18) Stam J：Thrombosis of the cerebral vein and sinuses. *N Engl J Med* **352**：1791–1798, 2005
19) Martinelli I *et al*：Long–term evaluation of the risk of recurrence after cerebral sinus–venous thrombosis. *Circulation* **121**：2740–2746, 2010
20) Einhäupl K *et al*：EFNS guideline on the treatment of cerebral venous and sinus thrombosis in adult patients. *Eur J Neurol*. published online, 2010

はせがわ・やすひろ

長谷川泰弘　聖マリアンナ医科大学神経内科教授

1954 年，福岡市生まれ．
1980 年，鹿児島大学医学部卒業．1981 年，国立循環器病センター内科脳血管部門．1992 年，米国マサチューセッツ大学神経内科．1996 年，国立循環器病センター医長．2005 年より現職．専門は，脳卒中学，神経内科学．研究テーマは脳梗塞急性期治療．趣味は音楽．

「脳梗塞 t-PA 研究会」第 4 回研究集会

＜総　説＞

国内多施設共同登録研究 Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rt-PA Registry：全体成績とサブ研究の紹介

豊田　一則[1)]　古賀　政利[1)]　塩川　芳昭[2)]　中川原譲二[3)]　古井　英介[4)]
木村　和美[5)]　山上　宏[6)]　岡田　靖[7)]　長谷川泰弘[8)]　苅尾　七臣[9)]
奥田　聡[10)]　永沼　雅基[1)]　西山　和利[2)]　峰松　一夫[1)]

要旨：国内 10 施設が参加した厚生労働科学研究 Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rt-PA Registry に登録された急性期脳梗塞患者 600 例（女性 223 例，72±12 歳）の，遺伝子組み換え組織型プラスミノゲン・アクティベータ（recombinant tissue-type plasminogen activator: rt-PA）静注療法の治療成績を，紹介する．治療後 36 時間以内に 119 例（19.8％，16.8–23.2％）に頭蓋内出血を，8 例（1.3％，0.7–2.6％）に症候性頭蓋内出血（SITS-MOST 定義）を認めた．3 カ月後に 199 例（33.2％，95％ CI 29.5–37.0％）が完全自立（modified Rankin Scale [mRS] 0–1）した．欧州の rt-PA 適応基準にしたがって患者を限定すると，399 例中 162 例（40.6％，35.9–45.5％）が完全自立し，この割合は他の国内外の市販後調査成績と同等であった．サブ解析で，MRI 拡散強調画像での早期虚血所見や腎機能障害の存在が，治療成績と良く相関することを明らかにした．

Key words：acute stroke, chronic kidney disease, early ischemic change, stroke outcome, thrombolysis

（脳卒中 32：756–761，2010）

緒　言

脳卒中はわが国の国民病であり，その征圧は喫緊の課題である．とくに超急性期から急性期は治療介入による転帰改善効果がもっとも期待される時期であるが，同時期の危険因子管理の意義は国内外のいずれにおいても明らかでない．Stroke Acute Management with Urgent Risk-factor Assessment and Improvement（SAMURAI）研究は，筆者が主宰する厚生労働科学研究（循環器疾患等生活習慣病対策総合研究事業）「わが国における脳卒中再発予防のための急性期内科治療戦略の確立に関する研究」に参加する国内 10 施設による一連の多施設共同研究を指す．このうち SAMURAI rt-PA Registry では参加施設で遺伝子組み換え組織型プラスミノゲン・アクティベータ（recombinant tissue-type plasminogen activator: rt-PA）静注による治療を受けた急性期脳梗塞患者を登録し，その治療成績を明らかにするとともに，危険因子を含めた背景要

[1)]国立循環器病研究センター脳血管内科
[2)]杏林大学脳神経外科・脳卒中センター
[3)]中村記念病院脳神経外科脳卒中センター
[4)]財団法人広南会広南病院脳血管内科
[5)]川崎医科大学脳卒中医学
[6)]神戸市立医療センター中央市民病院脳卒中センター
[7)]国立病院機構九州医療センター脳血管センター脳血管内科
[8)]聖マリアンナ医科大学神経内科
[9)]自治医科大学循環器内科
[10)]国立病院機構名古屋医療センター神経内科
（2010 年 9 月 26 日受付，2010 年 9 月 27 日受理）

表1　600例のおもな背景所見および脳梗塞の性状

危険因子，合併症	
高血圧症	366例(61.0%)
糖尿病	110例(18.3%)
脂質代謝異常	125例(21.0%)
心房細動	258例(43.4%)
心不全(588例中)	51例(8.7%)
脳梗塞既往	109例(18.2%)
発症前 modified Rankin Scale 2以上	65例(10.8%)
冠動脈疾患既往	77例(13.1%)
発症前の抗凝固薬服用	53例(8.8%)
発症前の抗血小板薬服用	192例(32.0%)
脳梗塞の性状，来院時所見	
おもな血管閉塞部位(546例中)	
内頸動脈	91例(16.7%)
中大脳動脈主幹部(M1)	159例(29.1%)
中大脳動脈分枝(M2)	108例(19.8%)
脳底動脈	22例(4.0%)
脳梗塞亜病型	
心原性脳塞栓症	380例(63.3%)
アテローム血栓性梗塞	91例(15.2%)
ラクナ梗塞	29例(4.8%)
その他の脳梗塞	100例(16.7%)
CTでのASPECTS(501例)	10(8–10)*
拡散強調画像でのASPECTS(520例)	8(7–10)*
NIH Stroke Scale値	13(7.3–19)*
発症から治療開始までの時間	145分(121–166)*
rt-PA投与直前の降圧薬静注	164例(27.6%)
エダラボン静注	502例(83.7%)

*中央値(四分位値)

因が治療成績に及ぼす影響を調べている．2010年9月現在で，この登録研究から4編の原著論文が掲載または掲載許可された[1)~4)]．ここではこの4編の内容を簡潔に紹介する．

方　法

国内で急性期脳梗塞患者へのrt-PA静注療法が承認された2005年10月から2008年7月までの間に，研究班に所属する10施設でこの治療を受けた急性期脳梗塞患者を，後ろ向きに連続登録した．rt-PA静注療法の適応は適正治療指針に則って決定し，薬剤投与方法や治療前後の患者管理も適正治療指針の記載にしたがった[5)]．本研究の遂行にあたっては，参加施設ごとに倫理委員会の承認を得た．

結　果

(1)全体成績(文献1に基づく)

600例(女性223例，72±12歳)を登録した．同じ期間に国内で約13500例がrt-PA静注療法を受けており，本研究での登録症例はその4.4%にあたった．このうち422例(70.3%)が欧州での適応基準(換言すればSafe Implementation of Thrombolysis in Stroke-MOnitoring Study [SITS-MOST][6)]の登録基準：80歳以下，治療前NIHSS 25未満，糖尿病と脳梗塞既往の合併例以外など)を満たした．この600例のおもな背景所見および脳梗塞の性状を，表1に示す．

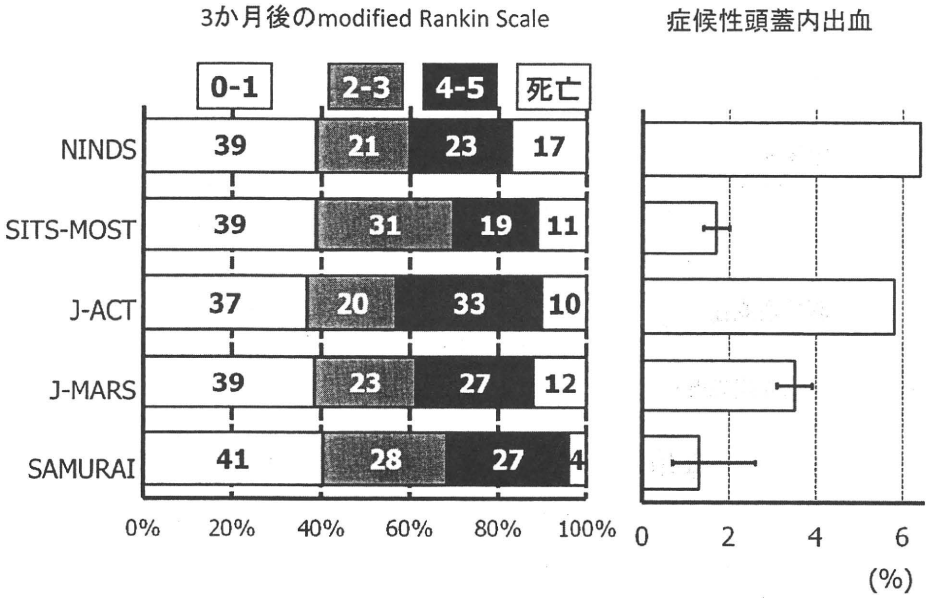

図1　国内外の臨床試験における3カ月後のmodified Rankin Scaleおよび36時間以内の症候性頭蓋内出血発症率

治療後36時間以内に119例（19.8%，16.8–23.2%）に頭蓋内出血を認め，そのうち30例がPH type I，21例がPH type IIであった．症候性頭蓋内出血は，NIH Stroke Scale値で1点以上の進行を症候性とみなすと23例（3.8%，2.6–5.7%）に，SITS-MOSTの定義に合わせて4点以上の進行を伴うPH type IIと定義すると8例（1.3%，0.7–2.6%）に認めた．3カ月後に199例（33.2%，95% CI 29.5–37.0%）が完全自立（modified Rankin Scale [mRS] 0–1）した．発症前に既にmRSが2以上であった65例を除くと，完全自立者は37.2%（33.2–41.4%）に達した．さらに欧州の適応基準にしたがって患者を限定すると，399例中162例（40.6%，35.9–45.5%）が完全自立した．また600例中43例が3カ月以内に死亡し，うち7例は症候性頭蓋内出血を起こした患者であった．この成績を米国National Institute of Neurological Disorders and Stroke（NINDS）主導による多施設共同臨床試験[7]，欧州での市販後調査SITS-MOST[6]，国内での第III相試験Japan Alteplase Clinical Trial（J-ACT）[8]，国内での市販後調査Japan post-Marketing Alteplase Registration Study（J-MARS）[9]と比べる（図1）．本研究やJ-MARSの結果は，対象患者を欧州の適応基準に限定して求めている．また症候性頭蓋内出血はNIH Stroke Scale値で4点以上の進行を基準としたが，NINDS試験のみは軽度の症状進行も含めている．rt-PA静注療法によって約4割の患者が3カ月後に完全自立することを期待でき，また症候性頭蓋内出血は高々6%程度であった．本研究では，出血発症率や死亡率が他研究よりも低かった．

(2) MRI拡散強調画像での早期虚血所見と治療成績（文献2に基づく）

このサブ研究では，MRI拡散強調画像（diffusion-weighted image: DWI）での早期虚血所見をAlberta Stroke Program Early CT Score（ASPECTS）を用いて評価し[10]，治療成績との関連を調べた．ASPECTSは，本来CTでの早期虚血所見を半定量評価する尺度である．一側の中大脳動脈領域を10カ所の関心領域に分け，いずれの場所にも早期虚血変化を認めない場合を10点とし，早期虚血変化を認めると領域ごとに1点ずつ減じる．

600例のうち，発症前のmRSが2以下で，rt-PA投与前にMRIを撮影した477例（女性161例，71±11歳）を対象とした．DWI-ASPECTSの中央値は8（四分位値7–10）であった．477例のうち40例（8.4%）にPH型の頭蓋内出血を認め，うち15例が症候性（NIH Stroke Scale値で4点以上の進行）であった．3カ月後に245例（51.4%）が機能的自立（mRS 0–2）し，29例（6.1%）が死亡した．

