Table 1. Characteristics of the Study Population.

		Cases		Controls	
		tAMD	PCV	Control 1*	Control 2†
No. of participants		401	510	336	1194
Age	Mean ± SD	77.38±8.39	74.98±7.77	74.16±8.42	50.34±15.9
Gender	Men	287	372	142	493
	Women	114	138	194	701

tAMD, typical age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; SD, standard deviation.
*Cataract patients without age-related maculopathy.
†Healthy Japanese individuals.
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another study in a larger cohort (n = 7723 and 2327) which involved the same population could not replicate the finding of the previous study [17,18]. Recently, Lee et al. have shown that *SERPING1* is positively associated with AMD in Caucasians [19], but whether this gene is truly associated with AMD remains controversial.

Furthermore, the association of *SERPING1* with AMD has been evaluated also in Asians. Lu et al. examined the association in 194 AMD patients and 285 controls and reported that *SERPING1* is not associated with AMD in the Chinese population [20]. The association between PCV and *SERPING1* has also been evaluated in a smaller Chinese cohort (118 patients and 115 controls), also with negative findings [21]. So far, all Asian studies for *SERPING1* did use smaller cohorts than those of original studies and not consider their statistical power. For evaluating the true gene-disease association, it would be helpful to replicate the positive association reported in previous studies using a different ethnic cohort. The aim of this study, which involved a relatively large number of participants, was to investigate whether the *SERPING1* gene variants are associated with typical AMD or PCV in a Japanese population.

Materials and Methods

All procedures in this study adhered to the tenets of the Declaration of Helsinki. This study was approved by the Ethics Committee of each institute involved (Kyoto University Graduate School and Faculty of Medicine, Ethics Committee, the Ethical

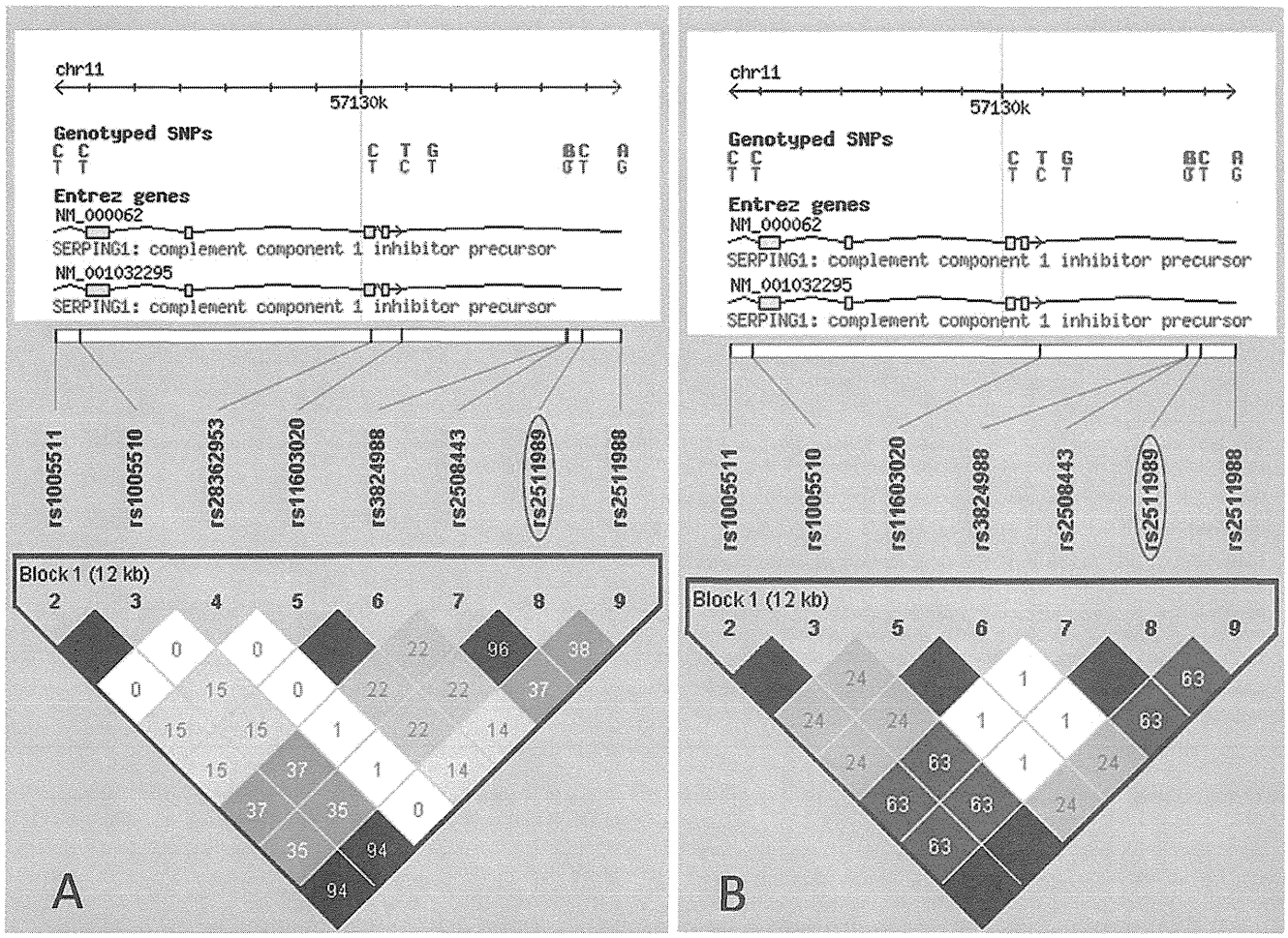

Figure 1. Linkage disequilibrium (LD) structure across the complement component 1 inhibitor (*SERPING1*) gene in Caucasian and Japanese populations. Genotype data were retrieved from HapMap CEU (Utah residents with ancestry from northern and western Europe; A) and JPT (Japanese in Tokyo, Japan; B) data sets. Haplotype blocks were determined using the "four-gamete rule" option in Haploview; all HapMap single nucleotide polymorphisms on *SERPING1* gene are in the same block in both populations. Each box provides estimated statistics of the coefficient of determination (r^2), with darker shades representing stronger LD.
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Committee of Fukushima Medical University, the Ethical Committee of Kobe City Medical Center General Hospital, the Ethical Committee of Ozaki Eye Hospital, the Ethical Committee of the Otsu Red Cross Hospital, the Ethical Committee of Nagahama City Hospital, and the Ethical Committee at Aichi Cancer Center). All of the patients were fully informed about the purpose and procedures of this study, and written consent was obtained from each.

In this study, 401 patients with typical AMD and 510 patients with PCV were recruited from the Department of Ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. The control group included 2 populations: (1) 336 individuals who underwent cataract surgery and had no age-related maculopathy (ARM) (Control 1) were recruited from the Department of Ophthalmology, Kyoto University Hospital, Ozaki Eye Hospital, Japanese Red Cross Otsu Hospital, and Nagahama City Hospital; and (2) 1194 healthy individuals who were recruited from the Aichi Cancer Center Research Institute as the general population control (Control 2). AMD and ARM were defined according to the International Classification System for ARM and AMD [22]. The diagnosis of PCV was based on indocyanine green angiography, which showed a branching vascular network that terminated in polypoidal swelling. Typical AMD were late AMD which showed classic choroidal neovascularization (CNV), occult CNV, or both. All diagnoses were made by 3 retina specialists (K.Y., A.T., and A.O.); a fourth specialist (N.Y.) was consulted when the subtype classification could not be decided on by the initial 3 reviewers. All of the subjects were unrelated and were of the Japanese descent.

Genomic DNAs were isolated from the peripheral blood of the subjects by using a DNA extraction kit (QuickGene-610L, Fujifilm, Minato, Tokyo, Japan). The samples of all the patients with typical AMD and PCV and of cataract patients were genotyped using a Taqman single nucleotide polymorphism (SNP) assay with the ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). The individuals recruited from the Aichi Cancer Center Research Institute were genotyped using Illumina Human-Hap 610 chips (Illumina Inc., San Diego, CA).

Linkage disequilibrium (LD) structures across the *SERPING1* gene were compared between the Caucasian and Japanese populations, using genotype data retrieved from the HapMap CEU and JPT data sets [23]. The retrieved data were loaded into Haploview to estimate LD parameters and to identify haplotype blocks [24]. Deviations in genotype distributions from the Hardy–Weinberg equilibrium (HWE) were assessed using the HWE exact test. Statistical analyses for differences in the observed genotypic distribution were performed by the chi square test for trend; logistic regression analysis was performed for age and gender adjustments. The statistical power calculation was performed using QUANTO version 1.2 [25]. P values less than 0.05 were considered statistically significant.

Results

The demographic details of the study population are presented in Table 1. Because all SNPs of the *SERPING1* gene are in the same haplotype block, rs2511989 was selected as the haplotype-tagging SNP; rs2511989 was reported to be associated with the risk of AMD in previous studies [16,19] (Fig. 1). Details of allele and genotype counts and summary statistics for rs2511989 are shown in Table 2. The success rate of genotyping of rs2511989 was 98.1%, and the distributions of the genotypes for all study groups were in the Hardy–Weinberg equilibrium ($P>0.05$). Although we compared the genotype distributions of rs2511989 in typical AMD and PCV patients against 2 independent control groups (cataract patients without ARM and healthy Japanese individuals), *SERPING1* rs2511989 was not significantly associated with typical AMD ($P = 0.932$ and 0.513, respectively); furthermore, it was not significantly associated with PCV ($P = 0.505$ and 0.141, respectively). After correction for age and gender differences based on a logistic regression model, the difference in the genotype distributions remained insignificant ($P>0.05$). Table 3 shows the odds ratios adjusted for age and gender under various genetic models. We could not find a significant association in any genetic model.

Next, we calculated our statistical power to detect an association of a risk allele with the odds ratio reported in the previous study that investigated the association of rs2511989 with developing AMD. When we targeted the original study reported by Ennis (odds ratio 0.63) [16], our sample size had more than 90% power to detect the association (Table 2). In addition, the statistical power calculation revealed that our sample size could detect the gene-disease association for an odds ratio of 0.797 by more than 80%.

