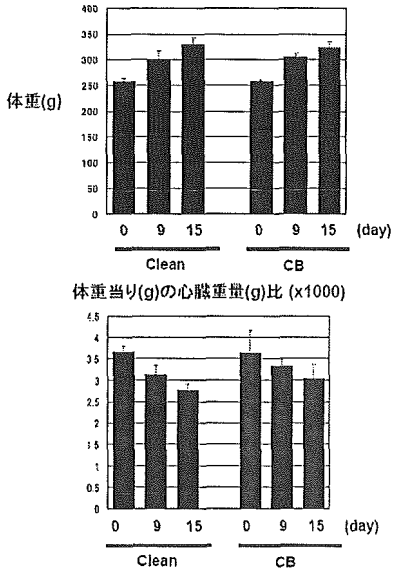
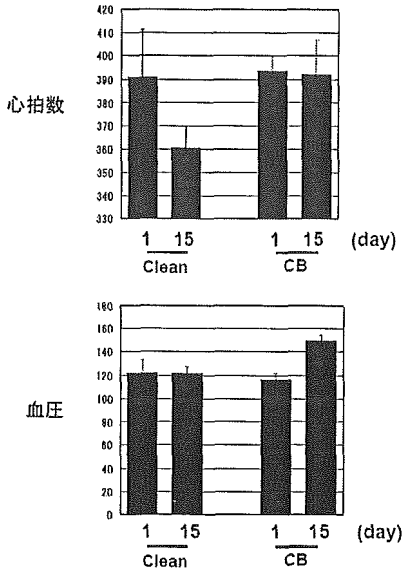
CB 曝露後のラットの体重はコントロール群の体重と比較するとほとんど差異が無かった。

しかし、体重当りの心重量比は CB 曝露の期間が長くなるにしたがって、コントロール群よりも増加することが判明した。そこで、尾部より血圧を測定した結果、心拍数はコントロール群では加齢とともに減少したのに対して、CB 曝露群のラットでは加齢にともなう心拍数の減少が見られなかった。尾部での血圧は CB 曝露群で有意に上昇することが判明した。これらのことから、CB 曝露群は CB 曝露の時間と共に、CB が体内（特に肺組織）に浸潤し、原因は未だ不明であるが血圧が上昇し、それにともなって心重量が増大していると考えられた。心疾患に関わるマーカー蛋白質の解析は今、遂行しているところである。





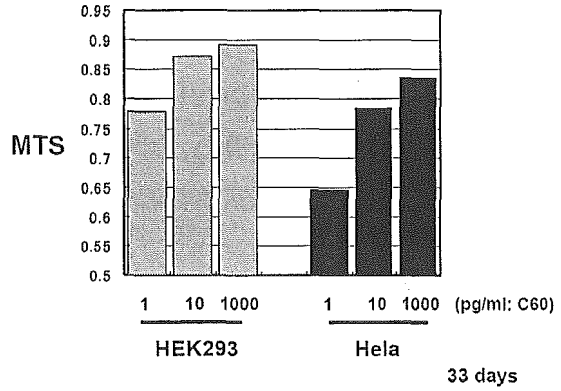
6．水溶性フラーレン｛C60(OH24)｝で長期間培養した細胞の小核試験

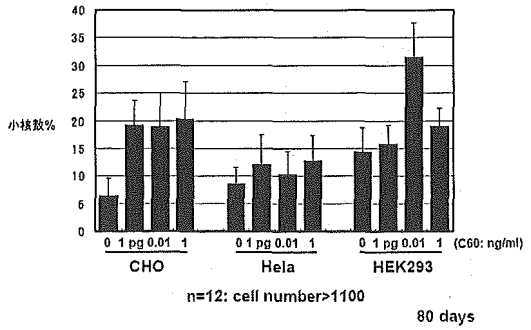
（測定方法）：CHO, Hela, HEK293 の各細胞株を水溶性フラーレン（0~1 ng/ml）添加の条件で約８０日間培養し、細胞増殖能を WST-8 で測定し、小核試験は Matsuoka 等の方法に従って行った。但し、核染色には DAPI を用いた。

結果

１）細胞増殖能

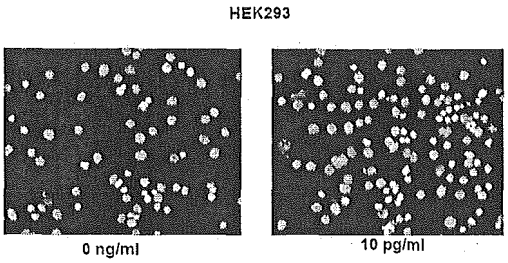
３３日間、水溶性フラーレン（1 pg~1 ng/ml）添加の条件で培養すると、水溶性フラーレンの濃度に依存して細胞増殖が亢進することが明らかになった。





2）小核試験

CHO, Hela, HEK293 の各細胞を低濃度の水溶性フラーレン（0~1 ng/ml）で８０日間培養後、細胞を固定し、核を DAPI で染色した後、蛍光顕微鏡で全体の細胞数の中の小核を持った細胞数をカウントし、小核数（%）として表した。細胞によって小核数は異なるが、CHO, Hela, HEK293 すべてで水溶性フラーレンによる核の異常が観察された。この結果から、我々の生活環境レベルのナノ粒子の濃度で長期間曝されると核が異常な細胞が増大し、癌を始めとする生活習慣病疾患へと進展する可能性が示唆された。

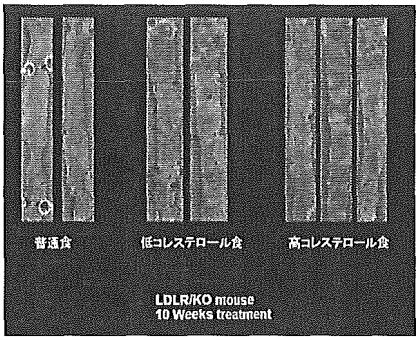


考察

化学物質の毒性試験で培養細胞を用いたアッセイは急性効果を調べている事例がほとんどである。今回、小核試験に用いた水溶性フラーレンの濃度は 1 pg/ml~1 ng/ml であり、この濃度は我々が生活している環境レベルの濃度に近いと考えられる。しかしながら、水溶性フラーレンを環境レベルの濃度で長期間細胞培養するとやがて細胞自身の性質が次第に変化し、より一層細胞増殖能を持ち、かつナノ粒子に耐性を持った細胞が出現することを明らかにした。この結果は、極低濃度のナノ粒子でさえも培養細胞に長期間曝露すると変異原として作用し得ることを示唆する。

7．動脈硬化発症モデルマウス；LDL receptor knockout mouse にカーボンブラック（CB）を長期間投与し、動脈硬化進展との関連性を検討する。

今、現在は動脈硬化巣の進展度を大動脈をオイルレッド O で染色することによって、定量的に評価することを試みている。



今後は二つの群に分け、気管内へ手動でCB(1 mg/匹)を毎週一回投与し、約１０週間続けた後動脈硬化巣の大きさを定量することにしている。また、以下の項目についても解析することにしている。

1）体重測定、尾部での血圧測定
2）各臓器重量
3）炎症性マーカーの測定：MCP-1, CRP, IL-6 の血中濃度を測定する。
4）凝固系因子の測定：PAI-1, tPA の血中濃度を測定する。
5）免疫組織学的検証：iNOS,Nitrotyrosin, eNOS の各臓器での発現量を抗体による免疫組織学的方法で解析する。

	動物数(匹)	
	曝露期間	
	10週間	10週間以上
普通食群	10	10
普通食+CB投与群	10	10
低コレステロール食群	10	10
低コレステロール食+CB群	10	10

D. 結論

平成１７年度では、主に培養細胞系で２種類のナノ粒子に対する急性効果を検討した。カーボンブラックはヒト培養内皮細胞に対して細胞傷害、増殖抑制、炎症性サイトカインの発現促進作用を有することを明らかにした。一方、水溶性フラーレンは内皮細胞に対し、細胞質内でのユビキチン化タンパク質の異常蓄積を誘導し、オートファジー依存的細胞死を誘発することを明らかにした。

血小板ではカーボンブラックは ADP による血小板凝集反応に影響を与えなかった。それに対して水溶性フラーレンは濃度依存的に ADP による血小板凝集反応を促進した。

さらに、内皮細胞と並んで動脈硬化発症に重要な役割を果たしているマクロファージにおいても内皮細胞と同様にナノ粒子処置により食胞が多数確認できた。しかし、ナノ粒子単独ではマクロファージに対し強い細胞障害作用を及ぼさなかったが、動脈硬化発症に関与する酸化 LDL が共存するとナノ粒子の細胞障害性が増強することが明らかになった。これらの結果から、ナノ粒子の動脈硬化発症過程における作用点は、初期段階に**血管内皮細胞**を障害し、その後**マクロファージ**を活性化させ、変性 LDL の細胞内への取り込みを促進し（泡沫化）、泡沫化した細胞から分泌された MMP によってプラークが破綻し、ADP などによって活性化、凝集した**血小板**が血栓を形成し、心筋梗塞へ進展すると推察している。細胞レベルの実験結果をより発展させるため、カーボンブラックのラットへの全身曝露実験を行った。詳細は今現在解析中である。１８年度以降は１７年度で行った実験項目を引き続き調査分析し、更に動脈硬化発症マウスでのナノ粒子の長期暴露、投与を試み、ナノ粒子の影響を実験動物レベルで詳細に調査検討する予定である。

E. 健康危険情報

健康を害することは本研究ではないと考えられる。

F. 研究発表

1. 論文発表

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2. Naoharu Iwai, Hiroaki Naraba. Polymorphisms in pre-microRNAs. Biochem. Biophys. Res. Commun. 2005; 331:1439-1444.

