods

Study Population

The selection criteria and design of the Suita Study have been described previously[16–18] The genotypes were determined in 1,875 subjects recruited from the Suita Study between April 2002 and February 2003. The MI group consisted of 353 (males 306, females 47) randomly selected inpatients and outpatients with documented MI who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003[19]

(Received April 1, 2005; revised manuscript received May 23, 2005; accepted June 2, 2005)

National Cardiovascular Center, Suita, Japan.
Mailing address: Naoharu Iwai, MD, Department of Epidemiology, Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita 565-8565, Japan. E-mail: iwai@ri.ncvc.go.jp

Table 1 Characteristics of the Study Population

	Control	MI	p-value
n	1,875	353	
Sex (% male)	46.5	86.69	<0.0001
Age (years)	64.5±0.25	58.8±0.59	<0.0001
BMI (kg/m²)	22.8±0.07	23.9±0.16	<0.0001
C-SM (%)	44.3	61.2	<0.0001
HT (%)	40.6	53.7	<0.0001
HLP (%)	27.8	51.7	<0.0001
HDL-C (mg/dl)	59.9±0.35	44.0±0.93	<0.0001
DM (%)	6.1	39.1	<0.0001

Data are mean±standard error. Differences between the 2 groups (control vs myocardial infarction (MI)) were calculated by t-test or χ^2 analysis. BMI, body mass index; C-SM, current smoking habit; HT, hypertension; HLP, hyperlipidemia; HDL-C, high-density lipoprotein cholesterol; DM, diabetes mellitus.

Table 2 Primers for Sequence Analysis

Probe	Sequence (5'-3')
P1S	GATATCAGCTGTCCCTCCCCACTG
E1S	CTCAGGGAAGTTTCCCATGACAAGG
E2S	CAGTAGAGAGCAGCTGCTGAGTACG
E3S	CCAAGTCTCCCTTACGCATCACCG
E4S	CTTGGGTCTTTTCCTGAAGTGC
E5S	GGAGCATTGTTGAGTCCAGGGAGC
P1A	GCACAACCTGCCCCTGTACAGGAAG
E1A	CAAAACCTTCAAGTTGCAGCCCTG
E2A	CACAAAGCCTCTCTGGTGAAGTCC
E3A	GCTCTCACCTCTCCAGGGCTTCAC
E4A	GCTCAGGGAAAGAAGAATCAGAGGTC
E5A	GGATTACAGGTATGAGCCACCACACC

Primers used for sequence analysis of a promoter region and all of the 5 exon regions including noncoding regions in ALOX5AP.

All the subjects enrolled in the present study provided written informed consent. The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center. The characteristics of the study population are shown in Table 1. Subjects with systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, and/or taking antihypertensive medication were categorized as having hypertension (HT). Subjects with fasting blood glucose ≥126 mg/dl, hemoglobinA1c ≥6.5%, and/or treatment for diabetes mellitus (DM) were categorized as having DM. Subjects with total cholesterol ≥220 mg/dl, triglycerides ≥150 mg/dl, and/or antihyperlipidemic medication were categorized as having hyperlipidemia (HLP).

DNA Studies

A promoter region and all of the 5 exon regions, including noncoding regions, were sequenced in 48 healthy subjects and 48 subjects with MI. Primers for sequencing are shown in Table 2. Eight polymorphisms were found and all the genotypes were determined by the TaqMan system in 1,875 control subjects and 353 subjects with MI. The probes and primers are shown in Table 3. HapA and HapB reported by DeCode,[6] which have been reported to be associated with MI and stroke, each consisted of 4 SNPs that were outside the area of our sequencing. To validate the possible importance of these haplotypes, the genotypes of 7 additional SNPs were also determined by the TaqMan system (Table 3).

Table 3 TaqMan Probes and Primers for *ALOX5AP* Genotyping

	Probe		Primer	
	VIC	FAM	F	R
At–607 G	*TTTTCTTGGAATTCAAAA*	*TTTCTTGGGATTCAAAA*	*AAACCTTATGTGGCTGCTACTTACC*	*GTGCCCCAATACTGTCCTTCT*
Tt–515C	*TGTGCTGTGTGTGTGT*	*TGCGTGCGTGTGTGT*	*TGGAATTCAAAAAAGAAGGACAGTA*	*GGGCAACAGAGCAACACTGTCT*
At162C	*CCCTTCATCAGGG*	*CCCTTCATTCAGGG*	*CAGCGTGGTCCAGATGGTA*	*TCCAAACAACCATCAAAGAAAATC*
At86–90C	*AGTCCTAATGCCTGATGTGT*	*AGTCCTAATGCCTGATGTTG*	*GCCTTTGAGCGGGTCTACACT*	*TTAAGGTTGGTCATCACTCCTAGAA*
Tt8233C	*AGCCAAGTTTCTGAGCG*	*TGAGCCAAGCTTCTGA*	*ACCACCCTTAATACCACATGTCTGT*	*AAGCCTCTCTGGTGAAGTCCAA*
Gt206164C	*CCTTCCCCCTCTCCAC*	*CCTTCCCCGCTCCCA*	*AGGGAAAGAAGAATCAGAGGTCCTA*	*CAGGAAGAGTGACAATTCAAACAGTA*
Ct2856969G	*CTTCTTTTTTGGAAGTGACT*	*CTTCTTTTTTGGAAGTGACT*	*GTCCGTTGCTGGCCATATTCA*	*TGGACATCGTCTTTATGTAGTTTTCAA*
At267949G	*CTATTCCCGTGCATTT*	*CTATTCCCGTGCATTT*	*GAAGAAAATGATGTCATGTCAGCTC*	*TGGTCACAAAACATCTTCAGAGAAC*
HapA1	*CCACTGTTGCCCAGTGG*	*AGCCACTGTTACCCAGTG*	*ATGATTCTTCTGACAGCATCAGCT*	*CATGTTGCTGTGTCCCATACATAGC*
HapA2, HapB2	*TGCAATTCTATTTAACCTCAA*	*TGCAATTCTATGTTAACCTCAA*	*TCACAAGATCCAGATGTATGTCCAA*	*ACTCTTAAGGTAGGTTCTATGGTTGCAA*
HapA3	*AGAGCGGTGTGATAA*	*CAGAGCGGCATGTGATAA*	*TCGGAGGCCTCGTGTTTCAG*	*CCAGGAGCCAAGCATTAGCA*
HapA4	*AATTGCTCGATGAGATCCT*	*AATTGCTCGATGAGATCC*	*TGCTTTAGTTCTTGACCTCACCAA*	*ACCATCTGGGTTCAAGAGAGAAAT*
HapB1	*CTGCTCTGGGCCTC*	*TGCCTCAGCCTCCCA*	*ACATCACCTAGTGTGTTGTGTAAGAAA*	*ACTGTCTTGAACTCCTGACCTCAG*
HapB3	*AACTGAGGGTAAAGATTC*	*AACTGAGGGTAAAGATT*	*TCTTTAACACCCTGTCTCCAAATACA*	*TGGTCCCTTCCAAAATTCATATG*
HapB4	*TTTTAAAAAACCTGAAAGGACCAC*	*TTTTAAAAAACTGAAAGGACCAC*	*TGCACCCCACAAATACCTACAA*	*ATCCTGATGGCCTGGCATT*

TaqMan probes and primers used to determine the genotypes of SNPs in ALOX5AP. The nucleotides of polymorphisms are underlined. HapA and HapB are defined as follows: HapA1, SG13S376; HapA2 and HapB, SG13S114; HapA3, SG13S89; HapA4, SG13S32; HapB1, SG13S377; HapB3, SG13S41; HapB4, SG13S35 (see Reference 1).

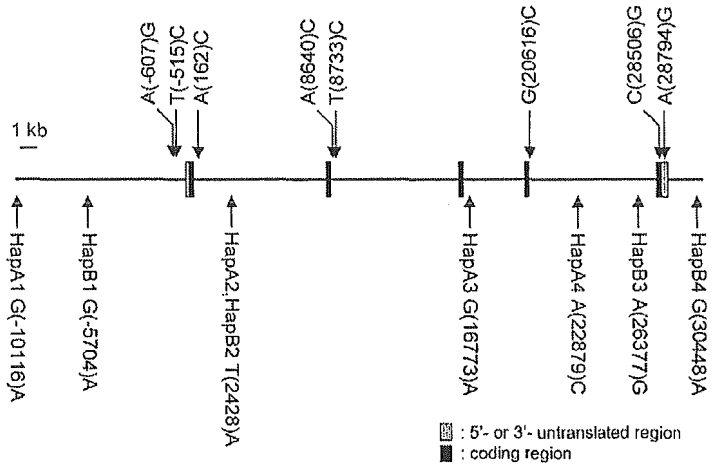

Fig 1. Schematic of the *ALOX5AP* gene and the positions of the determined polymorphisms. Gray boxes indicate the 5'- or 3'-untranslated regions, and black boxes indicate coding regions.

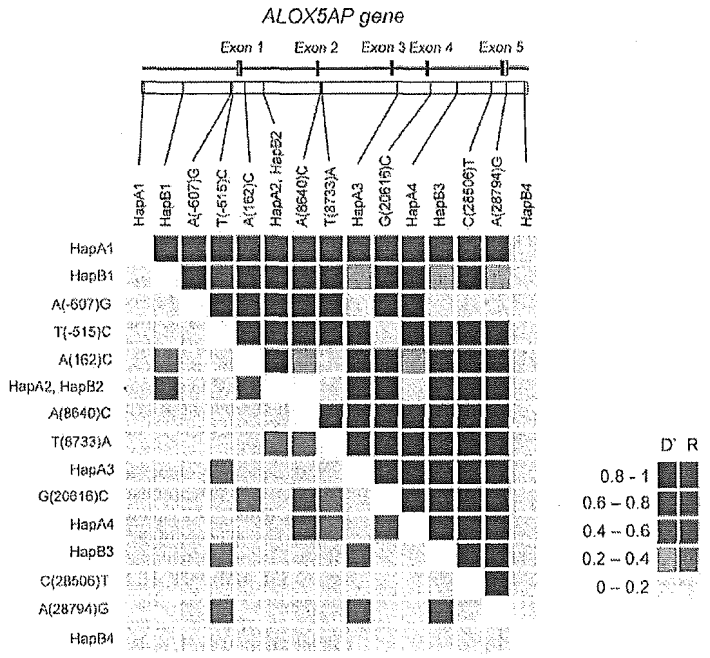

Fig 2. Linkage disequilibrium among the SNPs in the *ALOX5AP* gene. Two measures of LD are shown: D'-values in the upper right triangle and R-square values in the lower left triangle. Color-coded scales for D'-values or R-square values (measures of LD strength) are provided on the right.