Discussion

In the present study, we investigated whether *SERPING1* gene variants are associated with typical AMD or with PCV in a Japanese population. We selected rs2511989 as the haplotype-tagging SNP, because this has been reported to be positively associated with the risk of AMD in Caucasians. The results of this study showed that *SERPING1* rs2511989 was not associated with the risk for typical AMD in a Japanese population; thus, the results did not support the hypothesis that an association between the *SERPING1* gene and AMD exists. Our sample size had more than 90% power to detect the association determined in the previous

Table 2. *SERPING1* rs2511989 Genotypic Distributions and Results of Association Tests and Power Analysis.

						vs Control 1			vs Control 2		
		GG	GA	AA	MAF	P Value	Adjusted P*	Power†	P Value	Adjusted P*	Power†
Cases	tAMD	293	102	6	0.142	0.932	0.687	93.6%	0.513	0.860	99.3%
	PCV	380	125	5	0.132	0.505	0.855	95.7%	0.141	0.678	99.2%
Controls	Control 1	248	76	10	0.144	-	-	-	-	-	-
	Control 2	859	308	27	0.152	-	-	-	-	-	-

tAMD, typical age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; MAF, minor allele frequency.
*Adjusted for age and gender.
†Statistical power for detecting the association reported in the previous study (odds ratio 0.63).
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Table 3. Odds Ratios in Various Genetic Models.

		Adjusted Odds Ratio (95% Confidence Interval)*	
Group	**Model**	**vs tAMD**	**vs PCV**
Control 1	Additive	0.938 (0.687–1.281)	0.972 (0.72–1.312)
	Dominant	1.283 (0.746–2.204)	0.598 (0.338–1.056)
	Recessive	0.934 (0.783–1.114)	1.283 (0.746–2.204)
Control 2	Additive	1.034 (0.716–1.491)	0.933 (0.673–1.294)
	Dominant	0.940 (0.470–1.879)	0.709 (0.349–1.440)
	Recessive	1.025 (0.839–1.254)	0.983 (0.823–1.174)

*Adjusted for age and gender.
tAMD, typical age-related macular degeneration; PCV, polypoidal choroidal vasculopathy.
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study in a Caucasian cohort (odds ratio 0.63) [16]. Furthermore, we found no evidence to support the role played by *SERPING1* rs2511989 in the susceptibility to PCV, and this finding is in agreement with that of the previous study in a Chinese population [21].

The reported association between AMD and *SERPING1* rs2511989 is shown in Table 4. The size of our Japanese cohort was similar to that of the original study [16]. Furthermore, the statistical power calculation revealed that our sample size could detect the gene-disease association for an odds ratio of 0.797 by more than 80%. Had there been a true protective effect of *SERPING1* gene variants for developing AMD at the same level as was reported in previous studies [16,19], the statistical power of our study would have detected such an association. Differences in the ethnicities of subjects might be 1 reason for the difference observed between the results of this study in a Japanese cohort and those of the previous study in a Caucasian cohort. Frequency of the minor allele of rs2511989 was reportedly greater in the earlier study in a Caucasian population than that of the present study in a Japanese population. In fact, in reference to the allele frequency data from the HapMap, all genetic variants across the *SERPING1* gene showed smaller minor allele frequency in Japanese than in Caucasians.

Another possible explanation for the differences between our findings and those of other studies in different ethnic cohorts may include a difference in the phenotypes of AMD. Numerous studies have reported that distinguishing features of Asian AMD include male predominance, unilateral presentation, comparatively low incidence of soft drusen, and greater prevalence of neovascular AMD and PCV [26–29]. To address these concerns, we classified AMD patients into those with typical AMD and those with PCV, but the possible hidden differences in the phenotypes cannot be excluded. Alternatively, considering the fact that genetic variants that are associated with a particular disease in 1 population may not necessarily be associated in another population [30–32]; moreover, it is possible that gene-disease association of *SERPING1* in populations from East Asia is very weak or absent as compared with Caucasian populations.

In this study, we used general population-based controls (Control 2). The possibility exists that some of the eyes in the control 2 group might have or develop AMD or PCV, and this might be a possible explanation for the negative results in this study. However, because the prevalence of AMD in the general population is estimated to be 0.5% in the Japanese population [33], the loss of the statistical power of association analysis must be negligible. In addition, we also performed a subset analysis on

Table 4. Comparison of Association Observed between AMD and *SERPING1* rs2511989.

Subject Group	Current Study (JP)			Mayo Subjects (US)		AREDS Subjects (US)		Ennis et al. (UK)		Ennis et al. (US)		Lee et al. (US)		Lu et al. (CH)	
Subjects	**Case**	**Control 1**	**Control 2**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**
No. of participants	401	336	1194	470	310	1221	295	479	479	248	252	556	256	194	285
Allele count	G 688	572	2026	569	363	1435	357	597	500	322	282	669	283	336	493
	A 114	96	362	371	257	1007	233	355	454	174	222	413	229	52	69
Genotype count	GG 293	248	859	179	103	436	115	191	132	100	79	213	74	147	215
	GA 102	76	308	211	157	563	127	215	236	122	124	273	135	42	63
	AA 6	10	27	80	50	222	53	70	109	26	49	70	47	5	3
MAF	0.142	0.144	0.152	0.395	0.415	0.412	0.395	0.373	0.475	0.351	0.441	0.382	0.447	0.134	0.123
P values	-	0.932	0.513	-	0.46	-	0.41	-	5.4×10^{-6}	-	0.0037	-	0.011	-	0.61

MAF, minor allele frequency.
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controls 2 with 55 years of age or older to minimize the possibility that some of the eyes in the control group might develop AMD or PCV. However, no new significant differences in the genotypic distributions were found in the current study (data not shown). Thus, we concluded that the result of the analysis using control 2 is valuable as reference data which supports a lack of association between *SERPING1* and both typical AMD and PCV in a Japanese population. Another limitation is about geographical difference of Control 1, which may influence genetic background of the participants. However, because the Japanese population has been reported to have a rather small genetic diversity, according to data from the SNP discovery project in Japan [34], geographical difference should not be affect our statistical results.

In conclusion, this study showed a lack of association between *SERPING1* and both typical AMD and PCV in a Japanese population; thus, the results suggest that *SERPING1* does not play a significant role in the risk of developing AMD or PCV in Japanese.

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Author Contributions

Conceived and designed the experiments: IN KY HN NY. Performed the experiments: IN NG HN HH. Analyzed the data: IN RY. Contributed reagents/materials/analysis tools: IN KY RY NG HN HH AT AO MS TI AO KM KT FM NY. Wrote the paper: IN KY RY.

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The human *AIRE* gene at chromosome 21q22 is a genetic determinant for the predisposition to rheumatoid arthritis in Japanese population

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Rheumatoid arthritis (RA) is a typical complex trait and the major cause of chronic inflammation worldwide. Although multiple genetic loci have been shown for their association with the onset of RA, they cover only a part of its genetic components and are largely ethnicity-specific. To identify novel genetic factors related to the predisposition and prognosis of RA in Japanese, we conducted a large-scale genome-wide association (GWA) study. We performed a GWA analysis by scanning the genome of 1247 RA cases and 1486 controls for 277 420 single nucleotide polymorphisms (SNPs), followed by replication analysis using two independent sample sets consisting of 1865 cases and 1623 controls, and 2303 cases and 3380 controls. We identified two SNPs, rs2075876 and rs760426, in intron of the autoimmune regulator *AIRE* gene at chromosome 21q22 that showed strong associations with the disease ($P = 3.6 \times 10^{-9}$ and $P = 4.4 \times 10^{-8}$, respectively). Rs1800250, in exon7 of *AIRE*, was in strong linkage disequilibrium ($r^2 = 0.94$) with rs2075876 and introduced an amino acid alteration (S278R) in the SAND domain of the AIRE protein. *In silico* analysis showed the decreased transcription of *AIRE* by the risk allele of rs2075876 compared with the alternative allele ($P = 6.8 \times 10^{-5}$). No correlation was observed between the rs2075876 genotype and quantitative traits reflecting the progression of RA. As AIRE is a key molecule which regulates the expression and presentation of self-antigens in thymic negative selection, its downregulation by genetic polymorphisms may result in the survival of auto-reactive T cells to trigger auto-inflammation in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a major cause of chronic arthritis worldwide and results in severe functional impairment and joint destruction. The impairment of joints and disability for social activity bring strong social and economic impact (1). Both environmental and genetic factors are considered to be associated with its onset and progression (2). Twin studies of

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the European populations showed that ~60% of RA onset could be attributed to genetic factors (3). In them, *HLA-DRB1* is the strongest genetic component of the disease beyond ethnicity, and is estimated to correspond to 30–50% of the genetic components in Europeans (4). Although extensive genetic analyses including hypothesis-independent genome-wide association (GWA) studies identified >20 genes in Europeans (5–13) and 7 genes in East Asians (14–19) as genetic risk loci for RA, they account for only a part of its genetic components. Moreover, trans-ethnic comparison demonstrated that their association with RA is mostly specific to a particular ethnicity and as little as three genes, namely, *CCR6*, *STAT4* and *TNFAIP3*, have shown their association in both populations. These results strongly suggest the existence of additional susceptibility loci to RA in East Asian populations (14–19). By these reasons, we have conducted a GWA study using large DNA collections of Japanese RA patients.

RESULTS

GWA analysis

We performed a large-scale genome scan using a Japanese DNA collection (collection 1) consisting of 1247 RA cases and 1486 general population controls with Illumina Infinium arrays (Supplementary Material, Table S1). After a standard procedure of quality control (see Materials and Methods), 241 523 single nucleotide polymorphisms (SNPs) were examined for their association with RA. Quantile-quantile plot to estimate population stratification resulted in a small inflation factor ($\lambda = 1.05$). The strongest association was detected for markers in the *HLA* locus with the strongest *P*-value of 2.4×10^{-38} for rs9296015. Another known genetic determinant, *PADI4*, also showed strong association (strongest $P = 1.8 \times 10^{-8}$ for rs2240335). Also, a modest association was found in the *CCR6* gene (strongest $P = 9.7 \times 10^{-4}$ for rs1556413) (16). However, there was no evidence of association for *STAT4* and the disease in our study ($P > 0.070$). There were no other loci that showed significant association ($P < 2.1 \times 10^{-7}$) after Bonferroni's correction for multiple testing.

We then took a strategy to select candidate genes/markers for further genotyping analysis based on their functional relevance in the immune system. For this purpose, we generated a list of SNP markers showing potential association with the disease (nominal $P < 0.001$), and investigated their chromosomal locations and corresponding genes in the order of association strength. Among the top 471 SNPs with *P*-value smaller than 0.001, we found two SNPs located in intron of the *AIRE* gene at chromosome 21q22, which is known as an autoimmune regulator. They were rs2075876 and rs760426 with *P*-value of 5.1×10^{-4} and 2.0×10^{-4}, respectively, and were ~6.7 kb apart from each other and in moderate linkage disequilibrium (LD) ($r^2 = 0.63$, Fig. 1). We performed genotyping of these two markers using an additional DNA collection (termed as collection 2) consisting of 1865 cases and 1623 controls. All the RA cases and 855 controls were newly genotyped with the Taqman method, and the genotypes of the other 768 controls were extracted from genome scan results of other population-based genetic studies. We successfully confirmed the association of rs2075876 ($P = 5.1 \times 10^{-4}$) in collection 2. The other marker, rs760426, showed a moderate association ($P = 0.011$) (Table 1).