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6. Hiroaki Naraba, Naoharu Iwai. Assessment of MicroRNA System in Salt-sensitive Hypertension. Hypertens. Res. 2005;28:819-826.

7. Hideyuki Yamawaki, Naoharu Iwai. Mechanisms underlying nao-sized air-pollution-mediated progression of atherosclerosis-Carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells. Circ. J 2006; 70: 129-140.

8. 環境ナノ粒子の動脈硬化促進メカニズムの検討。山脇英之、丹羽保晴、丹羽勝利、岩井　直温。血圧（Journal of Blood Pressure）2006 年 3 月号掲載

G. 知的財産権の出願・登録状況

1．特許取得

特になし

2．実用新案登録

特になし

3．その他

特になし

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

研究論文雑誌

発表者氏名	論文タイトル名	発表雑誌	巻号	ページ	出版年
Naoharu Iwai, Naomi Yasui, HiroakiNaraba, Naomi Tago, Hideyuki Yamawaki, Hiroshi Sumiya.	Klk1 as one of the genes contributing to hypertension in Dahl salt sensitive rat.	Hypertension.	45	947-953	2005
Naoharu Iwai, Hiroaki Naraba.	Polymorphisms in pre-microRNAs.	BBRC	331	1439-1444	2005
Kazuaki Kajimoto, Keisuke Shioji, Chisaki Ishida, Yoshitaka Iwanaga, Yoshihiro Kokubo, Hitonobu Tomoike, Shun-ichi Miyazaki, Hiroshi Nonogi, Yoichi Goto, Naoharu Iwai.	Validation of the association between the gene encoding 5-lipoxygenase-activating protein and myocardial infarction in a Japanese population.	Circ. J.	69	1029-1034	2005
Kazuaki Kajimoto, Keisuke Shioji, Naomi Tago, Hitonobu Tomoike, Hiroshi Nonogi, Yoichi Goto, Naoharu Iwai.	Assessment of MEF2A mutations in myocardial infarction in Japanese patients.	Circ. J.	69	1192-1195	2005
Hiroaki Naraba, Yoshihiro Kokubo, Hitonobu Tomoike, Naoharu Iwai.	Functional Confirmation of Gitelman's Syndrome Mutations in Japanese.	Hypertens. Res.	28	805-809	2005
Hiroaki Naraba, Naoharu Iwai.	Assessment of MicroRNA System in Salt-sensitive Hypertension.	Hypertens. Res.	28	819-826	2005
Hideyuki Yamawaki, Naoharu Iwai.	Mechanisms underlying nao-sized air-pollution-mediated progression of atherosclerosis-Carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells.	Circ. J.	70	129-140	2005
山脇英之、丹羽保晴、丹羽勝利、岩井 直温	環境ナノ粒子の動脈硬化促進メカニズムの検討。	血圧	13	253-256	2006

III. 研究成果の刊行物·別刷

Klk1 as One of the Genes Contributing to Hypertension in Dahl Salt-Sensitive Rat

Naoharu Iwai, Naomi Yasui, Hiroaki Naraba, Naomi Tago, Hideyuki Yamawaki, Hiroshi Sumiya

Abstract—A genome-wide quantitative trait loci analysis for blood pressure was performed using 107 male F_2 rats derived from Dahl salt-sensitive and Lewis rats. Blood pressure was assessed by telemetry, and >400 microsatellite markers were used for genotyping. Two major quantitative trait loci for blood pressure were identified at chromosome 1 and chromosome 10. The expression levels of 366 transcripts around the chromosome 1 quantitative trait loci were assessed by RT-PCR, and we found that the *Klk1* (kallikrein 1) and *Ngfg* (nerve growth factor gamma) mRNA levels were significantly reduced in the kidneys of Dahl salt-sensitive rats compared with those in Lewis rats. The expression levels of kallikrein 1 protein were also suppressed in Dahl salt-sensitive rats compared with those in Lewis rats. Because the kallikrein–kinin system has been shown to be involved in renal function, including salt homeostasis, it is likely that the reduced expression of *Klk1* contributes to salt-sensitive hypertension in Dahl salt-sensitive rats. (***Hypertension.* 2005; 45:947-953.**)

Key Words: kallikreins ■ rats, Dahl ■ genetics

Dahl salt-sensitive rats have been widely used for the investigation of salt-sensitive hypertension. Sixteen genomic regions containing quantitative trait loci (QTLs) for blood pressure (BP) regulation have been reported in this strain.[1–3] However, the genes responsible for this salt-sensitive hypertension have not yet been confirmed, despite numerous genetic studies.

A possible explanation for the lack of identification of the responsible genes in rat genetic studies to date might be the incompleteness of the rat genome data. The Rat Genome Project is now almost complete,[4] and thus, it is now possible to obtain information on almost all of the protein-coding genes in candidate loci for a given phenotype.

Another drawback in previous rat genetic studies was the method used to assess BP. The BP levels of F_2 rats were usually measured either with a tail-cuff method or with a temporary inserted arterial catheter. The validity of these classic methods of BP measurement is not concretely established, and furthermore, largely dependent on the skill levels of the individual researchers. Moreover, with these methodologies, circadian rhythm changes in BP cannot be adequately assessed. In the present study, these drawbacks were overcome by using telemetry.

In the present study, a genome-wide QTL analysis for BP was performed using 107 male F_2 rats derived from Dahl salt-sensitive (DS) and Lewis (LEW) rats. The BP was measured by a telemetry system, and the genotypes of the F_2 rats were determined with >400 genetic markers throughout the genome. Major QTLs were identified for daytime and nighttime BP (systolic and diastolic) levels. Next, we assessed the expression levels of 366 transcripts in the prominent QTLs and identified 2 transcripts that were differentially expressed in the kidneys of the 2 strains.

Materials and Methods

Experimental Animals

DS and LEW rats were purchased from Sunplanet (Tokyo, Japan) and Charles River Japan (Yokohama, Japan), respectively. Rats were housed in a temperature-controlled room with the light on from 7:00 AM to 7:00 PM (daytime) and fed normal rat chow (0.5% NaCl; Clea Japan) and tap water ad libitum.

Male DS rats were mated with female LEW rats to produce F_1 rats, and F_1 rats were then intercrossed to produce an F_2 population consisting of 107 male rats. F_2 rats were started on an 8% NaCl diet (Oriental Yeast) at 5 weeks of age according to the protocol described by Rapp et al.[1] Radiotelemetry devices (Data Sciences International) were implanted into the lower abdominal aorta of F_2 rats at 9 weeks of age using sodium pentobarbital as an anesthetic agent (25 mg/kg IP). At 14 weeks of age, BP and heart rate were continuously measured for 44 hours (daytime 7:00 AM to 7:00 PM; nighttime 7:00 PM to 7:00 AM), and the data obtained during the latter 24 hours were used for analyses. The results were analyzed using Fluclet TM software (Dainippon Pharmaceutical).[5] The present study was conducted in accordance with current guidelines for the care and use of experimental animals of the National Cardiovascular Center.

