高血圧の薬理遺伝学

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ミレニアム・ゲノム・プロジェクトの最終目標は主要5大疾患（がん，高血圧，糖尿病，痴呆，喘息・アレルギー）の原因となる遺伝子を解明し，その遺伝子情報にもとづく個別化診療を確立することであった．高血圧の診療においてこのような個別化診療はまだ確立されていないが，その最も近道と考えられるのが薬剤感受性遺伝子の情報にもとづく降圧薬の選択である．本稿では高血圧領域における薬理遺伝学（pharmacogenetics/pharmacogenomics）の現況を紹介し，国立循環器病センターでの取り組みをふまえ，今後の展望を述べる．

はじめに

薬剤反応性の個人差には人種差のような集団として影響を及ぼす因子と，各個人の遺伝素因がもたらす因子により20～95%は規定され，これに年齢や薬物代謝に関わる臓器の機能，併用薬剤や治療，病気の程度など非遺伝因子が加わると考えられている[1]．古くから薬理遺伝学（pharmacogenetics）とは「薬剤の反応性における遺伝素因の関与を明らかにする学問」と定義され，その根本は，生まれつき個体に発現されている蛋白の構造，配置，濃度といった遺伝的素因がさまざまな部位での薬剤の効果に影響を及ぼしているといった概念にもとづいている[2]．

これまでにpharmacogenetics的手法により明らかにされた成果の多くは，薬剤代謝に関する代謝酵素遺伝子変異と薬剤の効果や副作用に関するものであった（薬物動態学：pharmacokinetics）．しかしながら降圧薬における遺伝子変異と降圧効果に関する報告は，降圧薬の薬理作用部位に関連する遺伝子の多型に関するpharmacodynamics（薬力学）への関与を検討したものがほとんどである．ヒトゲノム・シークエンスの完了を受けて，これまでわからなかった薬剤の作用部位や詳細な分子構造が明らかにされていくにつれ，それらを標的とした薬理ゲノミクス（pharmacogenomics）が急速に進歩している．高血圧の遺伝的素因の本体を明らかにし，遺伝子情報をもとに高血圧の個別化診療をおこなうことを最終目標にしたミレニアム・ゲノム・プロジェクト（MGP）が2005年3月で終了した[3]．

本稿では期待が高まるpharmacogenetics/pharmacogenomics的手法を用いた降圧薬選択の実現に向けて，MGPにおけるわれわれの取り組みと成果をふまえ，今後の展望について概説する．

KEY WORD
個別化診療，薬理遺伝学，薬理ゲノミクス，マイクロアレイ

1．これまでの降圧薬に対するpharmacogenetics/pharmacogenomics研究成果

一般に降圧薬を含む多くの薬剤では，遺伝因子を含め

種々の因子の薬剤効果への影響はそれぞれは小さいが，累積することにより薬効に影響が及ぶ正規分布型の反応を示すため，影響を及ぼす遺伝子変異や多型を同定するのは容易ではないとされる．これまでに報告された降圧薬の効果に関連する遺伝子多型の多くはpharmacodynamicsの観点より検討されてきた．その理由としてTurner[2]は以下のように推察している．「現在，多型により多様性のある薬物代謝酵素で代謝される降圧薬（ヒドララジン，メチルドパ，β遮断薬など）は多用されなくなっている．また効果や副作用の発現に個人差が大きい降圧薬は市場に生き残れない．さらに実験的にヒトの薬物代謝を評価する方法の確立に伴い，一つの遺伝子多型により多様性を示す酵素で，もっぱら代謝される降圧薬の出現の可能性は非常に低い，などの理由により，pharmacodynamicな機序が今現在広く使用されている降圧薬では，その効果の個人差に強く関与しているであろうと考えられているからである．」また，そのアプローチ法はほとんどが候補遺伝子アプローチである[3]．以下に各降圧薬の感受性遺伝子の現況を述べる．

2．サイアザイド系利尿薬（TZD）

サイアザイド系利尿薬（TZD）の効果に関与する遺伝子多型は数ヵ所についての報告がある．具体的にはG蛋白β3サブユニット遺伝子（*GNB3*）C825T多型[4]，α-アデュシン遺伝子（*ADD1*）Gly 460 Trp多型[5]，レニン・アンジオテンシン（RA）系遺伝子のACE遺伝子（*ACE*）I/D多型，ならびにアフリカ系米国人の女性ではアンジオテンシンIIタイプ1受容体遺伝子（*AT1R*）A1166C多型やアンジオテンシノーゲン（*AGT*）遺伝子G-6A多型，さらにはeNO遺伝子Glu 298 Asp多型でも報告されている．

*GNB3*のC825T多型はβ3-shortを生じ，高血圧の原因遺伝子変異の一つとも考えられている．*GNB3*は12p13領域に位置し，その第10エクソン上にC825Tは存在する．C825Tはサイレントな変異であるが，スプライシングの異常を生じ，その結果，第8，9エクソンの一部に対応する41残基を欠失するβ3-shortを産生する確率を上げると推定されている．β3-shortで欠失する部分はG蛋白αサブユニット（Gα）との相互作用に重要な場所に位置しているため，受容体によるGαの活性化を促進すると考えられている．この*GNB3*のC825T多型が低レニン活性と関連することが報告されたため，TZDの効果にも影響することが予測され197人のアフリカ系米国人と190人のコーカソイドで検討された結果，CC，CT，TTの順に有意にTZDによる降圧効果が良好であった[4]．

アデュシンは細胞膜骨格蛋白でαβのヘテロ2量体を形成する．Milan高血圧ラットの解析でαとβ-アデュシン遺伝子のミスセンス変異が腎臓でのNa再吸収亢進に関与し，高血圧を呈することから，ヒトにおいても*ADD1*の遺伝子多型と高血圧との関連が検討され，Gly 460 Trp多型で有意な関係が認められた．日本人でも低レニン性高血圧には有意な関連を示すため，食塩感受性に影響を及ぼしているものと考えられるが，高血圧・低レニンの多いTrp 460アレルの保有者ではTZDへの反応性が良好であった[6]．

さらに同グループは*ACE* I/D多型と*ADD1* Gly 460 Trp多型を組み合わせた場合，単独よりもTZDへの反応性を予測できると報告した．つまり，TZD感受性の強い*ADD1* Trp 460型と*ACE* I型の両方を有する群で最もTZD投与後の降圧効果が良好であった[7]．

これらの成績はすべて欧米からのもので，日本人で，ある程度大規模なTZDの効果に関連する遺伝子変異・多型の報告はなされていなかった．われわれ[8]は，76人の新規TZD服用患者の降圧効果から感受性遺伝子多型の同定を試みた．平均血圧で5 mmHg以上の降圧を認めた群を反応群と定義し，遺伝子多型は*GNB3* C825T，*ADD1* Gly 460 Trp，RA系や交感神経系関連遺伝子に加え，サイアザイド感受性Na-Cl共輸送体遺伝子（*TSC*），TZD感受性のGordon症候群の原因遺伝子である*WNK1*，*WNK4*，ミネラルコルチコイド受容体遺伝子などをダイレクト・シークエンスにより同定した一塩基多型（SNPs），合計17遺伝子，48多型をタイピングした．その結果，*TSC* C1784Tとβ3アドレナリン受容体遺伝子（*ADRB3*）T727C（Trp 64 Arg）の2 SNPsが有意な関連性を示した（図1）．しかしながら，前述した*GNB3* C825T，*ADD1* Gly 460 Trpでは有意な相関を認めな