機能的自立に回復した患者のDWI-ASPECTSの中央値は9（四分位値8–10）で，機能的自立に至らな

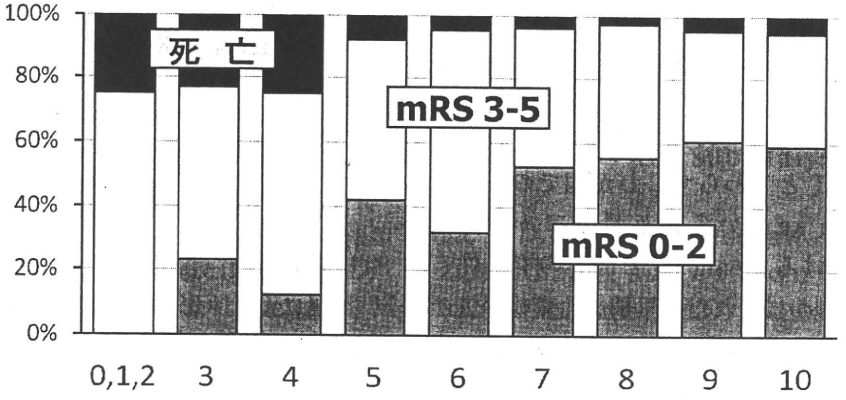

図2　MRI 拡散強調画像での ASPECTS と 3 カ月後の modified Rankin Scale

かった患者(中央値 8，四分位値 6–9)より高値であった(p<0.001)．図 2 に異なる DWI-ASPECTS を示す患者ごとの 3 カ月後 mRS を示す．DWI-ASPECTS 7 以上の患者群では機能的自立患者の割合は同程度であったが，6 以下の患者群では ASPECTS が低くなるにつれて機能的自立の割合が減った．多変量解析を用いると，DWI-ASPECTS 7 以上は他の背景要因で補正した後も，機能的自立との間に有意な関連を認めた(オッズ比 1.85，95% CI 1.07–3.24，p=0.029)．

3 カ月以内に死亡した患者の DWI-ASPECTS の中央値は 7(四分位値 4–9.5)で，生存した患者(中央値 9，四分位値 7–10)より低値であった(p=0.038)．図 2 において，DWI-ASPECTS 4 以下で死亡率が 20% を超えた．多変量解析で DWI-ASPECTS 4 以下は死亡との間に有意な関連を認めた(オッズ比 3.61，95% CI 1.23–9.91，p=0.021)．

同じく多変量解析を用いると，DWI-ASPECTS 5 以下は症候性頭蓋内出血との間に有意な関連を認めた(オッズ比 4.74，95% CI 1.54–13.64，p=0.008)．

(3) 腎機能障害と治療成績(文献 3 に基づく)

このサブ研究では，入院時の血中クレアチニン値を用いて推算糸球体濾過率(estimated glomerular filtration rate: eGFR) を算出し[11]，この値が 60 ml/min/1.73 m^2 未満の場合を腎機能障害ありと定義して，治療成績との関連を調べた．

600 例のうち，発症前 mRS が 4 以上の例などを除く 578 例(女性 206 例，71±12 歳)を対象とした．このうち 186 例(32.2%)が腎機能障害を有し，うち 4 例は維持血液透析を受けていた．腎機能障害患者と腎機能正常患者との間で，入院時 NIH Stroke Scale 値に有意差を認めなかった．

36 時間以内の頭蓋内出血は，腎機能障害患者に有意に多く(27.4%対 16.6%，p=0.004)，多変量解析を用いると腎機能障害は他の背景要因で補正した後も，頭蓋内出血との間に有意な関連を認めた(オッズ比 1.81，95% CI 1.16–2.84，p=0.009)．症候性頭蓋内出血に関しても，同様の関係を認めた．

腎機能障害患者は腎機能正常患者に比べて，3 カ月後の mRS が有意に高く(中央値 3，四分位値 1–5 対 2，1–4，p<0.001)，完全自立(mRS 0–1)した患者が少なく(25.8%対 38.0%，p=0.004)，転帰不良例(mRS 4–6：47.9 % 対 34.7 %，p=0.003) や死亡例(13.4 % 対 3.8%，p<0.001)が多かった．多変量解析で腎機能障害は転帰不良(オッズ比 1.55，95% CI 1.01–2.38，p=0.046)や死亡(オッズ比 2.94，95% CI 1.38–6.42，p=0.006)との間に有意な関連を認めたが，完全自立との間には有意な関連を認めなかった(オッズ比 0.70，95 % CI 0.44–1.09，p=0.114)

(4) スタチンの発症前ないし急性期服用と治療成績(文献 4 に基づく)

このサブ研究では，スタチン服用と治療成績との関連を調べた．発症前に 67 例(11.2%)が，急性期に 60 例(10.0%)がスタチンを服用した．多変量解析にてスタチンの発症前服用と頭蓋内出血との間に，有意な関連を認めなかった(オッズ比 1.46，95% CI 0.76–2.81)．同じく 3 カ月後の完全自立(mRS 0–1)に関しても，スタチンの発症前服用(オッズ比 1.05，95 % CI 0.55–2.01)，急性期服用(オッズ比 1.31，95 % CI 0.66–2.59)との関連はみられなかった．

考　察

SAMURAI rt-PA Registryの全体成績における完全自立患者の割合は，国内外の大規模市販後調査J-MARS，SITS-MOSTと変わらず，わが国でのみ採用されている0.6 mg/kgという投与量のアルテプラーゼが，海外における0.9 mg/kgと同等の有効性を示し得ることが明らかにされた．早期虚血変化の広がりの程度は虚血の強さを示唆しており，治療成績への関与が強いことも示された．わが国ではMRIが広く普及しており，DWIでの早期虚血所見やMRAでの血管閉塞部位の情報をもとに，rt-PAの治療適応をより適切に判定することが期待できる．ただしMRI精査に伴う治療開始の遅れを，最小限に食い止める必要がある．腎機能障害ないし慢性腎臓病が脳卒中の発症に関連することは，近年報告されているが，rt-PA治療の成績にも関連し得ることは興味深い．同じく脂質代謝も治療成績に影響する可能性が考えられるが，少なくとも今回示した研究結果でスタチン服用と治療成績の間に有意な関連を認めなかった．このサブ研究の主著者である牧原は，脂質諸値と治療成績の関連を，引き続き調査中である．

SAMURAI rt-PA Registryからは，他にも患者背景要因(危険因子，発症前服用薬など)と治療成績との関連を検討したいくつかの研究が国内学会，国際学会で発表されており，逐次論文化を進めている．

「脳梗塞t-PA研究会」第4回研究集会で発表した．

参考文献

1) Toyoda K, Koga M, Naganuma M, et al: Routine use of intravenous low-dose recombinant tissue plasminogen activator in Japanese patients: general outcomes and prognostic factors from the SAMURAI register. Stroke 40: 3591–3595, 2009
2) Nezu T, Koga M, Kimura K, et al: Pre-treatment ASPECTS on DWI predicts 3-month outcome following rt-PA: SAMURAI rt-PA Registry. Neurology 75: 555–561, 2010
3) Naganuma M, Koga M, Shiokawa Y, et al: Reduced estimated glomerular filtration rate is associated with stroke outcomes after intravenous rt-PA: the Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rt-PA Registry. Cerbrovasc Dis 2010, in press
4) 牧原典子，岡田　靖，古賀政利ら：rt-PA静注療法施行症例におけるスタチンの頭蓋内出血および転帰に及ぼす影響：Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rt-PA Registry. 臨床神経学 40: 225–231, 2010
5) 日本脳卒中学会医療向上・社会保険委員会rt-PA(アルテプラーゼ)静脈療法指針部会：rt-PA(アルテプラーゼ)静脈療法適正治療指針 2005年10月．脳卒中 27: 327–354, 2005
6) Wahlgren N, Ahmed N, Davalos A, et al: Thrombolysis with alteplase for acute ischaemic stroke in the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST): an observational study. Lancet 369: 275–282, 2007
7) The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group: Tissue plasminogen activator for acute ischemic stroke. N Engl J Med 333: 1581–1587, 1995
8) Yamaguchi T, Mori E, Minematsu K, et al for the Japan Alteplase Clinical Trial (J-ACT) Group: Alteplase at 0.6 mg/kg for acute ischemic stroke within 3 hours of onset: Japan Alteplase Clinical Trial. Stroke 37: 1810–1815, 2006
9) Nakagawara J, Minematsu K, Okada Y, et al: Thrombolysis with 0.6 mg/kg intravenous alteplase for acute ischemic stroke in routine clinical practice. The Japan post-Marketing Alteplase Registration Study (J-MARS). Stroke 41: 1984–1989, 2010
10) Barber PA, Demchuk AM, Zhang J, et al: Validity and reliability of a quantitative computed tomography score in predicting outcome of hyperacute stroke before thrombolytic therapy. ASPECTS Study Group. Alberta Stroke Programme Early CT Score. Lancet 355: 1670–1674, 2000
11) Matsuo S, Imai E, Horio M, et al: Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis 53: 982–992, 2009

Abstract

Stroke Acute Management with Urgent Risk-factor Assessment and Improvement (SAMURAI) rt-PA Registry: General results and subanalyses

Kazunori Toyoda, M.D.[1], Masatoshi Koga, M.D.[1], Yoshiaki Shiokawa, M.D.[2], Jyoji Nakagawara, M.D.[3], Eisuke Furui, M.D.[4], Kazumi Kimura, M.D.[5], Hiroshi Yamagami, M.D.[6], Yasushi Okada, M.D.[7], Yasuhiro Hasegawa, M.D.[8], Kazuomi Kario, M.D.[9], Satoshi Okuda, M.D.[10], Masaki Naganuma, M.D.[1], Kazutoshi Nishiyama, M.D.[2] and Kazuo Minematsu, M.D.[1]

[1]Department of Cerebrovascular Medicine, National Cerebral and Cardiovascular Center
[2]Departments of Neurosurgery and Stroke Center, Kyorin University School of Medicine
[3]Department of Neurosurgery and Stroke Center, Nakamura Memorial Hospital
[4]Department of Stroke Neurology, Kohnan Hospital
[5]Department of Stroke Medicine, Kawasaki Medical School
[6]Department of Neurology, Stroke Center, Kobe City General Hospital
[7]Department of Cerebrovascular Disease, National Hospital Organization Kyushu Medical Center
[8]Department of Neurology, St Marianna University School of Medicine
[9]Division of Cardiovascular Medicine, Department of Medicine, Jichi Medical University School of Medicine
[10]Department of Neurology, National Hospital Organization Nagoya Medical Center

Stroke Acute Management with Urgent Risk-factor Assessment and Improvement [SAMURAI] rt-PA Registry is a multicenter retrospective observational study from 10 Japanese stroke centers. A total of 600 patients (223 women, 72±12 years old) treated with intravenous alteplase (0.6 mg/kg) were studied. Symptomatic intracerebral hemorrhage within 36 hours with ≥4 point-increase from the baseline NIH Stroke Scale score developed in 8 patients (1.3%, 95% CI 0.7–2.6%). At 3 months, 199 patients (33.2%, 29.5–37.0%) had a modified Rankin Scale (mRS) score ≤1. Analysis of 399 patients with a premorbid mRS score ≤1 who met the criteria of the European license (≤80 years old, an initial NIHSS score ≤24, etc.) showed that 40.6% (35.9–45.5%) had a 3-month mRS ≤1. In the subanalyses from this registry, early ischemic change on diffusion-weighted imaging assessed by the Alberta Stroke Programme Early CT Score, as well as reduced estimated glomerular filtration rate, was associated with early intracerebral hemorrhage and 3-month outcomes of patients.