Statistical Analysis

Values are expressed as mean ± standard error of the mean. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc, Cary, NC, USA) unless otherwise stated. Multiple logistic analysis was performed to obtain predictors for MI. R-square values between polymorphisms and haplotype frequencies in the control and MI groups were analyzed using the SNPAlyze Pro

Table 4 Genotype Frequencies of Each Polymorphism in *ALOX5AP* in the Total Group and in Males

Polymorphism	Control			MI			p1 value	p2 value	Control males			MI males			p1 value	p2 value
	Major	Hetero	Minor	Major	Hetero	Minor			Major	Hetero	Minor	Major	Hetero	Minor		
A1–607G	1,943	31	1	342	11	0	0.1893	0.4430	854	17	0	295	11	0	0.1201	0.1167
T1–515C	1,859	15	0	346	2	0	0.6451	0.5695	863	8	0	299	2	0	0.6713	0.5876
A162C	491	939	445	105	179	68	0.1294	0.2706	240	428	203	93	156	63	0.4989	0.4470
A8640C	800	853	237	146	167	39	0.5230	0.5009	371	381	118	124	146	35	0.4035	0.4553
T8733C	605	903	364	113	179	61	0.5697	0.3391	294	413	163	97	157	52	0.5061	0.3207
G20616C	1,122	651	102	210	127	16	0.7331	0.8127	510	313	48	181	111	14	0.8151	0.7954
G28506G	1,862	12	0	353	0	0	0.0415	0.7168	866	5	0	306	0	0	0.0823	0.8010
A28794G	1,847	26	1	344	7	0	0.5980	0.7270	859	11	1	299	6	0	0.5133	0.5663
HapA1 (G–1911A)	1,853	1	0	352	0	0	0.5575	0.9375	871	0	0	305	0	0		
HapA2, HapB2 (T12420A)	792	944	239	138	173	39	0.2976	0.1489	370	384	117	123	145	35	0.4675	0.4425
HapA3 (G16773A)	1,848	26	1	346	7	0	0.6051	0.7313	859	11	1	300	6	0	0.5156	0.5703
HapA4 (A22879C)	800	892	242	145	168	39	0.4285	0.3791	373	382	116	123	146	36	0.4620	0.4838
HapB1 (G–5704A)	1,249	561	64	221	121	10	0.2426	0.1756	573	266	32	200	96	9	0.8119	0.8655
HapB3 (A26377G)	1,847	27	1	343	9	0	0.3032	0.7448	858	12	1	298	7	0	0.4560	0.5632
HapB4 (G30848A)	1,875	0	0	353	0	0			871	0	0	306	0	0		

Major, Hetero and Minor indicate major genotype, heterozygous genotype and minor genotype, respectively; for example, in the case of polymorphism A1–607G, major, hetero, and minor refer to the AA, AG, and GG genotypes, respectively. The numbers of each genotype of each SNP are shown. P1 values were calculated by χ^2 analysis and P2 values were calculated by multiple logistic analysis including age, sex, BMI, HT, HLP, DM, and C-SM as independent variables (See Table 1 for abbreviations).

statistical package (version 3.2, Dynacom Inc). Diplotypes were also estimated by the SNPAlyze Pro statistical package.

Results

Sequence analyses in 96 subjects revealed the existence of 8 polymorphisms in Japanese. The genotypes of these 8 polymorphisms were determined by the TaqMan system in 1,875 control subjects and 353 MI subjects. Seven additional genotypes were also determined by the TaqMan system to validate the possible importance of HapA and HapB, as reported by DeCode6 A schematic of *ALOX5AP* and its polymorphisms are shown in Fig 1. The LD values calculated by D'- or R-square values among these SNPs are shown in Fig 2.

Genotype frequencies in the control and MI groups are shown in Table 4. P1 values were calculated by chi-square analysis and P2 values were calculated by multiple logistic analysis including age, sex, body mass index (BMI), HT, HLP, DM, and current smoking (C-SM) as independent variables.

The allele frequencies of the SNPs comprising HapA and HapB were significantly different between the Icelandic and Japanese populations. The allele frequencies of HapA1, HapA3, HapB3, and HapB4 were significantly less in Japanese, and some of the HapA and HapB haplotype frequencies were too small for conducting meaningful association studies in Japanese. Thus, we conducted haplotype analyses based on the polymorphisms found in our study population.

The allele frequencies of the A162C, A8640C, T8733A, and G20616C polymorphisms exceeded 0.15 (Table 4). The polymorphisms A8640C, T8733A, and G20616C are in tight LD (Fig 2). Therefore, we constructed haplotypes with A162C and one of the polymorphisms from A8640C, T8733A, and G20616C, and compared haplotype frequencies between the control and MI groups. The most significant difference in haplotype frequency ($p<0.0001$ [1,000 permutations]) was observed in the haplotype constructed by the A162C and T8733A polymorphisms (Table 5). The haplotype (AA) was less frequent in the control than in the MI group (20.0% vs 25.8%, p=0.003 [1,000 permutations]. The haplotype (CA) was more frequent in the control than in the MI group (23.6% vs 16.9%, p=0.001 [1,000 permutations]. Similar trend was also observed in male subjects only (Table 5).

Next, diplotypes of the study population were estimated and the characteristics of the subjects with the homozygous genotype of the (CA) haplotype and the others are shown in Table 6. The influence of the haplotypes on susceptibility to MI was assessed by multiple logistic analysis in which age, sex, C-SM, BMI, HT, HLP, and DM were included as independent variables. The homozygous genotype of the (CA) haplotype was significantly associated with reduced risk for MI (p=0.0431, odds ratio=0.4436, 95% confidence interval=0.189–0.926) over other genotypes. However, multiple logistic analysis did not conclude that the homozygous genotype of the (AA) haplotype was associated with increased risk for MI (p=0.2901).

Discussion

The purpose of the present study was to validate in a Japanese population the association between *ALOX5AP*

Table 5 Frequencies of the Haplotype Constructed by the Polymorphisms A162C and T8733A in the Total Group and in Males

Haplotype	Overall (%)	Control (%)	MI (%)	Permutation p-value	Overall (%)	Control males (%)	MI males (%)	Permutation p-value
A162C/T8733A				<0.0001				0.043
AT	31.0	31.3	29.4	0.388	31.4	32.0	29.8	0.361
CT	25.6	25.2	27.8	0.196	26.1	25.5	27.5	0.421
AA	20.9	20.0	25.8	0.003	21.4	20.1	25.1	0.035
CA	22.6	23.6	16.9	0.001	21.1	22.3	17.6	0.03

The percentage of the haplotype constructed by the polymorphisms A162C and T8733A is indicated. Permutation p-values were calculated by 1,000 iterations of the permutation test using the SNPAlyze Pro statistical package.
The haplotypes [AT], [CT], [AA], and [CA] mean as follows: AT, A162 and T8733; CT, C162 and T8733; AA, A162 and A8733; CA, C162 and A8733, respectively.

variants and MI that has been recently reported in Caucasian populations.

Because the genetic contribution of a single gene to common disease susceptibility seems to be very low, as observed in the I/D polymorphism of the ACE gene in cardiovascular diseases;[20,21] validation studies in other populations are very important. However, some of the allele frequencies of the HapA and HapB haplotypes were too low in our study population and so we could not replicate the previous studies by DeCode.[9] However, the haplotype that was newly identified in our study population was revealed to be significantly associated with MI. Thus, the hypothesis that *ALOX5AP* contributes to the susceptibility for MI is validated and strengthened by the present study.

The precise mechanism of how variants of *ALOX5AP* confer susceptibility for atherothrombotic diseases remains to be determined. The biological significance of the haplotype defined by A162C and T8733A remains to be solved, because these 2 polymorphisms reside in intron regions. Future studies will be needed to investigate whether the haplotype influences the production of leukotrienes by neutrophils.

Identification of *ALOX5AP* as one of the genes contributing to MI may have clinical implications. Leukotriene antagonists are currently used to treat asthma and various allergic diseases.[22] It would be interesting to determine whether these clinically useful antagonists could be helpful for the secondary prevention of MI in selected subjects defined by haplotype.

Acknowledgments

We are deeply grateful to the following people for their support in our population survey: Dr Otosaburo Hishikawa, Dr Katsuyaki Kawanishi, Mr Shigeru Kobayashi, and members of the Saita City Medical Association. We are also thankful to the members of our attendants' society (Satsuki junya-kai) for their cooperation and assistance with our survey of risk factors and preventive activity on cardiovascular diseases. We also thank Professor Soichiro Kitamura, President of the National Cardiovascular Center, for considering our research work.

This study was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan (MPJ-3), a grant-in-aid from the Salt Science Research Foundation (No. 05C5), and a grant-in-aid from the Uehara Memorial Foundation.

Table 6 Characteristics of Subjects With the Homozygous Genotype of the [CA] Haplotype and the Others

Genotype	[CA][CA] homozygous	Others	p-value
n	113	2,115	
Sex (% male)	45.1	53.3	0.0920
Age (years)	62.3±1.06	63.7±0.24	0.2218
BMI (kg/m²)	23.1±0.29	22.9±0.07	0.5411
C-SM (%)	44.3	47.2	0.5453
HT (%)	37.2	43.0	0.2192
HLP (%)	28.3	31.8	0.4353
DM (%)	8.9	11.4	0.3846
MI (%)	8.0	16.3	0.0107

Data are mean±standard error. Differences between the 2 groups ([CA] [CA] homozygous vs the others) were calculated by t-test or χ^2 analysis.
BMI, body mass index; C-SM, current smoking habit; HT, hypertension; HLP, hyperlipidemia; HDL-C, high-density lipoprotein cholesterol; DM, diabetes mellitus.

References

1. Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D, et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 1992; 359: 641–644.
2. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 2002; 32: 650–654.
3. Mukae S, Aoki S, Itoh S, Sato R, Nishio K, Iwata T, et al. Mitochondrial 5178A/C genotype is associated with acute myocardial infarction. *Circ J* 2003; 67: 16–20.
4. Ozaki K, Inoue K, Sato H, Iida A, Ohnishi Y, Sekine A, et al. Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxin-alpha secretion in vitro. *Nature* 2004; 429: 72–75.
5. Wang X, Ria M, Kelmenson PM, Eriksson P, Higgins DC, Samnegard A, et al. Positional identification of TNFSF4, encoding OX40 ligand, as a gene that influences atherosclerosis susceptibility. *Nat Genet* 2005; 37: 365–372.
6. Helgadottir A, Manolescu A, Thorleifsson G, Gretarsdottir S, Jonsdottir H, Thorsteinsdottir U, et al. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet* 2004; 36: 233–239.
7. Dixon RA, Diehl RE, Opas E, Rands E, Vickers PJ, Evans JF, et al. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* 1990; 343: 282–284.
8. Voelkel NF, Stenmark KR, Reeves JT, Mathias MM, Murphy RC. Actions of lipoxygenase metabolites in isolated rat lungs. *J Appl Physiol* 1984; 57: 860–867.
9. Bray MA, Ford-Hutchinson AW, Smith MJ. Leukotriene B4: An inflammatory mediator in vivo. *Prostaglandins* 1981; 22: 213–222.
10. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002; 105: 1135–1143.
11. Murtagh BM, Anderson HV. Inflammation and atherosclerosis in acute coronary syndromes. *J Invasive Cardiol* 2004; 16: 377–384.
12. Bray MA, Cunningham FM, Ford-Hutchinson AW, Smith MJ. Leukotriene B4: A mediator of vascular permeability. *Br J Pharmacol* 1981; 72: 483–486.
13. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Freeman A, Showell HJ. Leukotriene B4 receptor antagonism reduces monocytic foam cells in mice. *Arterioscler Thromb Vasc Biol* 2002; 22: 443–449.
14. Spanbroek R, Grabner R, Lotzer K, Hildner M, Urbach A, Ruhling K, et al. Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *Proc Natl Acad Sci USA* 2003; 100: 1238–1243.
15. Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation* 1995; 92: 657–671.

16. Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: The Suita study. *Stroke* 1997; **28**: 518–525.
17. Shioji K, Mannami T, Kokubo Y, Inamoto N, Takagi S, Goto Y, et al. Genetic variants in PCSK9 affect the cholesterol level in Japanese. *J Hum Genet* 2004; **49**: 109–114.
18. Kokubo Y, Iwai N, Tago N, Inamoto N, Okayama A, Yamawaki H, et al. Association analysis between hypertension and CYBA, CLCNKB, and KCNMB1 functional polymorphisms in the Japanese population: The Suita Study. *Circ J* 2005; **69**: 138–142.
19. Shioji K, Mannami T, Kokubo Y, Goto Y, Nonogi H, Iwai N. An association analysis between ApoAI polymorphisms and the high-density lipoprotein (HDL) cholesterol level and myocardial infarction (MI) in Japanese. *J Hum Genet* 2004; **49**: 433–439.
20. Fildes JE, Walker AH, Densem CG, Deiraniya AK, Hutchinson IV, Leonard CT, et al. Angiotensin converting enzyme insertion/deletion polymorphism does not influence postcardiac transplantation hypertension onset or progression. *J Heart Lung Transplant* 2005; **24**: 406–410.
21. Sayed-Tabatabaei FA, Schut AF, Vasquez AA, Bertoli-Avella AM, Hofman A, Witteman JC, et al. Angiotensin converting enzyme gene polymorphism and cardiovascular morbidity and mortality: The Rotterdam Study. *J Med Genet* 2005; **42**: 26–30.
22. Busse WW, Gaddy JN. The role of leukotriene antagonists and inhibitors in the treatment of airway disease. *Am Rev Respir Dis* 1991; **143**: S103–S107.

Circ J 2005; **69**: 1192–1195

Assessment of MEF2A Mutations in Myocardial Infarction in Japanese Patients

Kazuaki Kajimoto, PhD; Keisuke Shioji, MD; Naomi Tago, BS; Hitonobu Tomoike, MD; Hiroshi Nonogi, MD; Yoichi Goto, MD; Naoharu Iwai, MD

Background Recently, a mutation in the human MEF2A gene was reported to be responsible for an autosomal dominant form of coronary artery disease, so the purpose of the present study was to assess the significance of MEF2A mutations in Japanese subjects with myocardial infarction (MI).

Methods and Results The study population consisted of 589 control subjects recruited from the Suita study and 379 subjects with MI. The promoter, all the exons, and 3'-UTR regions of MEF2A were sequenced in 190 subjects with myocardial infarction. We found 2 amino acid length polymorphisms, a 7-amino acid deletion polymorphism, and a nonsense mutation (R447X) in exon 12. The length and deletion polymorphisms did not confer susceptibility to MI. Although the nonsense mutation was detected in 1 subject with MI, and in none of the control subjects, the impact of this mutation does not appear to be great; the subject had the MI while in his 70s, had 2 major risk factors, and no family history of ischemic heart disease.

Conclusion MEF2A polymorphism does not contribute appreciably to MI in the Japanese population. (*Circ J* 2005; **69**: 1192–1195)

Key Words: MEF2A; Myocardial infarction; Polymorphisms

Myocardial infarction (MI) is a multifactorial disease caused by environmental and genetic factors. There is an increasing number of studies that have identified the genes contributing to ischemic heart diseases (IHD)[1–8] and recently, a mutation in the human *MEF2A* gene was reported as responsible for an autosomal dominant form of coronary artery disease (CAD)? The 7-amino acid deletion disrupts the nuclear localization of the mature protein and reduces MEF2A-induced transcriptional activation? The same authors have reported MEF2A missense mutations in 4 of 207 sporadic CAD cases and estimated that MEF2A mutations contribute to approximately 2% of CAD![9] On the other hand, Weng et al recently reported a lack of MEF2A mutations in 300 CAD cases![1] so the purpose of the present study was to assess the significance of MEF2A mutations in Japanese subjects with MI.

Methods

Study Population

The control group consisted of 589 subjects recruited from the Suita study who were at least 60 years of age with no cardiovascular disease and no family history IHD. The selection criteria and design of the Suita Study have been described previously![12–14] We excluded young subjects from the control group, because they might develop MI in their 50s and 60s. The MI group consisted of 379 randomly selected inpatients and outpatients with documented MI who were admitted to the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003 (MI group)![15] The characteristics of the study population are shown in Table 1. All subjects gave written informed consent and the present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center.

DNA Studies

A promoter region and all of the 12 exon regions were sequenced in 190 of the subjects with MI (Fig 1) and we found variations that altered the amino acid sequences in exon 12 only. Next, we sequenced exon 12 in 589 control subjects and the remaining 189 subjects with MI. The variations in exon 12 were all determined by sequencing.

Statistical Analysis

All statistical analyses were performed with the JMP

(Received May 30, 2005; revised manuscript received June 29, 2005; accepted July 5, 2005)

National Cardiovascular Center, Suita, Japan
Mailing address: Naoharu Iwai, MD, Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita 565-8565, Japan.
E-mail: iwai@ri.ncvc.go.jp

Table 1 Characteristics of the Study Population

	Control	MI	p-value
n	584	379	
Gender (% male)	49.3	85.5	<0.0001
Age (years)	70.6±0.3	58.0±0.4	<0.0001
BMI (kg/m^2)	22.67±0.12	23.83±0.115	<0.0001
TC (mg/dl)	207.7±1.4	199.7±2.3 (n=224)	0.0030
HDL-C (mg/dl)	58.8±0.6	43.0±1.1 (n=194)	<0.0001
Smoking			<0.0001
Current	93	228	
Past	163	74	
Never	328	77	
DM (%)	7.7	38.7	<0.0001
HT (%)	35.5	52.9	<0.0001

Data are mean±standard error. Differences between 2 groups (Control vs myocardial infarction (MI)) were calculated by t-test or χ^2 analysis.
BMI, body mass index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; DM, diabetes mellitus; HT, hypertension.

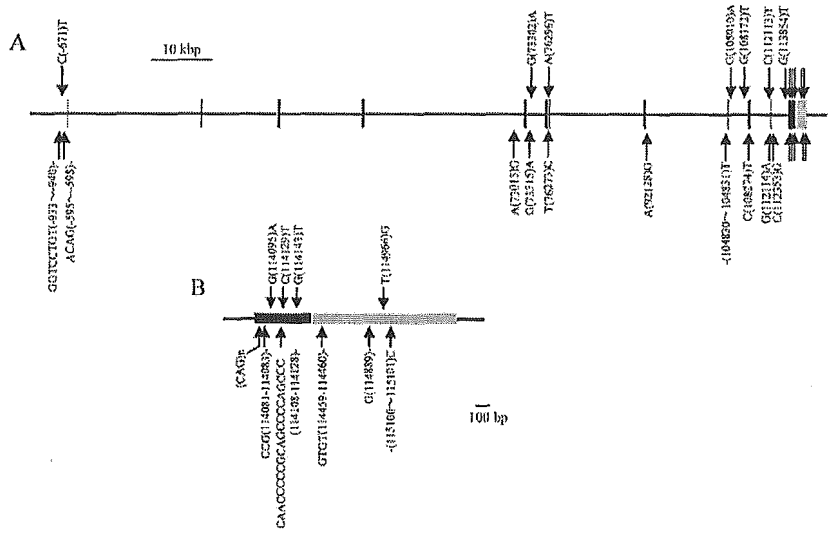

Fig 1. Scheme of the MEF2A gene. (A) Promoter region and all of the 12 exon regions are shown. (B) Expanded region of exon 12. The 5'- and 3'-UTR regions are indicated by gray boxes, and coding regions are indicated by black boxes. The 27 polymorphisms that were found are indicated by arrows.

statistical package (SAS Institute Inc, Cary, NC, USA) unless otherwise stated. Chi-squared analysis was performed to compare haplotype frequencies between the control and MI groups.

Results

We found 27 variations in MEF2A (Table 2), and 4 variations in exon 12 that altered the amino acid sequence of the MEF2A protein (Fig 2). The number of polyglutamine tandem repeats (region A) varied between 4 and 15 (genotype 1), and the number of proline tandem repeats (region B) varied between 4 and 5 (genotype 2). The 21-bp deletion (7-amino acid deletion), which was originally implicated in an autosomal dominant form of CAD[9] was also observed in region C (genotype 3) (Fig 2). We found one nonsense mutation (R447X) in exon 12 in a MI subject, and it was localized just downstream of the 21-bp deletion site (Fig 3).

The haplotype frequencies defined by the 3 genotypes are shown in Table 3: there were no significant differences between the control and MI groups.

Discussion

Wang et al reported that a mutation in the human MEF2A gene was responsible for an autosomal dominant form of CAD[9] but Weng et al could not find any MEF2A mutations in 300 cases of CAD[11] Thus, the association between mutations of MEF2A and CAD is controversial[16,17] and our results favors a lack of association.

Our results indicate that length polymorphisms in MEF2A do not contribute appreciably to MI in the Japanese population. Furthermore, the 21-bp deletion in MEF2A, which was originally implicated[9] did not seem to be associated with MI. The nonsense mutation (R447X) may affect susceptibility to MI, but the particular patient with this mutation had the MI in his 70s, and had no family history of IHD. He also had 2 risk factors: diabetes mellitus and smoking. Thus, the impact of this mutation does not appear to be great.

We sequenced all the coding regions of MEF2A in 190 subjects with MI and found neither missense nor nonsense mutations, except for R447X. Taking all our results together, MEF2A polymorphism does not appear to contribute appreciably to MI in the Japanese population.