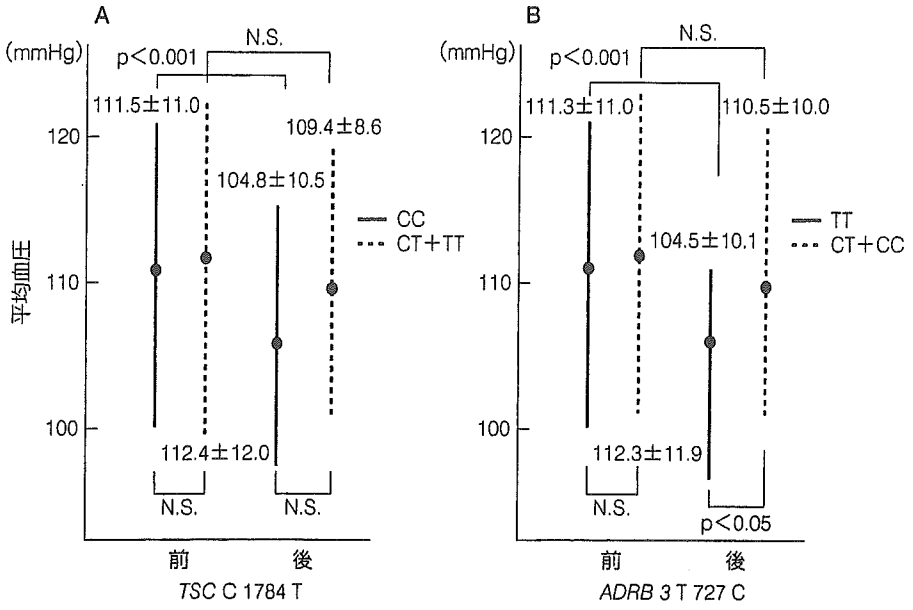

図 1. サイアザイド系利尿薬感受性遺伝子多型
TSC C 1784 T では CC 型の患者でサイアザイド系利尿薬投与により有意に降圧するが，T アレル保有者では有意な降圧が得られていない（A）．*ADRB3* T 727 C では TT 保有者がサイアザイド系利尿薬に感受性をもつ（B）．
(Matayoshi T *et al*, 2004[10]より引用)

かった．これらの多型は日本人高血圧への関与でも否定的な報告がされており[9][10]，人種差を反映している可能性がある．TSC 遺伝子多型の TZD の効果への関与は同時期にほかのわが国のグループからも報告されている[11]．

3．レニン・アンジオテンシン系抑制薬（ACE 阻害薬，ARB）

ACE 阻害薬，ARB の降圧効果に関連する遺伝子多型の報告の多くは RA 系遺伝子の解析に集中しているが，その結果は非常にばらついている[12]．*ACE* の I/D 多型は ACE 活性を規定しているとされ，D アレルで ACE 活性が I 型にくらべ高い．したがって DD 型の保有者は ACE 阻害薬の反応が良好であることが予想されるが，DD 型の頻度がモンゴロイドにくらべ多いコーカソイドを対象にした解析でさえむしろ negative な報告が多い．

われわれも 98 人の新規 ACE 阻害薬内服開始患者においてその降圧効果に関与する多型を RA 系遺伝子，カリクレイン・キニン系（カリクレイン-*KLK1*，ブラジキニン受容体），RA 系関連の血管作動物質（エンドセリン 1-*EDN1*，NO，ADMA），*GNB3* C 825 T，*ADD1* Gly 460 Trp などの遺伝子合計 19 多型を調べた結果，RA 系遺伝子にはいずれも有意な相関は認められず，女性でのみ *EDN1* Ly 198 Asn と *KLK1* Lys 186 Glu 多型に有意な関連を認めた[3]．なお ARB に対する感受性多型は後述する SILVHIA 研究で多数調べられている．

4．β 遮断薬

Jia ら[13]は Gsα 遺伝子第 5 エクソンの制限酵素 FokI で認識されるコドン 131 の同義置換多型が高血圧と関連し，β 遮断薬の降圧効果の大きい群で FokI 認識遺伝子型が多いことを見出した．Gsα 遺伝子は高血圧候補遺伝子であり，Gsα サブユニットは β 受容体の cAMP 生成に重要で血管拡張反応の刺激を担うとされるため興味深い．β_1アドレナリン受容体遺伝子（*ADRB1*）Gly 389 Arg 多型には陽性[14]と陰性[15]の両方の報告がある．また β_2アドレナリン受容体遺伝子（*ADRB2*）の SNP も SILVHIA 研究のマイクロアレイを用いた解析でアテノールの降圧効果に関与することが示された[16]．

5．Ca 拮抗薬

Ca 拮抗薬の降圧効果に明らかに関与する遺伝子多型は報告されていない[3)12)]．とくにジヒドロピリジン系 Ca 拮抗薬（d-CCB）はわが国のみならず国際的にも使用頻度が高い薬剤であるために感受性遺伝子が存在するなら，その同定における意義は大変大きいと考えられる．われわれはＬ型カルシウムチャネル遺伝子の多型に注目し，新規に d-CCB が処方された 185 人の本態性高血圧患者を対象として TZD のときと同様の降圧基準にて，反応（R）群，非反応（NR）群に分け，Ｌ型カルシウムチャネル・サブユニット遺伝子（α1S：*CACNA1S*，α1C：*CACNA1C*，α1D：*CACNA1D*）を選択し，アレル頻度の高い計 12 多型について解析した．この結果，*CACNA1S* のエクソン 3，G/A 多型，*CACNA1D* のイントロン 3，G/A 多型が R，NR 群の頻度ならびに d-CCB 投与後の血圧値にアレル間で有意な差を認めたため，d-CCB の応答性に影響を与えているものと考えている[17)]．

6．降圧薬感受性遺伝子同定のための臨床介入試験

1）SILVHIA 研究

スウェーデン・ウプサラ大学の研究グループは ARB（イルベサルタン）の降圧ならびに心肥大抑制効果に対する遺伝子多型の関与を SILVHIA（Swedish Irbesartan Left Ventricular Hypertrophy Investigation vs. Atenolol）研究として精力的に検討している[18)]．この研究ではイルベサルタンとアテノロールをそれぞれ 50 人程度の高血圧患者に 12 週間，単剤投与するといったプロトコールで，それぞれの薬剤の降圧効果，心肥大退縮作用を検討したものである．

イルベサルタンは軽～中等症高血圧患者の 40～50% で有効な降圧効果をもつ薬とされているが，*ACE* I/D 多型のII型を示す患者の 89% に拡張期血圧（DBP）で 10 mmHg 以上の降圧を認めた．一方，DD 型では 24% しか DBP＞10 mmHg 以上の降圧を示した患者はいなかった[19)]．同様にアルドステロン合成酵素遺伝子 C-344 T もイルベサルタンの降圧効果に有意な関連性を示していた[20)]．ほかの RA 系関連の遺伝子多型では降圧効果に有意性を認めたものはなかったが，アンジオテンシノーゲン遺伝子 T 174 M，M 235 T ならびにアンジオテンシン 1 型受容体遺伝子 A 1166 C はイルベサルタンの心肥大退縮作用に有意性を認めた[21)]．

またこの研究グループはマイクロアレイを用いた独自のタイピング法（Microarray based DNA polymerase assisted minisequencing single nucleotide primer extension assay with fluorescence detection）を開発し，SILVHIA 研究においてそれぞれの薬剤の降圧効果[16)]や心肥大退縮作用[22)]に関与する SNP を RA 系や交感神経系，血管作動性物質，脂質代謝などに関わる 25 遺伝子，74 SNPs で検討して複数の薬剤感受性遺伝子多型を同定している．今後，pharmacogenomics による個別化診療の実現に向けて，このような迅速に多数の遺伝子多型をタイピングする方法の開発が望まれていただけに，このマイクロアレイを用いたタイピング方法は今後急速に発展していくことが予測される．

2）GEANE 研究

降圧薬には抗がん剤のような重篤な副作用はほとんど認められないため，pharmacogenomics は降圧薬の効果，つまり薬剤応答性の予測のために応用される必要がある．ここまで述べてきたように，残念ながら各降圧薬でその効果にはっきりと関連性をもった遺伝子変異・多型の報告はまだ少なく，今後の更なる研究成果が待たれるところである．より関連性の強い薬剤応答性・感受性遺伝子の同定のためには，多数例の無治療高血圧患者に前向きに降圧薬を投与し，正確に降圧の程度を把握し，数多くの薬物代謝酵素や薬理作用機序関連の遺伝子多型との相関を検討する必要がある．

これまでわが国にこのような研究はなかったが，現在，国立循環器病センターでは全国の大学・医療センター計 8 施設とともに降圧薬感受性遺伝子多型同定のための多施設共同研究（GEANE 研究：Gene Evaluation for ANtihypertensive drug Effect）を開始した．GEANE 研究では，無投薬の軽～中等症本態性高血圧患者に TZD，ARB，長時間作用型 d-CCB を 3ヵ月ごとに少量から通常使用量に増量して内服してもらい，観察期も含め