(Jpn J Stroke 32: 756–761, 2010)

Hereditas 147: 313–319 (2010)

The haplotype of the CACNA1B gene associated with cerebral infarction in a Japanese population

MAI YAMAGUCHI[1,3], TOMOHIRO NAKAYAMA[2], ZHENYAN FU[3], NAOYUKI SATO[2], MASAYOSHI SOMA[4], AKIHIKO MORITA[1], SHIGEAKI HINOHARA[5], NOBUTAKA DOBA[5] and TOMOHIKO MIZUTANI[1]

[1]*Division of Neurology, Dept of Medicine, Nihon University School of Medicine, Tokyo, Japan*
[2]*Division of Laboratory Medicine, Dept of Pathology and Microbiology, Nihon University School of Medicine, Tokyo, Japan*
[3]*Division of Molecular Diagnostics, Dept of Advanced Medical Science, Nihon University School of Medicine, Tokyo, Japan*
[4]*Division of General Medicine, Dept of Medicine, Nihon University School of Medicine, Tokyo, Japan*
[5]*The Life Planning Center, Tokyo, Japan*

Yamaguchi, M., Nakayama, T., Fu, Z., Sato, N., Soma, M., Morita, A., Hinohara, S., Doba, N. and Mizutani, T. 2009. The haplotype of the CACNA1B gene associated with cerebral infarction in a Japanese population. – *Hereditas 147*: 313–319. Lund, Sweden. eISSN 1601-5223. Received February 10, 2009. Accepted May 26, 2009.

Cerebral infarction (CI) is thought to be a multifactorial disease that is affected by several environmental factors and genetic variants. N-type voltage-gated calcium channels (VGCCs), which are expressed primarily in the neurons, have various roles in neuronal functions and are especially involved with neurotransmitter release at the sympathetic nerve terminals. We considered the α1B subunit of the N-type voltage-gated calcium channel (CACNA1B) to be representative of the general characteristics of this channel type. The aim of the present study was to assess the association of the human CACNA1B gene with the occurrence of CI via a haplotype-based case-control study that used single nucleotide polymorphisms (SNPs) from the Japanese population. A total of 165 CI patients and 314 controls were enrolled in the case-controlled studies that examined three SNPs of the human CACNA1B gene (rs7042521, rs11137351, rs10780199). There were significant differences between the CI and control groups for the overall distribution of the genotypes and the presence of the recessive rs10780199. Multiple logistic regression analyses revealed that even after adjusting for confounding factors (odds ratio: 1.716), the frequencies of the A/G and G/G genotypes of rs10780199 in the CI group were significantly higher than those observed in the control group ($p=0.021$). Furthermore, the C-C-G and G-G-G haplotypes of rs7042521-rs11137351-rs10780199 were significantly more frequent in the CI group than in the control group ($p=0.024$ and $p<0.000$). In conclusion, significant differences were noted between the CI and control patients for the specific SNPs and haplotypes in the CACNA1B gene. The results indicate that these polymorphisms and haplotypes might be genetic markers for CI.

Tomohiro Nakayama, Division of Laboratory Medicine, Dept Pathology and Microbiology, Nihon University School of Medicine, Ooyaguchi-kamimachi, 30-1 Itabashi-ku, JP-173-8610 Tokyo, Japan. E-mail: tnakayam@med.nihon-u.ac.jp

Today, cerebral infarction (CI) is one of the main causes of death in the world, although the genetic basis of CI has yet to be determined. CI is now thought to be a multifactorial disease that is affected by several environmental factors and genetic variants (Hassan and Markus 2000).

The N-type voltage-gated calcium channels (VGCCs) are expressed primarily in neurons that have various functional roles, although they are especially involved with neurotransmitter release at the sympathetic nerve terminals (Hirning et al. 1988; Toth et al. 1993; Hong and Chang 1995; Vega et al. 1995; Serone and Angus 1999; Catterall et al. 2005). It has been demonstrated that N-type VGCCs regulate the systemic cardiovascular tone. Animal model studies have suggested that blocking the N-type VGCCs can decrease the neuronal damages associated with ischemic brain injury (Valentino et al. 1993; Pringle et al. 1996; Perez-pinzon et al. 1997; Takahara et al. 2004). Voltage-gated calcium channels, which include the N-type, are complex proteins composed of four or five distinct subunits that are encoded by multiple genes (Catterall 2000). Differences within the α1 subunit are related to the differences associated with the actual function, such as conduction pores, voltage sensors and gating apparatus. The α1B subunit is characterized as the N-type voltage-gated calcium channel. Therefore, the human gene encoded α1B subunit (CACNA1B) could be a CI candidate gene. However, at the present time there have been no genetic association studies that have examined the relationship between CI and CACNA1B.

The human CACNA1B gene is a single copy gene that spans about 244 kilobase pairs (kb), and is composed of 47 exons that are interrupted by 46 introns (Fig. 1). The gene is located on 9q34. The aim of the present study was to assess the association between the human CACNA1B gene and the occurrence of CI in the Japanese population via a haplotype-based case-control study that used single nucleotide polymorphisms (SNPs).

DOI: 10.1111/j.1601-5223.2009.02115.x

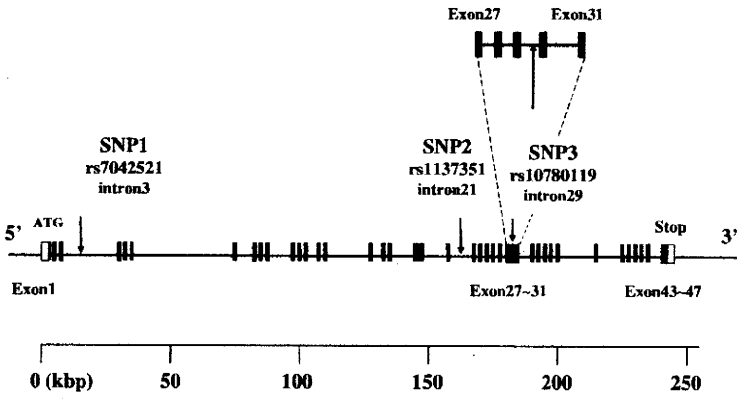

Fig. 1. Organization of the human CACNA1B gene and location of the SNPs. Boxes indicate exons and lines indicate introns. The three arrows show the location of the three SNPs.

MATERIAL AND METHODS

Subjects

For this case-control study, all patients and control subjects were recruited from the northern area of Tokyo. Subjects were selected from patients who had been admitted to our hospital (Nihon University Hospital in Tokyo) or community hospitals (in Tokyo) between 1995 and April 2005 (Aoi et al. 2004). Approximately 1000 CI patients consulted doctors or were admitted to our hospital during this period, with 25% patients agreeing to take part in our study. Control subjects were selected from outpatients who were present at the hospital during the same time period. All subjects in the study groups were Japanese, with 165 patients diagnosed with CI (mean age, 66.8±12.3 years). For the CI diagnosis, a board-certified neurologist evaluated new focal neurological deficits and symptoms using either CT or MRI. Electrocardiographic and echocardiographic findings for patients with cardioembolic CI diagnosed by CT, MRI, anamnesis of recent MI, valvular heart disease and atrial fibrillation were excluded from the current study. These findings were excluded, as we were unable to obtain informed consent from these subjects. Thus, the subjects enrolled in the CI group consisted of patients with non-cardioembolic CI, which included subjects with atherothrombotic and lacunar infarctions as determined by the Classification of the Cerebrovascular Disease III (Special report from the National Institute of Neurological Disorders and Stroke 1990). The CI group also included patients diagnosed on the basis of thrombotic and hemodynamic mechanisms.

The control group consisted of 314 healthy elderly Japanese (mean age, 78.0±4.2 years) who all volunteered to join the project. All of the control participants were members of the New Elder Citizenship project in Japan (Hinohara and Doba 2005). The mean age of the control group was older than that of the CI group and this control group was regarded as a super-control group (Haketa et al. 2004). All subjects lived in and around Tokyo and had vascular risk factors such as hypertension, hypercholesterolemia, or diabetes mellitus (DM) but no history of CI. All participants were confirmed to be grade 0 on the modified rankin scale. Since many cerebrovascular diseases occur late in life, healthy elderly subjects are more suitable than young or middle-aged subjects when determining phenotypes of diseases related to aging. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.

Biochemical analysis

Plasma concentrations of total cholesterol were measured using the methods of the Clinical Lab. Dept of Nihon Univ. Hospital (Nakayama et al. 2004a; Cha et al. 2007).

Genotyping

Based on the allelic frequency data for registered SNPs from the Natl Center for Biotechnology Information (NCBI) website and from the Applied Biosystems (Foster City, CA, USA) and Celera (Rockville, MD, USA) Discovery System, SNPs with minor allele frequencies > 20% were chosen for the study. This criterion was selected because SNPs with a high frequency of minor alleles are very useful genetic markers in genetic association studies. The accession numbers for the three selected SNPs in the human CACNA1B gene were as follows: rs7042521 (C___29084134_10), rs11137351 (C___280614_10) and rs10780199 (C___31373106_10) (Fig. 1). For the purposes of this study, the SNPs were designated as SNP1, SNP2 and SNP3, respectively. All SNPs were located in the intron region. Genotypes were determined using Assays-on-Demand kits (Applied Biosystems) together with TaqMan® PCR, as has been previously described (Maruyama et al. 2004; Nakayama et al. 2004b).

Linkage disequilibrium analysis and the haplotype-based case-control study

Based on the genotype data of the genetic variations, we performed a linkage disequilibrium (LD) analysis and a haplotype-based case-control study by applying SNPAlyze ver. 3.2 (Dynacom Co., Ltd., Yokohama, Japan) that employed the expectation–maximization (EM) algorithm (KOBAYASHI et al. 2005). D′ values >0.25 were used to assign SNP locations to one haplotype block.

Statistical analysis

Data are shown as the mean ± D. Hardy–Weinberg equilibrium (HWE) was assessed using χ^2 analysis (PARRA-ROJAS et al. 2006). The Mann–Whitney U-test was used to assess the differences for continuous variables in the clinical data between the CI and control groups. The Fisher's exact test was used to assess the differences in the categorical variables. Genotypes were classified into three distinct groups: overall, dominant, and recessive. The distribution of the three models of genotypes was examined with Fisher's exact test. In addition, multiple logistic regression analyses were performed to assess the contribution of confounders. Statistical analyses were performed with SPSS software for Windows, ver. 12 (SPSS Inc. USA). Statistical significance was established at $p<0.05$. Furthermore, a p-value of less than 0.05/n (n: numbers SNPs analyzed) was considered to be significant when correcting for the number of comparisons made (Bonferroni correction) (KOBAYASHI et al. 2005).

Pairwise linkage disequilibrium (LD) patterns for the CACNA1B gene were evaluated using $|D'|$ and r^2. The threshold value of the frequencies of the haplotypes included in the analysis was set to 1%. All haplotypes below the threshold value were excluded from the analysis. The overall distribution of haplotypes was analyzed using 2×m contingency tables, with a p-value of <0.05 considered to indicate statistical significance. The p-value of each haplotype was determined using a χ^2-analysis, a permutation method, and SNPAlyze version 3.2 (Dynacom Co.) (NAKAYAMA et al. 2004b).

