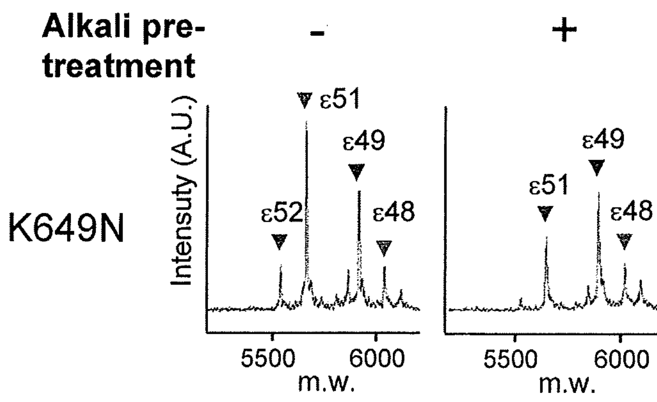
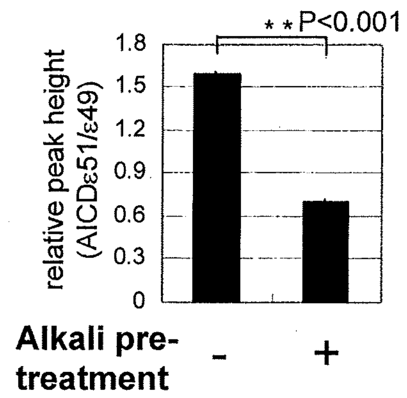
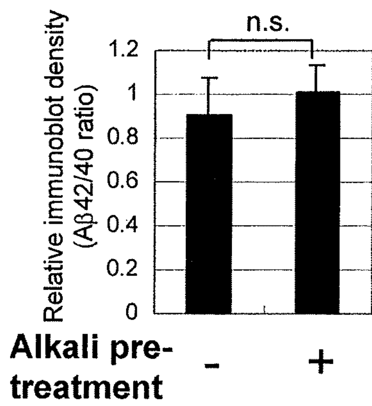
A





B



Mori et al., Figure 4

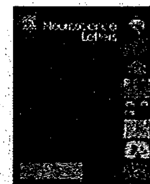
Author's personal copy

Neuroscience Letters 461 (2009) 177–180

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet



SORL1 is genetically associated with Alzheimer disease in a Japanese population

Ryo Kimura [a,b,*], Mitsuko Yamamoto [b], Takashi Morihara [b], Hiroyasu Akatsu [c], Takashi Kudo [b], Kouzin Kamino [b,d], Masatoshi Takeda [b]

[a] *Department of Psychiatry, Osaka General Medical Center, Osaka, Japan*
[b] *Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[c] *Choju Medical Institute, Fukushimura Hospital, Toyohashi, Aichi, Japan*
[d] *Shoraiso National Hospital, Yamatokoriyama, Nara, Japan*

ARTICLE INFO

Article history:
Received 5 April 2009
Received in revised form 18 May 2009
Accepted 1 June 2009

Keywords:
Alzheimer disease
Association
SORL1
Apolipoprotein E

ABSTRACT

A recent study reported that variants of the neuronal sortilin-related receptor gene (*SORL1*) increased the risk of late-onset Alzheimer disease (AD) in several populations. Here, we examined the risk effect in a large, well-characterized group of 437 late-onset AD patients and 451 control subjects in a Japanese population. Among eight single-nucleotide polymorphisms (SNPs) of the *SORL1* gene for which association has been reported, we found a significant association for four of them, located between exon 24 and intron 37. This risk was evident in non-carriers of the apolipoprotein E-ε4 allele, but not in its carriers. Our results support the evidence that genetic variants of *SORL1* affect susceptibility to late-onset AD.
© 2009 Elsevier Ireland Ltd. All rights reserved.

Alzheimer disease (AD) is the most common cause of dementia in the elderly and is characterized by progressive cognitive decline with brain atrophy. The main pathological features of AD are neurofibrillary tangles and senile plaques, caused by the progressive deposition of amyloid β protein (Aβ) in the brain. It is believed that β-amyloid contributes to the development of AD by disturbing synaptic function, leading to neuronal death [23]. In the case of late-onset AD (LOAD), possession of the e4 allele of the apolipoprotein E (APOE) gene (*APOE-ε4*) is the major genetic risk in multiple genetic backgrounds, suggesting that lipid metabolism is a major concern in the development of LOAD [6]. Recently, it was shown that the sortilin-related receptor 1 (*SORL1*) is genetically associated with late-onset AD in several populations [22]. SORL1 is a member of the low-density lipoprotein receptor family, which binds apolipoprotein E. SORL1 functions as a sorting and trafficking protein, guiding β-amyloid precursor protein (APP) into the recycling endosome pathways that result in reduced Aβ production [2,5]. In addition, *SORL1* gene expression was reduced in sporadic AD, but not in familial AD [7].

Here, we conducted a case-control study to investigate whether single-nucleotide polymorphisms (SNPs) of the *SORL1* gene are associated with the risk of LOAD among Japanese.

Patients with LOAD ($N=437$, mean age of onset of 72.0 ± 8.2 years, 69.6% female) were diagnosed as having definite or probable AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [17]. Control subjects ($N=451$, mean age 74.7 ± 5.9 years, 61.6% female) were recruited from among elderly people in the general population. Written informed consent to participate in this study was obtained, and peripheral blood was then drawn and subjected to DNA extraction. The patients and controls were age- and sex-matched between the two groups. The procedure used to obtain the specimens was approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine and the Choju Medical Institute of Fukushimura Hospital. Eight SNPs examined in the original report [22] were genotyped: the numbering of the SNPs in this study followed the description by Rogaeva et al. Genotyping was performed by a quantitative genotyping method using the TaqMan SNP Genotyping System [8]. All probes and primers were designed by the Assay-by-Design service of Applied Biosystems. The *APOE* genotype was determined by a PCR-RFLP method [8].

Hardy–Weinberg equilibrium tests were conducted for all SNPs for cases and controls. Allele and genotype frequencies were assessed for associations by the one-sided chi-square test. As we aimed to replicate published results by original report [22], normal P values which were less than 0.05 were considered statically significant. Odds ratios (OR) for risk allele carriers are presented with 95% confidence intervals. The linkage disequilibrium (LD) structure among the *SORL1* SNPs was examined using Haploview software (http://www.broad.mit.edu/haploview/haploview) [3]. Haplotype

* Corresponding author at: Department of Psychiatry, Osaka General Medical Center, 3-1-56 Bandai Higashi, Sumiyoshi-ku, Osaka 558-8558, Japan.
Tel.: +81 6 6692 1201; fax: +81 6 6606 7000.
E-mail address: kimura@psy.med.osaka-u.ac.jp (R. Kimura).

0304-3940/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2009.06.014

Author's personal copy

blocks were defined using the confidence intervals algorithm. Default settings were used in these analyses, which create 95% confidence bounds on D' to delineate SNP pairs in strong LD.

We genotyped eight SNPs in the *SORL1* gene: rs661057 (SNP4), rs668387 (SNP8), rs689021 (SNP9), rs12285364 (SNP12), rs2070045 (SNP19), rs3824968 (SNP23), rs2282649 (SNP24), and rs1010159 (SNP25). The genotype frequencies of all the SNPs followed the Hardy–Weinberg equilibrium. The allelic frequencies for the eight SNPs in both cases and controls are shown in Table 1. We observed significant associations for SNPs 19, 23, 24, and 25 in the allelic association test. In particular, SNP24, the T allele was more significantly associated with an increased risk of LOAD ($P = 0.009$, odds ratio = 1.6). The degree of linkage disequilibrium between the *SORL1* SNPs is shown in Fig. 1.

To examine the interaction between *SORL1* and *APOE*, we stratified according to the presence or absence of the *APOE-ε4* allele. In our datasets, the *APOE-ε4* allele was associated with LOAD ($P < 1 \times 10^{-10}$): compared to non-*APOE-ε4* carriers, the odds ratio for carrying one *APOE-ε4* allele was 4.3, and that for carrying two *APOE-ε4* allele was 28.4. In non-*APOE-ε4* carriers, three SNPs (23, 24, 25) showed significant association with LOAD (Table 2). However, no association was found in *APOE-ε4* carriers (Table 2), indicating that the association of *SORL1* is specific for non-*APOE-ε4* carriers in our dataset.

Our results provide an independent replication of the association between *SORL1* and LOAD in a Japanese population. The original report of this association included case-control studies in Northern Europeans, Caucasian Americans, Caribbean Hispanics, and Israeli Arabs [22]. Since then, replication studies in other cohort [10] have given both positive [4,11,12,14,15,18,26] and negative results [19]. We found that *SORL1* was genetically associated with LOAD, and this association was significant specifically in non-*APOE-ε4* carriers. This risk effect seems to be smaller than the effect of the *APOE* genotype because the odds ratio was close to 1.6. Although we did not find a risk effect of *SORL1* in *APOE-ε4* carriers, it is possible that sample size among individuals with *APOE-ε4* could have affected our results.

