表4 高齢者に対してとくに慎重な投与を要する薬物のリスト

薬物分類	薬物	医原性疾患・事故	摘要
強心配糖体	・ジゴキシン	・ジギタリス中毒	・腎機能低下が関与。低K血症が存在するときに感受性増大。血中薬物濃度のモニタリングが必要
降圧薬および抗不整脈薬	・α_1遮断薬	・転倒(起立性低血圧による)	・血管拡張作用による
	・β遮断薬	・転倒(徐脈による)，心不全，喘息増悪	・心機能抑制作用による
	・ジルチアゼム，ベラパミル	・転倒(徐脈による)，心不全	・心機能抑制作用による
利尿薬	・ループ利尿薬，サイアザイド	・尿失禁	・ADL低下の要因。褥瘡感染症誘発
		・便秘，麻痺性イレウス	・低カリウム血症による。腎機能をみながらKの補給
		・不整脈	・低カリウム血症による。腎機能をみながらKの補給
		・脳梗塞，腎機能障害	・脱水による。ヘマトクリットなど脱水の指標に注意
抗凝固薬	・ワーファリン	・出血	・肝でのビタミンK依存性凝固因子の合成の低下
抗生物質	・アミノ配糖体	・腎機能障害	・加齢に伴う腎機能低下
消炎鎮痛剤	・非ステロイド性消炎鎮痛薬(NSAID)	・消化管出血・穿孔	・消化管における粘膜保護因子(PGE_1)産生低下
		・低血圧，失神	・とくに坐薬では急激な血中濃度上昇により多発
睡眠薬	・ベンゾジアゼピン系(ジアゼパム，アルプラゾラム，ミダゾラム)	・転倒(筋弛緩と平衡機能障害による) ・健忘，注意力低下	・代謝酵素(CYP3A4)阻害による血中濃度の上昇
抗うつ薬	・四環系抗うつ薬（ミアンセリン，マプロチリンなど)	・薬剤性鎮静	
経口血糖降下薬	スルホニル尿素薬	・低血糖による健忘，注意力低下	ビグアナイド系は老年者では使用しづらい。
甲状腺ホルモン	レボチロキシン	・狭心症	少量より投薬開始，狭心症症状発現時は半量に減量

(詳細は「日本老年医学会編：高齢者の安全な薬物療法ガイドライン2005」の各項目を参照)

表5 高齢者に頻用される薬物の主な相互作用

薬物分類	影響を受ける薬物	影響を及ぼす薬物	副作用	機構
強心配糖体	・ジゴキシン	・利尿薬（サイアザイド，ループ利尿薬），アムホテリシンB	・ジギタリス中毒	・低K血症による心筋の感受性増大
		・カルシウム拮抗薬（ニフェジピン，ジルチアゼム）	・ジギタリス中毒	・クリアランス低下
		・抗不整脈薬(キニジン，アミオダロン，プロパフェノン，ベラパミル)	・ジギタリス中毒	・クリアランス低下
降圧薬	・ACE阻害薬	・カリウム保持性利尿薬 ・インドメタシン	・高K血症	・アルドステロン減少によるK貯留増大
	・β遮断薬	・シメチジン，ベラパミル，ジルチアゼム，プロパフェノン	・作用減弱 ・降圧効果増強，徐脈	・PG合成作用に拮抗 ・代謝阻害
利尿薬	・サイアザイド系利尿薬	・非ステロイド系抗炎症薬	・作用減弱，消失	・腎尿細管におけるPGE産生抑制
抗凝固薬	・ワーファリン	・非ステロイド性抗炎症薬，ST合剤	・作用増強（出血）	・相加作用，蛋白結合置換
		・プロパフェノン，シメチジン，エリスロマイシン	・作用増強（出血）	・代謝阻害
		・クロフィブラート	・作用増強（出血）	・蛋白結合置換，ビタミンK代謝回転への影響
		・ビタミンK	・作用減弱	・ビタミンK依存性の凝固因子の合成促進
抗生物質	・アミノグルコシド	・ループ利尿薬	・腎毒性の増強	
	・キノロン系（エノキサシン）	・抗炎症薬（フェンブフェン）	・痙攣	・痙攣域値低下
	・テトラサイクリン系，キノロン系	・金属含有製剤(Al, Ca, Mg, Fe)	・作用減弱	・錯体形成し吸収阻害
	・ペニシリン，パラアミノサリチル酸	・プロベネシド	・作用増強	・尿細管分泌阻害
消炎鎮痛薬	・NSAID	・プロベネシド	・作用増強	・尿細管分泌阻害
睡眠薬	・ベンゾジアゼピン系(ジアゼパム，アルプラゾラム，ミダゾラム)	・シメチジン，オメプラゾール	・作用増強(転倒・骨折)	・代謝酵素（CYP3A4）阻害による血中濃度の上昇
躁病薬	・炭酸リチウム	・サイアザイド，NSAID，ACE阻害薬	・リチウム中毒	・近位尿細管再吸収促進
気管支拡張薬	・テオフィリン	・キノロン系抗菌薬，エリスロマイシン，シメチジン，メキシレチン	・作用増強による中毒症状（頭痛，嘔気，心悸亢進）	・代謝阻害
抗アレルギー薬	・アステミゾール	・アゾール系，エリスロマイシン系抗真菌薬	・心毒性	・代謝阻害による未変化体濃度上昇
経口血糖降下薬	・スルホニル尿素薬	・β遮断薬	・低血糖	・β2遮断
		・サリチル酸，クロフィブラート	・低血糖	・蛋白結合置換
		・シメチジン	・低血糖	・代謝阻害
脂質異常症治療薬	・スタチン	・フィブラート系薬物，ニコチン酸	・CPK上昇	
甲状腺ホルモン	・T4製剤	・コレスチラミン，コレスチポール	・作用減弱・消失	・吸着による吸収阻害
抗精神病薬	・メトクロプラミド	・塩酸チアプリド，スルピリド	・錐体外路症状	・D2レセプター阻害の相加作用

3》高齢者において注意すべき薬物副作用と薬物の相互作用，および医原性疾患・事故

生体の加齢変化を背景として，高齢者ではこれ以外の各種薬剤の使用時にも特別の注意が必要となる。老年期疾患の改善をめざして投与されたはずの薬剤は，不用意な投薬による副作用によりしばしば高齢者の日常生活動作(activity of daily living；ADL)や生活の質（quality of life；QOL）を低下させ，ときには医原性疾患・事故につながる(表4)。さらに高齢者ではしばしば多剤が投与されており，薬物相互作用による副作用をしばしば認め，若年者に比べ高齢者ではその出現頻度は高く，また重篤化することが知られている[3]（表5)。この結果，“寝たきり”や認知症を，さらには各種感染症，廃用症候群を引き起こしかねない(図7)。高齢者では運動能の低下，恒常性維持機能の低下，知的機能の低下，失禁と相まって，医原性の疾患・事故がADL・QOL低下への悪循環をつくりだす。

以下に高齢者に頻発する代表的な医原性疾患・事故を取り上げる。

3-a 転倒

転倒は高齢者，とくに高齢女性では，大腿骨頸部骨折，脊椎圧迫骨折につながり“寝たきり”の直接の原因となる。反復する転倒の原因となる薬剤として睡眠薬，降圧薬(とくに起立性低血圧の原因となる中枢性降圧薬，α_1遮断薬)，非ステロイド性消炎鎮痛薬(とくに坐薬で用いる場合)による血圧降下，抗不整脈薬(ベラパミル，ジルチアゼム，β遮断薬)による徐脈，経口血糖降下薬による低血糖などがあげられる。

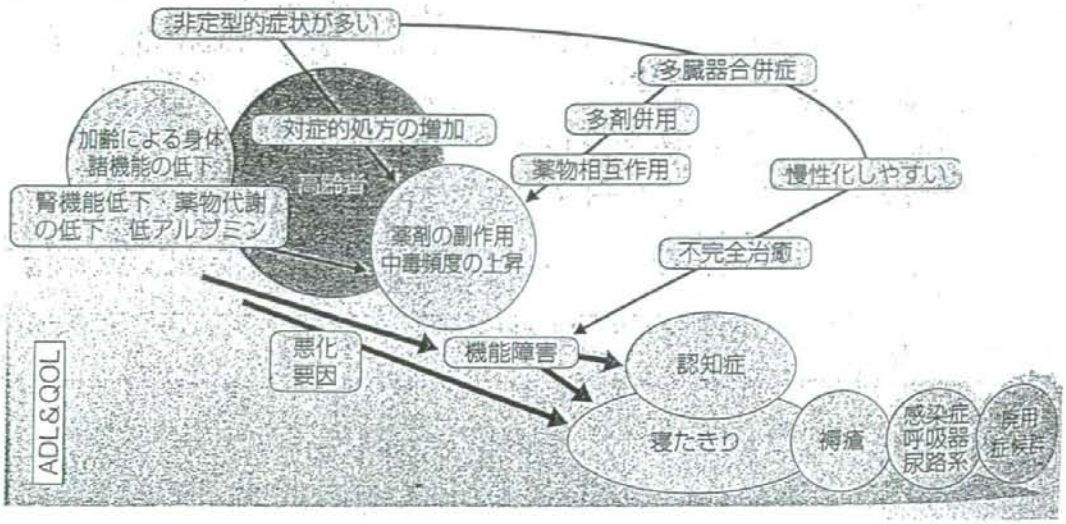
3-b 健忘症候群

医原性に健忘症状・注意力低下を引き起こす薬剤として睡眠薬，抗不安薬などの向精神薬，抗パーキンソン薬，抗甲状腺薬，経口血糖降下薬による低血糖などがあげられる。また，レセルピン，メチルドパなどでうつ状態を引き起こすことがある。

3-c 尿失禁

利尿薬，催眠薬などで尿失禁の原因となる。尿失禁はADL低下の要因であり，褥瘡発症の誘因となる。

図7 老年病の特徴と薬物療法



3-d 低栄養

非ステロイド性消炎鎮痛薬，ジゴキシン，その他の多くの薬剤の副作用として食欲不振は代表的なものであり，長期の漫然とした投薬は低栄養状態を引き起こす。

3-e 消化管出血・穿孔

非ステロイド性消炎鎮痛薬は高齢者においては高率に上部消化管出血を惹起する。これは高齢者においては消化管保護因子であるプロスタグランジン（とくにPGE1）の合成低下によるとされる。痛風薬であるプロベネシドの併用はこの副作用を増強させる。