RESULTS

Table 1 shows the characteristics of the study participants. The association studies included both control and CI groups. For the total participants, systolic blood pressure, diastolic pressure and plasma total cholesterol concentration were significantly higher in the CI group as compared to the control group. The mean age of the control group was older than that of the CI group and the control group was regarded as a super-control group (HAKETA et al. 2004). The incidence of hypertension, diabetes and hyperlipidemia for the total CI participants was also significantly higher as compared to that of the control group. There was no difference for the body mass index between the two groups.

Table 1. *Characteristics of study.*

	Control	CI	p-value
Number of subjects	314	165	
Age (years)	78.0±4.2	66.8±12.3	
BMI (kg m^{-2})	22.6±2.8	23.2±3.6	0.104
SBP (mmHg)	135.7±16.4	150.6±24.7	<0.001
Total cholesterol (mg dl^{-1})	215.8±33.0	194.2±47.8	<0.001
Hypertension	72.3	90.3	<0.001
DM (%)	2.2	17.6	<0.001
Hyperlipidemia	54.5	38.2	0.001

BMI, body mass index; SBP, systolic blood pressure; DM, diabetes mellitus; CI, cerebral infarction.
Values are the means±SD. P-values were analyzed between the CI group and the control
p-value of continuous variables calculated by Mann–Whitney U-test.
p-value of categorical variables calculated by Fisher's exact test.

The distributions of the genotypes and the two kinds of dominant or recessive models for the three SNPs in the 165 CI patients and in the 314 control subjects are summarized in Table 2. There were no markers that indicated any evidence of deviation from the Hardy–Weinberg equilibrium (data not shown). Significant genotype distribution differences were seen for rs10780199 (SNP3) between the total participants of the CI group and the control group ($p=0.032$). The distribution of the recessive model for SNP3, i.e. A/A versus A/G and G/G (SNP3R), differed significantly between the CI and the control groups ($p=0.009$). Furthermore, the χ^2-test performed after the Bonferroni correction ($p=0.05/3$) indicated that the A/G and G/G of the SNP3 were significantly higher in the CI group.

When the confounding factors, which included hypertension, total cholesterol and DM, were included within the multiple logistic regression analysis, significant differences were noted between the CI and control groups. In addition, the SNP3R was higher for the total CI participant group as compared to that observed for the control group ($p=0.021$), with the calculated odds ratio determined to be 1.716 (95% confidence interval: 1.086–2.712) (Table 3a). Hypertension, total cholesterol and DM were also found to be significantly different between the CI and the control groups. To investigate the interaction between the recessive model of SNP3 and the confounding factors, we used our multiple logistic regression model and enrolled interaction variables that included SNP3R×hypertension (Table 3b), SNP3R×total cholesterol and SNP3R×DM, respectively. No interactions

Table 2. *Genotype distribution in the control and CI.*

Variants		Total participants		
		Control	CI	p-value
		314	165	
rs7042521 (SNP1)	Genotype			
	G/G	95 (30.3%)	50 (30.3%)	0.935
	G/C	164 (52.2%)	84 (50.9%)	
	C/C	55 (17.5%)	31 (18.8%)	
	dominant			
	G/G, G/C	259 (82.5%)	134 (81.2%)	0.730
	C/C	55 (17.5%)	31 (18.8%)	
	recessive model			
	G/G	95 (30.3%)	50 (30.3%)	0.991
	G/C, C/C	219 (69.7%)	115 (69.7%)	
	Allele			
	G	354 (56.4%)	184 (55.8%)	0.856
	C	274 (43.6%)	146 (44.2%)	
rs11137351 (SNP2)	Genotype			
	C/C	91 (29.0%)	47 (28.5%)	0.154
	C/G	156 (49.7%)	94 (57.0%)	
	G/G	67 (21.3%)	24 (14.5%)	
	dominant			
	C/C, C/G	247 (78.7%)	141 (85.5%)	0.072
	G/G	67 (21.3%)	24 (14.5%)	
	recessive model			
	C/C	91 (29.0%)	47 (28.5%)	0.909
	C/G, G/G	223 (71.0%)	118 (71.5%)	
	Allele			
	C	338 (53.8%)	188 (57.0%)	0.352
	G	290 (46.2%)	142 (43.0%)	
rs10780199 (SNP3)	Genotype			
	A/A	115 (36.6%)	41 (24.8%)	0.032*
	A/G	163 (51.9%)	100 (60.6%)	
	G/G	36 (11.5%)	24 (14.5%)	
	dominant			
	A/A, A/G	278 (88.5%)	141 (85.5%)	0.333
	G/G	36 (11.5%)	24 (14.5%)	
	recessive model			
	A/A	115 (36.6%)	41 (24.8%)	0.009*#
	A/G, G/G	199 (63.4%)	124 (75.2%)	
	Allele			
	A	393 (62.6%)	182 (51.2%)	0.026*
	G	235 (37.4%)	148 (44.8%)	

*significant difference in distribution.
#significant difference after a correction for multiple testing (Bonferroni's correction).
p-value calculated by Fisher's exact test. CI, cerebral infarction.

Table 3a. *Multiple logistic regression analysis in CL.*

	Total			
		95% CI		
rsl0780199 (SNP3)	p-value	OR	lower limit	upper limit
AG+GG genotype	0.021	1.716	1.086	2.712*
Hypertension	0.000	3.626	1.975	6.659*
Hyperlipidemia	0.000	0.444	0.292	0.676*
DM	0.000	10.696	4.449	25.713*

*significant
CT, cerebral infarction; DM, diabetes mellitus; OR, odds

Table 3b. *The interaction between the AG+GG genotype and hypertension.*

	Total			
		95% CI		
rsl0780199 (SNP3)	p-value	OR	lower limit	upper limit
SNP3R×hypertension	0.731	1.26	0.338	4.697

were noted between the AG+GG genotype of SNP3 and these three confounding factors.

Figure 2 shows the pairwise LD patterns for the CACNA1B gene. Because the |D'| values of SNP1–SNP3 were ≥0.25, all polymorphisms were located in one haplotype block. Data from the International Human Haplotype Map (HapMap) Project (www.hapmap.org/index.html.en) also indicated that the three SNPs are included within one LD block. Since the r^2 values of SNP1–SNP2, SNP2–SNP3 and SNP1–SNP3 were ≥0.50, we constructed a haplotype-based association study using SNP1–SNP2–SNP3.

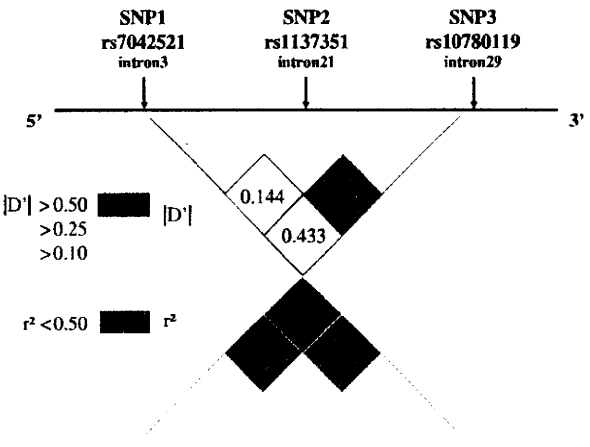

Fig. 2. Pairwise linkage disequilibrium (LD) analysis. Pairwise LD among the three marker pairs studied in the CACNA1B gene was evaluated by |D'| and r^2. D values >0.25 were used to assign SNP locations to one haplotype block. r^2 values <0.5 were selected as tagged. |D'| >0.5 and r^2 <0.5 are shown as deep gray-shaded values. |D'| >0.25 are shown as light gray-shaded values.

The haplotype-based case-control study was performed using SNP1, SNP2 and SNP3 for the total participants (Table 4). There was a significant difference in the overall distributions noted for the combination of SNP1–SNP2–SNP3 between the CI and control groups ($p < 0.001$). The C-C-G and G-G-G haplotypes were significantly more frequent in the CI group as compared to the control group ($p = 0.024$ and $p < 0.000$). Thus, the results suggest that the C-C-G and G-G-G haplotypes might be susceptibility haplotypes for CI.

DISCUSSION

The pathophysiology of CI involves a very complex process. When the cerebral blood flow decreases, it leads to the release of glutamate as a neurotransmitter from the nerve terminals and other brain cells (Choi 1988; Kobayashi and Mori 1998). These processes can induce calcium entry from outside of the nerve cells into the postsynaptic neurons, thereby threatening neuronal cell survival. Various models of neuronal ischemia have demonstrated that just prior to neuronal cell death there is a calcium influx that leads to an increase in the intracellular calcium (Choi 1988, 1995; Kristian and Siesjo 1998; Bano et al. 2005). Although various calcium channel blockers have been confirmed to have a neuro-protective effect not only in the in vitro models of ischemia but also in the in vivo models, there have been no reports that have clearly examined the relationship between CI and the gene for the VGCCs.

Genetic analyses of complex traits and diseases and population-based gene identification studies can be more easily performed when using SNPs versus other polymorphisms, such as microsatellite markers. In fact, SNPs with high genomic frequencies are particularly useful for susceptible gene discovery purposes. Moreover, because new SNP alleles arise as mutations at different loci and different points in time, and since they occur in such greater abundance as compared to the genomes, groups of neighboring SNPs may create a haplotypic diversity that can be exploited in direct association studies (Schork et al. 2000). Morris et al. found that analyses based on haplotypes have advantages over analyses based on individual SNPs in genes with multiple susceptibilities, particularly when the linkage disequilibria between the SNPs are weak (Morris and Kaplan 2002). When trying to determine a relationship between a genetic variation and phenotypes, a haplotype-based case-control study provides more information as compared to what can be obtained when only using a case-control study that employs a single SNP (Stephens et al. 2001; Zhang et al. 2002; Ma et al. 2005). Based on previous findings that have indicated that a haplotype analysis would be useful in the assessment of the association between haplotypes and CI (Nakayama et al. 2006), we attempted to perform a

Table 4. *Haplotypes that showed significant differences for the overall distribution and groups.*

	Overall		Distribution of individual				
Combination of	X^2	p	haplotype	control	CI	X^2	p
SNP1-SNP2-	24.6	<0.001*	G-C-	0.160	0.140	0.725	0.395
			C-C-	0.030	0.019	1.273	0.259
			G-G-	0.287	0.287	0.001	0.974
			C-G-	0.151	0.105	3.669	0.055
			G-C-	0.113	0.107	0.130	0.718
			C-C-	0.236	0.303	5.118	0.024*
			G-G-	0.000	0.023	15.255	0.000*
			C-G-	0.022	0.015	0.587	0.444

*shows significant difference for the susceptibility haplotypes of CI, cerebral

haplotype-based case-control study for the CACNA1B gene, which consisted of three SNPs.