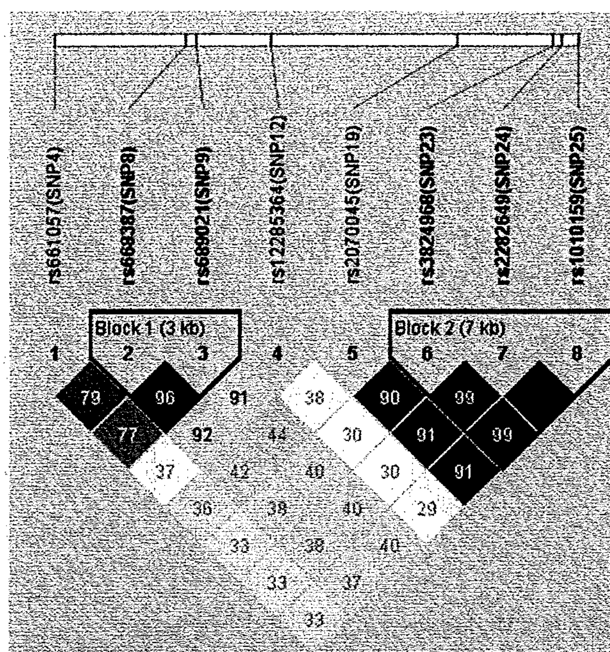


Fig. 1. Linkage disequilibrium between the *SORL1* SNPs. The number within each box represents the pairwise LD as assessed by D'.

A recent study found two SNPs (8 and 24) were significantly associated with AD in a Japanese population [13,25]. These results are similar to those of our study. Importantly, for SNP 24, the T allele was associated with AD in Caucasians as well as in the Japanese population. Therefore, our study provides evidence supporting the association of the *SORL1* gene with LOAD.

SORL1 is essential for protein transport among the plasma membrane, endosomes and late Golgi compartments [27]. In particular, SORL1 binds APOE and regulates intracellular trafficking

Table 1
Association analysis of LOAD in the *SORL1* gene.

SNP	SNP ID	SNP type	AD status	N	Genotype number			MAF	Allelic P	OR (95% CI)
					TT	TC	CC			
4	rs661057	Intron 1	LOAD	436	107	216	113	0.507	0.667	
			Controls	449	119	214	116	0.497		
					TT	TC	CC			
8	rs668387	Intron 6	LOAD	435	121	219	95	0.470	0.456	
			Controls	451	127	208	116	0.488		
					AA	AG	GG			
9	rs689021	Intron 6	LOAD	437	89	215	133	0.450	0.442	
			Controls	451	107	208	136	0.468		
					CC	CT	TT			
12	rs12285364	Intron 9	LOAD	436	291	129	16	0.185	0.075	
			Controls	451	326	112	13	0.153		
					GG	GT	TT			
19	rs2070045	Exon 25	LOAD	437	94	240	103	0.490	0.025	1.53 (1.13, 2.05)
			Controls	450	87	219	144	0.437		
					AA	AT	TT			
23	rs3824968	Exon 34	LOAD	436	98	243	95	0.503	0.022	1.57(1.16,2.12)
			Controls	451	91	223	137	0.449		
					CC	CT	TT			
24	rs2282649	Intron 38	LOAD	432	92	241	99	0.508	0.009	1.61 (1.19, 2.19)
			Controls	451	137	226	88	0.446		
					CC	CT	TT			
25	rs1010159	Intron 39	LOAD	432	98	241	93	0.506	0.019	1.58 (1.16, 2.14)
			Controls	450	91	223	136	0.450		

MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval.

Author's personal copy

Table 2
Genotypic and allelic distribution for the *APOE-ε4* negative and positive subgroups.

SNP	SNP ID	SNP type	AD status	Non-APOE-ε4 carriers							APOE-ε4 carriers					
				N	Genotype number			MAF	Allelic P	OR (95% CI)	*N*	Genotype number			MAF	Allelic P
					TT	TC	CC					TT	TC	CC		
4	rs661057	Intron 1	LOAD	223	50	115	58	0.482	0.639		213	57	101	55	0.495	0.433
			Controls	379	98	180	101	0.496			70	21	34	15	0.457	
					TT	TC	CC					TT	TC	CC		
8	rs668387	Intron 6	LOAD	222	67	113	42	0.444	0.125		213	54	106	53	0.498	0.736
			Controls	379	107	173	99	0.489			72	20	30	22	0.514	
					AA	AG	GG					AA	AG	GG		
9	rs689021	Intron 6	LOAD	223	38	115	70	0.428	0.163		214	51	100	63	0.472	0.777
			Controls	379	88	180	111	0.470			72	19	28	25	0.458	
					CC	CT	TT					CC	CT	TT		
12	rs12285364	Intron 9	LOAD	223	154	65	4	0.164	0.668		213	137	64	12	0.207	0.109
			Controls	379	272	97	10	0.154			72	54	15	3	0.146	
					GG	GT	TT					GG	GT	TT		
19	rs2070045	Exon 25	LOAD	223	45	121	57	0.473	0.218		214	49	119	46	0.507	0.149
			Controls	378	70	190	118	0.437			72	17	29	26	0.438	
					AA	AT	TT					AA	AT	TT		
23	rs3824968	Exon 34	LOAD	223	52	124	47	0.511	0.035	1.57 (1.06, 2.32)	213	46	119	48	0.495	0.362
			Controls	379	73	194	112	0.449			72	18	29	25	0.451	
					CC	CT	TT					CC	CT	TT		
24	rs2282649	Intron 38	LOAD	220	45	124	51	0.514	0.026	1.63 (1.10, 2.42)	212	47	119	46	0.498	0.212
			Controls	379	112	195	72	0.447			72	25	31	16	0.438	
					CC	CT	TT					CC	CT	TT		
25	rs1010159	Intron 39	LOAD	219	50	124	45	0.511	0.040	1.61 (1.08, 2.39)	213	48	119	46	0.505	0.269
			Controls	378	73	194	111	0.450			72	18	29	25	0.451	

MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval.

Author's personal copy

of β-amyloid precursor protein [20]. Several genome-wide studies have reported an association of LOAD with the dynamin-binding protein gene (*DNMBP*) and the dynamin2 gene (*DNM2*) [1,9]. These genes are involved in the pathway of vesicle trafficking and membrane transport [21,24]. Further, expression of *DNMBP* and *DYM2* mRNA in brain tissue from patients with LOAD was decreased, as was *SORL1* mRNA [1,7,9]. Therefore, trafficking and recycling dysfunction can lead to the development of LOAD. Furthermore, it has been recently reported that supplementation with DHA increases SORL1, leading to prevention of the progression of AD [16].

Our findings provide further evidence of genetic variations in *SORL1* affecting susceptibility to LOAD. Further studies using functional analysis, such as studies on cognitive behavior in AD mouse models and changes in pathological findings, are required in order to identify the role of *SORL1* in the development of AD.

Conflict of interest

We have no conflicts of interest to declare.

Acknowledgements

We thank Drs. Y. Ikejiri, T. Nishikawa, H. Yoneda, Y. Moto, A. Sawa, S. Fujinaga, T. Matsubayashi, K. Taniguchi, Y. Ikemura and T. Mori for clinical evaluation. This work was funded by the Future Program and the Japan Society for the Promotion of Science (JSPS), and by a Grant-in-Aid for Scientific Research on Priority Areas "Applied Genomics" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] N.J. Aidaralieva, K. Kamino, R. Kimura, M. Yamamoto, T. Morihara, H. Kazui, R. Hashimoto, T. Tanaka, T. Kudo, T. Kida, J. Okuda, T. Uema, H. Yamagata, T. Miki, H. Akatsu, K. Kosaka, M. Takeda, Dynamin 2 gene is a novel susceptibility gene for late-onset Alzheimer disease in non-APOE-epsilon4 carriers, J. Hum. Genet. 53 (2008) 296–302.

[2] O.M. Andersen, J. Reiche, V. Schmidt, M. Gotthardt, R. Spoelgen, J. Behlke, C.A. von Arnim, T. Breiderhoff, P. Jansen, X. Wu, K.R. Bales, R. Cappai, C.L. Masters, J. Gliemann, E.J. Mufson, B.T. Hyman, S.M. Paul, A. Nykjaer, T.E. Willnow, Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precu rsor protein, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 13461–13466.

[3] J.C. Barrett, B. Fry, J. Maller, M.J. Daly, Haploview: analysis and visualization of LD and haplotype maps, Bioinformatics 21 (2005) 263–265.

[4] K. Bettens, N. Brouwers, S. Engelborghs, P.P. De Deyn, C. Van Broeckhoven, K. Sleegers, SORL1 is genetically associated with increased risk for late-onset Alzheimer disease in the Belgian population, Hum. Mutat. 29 (2008) 769–770.

[5] C. Bohm, N.M. Seibel, B. Henkel, H. Steiner, C. Haass, W. Hampe, SorLA signaling by regulated intramembrane proteolysis, J. Biol. Chem. 281 (2006) 14547–14553.

[6] E.H. Corder, A.M. Saunders, W.J. Strittmatter, D.E. Schmechel, P.C. Gaskell, G.W. Small, A.D. Roses, J.L. Haines, M.A. Pericak-Vance, Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families, Science 261 (1993) 921–923.

[7] S.E. Dodson, M. Gearing, C.F. Lippa, T.J. Montine, A.I. Levey, J.J. Lah, LR11/SorLA expression is reduced in sporadic Alzheimer disease but not in familial Alzheimer disease, J. Neuropathol. Exp. Neurol. 65 (2006) 866–872.