We further examined whether or not the results of our study were reproducible in another Japanese RA GWA study of Biobank Japan Project recruiting 2303 cases and 3380 controls (termed as collection 3) (16). The statistical test again returned significant associations for these markers ($P = 3.6 \times 10^{-4}$ for rs2075876 and $P = 8.2 \times 10^{-4}$ for rs760426, Table 1). When the genotyping results of the three collections were pooled, the association *P*-value reached $P = 3.6 \times 10^{-9}$ for rs2075876 and $P = 4.4 \times 10^{-8}$ for rs760426 (Table 1).

We then investigated whether or not the association of the *AIRE* gene with RA was observed in Europeans. Our own genome scan results of German RA samples (I.M., M.L. and F.M., unpublished data) showed no associations for the SNP markers in the *AIRE* locus. Two large-scale GWA studies of European descents, namely, Wellcome Trust Case Control Consortium (9) and a meta-analysis of multiple GWA studies (12), did not identify *AIRE* as a risk locus, strongly suggesting its limited contribution to RA in East Asian populations.

Structure and organization of the human *AIRE* locus

LD structure of the chromosomal region containing rs2075876 and rs760426 was generated using Japanese HapMap results. As shown in Figure 1, rs2075876 and rs760426 are located in an LD block encompassing the 32 kb region between intron 5 of the *AIRE* gene and intron 12 of the liver phosphofructokinase *PFKL* gene. As the SNPs around the *PFKL* gene showed weaker association with RA ($P > 0.002$) than the two SNPs, we considered that the observed association with RA was most likely with the *AIRE* polymorphisms. However, both of these SNPs were located in intron and no other SNP markers in the genotyping arrays were mapped in this LD block and showed similar degree of association with RA. Hence, we searched for SNPs in dbSNP that were located in exons of *AIRE* and introduce functional alterations of the AIRE protein. There were five non-synonymous SNPs in the coding region of *AIRE* out of which rs1800520 in exon7 showed an allele frequency similar to that of rs2075876 (0.420). Rs1800520 introduced an amino acid alteration from serine to arginine at amino acid residue 278 (S278R). We genotyped rs1800520 in the DNA samples of all the cases ($n = 1865$) and a part of controls ($n = 855$) of collection 2 and found that rs1800520 was in strong LD with rs2075876 ($r^2 = 0.94$) and was also associated with RA ($P = 0.0071$).

AIRE polymorphism and expression

Although both rs2075876 and rs760426 are located in intron, they may have functional roles such as regulation of *AIRE* transcription. The correlation between these SNPs and transcription levels of *AIRE* was examined by using the expression profiles of 210 lymphoblastoid cells in Gene Expression Omnibus (GEO) database (20). As the result, the transcription of *AIRE* was decreased by the risk allele (A) of rs2075876 ($P = 6.8 \times 10^{-5}$, Fig. 2) but not by that of rs760426 ($P = 0.24$). Although we hypothesized the presence of a transcription factor-binding site around rs2075876, *in silico* study

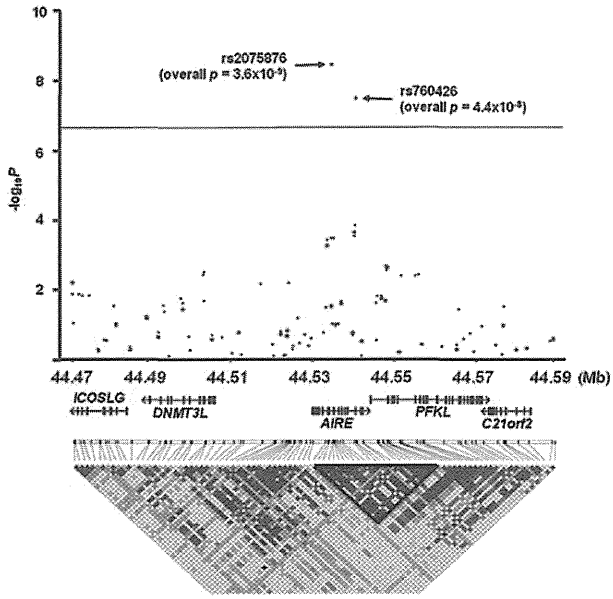

Figure 1. A schematic organization of the human *AIRE* locus at 21q22. *P*-values of the initial genome scan using collection 1 were calculated by the Trend χ^2 test and plotted in red circles. The blue circles indicate *P*-values obtained by imputation using HapMap Japanese results. Overall *P*-values of rs2075876 and rs760426 using the combined results of collections 1, 2 and 3 were also shown in green circles. A horizontal line indicates Bonferroni-adjusted $P = 2.5 \times 10^{-7}$. The structure and orientation of four genes were shown below the plots with their transcriptional orientations according to the NCBI Reference Sequence Build 36.3. LD blocks were generated according to the pairwise LD estimates of the SNPs in HapMap Japanese results.

did not predict a motif of transcription factor-binding site spanning rs2075876. Multiple nucleotide sequence alignment around rs2075876 showed a high degree of conservation among seven mammalian species (human, chimpanzee, rhesus macaque, bushbaby, horse, cow and dog). The corresponding region of rodents (mouse and rat) showed much weaker conservation (Supplementary Material, Fig. S1).

AIRE polymorphism and difference in clinical phenotypes and disease activity

RA is often subdivided into two groups based on the presence of circulating antibodies to citrullinated peptide antigen (ACPA), a specific predictive biomarker for destructive RA (21–22). In our patient collections (collection 1 and collection 2), there were 803 patients with ACPA quantification of which 176 patients were negative for ACPA. We compared the allele frequency of rs2075876 between APCA(+) and ACPA(−) groups and found no significant difference [0.39 for ACPA(+) and 0.40 for ACPA(−), $P = 0.66$]. We next tested whether rs2075876 was associated with the disease activity and prognosis. For this purpose, 212 RA patients for whom the quantitative DAS28 score was available were chosen to evaluate the correlation of RA activity and rs2075876 genotypes. Statistical analysis did not return correlations between rs2075876 genotypes and DAS28 (Supplementary Material, Fig. S2).

DISCUSSION

AIRE is a transcriptional regulator primarily expressed in medullary thymic epithelial cells (mTEC), and plays a functional role in thymocyte education and negative selection by controlling the expression of peripheral antigens in thymus (23). The expression of *AIRE* in non-thymic tissues is still controversial; some studies detected *AIRE* transcripts at a lower level in secondary lymphoid organs and in periphery while others did not (24–25), and the expression of the AIRE protein in such tissues is yet to be established. In human, dysfunction of *AIRE* caused a rare systemic multi-organ autoimmune disease known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) (26). However, the patients rarely show joint destruction as observed in RA (27). In mice deficient for *aire* which develop APECED-like multi-organ autoimmune features and do not manifest with arthritis, a dramatic decrease in the expression of type II collagen was observed in mTEC and the incidence and severity of collagen-induced arthritis were augmented when compared with the wild-type (28). Such observations indicate the possible involvement of *AIRE* in immunopathology both in the human and in the mouse. However, the involvement of *AIRE* in human multigenetic autoimmune diseases still remains to be elucidated. Our study is the first successful case which clearly showed the involvement of *AIRE* in systemic autoimmunity. The function of the AIRE protein in the secondary lymphoid organs is not fully understood. Elucidation of the functions of AIRE in peripheral organs may provide hints to the involvement of *AIRE* in the predisposition or progression in RA.

In silico analysis using the GEO database showed that the risk allele of rs2075876 decreased the transcription level of *AIRE*. This may cause lower expression of various peripheral tissue antigens (PTAs), resulting in the failure of negative selection in the thymus resulting in the survival of autoreactive T cells. Although low amount of *AIRE* transcripts in B-lymphocytes was detected in most of the reported experiments, the conclusive answer for the functional impact of rs2075876 to the immune regulation needs further studies using the tissues in which *AIRE* is strongly expressed. The S278R replacement by rs1800520 is located in the SAND domain, a conserved sequence motif in nuclear proteins including Sp100 family and plays a key role in transcription regulation. However, the SAND domain of AIRE lacks the canonical KDWF motif for the interaction with DNA. Also amino acid sequence alignment of the SAND domains in different nuclear proteins revealed that S278R was located at the poorly conserved carboxyl terminal (29). Moreover, the interaction of AIRE with histone H3 through a plant homeodomain finger was suggested to be important to up-regulation of PTA genes (30). On the other hand, an assessment of mRNA stability by a computerized modeling showed lower stability of *AIRE* mRNA with the risk allele of rs1800520 than the alternative allele, suggesting the possibility of shorter half-life of the transcripts and thus lower amount of the AIRE protein. As such, we cannot conclude whether or not these SNPs have functional impact to the regulation of *AIRE* expression. The existence of unidentified SNPs that are in strong LD with them and play important functional roles is also conceivable. Extensive analyses of the *AIRE* locus by fine mapping and

Table 1. Association analysis of two SNPs in the *AIRE* gene with RA in Japanese

rs2075876		Genotype counts			Frequency A	OR (95% CI)	*P*-value
		GG	GA	AA			
Collection 1	Case	480	554	201	0.39	1.22 (1.09–1.36)	5.1×10^{-4}
	Control	639	680	167	0.34		
Collection 2	Case	706	887	243	0.37	1.18 (1.07–1.31)	9.4×10^{-4}
	Control	710	671	192	0.34		
Collection 3	Case	905	1061	330	0.37	1.15 (1.07–1.25)	3.6×10^{-4}
	Control	1462	1506	398	0.34		
Combined study	Case	2091	2502	774	0.38	1.18 (1.11–1.24)	3.6×10^{-9}
	Control	2811	2857	757	0.34		
rs760426		Genotype counts			Frequency G	OR (95% CI)	*P*-value
		AA	AG	GG			
Collection 1	Case	464	559	219	0.40	1.23 (1.10–1.38)	2.0×10^{-4}
	Control	608	709	169	0.35		
Collection 2	Case	684	897	265	0.39	1.13 (1.03–1.25)	0.011
	Control	666	741	205	0.36		
Collection 3	Case	866	1078	357	0.39	1.14 (1.06–1.23)	8.2×10^{-4}
	Control	1408	1520	450	0.36		
Combined study	Case	2014	2534	841	0.39	1.16 (1.10–1.22)	4.4×10^{-8}
	Control	2682	2970	824	0.36		

OR, odds ratio; 95% CI, 95% confidence interval.