3-f 腎機能障害

抗菌剤，とくにアミノグルコシドによる腎障害は高齢者において多発する。とくにループ利尿薬と併用した場合高率に腎障害を招来する。

3-g ジゴキシン中毒

高齢者では心不全の治療目的に強心配糖体の使用頻度が増えるが，使用頻度の高いジゴキシンは親水性薬物であり，加齢に伴う腎機能の低下を反映して，ジゴキシン中毒が増加する。とくに低カリウム血症時(利尿薬の併用など)，およびカルシウム拮抗薬・抗不整脈薬の併用時に頻発する。高齢者ではジゴキシン使用時には血中濃度のモニタリングが必要である。

3-h ワルファリンによる出血

ワルファリン使用による出血(皮下出血，血尿，鼻出血，消化管出血，頭蓋内出血など多様)も高齢者で増加する。シメチジン，トルブタミドなどの併用によりその作用は増強される。プロトロンビン時間によるモニタリングが必要である。

その他，種々の医原性疾患・事故が高齢者では増加する(表2～5)。高齢者に対する投薬の際にはこれらの事情をふまえて一層の慎重さが望まれる。

4 高齢者への処方の工夫

投薬の際，複雑な投与回数・方法は高齢者では対応できない。処方はなるべく少なく，服用方法は朝夕2回，あるいは朝1回などの簡単な処方(simple)を心がける。降圧薬など長期に服用しなければならない薬物では最近1日1回服用の持効剤により，副作用の軽減，コンプライアンスの向上に役立っている。また高齢者では通常投与量ではしばしば過剰投与となることから成人投与量の半量あるいは1/4量程度の少量投与(small)が必要である。効果がなければいたずらに増量せず，短期間(short)で中止し，別の薬に変更するなどの注意が必要である。高齢者では，多病性のゆえに重複診療，重複投与の危険性が増す。後期高齢者医療制度では在宅高齢者に対して受け持ち医師の一元的な投薬内容の管理が求められている。また高齢者においては本人により服薬管理ができない場合も多く，家族とともに，薬剤師，訪問看護師など多職種による服薬管理，副作用の早期発見などが期待されている。

（森本茂人）

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High-density association study and nomination of susceptibility genes for hypertension in the Japanese National Project

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Essential hypertension is one of the most common, complex diseases, of which considerable efforts have been made to unravel the pathophysiological mechanisms. Over the last decade, multiple genome-wide linkage analyses have been conducted using 300–900 microsatellite markers but no single study has yielded definitive evidence for 'principal' hypertension susceptibility gene(s). Here, we performed a three-tiered, high-density association study of hypertension, which has been recently made possible. For tier 1, we genotyped 80 795 SNPs distributed throughout the genome in 188 male hypertensive subjects and two general population control groups (752 subjects per group). For tier 2 (752 hypertensive and 752 normotensive subjects), we genotyped a panel of 2676 SNPs selected (odds ratio $\geq$ 1.4 and $P \leq 0.015$ in tier 1) and identified 75 SNPs that showed similar tendency of association in tier 1 and tier 2 samples ($P \leq 0.05$ for allele frequency and $P \leq 0.01$ for genotype distribution tests). For tier 3 (619 hypertensive and 1406 normotensive subjects), we genotyped the 75 SNPs and found nine SNPs from seven genomic loci to be associated with hypertension

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($P \leq 0.05$). In three of these loci, the lowest *P*-values were observed for rs3755351 ($P = 1.7 \times 10^{-5}$) in *ADD2*, rs3794260 ($P = 0.0001$) in *KIAA0789* and rs1805762 ($P = 0.0003$) in *M6PR* when case–control comparison was made in the combined data. An SNP (rs3755351) within *ADD2* had the lowest *P*-value and its experiment-wide significance level is 0.13. Thus, these results have nominated several susceptibility genes for hypertension, and independent replication will clarify their etiological relevance.

INTRODUCTION

Essential hypertension (MIM 145500) is a multifactorial trait, in which interactions among genetic, environmental and demographic factors are involved. Substantial contribution of genetic factors to the overall disease etiology has been documented by a number of epidemiological studies. For example, family studies controlling for a common environment indicate that blood pressure heritability is in the range of 15–35% (1–3). Accordingly, considerable efforts have been made in the study of molecular genetics of hypertension, but the inherently complex nature has hampered progress in the elucidation of the genes involved (4). Over the last decade, multiple genome-wide linkage analyses have been conducted by using microsatellite markers to localize genes influencing hypertension status and/or blood pressure levels in a number of populations derived from various ethnic groups. Although no single study has so far yielded definitive evidence for 'principal' hypertension susceptibility gene(s), some of these studies provide consistency of linkage results in a few chromosomal regions (5–7). It is therefore assumed that multiple genes contribute to the etiology of hypertension independently or synergistically, with each gene exerting small effects under a certain environmental condition.

In parallel with family-based linkage analyses across the entire genome, population-based association studies have been performed, particularly focusing on individual candidate genes to search for genetic influences on hypertension. Association studies for mapping disease-related genes have recently gained popularity over traditional family-based linkage analyses mainly because of their far greater statistical power to detect the presence of genes with relatively 'minor' effects (8,9). Some researchers criticize the liability to false-positive or non-replicable claims. Nevertheless, population-based association studies have become an alternative and complementary approach to family-based linkage analyses in practice.

Given the limitation of statistical power that can be achieved by family-based linkage analyses with sample size practically collectable, population-based association studies are now underway in a genome-wide scale for a number of multifactorial diseases (10). Here, we performed a high-density association study of hypertension with a three-tiered genotyping approach in the Japanese population (Fig. 1).

RESULTS

Multi-tiered case–control study

We performed a large-scale case–control association study of hypertension using SNP markers selected from the Japanese SNP (JSNP) database (11,12). These SNP markers were distributed throughout the genome (Table 1). Only male hypertensive individuals were tested in tier 1, and a total of 80 795 SNPs distributed on 22 autosomes were used for the association study. Details of the high-throughput genotyping were same as previously described (13,14), and technical evaluation of our genotyping assay (e.g. overall success rate and accuracy of the genotyping assay) is shown in the supplementary material (Supplementary Explanation). JSNP had been developed as a database for the SNP discovery project with particular focus on common gene variations in the Japanese population. Although SNP marker resources used in the current study showed a certain degree of diversity in terms of the number of typed SNPs per gene locus, this partially reflected the variable size of re-sequenced fragments depending on the individual gene structure (12).

The gene-centered genome-wide exploratory test in tier 1 identified 2676 SNPs with odds ratio (OR) ≥ 1.4 and $P \leq 0.015$ in at least one test comparing allele frequency and/or genotype distribution (dominant or recessive models) between 188 hypertensive patients and 752 population control subjects in either of two panels (see Materials and Methods). In this exploratory test, the SNPs showing inverted tendency of OR between two pairs of case–control comparisons and significant deviations from Hardy–Weinberg equilibrium (HWE) in any panel ($P \leq 0.01$) were excluded. Subsequently, we performed a screening of these 2676 SNPs with 752 hypertensive patients and 752 normotensive controls in tier 2, which constituted the first 'case versus unaffected control' study panel, i.e. comparison between 940 cases and 752 controls, together with the 188 cases in tier 1. On the basis of relatively stringent criteria, we identified 75 SNPs that showed *P*-values of ≤ 0.01 for genotype distribution and *P*-values of ≤ 0.05 for allele frequency in the χ^2-test statistic. To further examine the association signals, we performed a replication study of these 75 SNPs with another panel of 619 hypertensive subjects and 1406 normotensive controls in tier 3. Cases and unaffected controls collected in tiers 2 and 3 were enrolled according to the identical criteria and their baseline characteristics are shown in Table 2. There were some trait differences in cases between tiers 2 and 3, such as blood pressure measurements and percentages of the subjects taking anti-hypertensive medication. This could be largely attributed to differences in sample enrollment settings between tiers 2 and 3; that is, cases in tier 3 were enrolled from either the annual medical checkup of a medical institution or the clinic practices of general practitioners, whereas a major part of cases in tier 2 were from the clinic practices of university hospitals. Among the 75 SNPs showing *P*-values between 0.05 and 4.4×10^{-5} in the first 'case versus unaffected control' study, only nine SNPs showed borderline association (at the level of $P \leq 0.05$) in

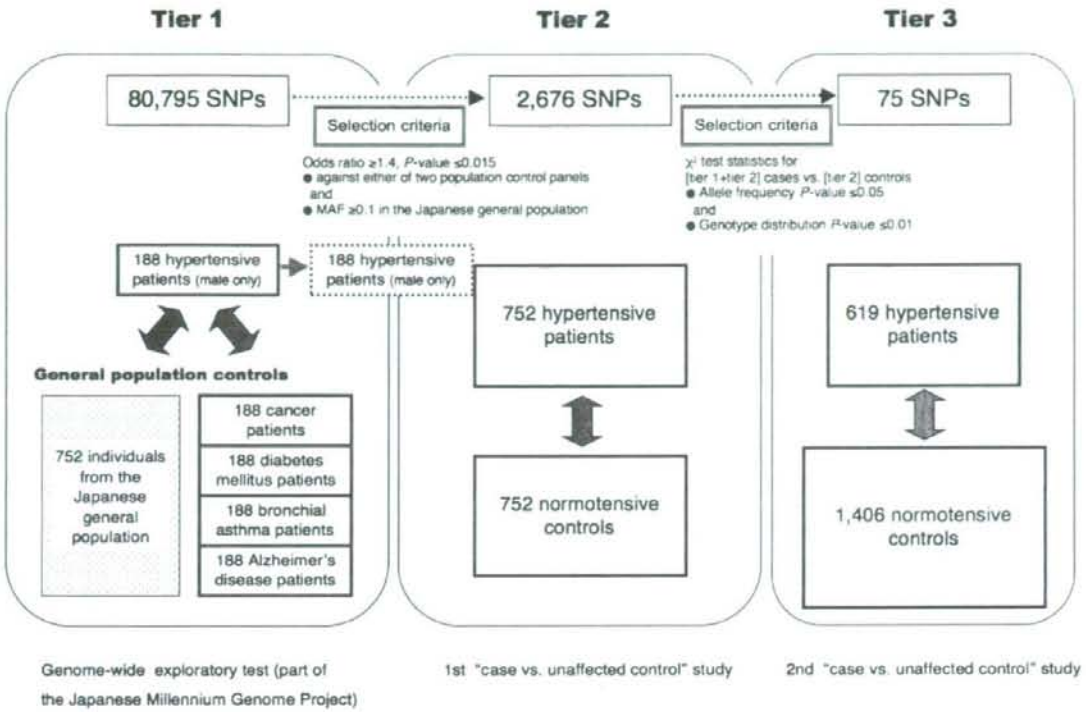

Figure 1. Schematic presentation of a three-tiered screening strategy in the present study. Gene-centered genome-wide exploratory test was performed in tier 1, followed by case–control study of disease-associated SNPs in tier 2 and tier 3 samples. A panel of 188 male hypertensive patients were compared with each of two population control panels in tier 1. Subsequently, 'case versus unaffected control' study was repeated twice to identify the best candidate SNPs. In transitions from tier 1 to tier 2, and from tier 2 to tier 3, the number of SNPs was reduced according to the selection criteria that we arbitrarily defined. See details in the Materials and Methods section.