インフォームド・コンセント（軽・中等症の未治療高血圧患者を対象とする）
↓　血圧測定，採血（一般生化学ーとくに血清K，脂質値，尿酸値，血糖値，遺伝子採血）
↓　観察期(1ヵ月)投薬順ランダマイゼーション(以下1．TZD，2．d-CCB，3．ARBの場合)
第1薬投薬（3ヵ月：1回/月受診）
↓　サイアザイド系利尿薬（インダパミド1mgより開始）
↓　Dose up（インダパミド2mgに増量）
↓　(インダパミド2mgを継続)，採血（一般生化学）
第2薬投薬（3ヵ月：1回/月受診）
↓　Ca拮抗薬（アムロジピン2.5mgより開始）
↓　Dose up（アムロジピン5mgに増量）
↓　(アムロジピン5mgを継続)，採血（一般生化学）
第3薬投薬（3ヵ月：1回/月受診）
↓　ARB（バルサルタン40mgより開始）
↓　Dose up（バルサルタン80mgに増量）
↓　(バルサルタン80mgを継続)，採血（一般生化学）
終了

図2．GEANE研究投薬プロトコール
GEANE研究は国立循環器病センター高血圧腎臓内科，同研究所，国立病院機構九州医療センター高血圧内科，大阪大学老年・腎臓内科学，金沢医科大学高齢医学，日本大学第2内科，九州大学臨床薬理学，愛媛大学老年医学，同第2内科の共同研究である．

合計10ヵ月間で投薬を終了するデザインで施行中である（図2）．降圧効果のみならず副作用や代謝性の異常も解析予定で，複数のSNPを検討し，降圧薬としてとくに重要と考えられる3種類の薬剤の感受性遺伝子多型，ならびに副作用関連遺伝子多型を検討する予定である．

本研究は（1）同一患者に3種類の降圧薬をクロスオーバーに内服させるため，その個人の正確な薬剤反応性が評価できる，(2) 多施設共同でおこなうことにより，従来の研究より多くの対象者での解析が期待できる，(3) 迅速遺伝子タイピング法を用いて複数の遺伝子多型の解析が可能である，といった特徴を有しているため，これにより同定された遺伝子多型を実際の臨床に応用し，個別化診療を確立することを構想している．

おわりに

ミレニアム・ゲノム・プロジェクトがおこなわれたことでわが国の疾患遺伝子研究は飛躍的に進歩した．高血圧の領域でも高血圧原因遺伝子多型や病態修飾遺伝子多型が多数同定されてきた．今後はこれらを臨床に応用していく必要がある．薬理遺伝学的なアプローチは，個別化診療確立に向けて最も臨床応用しやすい方法であると考えられるため，今後の急速な進歩が期待される．

謝　辞

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Original Article

Single Nucleotide Polymorphisms in the Interleukin-6 Gene Associated with Blood Pressure and Atherosclerosis in a Japanese General Population

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It is known that increased plasma levels of inflammatory markers, such as interleukin-6 (IL-6), are associated with atherosclerosis and myocardial infarction. The aim of this study was to reveal the contribution of the single nucleotide polymorphisms (SNPs) of the *IL-6* gene on the blood pressure regulation and progression of atherosclerosis in a general Japanese population. In order to evaluate the potential implications of genetic variability of the *IL-6* gene, we explored eight SNPs by direct sequencing for the entire coding region and the promoter region in the *IL-6* gene and genotyped two SNPs, −636G>C in the promoter region and 1691C>G in intron 3, for a total of 2,421 Japanese subjects (1,162 men and 1,259 women). As a consequence, −636 G>C was significantly associated with systolic blood pressure (SBP) and carotid intima-media thickness (IMT) in women, and 1691C>G showed a relationship with SBP and carotid IMT in men after adjustment for all confounding factors. Although neither SNP had a significant correlation to the prevalence of hypertension, the haplotype frequency analysis indicated that the number of hypertensive men with a G allele at both −636 and 1691 was significantly greater than the number of nonhypertensive men with this combination. Thus, these two SNPs in the promoter region and intron 3 of the *IL-6* gene might play a role in the blood pressure regulation and progression of atherosclerosis in the Japanese. (*Hypertens Res* 2005; 28: 35–41)

***Key Words*: interleukin-6, single nucleotide polymorphism, blood pressure, atherosclerosis, Japanese general population**

Introduction

Recent epidemiological studies have shown that chronic inflammation plays a key role in cardiovascular disease (CVD) (*1*). Several epidemiological studies show that increased plasma levels of inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) are associated with atherosclerosis, myocardial infarction (*2*), endothelial dysfunction (*3*) and high blood pressure (*4*, *5*).

IL-6 is a pleiotropic cytokine involved in not only immunity and inflammation but also bone metabolism and neural development (*6*). IL-6 also stimulates the proliferation of cultured vascular smooth muscle cells (*7*), indicating that this cytokine may play an important role in the development of arteriosclerosis.

Regarding the pathophysiological contribution of the gene polymorphisms of *IL-6* to CVD, the positive correlation between two single nucleotide polymorphisms (SNPs) in the promoter region of *IL-6* and CVD has been previously

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Table 1. Clinical Features of Study Participants

Variables	Men (n=1,162)	Women (n=1,259)	p
Age (years old)	61.0±12.2	58.9±11.7	<0.0001*
Body mass index (kg/m^2)	23.1±2.8	22.4±3.1	<0.0001*
SBP (mmHg)	129.2±19.1	128.5±21.1	0.443*
DBP (mmHg)	81.0±10.8	78.8±10.6	<0.0001*
TC (mg/dl)	204.5±32.0	215.9±32.9	<0.0001*
HDL cholesterol (mg/dl)	54.5±14.3	64.4±15.4	<0.0001*
Hypertension (%)	39.7	35.5	0.0343†
Diabetes mellitus (%)	9.0	3.4	<0.0001†
Hyperlipidemia (%)	35.2	51.4	<0.0001†
Current alcohol consumer (%)	71.5	29.3	<0.0001†
Current smoker (%)	38.2	8.7	<0.0001†
Antihypertensive medication (%)	17.7	17.2	0.7500†
−636G>C (n)			
GG	87	79	0.109†
GC	359	439	
CC	648	681	
1691C>G (n)			
CC	14	12	0.785†
GC	175	198	
GG	938	1,028	

Values are mean±SD or percentage. Hypertension: SBP ≥140 mmHg, DBP ≥90 mmHg or antihypertensive medication; diabetes mellitus: fasting plasma glucose ≥126 mg/dl, HbA1c ≥ 6.5% or antidiabetic medication; hyperlipidemia: TC ≥220 mg/dl, TG ≥150 mg/dl or antihyperlipidemia medication. *p was calculated by Student's t-test. †p was calculated by χ^2 test. SBP; systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride.

reported. An association of the −174G>C polymorphism has been found with systolic blood pressure (SBP) and diastolic blood pressure (DBP) in middle-aged healthy men (*8*). Several studies have suggested that the −174G allele was associated with a higher plasma level of IL-6 in patients with abdominal aortic aneurysms (*9*), in patients undergoing coronary artery bypass graft surgery (CABG) (*10*), and in healthy women (*11*). Another polymorphism, −572G>C, which was identical to −634G>C, was associated with the progression of diabetic nephropathy (*12*) and increased plasma levels of IL-6 after CABG (*10*). Functional studies using the reporter gene showed that −174G>C, −572G>C, and the A and T repeat variation, AnTn tract, which lies between the −174G>C and −572G>C, intricately cooperate in regulating *IL-6* gene expression (*13*).

Although several association studies on −174G>C in Caucasians have been reported, this polymorphism is recognized only with low allele frequency in South Chinese, Korean, and Japanese populations (*12*, *14–16*). In the present study, we identified the common SNPs of the *IL-6* gene in the Japanese, and investigated the association between SNPs of the *IL-6* gene and blood pressure and atherosclerosis in the Suita Study, which employed a representative general Japanese population.

Methods

Subjects

The selection criteria and design of the Suita Study have been previously described (*17–20*). The protocol of study was approved by the Ethics Committee of the National Cardiovascular Center. Only the participants who gave their written informed consent for genetic analysis were included in this study. DNA from leukocytes was collected from 2,421 participants who visited the Division of Preventive Cardiology at the National Cardiovascular Center between May 1996 and February 1998.