Acknowledgments

This study was supported by the Ministry of Health, Labour and Welfare, a grant-in-aid from the Salt Science Research Foundation (No.05C5), and a grant-in-aid from the Uehara Memorial Foundation. The Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

References

1. Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D, et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 1992; 359: 641–644.
2. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 2002; 32: 650–654.
3. Mukae S, Aoki S, Itoh S, Sato R, Nishio K, Iwata T, et al. Mitochondrial 5178A/C genotype is associated with acute myocardial infarction. *Circ J* 2003; 67: 16–20.
4. Ozaki K, Inoue K, Sato H, Iida A, Ohnishi Y, Sekine A, et al. Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxin-alpha secretion in vitro. *Nature* 2004; 429: 72–75.
5. Ogawa M, Abe S, Biro S, Saigo M, Kihara T, Setoyama S, et al. R353Q polymorphism, activated factor VII, and risk of premature myocardial infarction in Japanese men. *Circ J* 2004; 68: 520–525.
6. Wang X, Ria M, Kelmenson PM, Eriksson P, Higgins DC, Samnegard A, et al. Positional identification of TNFSF4, encoding OX40 ligand, as a gene that influences atherosclerosis susceptibility. *Nat Genet* 2005; 37: 365–372.
7. Ozturk O, Ulgen MS, Tekes S, Ozturk U, Topruk N. Influence of

Table 2 The 27 Variations Found in the MEF2A Gene

Region	Polymorphisms	Sequence around polymorphisms	Amino acid mutation
Promoter	GGTCCTGT(–933~–940)–	AGACATTTTAATTTCACCTC[GGTCCTGT/–] GGTCCTGTGGTCCTGTGATC	
	C(–671)T	TGGCCGACGCTTAAGGAAAA[C/T] AGAAAAGAACCTACATGAAT	
	ACAG(–595~–598)–	TTTATTAATGTGAATCATAG[ACAG/–] CCATCAGTCTTCTATTCATT	
Intron 4	A(73015)G	CCTGTAAGTACTTTTACTTT[A/G] CCTCTACTTTTTATTTGTTG	
Intron 5	G(73302)A	GGTGATGACAACAATAAGTA[G/A] AAGGGAAGAAATGCATTTTA	
	G(73315)A	ATAAGTAGAAGGGAAGAAAT[G/A] CATTTTATTAGTATTTTTTA	
Intron 6	T(76273)C	CTTTGATGAGCAAGAATCAC[T/C] GATACATAATTGGCCTCATT	
	A(76296)T	TACATAATTGGCCTCATTTT[A/T] AAGTATTTTTTTGAAAATCA	
Intron 8	A(92128)G	GTATTTGAAGAGACAAGTCT[A/G] TTTCAAATACACAAAAATCA	
	–(104830~104831)T	TAGTGTCCAAATATGTTTTT[T/–] CTAAAGAAATATTTTGTTTG	
Intron 9	G(105010)A	CGTGTTGTTATCTAACCAAT[G/A] TTCCCTTTGTTACACAAATT	
	G(108172)T	CAGTGCTTCAGAAAATGACA[T/G] TCATATGAAACTGTGAAAAA	
Exon 10	C(108274)T	TCTTTTTTGATCTCACAGAA[T/C] ACCCAGAGGATCAGTAGTTC	N297N
Intron 10	C(112113)T	AGGAACTTTTGCAGTAGCTA[C/T] GTAAAAAATAGATTCCGTA	
	G(112114)A	GGAACTTTTGCAGTAGCTAC[G/A] TAAAAAATAGATTCCGTATG	
Intron 11	C(112353)G	TTACTCCTGGCCTACACACT[C/G] TCTTTTCCTATCAGTGACAG	
	G(113854)T	CTCTGGGCCCTTTTCCATCA[G/T] GCAGTGTCTCTACTGTATCA	
Exon 12	(CAG)n(n=4–15) (114048–114080)	GTATGACCCCATCGGGCTTC[(CAG)n] CCGCCGCCACCACCGCAGCC	(Q)n (n=4–15)
	CCG(114081–114083)–	AGCAGCAGCAGCAGCAGCAG[CCG/–] CCGCCACCACCGCAGCCCA	(P)n (n=4 or 5)
	G(114095)A	CAGCAGCCGCCGCCACCACC[G/A] CAGCCCCAGCCACAACCCCC	P435P
	CAACCCCCGCAGCCCCAGC CC(114108–114128)–	CACCACCGCAGCCCCAGCCA[CAACCCCCGCA GCCCCAGCCC/–]CGACAGGAAATGGGGCGCTC	7 amino acid deletion
Exon 12	C(114129)T	AACCCCCGCAGCCCCAGCCC[C/T] GACAGGAAATGGGGCGCTCC	R447X
	G(114143)T	CAGCCCCGACAGGAAATGGG[G/T] CGCTCCCCTGTGGACAGTCT	G451G
3'-UTR	GTGT(114459–114460)-	ATATATGTATGTGGGTGTGA[-/GTGT] GTGTGTATGTGTGGGTGTGT	
	G(114889)-	AAAACAAACAAAACAAAAAA[G/-] CCCCACACATAACTGTTTTG	
	T(114966)G	TAGCTAATAAAGAAAGAGAA[T/G] AGAAAACACGCATGAGATAT	
	-(115100~115101)C	TCATATCTTAAAAATTAAAG[-/C] AAACTGATTTTAGCTCATGT	

In the polymorphisms found in the exons, mutations of the amino acid sequence are also indicated.

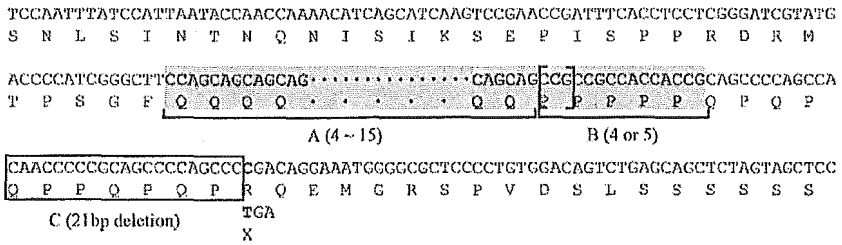

Fig 2. Nucleotide and amino acid sequences of the region containing the 4 variations in exon 12 of MEF2A. The gray box marks the polyglutamine and proline tandem repeats: the number of polyglutamine tandem repeats varies between 4 and 15, the number of proline tandem repeats varies between 4 and 5. The 21-bp deletion site is indicated by a box. The nonsense mutation (R447X) site is localized just downstream to the 21-bp deletion site.

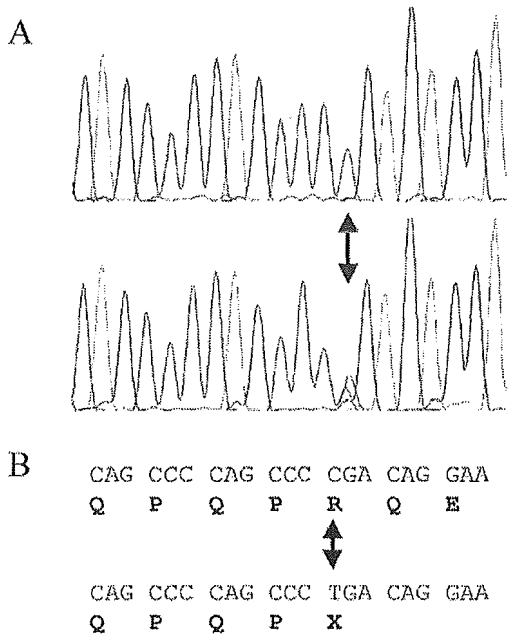

Fig 3. MEF2A nonsense mutation R447X in exon 12 in the subject with myocardial infarction (MI). (A) Sequence analysis of the control and MI subject indicated a C to T substitution at codon 447 in exon 12 of MEF2A. This mutation changes the amino acid residue arginine to stop codon (B).

angiotensin-converting enzyme I/D gene polymorphism on the right ventricular myocardial performance index in patients with a first acute anterior myocardial infarction. *Circ J* 2005; 69: 211–215.

8. Momiyama Y, Ohmori R, Nagano M, Kato R, Taniguchi H, Egashira T, et al. Polymorphism of the 3'-untranslated region of interleukin-12 p40 gene is not associated with the presence or severity of coronary artery disease. *Circ J* 2005; 69: 793–797.
9. Wang L, Fan C, Topol SE, Topol EJ, Wang Q. Mutation of MEF2A in an inherited disorder with features of coronary artery disease. *Science* 2003; 302: 1578–1581.
10. Bhagavatula MR, Fan C, Shen GQ, Cassano J, Plow EF, Topol EJ, et al. Transcription factor MEF2A mutations in patients with coronary artery disease. *Hum Mol Genet* 2004; 13: 3181–3188.

Table 3 Frequencies of Haplotypes Defined by the 3 Genotypes

Haplotype	*C (numbers)*	*% (row)*	*MI (numbers)*	*% (row)*
A4B5C+	*26*	*2.23*	*10*	*1.32*
A5B5C+	*2*	*0.17*	*5*	*0.66*
A5B4C+	*1*	*0.09*	*0*	*0.00*
A6B5C+	*2*	*0.17*	*2*	*0.26*
A7B5C+	*5*	*0.43*	*2*	*0.26*
A8B5C+	*3*	*0.26*	*8*	*1.05*
A9B5C+	*396*	*33.90*	*263*	*34.61*
A9B5C–	*3*	*0.26*	*3*	*0.39*
A9B4C+	*78*	*6.68*	*50*	*6.58*
A10B5C+	*140*	*11.99*	*96*	*12.63*
A10B4C+	*0*	*0.00*	*2*	*0.26*
A11B5C+	*475*	*40.67*	*298*	*39.21*
A11B4C+	*6*	*0.51*	*1*	*0.13*
A12B5C+	*5*	*0.43*	*6*	*0.79*
A12B4C+	*0*	*0.00*	*1*	*0.13*
A14B5C+	*22*	*1.88*	*11*	*1.45*
A15B5C+	*4*	*0.34*	*2*	*0.26*
	1,168		*760*	

The frequencies of haplotypes defined by genotypes 1–3 in the control (C) or myocardial infarction (MI) groups are shown. The haplotypes are defined as follows: A represents the number of polyglutamine tandem repeats between 4 and 15 (region A in Fig 2); B represents the number of proline tandem repeats between 4 and 5 (region B); and C+ or – represents the existence or deletion of the 21-bp nucleotide (region C).

11. Weng L, Kavaslar N, Ustaszewska A, Doello H, Schackwitz W, Hebert S, et al. Lack of MEF2A mutations in coronary artery disease. *J Clin Invest* 2005; 115: 1016–1020.
12. Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: The Suita study. *Stroke* 1997; 28: 518–525.
13. Shioji K, Mannami T, Kokubo Y, Inamoto N, Takagi S, Goto Y, et al. Genetic variants in PCSK9 affect the cholesterol level in Japanese. *J Hum Genet* 2004; 49: 109–114.
14. Kokubo Y, Iwai N, Tago N, Inamoto N, Okayama A, Yamawaki H, et al. Association analysis between hypertension and CYBA, CLCNKB, and KCNMB1 functional polymorphisms in the Japanese population: The Suita Study. *Circ J* 2005; 69: 138–142.
15. Shioji K, Mannami T, Kokubo Y, Goto Y, Nonogi H, Iwai N. An association analysis between ApoA1 polymorphisms and the high-density lipoprotein (HDL) cholesterol level and myocardial infarction (MI) in Japanese. *J Hum Genet* 2004; 49: 433–439.
16. Wang Q, Rao S, Topol EJ. Miscues on the "lack of MEF2A mutations" in coronary artery disease. *J Clin Invest* 2005; 115: 1399–1400.
17. Weng L, Cohen J, McPherson R, Pennacchio LA. Response to Wang et al. *J Clin Invest* 2005; 115: 1400.