the second 'case versus unaffected control' study (Fig. 2). Of these, we found six SNPs that showed *P*-values of ≤ 0.05 for both genotype distribution and allele frequency in the χ^2-test statistic (Table 3). rs3755351 and rs3771426 were located within the assumed intron 1 of *ADD2*, rs3787240 and rs3787241 were located in the same intron of *EYA2*, and the remaining two SNPs—rs3794260 and rs1805762—each located in *KIAA0789* and *M6PR*. To adjust for three covariates—age, gender and body mass index (BMI), we also performed logistic regression analysis for the significant SNPs (Supplementary Material, Table S1). With consideration of genetic model consistency, an SNP (rs3755351) showed the strongest association in the identical model (an additive model by logistic regression analysis) among three tiers. Further details of the association results are described in the Discussion.

SNP discovery and further test of association in three selected genes

Because a group of SNPs from three genes, *ADD2*, *KIAA0789* and *M6PR*, were particularly noted for their significant association with hypertension (Table 3), we searched for potentially functional SNPs by re-sequencing the 5′- and 3′-untranslated regions, all exons and exon–intron borders of the individual loci, on the basis of the gene structure deposited in the human genome database (http://www.ncbi.nlm.nih.gov/). We detected a total of 74 SNPs—25 SNPs in *ADD2*, 40 SNPs in *KIAA0789* and 9 SNPs in *M6PR*—and thereby selected 25 tag SNPs for genotyping 2025 subjects in tier 3 (see Supplementary Material, Table S2A, B and C). Apart from four SNPs which had been already included in the JSNP screening marker set, we found four additional SNPs, two in *ADD2* (rs2024453 and rs10084293) and one each in *KIAA0789* (rs9739493) and *M6PR* (rs1805725), to be significantly associated with hypertension (Table 3). Thus, in each gene, we identified at least two SNPs showing modest evidence of association with hypertension ($P \leq 0.05$ level in tier 3) but these SNPs did not necessarily belong to the same linkage disequilibrium (LD) block (Fig. 3 and LD group in Supplementary Material, Table S2A, B and C). The analysis of haplotypes inferable from tag SNPs did not show more significant disease association than the analysis of individual SNPs in any of three genes tested (data not shown).

Consideration of study power and multiple testing

We first estimated a type I error probability for the three-tiered screening to be 6.8×10^{-5}: 0.036 for tier 1, 0.0009 for tiers 1

Table 1. Summary of SNPs genotyped in tier-1 screening and genome coverage estimated by HapMap data

Chromosome	From JSNP screening markers		JSNP overlapped with HapMap (overlap)	From HapMap data (Release 21, JPT)			
	Total SNPs in JSNP	Proportion of SNPs unique to JSNP		SNPs in close LD ($r^2 \geq 0.8$) with overlap	(HapMap total−NA) SNPs	Coverage estimate: SNPs in LD ($r^2 \geq 0.8$)/(HapMap total−NA) SNPs	Total SNPs in HapMap
1	8378	0.370	5281	26 236	113 362	0.231	139 002
2	7336	0.293	5189	28 763	123 447	0.233	160 546
3	5128	0.358	3290	17 494	91 985	0.190	125 160
4	3172	0.366	2010	11 998	74 080	0.162	114 809
5	4973	0.311	3427	18 432	91 206	0.202	122 243
6	6220	0.272	4527	26 182	110 532	0.237	134 177
7	5813	0.358	3731	18 241	81 727	0.223	99 808
8	2388	0.246	1800	12 644	80 400	0.157	111 953
9	2818	0.218	2203	12 358	70 091	0.176	91 908
10	3159	0.322	2141	13 552	81 103	0.167	100 771
11	3636	0.248	2735	14 953	75 147	0.199	95 905
12	3816	0.223	2964	15 188	73 983	0.205	89 436
13	1291	0.290	917	6717	48 622	0.138	75 956
14	2913	0.219	2275	11 583	50 769	0.228	62 203
15	2311	0.194	1863	9903	46 599	0.213	54 210
16	2677	0.268	1959	8227	43 415	0.189	51 865
17	3246	0.258	2408	10 050	38 550	0.261	41 725
18	1243	0.207	986	6370	41 494	0.154	56 203
19	3392	0.308	2346	7626	25 524	0.299	26 949
20	2588	0.402	1548	8784	41 725	0.211	45 582
21	1761	0.275	1276	5777	23 465	0.246	26 892
22	2536	0.280	1825	7569	24 402	0.310	25 077
Total	80 795	0.298	56 701	298 647	1 451 628	0.206	1 852 380

The numbers of SNPs genotyped in tier-1 screening are demonstrated for each chromosome. Genome coverage was assessed with the HapMap data from JPT ($n = 45$); that is, the proportion of HapMap SNPs showing high r^2 (≥ 0.8) to one of the SNPs genotyped in this study (which are all derived from JSNP) is calculated. Because substantial part of the SNPs have turned out to be unique to JSNP, those overlapping with the HapMap SNPs, in the 'overlap' column, are used to estimate genome coverage. Here, NA represents a category of SNPs which have been mapped to the genome (NCBI B35) but do not have LD information against the HapMap SNPs. In this context, it is appropriate to reduce this NA SNPs from total SNPs deposited in the HapMap data when estimating genome coverage and we therefore use the number of SNPs (HapMap total−NA) as a denominator.

and 2 combined and 0.076 for tier 3 screening. Then, we estimated overall sensitivities (which could represent the statistical power) to be 0.10–0.45, 0.04–0.23 and 0.01–0.08 for a disease-associated SNP of OR = 1.4, 1.3 and 1.2, respectively, assuming the disease allele frequency within 0.1–0.9, the disease prevalence of 0.25 and the multiplicative genotype model. Since we had adopted relatively generous criteria for screening association signals, we evaluated the false discovery rate (FDR) to account for multiple testing (15). FDR for the nine SNPs found as significant was 0.69. A multi-staged screening in the current study could be largely categorized into two steps: tiers 1 and 2 (which constitute the first 'case versus unaffected control' study) and tier 3 (which constitutes the second 'case versus unaffected control' study). We therefore assessed experiment-wise type I errors with particular focus on the last-stage screening in tier 3. By permutation, the chance of observing a *P*-value of 0.0019 (for allele frequency test at rs3755351 in *ADD2*) in tier 3 was estimated to be 0.13.

DISCUSSION

With the recent advent of high-throughput genotyping technologies and high-resolution maps of SNP markers, it is expected that genome-wide association studies allow us to identify systematically the contributions of common genetic variations to human multifactorial diseases (16–18). In this line, our study has attempted to discover common hypertension susceptibility gene variants via a gene-centered genome-wide association design for the first time. Despite the modest genetic impacts assumed for hypertension, e.g. the λ-values (the relative risk for siblings of the affected probands) have been reported to be approximately 4 (19), we have nominated several susceptibility genes for hypertension (Table 3). Among these genes, findings for *ADD2* and *KIAA0789* are particularly noteworthy, because the former has been known to be a physiological candidate gene for hypertension and the latter is a novel gene with as-yet unknown physiological function.

Through a multi-tiered screening, nine SNPs derived from seven distinct gene loci have remained to show some evidence of association out of the 80 795 SNPs initially screened. Although the selection criteria were arbitrarily defined in the present study, a small percentage of the SNPs have passed the criteria in transitions from tier 1 to tier 2 (3.3%) and from tier 2 to tier 3 (2.8%). In the *ADD2* gene, for example, the minor allele frequency (MAF) of rs3755351 is lower in case groups (0.14–0.19) than that in control groups (0.21–0.22) throughout three tiers. A *P*-value of 1.7×10^{-5} and an OR of 1.30 (95% CI 1.15–1.46) are attained for allele frequency comparison of rs3755351 when the subjects studied in different tiers are combined and finally categorized into

Table 2. Clinical characteristics of participants

Variables	Case group Tier 2 panel	Tier 3 panel	Control group Tier 2 panel	Tier 3 panel
Number of subjects (female/male)	752 (353/399)	619 (280/339)	752 (366/386)	1406 (650/756)
Present age, year	62.4 ± 10.3	54.1 ± 8.4[a]	62.0 ± 8.7	58.4 ± 6.6
Age of onset, year	47.3 ± 10.2	43.2 ± 9.9	—	—
Current BMI, kg/m^2	23.9 ± 3.2	25.1 ± 3.6[a]	22.5 ± 2.8	22.4 ± 2.7
Smoking[b]				
None, %	48.6	61.6	66.0	58.6
Previous smoker, %	—	17.0	—	10.2
Current smoker, %	51.4	21.4	34.0	31.2
Blood pressure				
Systolic blood pressure, mmHg	146.4 ± 19.5[a]	150.9 ± 19.3[a]	113.8 ± 9.8	114.4 ± 10.1
Diastolic blood pressure, mmHg	86.4 ± 13.0[a]	91.4 ± 12.2[a]	69.8 ± 7.7	70.3 ± 7.2
Treatment of hypertension, %	92.6	75.4	—	—
Blood chemistry				
Serum creatinine, mg/dl	0.87 ± 0.69[a]	0.75 ± 0.50[c]	0.73 ± 0.18	0.70 ± 0.23
Fasting plasma glucose, mg/dl	105.3 ± 28.7	109.0 ± 31.0[a]	104.0 ± 41.9	99.2 ± 22.7
Serum total cholesterol, mg/dl	204.4 ± 31.1[c]	213.4 ± 33.8	209.2 ± 33.7	215.6 ± 34.1
Serum triglyceride, mg/dl	129.8 ± 82.2[a]	141.3 ± 124.6[a]	108.1 ± 67.1	110.1 ± 71.6
Serum HDL cholesterol, mg/dl	56.2 ± 16.7[a]	61.9 ± 19.5	60.6 ± 16.0	63.5 ± 17.5

Values are means ± SD.
For some variables, subjects with insufficient information are not included in the calculation.
[a]$P < 0.001$, case group versus control group by the unpaired *t*-test in each tier.
[b]Because of differences in the questionnaire, smoking status is categorized into two groups (non-smoker or smoker) in the tier 2 panel.
[c]$P < 0.01$, case group versus control group by the unpaired *t*-test in each tier.

the case (tiers 1–3) and unaffected control (tiers 2 and 3) groups. None of our results appears to be significant with the use of a strict Bonferroni correction, a very conservative evaluation of significance, and further replication in an independent population is indispensable.