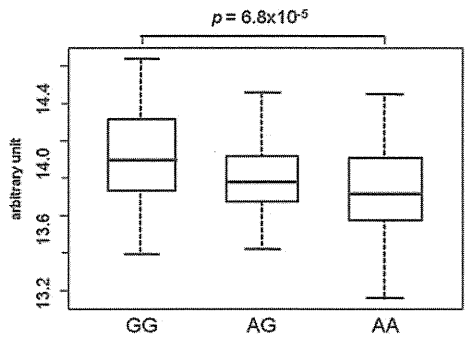

Figure 2. Comparison of the expression levels of *AIRE* among three subgroups of cell lines according to the genotype of rs2075876. 'G' and 'A' correspond, respectively, to the risk and the alternative alleles of rs2075876.

extensive sequencing in combination with examination of promoter activity will answer this question.

There was no association between *AIRE* and RA in Europeans even in the large-scale meta-analysis of GWA studies with a strong detection power (12). Although the frequency of the risk allele of rs2075876 is much lower in Caucasians (0.15 in Caucasian HapMap results and 0.097 in our own genome scan results) compared with that of the current study (0.34), this does not fully explain the lack of association in Europeans. This suggests that the association of *AIRE* with RA is, like that of *PADI4*, specific to East Asian populations including Japanese. The future validation study using other Asian population will address this issue.

MATERIALS AND METHODS

Study subjects

RA collections 1–3 consisted of 1247 affected individuals and 1486 controls, 1865 cases and 1623 controls, and 2303 cases and 3380 controls, respectively (summarized in Supplementary Material, Table S1). The case subjects of collections 1 and 2 were recruited at the rheumatology departments of Kyoto University Hospital, Dohgo Spa Hospital, Sagamihara National Hospital, Tokyo University Hospital and Tokyo Women's Medical University. The control subjects for collection 1 were from Aichi Cancer Center Hospital and Research Institute and the Department of Ophthalmology and Visual Science at Kyoto University Hospital. DNA samples of healthy Japanese volunteers in collection 2 were from Pharma SNP Consortium (31) and the Center for Genomic Medicine, Graduate School of Medicine, Kyoto University. The case and control subjects in collection 3 were recruited in the Biobank Japan Project at the Institute of Medical Science, the University of Tokyo; the Department of Allergy and Rheumatology, Graduate School of Medicine, the University of Tokyo (32). All cases fulfilled the revised criteria (1987) of the American College of Rheumatology for RA. Among the RA cases, DAS28 score for RA activity in 212 RA patients was obtained at each institution. Written informed consent was obtained from all the participants at the institute of sample collection after being approved for genetic studies by the local ethical committee.

GWA analysis

Genome scan for collection 1 was performed using Infinium Technology (Illumina Inc., San Diego, CA, USA). Case subjects were genotyped with Human-Hap300 (version 1.0, 302 627 SNPs), Human CNV370-Duo (version 1.0, 332 270 SNPs) or Human610-Quad (version 1.0, 577 348 SNPs). For control subjects, they were genotyped with Human610-Quad (version 1.0, 577 348 SNPs) and HumanHap550 (version 3.0, 547 163 SNPs). For validation analysis, Taqman

technology (Life Technologies Corp., Foster City, CA, USA) was employed.

Quality control and statistical tests for case–control association

A total of 277 420 SNPs that were common among the four types of arrays described above were selected for the association study. One thousand two hundred and forty-six cases and 1486 controls with call rate being >0.90 and not showing high degree of kinship (PI_HAT < 0.10 by PLINK) were examined for the association analysis. A total of 241 523 SNPs with call rate >0.95 for both cases and controls and minor allele frequency >0.05 either in the case or in the control were used for the analysis. The case–control association was examined with the Cochran–Armitage trend for each collection as well as for the combined pooled study. Population stratification was examined and corrected with Genomic Control. SNPs that showed P-value $<10^{-3}$ were selected as candidates for further evaluation. SNPs in the *HLA*, *PADI4* and *CCR6* loci were not selected for validation studies. Haploview version 4.1 software (33) was used for LD evaluation, and MapViewer (build 36.3) (34) was used to identify the location and structure of the genes in the region.

Analysis of *AIRE* expression

A gene-expression data set in lymphoblastoid cell lines derived from 210 unrelated HapMap populations was obtained from GEO database (20). The correlation between the expression of *AIRE* and genotypes of SNPs in the region was examined using the calculation program recommended by GEO. The association P-values were obtained by the Joncheere–Terepstra method using R software or SPSS (version 18).

Bioinformatics analysis

Genome sequence alignment of 14 placental mammals was obtained from the UCSC genome browser (http://genome.ucsc.edu). Motif search was carried out by the Jaspar database (35) (http://jaspar.cgb.ki.se) using 'Jasper Core Subset' which contains 138 matrices for known *cis*-acting elements. The matrices were converted into bit scores and used to search against the genomic sequences around the SNP of interest. Identification of orthologs of the *AIRE* gene in different mammals and multiple nucleotide sequence alignment was performed using KEGG SSDB Database (www.genome.jp/kegg/ssdb).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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APPENDIX

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Identification of blood biomarkers of aging by transcript profiling of whole blood

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ABSTRACT

Immunological changes that inevitably occur with aging are related to the onset of various diseases including autoimmune diseases, immunodeficiency, as well as other age-reflecting (AR) diseases. They are becoming serious problems in the global trend of longevity. To understand the AR changes, we searched for genes whose expression profiles in the whole peripheral blood change dramatically as a function of age using the Agilent whole human genome 44K microarray. After examining two cohorts consisting of 154 healthy people between age 23 and 77, we discovered 16 transcripts strongly and reproducibly correlated with age. Analysis using a publicly available gene expression dataset for a variety of human immune cells revealed that some of these transcripts were highly expressed in specific cell types whose number and function are known to change with age. This analysis shed light on the molecular mechanism of AR immunological system changes. Because of its simplicity, the assay system is expected to be useful for understanding individual health conditions.

1. Introduction

Senescent immune imbalance has been well known and is becoming a serious problem in the global trend of longevity. In addition to the typical disease-associated problems such as autoimmune diseases and immunodeficiency, the immune system of our body is steadily following paths of transition from the young to elderly. Causes, consequences, as well as mechanism of the immune system transition have been studied intensively, but these works have not reached systematic studies because of enormous variety of individuality [1–3].

One approach to this problem is to focus on age-reflecting (AR) shift of transcript balance in the peripheral blood mononuclear cells regardless of gender. Although factors that can affect the profile may be countless, such as those organ activities wherein immune cells are produced, nursed, educated, or mobilized to cope with changes of our physiological condition such as infection, damage of the body parts, hormone balance and drug-intake: These factors should be eliminated when we attempt to compare "normal" transcriptome profiles of man.

We undertook transcriptome studies with whole peripheral blood of normal, healthy people. Because isolation of peripheral blood mononuclear cells (PBMC) for analyses introduces technology-associated uncertainties, such as loss of significant fraction of white blood cells, loss of fragile transcriptome during the purification process, etc., we took whole peripheral blood as the starting material for this study.

Using simple correlation analyses, we compared blood from young, mature, and senior people for AR transcripts. We used two cohorts consisting of 154 healthy people, male and female, between age 23 through 77. Excluded were those regularly taking drugs, having been treated by doctors, or having biased biochemical data. We discovered 16 transcripts that behaved in AR fashion: Some were more abundant in older individuals (AR-increase), whereas others were less abundant in older individuals (AR-decrease). Here, we report results of the analyses, and describe some properties of these AR transcripts.

2. Materials and methods

2.1. Participants

Approval for the study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science. Blood samples were obtained from healthy volunteers at two related health examination sites in Ishikawa Prefecture: Hokkoku Clinic (site H), Public Central Hospital of Matto Ishikawa (site M). Of these volunteers, none needed routine doctor's visit nor were treated with any drugs. Informed consent was obtained from all subjects, and clinical data were collected.

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2.2. RNA extraction from blood

Blood samples were collected in PAXgene Blood RNA tubes (BD, NJ, USA), incubated and stored according to the manufacturer's protocol. Total RNA was extracted using the PAXgene Blood RNA Kit (QIAGEN, CA, USA) following the manufacturer's instructions. RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

2.3. Microarray and data preprocessing

Cy3-labeled cRNA was synthesized from 250 ng of total RNA using Agilent Low RNA Input Linear Amplification Kit PLUS, One-Color (Agilent Technologies). After checking the quality and quantity of the cRNA using a NanoDrop ND-1000 spectrophotometer and an Agilent Bioanalyzer, the cRNA was hybridized at 65 °C for 17 hours to an Agilent Whole Human Genome Microarray 4 × 44K (Design ID: 014850). After washing, microarrays were scanned using an Agilent DNA microarray scanner (Agilent Technologies). All procedures from labeling to scanning were performed according to the manufacturer's instructions. Intensity values of each scanned feature were quantified using Agilent Feature Extraction Software, which performs background subtractions. Normalization was performed using Agilent GeneSpring GX version 11.0.2. (per chip normalization: 75 percentile shift; per gene normalization: none).

2.4. Extraction of the age-reflecting transcripts

Analysis was performed with probes annotated with Entrez Gene ID. Only those probes whose expression data were available at least 1 sample were taken for further analyses. Because the analyses were done on different occasions on samples from sites H and M, the batch cluster effect could give rise to bias to the microarray data. Therefore, we handled the data from the two sites independently to identify the AR transcripts. With this precaution, we selected transcripts whose expression levels correlated with age irrespectively of the two cohort sites. To further clarify the issue, Pearson's correlation and its significance between normalized gene expression levels and age were calculated under the R environment (http://cran.at.r-project.org), and transcripts with false discovery rate (FDR) of 0.25 or less in both sites were selected as the AR transcripts. We further confirmed the findings by taking GSE23515 in NCBI Gene Expression Omnibus (GEO) database [4] that included peripheral blood microarray datasets with age information.

2.5. Hierarchical clustering analysis

Hierarchical clustering analyses based on per gene normalization (divided by the median value of all samples for each transcripts) were performed by Spearman rank correlation similarity metric and complete linkage clustering algorithm using TIGR MultiExperiment Viewer software (http://www.tm4.org/). Distance threshold for dividing the cluster was set to 0.65.