Original Article

Functional Confirmation of Gitelman's Syndrome Mutations in Japanese

Hiroaki NARABA, Yoshihiro KOKUBO, Hitonobu TOMOIKE, and Naoharu IWAI

Gitelman's syndrome is an autosomal recessive inherited renal tubular disorder resulting from loss-of-function mutations in the thiazide-sensitive sodium chloride cotransporter gene (*SLC12A3*). We have previously reported that the combined allele frequency for the reported Gitelman's syndrome mutations is 0.0321. However, almost all of the reported Gitelman's syndrome mutations were from case reports without functional confirmation. In the present study, we assessed the functionality of the two most prevalent mutations in Japanese, T180K and L849H, using a mammalian cell expression system. Human *SLC12A3* cDNA was transiently expressed in Chinese hamster ovary (CHO) cells under the control of a cytomegalo virus (CMV) promoter. The T180K and L849H mutations were introduced by site-directed mutagenesis. The activity of the Na^+-Cl^- cotransporter was assessed by measuring tracer $^{22}Na^+$ uptake. While the T180K variation was just a polymorphism, the L849H mutation was confirmed to be a loss-of-function mutation and appears to be responsible for the Gitelman's syndrome. This observation may have very important clinical implications, since the allele frequency of this variation is 0.0126. (*Hypertens Res* 2005; 28: 805–809)

***Key Words*: *SLC12A3*, Gitelman's syndrome, blood pressure**

Introduction

Gitelman's syndrome (GS) is an autosomal recessive inherited renal tubular disorder resulting from loss-of-function mutations in the thiazide-sensitive sodium chloride cotransporter gene (*SLC12A3*) (*1*). The clinical features of GS resemble the effects of thiazide administration, and the reduced salt reabsorption seen in GS seems to be associated with resistance to hypertension (*2*).

In the previous study, we established a TaqMan system for 9 GS mutations reported in Japanese (T180K, A569V, L623P, R642C, L849H, A242V, F846X, R955Q, and an 18-bp insertion at 794–811), and investigated the frequencies of these mutations in the Suita Study. We found 56 subjects heterozygous with the 180K allele, 14 subjects heterozygous with the 569V allele, 1 subject with the 623P allele, 1 subject with the 642C allele, 47 subjects with the 849H allele, and 1 compound heterozygous subject with the 180K and 849H alleles, in total of 1,852 subjects (*3*). Thus, the combined allele frequency of the GS mutations was 0.0321. However, identification of these mutations is all based on case reports; functional analyses of the mutations have never been performed. The allele frequencies of the T180K and L849H mutations are 0.0151 and 0.0126, respectively, and these were the two most frequent mutations in our study population. The purpose of the present study was to determine whether these two GS mutations were really loss-of-function mutations. We also investigated phenotypes of subjects heterozygous with these GS mutations.

From the National Cardiovascular Center, Suita, Japan.

The present study was supported by research grants from the Ministry of Health, Labor and Welfare, the Salt Science Research Foundation (05C5) and the Uehara Memorial Foundation.

Address for Reprints: Naoharu Iwai, M.D., Ph.D., Department of Epidemiology, Research Institute, Division of Cardiology, National Cardiovascular Center, 5–7–1 Fujishirodai, Suita 565–8565, Japan. E-mail: iwai@ri.ncvc.go.jp

Received June 30, 2005; Accepted in revised form August 7, 2005.

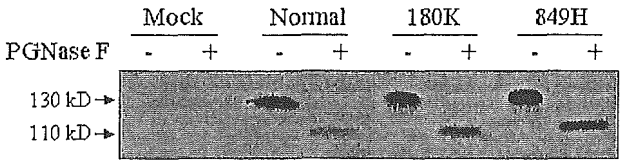

Fig. 1. *Western blot analysis of expressed mutants. Membrane fractions from Chinese hamster ovary cells transfected with empty (Mock), wild-type SLC12A3 (Normal), or mutant (180K and 849H) vector were treated with* N*-glycosidase F (PGNase F) and subjected to Western blotting with polyclonal antibodies that recognize the V5 tag as described in the Methods section.*

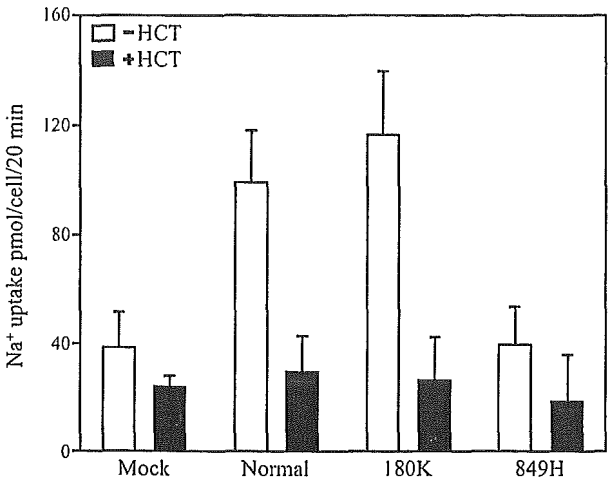

Fig. 2. *Thiazide-sensitive $^{22}Na^+$ uptake in Chinese hamster ovary cells (CHO). CHO cells were transiently transfected with empty (Mock), wild-type SLC12A3 (Normal), or mutant (180K and 849H) vector and analyzed for $^{22}Na^+$ uptake 36 h after transfection as described in the Methods section. Uptake was assessed in the absence (white bars) or presence (solid bars) of 100 μmol/l hydrochlorothiazide (HCT) in the uptake medium. Each bar represents the mean±SEM of four wells from three different experiments (n=12). The mean value of the $^{22}Na^+$ uptake in the presence of HCT was considered as a background value, and was subtracted from each value in the absence of HCT. One way ANOVA indicated that the values of the four groups were significantly different ($p<0.0001$). Subsequent Tukey-Kramer's HSD test indicated that the values of the 849H allele were significantly different from those of the normal allele ($p<0.01$).*

Methods

Construction of the SLC12A3 Expression Vector

Human *SLC12A3* cDNA was synthesized by reverse transcription–polymerase chain reaction (RT-PCR) amplification from a human kidney and was subcloned into the pcDNA-3.1/V5-His vector (Invitrogen, Carlsbad, USA). The V5-His epitope was fused to the carboxyl terminal of the human *SLC12A3* cDNA. The T180K and L849H mutations were introduced by using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, USA).

Cell Culture and Transient Transfection

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 unit/ml of penicillin, and 100 μg/ml streptomycin. Cells were transiently transfected with 1 μg of *SLC12A3* expression plasmids and 0.1 μg of a green fluorescent protein expression vector, phMGFP vector (Promega, Madison, USA), using Lipofectamine^{2000} (Invitrogen) according to the manufacturer's recommendations. Transgene expression was normalized from the fluorescence intensity of the green fluorescent protein.

$^{22}Na^+$ Uptake Assay

Functional analysis of the Na^+-Cl^- cotransporter was assessed by measuring tracer $^{22}Na^+$ uptake as described by De Jong *et al.* (*4*). At 36 h after transfection, cells were transferred to Cl^--free medium containing 5 mmol/l HEPES/Tris (pH 7.4), 96 mmol/l sodium gluconate, 2 mmol/l potassium gluconate, 1.8 mmol/l calcium gluconate, 1 mmol/l $Mg(NO_3)_2$, 2.5 mmol/l sodium pyruvate, and 5 μg/ml gentamycin and incubated for 3 h. Cells depleted of Cl^- were transferred to uptake medium containing 20 mmol/l HEPES/Tris (pH 7.4), 72 mmol/l *N*-methyl-D-glucosamine-HCl, 48 mmol/l NaCl, 5 mmol/l KCl, 2 mmol/l $Na_2H_2PO_4$, 1 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 0.5 mmol/l ouabain, 100 μmol/l amiloride, 100 μmol/l bumetanide, and 1 μCi/ml of $^{22}Na^+$ and incubated for 20 min at room temperature with or without 100 μmol/l hydrochlorothiazide. Under these conditions, $^{22}Na^+$ uptake increased nearly linearly between 5 and 30 min of incubation time. The uptake reaction was stopped by washing the cells six times with ice-cold uptake medium. After the cells were dissolved in 10% sodium dodecyl sulfate, tracer radioactivity was determined by a γ counter.

Immunoblotting

The CHO cells were harvested, suspended, and broken by sonication in ice-cold buffer (0.32 mol/l sucrose, 5 mmol/l Tris-HCl, pH 7.5, and 2 mmol/l EDTA). The cell lysate was centrifuged at 3,000 × *g* for 10 min. Then the supernatant was centrifuged at 100,000 × *g* for 1 h. The pellet was resuspended in buffer containing 5 mmol/l Tris-HCl, pH 7.5, and 2 mmol/l EDTA and used as the membrane fraction. The membrane fraction was subjected to digestion with 0.25 unit of *N*-glycosidase F (Roche Molecular Biochemicals, Mannheim,

Table 1. Characteristics According to Genotypes

	T180K			L849H		
	TT	TK	*p*	LL	LH	*p*
N	3,510	106		3,534	82	
Age	64.7±11.3	65.2±10.4	ns	64.7±11.3	62.6±10.6	0.09
BMI (kg/m^2)	22.8±3.1	22.7±3.0	ns	22.8±3.1	22.5±3.1	ns
SBP (mmHg)	129.3±19.7	132.1±19.6	ns	129.4±19.6	128.2±18.2	ns
DBP (mmHg)	77.7±10.2	77.6±10.1	ns	77.8±10.2	77.2±9.7	ns
ResSBP (mmHg)	−0.1±19.4	2.4±17.6	ns	0.0±17.6	0.6±16.8	ns
ResDBP (mmHg)	0.0±9.7	0.1±10.0	ns	0.0±9.7	−0.3±9.2	ns
HTN (%)	29.7	32.1	ns	29.9	29.3	ns
K (mmol/l)	4.3±0.3	4.3±0.3	ns	4.3±0.3	4.2±0.2	0.03
Mg (mg/dl)	2.5±0.4	2.5±0.4	ns	2.5±0.4	2.5±0.4	ns
U-pH	6.0±0.8	6.1±0.9	ns	6.0±0.8	6.2±0.9	0.06

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ResSBP, residuals of SBP adjusting for age and BMI; ResDBP, residuals of DBP adjusting for age and BMI; HTN, prevalence of hypertension; K, serum potassium level; Mg, serum Mg level; U-pH, urine pH. Values are expressed as mean±SD.