The characteristics of the subjects analyzed in the present study are summarized in Table 1. Blood pressure was measured in the subjects after at least 10 min of rest in a sitting position. The blood pressure value is the mean of two physician-obtained measurements (recorded >3 min apart). Carotid intima-medial thickness (IMT) was measured by ultrasonography using previously described methods (*21*) as an indicator of atherosclerosis. Hypertension was defined as SBP ≥140 mmHg or DBP ≥90 mmHg, or the current use of antihypertensive medication, diabetes mellitus (DM) was defined as fasting blood glucose ≥126 mg/dl, HbA1c ≥6.5%

Table 2. Identified Polymorphisms in the *IL-6* Gene

SNPs	Amino acid	Region	Allele 1	Hetero	Allele 2	Total	Allele frequency		Flanking sequence	dbSNP ID
Allele 1>Allele 2	change		Homo		Homo		Allele 1	Allele 2		
−636G>C*,†		promoter	7	16	25	48	0.313	0.688	tctacaacagcc[g/c]ctcacagggaga	
1338C>T*		intron 2	8	14	23	45	0.333	0.667	ttttcttagaga[c/t]tttcctggctgt	
1368G>T*		intron 2	8	15	23	46	0.337	0.663	aacaatgaaaag[g/t]ccctctagtggt	rs2066992
1394A>G		intron 2	44	2	0	46	0.978	0.022	tttgttttaggg[a/g]cacttaggtgat	
1691C>G†		intron 3	39	7	0	46	0.924	0.076	tgaggaggccaa[c/g]ttcaagcttttt	rs2069840
2099T>G		intron 3	45	1	0	46	0.989	0.011	tatttaaaatgg[t/g]gctgtccaatgt	
4158T>A	Asp162Glu	exon 5	33	3	0	36	0.958	0.042	aaagaatctaga[t/a]gcaataaccacc	
4415G>A	3′ UTR	exon 5	34	1	0	35	0.986	0.014	ggagaactaaaa[g/a]tatgagcgttag	

*These SNPs are in linkage disequilibrium ($r^2 > 0.95$). †These SNPs were used for genotyping analysis. The A of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomeclature Working Group (*Hum Mut* 1998; **11**: 1–3). The genome sequence retrieved from GenBank (accession ID: NT_007819.14, GI: 37538470) was used as a reference sequence. IL-6, interleukin-6; SNP, single nucleotide polymorphism.

or the current use of insulin or oral anti-diabetic agents, and hyperlipidemia was defined as total cholesterol ≥ 220 mg/dl or triglyceride ≥ 150 mg/dl, or the current use of antihyperlipidemia medication at the time of the first examination.

Direct Sequencing for the Detection and Genotyping of Polymorphisms in the *IL-6* Gene

We sequenced the entire coding region of the *IL-6* gene and the 5′-flanking region upstream, approximately 1.5 kb from the transcription start site, using DNA samples from 48 volunteers after obtaining their written informed consent. The method of direct sequencing has been previously described (*22*). The identified polymorphisms were numbered from the A of the initiator codon, as recommended by the Nomenclature Working Group (*23*). The genotype of the SNPs having a 5% or greater minor allele frequency was determined by the TaqMan-polymerase chain reaction (PCR) system (*24*). The sequences of PCR primers and probes for the TaqMan-PCR method were as follows. For −636G>C, the primers were 5′-GTAACTGCACGAAATTTGAGGGT-3′ (sense) and 5′-GTTTCCTCTGACTCCATCGCA-3′ (antisense), and the probes were Fam-ACAGCCCCTCACAGG-MGB (for the C allele) and Vic-ACAGCCGCTCACAG-MGB (for the G allele). For 1691C>G, the primers were 5′-TCTGGCCAT ACCTGTCCAAGA-3′ (sense) and 5′-CAGCAACAAAAAG TGGGTAAATGT-3′ (antisense), and the probes were Fam-AAGCTTGAAGTTGGCCT-MGB (for the C allele) and Vic-AAGCTTGAACTTGGCCT-MGB (for the G allele).

Statistical Analysis

The association of genotypes with blood pressure and IMT was investigated by ANCOVA considering potential confounding factors. For multivariate risk predictors, the adjusted odds ratios were given with 95% confidence intervals. The relationship between genotype and hypertensive risk was expressed as an odds ratio adjusted by possible confounding factors. All analyses were performed with SAS statistical software (release 8.2; SAS Institute Inc., Cary, USA). Linkage disequilibrium (LD) was evaluated by obtaining r^2 values between polymorphisms, and haplotype analysis was performed using the program SNPAlyze, ver. 3.1Pro (DYNACOM Co., Ltd., Mobara, Japan).

Results

Polymorphisms in the *IL-6* Gene

Direct sequencing using DNA samples from 48 volunteer subjects identified eight SNPs in the *IL-6* gene, including one SNP in the promoter region, one missense mutation in exon 5, one SNP in the 3′-untranslated region, and five SNPs in introns (Table 2). One SNP, −636G>C, was identical to the previously described −572G>C (−634G>C) (*13*, *16*). Three SNPs, −636G>C, 1338C>T and 1368G>T, were tightly in LD ($r^2 > 0.95$). An AnTn polymorphism in the promoter region was also detected, but it was difficult to detect the precise number of A and T residues by direct sequencing. We could not detect −174G>C in our Japanese population, as reported previously (*12*, *16*). A missense mutation, 4158T>A (Asp162Glu), may be functionally important. However, since the allele frequency of this mutation was low, this SNP was not genotyped in the present study. Finally, −636G>C in the promoter region and 1691C>G in intron 3, both of which showed a minor allele frequency of 0.05 or more, were selected for genotyping.

Study Population

The clinical characteristics of the study subjects, 1,162 men and 1,259 women, are shown in Table 1. There were significant differences between men and women in some variables, including age, body mass index, DBP, serum total choles-

Table 3. Multivariate-Adjusted Blood Pressure Levels on Genotype of SNPs in *IL-6* Gene

SNP	Men			Women		
	Genotype group		*p*	Genotype group		*p*
-636G>C	GG	GC+CC		GG	GC+CC	
DBP (mmHg)	80.5±1.1	81.0±0.3	0.656	80.4±1.1	78.8±0.3	0.160
SBP (mmHg)	127.9±1.8	129.3±0.5	0.461	133.3±2.0	128.4±0.5	0.017
	GG+GC	CC		GG+GC	CC	
DBP (mmHg)	81.1±0.5	80.9±0.4	0.656	78.6±0.4	79.1±0.4	0.383
SBP (mmHg)	129.7±0.8	128.8±0.7	0.363	128.7±0.8	128.8±0.7	0.935
1691C>G	CC	CG+GG		CC	CG+GG	
DBP (mmHg)	81.0±0.3	81.7±0.7	0.360	78.9±0.3	78.6±0.7	0.727
SBP (mmHg)	128.9±0.5	131.2±1.2	0.072	128.6±0.6	127.8±1.2	0.571
	CC+CG	GG		CC+CG	GG	
DBP (mmHg)	81.1±0.3	81.1±2.7	0.982	78.8±0.3	80.3±2.8	0.597
SBP (mmHg)	129.3±0.5	126.7±4.4	0.559	128.5±0.5	123.8±5.1	0.356

Values are mean±SD. All adjusted for age, body mass index, hyperlipidemia, diabetes mellitus, smoking, drinking, and antihypertensive medication. Il-6, interleukin-6; SNP, single nucleotide polymorphism; DBP, diastolic blood pressure; SBP, systolic blood pressure.

terol, and high desity lipoprotein cholesterol levels, prevalence of hypertension, DM, and hyperlipidemia, and percentage of current alcohol drinking and smoking.

Association of the Polymorphisms with Blood Pressure

DBP and SBP levels were evaluated in men and women by genotypes after adjustment for the confounding risk variables. After adjustment for age, the -636G>C polymorphism was significantly associated with both SBP and DBP in women, and the 1691C>G polymorphism was significantly associated with SBP in men (data not shown). After adjustment for all confounding factors, only -636G>C was significantly associated with SBP in women (p=0.017) (Table 3). Mean SBP levels of women with the GG genotype were approximately 5 mmHg higher than those of women with the C allele. There were no significant positive associations between -636G>C and blood pressure in men. In order to exclude the influence of antihypertensive medication, we performed the same analysis in subjects who did not receive medication. There was no difference in the distribution of the genotypes of the two poly-

Table 4. Multivariate-Adjusted Blood Pressure Levels in Subjects without Antihypertensive Medication

SNP	Genotype group		*p*	Adj
-636G>C Women	GG (n=59)	GC+CC (n=932)		
DBP (mmHg)	80.2±1.3	77.4±0.3	0.037	age
	79.7±1.3	77.4±0.3	0.073	all
SBP (mmHg)	130.5±2.3	124.2±0.6	0.008	age
	130.2±2.3	124.3±0.6	0.012	all
1691C>G Men	CC (n=780)	CG+GG (n=145)		
DBP (mmHg)	79.9±0.4	80.4±0.9	0.620	age
	79.9±0.4	80.6±0.8	0.419	all
SBP (mmHg)	125.5±0.6	128.4±1.4	0.045	age
	125.4±0.6	128.6±1.3	0.027	all

Values are mean±SD. All adjusted for age, body mass index, hypertension, diabetes mellitus, smoking, and drinking. SNP, single nucleotide polymorphism; Adj, adjustment; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Table 5. Odds Ratio of -636G>C and 1691C>G for Hypertension

SNP	Genotype group	OR (95% CI)	*p*
-636G>C			
Men	GG	1	
	GC+CC	1.10 (0.63–1.93)	0.745
	GG+GC	1	
	CC	1.30 (0.95–1.77)	0.103
Women	GG	1	
	GC+CC	1.64 (0.90–2.98)	0.105
	GG+GC	1	
	CC	0.92 (0.67–1.26)	0.605
1691C>G			
Men	CC	1	
	CG+GG	0.69 (0.46–1.02)	0.065
	CC+CG	1	
	GG	2.04 (0.43–10.00)	0.371
Women	CC	1	
	CG+GG	1.09 (0.72–1.64)	0.69
	CC+CG	—	
	GG	—	—

*Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence intervals.