The candidacy of *ADD2* as a hypertension susceptibility gene has been supported by several physiological and biochemical findings (20–22), together with some evidence from the studies of molecular genetics (23–27). Adducin is a ubiquitously expressed membrane-skeleton heteromeric protein composed of different subunits, α-, β- and γ-subunits. It is known to play a substantial role in the regulation of membrane ion transport. Point mutations of the α- and β-adducins account for up to 50% of the blood pressure difference between Milan hypertensive and normotensive rat strains, probably via the modulation of the Na^+–K^+ ATPase activity (one of major Na^+-channels) in the kidney (23,24). In this line, of note is the fact that β-adducin-deficient mice show significant increases in systolic and diastolic blood pressures and pulse pressure (21). The human homolog of β-adducin spans over 100 kb on chromosome 2p13 and comprises 17 exons. It has been reported that a common SNP (rs4984) identified at position 1797 in exon15 is associated with an increased risk of hypertension under certain pathological conditions in European populations (25–27), whereas this SNP itself is not polymorphic in Asian populations (http://www.ncbi.nlm.nih.gov/SNP/). Also, it has to be noted that one previous study (28) showed significant evidence for hypertension linkage in the 2p13 region (a peak of 2.84 LOD at 93 cM), where the *ADD2* locus is exactly located among several positional candidate genes. Despite our investigation in the *ADD2* locus, we could not find either a clear LD block-like structure or potentially functional SNPs in the vicinity of three disease-associated SNPs (rs2024453, rs3755351 and rs3771426), which are located in the putative promoter region and intron 1, apart from rs10084293 located within an LD block of *ADD2* (Fig. 3). We have assessed the independence of multiple associated SNPs in *ADD2* by logistic regression analysis and have found that the observed association in this gene could be explained principally by the most significant SNP (rs3755351) (see Supplementary Explanation). Once these associations are validated in an independent study panel, further extensive searches of functional SNPs in the *ADD2* locus are warranted.

Our high-density association study has also highlighted the *KIAA0789* gene located on chromosome 12q23.3. This gene encodes a hypothetical protein, LOC9671, which is expressed principally in the central nervous system and modestly in the pancreas (unpublished data). The predicted gene structure of *KIAA0789* involves 9 exons, spanning ~120 kb. There is a clear LD block in the 5′ region of the putative exon 1 (~3.8 kb in size), whereas we have found two other LD block-like structures within the *KIAA0789* gene (Fig. 3). Two disease-associated SNPs (rs3794260 and rs9739493) have turned out to reside in different LD blocks, and the construction of their haplotypes does not seem to provide much additional information on disease association. Although the precise gene structure and gene function remain unknown, *KIAA0789* appears to contain a carboxy-binding WSC domain, and its homologs are likely to exist in mice and rats according to the database information (http://www.ncbi.nlm.nih.gov/). Again, detailed investigation including independent

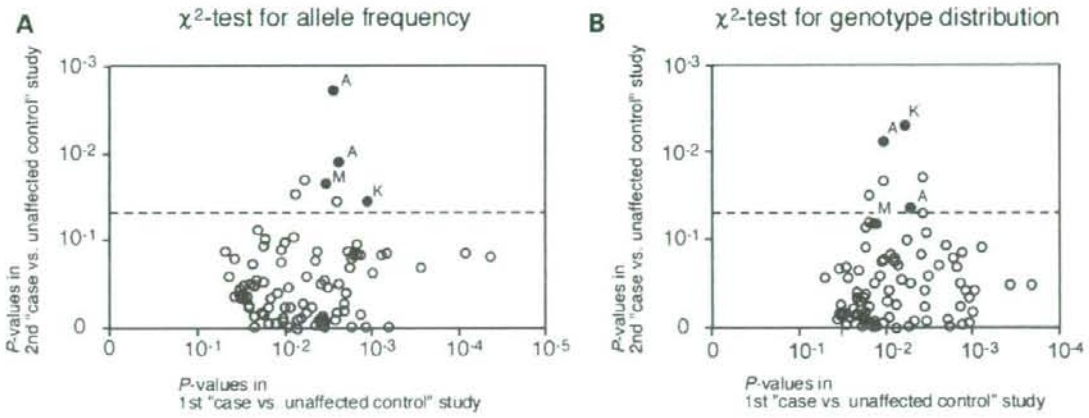

Figure 2. Statistical significance of χ^2-test of disease association in the two-staged 'case versus unaffected control' study. $-\mathrm{Log}_{10}$ *P*-values were used instead of raw *P*-values in each scatter plot. The dashed lines indicate $P = 0.05$. (**A**) As for SNPs genotyped in the second 'case versus unaffected control' panel, *P*-values for allele frequency in the second 'case versus unaffected control' panel were plotted against those in the first 'case versus unaffected control' panel, where SNPs located in three gene loci—*ADD2*, *KIAA0789* and *M6PR*—are depicted with solid circles to which the following symbols are attached: A, *ADD2*; K, *KIAA0789*; M, *M6PR*. (**B**) As for SNPs genotyped in the second 'case versus unaffected control' panel, *P*-values for genotype distribution ([2 × 3] contingency table) in the second 'case versus unaffected control' panel were plotted against those in the first 'case versus unaffected control' panel, where SNPs located in three gene loci—*ADD2*, *KIAA0789* and *M6PR*—are depicted with solid circles as mentioned earlier.

replication of disease association will lead us to clarify the etiological relevance of *KIAA0789* to hypertension.

Another, potential disease association, though modest statistical significance, has been found for *M6PR*. The *M6PR* gene encodes a cation-dependent receptor for mannose-6-phosphate groups on lysosomal enzymes and plays a critical role in the segregation and targeting of lysosomal enzymes to lysosomes. Thus far, no functional relation between *M6PR* and hypertension has been reported. Similar to *KIAA0789*, this gene could also allow us to identify a novel, as-yet unnoticed blood pressure regulatory mechanism.

We should bear in mind several limitations inherent in the present study. First, the level of genome coverage is an issue of heated debate (10,18). Some people may argue that our *a priori* marker selection strategy is gene-centric without utilizing LD information and hence it is not sufficient to pick up as many modest associations as possible in genome-wide searches of hypertension susceptibility genes. A comprehensive framework of common variations throughout the human genome has been made available by the recent completion of the International HapMap Project (29). On the basis of our assessment, the JSNP screening markers in this study cover 20.6% of the HapMap SNPs, whereas a substantial proportion (~30%) of SNPs appear to be unique to JSNP (Table 1 and Supplementary Material, Fig. S1). Under these circumstances, an ideal set of SNPs for our study would encompass deliberately selected tag SNPs (principally common genetic variants) and additional 'singleton' SNPs (sometimes rare genetic variants). Besides this argument of tag SNPs, there are two points of weakness regarding genome coverage as follows: (i) sex chromosome markers have been excluded from the analysis because of the pre-determined policy of multi-disease collaborative study in the Japanese Millennium Genome Project, and (ii) a substantial part of the expressed human genes is not covered by the JSNP database (11), in which the fundamental SNP data were almost fixed in the middle of 2003. Second, the statistical power attainable by our study panel needs to be taken into consideration. For the last few years, genotype costs have fallen dramatically, yet present economic and experimental conditions make it necessary, in practice, to reduce the number of genotyped samples down to a moderately sized case group (188 subjects in our study) at the initial screening with approximately 80 000 SNPs. We arbitrarily set the selection criteria of $\mathrm{OR} \geq 1.4$ and $P \leq 0.015$ in transition from tier 1 to tier 2, where the overall statistical power is estimated to be 10–45% for a disease-associated SNP of $\mathrm{OR} = 1.4$ and 1–8% for that of $\mathrm{OR} = 1.2$, assuming the disease allele frequency within 0.1–0.9 and the disease prevalence of 0.25. Thus, it is likely that our study design allows for capturing less than half of the true disease associations particularly with regard to modest genetic susceptibility. Third, ethnic diversity has not been tested within the scope of the present study. Instead of using commercially available SNP sets aimed at full genomic coverage, we have attempted to focus on potentially functional variants and also relatively common SNPs ($\mathrm{MAF} \geq 0.1$) in the Japanese population. Accordingly, some of disease-associated SNPs listed in Table 3 may be rare or not polymorphic in the other ethnic groups. To clarify allele frequency representation of individual loci and etiological impacts attributable to them, further examination is required in the context of ethnic diversity.