Genotyping and QTL Analysis

Genotyping was performed by PCR using appropriate PCR primer pairs (custom-made by Amersham Pharmacia Biotech), based on information from the Rat Genome Database (http://rgd.mcw.edu/) and Ratmap (http://ratmap.gen.gu.se/). We found sequence variations between DS and LEW rats in several genes, and these

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Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000161969.65767.0d

TABLE 1. Primers for Members of the Kallikrein Gene Family

	Gene Name	Other Name(s)	Expression	Sense Primer	Antisense Primer
1	LOC308562	KLK14	ND	CATGTTGAGGACATCAAAGCAAG	CCGCACTTTCCTCTGTAGCTTC
2	LOC292848	KLK13	ND	GAACATCAAGATTGAGAAGGCCC	GCTGGACAGGAGACTTCAACTCC
3	LOC308564	KLK-L5	ND	CATCAGCTTGACCTATGCTGTCC	GACCGAGGGAACCAGTTAGTTG
4	LOC292849	hippostasin prostate type	ND	GGCCATGATGATTCTCCGATTC	CGCACAGCTCGGGTAATGAAG
5	LOC361562		ND	GAACTGGAGGTTATGGAAGAGGG	GTCACAGCCCTCTCTGAGCC
6	LOC292850	KLK10	ND	CTCTCCTCAGCCAGAAGCAG	GGATGCATTCTCAGAGGTGTG
7	LOC292851	KLK-L3	ND	TAGTGCTGTTCTCTCTGCTGGC	GCATGGTGTCAAATAAGCTTGTTG
8	LOC308565	KLK8	ND	CAATCCAGACGTGGATCCTTC	CGAGGTTTGCTGAGTTCTGC
9	LOC292852	Thymopsin	ND	GAGGATCAGAGTGCCCAGAGG	GGACACCAGACCTTGAAGGGTG
10		KLK6	ND	CTGAGACTTTCCAAAGGCAAATC	CTGGACATCTGAAGGCTCTGAG
11	LOC308566	KLK5	ND	GAGGCCACCCTTACTATCAGAACTC	GAACATCTGCTGCCCAGACTC
12	LOC292855	KLK12	ND	CTCTTCCTGTCCGTGGGACGAAT	CATCTGTCACCATCTGTGTGTG
13	LOC292858	KLK10	ND	CCAGGCCAGATAAGTCTCTAAGTGC	GGTAATTCCCAATTCAGGGGTTTG
14	LOC292861	KLK4	ND	CACTACAACCATACCGGTATCTGAC	GGTAATTCTGATATAAGGGGTTTT
15	Ton	Tonin	ND	CCCTAGTCCTGTCCGTGGGACGAAT	CTTACTACCATCTCAGAGGGGTTG
16	LOC292866	KLK8	ND	TCCTCATCCTGTCCCTGGGATGGAA	GGAAATTCCCATTTGAGGGGTGTG
17	LOC292868	S3 KLK/KLK9	ND	CCTGCTGCTACTGGACACACGATAT	CTTAATTTCATCTCAGAGGGGTTG
18	LOC365240		ND	CATACAGTGCCTTCATCAGCC	GTGGCAGAATCAGTCAATGGC
19	KLK1	KLK7	D<L	CCTGATCCTATTCCTCGACCTGTCCCTG	GTAGATGGCTGGCATGTTGGTTTTGG
20	LOC292872	S1/KLK3	ND	TGTCTGTACGGACTCTTATTCACAGA	GGGAATTCCCACTCAGAGGGGTTG
21	LOC292873	Prostin	ND	GAGCCCACTGAGGAGGCTAAG	GTTGTTCTGGGCCATCGAAC
22	Ngfg	KLK1	D<L	CCTGCTGCTCCTGCATGCCTGTTAC	CTTAATTCCAATCCGTCAGGTGTG

Blast analysis in the rat genome with the Klk1 sequence identified 22 homologous sequences. Primers were set on separate exons when possible transcripts have introns, and were selected to gene-specific sites to avoid cross-amplification from homologous genes. Expression was determined by RT-PCR in the kidneys.

ND indicates not detected.

polymorphisms were also included in the genotyping data. The raw data are provided as a supplement (supplemental Table I, available online at http://hyper.ahajournals.org). We separately analyzed QTLs for daytime systolic BP (D-SBP), diastolic BP (D-DBP), nighttime systolic BP (N-SBP), and diastolic BP (N-DBP) levels using MapManager QTLb20.[6] We first performed Quick Test to obtain significant thresholds for BP values. The likelihood ratio statistics (LRSs) for suggestive, significant, and highly significant loci were calculated to be 12.3, 17.7, and 25.9, respectively. Next, we performed marker regression for these 4 BP values. The most significant QTL was added to the background, and a second marker regression was performed to obtain the second QTL, which was added to the background to obtain the third QTL. We obtained 1 significant (chromosome 1) and 1 or 2 suggestive QTLs (chromosome 10 and chromosome 12) for these 4 BP values. Interval mapping of chromosome 1 was performed (free model) with other QTLs included as background.

RT-PCR Analysis of Expression Levels of Transcripts

For the screening of differentially expressed transcripts, we analyzed the expression levels of transcripts in the kidneys of DS and LEW rats (5 weeks of age) on a normal diet (n=2 in each group). The expression levels of transcripts were assessed by simple RT-PCR at 25 and 32 amplification stages because overamplification might obscure possible differences in expression levels. A 2.5-fold difference can be reliably detected by this method, provided the expression level of the target mRNA is between the *Ngfg* (nerve growth factor gamma) mRNA level and 1/1000 of that level (supplemental Figure I). After this initial screening, several possible transcripts that might be differentially expressed were subjected to precise estimation by competitive RT-PCR analyses with 18s ribosomal RNA as an internal control (QuantumRNA 18s Internal Standards Kit; Ambion). Because the 18s ribosome RNA level is extremely high, the PCR primers for 18s cDNA amplification are diluted by 18s competimers to reduce the efficiency of amplification to attain a comparable level to the target. The 18s competimers are modified at their 3′ ends to block extension by DNA polymerase. The ratios of the 18s primers to 18s competimers were 3:7 for the *Klk1* (kallikrein 1) and *Ngfg* mRNAs. The relative intensity of the PCR product from the *Klk1* (721-bp) or *Ngfg* (529-bp) mRNA to the PCR product from 18s RNA (488-bp) was assessed by a densitometer. This assessment was performed within a range in which a linear relationship was maintained between the expression levels and the ratios of the PCR products. The expression levels were assessed in the kidneys of 5-week-old DS rats, 5-week-old LEW rats, 14-week-old DS rats under normal diet, 14-week-old LEW rats under normal diet, 14-week-old DS rats under a high-salt diet, and 14-week-old LEW rats under a high-salt diet (n=4 in each group).

The 366 transcripts that were analyzed and their amplification primers are provided as a supplement (supplemental Table II). Sense and antisense primers were derived from separate exons when the transcripts had introns. RNA samples were briefly treated with DNaseI and extracted with phenol-chloroform before reverse transcription to prevent possible amplification from contaminated genome DNA, which might obscure the expression levels of the intron-less transcript. Primers for the kallikrein gene family were derived from gene-specific sequence regions (Table 1) to avoid cross-amplification of homologous sequences, which had been confirmed by direct sequencing of the PCR products.

TABLE 2. Marker Regression Analysis of BP

BP		Marker	LRS	BP		Marker	LRS
D-SBP	Ch1	*D1Rat410*	18.4	N-SBP	Ch1	*D1RatArb33*	14.8
		*D1Rat27**	18.8			*D1Rat410*	19.1
		Ngfg	14.6			*D1Rat27**	20.4
		D1Mgh7	14.6			*Ngfg*	15.9
		Igf1r	13.1			*D1Mgh7*	14.3
		D1Rat269	13.2			*Igf1r*	12.8
		D1Rat35	13.5			*D1Rat269*	12.4
		Iqgap1	16.0			*D1Rat35*	12.8
		Pex11a	16.6			*Pex11a*	13.3
		NTRK3	16.9			*NTRK3*	12.8
					Ch10	*Pex12*	12.7
D-DBP	Ch1	*D1Rat410*	15.0	N-DBP	Ch1	*D1Rat410*	13.6
		*D1Rat27**	16.8			*D1Rat27**	15.4
		Ngfg	14.1			*Ngfg*	13.4
		D1Mgh7	12.4				
					Ch10	*D10Rat98*	13.6
	Ch10	*D10Rat98*	14.5			*Pex12*	13.3
		Pex12	14.9				
	Ch12	*D12Arb6*	13.7				

MapManagerQTX quick test indicated that the LRS values were 12.3, 17.7, and 25.9 for suggestive loci, significant loci, and highly significant loci, respectively. Marker regression analysis was performed using these 4 BP values. The most significant QTL was added to the background (indicated by *), and a second marker regression was performed to obtain the second QTL, which was added to the background to obtain the third QTL. We obtained 1 significant (chromosome 1) and 1 or 2 suggestive QTL (chromosome 10 and chromosome 12) for these 4 BP values.

Western Blotting

Rabbit polyclonal antibody against rat urinary kallikrein was obtained from Merck Biosciences (formerly Calbiochem), which recognizes the active and inactive (prepro) forms.