Table 6. Haplotype Frequency of *IL-6* Gene in Hypertensives (HT) and Normotensives (NT)

Haplotype	-636/1691	Men				Women			
		HT (866 alleles)	NT (1,300 alleles)	χ^2	p	HT (854 alleles)	NT (1,532 alleles)	χ^2	p
1	C/C	73.1	77.2	4.63	0.031	74.1	75.5	0.52	0.47
2	G/C	17.8	16.1	1.08	0.298	17.9	16.4	0.84	0.36
3	G/G	9.1	6.8	4.04	0.044	7.8	8.0	0.03	0.874
4	C/G	0.0	0.0	—	—	0.1	0.1	0.18	0.675

Haplotype frequencies are expressed as percentage.

Table 7. Multivariate-Adjusted Carotid IMT on Genotype of SNPs in *IL-6* Gene

SNP	Men				Women			
	Genotype group			p	Genotype group			p
-636G>C	GG	GC	CC		GG	GC	CC	
Mean IMT (mm)	0.91±0.01	0.91±0.01	0.90±0.00	0.288	0.89±0.01	0.86±0.00	0.85±0.00	0.005
Max-IMT (mm)	1.70±0.08	1.78±0.04	1.70±0.03	0.357	1.47±0.06	1.36±0.02	1.33±0.02	0.025
1691C>G	CC	CG	GG		CC	CG	GG	
Mean IMT (mm)	0.91±0.00	0.92±0.01	0.95±0.03	0.021	0.86±0.00	0.86±0.01	0.86±0.03	0.538
Max-IMT (mm)	1.71±0.03	1.86±0.06	1.60±0.22	0.108	1.34±0.02	1.38±0.04	1.26±0.15	0.515

Values are mean±SD. Adjusted for age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, smoking, and drinking. IMT, intima-medial thickness; SNP, sigle nucleotide polymorphism; Max-IMT; maximum-IMT.

morphisms among the subjects administered antihypertensive drugs. After adjustment for all confounding factors, -636G>C and 1691C>G were significantly associated with SBP in women and men, respectively (Table 4).

The association between these two genotypes and hypertension was not significant in either men or women (Table 5). Table 6 indicates the results of haplotype frequency analysis for the *IL-6* gene polymorphisms between hypertensives and normotensives. We identified that haplotypes 1 and 3 had significantly lower ($p=0.031$) and higher ($p=0.044$) frequency in hypertensive men than in normotensive men, respectively (Table 6). In other words, the prevalence of men with a G allele at both -636 and 1691 in the *IL-6* gene was significantly higher in the hypertensives. In contrast, there was no difference in the haplotype frequency between hypertensive and normotensive women.

Association of the Polymorphisms with Carotid IMT

We also evaluated the relationship between the *IL-6* gene polymorphisms and mean IMT and maximum-IMT. In men, 1691G was associated with greater mean IMT. In women, on the other hand, mean IMT and maximum-IMT significantly decreased as the copy number of the C allele at -636 decreased (Table 7).

Discussion

In the present study, we searched for polymorphisms in the coding and promoter regions of the *IL-6* gene by direct sequencing and identified eight SNPs. Among them, two polymorphisms, -636G>C in the promoter and 1691C>G in intron 3, had positive associations with blood pressure and carotid atherosclerosis in a large-scale Japanese general population. Specifically, -636G>C in women and 1691C>G in men were significantly associated with SBP, mean IMT and maximum-IMT.

Although women with GG of -636G>C showed significantly higher SBP than the C carriers, there was no significant association with the prevalence of hypertension. Nakajima *et al.* previously reported that only -636G>C of *IL-6* showed a trend of association with hypertension in about 300 Japanese women (*16*). Our present study supports their results: no relationship was observed between the haplotypes of *IL-6* and the morbidity of hypertension in the haplotype frequency analysis in women.

This is the first report to show a positive relationship between 1691C>G in intron 3 of *IL-6* and clinical features. Among non-medicated subjects, men with the G allele of 1691C>G showed a significantly higher SBP than did subjects with the CC genotype. Although this SNP did not seem to involve the prevalence of hypertension by itself, the frequency of the haplotype that contains the G allele at both -636 and 1691 was significantly higher in hypertensive than in normotensive men. This suggested that these SNPs should have a cooperative influence on the prevalence of hypertension in men, although each SNP has only a weak effect on blood pressure.

Inflammation is closely related to atherosclerosis (*1*). Recent studies have suggested that the −174G>C polymorphism in the *IL-6* promoter was associated with myocardial infarction in subjects from Northern Ireland and France (*25*), with coronary heart disease in British subjects under pravastatin treatment (*26*), and with ischemic stroke in Italians (*27*). However, this SNP, −174G>C, could not be identified in Japanese. Instead, we found that −636G>C and 1691C>G in the *IL-6* gene were associated with carotid IMT in women and men, respectively. Since a significant association was observed by multivariate analysis with adjustment for confounding risk factors, including hypertension, these two SNPs appear to be independent risk factors of atherosclerosis. Regarding the significant association between *IL-6* gene polymorphisms and blood pressure in men and women, it is possible that atherosclerosis may intervene in the positive relationships between −636G>C and SBP in women and between 1691C>G and SBP in men.

It remains unclear why the influence of SNPs on clinical features differed by gender. It is also unclear whether these SNPs are "functional" or just risk markers. Several studies have reported that the combination of polymorphisms in the promoter region of *IL-6* affects its gene expression (*13*, *28*, *29*). It has been shown that East Asians and African-Americans have different genotype patterns in *IL-6* compared with Caucasians (*12*, *14–16*, *30*). The −174G>C polymorphism in the promoter region of *IL-6* is considered to regulate *IL-6* production (*9*, *26*, *31*) and is associated with juvenile chronic arthritis (*28*) and myocardial infarction in Caucasians (*25*). In the present study, −174G>C was not identified, although this SNP is commonly detected with about a 40% prevalence in Caucasians (*13*, *25*, *28*, *31*, *32*). In contrast, −636G>C, which we clarified to have a significant association with blood pressure and carotid IMT in Japanese women, has been recognized with only about a 5% prevalence in Caucasians (*13*, *25*, *32*). In the African-American population, −636G>C is common (9.5%) and −174G>C is rare (4%), findings which are very different from those for Caucasians (*30*); however, circulating *IL-6* levels in African-Americans are similar to those in Caucasians (*31*). Thus, −174G>C is important in the regulation of *IL-6* production in Caucasians, but other gene polymorphisms, such as −636G>C, may play important roles in *IL-6* production in East Asian and African-American populations. It is necessary to investigate the correlation between the plasma levels of *IL-6* and two SNPs, −636G>C and 1691C>G, in the *IL-6* gene. We also need to clarify whether these SNPs contribute to the morbidity of atherosclerotic CVD by prospective studies in Japanese.

In conclusion, two SNPs, one in the promoter region and the other in intron 3 of the *IL-6* gene, may be involved in blood pressure regulation through their contribution to atherosclerosis in Japanese.