During our preparation of this report, two genome-wide association studies for hypertension and/or blood pressure have been performed in Caucasians (30,31). When our results are compared with public data sets for these association statistics, a few SNPs in the regions of interest appear to show a tendency of association with hypertension or blood pressure;

Table 3. Summary of genomic SNPs associated with hypertension status in two-staged 'case versus unaffected control' study

dbSNP number	Gene symbol	Major/minor allele	Orientation	Minor allele frequency						Association analysis												
				Case			Control			*P*-value in the first 'case (tiers 1 and 2) versus unaffected control (tier 3)' study				*P*-value in the second 'case (tier 3) versus unaffected control (tier 3)' study				Total subjects: case (tiers 1–3) versus unaffected control (tiers 2 and 3)				
				Tier 1	Tier 2	Tier 3	Tier 1[a] (Ref. 1/ Ref. 2)	Tier 2	Tier 3	[2 × 3] Contingency	Dominant	Recessive	Allele	[2 × 3] Contingency	Dominant	Recessive	Allele	[2 × 3] Contingency *P*-value	Dominant *P*-value	Recessive *P*-value	Allele frequency model *P*-value	OR (95% CI)[b]
rs3755351	ADD2	C/A	fwd	0.14	0.19	0.17	(0.22/0.21)	0.22	0.21	0.011	0.041	0.007	0.003	0.007	0.071	0.003	0.002	0.00099	0.009	0.00006	0.00002	1.30 (1.15–1.46)
rs3771426	ADD2	T/C	fwd	0.13	0.16	0.16	(0.19/NA)	0.2	0.19	0.005	0.066	0.016	0.005	0.043	0.093	0.025	0.012	0.0003	0.003	0.0007	0.00008	1.28 (1.13–1.45)
rs2024453	ADD2	T/C	rev	—	—	0.29	—	—	0.33	—	—	—	—	0.034	0.013	0.132	0.023	—	—	—	—	—
rs10084293	ADD2	G/A	rev	—	—	0.44	—	—	0.48	—	—	—	—	0.089	0.083	0.063	0.027	—	—	—	—	—
rs3794260	KIAA0789	G/A	fwd	0.14	0.17	0.17	(0.21/0.18)	0.21	0.20	0.006	0.161	0.002	0.001	0.005	0.001	0.256	0.035	0.0008	0.006	0.001	0.0001	1.26 (1.12–1.42)
rs9734491	KIAA0789	T/C	fwd	—	—	0.41	—	—	0.44	—	—	—	—	0.026	0.008	0.641	0.041	—	—	—	—	—
rs1805762	M6PR	C/G	rev	0.23	0.22	0.22	(0.25/0.24)	0.26	0.25	0.015	0.045	0.009	0.003	0.061	0.332	0.019	0.022	0.001	0.615	0.0003	0.0003	1.23 (1.10–1.37)
rs1805725	M6PR	T/G	fwd	—	—	0.54	—	—	0.49	—	—	—	—	0.007	0.002	0.123	0.005	—	—	—	—	—
rs3787240	EYA2	C/T	fwd	0.25	0.21	0.22	(0.19/0.18)	0.18	0.19	0.003	0.001	0.059	0.008	0.083	0.227	0.034	0.028	0.001	0.002	0.007	0.0010	0.82 (0.73–0.92)
rs3787241	EYA2	G/A	fwd	0.24	0.21	0.22	(0.19/0.19)	0.18	0.19	0.008	0.003	0.080	0.014	0.104	0.267	0.042	0.037	0.002	0.003	0.01	0.0017	0.83 (0.74–0.93)
rs3761987	—	T/A	fwd	0.43	0.37	0.37	(0.36/0.36)	0.33	0.34	0.004	0.001	0.101	0.004	0.019	0.566	0.015	0.134	0.0007	0.031	0.004	0.0016	0.86 (0.78–0.94)
rs3741691	THAP2	A/C	fwd	0.26	0.22	0.22	(0.21/0.20)	0.19	0.21	0.011	0.043	0.007	0.002	0.021	0.139	0.066	0.117	0.007	0.727	0.002	0.0064	0.85 (0.76–0.96)
rs1298463	CCDC131	A/G	fwd	0.26	0.22	0.22	(0.21/0.19)	0.19	0.21	0.016	0.032	0.010	0.004	0.033	0.182	0.073	0.312	0.012	0.736	0.0033	0.0090	0.86 (0.77–0.96)

Two SNPs of EYA2, rs3787240 and rs3787241, are located closely (only 296 bp apart) and in complete LD ($r^2 = 1.00$) to each other.

Also, rs3741691 and rs1298463 are located closely (44 kb apart) and have turned out to be in strong LD ($r^2 = 0.99–1.00$) to each other.

[a]In tier 1 control subjects, the figures in parentheses are minor allele frequencies (MAFs) calculated separately in the Ref. 1 panel—the other disease patients who can be regarded as arbitrary general controls; Ref. 2 panel—752 individuals from the Japanese general population (see Materials and Methods).

[b]The OR was calculated as the ratio of the odds of disease in chromosomes with major alleles relative to those without them.

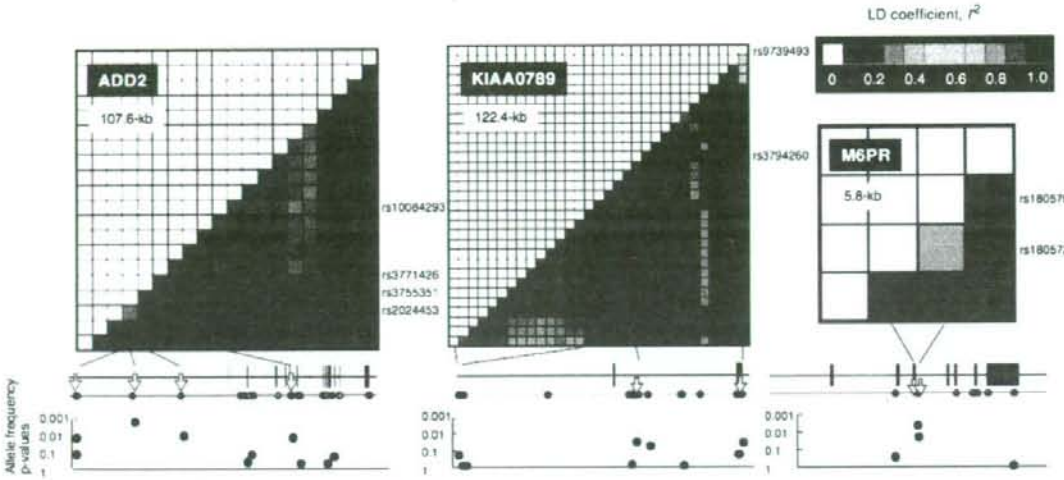

Figure 3. LD relations between SNPs in the *ADD2*, *KIAA0789* and *M6PR* genes (top) and disease association of markers from the corresponding genomic regions (bottom). In the top, the LD between a pair of markers is indicated by the color of the block above and to the left of the intersection of the markers. For the sake of readability, only the names of SNPs showing significant association are shown to the right of the vertical axis of the LD plot. The rest of the SNP information is described in Supplementary Material, Table S2A, B and C. In the upper bottom, the location of genetic markers studied in the corresponding genomic region is shown with relation to gene structure. Here, green and red circles indicate the SNPs with low (MAF < 0.05) and high (MAF ≥ 0.05) allele frequencies in the Japanese, respectively. In the lower bottom, $-\log_{10}$ *P*-values of the differences in allele frequencies between case and control subjects in tier 3 (i.e. the second 'case versus unaffected control' study) were plotted against the location of individual genetic markers genotyped.

for example, an SNP (rs17006246) in *ADD2*, which is in strong LD ($r^2 = 0.806$ and $D' = 1$ in the HapMap JPT population) with rs3755351, the most significant SNP in our study, is modestly associated with hypertension status ($P = 0.029$) in the Diabetes Genetics Initiative (DGI) study but the direction of effect is opposite between rs3755351 typed in this study and rs17006246 typed in the DGI study. On the other hand, rs1805740, in strong LD with an SNP (rs1805762) in *M6PR*, is modestly associated with hypertension status ($P = 0.036$) in the Wellcome Trust Case Control Consortium study with the same direction of effect as in this study (see Supplementary Material, Table S3).

In summary, our high-density association study provides a list of gene loci potentially predisposing people to hypertension, which awaits replication across populations. With the available samples, we have observed an association of SNPs including three SNPs clusters (or gene loci) in the Japanese populations. In face of the complex nature of disease etiology, it seems to be a formidable task but worth challenging that we eventually apply the SNPs information to improved prevention, diagnosis and treatment of hypertension.

MATERIALS AND METHODS

Study design

We performed a large-scale association study for genes susceptible to hypertension by using a three-tiered genotyping approach (tiers 1, 2 and 3) as depicted in Figure 1. All methods of the study were approved by the review committees of the individual institutions involved in the present study. All subjects provided written informed consent for participation.

In the gene-centered genome-wide exploratory test in tier 1, we carried out genotyping of 83 802 SNPs (3007 of which were excluded from the analysis because they are on sex chromosomes or in the unknown locations) using genomic DNAs from 188 Japanese male hypertensive patients and 752 unrelated Japanese individuals (referred to as general population controls) and another panel of 752 Japanese subjects (referred to as arbitrarily defined controls) who were affected with any of the other four common diseases including gastric cancer, diabetes mellitus, bronchial asthma and Alzheimer's disease; each of these was investigated as the 'Japanese Millennium Genome Project' (Fig. 1). The theoretical basis of adopting this exploratory test scheme was previously reported elsewhere (32). Cases were enrolled from the clinical practice or the annual medical checkup of university hospitals and medical institutions according to the uniformly defined criteria. These included (i) systolic blood pressure ≥ 160 mmHg, diastolic blood pressure ≥ 95 mmHg, or both on two consecutive visits for untreated subjects; (ii) patients receiving long-term antihypertensive treatments; (iii) no secondary form of hypertension as evaluated by an extensive workup; (iv) family history of hypertension, i.e. at least one hypertensive subjects detectable among parents and siblings of the participants; (v) an age of onset known to be between 30 and 59 years. Moreover, only male subjects with BMI < 25 kg/m^2 were selected in tier 1. We compared allele frequencies and/or genotype distributions in hypertensive patients and two population control panels and evaluated deviation from HWE at each of the genotyped loci. For the subsequent screening

in tier 2, we selected SNPs (i) with OR $\geq$ 1.4 and $P \leq 0.015$ against either of two population control panels and with concordant OR tendency against two control panels; (ii) with MAF $\geq$ 0.1 and (iii) not showing significant deviations ($P = 0.01$ level) from Hardy–Weinberg expectations in the patient or control panels.

In tier 2 (which comprised 752 hypertensive patients and 752 normotensive controls), we further tested the SNPs thus screened in tier 1, which effectively constituted the first 'case (tiers 1 and 2) versus unaffected control (tier 2)' study. Here, cases in tier 2 were selected according to the criteria (i)–(v) mentioned earlier for tier 1. Normotensive controls, on the other hand, were defined as follows: (i) systolic blood pressure $\leq$130 mmHg and diastolic blood pressure $\leq$85 mmHg without receiving antihypertensive treatments; (ii) age $\geq$50 years and (iii) no family history of hypertension. Both males and females were included in tier 2 without reference to BMI. We selected SNPs (i) with P-value $\leq$0.05 when comparing allele frequency; and (ii) with P-value $\leq$0.01 when comparing genotype distribution between (tiers 1 and 2) cases and (tier 2) controls by χ^2 test statistics.