LEW and DS rats (5 weeks old; n=4) were euthanized and kidneys were harvested. Kidneys were frozen in liquid N_2 and were homogenized in Triton-based lysis buffer (1% Triton X-100, 20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerol phosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, and a 0.1% protease inhibitor mixture [Nacarai Tesque]). The protein concentration was determined with the bicinchoninic acid method (Pierce). Equal amounts of proteins (60 μg) were separated by SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane (BioTrace NT; Pall Corporation). After blocking with 5% BSA, membranes were incubated with primary antibody (1:1000 dilution) at 4°C overnight, and membrane-bound antibodies were visualized by horseradish peroxidase–conjugated secondary antibodies (1:10 000 dilution; 1 hour) and an ECL system (Amersham Biosciences). The expression levels were determined by densitometry.

Results

Linkage to BP

A total of 418 polymorphic markers in all of the 107 F_2 rats were genotyped, and some of them gave identical genotype data. Thus, the data on 383 effective genotypes were finally obtained (supplemental Table I).

Table 2 gives the results of the analysis using the MapManager QTX program for linkage to BP in the F_2 (DS× LEW) population. Separate analyses were conducted of the D-SBP, D-DBP, N-SBP, and N-DBP values. Table 2 shows only the markers that yielded an LRS of at least a suggestive level of significance (ie, >12.3). The QTLs for N-SBP were around *D1Rat27* and *Pex12* (Ch10). Those for N-DBP were around *D1Rat27* and *D10Rat98*, those for D-SBP were around *D1Rat27*, and those for D-DBP were around *D1Rat27*, *Pex12*, and *D12Arb6*.

Figure 1 provides LRS plots for the linkage to BP for chromosome 1 controlled by other suggestive loci. The chromosome 1 region near *D1Rat27* is the most prominent and consistent QTLs for all of the BP values.

Identification of Candidate Genes in the Chromosome 1 Region

We next focused on the chromosome 1 region near *D1Rat27* (at 90.3 mol/L). A total of 366 transcripts were found from this region between *Cyp2a2* (82.1 mol/L) and *loc292934* (99.5 mol/L), and RT-PCR expression analysis was performed (supplemental Table II). The expression of 240 genes was detected in the kidney of the 366 possible transcripts. As shown in Figure 2, the *Klk1* and *Ngfg* mRNAs were found to be differentially expressed between the kidneys of DS and LEW rats. *Klk1* and *Ngfg* are members of a large kallikrein

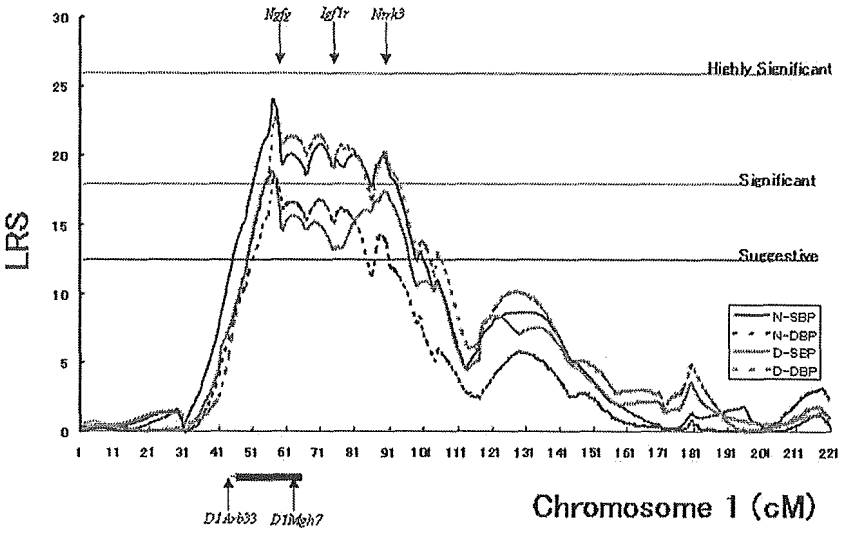

Figure 1. Interval mapping analysis of BP QTL on chromosome 1. Interval mapping analysis of BP values was performed on chromosome 1 with other suggestive loci included as background. LRS plots for BP values are shown. The region subjected to expression analysis is indicated as a horizontal bar between *D1Arb33* and *D1Mgh7*.

gene family. Gene-specific primers for the kallikrein gene family were set up as described in Materials and Methods (Table 1). Direct sequencing confirmed that the PCR products originated from the *Klk1* and *Ngfg* transcripts. Three-way ANOVA indicated that weeks of age (P=0.0023) and strain difference (P=0.0046) but not salt loading (P=0.8884) significantly affected the *Klk1* mRNA levels (P=0.0006). The *Klk1* mRNA level in LEW rats was 2.7-fold higher than that in DS rats. This differential expression was reconfirmed by the competitive RT-PCR method, in which a deletion mutant cRNA for Klk1 was used as an internal standard (supplemental Figure II).

Three-way ANOVA indicated that strain difference (P<0.0001) but not weeks of age (P=0.3629) or salt loading (P=0.3410) significantly affected the *Ngfg* mRNA levels (P=0.0005). The *Ngfg* mRNA level in LEW rats was 3.4-fold higher than that in DS rats at 5 weeks of age. The *Ngfg* mRNA level in LEW rats was 1.5-fold higher than that in DS rats at 14 weeks of age.

Sequence analysis of the entire *Klk1* and *Ngfg* sequences including the ≈1-kb promoter and ≈1-kb 3′ regions in DS and LEW rats revealed no sequence difference except in the number of TC repeats in intron 1 of *Ngfg*: the number was 24 and 26 in DS and LEW rats, respectively.

This differential expression was also confimed by Western blot analysis (Figure 3). A primary antibody recognizing rat urinary kallikrein corresponding to KLK1 was used. In DS rats, KLK1 expression was significantly suppressed

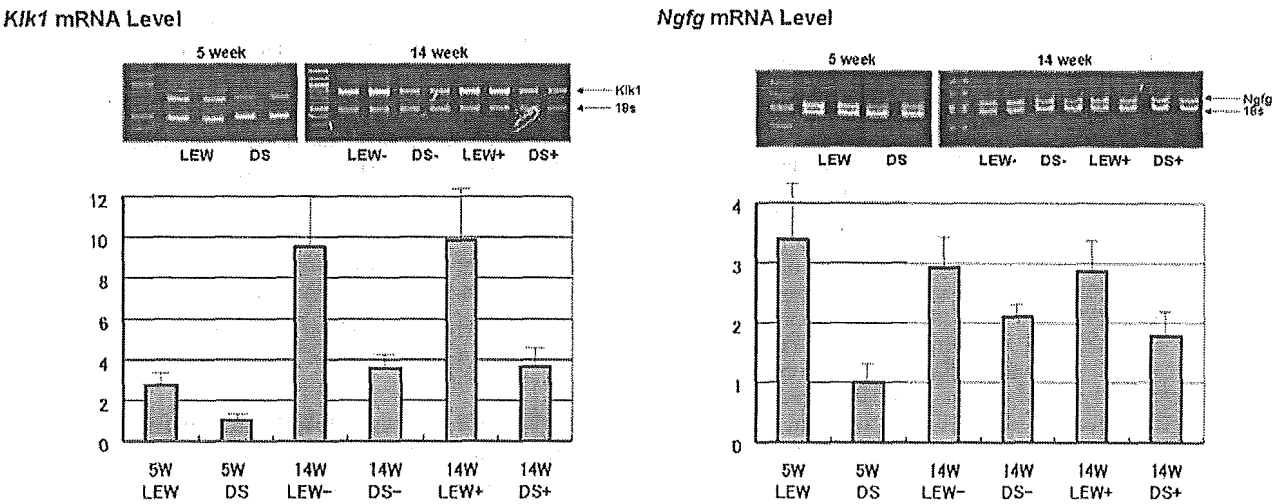

Figure 2. Expression levels of *Klk1* and *Ngfg* mRNA in the kidneys of DS and LEW rats. Expression levels of the *Klk1* and *Ngfg* mRNA were assessed by competitive RT-PCR using 18s ribosome RNA as an internal standard. The ratios of 18s primer to 18s competimer were 3:7 for the *Klk1* and *Ngfg* mRNAs. The size of the PCR products from the *Klk1*, *Ngfg*, and 18s RNA was 721 bp, 529 bp, and 488 bp, respectively. Three-way ANOVA indicated that weeks of age (P=0.0023) and strain difference (P=0.0046) but not salt loading (P=0.8884) significantly affected the *Klk1* mRNA levels (P=0.0006). Moreover, 3-way ANOVA indicated that strain difference (P<0.0001) but not weeks of age (P=0.3629) or salt loading (P=0.3410) significantly affected the *Ngfg* mRNA levels (P=0.0005). Each group contains 4 rats (n=4). 5W LEW indicates 5-week-old LEW rats under normal diet; 5DS, 5-week-old DS rats under normal diet; 14LEW−, 14-week-old LEW rats under normal diet; 14DS−, 14-week-old DS rats under a high-salt diet; 14LEW+, 14-week-old LEW rats under a high-salt diet; 14DS+, 14-week-old DS rats under a high-salt diet. The expression levels of the *Klk1* and *Ngfg* mRNAs in 5-week-old DS rats are arbitrary defined as 1.0. Vertical bars indicate SDs.