2.6. Assignment of the AR transcripts to blood immune cell types

Identification of the source cells expressing the AR transcripts was done with GSE22886, the sets of Affymetrix microarray data from a variety of resting and activated human immune cells in NCBI GEO [4,5]. Probe information was combined with Entrez Gene ID. There were no Affymetrix probes corresponding to AMZ1 (Gene ID: 155185). Replicated data with the same cell type were averaged. Cell types that exceeded 1.5 IQR of the upper quartile were defined as "Expected cell types".

2.7. Real-time quantitative reverse transcription PCR

cDNA was synthesized from 500 ng total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Gene expression was analyzed by real-time quantitative PCR using Applied Biosystems 7900HT real-time PCR system. Specific sets of primers and TaqMan probes were obtained from Applied Biosystems. Gene expression level of the target transcript (CD248) was normalized with the values of an endogenous control gene (GAPDH).

Table 1
Summary of demographics of the subjects enrolled in this study.

	Site H	Site M
Subject number	90	64
Gender		
Male	46	35
Female	44	29
Age	40 ± 10 (min: 23, max: 66)	53 ± 11 (min: 27, max: 77)
BMI (kg/m^2)	21.1 ± 2.3 (min: 16.3, max: 27.5)	22.2 ± 2.7 (min: 16.6, max: 28.5)
sBP (mmHg)	110 ± 11 (min: 90, max: 144)	121 ± 16 (min: 92, max: 156)
dBP (mmHg)	66 ± 9 (min: 50, max: 90)	76 ± 9 (min: 57, max: 97)
AST (IU/L)	20 ± 5 (min: 10, max: 36)	21 ± 5 (min: 13, max: 30)
ALT (IU/L)	18 ± 8 (min: 6, max: 39)	19 ± 6 (min: 10, max: 32)
Glucose (mg/dL)	88 ± 9 (min: 76, max: 127)	94 ± 9 (min: 79, max: 120)
HbA1c (%)	5.0 ± 0.2 (min: 4.5, max: 5.6)	5.2 ± 0.3 (min: 4.2, max: 6.1)
Cholesterol (mg/dL)	188 ± 27 (min: 123, max: 245)	196 ± 29 (min: 134, max: 248)
HDL (mg/dL)	67 ± 14 (min: 40, max: 108)	62 ± 15 (min: 30, max: 94)
Triglycerides (mg/dL)	75 ± 33 (min: 30, max: 192)	89 ± 38 (min: 40, max: 194)
RBC (10^4/μL)	463 ± 46 (min: 345, max: 577)	463 ± 35 (min: 404, max: 569)
Platelet (10^4/μL)	22.8 ± 6.0 (min: 11.3, max: 38.0)	23.9 ± 5.8 (min: 10.6, max: 47.6)
WBC (/μL)	5688 ± 1388 (min: 2900, max: 9300)	4998 ± 1235 (min: 2910, max: 7940)
Neutrophil (/μL)	3455 ± 1095 (min: 1372, max: 6125)	2927 ± 940 (min: 1118, max: 5057)
Eosinophil (/μL)	147 ± 109 (min: 9, max: 539)	151 ± 104 (min: 12, max: 572)
Basophil (/μL)	35 ± 21 (min: 7, max: 112)	21 ± 12 (min: 0, max: 52)
Monocyte (/μL)	296 ± 104 (min: 129, max: 644)	261 ± 75 (min: 90, max: 449)
Lymphocyte (/μL)	1755 ± 505 (min: 896, max: 3572)	1638 ± 499 (min: 698, max: 3008)

Data are expressed as mean ± SD.

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Table 2
List of 11 transcripts whose expression levels are positively correlated with age (AR-increase).

Agilent Probe ID	Gene name	Gene symbol	Gene ID	Site H		Site M		Correlation average	Expected cell types
				Correlation coefficient	FDR	Correlation coefficient	FDR		
A_24_P18137	Neurofilament, light polypeptide	NEFL	4747	0.505	2.17E-03	0.480	1.06E-01	0.492	Memory T-resting (naïve)[a]
A_23_P44674	Cysteine-rich protein 1 (intestinal)	CRIP1	1396	0.419	3.36E-02	0.468	1.07E-01	0.443	NA
A_32_P186731	Isthmin 1 homolog (zebrafish)	ISM1	140862	0.492	2.35E-03	0.391	2.13E-01	0.442	Memory T-resting (naïve)[a], CD4 T cell (naïve and resting)[a]
A_23_P62959	Pleckstrin homology-like domain, family A, member 3	PHLDA3	23612	0.498	2.17E-03	0.385	2.13E-01	0.441	Macrophage-7 day (of differentiatior
A_23_P215048	KIAA0408	KIAA0408	9729	0.419	3.36E-02	0.424	1.99E-01	0.422	B cell (naïve), Helper Th1-12 h (of differentiation)
A_23_P52610	Damage-specific DNA binding protein 2, 48 kDa	DDB2	1643	0.447	1.56E-02	0.396	2.13E-01	0.421	Helper Th2-48 h (of differentiation)
A_23_P92073	Poly(ADP-ribose) polymerase family, member 3	PARP3	10039	0.426	3.08E-02	0.405	2.07E-01	0.415	NA
A_23_P79482	Chimerin (chimaerin) 1	CHN1	1123	0.403	6.26E-02	0.369	2.44E-01	0.386	Memory T-resting (naïve)[a], Memory T-activated[a]
A_23_P391228	Mannosidase, endo-alpha-like	MANEAL	149175	0.381	1.10E-01	0.385	2.13E-01	0.383	NA
A_23_P23924	Calpain 2, (m/II) large subunit	CAPN2	824	0.347	2.12E-01	0.416	2.03E-01	0.382	NA
A_24_P383649	Archaelysin family metallopeptidase 1	AMZ1	155185	0.373	1.30E-01	0.364	2.49E-01	0.368	NA

Correlation coefficients were independently obtained with the site H dataset and site M datasets. Transcripts are arranged according to the "Correlation Average" from the two sites. Assignment of source cells expressing these transcripts are noted in the Materials and methods section.
[a] Over 3 IQR of the upper quartile.

Table 3
List of five transcripts whose expression levels are negatively correlated with age (AR-decrease).

Agilent Probe ID	Gene name	Gene symbol	Gene ID	Site H		Site M		Correlation average	Expected cell types
				Correlation coefficient	FDR	Correlation coefficient	FDR		
A_23_P52697	CD248 molecule, endosialin	CD248	57124	−0.604	3.80E-06	−0.467	1.07E-01	−0.535	CD8 T cell (naïve and resting)[a]
A_24_P314786	Solute carrier family 4, sodium bicarbonate transporter, member 10	SLC4A10	57282	−0.494	2.35E-03	−0.518	4.28E-02	−0.506	B cell (naïve), Memory B-IgM
A_24_P930111	Solute carrier family 4, sodium bicarbonate transporter, member 10	SLC4A10	57282	−0.485	2.90E-03	−0.496	8.62E-02	−0.491	B cell (naïve), Memory B-IgM
A_24_P348806	Pleckstrin homology domain containing, family A member 7	PLEKHA7	144100	−0.350	2.08E-01	−0.479	1.06E-01	−0.415	Plasma B-PBMC [a], Plasma B-bone marrow
A_23_P32444	Matrix-remodelling associated 8	MXRA8	54587	−0.369	1.38E-01	−0.364	2.49E-01	−0.366	Plasma B-bone marrow

Correlation coefficients were independently obtained with the site H dataset and site M dataset. Transcripts are arranged according to the "Correlation Average" from the two sites. Assignment of source cells expressing these transcripts are noted in the Section 2.
[a] Over 3 IQR of the upper quartile.

3. Results

3.1. Clinical characteristic of the two cohorts

Demography of participants in two cohorts of healthy individuals is shown in Table 1. Site H consisted of 46 males and 44 females. Site M consisted of 35 males and 29 females. The balance of gender is deemed reasonable. At site H, the average age of subjects was 40 ± 10, the age range from 23 to 66. At site M, the average age was 53 ± 11, and the age range from 27 to 77. The slight difference in average and distribution of age did not affect the AR studies. As noted, none of these volunteers were suffering from a disease, or had clinical data suggesting the need of medical attention. Bloods were collected using PAXgene Blood RNA tubes, RNA extracted and analyzed using Agilent 44K microarray.

3.2. Identifying the age-reflecting (AR) transcripts

We performed Pearson correlation analyses for the AR transcripts, independently with the H and M datasets. Although members in these two cohorts were not overlapping, we were able to obtain 16 common transcripts that show AR behavior (FDR $\leqslant$ 0.25). The results are shown in Tables 2 and 3, in which transcripts are ordered by the average of the correlation coefficient between the two cohort sites. In Table 2 are listed 11 transcripts whose expression levels are positively correlated with age (AR-increase). The correlation coefficients are 0.505 through 0.347 (FDR: 2.17E-3–2.12E-1) for site H, 0.480 through 0.364 (FDR: 1.06E-1–2.49E-1) for site M. Table 3, on the other hand, lists five transcripts that behave inversely, i.e. their expression levels are negatively correlated with age (AR-decrease). The correlation coefficients were −0.604 through −0.350 (FDR: 3.80E-6–2.08E-1) for site H, −0.518 through −0.364 (FDR: 4.28E-1–2.49E-1) for site M.

Scatter plots of the representative genes, NEFL and CRIP1 (the AR-increase transcripts), and CD248 and SLC4A10 (the AR-decrease transcripts) are shown in Fig. 1. The profiles from sites H and M are nearly superimposable.