In tier 3 (which comprised 619 hypertensive patients and 1406 normotensive controls), we performed the second 'case versus unaffected control' study to examine significant associations observed in tiers 1 and 2. The diagnostic criteria in tier 3 were identical to those in tier 2. For the assessment of assumptions when using statistical models in the present study, quantile–quantile plots of P-values were depicted for each stage of association test described in Supplementary Explanation.

No significant population stratification was observed for samples in tier 1 when it was assessed with the methods reported by Patterson *et al.* (33). However, the presence of population stratification was indicated for samples in the first stage 'case (tiers 1 and 2) versus unaffected control' study. We observed moderate bias in genotype frequency of some SNPs between the two tiers, which may have resulted from technical/experimental artifacts between genotyping of cases in tiers 1 and 2. Therefore, the trend test statistic at this analytical stage was corrected according to the significant eigenvector (see Supplementary Explanation). Stratification in tier 3 was not detected but could not be ruled out because of the relatively small number of SNPs ($n = 75$) genotyped in tier 3. As for the nine SNPs that showed significant disease association after multi-stage screening, they were not correlated with the significant eigenvector detected in tiers 1 and 2 cases and tier 2 controls. The P-values for nine SNPs were similar between the nominal and the EIGENSTRAT-corrected ones; for example, the nominal P-value was 0.0029 and the EIGENSTRAT-corrected P-value was 0.0069 at rs3755351 in *ADD2*.

SNP marker resource and genotyping

Most of the SNP markers used in the present study were same as the markers used in the previous reports (14) and derived from the JSNP database. The samples in tiers 1 and 2 were genotyped by PCR amplification of multiple genomic fragments with 20 ng of genomic DNA followed by characterization with the invader assay. Genotyping of the samples in tier 3 was undertaken using the TaqMan® SNP Genotyping Assays (Applied Biosystems). To secure the accuracy and completeness of genotyping, which is critical for large-scale studies (34), we attached a set of 'flags' to individual SNP data mainly dependent on the data completeness, after two independent investigators had checked the raw data robustness by looking at the scatter plot of the assay.

SNP discovery in the selected genes

Approximately 38 kb of genomic sequence spanning the exons and the 5′- and 3′-untranslated regions of three genes, *ADD2*, *KIAA0789* and *M6PR*, was re-sequenced in 48 Japanese control individuals to identify potentially functional SNPs. Since *KIAA0789* had not been fully annotated, the arbitrary positions of translation initiation sites were estimated according to the human genome database. From the SNPs thus identified, tag SNPs were selected for the three genes with the algorithm that we previously reported (35). These tag SNPs were then used for the case–control analysis in tier 3 to further examine association signals seen throughout the multi-staged screening. We deposited the identified SNP information in the NCBI's SNP database and also in our own database, JMDBase (Japan Metabolic Disease Database).

Statistical analysis

The SNPs were tested individually for the statistical significance of disease association with the χ^2-test statistic, which evaluated three inheritance models—[2 × 3] contingency table, dominant and recessive models—for genotype distributions and independence on [2 × 2] contingency table for allele frequencies. Here, the most significant P-values among three inheritance models were adopted for genotype distributions when we selected SNPs for screening in tier 3. The criteria for declaring suggestive evidence of disease association were arbitrarily set at each analytical stage as summarized in Figure 1, and they are described in the Results section. SNPs' genotype departures from HWE were tested using the χ^2-test with 1 degree of freedom.

In the three genes showing significant association signals, the extent of LD was measured in terms of an LD coefficient r^2 before the analysis of haplotype structure. Within each LD block, haplotypes were inferred from genotype data by the SNPHAP software for the case and control groups, respectively.

We randomly permutated the genotype of individuals across different panels, 100 times per SNP, and counted the ratio of permutations that fulfill the screening criteria. This ratio indicates the specificity of the study. According to the P-value distribution of the permutations, we evaluated the probability of observing an SNP with P-value no larger than the actual minimum. This probability indicates the experiment-wise P-value. For the specific prevalence and penetrance, we calculated genotype frequency and randomly generated genotypes according to their frequency. We generated genotypes for 1000 simulations of each panel and computed the ratio of simulations that could pass the screening. This ratio is considered the sensitivity of the study.

Values were expressed as means ± SD unless otherwise indicated.

Uniform resource locators

The JSNP database is available at http://snp.ims.u-tokyo.ac.jp/index.html. The National Center for Biotechnology Information's SNP database is available at http://www.ncbi.nlm.nih.gov/SNP/. The JMDBase is available at http://www.jmdbase.jp. SNPHAP is available at http://www.gene.cimr.cam.ac.uk/clayton/software/

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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Pulmonary venous flow and risk of cardiovascular disease in essential hypertension

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Objective The prognostic significance of the pulmonary venous flow in essential hypertensive patients was investigated.

Methods and results Doppler transthoracic echocardiograms were analyzed in 705 essential hypertensive subjects with no prior cardiovascular disease. At baseline, most subjects had 'normal diastolic function' or 'mild diastolic dysfunction'. During follow-up (mean, 32 months), 56 participants developed cardiovascular disease. Sex-specific median values were used to separate the higher group from the lower group of the peak velocity ratio of the pulmonary venous systolic to diastolic wave (*S/D*) (male <1.51, female <1.66), and of the transmitral velocity ratio of early diastolic to atrial filling (*E/A*) (male <0.84, female <0.82). Kaplan–Meier curves with log-rank tests showed significantly poorer event-free survival rates in the groups with high *S/D* ($P<0.01$) and low *E/A* ($P<0.01$), respectively. In multivariate Cox regression analysis, the *S/D* ratio (HR 1.07 for each 0.1 increase, $P=0.03$) or *E/A* ratio ($P<0.01$) was an independent predictor of cardiovascular disease events. When divided into four groups based on the respective sex-specific median levels of *S/D* in the *E/A* ≥ median and *E/A* <median groups, the group with high *S/D* and low *E/A* (*S/D*; male ≥1.77, female ≥1.81) had a significantly poorer event-free survival rate ($\chi^2=28.06$, $P<0.01$), and the adjusted-hazard ratio by multivariate Cox regression analysis was 2.16 (95% CI; 1.40–3.07, $P<0.01$).

Conclusion Increased *S/D* or decreased *E/A* is associated with an increased cardiovascular disease risk, and the combination of high *S/D* and low *E/A* may be a powerful predictor of cardiovascular disease in essential hypertension. Pulmonary venous flow evaluation may provide clinically important prognostic information in patients with essential hypertension. *J Hypertens* 26:798–805 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: cardiovascular disease, diastole, echocardiography, hypertension, predictor

Abbreviations: Ad, the duration of atrial filling wave; ANOVA, analysis of variance; ARdur, the duration of flow at atrial contraction; A-velocity, the peak of atrial diastolic phase filling; CI, confidence interval; CVD, cardiovascular disease; DcT, the deceleration time of early diastolic LV filling; E/A, the ratio of peak early to late diastolic filling velocity; E-velocity, the peak of early diastolic phase filling; HR, hazard ratio; LA, left atrial; LAD, left atrial dimension; LV, left ventricular; LVMI, left ventricular mass index; MVF, mitral valve flow; PVa, pulmonary vein atrial reversal; PVd, peak diastolic forward flow velocity; PVF, pulmonary venous flow; PVs, peak systolic forward flow velocity; S/D, the ratio of the pulmonary venous systolic velocity to diastolic velocity

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Introduction

Cardiovascular disease (CVD) such as congestive heart failure, coronary artery disease, and hypertension often leads to systolic and diastolic ventricular dysfunction. It is now recognized that patients with normal systolic function can have marked impairment of diastolic function (isolated diastolic dysfunction) [1]. Comprehensive Doppler echocardiography can now characterize diastolic function directly in addition to measuring systolic function. Doppler left ventricular (LV) diastolic filling indices, especially the ratio of peak early to late diastolic filling velocity (*E/A*), have gained wide acceptance as simple and commonly used indices to assess diastolic dysfunction. Previous studies have reported independent prognostic information on diastolic dysfunction in different clinical settings and populations, such as congestive heart failure [2], myocardial infarction [3,4], and the elderly [5]. Additionally, two recent reports from the PIUMA study [6] and the Strong Heart Study [7] pointed out the prognostic value of the transmitral *E/A* ratio in hypertensive patients and in the general population, respectively.

Noninvasive assessment of pulmonary venous flow (PVF) using pulse-wave Doppler transthoracic echocardiography is proposed as a useful measurement in various disease states [8,9]. Of these measures, the ratio of systolic velocity to diastolic velocity (*S/D*) by assessment of the pulmonary veins is a commonly used index to assess diastolic filling in PVF. Individuals with low *S/D* are considered to have a restrictive filling pattern, whereas those with high *S/D* have impaired early diastolic

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relaxation [8]. Previous studies have explored the prognostic value of PVF by categorizing diastolic dysfunction according to the progression of diastolic dysfunction [10], or in patients with systolic dysfunction [11,12]. These results seem to confirm the association between restrictive LV filling and higher CVD risk. Even in essential hypertensive patients with LV hypertrophy, however, a restrictive LV filling pattern is very uncommon [13], and diastolic dysfunction in hypertensive patients without heart failure is usually characterized by abnormally prolonged relaxation [14,15], that is decreased peak diastolic forward flow velocity (PV_d) and increased peak systolic forward flow velocity (PV_s), resulting in an increased *S/D* ratio [16]. Therefore, this study was undertaken to identify the clinical significance of PVF, in middle-aged and elderly essential hypertensive subjects, to determine its impact on prognosis. In addition, we further examined whether assessment of the PVF velocity pattern adds to the prognostic information provided by *E/A* ratio.

Methods

Study subjects

This study enrolled essential hypertensive patients in normal sinus rhythm, who had good-quality echocardiographic recordings, and monitored for a mean follow-up of 32.0 ± 18.0 months retrospectively. In our laboratory (the National Cardiovascular Center in Osaka, Japan), all hypertensive patients attended the echocardiography laboratory, and echocardiographic data were routinely collected consecutively. Of 865 patients at the time of the baseline examination, we excluded 160 patients (18.5%) for the following reasons: ischemic heart disease, acute coronary syndrome, congestive heart failure [New York Heart Association (NYHA) class II or greater], valvular heart disease, old cerebral infarction, history of transient ischemic attack, secondary hypertension, moderate or severe aortic or mitral regurgitation, heart rate over 100 bpm, or low ejection fraction (< 45%), or those with undetectable PVF throughout the cardiac cycle or absent reversal. After these exclusions, 705 hypertensive patients (350 women) remained. The patients with missing data for PVF were ($n = 126$, 14.6%), on average, obese and had excessive wall motion noise associated with atrial contraction compared with patients for whom PVF was available.