Germany) for 12 h at 37°C. The reaction was terminated with 6 mmol/l Tris-HCl (pH 6.8), 1% SDS, 1 mmol/l DTT, and boiling for 3 min. Samples were separated by SDS-PAGE and electroblotted onto a PVDF membrane. The protein blot was blocked with 5% nonfat dry milk in Tris-buffered saline (10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl) at room temperature for 2 h. The blots were then probed with anti-V5 antibody (Invitrogen) diluted 1:2,000 in Tris-buffered saline for 2 h at room temperature, washed five times with Tris-buffered saline, probed with horseradish peroxidase-conjugated secondary antibody in blocking buffer, and washed as before. The signals were detected using an enhanced chemiluminescence Western blotting kit (Amersham Biosciences, Uppsala, Sweden).

Study Population

The selection criteria and design of the Suita Study have been reported previously (*5–8*). The genotypes of the T180K and L849H mutations were determined by the TaqMan™ method in 3616 consecutive subjects enrolled between April 2002 and March 2004, who gave their informed consent for genetic analysis. The ethics committee of the National Cardiovascular Center approved the study protocol.

Statistical Analysis

Values are expressed as the mean±SD or the mean±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute, Inc., Cary, USA). Residuals of blood pressure values were calculated by adjusting for age and body mass index (BMI). Residuals represent the difference between the actual blood pressure value for each observation and the value predicted on the basis of age and BMI. For the $^{22}Na^+$ uptake assay, one way ANOVA followed by Tukey-Kramer's HSD test ($p<0.01$) was applied.

Results

Expression and Maturation of SLC12A3 in Transiently Transfected CHO Cells

To assess the expression of wild-type and mutant SLC12A3 protein transfected in CHO cells, membrane fractions were subjected to Western blotting with polyclonal antibodies that recognize the V5 tag. Bands of approximately 130 kD appeared in the wild-type and the 180K and 849H mutants (Fig. 1). The SLC12A3 cotransporter is a glycoprotein. The *N*-linked glycosylation of membrane proteins may play a role in protein folding and membrane targeting (*9*). The effect of glycosylation on the functional activity of SLC12A3 has been described previously (*10*). For determination of the maturation of SLC12A3, membrane fractions were treated with *N*-glycosidase F (PGNase F), which cleaves all types of *N*-glycans. Bands of 130 kD nearly disappeared after treatment with PGNase F, and digested proteins with a molecular size of approximately 100 kD migrated (Fig. 1). These data demonstrate that the wild-type and mutant SLC12A3 cotransporters are expressed as glycosylated proteins on the cell surface of CHO cells.

Thiazide-Sensitive $^{22}Na^+$ Uptake in Transiently Transfected CHO Cells

The functionality of the SLC12A3 cotransporter expressed in CHO cells was determined by measuring thiazide-sensitive $^{22}Na^+$ uptake. Figure 2 shows that $^{22}Na^+$ uptake was significantly increased in cells expressing the wild-type SLC12A3 cotransporter as compared with the corresponding mock-transfected cells. Importantly, hydrochlorothiazide (100

μmol/l) reduced $^{22}Na^+$ uptake to the levels observed in mock-transfected cells. Then, we investigated the $^{22}Na^+$ uptake of the mutant SLC12A3 proteins. As shown in Fig. 2, the $^{22}Na^+$ uptake of the 180K mutant SLC12A3 protein was not decreased as compared with that of the normal type. However, the $^{22}Na^+$ uptake of the 849H mutant SLC12A3 protein was significantly reduced ($p<0.01$, one-way ANOVA followed by Tukey-Kramer's HSD test, compared to the normal allele SLC12A3 protein) to almost none, although the mutant protein was expressed at the cell surface (Fig. 1). Thus, it was confirmed that the L849H mutation is a loss-of-function mutation.

Characteristics of Heterozygous Subjects with the L849H Allele

Once the L849H mutation was confirmed as a loss-of-function mutation, we reassessed the characteristics of subjects with the L849H allele. Of the 3,616 subjects enrolled in this study, 82 subjects were heterozygous with the L849H allele. The allele frequency is 0.0113. Table 1 shows the characteristics of these heterozygous subjects. The serum potassium levels were slightly lower in the heterozygous subjects, and a tendency of metabolic alkalosis (high urine pH) was also observed in the heterozygous subjects. However, serum magnesium levels and blood pressure levels were not lower in the heterozygous subjects than in the wild-type subjects. Thus, heterozygous subjects with the 849H allele did not seem to exhibit mild GS features.

Discussion

We have previously reported that the combined allele frequency for the reported GS mutations is 0.0321 (*3*). However, almost all of the reported GS mutations were from case reports without functional confirmation (*2*, *3*). It is possible that the reported mutations were simply polymorphisms and the true mutations were in linkage with the polymorphisms in the reported pedigree. Without functional confirmation, we cannot neglect this hypothesis. Thus, in the present study, we assessed the functionality of the two most prevalent mutations, T180K and L849H, which are suspected to be responsible for GS.

Although the CHO cell is not a tubular cell that naturally expresses the SLC12A3 cotransporter, we chose this cell line for its high efficiency in transfection and its lack of intrinsic activity of the cotransporter. Previously, the transporter activity has been assessed using a Xenopus oocyte system. However, the Xenopus oocyte is not a mammalian cell and may have a different protein processing system. As shown in the present study, the activity of the normal type SLC12A3 cotransporter was reliably assessed in the CHO system.

The present study suggests that the T180K variation is simply a polymorphism and may not be responsible for GS. Moreover, the T180K polymorphism resides in the second transmembrane domain, and the polymorphism does not seem to influence the hydropathy plot of the protein (data not shown). There has been only one study in which the T180K polymorphism (mutation) was reported to be responsible for GS (*11*). The affected families were collected at Tokyo or Tochigi (not specified in the paper). An unknown mutation in linkage disequilibrium with this polymorphism may be responsible for GS. This might be confirmed by re-sequencing the entire *SLC12A3* (including the promoter and intronic regions) in the affected families.

On the other hand, the L849H mutation has been revealed to be a loss-of-function mutation and appears to be responsible for GS. Moreover, this mutation has been reported to be responsible for GS in at least three reports (*11–13*). Monkawa *et al.* (*11*) reported that 1 of their 6 subjects with GS (from Tokyo and Tochigi) was heterozygous for this mutation, Fukuyama *et al.* (*12*) reported that 1 of their 7 GS subjects (from Okinawa) was heterozygous for it, and Maki *et al.* (*13*) reported that 2 of their 8 GS subjects (from Akita) showed compound heterozygosity for the mutation. This reproducibility further strengthens the hypothesis that the L849H mutation is responsible for GS. The L849H mutation resides in the carboxyl-terminal cytoplasmic region of the SLC12A3 protein. Although the function of this domain remains to be clarified, four GS mutations (2543–2544 delTT, L849H, R955Q, R1008X) have been reported in this domain in Japanese. We have also confirmed that the R1008X mutation is really a loss-of-function mutation (data not shown). Thus, this carboxyl-terminal cytoplasmic domain seems to play important roles in the functions of SLC12A3, although the precise roles remain to be determined (*14*).

The subjects heterozygous with the 849H allele did not seem to exhibit mild GS features. However, it is possible that those heterozygous subjects may mask GS features by compensating for their tubular derangement by increasing their sodium uptake, as reported by Cruz *et al.* (*2*), and may exhibit GS features under a low sodium diet or diuretics administration. This awaits further investigation.

Acknowledgements

We thank Ms. Naomi Tago for her technical assistance. We also thank Professor Yoshihisa Kurachi for helpful discussions.

References

1. Simon DB, Nelson-Williams C, Bia MJ, *et al*: Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* 1996; **12**: 24–30.
2. Cruz DN, Simon DB, Nelson-Williams C, *et al*: Mutations in the Na-Cl cotransporter reduce blood pressure in human. *Hypertension* 2001; **37**: 1458–1464.
3. Tago N, Kokubo Y, Inamoto N, *et al*: A high prevalence of Gitelman's syndrome mutations in Japanese. *Hypertens Res* 2004; **27**: 327–331.

4. De Jong JC, Willems PH, Goossens M, *et al*: Effects of chemical chaperones on partially retarded NaCl cotransporter mutants associated with Gitelman's syndrome in a mouse cortical collecting duct cell line. *Nephrol Dial Transplant* 2004; **19**: 1069–1076.
5. Mannami T, Baba S, Ogata J: Potential of carotid enlargement as a useful indicator affected by high blood pressure in a large general population of a Japanese city: the Suita study. *Stroke* 2000; **31**: 2958–2965.
6. Ono K, Iwanaga Y, Mannami T, *et al*: Epidemiological evidence of an association between *SLC6A2* gene polymorphism and hypertension. *Hypertens Res* 2003; **26**: 685–689.
7. Iwai N, Katsuya T, Mannami T, *et al*: Association between SAH, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. *Circulation* 2002; **105**: 41–47.
8. Shioji K, Kokubo Y, Mannami T, *et al*: Association between hypertension and the α-adducin, β1-adrenoreceptor and G-protein β3 subunit genes in the Japanese population; the Suita Study. *Hypertens Res* 2004; **27**: 31–37.
9. Freeze HH: Human disorders in *N*-glycosylation and animal models. *Biochim Biophys Acta* 2002; **1573**: 388–393.
10. De Jong JC, Van Der Vliet WA, Van Den Heuvel LP, *et al*: Functional expression of mutations in the human NaCl cotransporter: evidence for impaired routing mechanisms in Gitelman's syndrome. *J Am Soc Nephrol* 2002; **13**: 1442–1448.
11. Monkawa T, Kurihara I, Kobayashi K, Hayashi M, Saruta T: Novel mutations in thiazide-sensitive Na-Cl cotransporter gene of patients with Gitelman's syndrome. *J Am Soc Nephrol* 2000; **11**: 65–70.
12. Fukuyama S, Okudaira S, Yamazato S, Yamazato M, Ohta T: Analysis of renal tubular electrolyte transporter genes in seven patients with hypokalemic metabolic alkarosis. *Kidney Int* 2003; **64**: 808–816.
13. Maki N, Komatsuda A, Wakui H, *et al*: Four novel mutations in the thiazide-sensitive Na-Cl co-transporter gene in Japanese patients with Gitelman's syndrome. *Nephrol Dial Transplant* 2004; **19**: 1761–1766.
14. Gamba G: Molecular physiology and pathophysiology of electroneutral cation-chroride cotransporters. *Physiol Rev* 2005; **85**: 423–493.