To further confirm these findings, we searched the NCBI GEO databases [4] for similar datasets. We found that the GSE23515 data fitted well. Relevant parts of the data are copied in Supplementary

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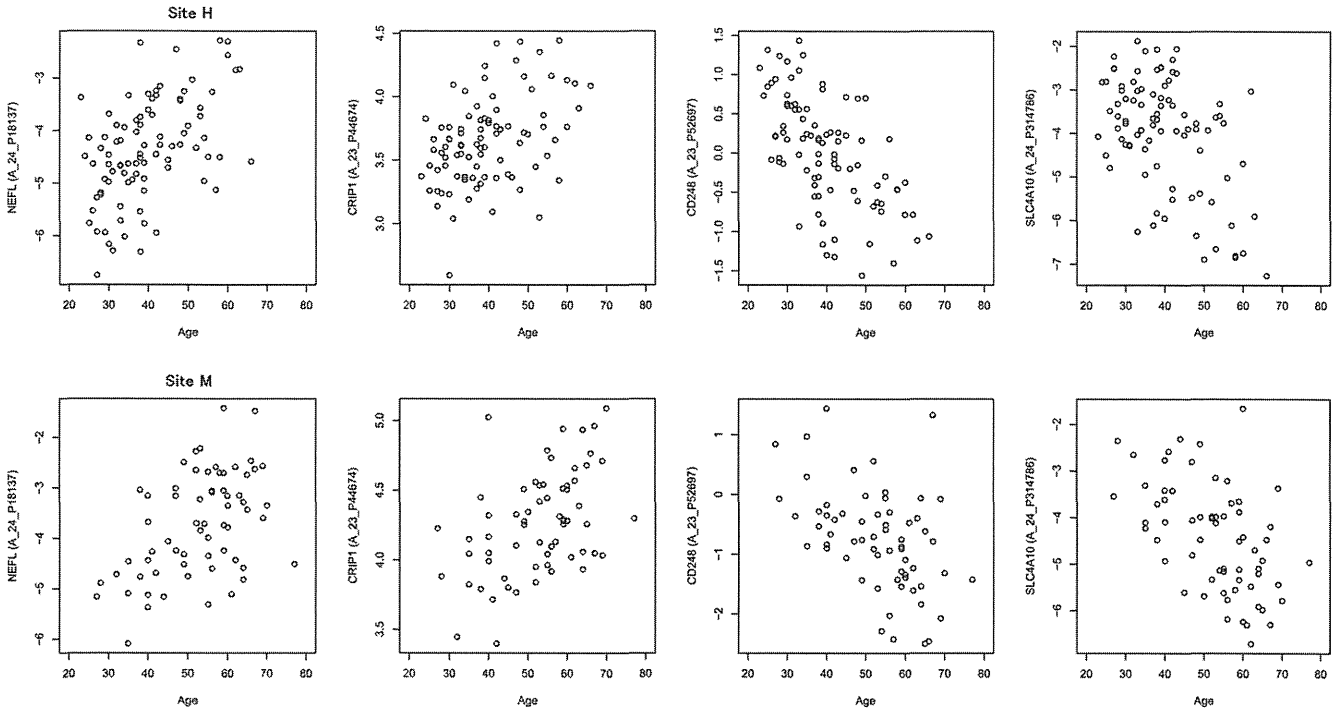

Fig. 1. Representative scatter plots showing correlations between age and some of the AR transcripts. Upper panel shows data from site H, and lower panel data from site M.

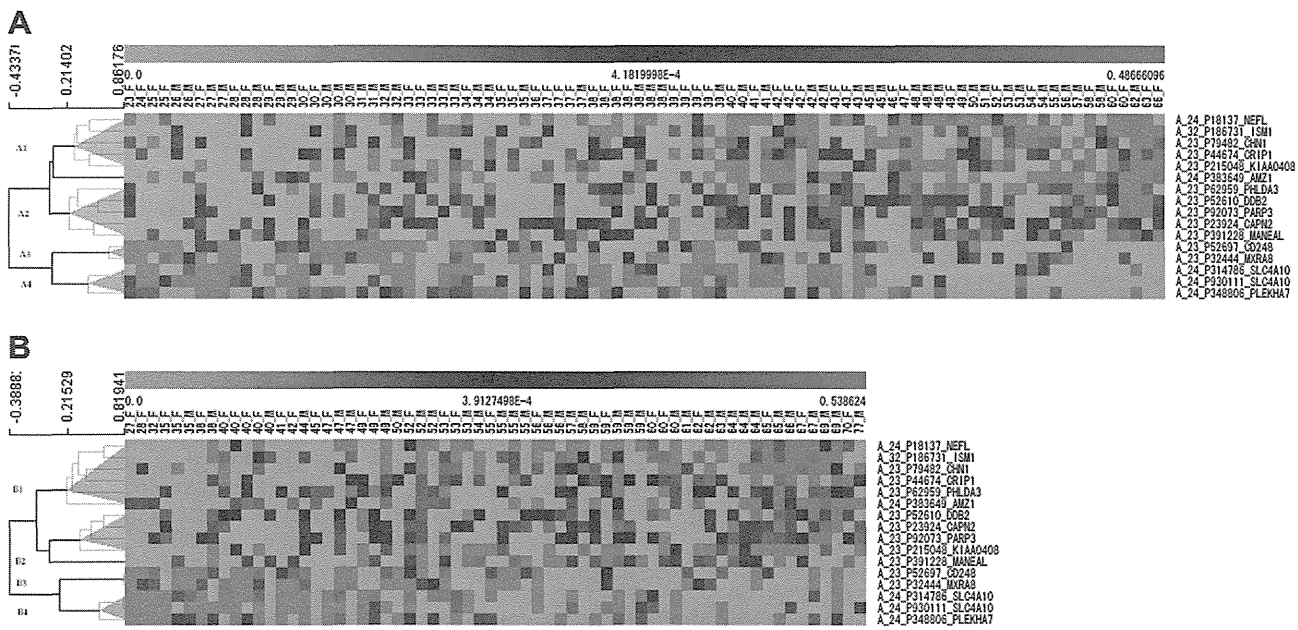

Fig. 2. Hierarchical clustering analysis of 16 AR transcripts based on the expression levels of (A) site H with 90 samples and (B) site M with 64 samples. Details of clustering analyses are described in Materials and methods section. Sample labels represent age and gender.

Tables 1 through 3. Although the subject number is only 24, and the age range is from 21 to 64, the dataset can serve as a perfect source of AR transcripts, and the results showed excellent agreement with ours. For comparison, scattergrams with the four of the transcripts (NEFL, CRIP1, CD248, SLC4A10) are displayed (Supplementary Fig. S1).

3.3. Hierarchical clustering analysis of the AR transcripts

To examine the uniformity/heterogeneity of these and other AR transcripts, we performed hierarchical cluster analysis as shown in Figs. 2A (site H) and 2B (site M). The AR-increase transcripts were grouped into four clusters in Figs. 2A and 2B. Members in cluster A1 show excellent agreement with those in cluster B1. Similarly, members in cluster A2 almost perfectly match with members in clusters B2. As with AR-decrease transcripts, members in A3 and A4 clusters almost perfectly have their counterparts in clusters B3 and B4, respectively. Having these transcripts been extracted independently from the sites H and M, it is highly likely that the AR-increase and AR-decrease transcripts are robust markers, consisting of at least two groups each.

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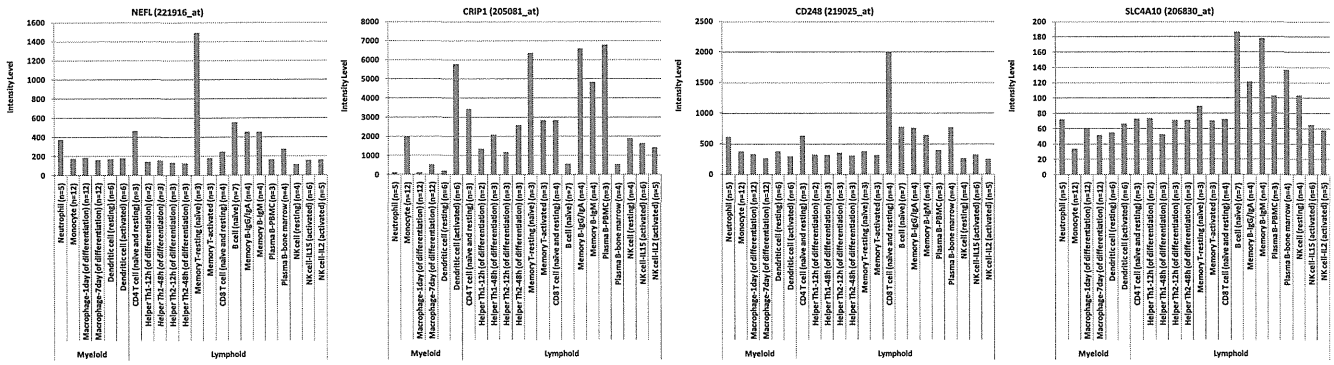

Fig. 3. Expression profiles of the top AR transcripts, NEFL and CRIP1 (AR-increase), and CD248 and SLC4A10 (AR-decrease) in various immune cells under variety of conditions. Texts in parentheses next to the gene symbols indicate Affymetrix probe ID. Data were taken from [4,5].

3.4. Assignment of the AR transcripts to blood immune cell types

Abbas et al. [5] examined gene expression profiles of immune cells under variety of conditions. Although their data were obtained with cultured cells, we took it liberty to refer to these data for assigning our AR transcripts. The selected four AR transcripts in Fig. 1 are displayed in Fig. 3. Other transcripts in Tables 2 and 3 are collectively displayed in Supplementary Fig. S2 in a similar way. The names of referred cells thus obtained are displayed in Tables 2 and 3. Regardless of AR-increase or AR-decrease, significant fractions of the AR transcripts are highly associated with T lymphocytes and B lymphocytes. For example, the top AR-increase transcript, neurofilament light polypeptide (NEFL), is specifically expressed in the memory T resting (naïve) cell, and the top AR-decrease transcript, endosialin (CD248), is highly expressed in the CD8 T (naïve and resting) cell.

3.5. Real-time RT-PCR verification of the AR transcripts

We took CD248, the transcript that showed the strongest correlation with age in microarray expression, and examined it in real-time RT-PCR for verification. We were able to show the expected correlation between this gene expression and age (Fig. 4).

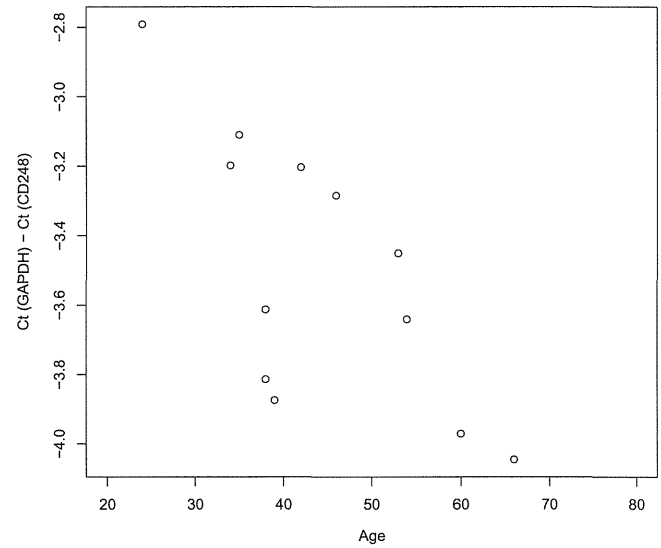

Fig. 4. Real-time RT-PCR verification of the correlation between age and the gene expression level of CD248. Data are shown in scatter plot. The gene expression level of CD248 was normalized to the expression of GAPDH (endogenous control gene).