Our results showed that the SNP3R frequency for the total CI participants was significantly higher than that observed in the control group. Furthermore, the C-C-G and G-G-G haplotypes of rs7042521–rs11137351–rs10780199 were significantly more frequent in the total CI participant group as compared to the control group. These results indicate that a susceptibility SNP and haplotypes exist for CI. Since CI is thought to be multifactorial disease that is not caused by a single mutation, our results indicate that these polymorphisms and haplotypes might very well be genetic markers for CI. While in this study we found that the G-G-G haplotype exhibited a greater frequency in the CI groups versus the control groups, a larger number of cases need to be analyzed to be able to conclusively determine whether these differences are truly significant. In addition, since the CI sample size was smaller than that for the control, differences between others haplotypes of the CI group and the control group might also exist. This is one of the challenges that our current line of investigations has been dealing with, and is one that will require us to collect a larger number of cases in the future if we are to be able to conclusively demonstrate these differences exist.

To directly elucidate the physiological properties of CACNA1B, Ino et al. generated mice that were genetically CACNA1B deficient (Ino et al. 2001). They reported that the N-type VGCCs have an important role within the sympathetic nervous system for circulatory regulation. The baroreflex response mediated by the sympathetic nervous system was markedly reduced after bilateral carotid occlusion in mice. Interestingly, sustained elevation of the heart rate and blood pressure has been demonstrated to occur in mutant mice. One of the major risk factors for CI is the elevation of blood pressure and hypertension (Lawes et al. 2004). On the basis of this original report, we suspected that the blood pressure factor might be associated with the occurrence of CI and the human CACNA1B gene in Japanese subjects. However, our assessment of interactions between the confounding factors confirmed that there were no interactions noted for the occurrence of CI, hypertension and the frequency of the SNP3R factors (Table 3b). Thus, our results suggest that the CACNA1B gene variations are significantly associated with the high CI risk in an independent manner and, therefore, these variations can be used as genetic markers for CI.

It should be noted that there were some limitations associated with our current study. For example, case-control studies sometimes exhibit pseudo-positive results due to sample scales or the selection of genetic markers. Our results indicated that for the three SNPs that we selected, there was an associated higher CI risk for one of the CACNA1B genotypes and the haplotype variations. Since the genotypes of other two SNPs were not found to be positive, this confirms that our results can be considered to be reliable. However, in order to conclusively confirm the reliability of the present data, familial linkage studies along with transmission disequilibrium tests will need to be performed in future experiments.

In conclusion, the rs10780199, and the C-C-G and G-G-G haplotypes in the human CACNA1B gene demonstrated that significant differences exist between the CI and control patients. These results indicate that the polymorphisms and the haplotypes might be genetic markers for CI.

Acknowledgements – The authors would like to thank Ms. K. Sugama for her excellent technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Nihon University School of Medicine, Kissei Pharmaceutical Co., Ltd., and Toray-Astellas Pharma Inc., Japan.

REFERENCES

Aoi, N., Soma, M., Nakayama, T. et al. 2004. Variable number of tandem repeat of the 5'-flanking region of type-C human natriuretic peptide receptor gene influences blood pressure levels in obesity-associated hypertension. – Hypertens. Res. 27: 711–716.

Bano, D., Young, K. W., Guerin, C. J. et al. 2005. Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. – Cell 120: 275–285.

Catterall, W. A. 2000. Structure and regulation of voltage-gated Ca2+ channels. – Annu. Rev. Cell. Dev. Biol. 16: 521–555.

Catterall, W. A., Perez-Reyes, E., Snutch, T. P. et al. 2005. International Union of Pharmacology. XLVIII. Nomenclature and structure–function relationships of voltage-gated calcium channels. – Pharmacol. Rev. 57: 411–425.

Cha, M. H., Kim, K. S., Suh, D. et al. 2007. Effects of genetic polymorphism of uncoupling protein 2 on body fat and calorie restriction-induced changes. – Hereditas 144: 222–227

Choi, D. W. 1988. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. – Trends Neurosci. 11: 465–469.

Choi, D. W. 1995. Calcium: still center-stage in hypoxic-ischemic neuronal death. – Trends Neurosci. 18: 58–60.

Haketa, A., Soma, M., Nakayama, T. et al. 2004. Two medium-chain acyl-coenzyme A synthetase genes, SAH and MACS1, are associated with plasma high-density lipoprotein cholesterol levels, but they are not associated with essential hypertension. – J. Hypertens. 22: 1903–1907.

Hassan, A. and Markus, H. S. 2000. Genetics and ischaemic stroke. – Brain 123 (Pt. 9.): 1784–1812.

Hinohara, S. and Doba, N. 2005. The future profile of health promotion and disease prevention in Japan: based on the study of seniors over age 75. – Methods Inf. Med. 44: 342–347.

Hirning, L. D., Fox, A. P., McCleskey, E. W. et al. 1988. Dominant role of N-type Ca2+ channels in evoked release of norepinephrine from sympathetic neurons. – Science 239: 57–61.

Hong, S. J. and Chang, C. C. 1995. Calcium channel subtypes for the sympathetic and parasympathetic nerves of guinea-pig atria. – Br. J. Pharmacol. 116: 1577–1582.

Ino, M., Yoshinaga, T., Wakamori, M. et al. 2001. Functional disorders of the sympathetic nervous system in mice lacking the alpha 1B subunit (Cav 2.2) of N-type calcium channels. – Proc. Natl Acad. Sci. USA 98: 5323–5328.

Kobayashi, T. and Mori, Y. 1998. Ca2+ channel antagonists and neuroprotection from cerebral ischemia. – Eur. J. Pharmacol. 363: 1–15.

Kobayashi, Y., Nakayama, T., Sato, N. et al. 2005. Haplotype-based case-control study revealing an association between the adrenomedullin gene and proteinuria in subjects with essential hypertension. – Hypertens. Res. 28: 229–236.

Kristian, T. and Siesjo, B. K. 1998. Calcium in ischemic cell death. – Stroke 29: 705–718.

Lawes, C. M. M., Bennett, D. A., Feigin, V. L. et al. 2004. Blood pressure and stroke: an overview of published reviews. – Stroke 35: 776–785.

Ma, L., Xue, Y., Liu, Y. et al. 2005. Polymorphism study of seven SNPs at ADH genes in 15 Chinese populations. – Hereditas. 142: 103–111.

Maruyama, A., Nakayama, T., Sato, N. et al. 2004. Association study using single nucleotide polymorphisms in the estrogen receptor beta (ESR2) gene for preeclampsia. – Hypertens. Res. 27: 903–909.

Morris, R. W. and Kaplan, N. L. 2002. On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. – Genet. Epidemiol. 23: 221–233.

Nakayama, T., Soma, M., Kanmatsuse, K. et al. 2004a. The microsatellite alleles on chromosome 1 associated with essential hypertension and blood pressure levels. – J. Hum. Hypertens. 18: 823–828.

Nakayama, T., Soma, M., Sato, N. et al. 2004b. An association study in essential hypertension using functional polymorphisms in lymphotoxin-alpha gene. – Am. J. Hypertens. 17: 1045–1049.

Nakayama, T., Asai, S., Sato, N. et al. 2006. Genotype and haplotype association study of the STRK1 region on 5q12 among Japanese: a case-control study. – Stroke 37: 69–76.

Parra-Rojas, I., Ruíz-Madrigal, B., Martínez-López, E. et al. 2006. Influence of the -308 TNF-alpha and -174 IL-6 polymorphisms on lipid profile in Mexican subjects. – Hereditas 143: 167–172.

Perez-Pinzon, M. A., Yenari, M. A., Sun, G. H. et al. 1997. SNX-111, a novel, presynaptic N-type calcium channel antagonist, is neuroprotective against focal cerebral ischemia in rabbits. – J. Neurol. Sci. 153: 25–31.

Pringle, A. K., Benham, C. D., Sim, L. et al. 1996. Selective N-type calcium channel antagonist omega conotoxin MVIIA is neuroprotective against hypoxic neurodegeneration in organotypic hippocampal-slice cultures. – Stroke 27: 2124–2130.

Schork, N. J., Fallin, D., Lanchbury, J. S. 2000. Single nucleotide polymorphisms and the future of genetic epidemiology. – Clin. Genet. 58: 250–264.

Serone, A. P. and Angus, J. A. 1999. Role of N-type calcium channels in autonomic neurotransmission in guinea-pig isolated left atria. – Br. J. Pharmacol. 127: 927–934.

Special report from the National Institute of Neurological Disorders and Stroke 1990. Classification of cerebrovascular diseases. – Stroke 21: 637–676.

Stephens, J. C., Schneider, J. A., Tanguay, D. A. et al. 2001. Haplotype variation and linkage disequilibrium in 313 human genes. – Science 293: 489–493.

Takahara, A., Konda, T., Enomoto, A. et al. 2004. Neuroprotective effects of a dual L/N-type Ca(2+) channel blocker cilnidipine in the rat focal brain ischemia model. – Biol. Pharm. Bull. 27: 1388–1391.

Toth, P. T., Bindokas, V. P., Bleakman, D. et al. 1993. Mechanism of presynaptic inhibition by neuropeptide Y at sympathetic nerve terminals. – Nature 364: 635–639.

Valentino, K., Newcomb, R., Gadbois, T. et al. 1993. A selective N-type calcium channel antagonist protects against neuronal loss after global cerebral ischemia. – Proc. Natl Acad. Sci. USA 90: 7894–7897.

Vega, T., De Pascual, R., Bulbena, O. et al. 1995. Effects of omega-toxins on noradrenergic neurotransmission in beating guinea pig atria. – Eur. J. Pharmacol. 276: 231–238.

Zhang, K., Calabrese, P., Nordborg, M. et al. 2002. Haplo-type block structure and its applications to association studies: power and study designs. – Am. J. Hum. Genet. 71: 1384–1391.

Common Variants in the ATP2B1 Gene Are Associated With Susceptibility to Hypertension

The Japanese Millennium Genome Project

Yasuharu Tabara, Katsuhiko Kohara, Yoshikuni Kita, Nobuhito Hirawa, Tomohiro Katsuya, Takayoshi Ohkubo, Yumiko Hiura, Atsushi Tajima, Takayuki Morisaki, Toshiyuki Miyata, Tomohiro Nakayama, Naoyuki Takashima, Jun Nakura, Ryuichi Kawamoto, Norio Takahashi, Akira Hata, Masayoshi Soma, Yutaka Imai, Yoshihiro Kokubo, Tomonori Okamura, Hitonobu Tomoike, Naoharu Iwai, Toshio Ogihara, Itsuro Inoue, Katsushi Tokunaga, Toby Johnson, Mark Caulfield, Patricia Munroe on behalf of the Global Blood Pressure Genetics Consortium, Satoshi Umemura, Hirotsugu Ueshima, Tetsuro Miki

Abstract—Hypertension is one of the most common complex genetic disorders. We have described previously 38 single nucleotide polymorphisms (SNPs) with suggestive association with hypertension in Japanese individuals. In this study we extend our previous findings by analyzing a large sample of Japanese individuals (n=14 105) for the most associated SNPs. We also conducted replication analyses in Japanese of susceptibility loci for hypertension identified recently from genome-wide association studies of European ancestries. Association analysis revealed significant association of the *ATP2B1* rs2070759 polymorphism with hypertension ($P=5.3\times10^{-5}$; allelic odds ratio: 1.17 [95% CI: 1.09 to 1.26]). Additional SNPs in *ATP2B1* were subsequently genotyped, and the most significant association was with rs11105378 (odds ratio: 1.31 [95% CI: 1.21 to 1.42]; $P=4.1\times10^{-11}$). Association of rs11105378 with hypertension was cross-validated by replication analysis with the Global Blood Pressure Genetics consortium data set (odds ratio: 1.13 [95% CI: 1.05 to 1.21]; $P=5.9\times10^{-4}$). Mean adjusted systolic blood pressure was highly significantly associated with the same SNP in a meta-analysis with individuals of European descent ($P=1.4\times10^{-18}$). *ATP2B1* mRNA expression levels in umbilical artery smooth muscle cells were found to be significantly different among rs11105378 genotypes. Seven SNPs discovered in published genome-wide association studies were also genotyped in the Japanese population. In the combined analysis with replicated 3 genes, *FGF5* rs1458038, *CYP17A1*, rs1004467, and *CSK* rs1378942, odds ratio of the highest risk group was 2.27 (95% CI: 1.65 to 3.12; $P=4.6\times10^{-7}$) compared with the lower risk group. In summary, this study confirmed common genetic variation in *ATP2B1*, as well as *FGF5*, *CYP17A1*, and *CSK*, to be associated with blood pressure levels and risk of hypertension. (***Hypertension*. 2010;56:973-980.**)

Key Words: hypertension ■ genetic variation ■ ATP2B1 ■ Millennium Genome Project ■ Global BPgen

Because of its large impact on a number of cardiovascular diseases, hypertension is a major contributor to global health burden. Because hypertension is one of the most prevalent complex genetic disorders, with a heritability of ≤60% based on the estimation by 24-hour blood pressure (BP) readings,[1] numerous studies, including recent genome-wide association studies (GWAS),[2–6] have attempted to identify genetic variation associated with human BP levels.