Original Article

Assessment of the MicroRNA System in Salt-Sensitive Hypertension

Hiroaki NARABA* and Naoharu IWAI*,**

Most animal microRNAs are imperfectly complementary to their mRNA targets and inhibit protein synthesis through an unknown mechanism. MicroRNAs have been reported to play important roles in a number of biological processes. We assessed the microRNA system in Dahl salt-sensitive rats in order to investigate possible roles of microRNA in salt-sensitive hypertension. We constructed microRNA libraries from the kidneys of Dahl salt-sensitive and Lewis rats taking normal or high-salt diets (4 groups), and identified 91 previously reported and 12 new microRNAs expressed in the kidney. We then used Northern blotting to assess the expression levels of 118 microRNAs in the kidneys and heart ventricles. No significant differences in microRNA expression profiles were observed among the 4 groups. Thus, the microRNA system seemed to be unlikely to contribute to salt-sensitive hypertension in Dahl salt-sensitive rats. (*Hypertens Res* 2005; 28: 819–826)

***Key Words*: microRNA, hypertension, Dahl salt-sensitive rat, hypertrophy**

Introduction

MicroRNAs constitute a growing class of non-coding RNAs (*1*, *2*). Most animal microRNAs are imperfectly complementary to their mRNA targets and inhibit protein synthesis through an unknown mechanism (*3*). MicroRNAs have been reported to modulate hematopoietic lineage differentiation (*4*), adipocyte differentiation (*5*), insulin secretion (*6*) and HIV-1 transcription (*7*), thus indicating that they could play important roles in a number of biological processes. The high degree of phylogenetic conservation of pre-microRNA sequences also supports the importance of this biological system (*8*).

Dahl salt-sensitive rats (DS) are one of the most prevalently used animal models of salt-sensitive hypertension (*9–12*). Although more than 16 quantitative trait loci (QTLs) have been identified in this model (*13*), most of the genes responsible for hypertension have not yet been identified (*14*). The precise mechanism of the increased salt retention in the kidneys of DS remains to be elucidated (*15*, *16*).

As described above, the microRNA system has been recognized to play important roles in various biological processes. It is thus possible that irregularities in this microRNA system are involved in the pathogenesis of hypertension in DS. The purpose of the present study was to address this hypothesis.

A microRNA cDNA library was constructed in order to identify the major microRNAs expressed in the kidney. The expression levels of more than 100 microRNA species were then assessed in the kidneys of DS and Lewis rats (LW). Moreover, microRNA expression profiles in the heart, one of the target organs of hypertension, were also investigated. A similar strategy has been employed by Poy *et al.* (*6*). They have identified 67 different microRNAs in the miRNA library

From the *Department of Epidemiology, Research Institute and **Department of Cardiology, National Cardiovascular Center, Suita, Japan.

The present study was supported by research grants from the Salt Research Foundation (05C5), from the Ministry of Health, Labor, and Welfare, and from the Uehara Memorial Foundation.

Address for Reprints: Naoharu Iwai, M.D., Ph.D., Department of Epidemiology, Research Institute, National Cardiovascular Center, 5–7–1 Fujishirodai, Suita 565–8565, Japan. E-mail: iwai@ri.ncvc.go.jp

Received May 11, 2005; Accepted in revised form July 28, 2005.

Table 1. MicroRNA (miRNA) Sequences Identified by Cloning from Kidney of Dahl Salt-Sensitive and Lewis Rats

miRNA[a]	Sequence (5′ to 3′)[b]	LW−[e]		LW+[e]		DS−[e]		DS+[e]	
		No. clones[c]	%[d]	No. clones[c]	%[d]	No. clones[c]	%[d]	No. clones[c]	%[d]
rno-let-7a	ugagguaguagguuguauaguu	6	1.6	16	4.0	7	1.9	4	1.0
rno-let-7b	ugagguaguagguuguguggguu	17	4.5	8	2.0	20	5.6	9	2.4
rno-let-7c	ugagguaguagguuguaugguu	12	3.2	10	2.5	17	4.7	8	2.1
rno-let-7d	agagguaguagguugcauagu			3	0.8	2	0.6		
rno-let-7e	ugagguaggagguuguauagu			1	0.3	3	0.8		
rno-let-7f	ugagguaguagauuguauaguu	10	2.7	3	0.8	3	0.8	7	1.8
mmu-let-7g[f]	ugagguaguaguuuguacagu	5	1.3	11	2.8	5	1.4	6	1.6
rno-let-7i	ugagguaguaguuugugcu			1	0.3	3	0.8	1	0.3
rno-miR-10a	uacccuguagauccgaauuugug	8	2.1	11	2.8	7	1.9	6	1.6
rno-miR-10b	uacccuguagaaccgaauuugu	5	1.3	3	0.8	7	1.9	4	1.0
mmu-miR-15a[f]	uagcagcacauaaugguuugug	5	1.3	3	0.8	2	0.6	4	1.0
rno-miR-15b	uagcagcacaucaugguuuaca	1	0.3					1	0.3
rno-miR-16	uagcagcacguaaauauuggcg	2	0.5	5	1.3	7	1.9	5	1.3
rno-miR-19b	ugugcaaauccaugcaaaacuga	1	0.3					1	0.3
rno-miR-20	uaaagugcuuauagugcaggua							1	0.3
rno-miR-21	uagcuuaucagacugauguuga	18	4.8	19	4.8	21	5.8	44	11.5
rno-miR-22	aagcugccaguugaagaacugu			1	0.3	1	0.3	1	0.3
rno-miR-23a	aucacauugccagggauuucc			1	0.3	3	0.8	1	0.3
rno-miR-23b	aucacauugccagggauuaccac	1	0.3	3	0.8	1	0.3	1	0.3
rno-miR-24	uggcucaguucagcaggaacag	6	1.6	4	1.0	8	2.2	6	1.6
rno-miR-25	cauugcacuugucucggucuga			1	0.3	1	0.3		
rno-miR-26a	uucaaguaauccaggauaggcu	6	1.6	5	1.3	8	2.2	6	1.6
rno-miR-26b	uucaaguaauucaggauagguu	61	16.3	65	16.3	50	13.9	59	15.5
rno-miR-27a	uucacaguggcuaaguuccgc			2	0.5	2	0.6	3	0.8
rno-miR-27b	uucacaguggcuaaguucug	1	0.3	2	0.5	3	0.8	3	0.8
rno-miR-28	aaggagcucacagucuauugag	1	0.3						
rno-miR-29a	cuagcaccaucugaaaucgguu	21	5.6	32	8.0	17	4.7	26	6.8
rno-miR-29b	uagcaccauuugaaaucagugu	14	3.7	19	4.8	6	1.7	14	3.7
rno-miR-29c	uagcaccauuugaaaucgguua	9	2.4	7	1.8	8	2.2	9	2.4
rno-miR-30a-5p	uguaaacauccucgacuggaagc	5	1.3	6	1.5	8	2.2	3	0.8
rno-miR-30a-3p	cuuucagucggauguuugcagc	1	0.3	8	2.0	1	0.3	2	0.5
rno-miR-30b	uguaaacauccuacacucagc			2	0.5	2	0.6	1	0.3
rno-miR-30c	uguaaacauccuacacucucagc	11	2.9	6	1.5	10	2.8	8	2.1
rno-miR-30d	uguaaacaucccccgacuggaag	5	1.3	1	0.3	3	0.8	3	0.8
rno-miR-30e	uguaaacauccuugacugga	1	0.3			2	0.6	3	0.8
rno-miR-32	uauugcacauuacuaaguugc							2	0.5
rno-miR-33	gugcauuguaguugcauug	8	2.1	1	0.3	5	1.4	2	0.5
cel-miR-83[f]	uagcaccauauaaaauucaguaa			1	0.3		0.0		
cel-miR-84[f]	ugagguaguauguaauauugu					1	0.3		
rno-miR-92	uauugcacuugucccggccug	2	0.5						
rno-miR-96	uuuggcacuagcacauuuuugcu			1	0.3			1	0.3
rno-miR-98	ugagguaguaaguuguauuguu					1	0.3		
rno-miR-99a	aacccguagauccgaucuugug					2	0.6	1	0.3
rno-miR-99b	cacccguagaaccgaccuugcg	2	0.5						
rno-miR-101	uacaguacugugauaacugaag	6	1.6	7	1.8	4	1.1	5	1.3
rno-miR-101b	uacaguacugugauagcugaag			1	0.3	1	0.3	1	0.3

Table 1. (Continued)

rno-miR-103	agcagcauuguacagggcuauga			3	0.8	1	0.3	1	0.3
rno-miR-106b	uaaagugcugacagugcagau			1	0.3				
rno-miR-125a	ucccugagacccuuuaaccugug	3	0.8	2	0.5	1	0.3	2	0.5
rno-miR-125b	ucccugagacccuaacuuguga			1	0.3	2	0.6	1	0.3
rno-miR-126	ucguaccgugaguaauaaugc	2	0.5	3	0.8	1	0.3	5	1.3
rno-miR-126*	cauuauuacuuuugguacgcg	60	16.0	60	15.0	59	16.4	44	11.5
rno-miR-130a	cagugcaauguuaaaagggc	2	0.5	3	0.8	1	0.3		
rno-miR-130b	ugcaaugaugaaggcuuau							1	0.3
rno-miR-135a	uauggcuuuuuauuccuauguga					1	0.3		
rno-miR-139	ucuacagugcacgugucu			1	0.3				
rno-miR-140	agugguuuuacccuaugguag	2	0.5	1	0.3	2	0.6	3	0.8
rno-miR-142-3p	uguaguguuuccuacuuuaugga	5	1.3	7	1.8	2	0.6	18	4.7
rno-miR-142-5p	cauaaaguagaaagcacuac	6	1.6			3	0.8	5	1.3
rno-miR-143	ugagaugaagcacuguagcuca	3	0.8	3	0.8	1	0.3	2	0.5
rno-miR-145	guccaguuuucccaggaaucccuu			1	0.3	1	0.3		
rno-miR-146	ugagaacugaauuccauggguu	3	0.8	7	1.8	5	1.4	10	2.6
rno-miR-150	ucucccaacccuuguaccagug					1	0.3		
rno-miR-151*	ucgaggagcucacagucuagua			1	0.3				
mmu-miR-182[f]	uuuggcaaugguagaacucaca					1	0.3		
rno-miR-183	uauggcacugguagaauucacug	1	0.3	1	0.3			1	0.3
rno-miR-185	uggagagaaaggcaguuc							1	0.3
rno-miR-186	caaagaauucuccuuuugggcuu			1	0.3			1	0.3
mmu-miR-189[f]	gugccuacugagcugauaucagu							1	0.3
rno-miR-191	caacggaaucccaaaagcagcu	1	0.3						
rno-miR-192	cugaccuaugaauugacagcc	2	0.5	3	0.8	2	0.6	3	0.8
rno-miR-193	aacuggccuacaaagucccag	4	1.1	4	1.0			2	0.5
rno-miR-194	uguaacagcaacuccaugugga	2	0.5	1	0.3	2	0.6	1	0.3
rno-miR-195	uagcagcacagaaauauuggc					1	0.3		
rno-miR-196a	uagguaguuucauguuguugg	5	1.3	4	1.0	3	0.8	1	0.3
mmu-miR-199a*[f]	uacaguagucugcacauugguu	1	0.3	5	1.3	1	0.3	2	0.5
rno-miR-200a	uaacacugucugguaacgaugu	13	3.5	6	1.5	8	2.2	5	1.3
rno-miR-200b	cucuaauacugccugguaaugaug	2	0.5	2	0.5	1	0.3	1	0.3
rno-miR-203	gugaaauguuuaggaccacuag	1	0.3	1	0.3				
rno-miR-206	uggaauguaaggaagugugugg					1	0.3		
rno-miR-210	cugugcgugugacagcggcug							1	0.3
rno-miR-214	acagcaggcacagacaggcag					2	0.6		
rno-miR-218	uugugcuugaucuaaccaugu	3	0.8	2	0.5	1	0.3	3	0.8
rno-miR-223	ugucaguuugucaaauacccc			1	0.3				
rno-miR-301	cagugcaauaguauugucaaagcau	2	0.5	1	0.3				
rno-miR-322	aaacaugaagcgcugcaaca			1	0.3	1	0.3	1	0.3
rno-miR-324-3p	ccacugccccaggugcugcugg							1	0.3
rno-miR-338	uccagcaucagugauuuuguuga			1	0.3				
rno-miR-342	ucucacacagaaaucgcacccguc					1	0.3	2	0.5
rno-miR-345	ugcugaccccuaguccagugc			1	0.3				
rno-miR-351	ucccugaggagcccuuugagccug					1	0.3		
Total		374	100	399	100	359	100	381	100