4. Discussion

Senescence of man and dynamic shift of immune activity have attracted researchers. Studies have been conducted to search for an age-dependent correlation with immune cell differentiation, production, cell population shift in organs or in blood stream [6]. Microarray technologies, which allow transcriptome analyses, have opened the door to a new era in this research field.

We attempted to extract age-reflecting (AR) transcripts in human peripheral blood. The profile of transcripts in the peripheral blood has been believed to be very complex, as population of cells will be unique from a person to person, and will reflect the host's physiological condition, such as disease, infection, injury, fatigue, hormone unbalance and so on, under bewilderingly complex transcriptional controls of immune cells. Nevertheless, we were able to extract AR marker transcripts through cohort studies, independently ran at two sites (90 and 64 participants, age 23–77) using simple correlation analysis between transcripts and age. We found 16 AR transcripts, 11 of which are AR-increase and 5 AR-decrease. Analyses of an another dataset obtained from NCBI GEO have yielded essentially the same results, indicating that these AR transcripts represent a set independent of ethnicity, and can be taken as AR biomarkers of man. Whitney et al. [1] and Eady et al. [7] have attempted similar approaches. Unfortunately, the former authors failed to present a gene list, and the latter authors handled too few samples for qualified data. Furthermore, the latter authors employed isolated peripheral blood mononuclear cells (PBMC), that are liable to errors because of some cell loss, degradation of some mRNA and changes in active genes during purification. As noted, the peripheral blood transcriptome profile is highly affected by donor's physiological condition, such as health condition, exercise, food intake, day rhythm, and many more. Despite these possible factors for complication, our AR transcripts gave rise to reproducible results after taking appropriate precautions.

Most of the genes for AR transcripts are well known, although their roles in AR phenotypes have not been well established. The 16 transcripts do not match the hypothetical longevity genes deduced from SNPs by Sebastiani [8], nor are included in the reference-driven pathway members around sirtuin [9], mTOR [10], Klotho [11]. Though not proved, the 16 AR transcripts may be biomarkers some of which reflect the population change of cells in the blood in the course of aging, and some other reflect gene expression control change within cells. When we examine the 16 transcripts in the GSE22886 dataset in conjunction with the cognate cells, many of them are expressed highly and preferentially in T or B lymphocytes.

Although the roles of the 16 AR marker transcripts in the process of senescence wait further elucidation, we can make some

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inference based on the four clusters of transcripts shown in Fig. 2A and B. Members in Group 1 (A1 and B1 clusters) show AR-increase. At least three of these genes (NEFL, CHN1, ISM1) are uniquely expressed (Over 3 IQR of the upper quartile) in memory T cells (Fig. 3 and Supplementary Fig. S2). Notice that the number of these cells shows increase with aging [12]. Thus, genes in this group may reflect changes in memory T cell number in the aging process.

Group 2 consisted of genes in A2 and B2 clusters. These genes also showed AR-increase in expression. DDB2 and PARP3 included in this group are known for their DNA-repair activities [13,14] or for involvement in cellular accumulation of reactive oxygen species [15], deeply associated with premature senescence. We may be able to infer that the Group 2 genes reflect cellular activity change, rather than cell population shift, caused by senescence.

Group 3 consisted of genes in A3 and B3 clusters. These genes showed AR-decrease expression. No definitive AR functions have been reported for the genes in this group, but CD248 may need special attention: This gene is specifically-expressed in CD8 T cell (Fig. 3), and is active [16]. This gene is also expressed in endothelial progenitor cells [17]. Both cells decreased in number with aging [18,19].

Genes in Group 4, consisting of A4 and B4 clusters, also showed AR-decrease in expression. In cell type assignment analysis, SLC4A10 (Fig. 3) and PLEKHA7 (Supplementary Fig. S2) were highly expressed in B cells. These findings suggest that the decline of gene expression in Group 4 with aging may reflect age-related changes in B cell function.

Because of the paucity of data on gene expression in different cell types in peripheral blood, our argument based on known gene functions in known cell, as above, is incomplete. Nevertheless, we argue that the cluster structures of the 16 transcripts assigned to the four groups (Fig. 2) are stable and relatively homogeneous, demonstrating that a significant number of genes in each group show similar clustering profile. Thus, it may be allowed to take one or several well-characterized genes in each group, such as NEFL (Group 1), DDB2 (Group 2), CD248 (Group 3), and SLC4A10 (Group 4), as representing majority of the genes in each of the four groups. We will be able to discover more AR genes as we extend this line of works. As discussed, some of them may reflect dynamic change of immune cell population, and others may represent emerging/recessing function of immune cells. Further studies on the function of these AR genes and their assignment to cells will provide insights into the roles played by immune system in conjunction with aging of man. In addition, this easy assay system could be used for monitoring the health condition of individuals: For example, the stable balance of the marker set may be taken as reflecting a stable health condition, whereas their distorted proportion could be taken as an alarm of physiological change of the examinee. Notice that fatigue, dementia, unnoticed cancer, drug or supplement intake, and many other temporary or long-lasting physiological changes may induce a balance shift.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.018.

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ORIGINAL ARTICLE
Pulmonary Circulation

Double-Blind, Placebo-Controlled Clinical Trial With a Rho-Kinase Inhibitor in Pulmonary Arterial Hypertension

– A Pilot Efficacy Trial –

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Background: We have previously demonstrated that long-term inhibition of Rho-kinase ameliorates pulmonary arterial hypertension (PAH) in animal models. In the present study, we examined the clinical effects of mid-term oral treatment with an extended release formulation of AT-877 (fasudil hydrochloride), a specific Rho-kinase inhibitor (AT-877ER) on PAH.

Methods and Results: 23 PAH patients were treated with either placebo (10/2 females/males, 51±16 years, idiopathic PAH (IPAH) in 6, PAH associated with connective tissue disease (CTD-PAH) in 3, PAH with congenital heart disease (CHD-PAH) in 2, and portal PAH in 1) or AT-877ER (6/5 females/males, 47±14 years, IPAH in 2, CTD-PAH in 5, and CHD-PAH in 4); 3 patients were excluded. We performed a 6-min walk test and right heart catheterization in the remaining 20 patients, before and 3 months after the treatment (placebo n=11, AT-877ER n=9). Although there were no significant differences between the 2 groups for the 6-min walk distance, pulmonary hemodynamics tended to be improved in the AT-877ER group, especially the prevalence of improved cardiac index from baseline, which was significantly higher in the AT-877ER than in the placebo group. In the AT-877ER group, serum levels of hydroxyfasudil, an active metabolite of AT-877ER tended to correlate with improvements in the cardiac index and mean pulmonary artery pressure.

Conclusions: Mid-term treatment with oral AT-877ER showed additional improvement in pulmonary hemodynamics in patients with PAH. (*Circ J* 2013; **77:** 2619–2625)

Key Words: Pulmonary arterial hypertension; Rho-kinase inhibitor; Signal transduction

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Pulmonary arterial hypertension (PAH), defined as mean pulmonary arterial pressure (PAP) ≥25 mmHg at rest during right heart catheterization,[1–3] is a fatal disease caused by small pulmonary artery obstruction from vascular proliferation and remodeling.[4] PAH is characterized by elevated PAP and increased pulmonary vascular resistance (PVR), frequently leading to right-sided heart failure and death.[4–6] The pathological changes of the pulmonary arteries in PAH include endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells (VSMC), and migration of inflammatory cells.[4,6,7] Anticoagulant agents, vasodilators and lung transplantation are currently used for the treatment of PAH, but more effective treatment needs to be developed.[6,8,9]

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In mid-1990s, 2 Japanese groups and 1 Singapore group independently identified Rho-kinase/ROK/ROCK as an effecter of the small GTP-binding protein Rho,[10–12] which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expressions.[13–15] We and others have demonstrated that Rho-kinase activation is substantially involved in the pathogenesis of cardiovascular diseases. First, the Rho-kinase pathway plays an important role in various cellular functions in response to various vasoactive substances.[14] Second, the so-called pleiotropic effects of statins, especially of high-doses of statins, may be mediated, at least in part, by their inhibitory effects on Rho, with a resultant inhibition of Rho-kinase.[14] Third, the effectiveness of AT877 (fasudil hydrochloride), a specific Rho-kinase inhibitor, for PAH has been demonstrated.[6,16–20]

In the present study, we examined the effects of mid-term oral treatment with an extended-release formulation of AT-877 (AT-877ER) in patients with PAH.

Methods

This study was a phase IIa clinical trial with fasudil for pulmonary arterial hypertension conducted by Asahi Kasei Pharma Corporation (Tokyo, Japan).

The ethics committees of all participating institutes approved the study protocol and all patients provided written informed consent. This report follows the recommendations of the 2010 Consolidated Standards of Reporting Trials Statement.

Study Patients

Patients with PAH were included when they had a baseline 6-min walk distance of ≥150 m with WHO functional class I–III. However, patients were excluded if they had received treatment with epoprostenol sodium, vardenafil hydrochloride hydrate, or tadalafil, or if they had changed dosages of bosentan, beraprost sodium, sildenafil citrate, warfarin potassium, calcium antagonists, cardiac glycosides or diuretics and/or doses of oxygen and nitrogen monoxide within 30 days, or had been started on any such regimens within 30 days prior to consent (for warfarin potassium, however, dosage modification as adjustment of the international normalized ratio of prothrombin was allowed). Further, patients were excluded if they had received concomitant treatment with bosentan and sildenafil citrate within 30 days prior to their informed consent. Patients with serum creatinine levels exceeding the upper limit of the study site's reference range were also excluded.

Study Design

From the viewpoint of feasibility, the sample size was planned as 30 patients in total (10 patients for WHO functional class I and 20 for functional classes II–III. The present study was designed as a 3-month, double-blind, randomized, placebo-controlled, multicenter trial in which 14 PAH centers in Japan participated. All patients were hospitalized 3–6 days before the first examination (day 1) and the last examination (week 12) (**Figure S1**). Administration of the study drug was started and ended during the hospitalization periods. Patients received either AT-877ER or placebo capsule twice daily (Asahi Kasei Pharma Corporation, Tokyo, Japan) for 12 weeks in a blind manner (**Figure S1**). The dosage of AT-877ER was increased every 3 days in a stepwise manner from 2 to 6 capsules/day (**Figure S1**). All patients were administered 2 capsules/day until day 4, when the dose was increased by the investigator's decision to 4 capsules/day. Until day 7, 4 capsules/day were given and the next doses were decided by investigators on day 7. Before increasing the study drug on days 4, 7, and 10, investigators checked the safety of the treatment in each subject and determined the subsequent treatment plan as follows. Whenever it was difficult to follow the intended regimen because of adverse effects or other reasons, the situation was required to be judged as "continuation at the dose level at the time of occurrence of the adverse drug reaction", "continuation with reduced dosage", or "discontinuation of study treatment". The treatment was randomized according to the 6-min walk distance, with drugs prescribed at baseline as stratifying factors, and used minimization with a randomized method. The 6-min walk distance was assessed before drug administration and at 4, 8 and 12 weeks of administration of the study drug. Cardiac catheterization was performed on the first and last days of the treatment protocol (**Figure S1**).