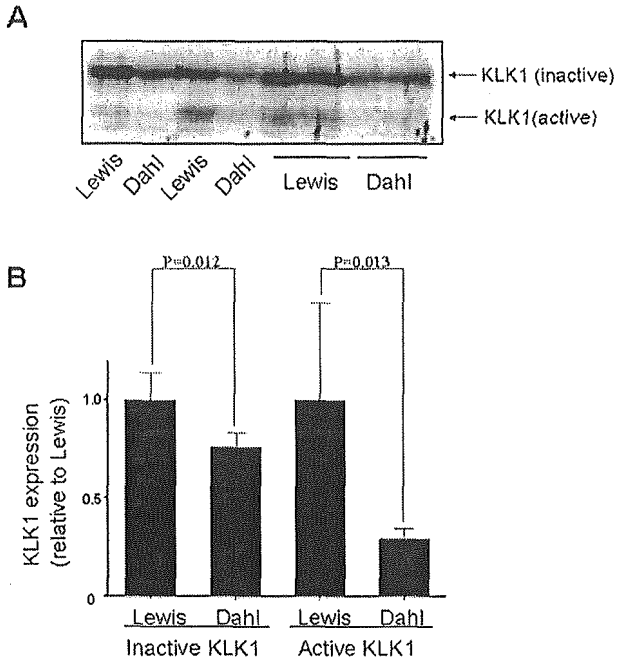

Figure 3. Expression levels of KLK1 protein in the kidneys of DS and LEW rats. KLK1 protein expression was assessed in the kidneys of DS and LEW rats (n=4) by Western blot analysis. A, Top, Upper (≈44-kDa) and lower (≈38-kDa) bands represent prepro(inactive) and active KLK1, respectively. B, Bottom, KLK1 expression is shown as fold change relative to LEW rats (n=4). Vertical bars indicate SDs.

compared with LEW rats. It should be noted that prepro-kallikrein (44 kDa) and active kallikrein (38 kDa)[7] were detected by this antibody, and the expression levels of both forms, especially those of active form, were suppressed in DS rats.

Discussion

In the present study, a genome-wide F_2 (DS X LEW) analysis with ≈400 markers and telemetry-based BP assessment was performed. The major QTLs for N-SBP, N-DBP, D-SBP, and D-DBP were identified. The most prominent and consistent QTLs for BP were identified on chromosome 1 near the kallikrein gene family. RT-PCR analyses showed that 2 transcripts in this region, namely *Klk1* and *Ngfg* mRNA, were differentially expressed in the kidneys of the 2 strains.

The kallikrein–kinin system has long been suspected to be involved in salt-sensitive hypertension in Dahl rats.[8,9] Knockout mice that lack a bradykinin-B2 receptor have been shown to have salt-sensitive hypertension.[10–12] A rat strain inbred for low urinary kallikrein excretion has also been reported to show salt-sensitive hypertension.[13] As clarified in the present study, *Klk1* mRNA expression levels in the kidney were lower in DS rats than in LEW rats. Therefore, it is likely that *Klk1* contributes to hypertension in DS.

Lower urinary excretion of kallikrein-like activity has been reported in DS rats,[8,9] and adenoviral transfer of the human kallikrein 1 gene has been reported to ameliorate hypertension and hypertension-associated target organ damage in DS rats.[14,15] Based on these reports, it is likely that the low expression of *Klk1* in DS rats is responsible for salt-sensitive hypertension. On the other hand, mice lacking *Klk1* have been reported to exhibit cardiovascular abnormalities with normal BP.[16,17] The hearts of these mice exhibited septum and posterior wall thinning and a tendency for dilatation, which led to decreased cardiac function. Thus, the normal BP in these mice, despite increased vascular resistance,[17] reflects a reduced cardiac function. Therefore, this knockout model is not necessarily inconsistent with our hypothesis that the reduced *Klk1* expression in DS rat kidneys is responsible for salt-sensitive hypertension in DS rats.

The LRS plots for BP values of Ch1 (Figure 1) are prominent and wide. Rapp et al reported the existence of 3 QTLs (QTL1a, QTL1b, and QTL2) on Ch1 on the basis of results in Dahl rat congenic strains with an introgressed LEW rat Ch1 segment.[18] In these congenic experiments, the *Klk1* locus seemed to have only a slight effect on BP (≈10mm Hg, congenic line (Ch1X3)X12; Figure 2[2]), and an adjacent locus distal to the *Klk1* locus appeared to be important (QTL1a). If 2 responsible genes reside in a very close range, the QTL plot will not give 2 separate peaks, but rather only 1 large pseudopeak. In such a case, it would not be possible to determine the precise location of the responsible genes only by linkage.

Thus, it is possible that the reduced expression of *Klk1* in DS rats may have only modest effects on BP, and another gene near the *Klk1* locus, which might have escaped the systematic expression analysis performed in the present study, might have profound effects on BP. Indeed, the expression analysis in the present study has several limitations; for example, subtle changes cannot be detected by the PCR-based expression analysis, the target tissue might not be the kidney, and the target gene might not be differentially expressed.

The unsolved problem of the present study is the lack of any significant sequence variations in *Klk1* and *Ngfg* loci despite differential expression patterns. There would seem to be 2 possible explanations. One is that the regulation of *Klk1* might be influenced by regulatory sequences acting over near-megabase distances. Recently, the existence of regulatory sequences acting over near-megabase distances has been suggested.[19] The size of genomic regions functionally linked to a particular gene may thus need to be considerably expanded.[19,20] The other explanation is that the downregulation of *Klk1* in DS rats may be attributable to another gene near the *Klk1* locus. The rat kallikrein gene family apparently consists of at least 22 genes encompassing >1 Mb.[21–23] The physiological significance of each member in this family has not been determined. It is also possible that *Klk1* expression may be modified to compensate for derangement in other members of this gene family. Such a compensatory decrease in *Klk1* or *Ngfg* could lead to salt-sensitive hypertension in the Dahl rat.

Whatever the reason for the downregulation of *Klk1* in DS rats, it has relevance to salt-sensitive hypertension because adenoviral transfer of human kallikrein 1 gene has been reported to ameliorate hypertension and hypertension-associated target organ damage in DS rats.[14,15] Based on the

latter hypothesis, *Klk1* might not be a causative gene but might be an effector for salt-sensitive hypertension.

Ngfg is also a member of the kallikrein gene family, and the physiological substrates for *Ngfg* have not been confirmed.[21–23] It is currently unclear whether the differential expression of *Ngfg* between the 2 strains might be related to salt-sensitive hypertension. *Ngfg* and *Klk1* were found to be downregulated in DS, although no significant sequence difference was observed between DS and LEW rats. Because the existence of large-scale copy number polymorphism has been reported in the human genome,[24] we assessed the copy number of the *Klk1* locus in DS and LEW rats, and found no significant difference. Coordinate suppression of *Klk1* and *Ngfg* might support either a compensation mechanism or the existence of long-range enhancers, as mentioned above.

In the present study, an assessment was made of 4 components of BP values: N-SBP, N-DBP, D-SBP, and D-DBP. Unexpectedly, the major QTLs for these traits were almost identical. Thus, the present results exclude the possibility that nighttime and daytime BP might be influenced by completely different sets of genes.

This investigation focused on the most prominent QTLs for BP levels (ie, those around the *D1Rat27* locus). Although no assessment was made of the expression levels of transcripts around the second (*Pex12* [peroxisomal biogenesis factor 12] at Ch10) or third (*D12Arb6*) QTL, we noted the existence of differentially expressed transcripts in the kidneys of DS and LEW rats, namely *Pnpo* (pyridoxine 5′-phosphate oxidase) at Ch10 and *P2rx4* (purinergic receptor P2X, ligand-gated ion channel 4) at Ch12, the differences for which had been revealed by chance in our previous microarray analyses.[25] However, a more thorough assessment will be necessary to identify candidate genes in these QTLs that could contribute to hypertension.

Garrett et al reported the existence of 9 BP QTLs (Ch1, 2, 3, 5, 8, 10, 16, 17, and 18) using F_2 derived from DS X LEW, the same strains used in the present study. The most prominent BP QTLs were reported on Ch5 and 10. Of these 9 QTLs, the QTLs on Ch1, 5, 8, 10, and 17 were confirmed by the establishment of congenic strains.[1,26,27] In the present study, BP QTLs were found on Ch1, 10, and 12. The most prominent BP QTL was on Ch1. Garrett et al measured BP by the tail-cuff method, and BP measured by this method is likely to be influenced by stress (eg, by heating of the tails and constrains). On the other hand, the BP QTLs detected by the telemetry system are free from such stress. The discrepancy observed between the 2 studies may be mainly attributable to the difference in the methods of BP measurement.