[8] R. Kimura, K. Kamino, M. Yamamoto, A. Nuripa, T. Kida, H. Kazui, R. Hashimoto, T. Tanaka, T. Kudo, H. Yamagata, Y. Tabara, T. Miki, H. Akatsu, K. Kosaka, E. Funakoshi, K. Nishitomi, G. Sakaguchi, A. Kato, H. Hattori, T. Uema, M. Takeda, The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between beta-amyloid production and tau phosphorylation in Alzheimer disease, Hum. Mol. Genet. 16 (2007) 15–23.

[9] R. Kuwano, A. Miyashita, H. Arai, T. Asada, M. Imagawa, M. Shoji, S. Higuchi, K. Urakami, A. Kakita, H. Takahashi, T. Tsukie, S. Toyabe, K. Akazawa, I. Kanazawa, Y. Ihara, Dynamin-binding protein gene on chromosome 10q is associated with late-onset Alzheimer's disease, Hum. Mol. Genet. 15 (2006) 2170–2182.

[10] J.H. Lee, S. Barral, C. Reitz, The neuronal sortilin-related receptor gene SORL1 and late-onset Alzheimer's disease, Curr. Neurol. Neurosci. Rep. 8 (2008) 384–391.

[11] J.H. Lee, R. Cheng, L.S. Honig, J.P. Vonsattel, L. Clark, R. Mayeux, Association between genetic variants in SORL1 and autopsy-confirmed Alzheimer disease, Neurology 70 (2008) 887–889.

[12] J.H. Lee, R. Cheng, N. Schupf, J. Manly, R. Lantigua, Y. Stern, E. Rogaeva, Y. Wakutani, L. Farrer, P. St George-Hyslop, R. Mayeux, The association between genetic variants in SORL1 and Alzheimer disease in an urban, multiethnic, community-based cohort, Arch. Neurol. 64 (2007) 501–506.

[13] J.H. Lee, N. Shibata, R. Cheng, R. Mayeux, Possible association between SORL1 and Alzheimer disease? Reanalysing the data of Shibata et al., Dement. Geriatr. Cogn. Disord. 26 (2008) 482.

[14] Y. Li, C. Rowland, J. Catanese, J. Morris, S. Lovestone, M.C. O'Donovan, A. Goate, M. Owen, J. Williams, A. Grupe, SORL1 variants and risk of late-onset Alzheimer's disease, Neurobiol. Dis. 29 (2008) 293–296.

[15] F. Liu, A. Arias-Vasquez, K. Sleegers, Y.S. Aulchenko, M. Kayser, P. Sanchez-Juan, B.J. Feng, A.M. Bertoli-Avella, J. van Swieten, T.I. Axenovich, P. Heutink, C. van Broeckhoven, B.A. Oostra, C.M. van Duijn, A genomewide screen for late-onset Alzheimer disease in a genetically isolated Dutch population, Am. J. Hum. Genet. 81 (2007) 17–31.

[16] Q.L. Ma, B. Teter, O.J. Ubeda, T. Morihara, D. Dhoot, M.D. Nyby, M.L. Tuck, S.A. Frautschy, G.M. Cole, Omega-3 fatty acid docosahexaenoic acid increases SorLA/LR11, a sorting protein with reduced expression in sporadic Alzheimer's disease (AD): relevance to AD prevention, J. Neurosci. 27 (2007) 14299–14307.

[17] G. McKhann, D. Drachman, M. Folstein, R. Katzman, D. Price, E.M. Stadlan, Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease, Neurology 34 (1984) 939–944.

[18] Y. Meng, J.H. Lee, R. Cheng, P. St George-Hyslop, R. Mayeux, L.A. Farrer, Association between SORL1 and Alzheimer's disease in a genome-wide study, Neuroreport 18 (2007) 1761–1764.

[19] R.L. Minster, S.T. DeKosky, M.I. Kamboh, No association of SORL1 SNPs with Alzheimer's disease, Neurosci. Lett. 440 (2008) 190–192.

[20] K. Offe, S.E. Dodson, J.T. Shoemaker, J.J. Fritz, M. Gearing, A.I. Levey, J.J. Lah, The lipoprotein receptor LR11 regulates amyloid beta production and amyloid precursor protein traffic in endosomal compartments, J. Neurosci. 26 (2006) 1596–1603.

[21] G.J. Praefcke, H.T. McMahon, The dynamin superfamily: universal membrane tubulation and fission molecules? Nat. Rev. Mol. Cell Biol. 5 (2004) 133–147.

[22] E. Rogaeva, Y. Meng, J.H. Lee, Y. Gu, T. Kawarai, F. Zou, T. Katayama, C.T. Baldwin, R. Cheng, H. Hasegawa, F. Chen, N. Shibata, K.L. Lunetta, R. Pardossi-Piquard, C. Bohm, Y. Wakutani, L.A. Cupples, K.T. Cuenco, R.C. Green, L. Pinessi, I. Rainero, S. Sorbi, A. Bruni, R. Duara, R.P. Friedland, R. Inzelberg, W. Hampe, H. Bujo, Y.Q. Song, O.M. Andersen, T.E. Willnow, N. Graff-Radford, R.C. Petersen, D. Dickson, S.D. Der, P.E. Fraser, G. Schmitt-Ulms, S. Younkin, R. Mayeux, L.A. Farrer, P. St George-Hyslop, The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease, Nat. Genet. 39 (2007) 168–177.

[23] D.J. Selkoe, Alzheimer's disease is a synaptic failure, Science 298 (2002) 789–791.

[24] S. Sever, Dynamin and endocytosis, Curr. Opin. Cell Biol. 14 (2002) 463–467.

[25] N. Shibata, T. Ohnuma, H. Baba, S. Higashi, K. Nishioka, H. Arai, Genetic association between SORL1 polymorphisms and Alzheimer's disease in a Japanese population, Dement. Geriatr. Cogn. Disord. 26 (2008) 161–164.

[26] E.K. Tan, J. Lee, C.P. Chen, Y.Y. Teo, Y. Zhao, W.L. Lee, SORL1 haplotypes modulate risk of Alzheimer's disease in Chinese, Neurobiol. Aging (2007).

[27] H. Yamazaki, H. Bujo, J. Kusunoki, K. Seimiya, T. Kanaki, N. Morisaki, W.J. Schneider, Y. Saito, Elements of neural adhesion molecules and a yeast vacuolar protein sorting receptor are present in a novel mammalian low density lipoprotein receptor family member, J. Biol. Chem. 271 (1996) 24761–24768.

J Bone Miner Metab
DOI 10.1007/s00774-009-0117-z

ORIGINAL ARTICLE

Regulation of ER molecular chaperone prevents bone loss in a murine model for osteoporosis

Shin-ichiro Hino · Shinichi Kondo · Kazuya Yoshinaga · Atsushi Saito · Tomohiko Murakami · Soshi Kanemoto · Hiroshi Sekiya · Kazuyasu Chihara · Yuji Aikawa · Hideaki Hara · Takashi Kudo · Tomohisa Sekimoto · Taro Funamoto · Etsuo Chosa · Kazunori Imaizumi

Received: 8 April 2009 / Accepted: 1 July 2009
© The Japanese Society for Bone and Mineral Research and Springer 2009

Abstract Endoplasmic reticulum (ER) stress response is important for protein maturation in the ER. Some murine models for bone diseases have provided significant insight into the possibility that pathogenesis of osteoporosis is related to ER stress response of osteoblasts. We examined a possible correlation between osteoporosis and ER stress response. Bone specimens from 8 osteoporosis patients and 8 disease-controls were used for immunohistochemical analysis. We found that ER molecular chaperones, such as BiP (immunoglobulin heavy-chain binding protein) and PDI (protein-disulfide isomerase) are down-regulated in osteoblasts from osteoporosis patients. Based on this result, we hypothesized that up-regulation of ER molecular chaperones in osteoblasts could restore decreased bone formation in osteoporosis. Therefore, we investigated whether treatment of murine model for osteoporosis with BIX (BiP inducer X), selective inducer BiP, could prevent bone loss. We found that oral administration of BIX effectively improves decline in bone formation through the activation of folding and secretion of bone matrix proteins. Considering these results together, BIX may be a potential therapeutic agent for the prevention of bone loss in osteoporosis patients.

Keywords Osteoporosis · Osteoblast · BiP · BIX · Endoplasmic reticulum stress response

S. Hino · S. Kondo · K. Yoshinaga · A. Saito ·
T. Murakami · S. Kanemoto · H. Sekiya · K. Chihara ·
Y. Aikawa · K. Imaizumi (✉)
Division of Molecular and Cellular Biology,
Department of Anatomy, Faculty of Medicine,
University of Miyazaki, Kihara 5200, Kiyotake,
Miyazaki 889-1692, Japan
e-mail: imaizumi@med.miyazaki-u.ac.jp

H. Hara
Department of Biofunctional Evaluation,
Molecular Pharmacology, Gifu Pharmaceutical University,
5-6-1 Mitahora-higashi, Gifu 502-8585, Japan

T. Kudo
Department of Clinical Neuroscience, Psychiatry,
Graduate School of Medicine, Osaka University,
2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

T. Sekimoto · T. Funamoto · E. Chosa
Department of Orthopaedic Surgery, Faculty of Medicine,
University of Miyazaki, Kihara 5200, Kiyotake,
Miyazaki 889-1692, Japan

Introduction

When cells synthesize secretory proteins in amounts that exceed the capacity of the folding apparatus, unfolded proteins accumulate in the endoplasmic reticulum (ER). To alleviate such a stressful situation (ER stress), eukaryotic cells activate a series of self-defense mechanisms referred to collectively as the ER stress response or unfolded protein response (UPR) [1–3]. A malfunction of the UPR caused by aging, genetic mutations, or environmental factors can result in various diseases such as diabetes and neurodegenerative disorders [4, 5].