[a]miRNA genes are named according to the miRNA Registry from the Sanger Institute (http://www.sanger.ac.uk/Software/Rfam/). [b]The longest representative from each miRNA sequence is presented. [c]Number of clones found in each library. [d]Percentage of clones in each library. [e]Dahl salt-sensitive (DS) and Lewis rats (LW) were fed a 0.2% (-) or 8% (+) NaCl diet. [f]No homologous sequences were found in rat miRNA Registry, but identical sequences are present in other organisms (mouse, mmu; *C. elegance*, cel).

form murine pancreatic β-cell line MIN6 and murine pancreatic α-cell line TC1. And one of them, the islet-specific miR-375 (miR means microRNA), has been found to be involved in insulin secretion (*6*).

Methods

Animal Studies

DS and LW rats were obtained from Charles River Laboratories (Yokohama, Japan) and Oriental Yeast, Co., Ltd. (Tokyo, Japan), respectively. Rats were weaned at 4 weeks of age and then fed a 0.2% or 8% NaCl diet (Oriental Yeast, Co., Ltd.) for a period of 9 weeks (*14*, *17*). We compared the expression levels in DS and LW, since we have previously performed genetic linkage analyses in the F2 rats derived from DS and LW (*14*). All rats were killed by cervical dislocation following a brief period of ether anesthesia, and the kidneys and hearts were rapidly removed. Total RNA was extracted from the kidneys and the left ventricles using Trizol reagent (Invitrogen, Carlsbad, USA). The present study was conducted in accordance with current guidelines for the care and use of experimental animals of the National Cardiovascular Center.

Cloning of MicroRNAs

Cloning of microRNAs was essentially performed as described by Lagos-Quintana *et al.* (*18*). Total RNA (500 μg) was separated on a 15% denaturing polyacrylamide gel and RNA fractions of between 18 and 25 bases were selected. A 5′-phosphorylated 3′-adaptor oligonucleotide (5′-pUUUaac cgcgaattccagx: uppercase, RNA; lowercase, DNA; p, phosphate; x, 3′-Amino-Modifier C-7; Integrated DNA Technologies, Inc., Coralville, USA) and a 5′-adaptor oligonucleotide (5′-acggaattcctcactAAA: uppercase, RNA; lowercase, DNA) were directionally ligated to the small RNAs. Adaptor-ligated RNA was amplified by reverse transcription and polymerase chain reaction (RT-PCR) with a 3′-primer (5′-CAGCCAA CAGGCACCGAATTCCTCACTAAA) and a 5′-primer (5′-GACTAGCTTGGTGCCGAATTCGCGGTTAAA). Polymerase chain reaction (PCR) products were then digested with *Ban*I and concatamerized using T4 DNA ligase. Concatamers ranging in size from 500 to 1,000 bp were separated on agarose gel and directly ligated in a pCR3.1-TOPO vector. Plasmid inserts were amplified by PCR using primers to vector sequences and were subjected to direct sequencing. Sequences from cloned microRNAs were grouped by sequence identity, and compared with a public database, the microRNA registry (http://www.sanger.ac.uk/Software/Rfam), in order to identify the clones. Unknown sequences, including sequences homologous to particular microRNAs, were searched using the Basic Local Alignment Sequence Tool (BLAST) algorithm, available at the National Center for Biotechnology Information (NCBI). The flanking sequences of the novel microRNAs were used to predict the secondary structure with the RNAfold program of the Vienna RNA package (http://www.tbi.univie.ac.at/). All of the novel microRNAs were submitted to the microRNA Registry website for official annotation.

Northern Blot Analysis

Fractionated total RNA was transferred to a Zetaprobe membrane (Biorad, Hercules, USA) and cross-linked to the membrane by UV irradiation. The resulting blots were then probed with StarFire probes corresponding to various microRNAs, which were labeled with [α-^{32}P]dATP. Prehybridization and hybridization were carried out as described previously (*8*). Equal amounts of RNA from the five rats in each group were pooled and these samples (10 μg) were used for first screening. We assessed the expression levels in the four groups: DS with high-salt diet (DS+), DS with normal-salt diet (DS−), LW with high-salt diet (LW+), and LW with normal-salt diet (LW−). When there was an apparent difference in expression among the four groups, the expression levels were reassessed with other Northern strips. Since the expression levels of several microRNAs (miR-30c, miR-151*, miR-214, miR-223, miR-322, Can-1, Can-7) were reconfirmed to differ among the four groups, the expression levels of these microRNAs were assessed in individual rats (n=5).

Results and Discussion

If the microRNA system were involved in the pathogenesis of hypertension, some microRNAs would be differentially expressed in the kidneys of hypertensive and normotensive rats. We initially investigated the microRNAs that were abundantly expressed in rat kidneys by constructing microRNA libraries. Four microRNA libraries were constructed from a DS given a high-salt diet (DS+), a DS given a normal diet (DS−), an LW given a high-salt diet (LW+) and an LW given a normal diet (LW−). About 400 tags for microRNAs were sequenced and identified in each library, as shown in Table 1.

We identified 84 previously reported microRNA species and 7 microRNAs that have not been reported in rats (mmu-let-7g, mmu-miR-15a, cel-miR-83, cel-miR-84, mmu-miR-182, mmu-miR-189, mmu-miR-199a*). We also identified 12 possible microRNA species, designated candidates (Can) 1 to 12 (Table 2). All of these new microRNA sequences were found in the rat genome. The sequences surrounding these potential microRNAs showed a stem-loop structure, which is a prerequisite for microRNA processing. Moreover, expression of these possible microRNAs was confirmed by Northern blotting (Fig. 1). Some species of microRNA (miR-21, miR-26b, miR-29a, miR-126*) were abundantly expressed (>5%) in the kidney. The number of tags corresponding to miR-21 in the DS+ rat seemed to be larger than those in the other rats. Although the Northern blot analysis confirmed that the expression level of miR-21 in this DS+ rat was indeed higher than that in the other rats, this was not consistently

Table 2. Candidate MicroRNAs (miRNAs)

Temporary name[a]	No. of clones[b] LW−	LW+	DS−	DS+[c]	Sequence (5′ to 3′)[d]	Harpin precursor[e]	Chromosome[f] Rat	Human	Mouse	Northern[g]
Can-1	6	2	2	1	acuggacuuggagucagaaggc	−c u guuacc 5′gggcu cugacuccaggucc gugu u 3′uccgg gacugagguucagg cacg c aa u auaaag	18	5	18	+
Can-2	3	2	2	2	cagcagcaauucauguuuugga	a aa guauu 5′ugcagc gc uucauguuuugga g 3′acgucg cg aaguacaaaacuu c − −− ggaac	X	X	X	+
Can-3	−	2	1	2	auauaauacaaccugcuaagug	au c u 5′augg auaauacaac ugcuaagugu c 3′uguu uauuauguug acgauucacg u au c a	X	X	X	+
Can-4	1	−	−	1	gucaacacuugcugguuucc	u g a −− g 5′ag gggggagccag aagu uugauguu ucu c 3′uc cuccuuugguc uuca aacugcga gga c u g c uu −	X	X	X	+
Can-5	1	−	−	1	uuaagacuugcagugauguuu	u ua a gcuccu 5′gc gu agacuugc gugauguuua c 3′cg cg ucugaacg cacuacaagu u u ug a guaccu	3	20	2	+
Can-6	−	−	−	1	ugaccgauuucuccuggugguuc	5′cu ucc c gucu gaccgauuuc ugguguu aga g uuggcuaaag accacga ucu u 3′ua uuu − guuu	13	1	12	+
Can-7	1	−	1	1	aaaccguuaccauuacugag	a a 5′agg aaccguuaccauuacug g 3′ucu uugguaaugguaaugau u c u	10	17	11	+
Can-8	−	1	1	−	uauucauuuacucccagccua	5′cu −− a ggcuggga aaauga auagaaa c ccgaccccu uuuacu uaucuua a 3′au ca ---- u u	13	1	1	+
Can-9	−	1	−	−	aaucauucacggacaacacuu	a− ug uuua 5′aagaga guuguucgugg gauucgc c 3′uuuuuu caacaggcacu cuaagca u ca ua gugu	6	14	12	+
Can-10	−	1	−	1	cagcagcacacuguggguuugua	a− −−− −− uac ac 5′cc gc agcac ac uguggguuug ggc u 3′gg cg uuguggug acaccaaac ccg g ag aga uc cugca gu	10	17	11	+
Can-11	−	−	−	1	aguucuucaguggcaagcuuu	− a u ccu 5′aguaguucuucag uggca gcuuua gu g 3′uugucaagaaguu accgu cgaaau cg a g − − accc	10	17	11	+
Can-12	10	5	3	5	uaauacugucugguaaugccgu	au c ug cugga 5′gg gu uuaccagaca guuagau u 3′cu cg aauggucugu uaaucug g gc u ca ucuau	5	−	4	+

[a]miRNA candidates that were newly identified in this study are listed. [b]Number of clones found in each library. [c]Lewis (LW) or Dahl salt-sensitive rats (DS) were fed a 0.2% (−) or 8% (+) NaCl diet. [d]The longest representative from each miRNA sequence is presented. [e]Predicted structures of miRNA precursors. The miRNA sequences are underlined. [f]Chromosome number locating miRNA precursors are presented. [g]All miRNA candidates were detected by Northern blotting.