Received March 16, 2010; first decision April 11, 2010; revision accepted September 1, 2010.

From the Departments of Basic Medical Research and Education (Y.T.), Geriatric Medicine (K.K., J.N., T.Mik.), Community Medicine (R.K.), Ehime University Graduate School of Medicine, Toon, Japan; Division of Anti-Aging and Genomics (Y.T., K.K., T.Mik.), Ehime Proteo-Medicine Research Center, Toon, Japan; Department of Health Science (Y.Ki., T.Oh., N.Takas., H.U.), Shiga University of Medical Science, Otsu, Japan; Division of Nephrology and Hypertension (N.H.), Yokohama City University Medical Center, Yokohama, Japan; Department of Medical Science and Cardiorenal Medicine (S.U.), Yokohama City University Graduate School of Medicine, Yokohama, Japan; Department of Geriatric Medicine (T.K., T.Og.), Osaka University Graduate School of Medicine, Suita, Japan; Departments of Planning for Drug Development and Clinical Evaluation (T.Oh.) and Clinical Pharmacology and Therapeutics (Y.I.), Tohoku University Graduate School of Pharmaceutical Science and Medicine, Sendai, Japan; Departments of Genomic Medicine (Y.H., N.I.), Molecular Biology (T.Mo.), Molecular Pathogenesis (T.Miy.), Preventive Cardiology (Y.Ko., T.Ok., H.T.), National Cerebral and Cardiovascular Research Center, Suita, Japan; Division of Molecular Life Science (A.T., I.I.), School of Medicine, Tokai University, Isehara, Japan; Divisions of Laboratory Medicine (T.N.) and Nephrology and Endocrinology (M.S.), Department of Medicine, Nihon University School of Medicine, Tokyo, Japan; Departments of Clinical Studies and Genetics (N.Takah.), Radiation Effects Research Foundation, Hiroshima, Japan; Department of Public Health (A.H.), Graduate School of Medicine, Chiba University, Chiba, Japan; Department of Human Genetics (K.T.), Graduate School of Medicine, University of Tokyo, Tokyo, Japan; Clinical Pharmacology (T.J., M.C., P.M.), William Harvey Research Institute, Queen Mary University of London, London, United Kingdom.

Full author list of the Global BPgen consortium is given in the online Data Supplement.

Correspondence to Yasuharu Tabara, Department of Basic Medical Research and Education, Ehime University Graduate School of Medicine, Shitsukawa 454, Toon-City, Ehime 791-0295, Japan. E-mail tabara@m.ehime-u.ac.jp

***Hypertension* is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.110.153429**

Except for rare mendelian forms of hypertension,[7] the estimated effects of each genetic factor on BP levels have been found to be small in the general population (typically <1.0 mm Hg on systolic BP [SBP] and <0.5 mm Hg on diastolic BP [DBP] per risk allele). However, multiple risk alleles are known to have a cumulative impact on several complex traits, including BP and hypertension risk.[3] In addition, it is anticipated that identification of novel susceptibility genes would lead to further understanding of disease pathogenesis.

As a part of a series of nationally based cooperative projects, the Millennium Genome Project (Millennium GPJ), we conducted multiple candidate gene analyses to identify susceptible genes and polymorphisms for hypertension. In a previously reported study,[6] we focused on 307 genes, which were genes encoding components of signal transduction pathways potentially related to BP regulation, including receptors, soluble carrier proteins, binding proteins, channels, enzymes, and G proteins. That study identified 38 single nucleotide polymorphisms (SNPs) as suggestively associated with hypertension by analysis of 758 hypertensive patients and 726 normotensive controls.[6] To extend our previous study, we have now genotyped all 38 of the SNPs in a replication panel composed of 1929 hypertensives and 1993 normotensives and have taken forward validated SNPs with further genotyping in a large Japanese genetic epidemiological cohort sample (n=14 105). An in silico validation analysis of our most promising loci was performed using the Global Blood Pressure Genetics (Global BPgen) consortium data set, a large-scale GWAS of samples of European descent.[2] Furthermore, we also conducted a replication analysis of recent European GWAS-derived susceptible loci for hypertension from Global BPgen[2] and CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) GWAS[3] in a Japanese large-scale general population sample (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).

Methods

Case and Control Subjects (Screening Panel)

Details of the screening panel subjects have been described previously.[6] Briefly, hypertensive patients and normotensive controls were recruited in the Asahikawa, Tokyo, Osaka, and Hiroshima regions of Japan according to the following criteria. Hypertensive subjects (n=758) had a previous diagnosis of hypertension at between 30 and 59 years of age and were either being treated with antihypertensive medication or had a SBP >160 mm Hg and/or DBP >100 mm Hg. They had a family history of hypertension in their parents and/or siblings and were not obese (body mass index [BMI] <25 kg/m^2). Normotensive controls (n=726) aged >45 years were recruited from the same regions. These individuals have never been treated with antihypertensive medications, and their SBP was <120 mm Hg and DBP <80 mm Hg. They had no family history of hypertension. All of the subjects were unrelated and were native Japanese.

Cohort-Based Population Samples

Seven independent study cohorts for cardiovascular diseases and related risk factors were combined to compose a large-scale Japanese genetic epidemiological population sample of 14 105. The Ohasama, Shigaraki, Takashima, Suita, and Nomura studies are general population-based genetic epidemiological studies. The study subjects were recruited via a medical checkup process for community residents. The 2 other cohorts, Yokohama and Matsuyama, are derived from employees of large manufacturing industries. The clinical parameters used in this study were obtained from personal health records during annual medical checkups. Further details of the study cohorts are described in the online Data Supplement.

Nested Case and Control Subjects Derived From the Cohort-Based Sample (Replication Panel)

Hypertensive cases and normotensive controls were chosen from the cohort-based population samples described above (n=11 569; the Suita study was excluded because of ethical issues). The selection criteria of the hypertensive and normotensive subjects were as follows: hypertensive subjects (n=1929) aged ≤64 years and either treatment with antihypertensive medication and/or SBP >160 mm Hg and/or DBP >90 mm Hg; normotensive subjects (n=1993) aged ≥40 years and having SBP <120 mm Hg and DBP <80 mm Hg; and no current use of antihypertensive medication and free from any history of cardiovascular disease.

Global BPgen (In Silico) Analyses

To investigate cross-validation of the most promising SNPs, we obtained results for 4 SNPs in the *ATP2B1* gene from the Global BPgen consortium, a study that is composed of 17 GWAS studies with 34 433 individuals of European descent. A detailed description of the study design and phenotype measurement for all of the cohorts has been reported previously.[2]

Validation of Published BP Polymorphisms in the Japanese Millennium Cohort

Thirteen loci have been identified recently and robustly validated for association with BP and hypertension in recent large-scale GWAS of European samples, by the Global BPgen consortium[2] and the CHARGE consortium.[3] From the associated SNPs reported at these 13 loci, we selected SNPs expected to have minor allele frequencies in Japanese samples >0.10, based on the HapMap database (JPT only, Public Release No. 27)[8]: *FGF5* rs1458038, *CYP17A1* rs1004467, *CSK* rs1378942, *PLCD3* rs12946454, *PLEKHA7* rs381815, *ULK4* rs9815354, and *CSK-ULK3* rs6495122. These 7 SNPs were genotyped in the Japanese population-based cohort sample to test whether the same associations exist in samples of Japanese ancestry.

Genotyping

Genomic DNA was extracted from peripheral blood. All of the SNPs were analyzed by TaqMan probe assays (Applied Biosystems Co, Ltd) using commercially available primers and probes purchased from the Assay-on-Demand system. The fluorescence level of PCR products was measured using an ABI PRISM 7900HT sequence detector.

Ethical Considerations

All of the study procedures were approved by the ethics committee of each university or research institute. Written informed consent was obtained from all of the participating subjects.

Ex Vivo Expression Analysis of ATP2B1 mRNA

Umbilical artery smooth muscle cells were isolated from umbilical cords obtained at delivery (n=34). Expression levels of ATP2B1 mRNA were analyzed by RT-PCR using a relative quantification method. Further details of the ex vivo expression analysis are described in the online Data Supplement.

Statistical Analysis

At each SNP, frequency differences in each genotype among hypertensive and normotensive subjects were assessed using a χ^2 test. Linkage disequilibrium (LD) coefficients were calculated using the Haploview software (Broad Institute).[9] Adjusted odds ratios for hypertension, as well as coefficients and SEs for SBP and DBP, were calculated using logistic and linear multiple regression analysis,

Table 1. Association of ATP2B1 SNPs With Hypertension in the Screening and Replication Panels

			Screening Panel						Replication Panel						
SNP	Genotype		Genotype Frequency			HWE	Call Rate	Odds (*P*)	Genotype Frequency			HWE	Call Rate	Odds (*P*)	Overall Odds (*P*)
rs1401982	AA/AG/GG	HT	318	328	92	0.603	96.3	1.28 (0.001)	825	833	247	0.108	98.7	1.25 (3.0×10^{-6})	1.26 (1.5×10^{-8})
		NT	249	324	118	0.474			699	961	305	0.397			
rs2681472	AA/AG/GG	HT	335	321	90	0.334	97.8	1.26 (0.003)	846	832	242	0.095	99.5	1.26 (1.0×10^{-6})	1.26 (8.7×10^{-9})
		NT	267	328	111	0.539			715	966	303	0.431			
rs2070759	GG/GT/TT	HT	216	379	151	0.515	97.6	1.16 (0.045)	582	896	399	0.118	97.2	1.18 (4.4×10^{-4})	1.17 (5.3×10^{-5})
		NT	186	341	175	0.454			507	956	474	0.579			
rs11105364	TT/TG/GG	HT	335	322	88	0.432	97.2	1.29 (0.001)	846	834	236	0.171	99.3	1.25 (2.4×10^{-6})	1.26 (4.1×10^{-9})
		NT	261	323	113	0.438			729	947	303	0.874			
rs11105378	CC/CT/TT	HT	359	301	76	0.276	97.3	1.37 (6.3×10^{-5})	868	821	217	0.280	98.8	1.28 (1.4×10^{-7})	1.31 (4.1×10^{-11})
		NT	280	320	108	0.295			746	922	300	0.586			

The screening panel is composed of 758 middle age–onset severe hypertensive patients and 726 middle-aged to elderly evidently normotensive controls (Table S4). The replication panel consists of 1929 hypertensive cases, and 1993 normotensive controls selected from 11 569 cohort sample were enrolled (Table S2). ORs and *P* values for allelic model are shown.

adjusting for sex, age, age^2, BMI, and cohort variables, using additive (1 degree of freedom) and genotypic (2 degrees of freedom) genetic models. Adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15 mm Hg for SBP and +10 mm Hg for DBP).[10] The Global BPgen data and statistical methods have been described elsewhere.[2] Meta-analysis was performed assuming fixed effects and using inverse variance weights. An unweighted genetic risk score based on 4 SNPs (*ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1* rs1004467, and *CSK* rs1378942) was calculated by adding the number of risk alleles showing higher BP values. Risk allele of each SNP was defined as follows: *ATP2B1*, C allele; *FGF5*, T allele; *CYP17A1*, A allele; and *CSK*, C allele. The *CSK-ULK3* SNP rs6495122 showing positive association with BP trait and hypertension was not included in the calculation of genetic risk score, because the strong LD with the *CSK* SNP rs1378942 (D′=0.884; r^2=0.731) is most parsimoniously explained by both SNPs tagging a single risk variant. Differences in mRNA expression levels among the *ATP2B1* rs1105378 genotype were assessed by ANOVA. The statistical analyses were performed using a commercially available statistical software package (JMP version 8, SAS Institute).