Osteoporosis is characterized by reduced bone mass, alterations in the microarchitecture of bone tissue, reduced bone strength, and an increased risk of fracture [6]. Many factors influence the risk of osteoporosis, including genetics, diet, physical activity, medication, and coexisting diseases, but the pathogenesis of osteoporosis still remains unclear. A very large number of genes have been identified as possible candidates for the regulation of bone mass and susceptibility to osteoporotic fractures [7]. Wolcott-Rallison syndrome

Published online: 17 September 2009

(WRS) is a rare autosomal recessive disorder that is characterized by permanent neonatal insulin-dependent diabetes, severe epiphyseal dysplasia, and osteoporosis [8]. The syndrome results from mutations in the gene encoding the eukaryotic translation initiation factor 2-kinase 3 (EIF2AK3, also called PERK or PEK), one of the major ER stress transducers [9]. EIF2AK3 deficient mice also exhibited skeletal defects included deficient mineralization, osteoporosis and abnormal compact bone development [10]. ATF4 is another ER stress-related gene and is a substrate for RSK2 (ribosomal serine/threonine kinase 2). ATF4-deficient mice showed a delay in ossification and osteopenia [11]. Patients with Coffin-Lowry syndrome, which is an X-linked disease characterized by mental retardation, delayed bone age, delayed closure of the fontanelle, and short stature, have mutations in RSK2. Both ATF4−/− mice and RSK2−/− mice showed disturbance in type I collagen production [11]. Furthermore, abnormal ER retention of type I procollagen was observed in PERK−/− osteoblasts [12]. Taken together, normal ER stress responses could play important roles in type I collagen synthesis and secretion.

The phenotypes of these murine models and human bone disease suggest that pathogenesis of bone disease, such as osteopenia and osteoporosis, is related to ER stress response of osteoblasts. However, little has been reported on the relationship between osteoporosis and ER stress response. In this report, we analyzed whether development of osteoporosis correlates with ER stress response in osteoblasts of osteoporosis patients.

BiP (immunoglobulin heavy-chain binding protein) is one of the ER molecular chaperones, and is induced in response to ER stress. BiP serves to restore folding of misfolded or incompletely assembled proteins [13, 14] and expression of BiP protects against various types of cell death induced by ER stress [15, 16]. A chemical compound, BIX (BiP inducer X), 1-(3,4-dihydroxyphenyl)-2-thiocyanato-ethanone (Fig. 3a), was developed as an inducer of *BiP* mRNA [17]. In this paper, we also investigated whether treatment of murine model for osteoporosis with BIX could effectively prevent bone loss.

Materials and methods

Patient details

Clinical osteoporosis was determined from the bone mineral density (BMD) by radiographic absorptiometry or dual energy X-ray absorptiometry (DXA). Bone samples used in this study were from 8 osteoporosis patients aged 53–88 years (Table 1, patient I–P). As control samples, 8 bone specimens from disease-controls were collected aged 35–77 years, undergoing surgical procedures such as amputation for hip osteoarthritis (Table 1, patient A–H).

Histological processing

Bone specimens were fixed at the earliest possible opportunity in 10% neutral formalin and then decalcified with 10% EDTA. Hematoxylin–Eosin (HE) was performed using 6 μm paraffin sections and standard protocols.

For immunohistochemistry, dewaxed paraffin sections were pretreated with 3% hydrogen peroxide in PBS for 5 min and treated with 1% bovine serum albumin in PBS for 60 min. Sections were subsequently incubated with mouse anti-KDEL antibody (MBL) or mouse anti-PDI antibody (Affinity BioReagents) followed by an incubation with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (GE Healthcare). The immunoreaction was visualized with DAB stain (Sigma). For immunofluorescence, primary antibodies were visualized with alexa-conjugated goat anti-mouse IgG (Molecular Probes). Stained sections were viewed using a confocal microscope (FV1000D, OLYMPUS).

Quantification of immunofluorescence

To quantify the expression levels of BiP and PDI, fluorescence images of stained sections were measured by Lumina Vision software (Mitani Corporation). Obtained fluorescence intensity was normalized to the total surface area of osteoblasts.

Cell culture materials

Primary cultures of osteoblasts were prepared from the calvaria of postnatal 4 days in C57BL/6 mice and were grown in alpha modified Eagle's medium supplemented with 10% fetal calf serum. To confirm that the osteoblastic genes were expressed in primary culture, the mRNAs for *Alkaline phosphatase* (*Alp*) and *osteocalcin* were examined using RT-PCR at 14 days after the plating of cells. The amount of secreted osteopontin in the supernatants from primary osteoblasts was determined by ELISA using commercial kits (R&D Systems). The primers used for amplification were as follows: Alp, 5′-GCCCTCTCCAAGACATATA-3′ and 5′-CCATGATCACGTCGATATCC-3′; osteocalcin, 5′-AA GCAGGAGGGCAATAAGGT-3′ and 5′-AGCTGCTGTG ACATCCATAC-3′.

RNA extraction and real-time RT-PCR

Female ICR mice (7-week old) were orally administered BIX at 10 or 30 mg/kg or vehicle for 24 h, then the tibia from the hind limb was collected. Total RNA was isolated

Table 1 Information on osteoporosis patients and controls

Patient	Disease	Osteoporosis (+/−)	Site of biopsy	Sex (M/F)	Age (years)	'Active' osteoblast	Remarks
A	Knee osteoarthritis	−	Condyle of femur	F	77	++	Figs. 1, 2
B	Hip osteoarthritis	−	Femur head	F	68	++	
C	Hip osteoarthritis	−	Femur head	M	58	++	
D	Hip osteoarthritis	−	Femur head	M	60	++	
E	Hip osteoarthritis	−	Femur head	F	78	+	
F	Hernia of intervertebral disk (L5/S1)	−	Spine (L5)	M	35	+	
G	Hernia of intervertebral disk (L4/L5)	−	Spine (L5)	M	72	+	
H	Hernia of intervertebral disk (L4/L5)	−	Vertebral arch (L4)	M	52	++	
I	Osteoporosis (mild)	+	Tibia	M	78	−	
J	Osteoporosis (mild)	+	Femur head	F	85	+	
K	Osteoporosis	+	Condyle of femur	F	88	−	
L	Osteoporosis	+	Spine (L4)	M	71	−	
M	Osteoporosis/rheumatoid arthritis	+	Condyle of femur	F	66	+	
N	Osteoporosis/rheumatoid arthritis	+	Condyle of femur	F	53	−	
O	Osteoporosis/knee osteoarthritis	+	Femur neck	F	81	−	
P	Osteoporosis	+	Spine (L2)	F	69	+	

++, Large population of active osteoblasts; +, small population of active osteoblasts; −, no active osteoblast (bone lining cell only)

from the tibia using an RNeasy Mini kit (Qiagen) and reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems). TaqMan real-time PCR was performed using specific primer sets of BiP and β-actin. The primers used for amplification were as follows: BiP, 5′-GTTTGCTGAGGAAGACAAAAAGCTC-3′ and 5′-CACTTCCATAGAGTTTGCTGATAATTG-3′; β-actin, 5′-TCCTCCCTGGAGAAGAGCTAC-3′ and 5′-TCCTGCTTGCTGATCCACAT-3′ Expression of mRNA for BiP and β-actin endogenous control was measured in each specimen with real-time PCR on an ABI PRISMs 7900HT Sequence Detection System (Applied Biosystems).

Ovariectomized mice

As a model for osteoporosis (estrogen deficiency), female ICR mice (20 to 36-week old) were either ovariectomized (OVX) or sham operated. After surgery, the mice were orally administered 10 or 30 mg/kg/day BIX for 8 weeks. The vehicle control group received 0.5% sodium calboxymethylcellulose solution. As a positive control, OVX mice were administered 17β-estradiol. Body weight and bone length were measured. The whole tibial bone mineral densities (BMD) were measured using a DCS-600EX (Aloka).

Statistics

Data are presented as the mean ± SE. The statistical significance of differences was evaluated using the Student's *t* test or Aspin-Welch's *t* test.

Ethics

Full consent was obtained prior to surgery and these studies were approved by the Miyazaki University Ethics Committees (date of issue: 5 February 2007, registration number: 323).