Perspectives

The Rat Genome Project is almost complete,[4] and precise maps of protein-coding genes are now available. Furthermore, the rat is an appropriate animal for the physiological assessment of cardiovascular functions. Therefore, the rat is a suitable model animal for identifying genes that are related to cardiovascular diseases by means of a positional cloning strategy. On the other hand, the existence of various noncoding RNAs has been confirmed, including microRNAs, and it is highly probable that these noncoding RNAs are involved in gene regulation and gene–gene interactive network regulation.[28,29] Moreover, the existence of large-scale copy number polymorphism in the human genome has also been reported.[24] An expression analysis of just the protein-coding genes might not be sufficient for the identification of genes that contribute to salt-sensitive hypertension.

Acknowledgments

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Polymorphisms in human pre-miRNAs

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Abstract

MicroRNAs constitute a growing class of non-coding RNAs that are thought to regulate gene expression via translational repression. MicroRNAs are initially transcribed as several hundred-nucleotide pri-miRNAs and are then processed to ~60-nucleotide hairpin pre-miRNAs. We hypothesized that polymorphisms in both pre-miRNA and mature microRNA modify various biological processes by influencing the processing and/or target selection of microRNAs. In the present study, we sequenced 173 human pre-miRNA genome regions in 96 subjects and found 10 polymorphisms in the 10 pre-miRNA hairpin regions. Although most of these polymorphisms seem to have no effect on microRNA processing, we identified a C to A polymorphism in the mature miR-30c-2 sequence. This polymorphism may alter target selection and thus exert profound biological effects. To the best of our knowledge, this is the first report of polymorphisms in pre-miRNAs.

Keywords: MicroRNA; Polymorphism; Non-coding RNA; Genetic; Epidemiology; TaqMan method

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 unknown mechanisms. Several microRNAs have been reported to modulate hematopoietic lineage differentiation [3], adipocyte differentiation [4], and insulin secretion [5], indicating that they could play important roles in numerous biological processes. The high degree of phylogenetic conservation in pre-miRNA sequences also supports the importance of this biological system [6].

MicroRNAs are initially transcribed as several hundred-nucleotide pri-miRNAs and are processed to ~60-nucleotide hairpin structure called pre-miRNAs [7]. Mutational analysis of the extended miR-30a stem–loop has shown that disruption of the stem blocks microRNA production [8]; however, the actual sequence of the stem does not appear to be functionally relevant nor does the sequence of the terminal loop appear to be important.

We hypothesized that polymorphisms in pre-miRNA and/or mature microRNA modify various biological processes by influencing the processing and/or target selection of microRNAs. However, the existence of polymorphisms in the human microRNA system has not yet been reported. In the present study, 173 human pre-miRNA genome regions in 96 subjects were sequenced and 10 polymorphisms in 10 pre-miRNA hairpins were identified. Furthermore, a C to A polymorphism in the mature miR-30c-2 sequence was also identified.

Materials and methods

Sequence analysis of microRNA genes. The pre-miRNA sequences were obtained from the microRNA registry (http://www.sanger.ac.uk/Software/Rfam/), and the corresponding genome regions were obtained by Blast analysis (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html). Genome regions corresponding to the 173 pre-miRNAs were sequenced (Table 1). The polymorphisms outside pre-miRNA region will be provided on request. Sequence analyses were performed

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Table 1
List of the 173 microRNA genes investigated in this study

let7a-1	let7a-2	let7a-3	let7b	let7c	let7d	let7e	let7f2	let7g	let7i
miR-1-1	miR-1-2	miR-7-1	miR-7-2	miR-7-3	miR-9-1	miR-9-2	miR-9-3	miR-10b	miR-15a
miR-15b	miR-16-1	miR-16-2	miR-17	miR-18	miR-20	miR-21	miR-23a	miR-23b	miR-24-1
miR-24-2	miR-26a-1	miR-26a-2	miR-26b	miR-27a	miR-29b-2	miR-29c	miR-34a	miR-34b	miR-34c
miR-30a	miR-30b	miR-30c-1	miR-30c-2	miR-30d	miR-30e	miR-31	miR-32	miR-92-1	miR-92-2
miR-93	miR-95	miR-98	miR-99a	miR-99b	miR-100	miR-101-1	miR-101-2	miR-103-2	miR-105-1
miR-105-2	miR-106a	miR-106b	miR-107	miR-122a	miR-124a-1	miR-124a-2	miR-124a-3	miR-125a	miR-125b-1
miR-126	miR-127	miR-128a	miR-128b	miR-129-2	miR-130a	miR-130b	miR-132	miR-133a-2	miR-133b
miR-134	miR-135a-1	miR-135a-2	miR-135b	miR-136	miR-137	miR-138-1	miR-138-2	miR-139	miR-140
miR-142	miR-143	miR-144	miR-145	miR-146	miR-147	miR-148a	miR-148b	miR-149	miR-150
miR-151	miR-153-1	miR-153-2	miR-154	miR-155	miR-181a	miR-181b-1	miR-181b-2	miR-181c	miR-182
miR-183	miR-184	miR-185	miR-186	miR-187	miR-188	miR-190	miR-191	miR-192	miR-193
miR-194-1	miR-194-2	miR-195	miR-196a-2	miR-196b	miR-197	miR-198	miR-199a-2	miR-199b	miR-19b-1
miR-19b-2	miR-200a	miR-200b	miR-200c	miR-203	miR-204	miR-205	miR-206	miR-208	miR-210
miR-211	miR-212	miR-213	miR-214	miR-215	miR-217	miR-218	miR-218-1	miR-218-2	miR-219-1
miR-219-2	miR-220	miR-221	miR-222	miR-223	miR-224	miR-296	miR-299	miR-301	miR-302
miR-323	miR-324	miR-326	miR-328	miR-330	miR-331	miR-335	miR-337	miR-338	miR-339
miR-340	miR-342	miR-375							

Genome regions corresponding to the 173 pre-miRNAs were sequenced. Sequence analyses were performed in 96 subjects randomly selected from the Suita study.

in 96 subjects randomly selected from the Suita study, an epidemiological cohort representing the general population in Japan. Details of this epidemiological cohort have been described elsewhere [9,10]. All of the participants were Japanese, and only those who gave their written informed consent for genetic analysis were included. The Ethics Committee of the National Cardiovascular Center approved the study protocol.

Estimation of the frequency of C to A polymorphism in miR-30c-2. The frequency of C to A polymorphism in miR-30c-2 was estimated by the TaqMan method (ABI) in 3631 subjects recruited from the Suita study. The probes for the C and A alleles were VIC-CTGTAAACA TCCTACACTCT and FAM-CTGTAAACATACTACACTCTC, respectively. The primers for amplification were 5′-CCTAGAGAG CACTGAGCGACAGA-3′ (forward) and 5′-TCTCCCAGCTTTC TTACTTTCCA-3′ (reverse). Frequencies of other polymorphisms in the pre-miRNAs were also estimated by the TaqMan method in 1775 subjects recruited from the Suita study. The details of the TaqMan methods used for other polymorphisms will be provided on request.

Establishment of an expression system for miR-30c-2. The miR-30c-2 region was amplified by PCR (forward: 5′-CCGTGAGTGTGCA AGCTCAAAGCTA-3′, reverse: 5′-CAGATCAGACTGCAGCAAC CCAC-3′), and PCR products (712 bp) corresponding to the C and A pre-miR-30c-2 were subcloned into pcDNA3.1 (Invitrogen) under control of the CMV promoter. Transient expression in CHO and HEK293 cells was performed using LipofectAmine2000 according to the manufacturer's recommendations.

Northern blot analysis. Ten micrograms of total RNA from 20 tissues (Human Total RNA Master Panel II, BD Biosciences) was transferred to a Zetaprobe membrane (BioRad) and cross-linked to the membrane by UV irradiation. The resulting blots were then probed with antisense StarFire probes (Integrated DNA Technologies) labeled with [α-^{32}P]dATP. The sequences were 5′-GCTGAGAGTGTAGG ATGTTTACA-3′ for the detection of mature miR-30c, 5′-CAGCCT TCTCCCAGCTTTCTTACTTTCCAC-3′ for the detection of pre-miR-30c-2, and 5′-CAACCCTCTCCCAGCCACCTTGAGCTCA CA-3′ for the detection of pre-miR-30c-1. The probe sequences for detection of the pre-forms of miR-30c-1 and miR-30c-2 correspond to specific regions of each stem–loop structure to prevent cross-hybridization. The membrane was hybridized for 24 h at 42 °C in 7% SDS/0.2 M Na_2PO_4, pH 7.2, and washed with 0.1% SDS/2× SSPE for 20 min at room temperature followed by 0.1% SDS/1× SSPE for 10 min at room temperature. Signals were analyzed using a BAS2500 image analyzer (Fuji Photo Film).