Diagnosis of Pulmonary Hypertension

PAH was defined as mean PAP ≥25 mmHg with pulmonary capillary wedge pressure (PCWP) ≤15 mmHg at rest.[1,2,6] Connective tissue disease and liver disease were diagnosed clinically and by blood tests. Congenital heart disease was diagnosed by echocardiography, and chronic thromboembolic pulmonary hypertension was diagnosed by ventilation-perfusion RI scans and computed tomography (CT).[6] Pulmonary function tests, arterial blood gases, chest X-ray and CT scan were used to diagnose lung disease and hypoxia. When the aforementioned abnormalities were ruled out, the patients were diagnosed as having idiopathic PAH (IPAH).[2,7] Heritable PAH was diagnosed as IPAH with a family history of PAH.[2,7,21]

Data Collection

Baseline demographic information (including age, sex, height and body weight), clinical diagnosis, comorbidities (connective tissue disease, liver disease, congenital heart disease, and thyroid dysfunction) and hemodynamic data from catheterization were recorded for each patient. Hemodynamic parameters examined included PCWP, PAP, right atrial pressure (RAP), cardiac output (CO), cardiac index (CI), systolic, diastolic and mean blood pressures, PVR, systolic vascular resistance, and mixed venous oxygen saturation. Blood data, including serum levels of creatinine and N-terminal pro-brain natriuretic peptide (NT-pro-BNP), plasma levels of BNP and other renal and liver functions, and urinary data were also collected.

To measure CO, both thermodilution and the Fick method were performed in 7 patients in the placebo group and in 4 in the AT-877ER group. In the present study, thermodilution data

Table 1. Baseline Characteristics of the Patients

	Placebo (n=12)	AT-877ER (n=11)
Age (years)	51.4±16.2	47.4±14.2
Female/Male (n)	10/2	6/5
Weight (kg)	51.0±7.9	62.9±12.6
WHO functional class (n)		
I	2	0
II	9	9
III	1	2
IV	0	0
Cause of PAH (n)		
Idiopathic	6	2
Connective tissue disease	3	5
Congenital heart disease	2	4
Portal hypertension	1	0
Background therapy		
Naive	2	0
Naive within 1 month of written informed consent	1	1
Beraprost alone	4	3
Bosentan	4	5
Sildenafil	1	2
Diuretics		
Without	8	5
With	4	6
Warfarin potassium		
Without	5	5
With	7	6
Oxygen therapy		
Without	5	3
With	7	8
Duration of PAH (years)		
<1	5	4
1–10	4	4
≥10	3	3
Pulmonary arterial pressure (mmHg)	47.2±14.3	40.5±17.2
Pulmonary vascular resistance (dyne·s^{-1}·cm^{-5})	865.7±476.9	687.9±550.3
Cardiac index (L·min^{-1}·m^{-2})	2.456±0.542	2.609±1.035
Arterial oxygen saturation (%)	95.56±2.58	94.63±2.30
Oxygen saturation of pulmonary artery (%)	70.18±6.36	70.30±6.18
6-min walk distance (m)	397.3±107.7	392.9±107.5
BNP (pg/ml)	119.3±140.5*	107.2±236.3
Creatinine (mg/dl)	0.65±0.11	0.77±0.18

Data are shown as mean±SD. *n=11.
BNP, B-type natriuretic peptide; PAH, pulmonary arterial hypertension.

acquired by the same technique during cardiac catheterization on the first and the last days of the treatment protocol took precedence over that acquired with the Fick method.

Blood samples were taken before oral administration of placebo or AT-877ER to measure the blood concentration of hydroxyfasudil, an active metabolite of fasudil, on the same day as cardiac catheterization was performed.[18]

Efficacy Endpoint

Efficacy was judged as a change in pulmonary hemodynamics and 6-min walk distance from baseline after 12 weeks of therapy.

Statistical Analysis

The analysis data set comprised all randomized patients who received at least 1 dose of the study medications. No estimation of missing data was performed. Demographics and baseline characteristics were summarized with descriptive statistics, including mean and standard deviation (SD) for continuous variables or counts and percentages for categorical variables. Changes from baseline for the hemodynamic parameters and 6-min walk distance were summarized with mean and SD. Student's t-test was performed for comparison of the AT-877ER and placebo treatment groups, and 2-sided 95% confidence intervals for the difference between treatment groups were calculated. Percent changes from baseline were also analyzed in a similar manner. Treatment comparison of the pro-

Table 2. Changes in Cardiac Hemodynamics and 6-min Walk Distance in the Placebo and AT-877ER Groups at Week 12

	Change from baseline		Difference between groups	
	Placebo (n=11)	AT-877ER (n=9)	Difference (95% CI)	P value*
Mean pulmonary arterial pressure (mmHg)	2.2±8.6 (11)	−0.6±2.9 (9)	−2.7 (−8.7 to 3.2)	0.3398
Pulmonary vascular resistance (dyne·s^{-1}·cm^{-5})	72.2±252.1 (11)	−31.8±137.6 (9)	−104.0 (−301.5 to 93.4)	0.2829
Cardiac index (L·min^{-1}·m^{-2})	0.09±0.397 (11)	0.368±0.496 (9)	0.278 (−0.141 to 0.697)	0.1805
Arterial oxygen saturation (%)	−0.48±3.61 (10)	−0.96±2.68 (9)	−0.48 (−3.58 to 2.63)	0.7507
Oxygen saturation of pulmonary artery (%)	−0.53±4.75 (11)	−2.10±3.48 (9)	−1.57 (−5.57 to 2.42)	0.4192
6-min walk distance (m)	31.3±47.9 (12)	18.9±32.3 (9)	−12.4 (−51.1 to 26.4)	0.5131

Data are shown as mean±SD. *t-test.
95% CI, 95% confidence interval.

Table 3. Categorical Counting for Cardiac Index

	Change from baseline >0		Difference between groups	
	Placebo (n=11) % (n/N)	AT-877ER (n=9) % (n/N)	Difference (95% CI)*	P value#
Cardiac index (L·min^{-1}·m^{-2})	45.5% (5/11)	88.9% (8/9)	43.4% (7.6 to 79.3)	0.0428

*No continuity correction; #Chi-square test.

portion of patients who showed improvement in their CI from baseline was performed using the chi-square test. No multiplicity adjustment was performed. All statistical analyses were performed using SAS (version 9.1.3 or later, SAS Institute, Cary, NC, USA).

Results

Patient Enrollment

Of the 34 patients included in this trial, 32 were enrolled for randomization of treatment with either AT-877ER or placebo (Figure S2); 2 patients failed to meet the inclusion criteria. After randomization, 23 of the 32 patients started receiving the study drug, because 5 patients in the AT-877ER group and 4 in the placebo group were excluded according to the inclusion/exclusion criteria (mean PAP <25 mmHg in 4 in the AT-877ER group and in 3 in the placebo group; PCWP >15 mmHg in 1 in the placebo group; serum creatinine level exceeding the upper limit in 1 in the AT-877ER group) (Figure S2). Of the 23 patients, 11 were randomized into the AT-877ER group and 12 as the placebo group (Figure S2), and of them, 9 patients in the AT-877ER group and 11 in the placebo group completed the treatment; 2 patients in the AT-877ER group discontinued the treatment because of the adverse events of renal impairment and heart failure death, respectively, and 1 in the placebo group because of an investigator's decision (Figure S2).

Baseline Patient Characteristics

There were 2 males and 10 females in the placebo group and 5 males and 6 females in the AT-877ER group (Table 1). Age, WHO functional class, type of PAH, combination treatment, and pulmonary hemodynamics are listed in Table 1. In the placebo group, 6 had IPAH, 3 had PAH associated with connective tissue disease (CTD-PAH), 2 had PAH with congenital heart disease (CHD-PAH), and 1 had portal hypertension PAH. In the AT-877ER group, there were 2 cases of IPAH, 5 of CTD-PAH, and 4 of CHD-PAH. There were 3 naive patients in the placebo group and 1 in the AT-877ER group (Table 1).

Tolerance of the Trial Drugs

In the placebo group, 1 patient received 2 capsules/day, 2 patients had 4 capsules/day, and 8 patients had 6 capsules/day, while in the AT-877ER group, 3 patients received 2 capsules/day, 3 patients had 4 capsules/day, and 3 patients had 6 capsules/day; 2 of these patients discontinued the treatment because of renal impairment and heart failure death, respectively, both on day 10. In the placebo group of 6 capsules/day, 1 patient discontinued the treatment on day 84 because of an investigator's decision.

Hemodynamic Parameters

Baseline mean PAP and PVR were lower and baseline CI was higher in the AT-877ER group than in the placebo group (Table 1). After the 3-month study period, mean PAP and PVR tended to be improved in the AT-877ER group compared with the placebo group (Table 2; Tables S1,S2). The incidence of a CI change from baseline was significantly improved in the AT-877ER group compared with the placebo group (Table 3).

Serum Levels of Hydroxyfasudil and Pulmonary Hemodynamics in the AT-877ER Group

In the AT-877ER group, serum levels of hydroxyfasudil, an active metabolite of fasudil, were dose-dependent of AT-877ER (Figure A). Further, serum levels of hydroxyfasudil tended to correlate with the improvements in CI (Figure B) and mean PAP (Figure C), but not of PVR (Figure D).

Safety

Adverse events occurred in all patients in the treatment and placebo groups (Table S3). The patient in the AT-877ER group who died had comorbid heart failure, and a causal relationship with the study drug was ruled out. Three patients experienced serious adverse events other than death (1 in the AT-877ER group and 2 in the placebo group). Pulmonary edema and pleural effusion occurred in 1 patient in the AT-877ER group with resultant death and a causal relationship with the study drug was not definite but possible. Idiopathic thrombocytopenic purpura and increased BNP occurred in 2 patients, respectively, in the placebo group.