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Insertion/deletion polymorphism in clusterin gene influences serum lipid levels and carotid intima-media thickness in hypertensive Japanese females

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Abstract

Clusterin has been implicated in lipid metabolism and atherogenesis, however, the influence of genetic variation has not been examined in Japanese. In this study, we identified 11 single nucleotide polymorphisms (SNPs) of clusterin gene by direct sequencing. Among them, one promoter SNP (−4453T > G), one missense SNP (4183G > A), and 2 common SNPs (5608T > C and 6316delT) were genotyped in 525 asymptomatic hypertensives not treated with lipid lowering agents. −4453T > G, 4183G > A, and 5608T > C showed no correlation with the clinical characteristics, however, in the 6316delT, an insertion (I)/deletion (D) polymorphism, D/D subjects had significantly higher levels of total cholesterol and low-density lipoprotein (LDL)-cholesterol than I/I subjects in females but not in males. Female subjects with the D allele (D/D + I/D) had greater intima-media thickness of the carotid artery than I/I subjects. In a multiple logistic regression analysis, the D allele of 6316delT was detected as an independent predictor for the plaque prevalence. In conclusion, the clusterin gene polymorphism may contribute to the serum lipid levels and the progression of carotid atherosclerosis in hypertensive Japanese females.

Keywords: Atherosclerosis; Carotid arteries; Hypertension; Risk factors; Clusterin; Apolipoprotein J; Polymorphism

Clusterin [also called apolipoprotein (apo) J, complement cytolysis inhibitor, sulfated glycoprotein-2, testosterone repressed message-2, and glycoprotein III] is an 80-kDa heterodimeric glycoprotein consisting of two subunits. Clusterin expression is observed in a variety of tissues, especially in injured site, and has been reported to show various functions such as the induction of nodule formation in culture cells, suppression of apoptosis induced by a variety of stressors, and inhibition of complement-mediated cell lysis [1].

Recent reports provide the evidence that clusterin is implicated in atherogenesis. In human aorta, clusterin localizes in atherosclerotic lesions but not in normal area, and the expression level of clusterin increases during progression of atherosclerosis [2]. In animal models, mRNA levels of clusterin transiently increased in rat aorta after balloon injury [3]. Furthermore, clusterin has been demonstrated to inhibit migration, adhesion, and proliferation [4], and induce differentiation in vascular smooth muscle cells [5]. We also reported that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, a potent endogenous ligand for peroxisome proliferator-activated receptor-γ, promotes the differentiation of vascular smooth muscle cells

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through the clusterin induction [6]. These findings suggest that clusterin plays an important role in the development of vascular disease.

Clusterin has been reported to be present in HDL-cholesterol together with apo A-I and cholesterol ester transport protein in circulating blood [7]. In an in vitro experiment, clusterin promotes the efflux of cholesterol from lipid-loaded mouse macrophages [8]. Few researchers have investigated the clusterin gene polymorphisms. Kamboh et al. [9] reported the existence of polymorphisms of the human clusterin gene in US and Nigerian blacks, however, found no significant correlation with serum lipid levels. Nestlerode et al. [10] reported that two polymorphisms of the clusterin gene [Asn317His (10580A > C), Asp328Asn (10613G > A)] found in African blacks were associated with serum HDL-cholesterol levels. Tycko et al. [11] investigated the relationships between the clusterin polymorphism and Alzheimer's disease, although they found no association. However, no evidence so far has been presented. Furthermore, clusterin polymorphism in Japanese has not been examined yet and its association with serum lipid levels and atherosclerosis remains unknown.

The aim of this study was, therefore, to identify polymorphisms of the clusterin gene in Japanese and to examine the associations among clusterin polymorphisms, serum lipid levels, and the carotid atherosclerosis. The severity of carotid atherosclerosis was quantitatively determined as the intima-media thickness of the carotid artery (CCA-IMT) and the presence of plaque.

Methods

Subjects. Nine hundred and fifty three patients with essential hypertension participating in an annual examination at the outpatient clinic of the Division of Hypertension and Nephrology, National Cardiovascular Center, Suita, Japan, between April 2002 and March 2003 were initially enrolled in this study. Subjects who were receiving lipid-lowering agents were excluded from the analysis. Patients with severe hyperlipidemia [total cholesterol $\geqslant$7.8 mmol/L (300 mg/dL) or triglyceride $\geqslant$4.5 mmol/L (400 mg/dL)], severe diabetes (HbA1c $\geqslant$8.0% or under insulin treatment), and severe renal insufficiency [serum creatinine $\geqslant$265 μmol/L (3.0 mg/dL)] were also excluded. Finally, 525 patients (284 men and 241 women) were analyzed. All patients were treated with antihypertensive agents [angiotensin II type I receptor blockers (ARBs), angiotensin-converting enzyme inhibitors (ACEIs), calcium channel blockers (CCBs), β-adrenergic receptor blockers (BBs), α-adrenergic receptor blockers (ABs), and diuretics). Hypertension was defined as a systolic blood pressure (BP) $\geqslant$140 mmHg and diastolic BP $\geqslant$90 mmHg. Hyperlipidemia was defined as a total cholesterol level $\geqslant$5.7 mmol/L (220 mg/dL) and/or triglyceride level $\geqslant$1.7 mmol/L (150 mg/dL). Diabetes was defined as a fasting plasma glucose (FPG) level $\geqslant$7.0 mmol/L (126 mg/dL) or treated with oral agents. Written informed consent was obtained from all patients. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Clinical parameters. At the time of the physical examination, blood pressure, body mass index (BMI), and a hematological and biochemical profile were determined. The measurements were performed in the morning after an overnight fast. Information on age and smoking status was obtained through a questionnaire and interview. BP was measured after 15 min quiet rest in the supported right arm of seated subjects with a mercury sphygmomanometer cuff-size adjusted to the arm's circumference. Three measurements made at intervals of more than 2 min were averaged. Total cholesterol, HDL-cholesterol, and triglyceride levels were enzymatically determined using an autoanalyzer. LDL-cholesterol was estimated using Friedewald's formula. FPG was determined by standard laboratory methods.

Screening of mutations in clusterin gene. Venous blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes using an NA-3000 nucleic acid isolation system (KURABO, Japan) and stored at −80 °C prior to use. We first sequenced 96 samples from healthy individuals after obtaining written informed consent. All exons and a part of the introns including promoter region in the clusterin gene were amplified by the polymerase chain reaction (PCR) and sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA). Primer sequences used for PCR and sequencing are available on request. The obtained sequences were examined for the presence of mutations using Sequencher software (Gene Codes, MI), followed by visual inspection. We could identify 11 single nucleotide polymorphisms (SNPs) of the clusterin gene (Table 1). Each polymorphism was named according to the recommendations of the Nomenclature Working Group for human gene mutations [12].

Table 1
Identified polymorphisms in the human clusterin gene in 96 healthy volunteers

Polymorphism	Region	Amino acid change	Allele1		Allele2	Allele1 frequency	Allele2 frequency	dbSNP ID
			Homo	Hetero	Homo			
−4453T > G	Promoter		95	1	0	99.5	0.5	—
−4404G > A	Promoter		94	2	0	99.0	1.0	—
1577A > C	Exon 3	None	95	1	0	99.5	0.5	—
1673C > T	Intron 3		94	2	0	99.0	1.0	—
1774A > G[a]	Intron 3		50	39	7	72.4	27.6	rs1532278
4183G > A	Exon 4	Val128Ile	95	1	0	99.5	0.5	—
5608T > C[a]	Exon 5	None	51	38	7	73.0	27.0	rs7982
6316delT	Intron 6		19	48	29	44.8	55.2	rs3216167
10535C > T	Intron 6		94	2	0	99.0	1.0	—
10627C > T	Exon 7	None	94	2	0	99.0	1.0	—
12156G > A	Intron 8		95	1	0	99.5	0.5	—

dbSNP ID, a registered number in a database of single nucleotide polymorphism. Each polymorphism was named according to the recommendations of the Nomenclature Working Group for human gene mutations [12].

[a] SNPs showed the significant linkage disequilibrium.

Genotyping of polymorphisms. The polymorphisms were genotyped using the TaqMan-PCR system as described previously [13]. Among the 11 SNPs identified, considering allele frequency and linkage disequilibrium, 2 common SNPs (5608T > C and 6316delT), one promoter SNP (−4453T > G), and one SNP with amino acid change (4183G > A) were genotyped in 525 asymptomatic hypertensives not treated with lipid lowering agents. Another promoter SNP (−4404G > A) could not be determined due to the technical problem.