Hypertension was defined as systolic blood pressure of 140 mmHg or over or diastolic blood pressure of 90 mmHg or above on repeated measurements, or receiving antihypertensive treatment. Diabetes mellitus was defined according to the American Diabetes Association criteria [17]. Smoking status was determined by interview, and defined as follows: never-smoker, past-smoker (those who had a history of habitual smoking but had quit), and current-smoker. Ischemic heart disease was defined as a 75% or greater organic stenosis of at least one major coronary artery as confirmed by coronary angiography, or a history of myocardial infarction or percutaneous transluminal coronary angioplasty. All procedures in the present study were carried out in accordance with institutional and national ethical guidelines for human studies. All participants enrolled in this study were Japanese, and all gave informed consent to participate in this study.

Baseline clinical characteristics

After fasting overnight, blood pressure was measured with an appropriate arm cuff and a mercury column sphygmomanometer on the left arm after a resting period of at least 10 min in the supine position. After blood pressure measurement, venous blood sampling from all subjects was performed. Height and body weight were measured, and body mass index was calculated. The following parameters were also determined: total cholesterol, triglycerides, and high-density lipoprotein cholesterol.

Echocardiographic methods and calculation of derived variables

Imaging and Doppler echocardiography were performed in all study participants. Studies were performed using phased-array echocardiography with M-mode, two-dimensional, pulsed, and color-flow Doppler capabilities, as previously described [18,19]. The left atrial dimension (LAD), LV internal dimension and septal and posterior wall thickness were measured at end-diastole and end-systole according to the American Society of Echocardiography recommendations [20]. Color-flow Doppler recordings were used to check for aortic and mitral regurgitation, as previously described [21]. End-diastolic dimensions were used to calculate LV mass by a previously reported formula [22]. LV mass was considered to be an unadjusted variable, and was normalized by body surface area and expressed as LV mass index (LVMI). End- diastole and end-systole LV volumes, calculated by Teichholz's method [23], were used to calculate ejection fraction.

The LV diastolic filling pattern was recorded from the apical transducer position with the subject in the left lateral decubitus position, and with the sample volume situated between the mitral leaflet tips. The leading edge of the transmitral Doppler flow pattern was traced to derive the peak of early diastolic and atrial phase LV filling (*E*-velocity and *A*-velocity, respectively), *E/A* ratio, the deceleration time of early diastolic LV filling (*DcT*), and the duration of atrial filling wave (A_d).

Pulmonary venous flow velocity was also routinely recorded by placing a sample volume about 1 cm into the right superior pulmonary vein [24]. Pulmonary vein systolic (PV_s), diastolic (PV_d), *S/D* ratio, and atrial reversal (PV_a), as well as the duration of flow at atrial contraction (*ARdur*), were recorded. When a biphasic PV_s was detected, the highest peak velocity was used [24]. A_d and *ARdur* were measured as close to the zero baseline as possible from start to termination of flow at atrial

contraction after the P wave on the simultaneously recorded electrocardiogram, and the difference in A_d and *ARdur* was calculated ($ARdur - A_d$). All measurements were performed by one trained investigator who was blinded to the clinical data of the subjects.

Differentiation of diastolic filling patterns

Each participant was placed into one of the following categories of filling pattern after echocardiography: normal filling, $1.0 < E/A$ ratio < 1.5 and $140 < DcT < 220$ ms; impaired relaxation, E/A ratio ≤ 1.0 and $DcT \geq 220$ ms; pseudonormal filling $1.0 < E/A$ ratio < 1.5 and $140 < DcT < 220$ ms, but S/D ratio < 1; restrictive pattern, E/A ratio ≥ 1.5 and $DcT \leq 140$ ms [10,25,26].

Clinical endpoints

For survival analysis, observation began on the day of echocardiography with verified updates through March 2004. All subjects were followed at the National Cardiovascular Center in Osaka, and treated by implementation of standard lifestyle and pharmacologic measures. All participants were periodically referred to our institution for blood pressure control and other diagnostic procedures. The CVD events of interest in this study were myocardial infarction and angina pectoris confirmed by electrocardiographic changes, coronary angiography or myocardial scintigraphy findings, stroke confirmed by clinical symptoms, computed tomography and magnetic resonance angiography or cerebrovascular angiography findings, and congestive heart failure requiring hospitalization. Congestive heart failure was defined by the Framingham Heart Study criteria [27], which require the simultaneous presence of at least two major criteria, or one major criterion in conjunction with two minor criteria [28], and requiring treatment with diuretics, vasodilators, or antihypertensive drugs. The cause of death was classified as CVD if there was sudden death from CVD. All CVD events were determined by an independent review panel of physicians who were unaware of the echocardiographic and clinical findings. Furthermore, patients with clinical evidence of pneumonia or uremia were excluded. For patients who experienced multiple nonfatal episodes of CVD, the analysis included only the first event.

Statistical analysis

Data are presented as mean ± SD for continuous variables and as proportions for categorical variables. The relationships between the S/D ratio and various parameters were assessed using univariate linear regression analysis and Pearson's correlation coefficient. The subjects were divided into two groups according to whether their S/D ratio was below or above the median value by each sex, and then the significance of any differences between groups was evaluated using unpaired t test. Event-free survival analysis was performed using the Kaplan–Meier method to plot the cumulative incidence of CVD according to median value of the baseline S/D ratio or E/A ratio by each sex, and the groups were compared by the Mantel log rank test. Cox proportional hazard analysis was used to examine the association between variables and the cumulative incidence of CVD in crude and multivariate models, after accounting for relevant variables using a P-value of less than 0.05 as the selection criterion. These effects were measured by the hazard ratio (HR) and 95% confidence interval (CI) based on Cox regression models.

We next divided the participants into two groups using the median value of the E/A ratio by each sex, and then stratified the participants into four groups according to the respective sex-specific median values of the S/D ratio in participants with E/A ratio of median or higher or E/A ratio below the median. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison posttest was used to analyze data among the four groups. Event-free survival analysis was performed using the Kaplan–Meier method to plot the cumulative incidence of CVD. The relative risk of CVD events in the Cox proportional hazard analysis was assessed in crude and multivariate models, and the cumulative incidence of CVD was calculated using the group with low S/D and high E/A as a reference for each. A P-value less than 0.05 was considered to be statistically significant. All calculations were performed using a standard statistical package (JMP 4.0; SAS Institute, Cary, North Carolina, USA). The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Clinical features

Baseline clinical and biochemical characteristics of the study subjects are listed in Table 1. Overall, most of the total subjects had 'normal diastolic function' or 'mild diastolic dysfunction (impaired relaxation)' [10,25]. LVMI was significantly higher, and the S/D ratio was significantly lower in male than in female participants (LVMI 134.5 ± 32.8 versus 119.4 ± 33.5 g/m^2, S/D ratio 1.57 ± 0.43 versus 1.69 ± 0.39, $P < 0.01$ respectively). There was no significant difference in E/A ratio, DcT, PV_a, or $ARdur - A_d$ between male and female participants. We first examined simple correlations between the S/D ratio and various parameters after dividing the subjects into two groups according to sex. As expected, in both male and female participants, the S/D ratio was significantly associated with age (male $r = 0.45$, female $r = 0.36$, $P < 0.01$, respectively), E/A ratio (male $r = -0.60$, female $r = -0.54$, $P < 0.01$, respectively), DcT (male $r = 0.44$, female $r = 0.39$, $P < 0.01$, respectively), and peak PV_a-velocity (male $r = 0.35$, female $r = 0.23$, $P < 0.01$, respectively), but not LAD (male $r = 0.04$, female $r = 0.08$) and LVMI (male $r = 0.06$, female $r = 0.09$). A significant association between E/A ratio and heart rate ratio was found (male $r = -0.14$, female $r = -0.16$, $P < 0.01$, respectively). The

Table 1 Baseline clinical characteristics of study participants

Variables	Total	*S/D* < median Male < 1.51 Female < 1.66	*S/D* ≥ median Male ≥ 1.51 Female ≥ 1.66
n	705	354	351
Age (years)	61.6 ± 11.9	57.7 ± 13.2	65.4 ± 8.9†
Male (%)	50.2	50.3	50.4
Body mass index (kg/m²)	24.3 ± 3.3	24.2 ± 3.5	24.3 ± 3.2
Duration of hypertension (years)	14.8 ± 10.4	13.7 ± 10.4	15.8 ± 10.4†
Smoking status (%)			
Never/past/current	52.3/30.2/16.6	56.0/25.3/18.7	51.0/34.5†/14.5
Systolic blood pressure (mmHg)	144.2 ± 15.6	142.8 ± 15.1	145.5 ± 16.1*
Diastolic blood pressure (mmHg)	81.5 ± 10.6	81.7 ± 11.1	81.1 ± 10.2
Pulse pressure (mmHg)	62.7 ± 14.0	61.1 ± 13.7	64.3 ± 14.1†
Heart rate (bpm)	66.9 ± 8.8	66.4 ± 9.1	67.2 ± 8.1
Diabetes (%)	23.4	21.5	25.4
Total cholesterol (mmol/l)	5.22 ± 0.82	5.24 ± 0.82	5.21 ± 0.82
Triglycerides (mmol/l)	1.51 ± 1.05	1.52 ± 1.20	1.49 ± 0.88
High-density lipoprotein cholesterol (mmol/l)	1.31 ± 0.40	1.35 ± 0.41	1.27 ± 0.37†
LAD (cm)	3.63 ± 0.47	3.61 ± 0.48	3.65 ± 0.45
LVMI (g/m²)	126.9 ± 34.0	123.1 ± 35.0	130.4 ± 32.5†
Ejection fraction (%)	71.7 ± 7.9	71.1 ± 8.1	72.2 ± 7.7
Peak E-velocity (m/s)	0.70 ± 0.16	0.76 ± 0.16	0.65 ± 0.15†
Peak A-velocity (m/s)	0.83 ± 0.19	0.78 ± 0.17	0.88 ± 0.19†
E/A ratio	0.88 ± 0.27	1.01 ± 0.28	0.76 ± 0.20†
DcT (ms)	232.2 ± 48.6	215.7 ± 39.4	248.6 ± 50.9†
A_d (ms)	145.7 ± 22.0	143.0 ± 21.6	148.4 ± 22.3†
Peak PV_s velocity (m/s)	0.63 ± 0.12	0.59 ± 0.11	0.67 ± 0.12†
Peak PV_d velocity (m/s)	0.40 ± 0.10	0.46 ± 0.11	0.35 ± 0.07†
S/D ratio	1.63 ± 0.42	1.31 ± 0.21	1.95 ± 0.32†
Peak PV_a velocity (m/s)	0.29 ± 0.08	0.27 ± 0.07	0.30 ± 0.09†
ARdur (ms)	116.0 ± 22.7	113.9 ± 23.1	118.3 ± 22.1†
ARdur − A_d (ms)	−29.7 ± 28.5	−29.3 ± 29.4	−30.0 ± 27.7
Diastolic filling patterns (%)			
Normal filling	25.0	37.8	12.0†
Impaired relaxation	73.3	58.8	88.0†
Pseudonormal filling	1.4	2.8	0†
Restrictive filling	0.3	0.6	0
Antihypertensive medication (%)	79.7	76.4	83.2*
Calcium channel blocker	66.5	62.5	70.5*
Beta-blocker	26.9	30.2	23.6*
ACEI or ARB	34.2	33.1	35.3
Diuretic	17.2	14.7	19.7