Results

Morphological analysis of osteoblasts from osteoporosis patients

Clinical osteoporosis was determined from the bone mineral density (BMD) by radiographic absorptiometry or dual energy X-ray absorptiometry (DXA) (Table 1, patient I–P). As control samples, 8 bone specimens from disease-controls were collected (Table 1, patient A–H). Compared with a previous report using human bone [18], there was no significant difference in the morphology between normal-control and 8 disease-control specimens. The reduction of bone mass with loss of trabecular bone and enlargement of medullary space were observed in all osteoporosis samples, as previously reported [19] (Fig. 1a, b). Moreover, consistent with a previous study [20], the bone marrow cavity was filled with adipocytes instead of stromal cells and hematopoietic cells. Although we could not detect any differences in the number of osteoblasts between osteoporosis and disease-control specimens, there was a morphological difference. Disease-control bone specimens showed a considerable

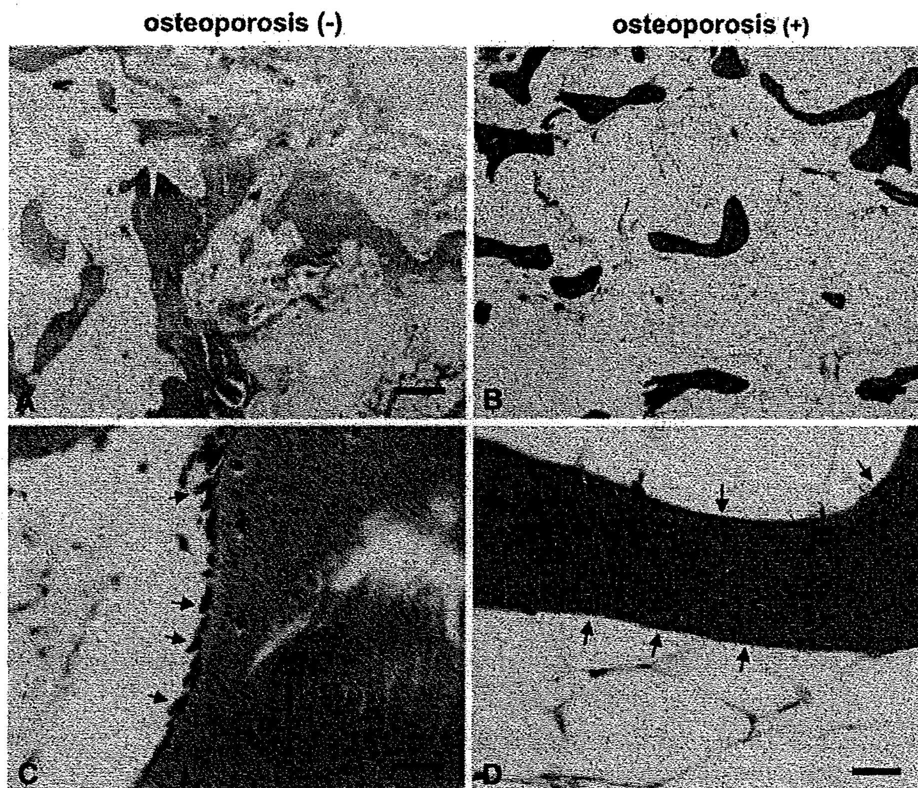


Fig. 1 Most osteoporosis osteoblasts are bone lining cells with decreased matrix synthesis. H&E staining of the condyle of the femur seen in controls (patient A; **a**) and osteoporosis patients (patient N; **b**). **c** Higher magnification of **a**. Osteoblasts with a cuboidal shape (*arrows*). **d** Higher magnification of **b**. Note, most osteoporosis osteoblasts are bone lining cells (*arrows*). *Scale bars* 500 μm (**a**, **b**), 50 μm (**c**, **d**)

population of 'active' osteoblasts with a cuboidal shape (Fig. 1c). On the other hand, notable flattened epithelium-like cells, called bone lining cells, were observed on the bone surface from osteoporosis patients (Fig. 1d). As shown in Table 1, osteoblasts from almost all patients that we examined showed this type of morphology.

Expression levels of ER stress-related genes are weaker in osteoporosis osteoblasts

To investigate whether ER stress occurs in human osteoblasts from osteoporosis patients, we next examined the expression level of the ER stress-related proteins BiP and PDI in osteoporosis and disease-control osteoblasts. Both BiP and PDI are up-regulated when ER stress occurs [21]. Immunohistochemical analysis using the anti-KDEL antibody which recognizes BiP protein, showed strong expression of the protein in disease-control osteoblasts (Fig. 2a, c). Immunoreactivity for BiP was weak in other cell types, such as osteocytes and blood cells. These staining patterns in disease-control osteoblasts showed that the level of BiP in the ER is high, indicating that there might be ER stress response that occurs physiologically in osteoblasts with a cuboidal shape. On the other hand, in osteoporosis osteoblasts, immunohistochemical analysis using the anti-KDEL antibody showed weak expression of the BiP protein in the cytosol around the nucleus (Fig. 2b, d). Strong immunoreactivity for PDI, which is also an ER stress-related protein, was observed in the ER of disease-control osteoblasts (Fig. 2e, g), but no immunoreactivity for PDI was observed in osteoblasts from osteoporosis patients (Fig. 2f, h). By quantitative analysis, as described in "Materials and methods", the expression levels of BiP and PDI were significantly decreased in osteoblasts from 8 osteoporosis patients compared with those from 8 disease-control specimens (Fig. 2i, j). These results indicate that ER stress response, including induction of ER molecular chaperones, is physiologically activated in cuboidal shaped active osteoblasts in the disease-controls. In contrast, it is down-regulated in bone lining cells from osteoporosis patients.

Based on these results, we hypothesized that induction of ER molecular chaperones could restore the decreased bone formation in osteoblasts from osteoporosis patients. Therefore, we next tested our hypothesis using a murine model for osteoporosis.

BIX induces BiP mRNA expression in mouse bone tissue

We used a chemical compound BIX (Fig. 3a), which has potential to induce BiP at the transcriptional level, for

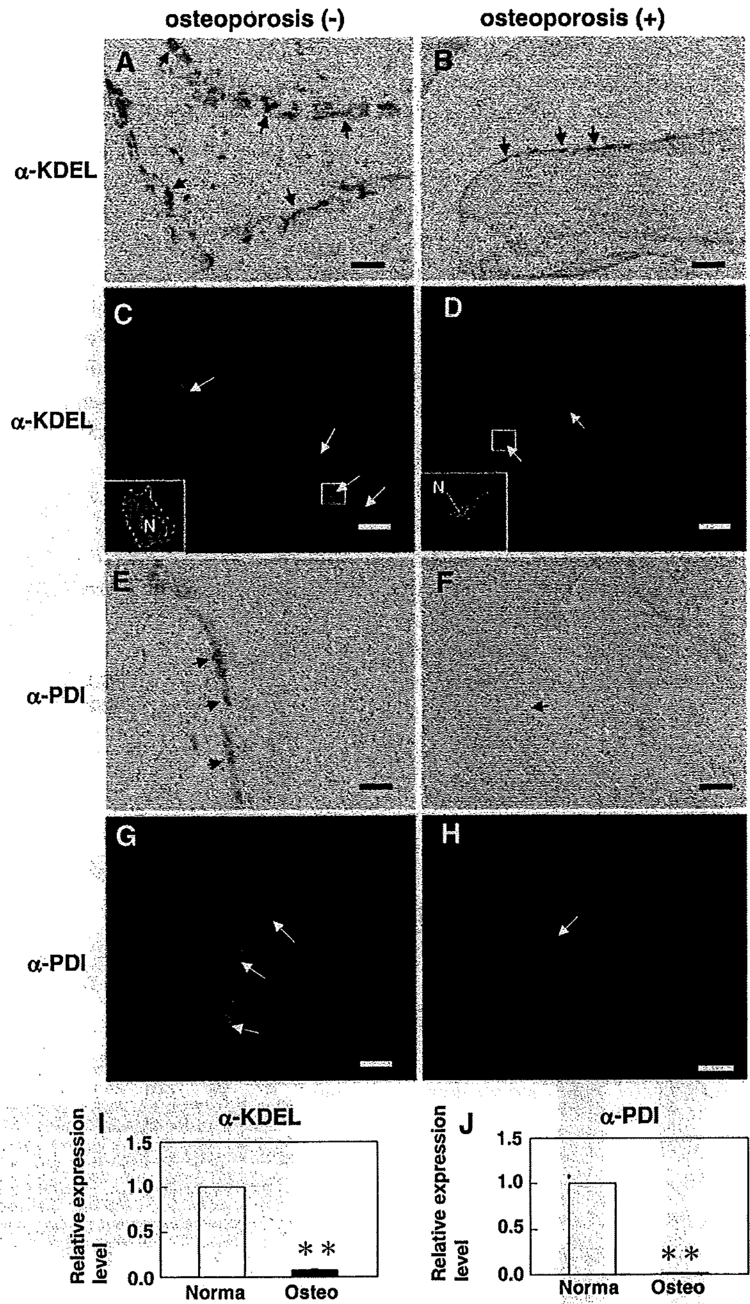


Fig. 2 Bone lining cells show weaker expression of ER stress-related genes. Immunohistochemistry for anti-KDEL that recognizes BiP, in the condyle of the femur seen in a control (patient A; **a**) and an osteoporosis patient (patient N; **b**). Immunofluorescence for anti-KDEL that recognizes BiP, in the spine seen in a control (patient G; **c**) and an osteoporosis patient (patient L; **d**). The *inset* is a higher magnification image of the *white box*. Cytosol and nucleus are encircled by dashed lines. *N* nucleus. Note, the immunoreactivity for KDEL (*arrows*) is weak in bone lining cells of the osteoporosis patient compared to the osteoblasts in the control. Immunohistochemistry for PDI protein in the condyle of the femur seen in a control (patient A; **e**) and an osteoporosis patient (patient N; **f**). Immunofluorescence for PDI protein, in the spine seen in a control (patient G; **g**) and an osteoporosis patient (patient L; **h**). Note, immunoreactivity for PDI (*arrows*) is notably weak in bone lining cells of the osteoporosis patient compared to control osteoblasts. **i** Quantitative analysis of BiP protein levels between (**c**) and (**d**). **j** Quantitative analysis of PDI protein levels between (**g**) and (**h**). These results shown (**i**, **j**) were normalized to the total surface area of osteoblasts. Note, there was no notable difference in the number of osteoblasts between osteoporosis patients and controls. The corresponding quantification results for BiP and PDI protein levels were expressed as the fold decrease compared with those in the control. Data are means ± SE, $n = 8$ (8 controls: patient A–H, 8 osteoporosis: patient I–P). $^{**}p < 0.01$ relative to the control. *Scale bars* 50 μm (**a**–**h**)

activation of declined osteoblasts. We first examined whether BIX induces *BiP* mRNA in mouse bone tissue. Mice were orally administered BIX at 10 or 30 mg/kg. At 24 h after the administration, total RNA was prepared from the tibia and then the expression levels of BiP were analyzed by real-time RT-PCR. As shown in Fig. 3b, *BiP* mRNA in tibia showed a tendency to increase in mice treated with BIX at 10 mg/kg and to significantly increase at 30 mg/kg, compared with the vehicle-treated group. These results indicate that oral administration of BIX induces *BiP* mRNA in mouse bone tissues.