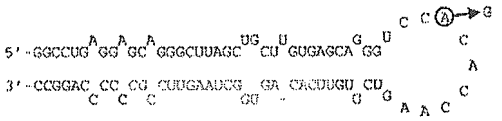
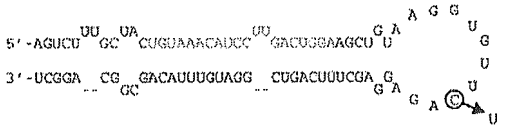
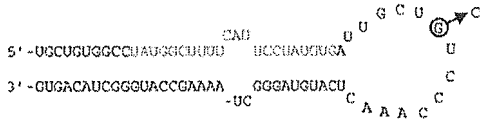
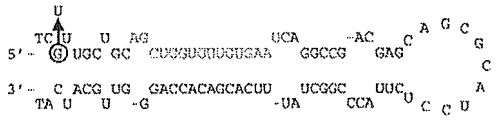
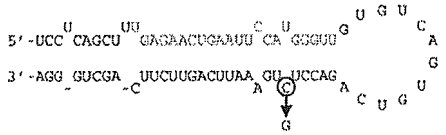
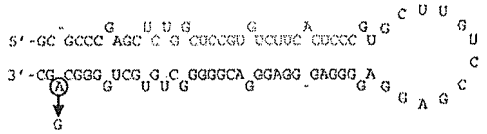
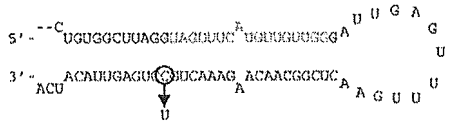
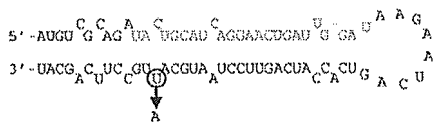
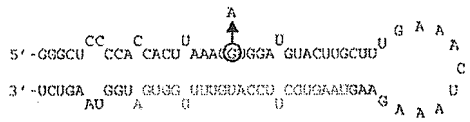
Influence of polymorphism on target selection. The possible target mRNAs of miR-30c were identified in a list of Human MicroRNA Targets [13], in which target sites of mRNAs for miR-30c (dominant C type) are predicted. The validity of these target sites as the target site for the A type variant of miR-30c was reassessed using open-source software for microRNA target prediction (miRanda, www.microrna.org). The algorithm uses dynamic programming to search for maximal local complementary alignments, corresponding to double-stranded antiparallel duplexes. A score of +5 was assigned for G:C and A:T pairs, +2 for G:U wobble pairs, and −3 for mismatched pairs, and the gap-open and gap-elongation parameters were set at −8 and −2, respectively. The thresholds for candidate targets were $S > 90$ and $\alpha G < -17$ kcal/mol, where S is the sum of the single-residue-pair match score over the alignment trace and αG is the free energy of duplex formation from the completely dissociated state, as calculated using the Vienna RNA secondary structure package [11].

Results and discussion

Of the 173 pre-miRNA regions, we identified 10 polymorphisms in the 10 pre-miRNA hairpin regions (Table 2). Zeng and Cullen [8] reported the sequence requirements for microRNA processing by introducing various mutations into an expression system for miR-30. They showed that mature microRNA production was highly dependent on the integrity of the precursor RNA stem, although the underlying specific sequence had little effect. They also showed that the specific sequence of the terminal loop only moderately affected microRNA production.

Polymorphisms in miR-27a, -30e, and -135b were observed in the terminal loop and were considered to be unimportant, based on the model of Zeng and Cullen [8]. Polymorphisms in miR-30c-2, -138-2, -146, -149, -196a-2, -217, and -302a were identified in the stem. The original sequences of the polymorphic sites in miR-149 and -217 give mismatches, and the polymorphisms also give mismatches. Therefore, these polymor-

Table 2
Polymorphisms found in the hairpins of pre-microRNAs

miRNA	Structure	Minor allele frequency
miR-27a		0.366
miR-30c-2		0.0006
miR-30e		0.003
miR-135b		0.014
miR-138-2		0.004
miR-146		0.393
miR-149		0.178
miR-196a-2		0.482
miR-217		0.0008
miR-302a		0.0003

Arrowheads indicate minor genotypes. Mature microRNAs are highlighted in red. Minor allele frequencies were determined by the TaqMan system in 1775 (3631 in the case of miR-30c) subjects recruited from the Suita study.

phisms do not alter the integrity of the stems and appear to be neutral. The original sequences of the polymorphic sites in miR-196a-2 and -302a are at G to C and G to U match sites, while the polymorphisms also give G to U and A to U match sites, respectively. Therefore, these two polymorphisms also appear to be neutral. The fact that most of the polymorphisms are neutral may reflect the importance of the microRNA system.

While miR-138-2 polymorphism disrupts base-pairing, the polymorphism at miR-146 gives a base-pairing

Table 3
Influence of the polymorphism on target selection

Target gene	C polymorphism (kCal/Mol)		A polymorphism (kCal/Mol)	
Glutamate receptor, ionotropic, N-methyl D-aspartate 2B (GRIN2B)	3' CGACUCUCACAUCCUACAAAUGU 5' 5' G-TGAGAGAGGGGGATGTTTCCA 3'	(-23.9)	3' CGACUCUCACAUCAUACAAAUGU 5' 5' G-TGAGAGAGGGGGATGTTTCCA 3'	(-17.03)
Ras homolog gene family, member B (RHOB)	3' CGACU-CUC-ACAUCCUACAAAUGU 5' 5' AATGGTGAGCT-TATGATGTTTACA 3'	(-17.8)	3' CGACU-CUC-ACAU-CAUACAAAUGU 5' 5' AATGGTGAGCT-TATG-ATGTTTACA 3'	(-15.54)
Semaphorin 3A (SEMA3A)	3' CG-ACUC-U-C-ACAUCCUACAAAUGU 5' 5' TCATG-GCATTATGT-GGATGTTTACA 3'	(-19.52)	3' CG-ACUC-U-C-ACAUCAUACAAAUGU 5' 5' TCATG-GCATTATGTGG-ATGTTTACA 3'	(-16.81)
Delta sleep inducing peptide, immunoreactor (DSIPI)	3' CGAC-U-C--UCACAUC-CUACAAAUGU 5' 5' TCTGTATCTTAGTGTAGCGATGTATACA 3'	(-23.85)	3' CGAC-U-C--UCACAUC-AUACAAAUGU 5' 5' TCTGTATCTTAGTGTAGCGATGTATACA 3	(-20.69)
CTD small phosphatase 1 (CTDSP1)	3' CGACUCUCAC-AUCCUACAAAUGU 5' 5' GGAGGGAGGGAAAGGATTTTTACA 3'	(-20.08)	3' CGACUCUCAC-AUCAUACAAAUGU 5' 5' GGAGGGAGGGAAAGGATTTTTACA 3'	(-13.21)
Collapsin response mediator protein 1 (CRMP1)	3' CGA-C-U-CUCACAUCC-UACAAAUGU 5' 5' G-TGGTACTAGTGT-GGTGTGTTTGCT 3'	(-19.02)	3' CGA-C-U-CUCACAUCAUACAAAUGU 5' 5' G-TGGTACTAGTGTGGTGTGTTTGCT 3	(-23.93)
Microtubule-associated protein, RP/EB family, member 2 (MAPRE2)	3' CGACUCUCAC-AUCCUACAAAUGU 5' 5' TTTGTG-GTGCTTTGATGTTTGCA 3'	(-18.65)	3' CGACUCUCAC-A-UCAUACAAAUGU 5' 5' TTTGTG-GTGCTTTG-ATGTTTGCA 3'	(-15.27)
Neuron navigator 1 (NAV1)	3' CGACUCUCACAUCCU---ACAAAUGU 5' 5' TCTG-GAGCCTGGGACCCTGTTTACA 3'	(-22.04)	3' CGACUCUC--ACAUC-A-UACAAAUGU 5' 5' TCTG-GAGCCTG-GGACCCTGTTTACA 3'	(-17.43)
Methionine adenosyltransferase II, alpha (MAT2A)	3' CGACUCUCACAUCCUA-CA-AA-UGU 5' 5' ACAGGGGGTCT-GGCTGGTGTTAACA 3'	(-18.12)	3' CGACUCUCACAUC-A-UACAAAUGU 5' 5' ACAGGGGGTCTGGCTGGTGTTAACA 3'	(-16.58)
MYC binding protein 2 (MYCBP2)	3' C-G-ACUCU-CACA-UCCUACAAAUGU 5' 5' GACATCAAACTTGTGAGG-TGTTTGCA 3'	(-17.01)	3' C-G-ACUCU-CACA-UCAUACAAAUGU 5' 5' GACATCAAACTTGTGAG-GTGTTTGCA 3'	(-14.66)
Septin 7 (SEPT7)	3' CGACUC-UCACAUCCU-ACAAAUGU 5' 5' TCTG-GTGGT-TT-GATTGTTTACA 3'	(-17.44)	3' CGACUC-UCACAUCAU-ACAAAUGU 5' 5' TCTG-GTGGT-TTG-ATTGTTTACA 3'	(-16.35)
A disintegrin-like and metalloprotease with thrombospondin type 1 motif 6 (ADAMTS6)	3' CG-ACUCUCACAUCCU-ACAAAUGU 5' 5' TCTTGA-AG-GT-GGGCTGTTTGCC 3'	(-18.44)	3' CG-ACUCUCACAUCAU-ACAAAUGU 5' 5' TCTTGA-AG-GTGG-GCTGTTTGCC 3'	(-14.61)