Results

Replication Genotyping

The clinical characteristics of the replication panel chosen from the cohort-based population samples (Table S1, available in the online Data Supplement) are shown in Table S2. Stringent case and control definitions, corresponding with the extreme upper ≈17% and lower ≈17% of the general population, were used to maximize power for fixed genotyping costs.[11] Thirty-six SNPs were successfully genotyped, and results for all of the SNPs are shown in Table S3. Significant association was observed for the *ATP2B1* rs2070759 polymorphism located in intron 8 ($P=4.4\times10^{-4}$; allele odds ratio [OR]: 1.18 [95% CI: 1.07 to 1.29]). Several other SNPs also showed marginally significant association; however, the *P* values did not reach statistical significance after application of Bonferroni correction for multiple comparisons (threshold: 0.05/36=0.0014; Table S3; we note that no other SNPs are significant if the less conservative Benjamini-Hochberg procedure is used to control the false discovery rate at 0.05). Although, the replication results in the less-strict nested case-control sample chosen from the same population sample have been reported in our previous article,[6] the association was recalculated to narrow down the SNPs to be applied to the following dense SNP analysis.

Dense SNP Analysis of the ATP2B1 Gene

To more precisely identify the SNP or SNPs increasing susceptibility for hypertension, we performed "de novo" genotyping of a dense SNP panel around marker rs2070759 in individuals from the original screening panel (Table S4).[6] Forty-one tag SNPs located in a 167-kb region around rs2070759 were selected using the HapMap database (Table S5).[8] Among the 27 SNPs polymorphic in our Japanese sample, the most significant association was observed with rs11105378; this yielded an allelic *P* value of 6.3×10^{-5} (OR: 1.37 [95% CI: 1.17 to 1.60]; Table 1 and Figure S2).

The most associated SNP and the 4 others from the dense SNP analyses were subsequently genotyped in the replication panel. Significant association of rs11105378 was confirmed in the replication panel with an allelic *P* value of 1.4×10^{-7} (OR: 1.28 [95% CI: 1.17 to 1.41]; Table 1). Meta-analysis of both study panels indicated significant association ($P=4.1\times10^{-11}$; OR: 1.31 [95% CI: 1.21 to 1.42]) and confirmed that the strongest association is seen for rs11105378. The D′ and r^2 measures of LD between rs2070759 and rs11105378 were 0.92 and 0.48, respectively. Other SNPs, rs1401982 (D′=0.99; r^2=0.64), rs2681472 (D′=0.99; r^2=0.61), rs11105364 (D′=0.97; r^2=0.59), located within the same LD block, were also significantly associated with hypertension (Table 1). The strong LD between associated SNPs suggests a single true association signal in this region.

We examined for possible association of SNPs in the *ATP2B4* gene, a well-investigated isoform of the *ATP2B1* gene, with hypertension in the screening panel. We observed no significant correlation with the 17 SNPs analyzed, which were selected using the HapMap database (Table S6).

Population-Based Meta-Analyses of ATP2B1 SNPs

The complete Japanese population-based sample was subsequently genotyped for the 4 most significant SNPs in

Table 2. Meta-Analysis of ATP2B1 SNPs With BP Traits

		Millennium GPJ			Global BPgen			CHARGE*			Pooled	
SNP	Coded Allele	n (Frequency)	Coefficient (SE), mm Hg	*P*	n (Frequency)	Coefficient (SE), mm Hg	*P*	n (Frequency)	Coefficient (SE), mm Hg	*P*	Coefficient (95% CI), mm Hg	*P*
SBP												
rs1401982	G	13 944	−1.22	1.8×10^{-7}	33 885	−0.30	0.022				−0.52	3.9×10^{-6}
		(0.376)	(0.23)		(0.385)	(0.13)					(−0.74 to −0.30)	
rs2681472	G	14 032	−1.33	1.2×10^{-8}	33 803	−0.62	5.2×10^{-4}	0.17	−1.29	3.5×10^{-11}	−1.03	9.9×10^{-20}
		(0.373)	(0.23)		(0.158)	(0.18)			(0.19)		(−1.26 to −0.81)	
rs11105364	G	14 013	−1.34	8.9×10^{-9}	33 877	−0.60	7.4×10^{-4}	0.17	−1.30	4.8×10^{-11}	−1.03	1.2×10^{-19}
		(0.364)	(0.23)		(0.179)	(0.18)			(0.19)		(−1.25 to −0.81)	
rs11105378	T	13 948	−1.33	1.5×10^{-8}	33 171	−0.59	0.001	0.16	−1.31	9.1×10^{-11}	−1.02	1.4×10^{-18}
		(0.360)	(0.23)		(0.158)	(0.18)			(0.20)		(−1.24 to −0.79)	
DBP												
rs1401982	G	13 944	−0.72	2.0×10^{-7}	33 898	−0.18	0.041				−0.34	8.1×10^{-6}
		(0.376)	(0.14)		(0.392)	(0.09)					(−0.49 to −0.19)	
rs2681472	G	14 032	−0.65	2.7×10^{-6}	33 829	−0.35	0.003	0.17	−0.64	3.7×10^{-8}	−0.54	9.7×10^{-15}
		(0.373)	(0.14)		(0.157)	(0.12)			(0.11)		(−0.68 to −0.41)	
rs11105364	G	14 013	−0.70	4.5×10^{-7}	33 898	−0.34	0.004	0.17	−0.63	1.2×10^{-7}	−0.54	7.5×10^{-14}
		(0.364)	(0.14)		(0.158)	(0.12)			(0.12)		(−0.68 to −0.40)	
rs11105378	T	13 948	−0.70	5.4×10^{-7}	33 183	−0.33	0.005	0.16	−0.62	3.1×10^{-7}	−0.54	1.6×10^{-13}
		(0.360)	(0.14)		(0.158)	(0.12)			(0.12)		(−0.68 to −0.39)	

Coefficients and SE for SBP and DBP were calculated under the additive model using multiple regression analysis adjusted for age, age^2, sex, and BMI. In both Millennium GPJ and Global BPgen, adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15 mm Hg for SBP and +10 mm Hg for DBP).[2] In the Japanese Millennium GPJ and also for some cohorts within Global BPgen, cohort variables were also adjusted to avoid residual population stratification.

*Results of the CHARGE Study were obtained from the published article.[3]

ATP2B1. To further validate and get more precise effect size estimates in Japanese, for this analysis, hypertensive cases were defined as individuals with treatment with antihypertensive medication, SBP ≥140 mm Hg, or DBP ≥90 mm Hg. The ORs for the 4 SNPs were all extremely similar (ranging from 1.19 to 1.21 under the additive model adjusted for age, age^2, sex, BMI, and cohort variables; see Table S7). These associations were replicated in the Global BPgen subjects of European descent; the pooled analysis demonstrated increased significance (rs1105378: OR: 1.17 [95% CI: 1.11 to 1.23]; $P=7.0\times10^{-10}$), as expected for a larger total sample size (n=28 866; Table S7).

We next evaluated the effect of the most associated SNP, rs11105378, on BP levels in the Millennium GPJ cohort (Table 2). We adjusted for several covariates that are associated with BP phenotypes: age ($r=0.362$; $P<0.001$ for SBP), BMI ($r=0.275$; $P<0.001$), and sex (male: 131.7±18.2; female: 128.6±20.8 mm Hg; $P<0.001$). In multiple regression analysis for BP levels, including also cohort indicator variables as covariates, the results for a 2-degree-of-freedom test with the TT genotype as a reference identified both the TC genotype (coefficient=+1.66 mm Hg; $P=2.2\times10^{-4}$) and CC genotype (+2.47 mm Hg; $P=4.9\times10^{-8}$) as independent determinants for SBP after adjustment. The TC (+0.91 mm Hg; $P=8.0\times10^{-4}$) and CC genotypes (+1.32 mm Hg; $P=1.8\times10^{-6}$) were also independently associated with DBP levels. We depict the covariate adjusted mean BP levels by rs11105378 genotype in Figure S3. Results of each cohort separately are summarized in Table S8. We next performed a meta-analysis of data from the Millennium GPJ and 2 large epidemiological studies (Global BPgen and CHARGE; Table 2). Results show the per-allele differences in SBP and DBP to be ≈1.0 and 0.5 mm Hg, respectively.

Genotype-Specific Differences in Ex Vivo Expression of ATP2B1 mRNA

Differences in *ATP2B1* mRNA expression in umbilical artery smooth muscle cells among rs11105738 genotype are shown in Figure 1. Assuming a recessive genetic model, cells homozygous for T allele showed significantly higher levels of

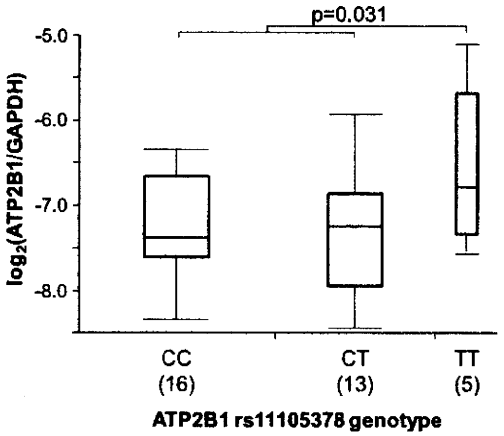

Figure 1. Ex vivo expression analysis of *ATP2B1* mRNA. Graphs depict the log^2 relative expression levels of the *ATP2B1* mRNA in umbilical artery smooth muscle cells obtained by normalizing to GAPDH. Genotype of *ATP2B1* rs11105378 of each sample was analyzed by direct sequencing using isolated genomic DNA from umbilical artery smooth muscle cells.