BIX promotes secretion of osteopontin

To investigate the effects of BIX on bone matrix formation of osteoblasts, we measured the amounts of secreted bone matrix protein from the primary osteoblasts. Initially, we tried to measure the secreted osteocalcin by ELISA. However, we could not consistently detect it in the supernatants of the primary culture of osteoblasts, because the levels of secreted osteocalcin were at very low. We then tried to measure osteopontin in the supernatants, and we could easily and reproducibly detect it. Therefore, we measured secreted osteopontin after treatment of primary cultured osteoblasts with 5 μM BIX for 12, 16, or 24 h. As shown in Fig. 4a, secretion of osteopontin was significantly elevated by the treatment with BIX for 24 h compared with that of the non-treated control. We confirmed that BIX induced *BiP* mRNA in the primary osteoblasts (Fig. 4b). In contrast, the treatment of BIX did not have an impact on the expression levels of *ALP* and *osteocalcin* mRNA (Fig. 4b), indicating that BIX could not affect the differentiation or maturation of osteoblasts. These results indicate that treatment with BIX enhances bone matrix secretion leading to augmentation of bone formation through facilitating protein folding in the ER of osteoblasts.

BIX prevents bone loss in ovariectomized mice

To examine the effects of BIX on bone formation in vivo, we used ovariectomized (OVX) osteoporosis mouse

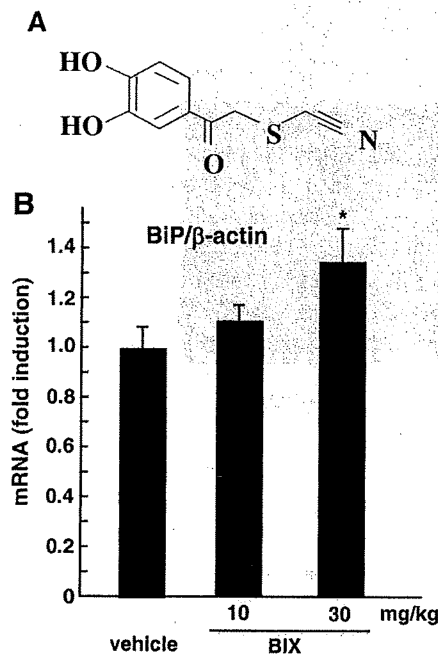


Fig. 3 BIX induces *BiP* mRNA in the tibia of mice. **a** Chemical structure of BIX (BiP inducer X), 1-(3,4-dihydroxy-phenyl)-2-thiocyanato-ethanone. **b** Real-time RT–PCR analysis of *BiP* mRNA in tibial bone. Mice were orally administered BIX (10 or 30 mg/kg) or vehicle for 24 h. Total RNA prepared from the tibia was subjected to real-time RT-PCR using specific primer sets of BiP and β-actin. The results shown were normalized by the *β-actin* mRNA levels, and the corresponding quantification results for the *BiP* mRNA levels were expressed as the fold induction compared with that of the vehicle control group. Data are means ± SE, $n = 5$. *$p < 0.05$ vs. vehicle (Student's *t* test)

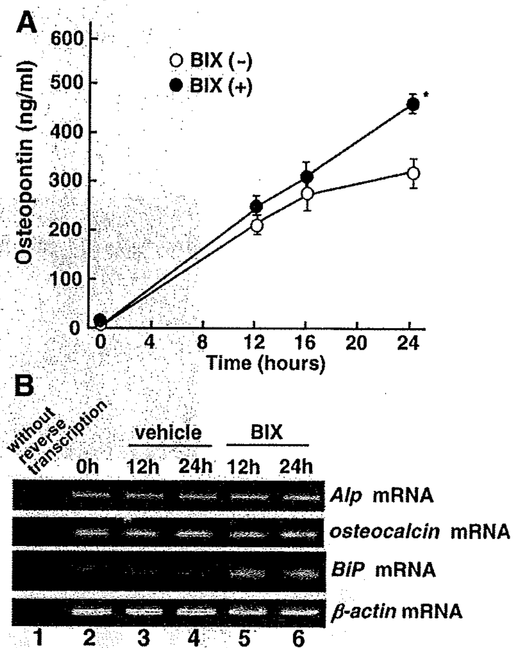


Fig. 4 BIX promotes secretion of osteopontin. **a** Primary cultured osteoblasts were treated with 5 μM BIX or without for 12, 16, or 24 h. Supernatants were analyzed for secretion of osteopontin by ELISA. Data are means ± SE from five independent experiments. *$p < 0.05$ vs. vehicle (Student's *t* test). **b** Total RNA prepared from the primary cultured osteoblasts was subjected to RT-PCR using specific primer sets of Alkaline phosphatase (Alp), osteocalcin, BiP, and β-actin. RT-PCR analysis of primary cultured osteoblasts at 14 days after the plating (*lane 2*). PCR reaction was subjected without reverse transcription as a control (*lane 1*). The expression levels of *Alp* and *osteocalcin* mRNAs were high, indicating that cultured cells prepared from the calvaria are sufficiently differentiated to mature osteoblasts. The effects of BIX on the Alp, osteocalcin, and BiP expression (*lane 3–6*). Primary cultured osteoblasts were treated with vehicle (*lane 3 and 4*) or 5 μM BIX (*lane 5 and 6*) for 12 or 24 h

models. OVX mice were orally administered BIX at 10 or 30 mg/kg for 8 weeks. The ovariectomy did not affect body weight change and bone length in each group (data not shown). The whole tibial bone mineral density (BMD) was significantly decreased in OVX mice compared with the sham-operated group (Fig. 5). Consistent with previous studies [22, 23], the BMD loss in OVX mice was recovered by treatment with 17β-estradiol, but this treatment caused hyperplasia of the bone matrix. The administration of BIX into sham-operated mice did not affect their bone formation (data not shown). The administration of BIX into OVX mice significantly prevented the whole tibial BMD loss (at 10 and 30 mg/kg BIX) (Fig. 5). However, treatment with 30 mg/kg BIX showed weak effects on the improvement of bone loss compared with 10 mg/kg BIX. Treatment with 30 mg/kg BIX caused a decrease of voluntary exercise, indicating that treatment with high doses of BIX could cause some toxic effects in mice. It is possible that this toxicity weakens the effects of BIX on the facilitation of bone formation. In addition, pathologically abnormal findings were not observed in bone tissues of BIX-treated mice, different from the case of treatment with 17β-estradiol. The administration of BIX did not lead to alteration of TRAP positive osteoclasts numbers (data not shown), suggesting that BIX does not affect the activities of osteoclasts. These results indicate that oral administration of BIX effectively improves decline in bone formation of osteoporosis mouse models through acting on osteoblasts.

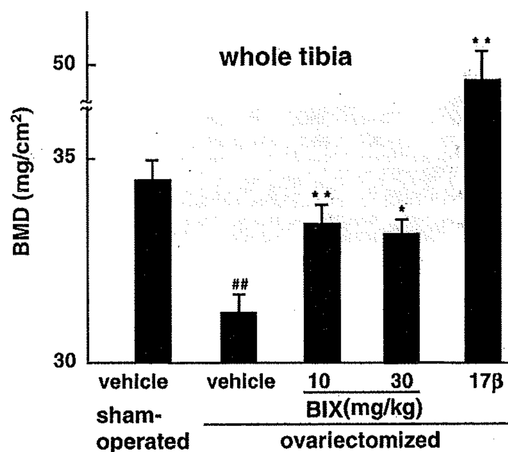


Fig. 5 BIX prevents bone loss in ovariectomized mice. Whole tibial BMD. Ovariectomized mice were orally administered 10 or 30 mg/kg/day BIX, or treated with 0.03 μg/mouse/day 17β-estradiol (17β) subcutaneously for 8 weeks after the operation. Data are means ± SE, $n = 11$ to 12. $^{\#\#}p < 0.01$ vs. sham vehicle (Student's t test). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. OVX vehicle (Student's t test, Aspin-Welch's t test)

Discussion

In the present study, we have shown that control osteoblasts with a cuboidal shape strikingly express ER stress-related genes such as BiP and PDI (Fig. 2a, c), suggesting that an ER stress response occurs in 'active' osteoblasts. High-level secretory protein synthesis and secretion could lead to an overload of the ER in active osteoblasts and this could cause ER stress. It is conceivable that activation of the ER stress response is a physiological adaptation for osteoblasts to properly fold and secrete massive amounts of proteins as a protection against ER overload, and that this response is essential for osteoblasts to synthesize bone matrix.