Carotid artery ultrasonography. Ultrasonography of bilateral carotid arteries was performed with a high-resolution Duplex scanner (SSA-390A; Toshiba Medical, Japan) using the probe at a frequency of 7.5 MHz for the B-mode scan. The subjects were investigated in the supine position. The carotid arteries were carefully examined with regard to wall changes from different longitudinal (anterior oblique, lateral, and posterior oblique) and transverse views. Each ultrasound image was recorded on a personal computer hard-disc with an on-line digital filing system, and intima-media complex thickness (IMT) was measured manually by off-line analysis. The IMT of the common carotid artery (CCA-IMT) was determined as the average of far-wall measurements from the images of three longitudinal scans in the right carotid artery measured at a site 10 mm below the carotid bulb. A plaque was defined as a focal thickening (≥1.2 mm) in bilateral common carotid arteries and bifurcations (near and far walls). Two independent sonographers who were masked from the clinical data performed the measurements. The intraobserver and interobserver coefficients of variation for CCA-IMT were 4.6% and 4.3%, respectively.

Statistical analysis. Values are expressed as means ± SD. All statistical analyses were performed using the JMP statistical software package (SAS institute, Gary, NC). Hardy–Weinberg equilibrium was assessed by χ^2 analysis. Linkage disequilibrium was performed using SNPAlyze Version 3.0 (Dynacom). To measure linkage disequilibrium between SNPs, Lewontin's D' was calculated. Differences among genotypes were analyzed by analysis of variance (ANOVA). Differences in variables between alleles were assessed with Student's t test. Predictive variables including the clusterin genotype for the plaque prevalence (≥1.2 mm) were analyzed by multiple logistic regression analysis. A value of $P < 0.05$ was considered statistically significant.

Table 2
Primers and probes for genotype determination

	Sequence
−4453T > G	
Sense	5′-GGTTAGCCCGGCTGTCTGT-3′
Antisense	5′-TCAGTAGGGCCAGGGAACTGT-3′
Probe for T	5′-Fam-CCAGTCCCAGACAC-MGB-3′
Probe for G	5′-Vic-CCAGTCCAAGACACA-MGB-3′
4183G > A	
Sense	5′-ATGTGTCCCCTTTTCACCTGG-3′
Antisense	5′-CCTGAAACAGACCTGCATGAAG-3′
Probe for T	5′-Fam-TTCTGCAGATGCGTGC-MGB-3′
Probe for C	5′-Vic-CTGCAGACGCGTGC-MGB-3′
5608 T > C	
Sense	5′-ATACACGAGGCTCAGCAGGC-3′
Antisense	5′-ATGAATTCTGTTGGCGGGTG-3′
Probe for T	5′-Fam-CACTTCCATAGCCCGG-MGB-3′
Probe for C	5′-Vic-ACTTCCACAGCCCGG-MGB-3′
6316delT	
Sense	5′-TGCGGATGAAGGACCAGTG-3′
Antisense	5′-GTCTGACTCCATAAAGGCAGCA-3′
Probe for insertion	5′-Fam-CCGTCCCCCCTGATCCCTTGT-TAMRA-3′
Probe for deletion	5′-Vic-CGTCCCCCCGATCCCTTGTG-TAMRA-3′

Results

Detection of genetic polymorphism in the clusterin gene

We found 11 SNPs in the human clusterin gene (8p21-12) in Japanese (Table 1). A strong linkage disequilibrium ($D' = 0.94$, $r = 0.92$) was shown between 1774A > G and 5608T > C, while no significant linkage disequilibrium was observed between other polymorphisms. Considering allele frequency and linkage disequilibrium, we selected two common SNPs, 5608T > C in exon 5 and 6316delT in intron 6, for genotyping in 525 hypertensive patients. The control for deviation from Hardy–Weinberg equilibrium gave non-significant results in 5608T > C (all: $\chi^2 = 2.764$, $P = 0.251$; men: $\chi^2 = 2.502$, $P = 0.286$; and women: $\chi^2 = 0.565$, $P = 0.754$) and 6316delT (all: $\chi^2 = 1.533$, $P = 0.465$; men: $\chi^2 = 1.173$, $P = 0.556$; and women: $\chi^2 = 0.561$, $P = 0.755$). We also genotyped one promoter SNP (−4453T > G) and one SNP with amino acid change (4183G > A), and found one heterozygote in −4453T > G and three heterozygotes in 4183G > A, respectively, however, they had no specific clinical characteristics (data not shown). Another promoter SNP (−4404G > A) could not be determined due to the technical problem. The sequences of the allele-specific probes and PCR primers used for the genotyping are listed in Table 2.

Association with serum lipid levels

Since a previous report suggested the correlation between clusterin gene polymorphisms and HDL-cholesterol level [10], we compared the serum levels of total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride among genotypes in each polymorphism. 5608T > C showed no significant relationship with serum lipid levels (data not shown). In 6316delT, serum lipid levels were not different in all genotypes in men, however, serum levels of total cholesterol and LDL-cholesterol were highest in subjects with the D/D genotype and lowest in I/I type in women (Table 3). Smoking status tended to increase in I/D and D/D patients compared to I/I patients, but not statistically significant. Other parameters including classical cardiovascular risk factors such as age, BP, BMI, and FPG were not significantly different among genotypes in both polymorphisms.

Association with carotid intima-media thickness

CCA-IMT was 0.92 ± 0.28 mm in men and 0.86 ± 0.30 mm in women, respectively. In a linear

Table 3
Characteristics of the patients classified by 6316delT polymorphism in clusterin gene

	Men			Women		
	I/I	I/D	D/D	I/I	I/D	D/D
No.	50	138	96	43	120	78
Age (year)	63.0 ± 8.9	64.3 ± 10.7	65.1 ± 9.9	65.6 ± 10.4	65.2 ± 9.6	64.0 ± 12.4
BMI (kg/m^2)	24.7 ± 3.3	24.2 ± 3.2	24.4 ± 3.4	23.5 ± 4.5	22.5 ± 4.8	24.1 ± 3.6
Current smoking (%)	48.0	56.5	54.2	13.9	16.7	19.2
Duration of HT (year)	15.7 ± 9.8	17.8 ± 11.6	18.1 ± 11.5	16.1 ± 11.8	17.1 ± 11.2	16.9 ± 10.9
Systolic BP (mmHg)	137.3 ± 16.4	139.5 ± 15.8	138.2 ± 17.3	142.0 ± 22.1	140.6 ± 19.6	143.4 ± 7.5
Diastolic BP (mmHg)	83.9 ± 8.4	84.0 ± 10.9	84.6 ± 12.1	82.1 ± 12.6	82.5 ± 11.4	84.7 ± 10.7
Heart rates (beats/min)	61.9 ± 11.0	60.8 ± 8.8	62.4 ± 10.2	63.2 ± 10.2	65.7 ± 12.3	64.2 ± 9.4
Total cholesterol (mmol/L)	4.96 ± 0.68	4.97 ± 0.73	4.99 ± 0.77	5.07 ± 0.81	5.33 ± 0.72	5.55 ± 0.80*
HDL cholesterol (mmol/L)	1.27 ± 0.36	1.23 ± 0.34	1.30 ± 0.37	1.42 ± 0.36	1.68 ± 0.32	1.52 ± 0.46
LDL cholesterol (mmol/L)	3.01 ± 0.65	3.06 ± 0.68	2.99 ± 0.72	3.05 ± 0.66	3.21 ± 0.81	3.48 ± 0.68*
Triglycerides (mmol/L)	1.51 ± 0.79	1.47 ± 0.71	1.52 ± 0.87	1.26 ± 0.61	1.23 ± 0.59	1.25 ± 0.56
FPG (mmol/L)	5.68 ± 0.84	5.87 ± 1.10	5.90 ± 1.09	5.41 ± 0.78	5.46 ± 0.96	5.38 ± 0.81
Antihypertensive agents (%)						
ARBs or ACEIs	58.0	55.8	52.1	51.2	42.3	47.5
CCBs	66.0	73.9	65.6	65.1	60.0	64.1
β-blockers	40.0	38.4	33.3	25.6	34.2	34.6
α-blockers	18.0	15.9	15.6	11.6	7.5	10.3
Diuretics	26.0	20.3	17.7	34.9	23.3	26.9

Values are represented as means ± SD or frequencies. BMI, body mass index; HT, hypertension; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FPG, fasting plasma glucose; ARBs, angiotensin II receptor blockers; ACEIs, angiotensin converting enzyme inhibitors; and CCBs, calcium channel blockers.
* $P < 0.05$ in ANOVA.

regression analysis, CCA-IMT was positively correlated with age and duration of HT, and inversely correlated with diastolic BP and HDL-cholesterol in men (Table 4). In women, age, BMI, duration of HT, systolic BP, and LDL-cholesterol were significantly associated. 5608T > C showed no significant difference in CCA-IMT among genotypes in both genders (data not shown). Although 6316delT polymorphism did not influence CCA-IMT in men, women with the D allele (I/D + D/D) (0.88 ± 0.31 mm) had significantly greater CCA-IMT than those with the I/I genotype (0.77 ± 0.19 mm) (Fig. 1). Furthermore, multiple logistic regression analysis including traditional cardiovascular risk factors and the treatment with ACEIs and/or ARBs revealed that D allele in 6316delT was independently associated with the plaque prevalence in women (Table 5). Age, duration of HT, and LDL-cholesterol level were also detected as an independent determinant.