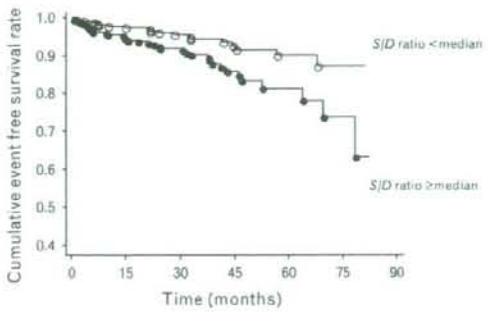
ACEI, angiotensin-converting enzyme inhibitor; A_d, the duration of atrial filling wave; ARB, angiotensin II receptor blocker; *ARdur*, the duration of flow at atrial contraction; A-velocity, the peak of atrial diastolic phase filling; *DcT*, the deceleration time of early diastolic LV filling; *E/A*, the ratio of peak early to late diastolic filling velocity; E-velocity, the peak of early diastolic phase filling; LAD, left atrial dimension; LVMI, left ventricular mass index; PV_a, pulmonary vein atrial reversal; PV_d, peak diastolic forward flow velocity; PV_s, peak systolic forward flow velocity; *S/D*, the ratio of the pulmonary venous systolic velocity to diastolic velocity. Data are mean ± SD or percentage. *$P < 0.05$ and †$P < 0.01$ versus *S/D* < median.

S/D ratio, however, was significantly associated with heart rate in male ($r = 0.11$, $P < 0.05$), but not in female participants ($r = 0.03$, NS).

Predictive value of *E/A* ratio and *S/D* ratio for cardiovascular disease

During the follow-up period, 56 patients (7.9%, 23 females) developed CVD. Specifically, there were 27 patients with nonfatal congestive heart failure, 11 with stroke, six with myocardial infarction, three with angina pectoris, and nine patients died from CVD causes. Among those who developed CVD, 50 patients had impaired relaxation, five with normal diastolic function and one with pseudonormal filling at baseline. The *S/D* ratio was significantly higher and the *E/A* ratio was significantly lower in patients who developed CVD during the follow-up period than in event-free subjects (*S/D* ratio 1.78 ± 0.39 versus 1.62 ± 0.42, *E/A* ratio 0.75 ± 0.18 versus 0.89 ± 0.28, $P < 0.01$, respectively). Because of the sex difference in *S/D* ratios, different median values for men and women were used to separate the high and low *S/D* ratio groups (male < 1.51, female < 1.66). The group with a high *S/D* ratio showed significantly older age, longer duration of hypertension, higher pulse pressure, LVMI, *DcT*, A_d, peak PV_a, and *ARdur*, and lower high-density lipoprotein cholesterol than those with a low *S/D* ratio (Table 1). Life table analysis of CVD throughout the follow-up period in the two groups based on the *S/D* ratio is plotted in Fig. 1. These curves illustrate the significantly poorer event-free survival in the group with a high *S/D* ratio. When the analysis was also performed by applying the sex-specific median values of *E/A* ratios to separate the high group from the low group of *E/A* ratio (male < 0.84, female < 0.82), the group with low *E/A* showed a significantly poorer event-free survival rate (log-rank $\chi^2 = 16.345$, $P < 0.01$). A univariate Cox proportional-hazard model showed that a high *S/D* ratio (HR 1.448, 95% CI 1.10–1.93, $P < 0.01$) and a low *E/A* ratio (HR 1.784, 95% CI 1.34–2.44, $P < 0.01$) were significant predictors of CVD events.

Fig. 1



Cardiovascular event-free survival in two groups with baseline peak velocity ratio of the pulmonary venous systolic to diastolic wave (*S/D*) < or ≥ median value (log-rank $\chi^2 = 7.101$, $P < 0.01$).

Furthermore, even when the *S/D* and *E/A* ratios were included in a univariate model as continuous variables, a significantly higher risk of CVD events was found in these ratios (*S/D* ratio, HR 1.09 for each 0.1 increase, 95% CI 1.03–1.15; *E/A* ratio, HR 0.75 for each 0.1 increase, 95% CI 0.66–0.86, $P < 0.01$ respectively). Other variables in this study that significantly predicted CVD events included LVMI (HR 1.02 for each 1.0 g/m^2 increase, 95% CI 1.01–1.02, $P < 0.01$), LAD (HR 1.01 for each 0.1 cm increase, 95% CI 1.00–1.01, $P < 0.01$), age (HR 1.06 for each 1-year increase, 95% CI 1.03–1.09, $P < 0.01$), diabetes (HR 1.45 for yes, 95% CI 1.10–1.89, $P = 0.010$), and pulse pressure (HR 1.03 for each 1 mmHg increase, 95% CI 1.02–1.05, $P < 0.01$). Sex, body mass index, duration of hypertension, smoking status, systolic and diastolic blood pressure, heart rate, total cholesterol, triglycerides, high-density lipoprotein cholesterol, ejection fraction, and $ARdur - A_d$ were included in the model, but failed to be significant predictors of CVD events. After adjusting for other risk factors (LVMI, age, diabetes, pulse pressure, and LAD) in multivariate Cox regression analysis, independence of the *S/D* (HR 1.07 for each 0.1 increase, 95% CI 1.01–1.14, $P = 0.033$) or *E/A* ratios (HR 0.82 for each 0.1 increase, 95% CI 0.71–0.94, $P < 0.01$) as predictors of CVD events was found. Because heart rate is one of the most important physiological correlates of all parameters of diastolic function, we further performed the multivariate Cox regression analysis after heart rate was added in the model, and found that the *S/D* (HR 1.07 for each 0.1 increase, 95% CI 1.00–1.14, $P = 0.039$) or *E/A* (HR 0.83 for each 0.1 increase, 95% CI 0.71–0.95, $P < 0.01$) ratios were independent predictors of CVD events.

Predictive value of *DcT*, PV_a, and $ARdur - A_d$ for cardiovascular disease

DcT was significantly longer in patients who developed CVD during follow-up (252.4 ± 46.2 versus 230.4 ± 48.4 ms, $P < 0.01$), whereas PV_a and $ARdur - A_d$ were not significantly longer in these patients (PV_a 28.79 ± 7.03 versus 28.69 ± 7.93 m/s, $P = 0.926$; $ARdur - A_d$ −31.61 ± 27.21 versus −29.50 ± 28.62 ms, $P = 0.592$). Even though the prognostic value of *DcT* was significant in a univariate model (HR 1.60 for each 50 ms increase, 95% CI 1.28–1.99, $P < 0.01$), the independence of *DcT* as a predictor of CVD events was lost in the multivariate model (HR 1.29 for each 50 ms increase, 95% CI 0.99–1.66, $P = 0.063$).

Incidence of cardiovascular disease jointly with *S/D* ratio and *E/A* ratio

To assess the combined effects of the *S/D* and *E/A* ratios, we constructed survival curves after dividing the subjects into two groups using the median value of the *E/A* ratio by each sex, and then stratified the subjects into four groups according to the sex-specific median values of the *S/D* ratio in the group with *E/A* ratio of median or above (*S/D* ratio male 1.31, female 1.51) and that with *E/A* ratio under the median (*S/D* ratio male 1.77, female 1.81). As a result, the participants were divided into four groups as follows; low *S/D* and high *E/A*, high *S/D* and high *E/A*, low *S/D* and low *E/A*, and high *S/D* and low *E/A*. The baseline clinical and biochemical characteristics of the study subjects are shown in Table 2. Compared with the group with low *S/D* and high *E/A*, the group with high *S/D* and low *E/A* showed an increased risk of cardiovascular morbidity, such as significantly longer duration of hypertension, higher prevalence of diabetes, higher pulse pressure, and worse dyslipidemia. Life table analyses of CVD throughout the follow-up period according to the four groups of baseline *E/A* and *S/D* ratios are plotted in Fig. 2. These curves illustrate the significantly poorer event-free survival in the group with high *S/D* and low *E/A*. We next performed Cox regression analysis to examine whether the influence of a high *S/D* ratio and low *E/A* ratio on CVD events was independent of other risk factors (Table 3). The risk of CVD was significantly higher in the group with high *S/D* and low *E/A* compared with that in the group with low *S/D* and high *E/A* (HR 2.66). In multivariate Cox regression analysis including LVMI, age, diabetes, pulse pressure, and LAD, the combination of high *S/D* ratio and low *E/A* ratio was an independent predictor of CVD (HR 2.16). Furthermore, even when the group with low *S/D* and low *E/A* was used as a reference, the group with high *S/D* and low *E/A* had a significantly higher risk of CVD events in univariate Cox regression analysis (HR 1.55, 95% CI 1.13–2.19, $P < 0.01$) and in a multivariate model (HR 1.48, 95% CI 1.07–2.10, $P < 0.02$).

In addition, we further performed multivariate Cox regression analysis after heart rate was added in the model, and found that the risk of CVD was significantly higher in the group with high *S/D* and low *E/A* than that in the group with low *S/D* and high *E/A* (HR 2.14, 95% CI 1.38–3.66, $P < 0.01$).