In osteoporosis patients, most osteoblasts are bone lining cells and there are few active osteoblasts (Fig. 1d), suggesting that the loss of active osteoblasts is associated with the pathogenesis of osteoporosis and causes a reduction in matrix synthesis. Further, bone lining cells showed a weaker expression of ER stress-related genes such as BiP and PDI (Fig. 2b, d) and down-regulated ER stress response. The results are consistent with a declining bone matrix synthesis in osteoporosis bone lining cells. Taken together, folding capacity of secreted proteins in the ER could be declined in osteoblasts from the patients followed by augmentation of reduced bone formation.

It is well known that loss of estrogen is an important risk factor for pathogenesis of osteoporosis. Ovariectomy in the mice results in an increase in bone turnover rate and significant loss of trabecular bone, vertebral bodies and the metaphysis of long bones [24]. In this study, we also observed ovariectomy caused decrease in whole tibial BMD involving trabecular bone loss as shown in previous studies. Oral administration of BIX prevented whole tibial BMD loss of this mouse model. BIX is an inducer of ER molecular chaperone BiP. BiP has a potential to facilitate folding of proteins in the ER. The possible mechanisms responsible for prevention of bone loss and acceleration of bone formation by BIX are that induced BiP could increase folding capacity in the ER followed by facilitating secretion of bone matrix proteins from osteoblasts. This hypersecretion of bone matrix proteins by BIX could lead to an increase in the bone formation rate that superpasses bone resorption by osteoclasts. Although various agents have been developed for the treatment of osteoporosis, the majority of these reduce bone resorption by affecting osteoclast activity. Considering our finding that BIX accelerates the osteoblast-mediated bone formation, BIX could be an alternative therapeutic agent for high-turnover osteoporosis.

Acknowledgments We would like to thank Keisuke Goto for performing the surgeries and kindly making tissue available from

patients, as well as Ai Ikeda and Tomoko Kawanami for their technical assistance in the laboratory. This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, the Takeda Science Foundation, and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

References

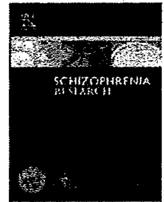
1. Harding HP, Calfon M, Urano F, Novoa I, Ron D (2002) Transcriptional and translational control in the mammalian unfolded protein response. Annu Rev Cell Dev Biol 18:575–599
2. Patil C, Walter P (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr Opin Cell Biol 13:349–355
3. Schröder M, Kaufman RJ (2005) ER stress and the unfolded protein response. Mutat Res 569:29–63
4. Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk−/− mice reveals a role for translational control in secretory cell survival. Mol Cell 7:1153–1163
5. Zhao L, Longo-Guess C, Harris BS, Lee JW, Ackerman SL (2005) Protein accumulation and neurodegeneration in the woozy mutant mouse is caused by disruption of SIL1, a cochaperone of BiP. Nat Genet 37:974–979
6. Kanis JA, Melton LJIII, Christiansen C, Johnston CC, Khaltaev N (1994) The diagnosis of osteoporosis. J Bone Miner Res 9:1137–1141
7. Ralston SH, de Crombrugghe B (2006) Genetic regulation of bone mass and susceptibility to osteoporosis. Genes Dev 20:2492–2506
8. Wolcott CD, Rallison ML (1972) Infancy-onset diabetes mellitus and multiple epiphyseal dysplasia. J Pediatr 80:292–297
9. Delépine M, Nicolino M, Barrett T, Golamaully M, Lathrop GM, Julier C (2000) EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott–Rallison syndrome. Nat Genet 25:406–409
10. Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR (2002) The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. Mol Cell Biol 22:3864–3874
11. Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, Li L, Brancorsini S, Sassone-Corsi P, Townes TM, Hanauer A, Karsenty G (2004) ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. Cell 117:387–398
12. Wei J, Sheng X, Feng D, McGrath B, Cavener DR (2008) PERK is essential for neonatal skeletal development to regulate osteoblast proliferation and differentiation. J Cell Physiol 217:693–707
13. Yoshida H (2007) ER stress and diseases. FEBS J 274:630–658
14. Ni M, Lee AS (2007) ER chaperones in mammalian development and human diseases. FEBS Lett 581:3641–3651
15. Katayama T, Imaizumi K, Sato N, Miyoshi K, Kudo T, Hitomi J, Morihara T, Yoneda T, Gomi F, Mori Y, Nakano Y, Takeda J, Tsuda T, Itoyama Y, Murayama O, Takashima A, George-Hyslop PS, Takeda M, Tohyama M (1999) Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. Nat Cell Biol 1:479–485
16. Rao RV, Peel A, Logvinova A, del Rio G, Hermel E, Yokota T, Goldsmith PC, Ellerby LM, Ellerby HM, Bredesen DE (2002) Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. FEBS Lett 514:122–128
17. Kudo T, Kanemoto S, Hara H, Morimoto N, Morihara T, Kimura R, Tabira T, Imaizumi K, Takeda M (2008) A molecular chaperone inducer protects neurons from ER stress. Cell Death Differ 15:364–375
18. Nampei A, Hashimoto J, Hayashida K, Tsuboi H, Shi K, Tsuji I, Miyashita H, Yamada T, Matsukawa N, Matsumoto M, Morimoto S, Ogihara T, Ochi T, Yoshikawa H (2004) Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone. J Bone Miner Metab 22:176–184
19. Jowsey J, Phil D, Kelly PJ, Riggs BL, Bianco AJ Jr, Scholz DA, Gershon-Cohen J (1965) Quantitative microradiographic studies of normal and osteoporotic bone. J Bone Joint Surg Am 47:785–806
20. Justesen J, Stenderup K, Ebbesen EN, Mosekilde L, Steiniche T, Kassem M (2001) Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. Biogerontology 2:165–171
21. Kondo S, Murakami T, Tatsumi K, Ogata M, Kanemoto S, Otori K, Iseki K, Wanaka A, Imaizumi K (2005) OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. Nat Cell Biol 7:186–194
22. Takano-Yamamoto T, Rodan GA (1990) Direct effects of 17β-estradiol on trabecular bone in ovariectomized rats. Proc Natl Acad Sci USA 87:2127–2176
23. Kawaguchi H, Pilbeam CC, Vargas SJ, Morse EE, Lorenzo JA, Raisz LG (1995) Ovariectomy enhances and estrogen replacement inhibits the activity of bone marrow factors that stimulate prostaglandin production in cultured mouse calvariae. J Clin Invest 96:539–548
24. Omi N, Ezawa I (1995) The effect of ovariectomy on bone metabolism in rats. Bone 17:163S–168S

Schizophrenia Research 109 (2009) 80–85

Contents lists available at ScienceDirect

Schizophrenia Research

journal homepage: www.elsevier.com/locate/schres



Association study of the *G72* gene with schizophrenia in a Japanese population: A multicenter study

Kazutaka Ohi [a,d], Ryota Hashimoto [a,b,d,*], Yuka Yasuda [a,b,d], Tetsuhiko Yoshida [a,d], Hidetoshi Takahashi [a,d], Naomi Iike [a,d], Motoyuki Fukumoto [a,d], Hironori Takamura [a,d], Masao Iwase [a], Kouzin Kamino [a,e], Ryouhei Ishii [a], Hiroaki Kazui [a], Ryuji Sekiyama [a], Yuri Kitamura [c], Michiyo Azechi [a], Koji Ikezawa [a], Ryu Kurimoto [a], Eiichiro Kamagata [a], Hitoshi Tanimukai [a], Shinji Tagami [a], Takashi Morihara [a], Masayuki Ogasawara [a], Masayasu Okochi [a], Hiromasa Tokunaga [a], Shusuke Numata [f], Masashi Ikeda [d,g], Tohru Ohnuma [h], Shu-ichi Ueno [f], Tomoko Fukunaga [a], Toshihisa Tanaka [a], Takashi Kudo [a,b], Heii Arai [h], Tetsuro Ohmori [f], Nakao Iwata [d,g], Norio Ozaki [d,i], Masatoshi Takeda [a,b]

[a] *Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[b] *The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[c] *Department of Physiology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan*
[d] *CREST (Core Research for Evolutionary Science and Technology), JST (Japan Science and Technology Agency), Kawaguchi, Saitama, Japan*
[e] *Shoraiso National Hospital, Yamatokoriyama, Nara, Japan*
[f] *Department of Psychiatry, Tokushima University Graduate School of Medicine, Kuramoto, Tokushima, Japan*
[g] *Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi, Japan*
[h] *Department of Psychiatry, Juntendo University School of Medicine, Bunkyo, Tokyo, Japan*
[i] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan*

ARTICLE INFO

Article history:
Received 6 August 2008
Received in revised form 17 January 2009
Accepted 26 January 2009
Available online 23 February 2009

Keywords:
Schizophrenia
G72
D-amino acid oxidase activator (DAOA)
Association study
Single nucleotide polymorphism (SNP)
NMDA receptor