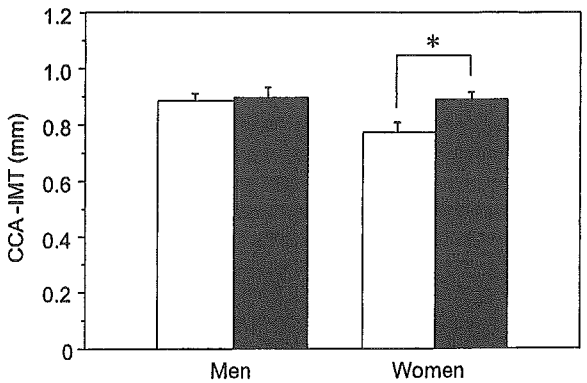

Fig. 1. The values of CCA-IMT in subjects without (I/I, open bar) or with (I/D + D/D, closed bar) the D allele of 6316delT. Error bars represent SE. *$P < 0.05$.

Table 4
Simple regression analyses for CCA-IMT

	Men		Women	
	R	*P* value	*R*	*P* value
Age	0.329	<0.001	0.224	<0.001
BMI	0.051	0.392	0.152	0.018
Current smoking (Yes, 1; No, 0)	0.116	0.051	0.042	0.515
Duration of HT	0.199	<0.001	0.269	<0.001
Systolic BP	0.061	0.331	0.133	0.038
Diastolic BP	−0.171	0.006	0.082	0.206
LDL cholesterol	0.056	0.345	0.156	0.015
HDL cholesterol	−0.152	0.010	−0.066	0.311
FPG	0.095	0.093	0.109	0.073
D allele (Yes, 1; No, 0)	0.014	0.817	0.179	0.005

Discussion

We identified 11 SNPs including three common variants in Japanese. Although 5608T > C was already reported in Caucasian and African-Americans by Tycko et al. (they defined as the allele VB) [11], and 1774A > G and 6316delT were registered in a database of single nucleotide polymorphism (db SNP), the other

Table 5
Multiple logistic regression analysis of factors affecting the presence of plaque in women

	Odds ratio (95% CI)	*P* value
Age	1.73 (1.22–2.43)	0.001
BMI	1.06 (0.78–1.45)	0.687
Current smoking (Yes, 1; No, 0)	1.20 (0.88–1.63)	0.245
Duration of HT	1.46 (1.05–2.02)	0.019
Systolic BP	1.24 (0.91–1.68)	0.373
LDL cholesterol	2.00 (1.46–2.74)	<0.001
FPG	0.96 (0.70–1.32)	0.756
Treatment with ACEIs and/or ARB (Yes, 1; No, 0)	1.03 (0.76–1.38)	0.866
D allele (Yes, 1; No, 0)	1.51 (1.11–2.04)	0.008

95% CI, 95% confidence interval. Adjusted odds ratios for continuous variables represent a difference of 1 SD.

eight polymorphisms were newly identified. The two SNPs, Asn317His (10580A > C) and Asp328Asn (10613G > A), which have been reported to influence serum level of HDL-cholesterol in African blacks [10], were not observed in Japanese. The allele frequencies of these coding variants were more frequent in African-Americans than Hispanics and were rare in Caucasians [10]. There are differences among races in clusterin gene polymorphism.

In the present study, only the 6316delT polymorphism in intron 6 was associated with serum levels of total cholesterol and LDL-cholesterol in women among our genotyped SNPs in Japanese hypertensive patients, although it did not influence the levels of HDL-cholesterol. It has been reported that clusterin exists in plasma HDL subfractions containing apo A-I and cholesterol ester transfer protein in circulating blood [7], and that clusterin influences the lipid transport [8,10]. However, raised clusterin levels have been reported in patients with normal levels of HDL-cholesterol and angiographically documented coronary artery disease who had lower HDL-cholesterol levels than healthy subjects [14,15]. Furthermore, circulating clusterin levels are normal in patients with Tangier disease deficient in apo A-I and HDL-cholesterol [16], suggesting that clusterin is also regulated independently of apo A-I and HDL-cholesterol.

The association between 6316delT and serum lipid levels was not observed in men. We cannot explain the reason for this difference between genders at this stage. It has been reported that androgen, a member of sex hormone, influences clusterin secretion. Clusterin is also called testosterone repressed prostate message-2 and its expression increases in androgen-depleted conditions [17]. The serum concentration of clusterin increases during prostatic regression [18]. Although our population did not include the apparent illness, the asymptomatic hormonal disturbance specific in men may influence the clusterin function, resulting in a distinct association of 6316delT with serum lipids between genders.

Higher total cholesterol and LDL-cholesterol levels in D allele females in our present study suggested that this deletion influences the possible function of clusterin in lipid metabolism besides cholesterol transport. The measurement of serum clusterin concentration can be raised as a useful method to clarify the direct effect of clusterin polymorphism on its function, however, we could not measure it in the present study. To indicate more direct contribution of genetic polymorphism to clusterin function, such future studies are required.

Numerous genetic factors associated with lipid synthesis such as the apo E, apolipoprotein gene cluster (apo A1/C3/A4/A5) on chromosome 11 and lipoprotein lipase have been known as a cause of hyperlipidemia. Polymorphisms of apo E [19] and lipoprotein lipase [20] genes are also known to be independent risk factors for cardiovascular disease. In the present study, female hypertensive patients with the D allele of 6316delT (I/D + D/D) who showed an increase in total cholesterol and LDL-cholesterol levels had higher CCA-IMT values than I/I type individuals (Fig. 1). Although our subjects had almost normal levels of serum cholesterol and LDL-cholesterol, an increase in the number of cardiovascular events was shown in subjects with total cholesterol level > 5.2 mmol/L and LDL cholesterol level > 2.6 mmol/L [21]. The progressed carotid atherosclerosis in subjects with the D allele of 6316delT shown in the present study may be partially due to the difference in LDL cholesterol levels between genotypes.

Previous studies have reported that polymorphisms in intron play an important role in pre-mRNA splicing. In intron, there are three indispensable regions, i.e., 5′ splice site, 3′ splice site, and branch point. Between 3′ splice site and branch point, polypyrimidine tract which contributes to the selection of branch point exists and several polymorphisms in the intronic polypyrimidine tract have been reported to influence the mRNA splicing [22,23]. Clusterin gene has been reported to form several splicing variants [24,25]. The protein synthesis inhibitors and heat shock protein also influence the alternative splicing process of clusterin gene [26,27]. The 6316delT polymorphism locates 35 base pair downstream of the exon 3 and leads to variation in the polypyrimidine tract length. Although we are not able to conclude whether this variant directly affects the serum lipid levels and carotid atherosclerosis at this stage, this mutation may influence the clusterin expression through the modification of mRNA splicing. More detailed studies are required to clarify the functional mechanism of 6316delT mutation.

Our study has several limitations. First, we sequenced all coding exons, part of introns which includes the promoter in the present study. However, it is uncertain whether functional mutations exist in 5′-upstream region beyond our sequencing region or in unsequenced introns that may create a new splice site. Second, all

patients were treated with antihypertensive agents. Several antihypertensive agents have been reported to suppress vascular remodeling besides BP-lowering effects, especially in ARBs [28] and ACEIs [29]. In our subjects, however, there was no significant difference in the treated ratio of antihypertensive agents among genotypes (Table 2). Furthermore, in a multiple logistic regression analysis including the treatment with ARBs and/or ACEIs, D allele of 6316delT was detected as an independent factor for determining the plaque prevalence, suggesting that antihypertensive therapy did not influence our results.

In conclusion, we identified 11 clusterin gene polymorphisms in Japanese. We also found that the frequent 6316delT polymorphism in the clusterin gene significantly influenced the serum levels of total cholesterol and LDL-cholesterol, CCA-IMT, and plaque prevalence in Japanese hypertensive females. Although further studies are required to clarify the functional mechanism of this mutation, our findings suggest the importance of common genetic variations in clusterin in determining the serum lipid levels and carotid atherosclerosis.

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