Table 3. Meta-Analysis of SNPs With BP Traits

SNP	Coded Allele	Millennium GPJ n (Frequency)	Millennium GPJ Coefficient (SE), mm Hg	Millennium GPJ P	Global BPgen n (Frequency)	Global BPgen Coefficient (SE), mm Hg	Global BPgen P	Pooled Coefficient (95% CI), mm Hg	Pooled P
Systolic BP									
FGF5	T	13 826	1.33	1.6×10^{-8}	30 850	0.62	1.6×10^{-6}	0.81	1.1×10^{-11}
rs1458038		(0.343)	(0.23)		(0.275)	(0.14)		(0.58 to 1.05)	
CYP17A1	A	14 007	0.89	2.3×10^{-4}	33 735	0.94	1.0×10^{-5}	0.92	6.2×10^{-9}
rs1004467		(0.680)	(0.24)		(0.901)	(0.21)		(0.61 to 1.23)	
CSK	C	13 920	0.77	0.007	34 126	0.62	2.4×10^{-6}	0.65	4.2×10^{-8}
rs1378942		(0.803)	(0.28)		(0.36)	(0.13)		(0.42 to 0.88)	
PLCD3	T	14 003	0.11	0.703	32 120	0.68	3.9×10^{-6}	0.57	2.5×10^{-5}
rs12946454		(0.831)	(0.30)		(0.28)	(0.15)		(0.30 to 0.83)	
PLEKHA7	T	14 030	0.11	0.687	33 706	0.52	2.6×10^{-4}	0.44	4.7×10^{-4}
rs381815		(0.199)	(0.28)		(0.26)	(0.14)		(0.19 to 0.68)	
CSK-ULK3	A	14 014	0.68	0.017	33 308	0.47	2.4×10^{-4}	0.51	1.7×10^{-5}
rs6495122		(0.812)	(0.28)		(0.45)	(0.13)		(0.28 to 0.74)	
ULK4	A	13 976	−0.67	0.059	32 034	0.17	0.297	0.01	0.950
rs9815354		(0.116)	(0.35)		(0.18)	(0.17)		(−0.29 to 0.31)	
DBP									
FGF5	T	13 826	0.73	1.8×10^{-7}	30 850	0.55	1.5×10^{-8}	0.61	6.1×10^{-14}
rs1458038		(0.343)	(0.14)		(0.275)	(0.10)		(0.45 to 0.77)	
CYP17A1	A	14 007	0.29	0.047	33 735	0.40	5.4×10^{-3}	0.35	4.9×10^{-4}
rs1004467		(0.680)	(0.14)		(0.901)	(0.14)		(0.15 to 0.54)	
CSK	C	13 920	0.41	0.015	34 126	0.48	5.9×10^{-8}	0.46	5.2×10^{-9}
rs1378942		(0.803)	(0.17)		(0.36)	(0.09)		(0.31 to 0.62)	
PLCD3	T	14 003	0.14	0.426	32 120	0.34	5.7×10^{-4}	0.30	1.9×10^{-4}
rs12946454		(0.831)	(0.18)		(0.28)	(0.09)		(0.14 to 0.46)	
PLEKHA7	T	14 030	0.13	0.437	33 706	0.23	0.014	0.20	0.018
rs381815		(0.199)	(0.17)		(0.26)	(0.10)		(0.04 to 0.37)	
CSK-ULK3	A	14 014	0.38	0.027	33 308	0.35	4.2×10^{-5}	0.36	7.4×10^{-6}
rs6495122		(0.812)	(0.17)		(0.45)	(0.09)		(0.20 to 0.51)	
ULK4	A	13 976	0.21	0.325	32 034	0.40	2.9×10^{-4}	0.36	2.3×10^{-4}
rs9815354		(0.116)	(0.21)		(0.18)	(0.11)		(0.17 to 0.55)	

ATP2B1 mRNA as compared with cells carrying 1 or 2 C alleles ($P=0.031$; see Figure 1). Under an additive genetic model, the overall P value was marginally significant ($P=0.091$).

Replication Analysis of European GWAS-Derived Susceptible SNPs in Japanese

We next conducted a replication analysis in the Millennium GPJ, in which we tested associated SNPs identified in recent large-scale European GWAS by the Global BPgen[2] and the CHARGE consortia.[3] From the 7 most promising SNPs of which the minor allele frequency in Japanese was >0.10 based on the HapMap database, 4 SNPs, namely, *FGF5* rs1458038, *CYP17A1* rs1004467, *CSK* rs1378942, and *CSK-ULK3* rs6495122, showed significant association in either binary trait analyses (Tables S9) or quantitative trait analysis (Table 3 and S10). The most significant association was observed with *FGF5* rs1458038; this yielded a P value of 1.6×10^{-8} (+1.33 mm Hg) with SBP and 1.8×10^{-7} (+0.73 mm Hg) with DBP in the Millennium GPJ cohort, and the effect size was greater than that of Europeans (Table 3). Meta-analysis of both study panels with data from Global BPgen indicated further significant associations.

Multiple Regression Analysis for BP Trait and Hypertension in Japanese

To clarify whether the 4 susceptibility SNPs (*ATP2B1*, *FGF5*, *CYP17A1*, and *CSK*) were independently associated with BP traits and hypertension, multiple regression analysis was performed with possible covariates (Table S11). After adjustment for age, age^2, sex, BMI, and drinking habits, this analysis confirmed that all 4 of the SNPs were independent determinants for both BP traits and hypertension.

Combined Effect of Risk Genotypes on Hypertension

A risk score for 4 susceptible genotypes was calculated to evaluate their combined effects on hypertension. ORs asso-

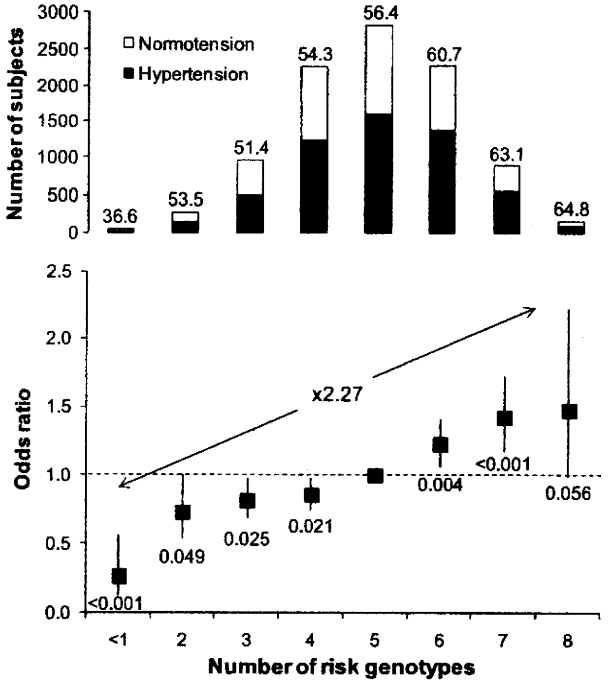

Figure 2. ORs for hypertension according to the number of risk genotypes Number of risk genotype was calculated by the following 4 SNPs: *ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1*, rs1004467, and *CSK* rs1378942. Hypertensive subjects were defined as being treated with antihypertensive medication, SBP ≥140 mm Hg, or DBP ≥90 mm Hg; normotensive subjects were defined as all not treated with antihypertensive medication, SBP ≤120 mm Hg, and DBP ≤85 mm Hg.[2] Adjusted OR for hypertension and BP levels were calculated using logistic and linear multiple regression analysis, adjusting for sex, age, age^2, BMI, and cohort variables. Frequency of hypertension and *P* values for the hypertension odds are shown in the top of column and the bottom of square, respectively.

ciated with increasing number of risk genotypes in a covariates adjusted logistic regression model are depicted in Figure 2 (overall P value was 5.4×10^{-5}). Compared with the reference group (5 risk genotypes), individuals carrying 7 or 8 risk genotypes had higher risk (OR: 1.43 [95% CI: 1.20 to 1.72]; $P=1.0\times10^{-4}$) in contrast to the lower OR of individuals with ≤2 risk genotypes (OR: 0.63 [95% CI: 0.47 to 0.85]; $P=0.020$). The OR of the high-risk group was raised to 2.27 (95% CI: 1.65 to 3.12; $P=4.6\times10^{-7}$) compared with the lowest risk group. Adjusted per-allele OR for hypertension was 1.17 (95% CI: 1.12 to 1.21; $P=4.0\times10^{-15}$). The distribution of the Japanese population sample among the number of risk genotypes is shown in Figure S4.

Discussion

The present study has identified SNPs located upstream or within the *ATP2B1* gene as strong susceptibility polymorphisms for hypertension in Japanese. These are findings that have also been reported recently in individuals of European descent[3] and in Koreans.[4] Although numerous studies have attempted to identify genetic markers for hypertension over the past 2 decades, there has been little cross-validation of loci in different ethnic groups so far except for mendelian forms of hypertension. The SNPs in *ATP2B1* identified in this study showed significant association in large-scale studies in populations with different ancestries and using different discovery approaches, including GWAS in the CHARGE consortium and the Korean study and an independent candidate gene analysis in our present study. Similar findings in different ethnic groups with different methods further strengthen these findings and indicate the *ATP2B1* gene region as a susceptibility locus of likely global significance for BP variation and development of hypertension. Two replication results very recently reported by another Japanese group[12] and a Korean group[13] also indicated the disease susceptibility of *ATP2B1* SNPs located in the same LD block.

No biological data have been provided whether SNP rs1105378 or other SNPs in strong LD have any effect on the transcriptional activity or transcriptional regulation of the *ATP2B1* gene. Furthermore, although alternative splicing has been found to generate several variants of *ATP2B1* mRNA,[14] the SNP associations that we have observed do not shed light on whether this is a potential mechanism for affecting BP. Our data first showed that the effect of SNPs on ATP2B1 gene expression levels is a potential mechanism by which disease-associated SNP alleles cause the phenotypic changes. Changes in the *ATP2B1* gene product levels are involved in BP regulation. We found no microRNA harboring rs11105378 in the miRBase database.[15]

The *ATP2B1* (so-called *PMAC1*) gene encodes the plasma membrane calcium ATPase isoform 1, which removes bivalent calcium ions from eukaryotic cells against very large concentration gradients and plays a critical role in intracellular calcium homeostasis. Although pathophysiological implications of *ATP2B1* gene products on the development of hypertension are uncertain, it has been reported that inhibition of *ATP2B1* by the selective inhibitor caloxin 2A1 showed endothelium-dependent relaxation of rat aorta by increasing cytosolic Ca^{2+} concentration and consequent activation of endothelial NO synthase.[16] Other information on the role of *ATP2B1* has been obtained from experiments using bladder smooth muscle cells: contractility measurements on these cells have documented the important role of *ATP2B1* in the extrusion of Ca^{2+} after carbachol stimulation or depolarization with potassium chloride.[17] These reports suggest altered vascular reactivity as a plausible explanation for disease susceptibility of *ATP2B1* gene.

In mammals, calcium ATPase isoforms are encoded by ≥4 separate genes (*ATP2B1* to *ATP2B4*).[18] It has been reported that overexpression of the human *ATP2B4* gene in arterial smooth muscle cells in mice increases vascular reactivity and BP partly because of negative regulation of neuronal NO synthase.[19] We, therefore, examined the possible association of *ATP2B4* gene polymorphisms with hypertension by using the screening panel. However, no significant correlation was observed in the 17 SNPs analyzed, which were selected by reference to the HapMap database. The pathophysiological association of plasma membrane Ca^{2+} pump with BP regulation may be isoform specific.

Numerous studies, including the recent GWAS,[3-6] have attempted to identify genetic variations associated with human BP levels. At present, it is not clear to what extent findings from GWAS in one population can be extrapolated