Sequence alignment and energy score are shown. C to A polymorphism in miR-30c is highlighted in red.

state. These two polymorphisms alter the integrity of the stem and thus may influence processing of these microRNAs. Recently, it was reported that pri-miRNA is processed by Drosha to produce pre-miRNA [12]. According to that report, Drosha cuts ~22 nt from the terminal loop–stem junction to produce pre-miRNA. Moreover, a mostly double-stranded, extended region (~10 bp) beyond the cleavage sites of pre-miRNA is essential for efficient processing. In the present study, miR-138-2 was revealed to have a polymorphism at 8 bp beyond the pre-miRNA cleavage site. Therefore, the polymorphism in miR-138-2 is likely to influence optimal microRNA processing by Drosha from pri-miRNA.

The C to A polymorphism in miR-30c-2 resides in the mature microRNA sequence (Table 2). This polymorphism would theoretically alter the selection of mRNA targets and may thus be considered to have profound biological influence. Indeed, more than 50 mRNAs have been predicted as targets of miR-30c [13], and certain selected predicted targets are shown in Table 3. According to the miRanda algorithm, 10 of the 12 predicted targets were not considered likely targets for the A-type variant miR-30c (Table 3). Only DSIPI and CRMP1 seem to be targets for the A-type variant miR-30c. On the other hand, it is also likely that the variant microRNA acquires new mRNAs as targets due to this single-nucleotide change.

MiR-30c may be produced from either pri-miR-30c-1 or pri-miR-30c-2 RNAs. If pri-miR-30c-2 were not transcribed in humans, this polymorphism would have no biological significance. However, Northern blot analysis indicated that pre-miR-30c-2 is expressed in human tissues (Fig. 1B), which strongly suggests that some part of miR-30c is derived from the miR-30c-2 gene. However, we failed to detect possible pre-miR-30c-1 at sizes between 60 and 70 nt. The details of the hybridized signal around 80–100 nt remain to be determined (Fig. 1C). It is uncertain whether the miR-30c-1 region is expressed as pre-miRNA and is processed to mature miR-30c.

The C to A polymorphism in miR-30c-2 disrupts the base-pairing in the stem and hence affects stem integrity.

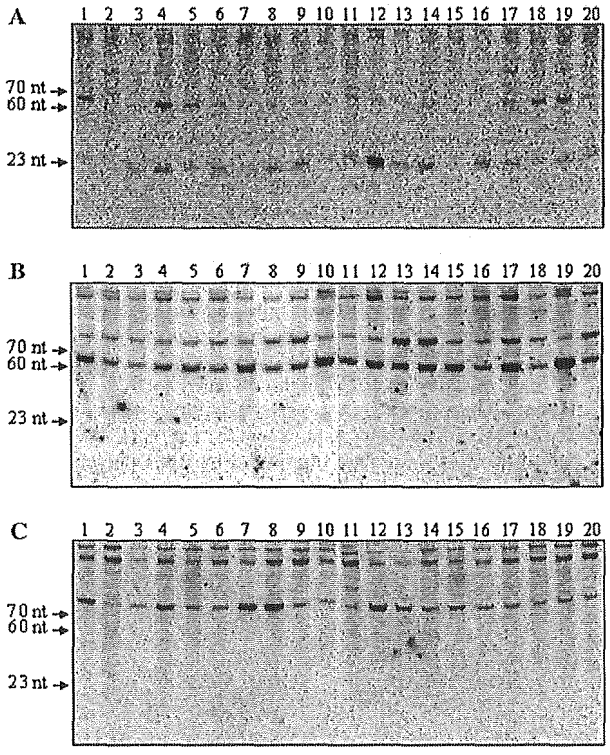


Fig. 1. Expression profile of miR-30c in human tissues analyzed by Northern blotting. Filters (A–C) were obtained by hybridization with the mature miR-30c probe, pre-miR-30c-2 probe, and pre-miR-30c-1 probe, respectively. A 23-nucleotide mature miR-30c was detected (A). A 65-nucleotide pre-miR-30c-2 was detected by the pre-miR-30c-2-specific probe (B), but putative pre-miR-30c-1 (around 65 nt) was not detected (C). Lanes: 1, adrenal gland; 2, bone marrow; 3, brain cerebellum; 4, whole brain; 5, fetal brain; 6, fetal liver; 7, heart; 8, kidney; 9, lung; 10, placenta; 11, prostate; 12, salivary gland; 13, skeletal muscle; 14, spleen; 15, testis; 16, thymus; 17, uterus; 18, colon; 19, small intestine; 20, stomach. The positions of 23, 60, and 70 nt were confirmed by an RNA marker.

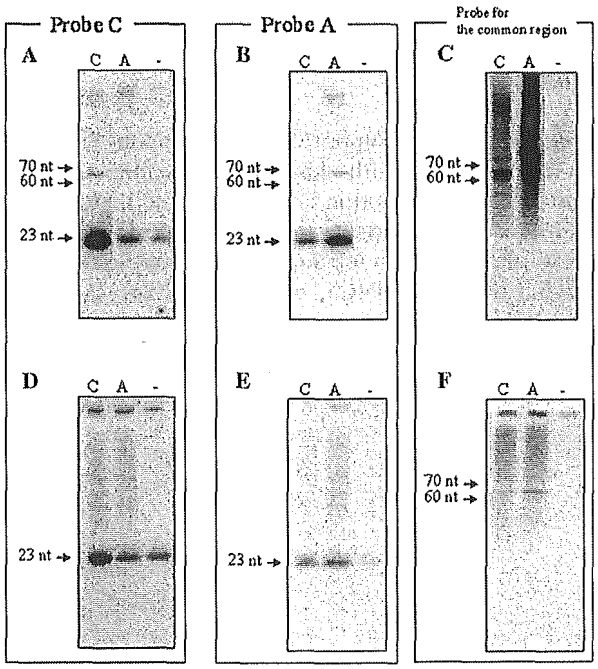


Fig. 2. Influence of the polymorphism on the processing of miR-30c-2. CHO-K1 (A–C) and HEK293 (D–F) cells were transiently transfected with a C or A-type pre-miR-30c-2 expression vector. (–) Mock transfection. Expression of pre- and mature-miR-30c-2 was examined using an antisense C-type probe (A,D), an antisense A-type probe (B,E), or a pre-miR-30c-2-specific probe lacking the polymorphic site (C,F). The mature miR-30c was produced from the natural C-type expression vector (A,D). The variant miR-30c was also produced from the variant A-type expression vector (B,E).

However, the variant A-type miR-30c-2 was also processed to give mature variant miR-30c (Figs. 2B and E). A larger amount of aberrantly processed products appears to be produced from the A-type expression vector (Figs. 2C and F), which suggests inefficient processing of the A-type pri-miRNA for miR-30c-2.

TaqMan analysis indicated that 11 subjects had a heterozygous genotype (CA genotype, C is the major allele) for the miR-30c-2 gene among the 3631 subjects recruited from the general Japanese population. These 11 subjects appeared healthy and were not associated with particular phenotypes in terms of age, height, weight, blood pressure, heart rate, or past and present illness (data not shown). The influence of the heterozygous state in miR-30c-2 may be very subtle, and scrupulous observation of the homozygous state over time may be necessary to confirm associated phenotypes.

In the present study, analyses were confined to the pre-miRNA regions. Recently, pri-miRNAs have been reported to be poly(A)$^+$ RNA transcribed by RNA polymerase II [14]. Therefore, variations in the promoter region may also be important for microRNA expression levels. Clarification of the pri-miRNA structures is required to fully assess the effects of genetic variations on the microRNA system.

Acknowledgments

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