ABSTRACT

G72 is one of the most widely tested genes for association with schizophrenia. As G72 activates the D-amino acid oxidase (DAO), *G72* is termed D-amino acid oxidase activator (DAOA). The aim of this study is to investigate the association between *G72* and schizophrenia in a Japanese population, using the largest sample size to date (1774 patients with schizophrenia and 2092 healthy controls). We examined eight single nucleotide polymorphisms (SNPs), which had been associated with schizophrenia in previous studies. We found nominal evidence for association of alleles, M22/rs778293, M23/rs3918342 and M24/rs1421292, and the genotype of M22/rs778293 with schizophrenia, although there was no association of allele or genotype in the other five SNPs. We also found nominal haplotypic association, including M15/rs2391191 and M19/rs778294 with schizophrenia. However, these associations were no longer positive after correction for multiple testing. We conclude that *G72* might not play a major role in the risk for schizophrenia in the Japanese population.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Schizophrenia (MIM181500) is a common neuropsychiatric disorder affecting 0.5–1% of the general population worldwide. Family, twin, and adoption studies of schizophrenia have indicated that there is a strong genetic factor with an estimated heritability of approximately 80% (Cardno and Gottesman, 2000). Several genome-wide linkage scan

* Corresponding author. The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Osaka University Graduate School of Medicine, D3, 2-2, Yamadaoka, Suita, Osaka, 5650871, Japan. Tel.: +81 668793074; fax: +81 668793059.
E-mail address: hashimor@psy.med.osaka-u.ac.jp (R. Hashimoto).

0920-9964/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.schres.2009.01.019

studies of whole-genome linkage scans show suggestive linkages to schizophrenia on chromosomes 1q, 3p, 5q, 6p, 8p, 11q, 13q, 14p, 20q and 22q (Owen et al., 2004). Chumakov et al. (2002) focused on chromosome region 13q22–q34, which have suggested by a number of linkage studies (Blouin et al., 1998; Brzustowicz et al., 1999; Chumakov et al., 2002; Lin et al., 1995). They built a map of 191 single nucleotide polymorphisms (SNPs) in a 5-Mb segment on 13q34 and found robust evidence for genetic association between schizophrenia and several SNPs in the narrowed 65-kb region. Two overlapping genes, *G72* (MIN 607408) and *G30* (MIN 607415), which are transcribed in opposite directions and span approximately 29 and 47 kb of genomic sequences, were annotated in this region (Chumakov et al., 2002). In vitro translation of these genes resulted in a product for *G72* only. Chumakov et al. (2002) demonstrated that the *G72* protein (i.e. LG72), which is only known in higher primates, acts as an activator of the *DAO* protein. The *G72* protein was therefore referred to as *DAO* activator (*DAOA*). Gene expression analysis of *G72* in postmortem dorsolateral prefrontal cortices showed a tendency toward increased expression of *G72* mRNA in schizophrenia than that in control (Korostishevsky et al., 2004), although the reported increase of *G72* expression has yet to be replicated. Furthermore, the activity of DAO was also increased in postmortem cortices from patients with schizophrenia (Madeira et al., 2008). D-serine is an agonist at the glycine modulation site of the N-methyl-D-aspartate (NMDA) -type glutamate receptor and plays a role in neuronal migration and cell death (Scolari and Acosta, 2007). As DAO oxidizes and degrades D-serine, DAO is considered to modulate NMDA function in cortex. Lower serum level of D-serine was revealed in patients with schizophrenia as compared to that in healthy controls. Furthermore, administration of D-serine as add-on medication reduced parts of the symptoms of schizophrenia (Boks et al., 2007). Chumakov et al. (2002) hypothesized that the activation of DAO activity by a *G72* protein product might promote degradation of D-serine and cause a hypofunction of glutamate-signaling through the NMDA receptor in schizophrenia. However, the potential relationship between *G72* and NMDA receptor system still lacks supporting evidence.

Significant associations of *G72* with schizophrenia have been reported in various populations other than Japanese, such as French Canadians, Russians, German, Palestinian Arabs, South African, Ashkenazic Jewish, Chinese, Taiwanese, Scottish, Korean and Irish (Addington et al., 2004; Chumakov et al., 2002; Corvin et al., 2007; Fallin et al., 2005; Hall et al., 2004; Hong et al., 2006; Korostishevsky et al., 2004, 2006; Ma et al., 2006; Schumacher et al., 2004; Shin et al., 2007; Shinkai et al., 2007; Wang et al., 2004; Yue et al., 2006, 2007; Zou et al., 2005). The majority of replication studies of *G72* have indicated significant associations of alleles, genotypes or haplotypes with schizophrenia. However, a minority have reported no association between *G72* and schizophrenia (Bakker et al., 2007; Goldberg et al., 2006; Liu et al., 2006; Mulle et al., 2005; Sanders et al., 2008; Vilella et al., 2008; Williams et al., 2006; Wood et al., 2007). Associations of this gene were also reported with bipolar disorder (Chen et al., 2004; Hattori et al., 2003; Prata et al., 2008; Schumacher et al., 2004; Williams et al., 2006), major depression (Rietschel et al., 2008) and panic disorder (Schumacher et al., 2005).

In this study, we examined possible association between G72 polymorphisms and schizophrenia in a large Japanese population.

2. Materials and methods

2.1. Subjects

The subjects for this study consisted of 1774 patients with schizophrenia [males: 55.5%, mean age of 45.6 years (SD 15.1)] and 2092 healthy controls [males: 49.3%, mean age of 45.0 years (SD 19.7)], which is the largest sample size to date for *G72* association study. There was no significant difference in age between patients and controls groups ($P=0.30$), while the sex ratio differed significantly between groups ($P=0.00014$). All subjects were biologically unrelated Japanese and were recruited at four geographic regions, which were located on the main islands in Japan: Osaka, Aichi, Tokushima and Tokyo. There is little possibility for great ethnic/genetic difference among these regions for feature of homogeneous race in Japan (Yamaguchi-Kabata et al., 2008). Cases were recruited from both outpatients and inpatients at university hospitals and related psychiatric facilities. Controls, including hospital and institutional staffs, were recruited from local advertisements. Each patient with schizophrenia had received a diagnosis and assessment by at least two trained psychiatrists as a part of routine clinical diagnosis and treatment at the university hospitals and the related psychiatric facilities, according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interviews and other available information including medical records and other research assessments. No patient was diagnosed on the basis of medical records alone. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had received psychiatric medication. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Fujita Health University, Nagoya University, Tokushima University and Juntendo University.

2.2. SNP genotyping and genomic sequencing

Eight SNPs, rs3916965 (M12), rs3916967 (M14), rs2391191 (M15), rs778294 (M19), rs3916970 (M20), rs778293 (M22), rs3918342 (M23) and rs1421292 (M24), were selected from the genomic region of the *G72* gene and its flanking regions. The designations of the SNPs in parentheses are according to Chumakov et al. (2002). To examine the association between schizophrenia and previously associated SNPs in a Japanese cohort, we chose eight SNPs, which had been associated with schizophrenia in previous studies, although our study design using these SNPs does not provide complete *G72* gene coverage. The positions of the eight SNPs analyzed in the present study are indicated in Supplementary Fig. 1. Venous blood was collected from the subjects and genomic DNA was

Table 1
SNP genotype and allele distribution in patients with schizophrenia and controls.

Marker position		M/m[c]	SCZ (%)			CON (%)			MAF		Genotypic	Allelic	OR
SNP number[a]	Kb[b]		M/M	M/m	m/m	M/M	M/m	m/m	SCZ (%)	CON (%)	P-value[d] ($df=2$)	P-value[d] ($df=1$)	
M12	0	A/G	57.1	35.5	7.4	55.9	37.6	6.5	25.1	25.3	0.26	0.87	0.99
M14	14	G/A	55.3	36.7	8.0	53.7	39.2	7.1	26.3	26.7	0.21	0.75	0.98
M15	2	A/G	55.1	37.0	7.9	54.3	38.8	6.9	26.4	26.3	0.34	0.91	1.01
M19	23	G/A	72.9	24.4	2.7	71.5	25.9	2.6	14.9	15.5	0.55	0.43	0.95
M20	12	A/G	39.0	46.1	14.9	40.9	45.3	13.8	38.0	36.4	0.4	0.17	1.08
M22	15	A/G	54.7	38.7	6.6	58.9	35.0	6.1	26.0	23.6	**0.034**	**0.019**	1.13
M23	17	T/C	31.7	49.4	18.9	34.7	48.3	17.0	43.6	41.2	0.09	**0.030**	1.11
M24	12	A/T	24.9	50.6	24.5	28.1	49.0	23.0	49.8	47.4	0.07	**0.037**	1.10

SCZ, patients with schizophrenia; CON, healthy controls; m, minor allele; M, major allele; MAF, minor allele frequency; OR, odds ratio.
[a]The db SNP IDs equivalent to the M–SNP IDs designed by Chumakov et al. (2002) are the following: M12 (rs3916965), M14 (rs3916967), M15 (rs2391191), M19 (rs778294), M20 (rs3916970), M22 (rs778293), M23 (rs3918342), and M24 (rs1421292).
[b]Distances inter-SNPs are shown (Kb).
[c]The first shown alleles are major allele. All the alleles are represented according to the forward DNA sequence to make them comparable with the previous published data.
[d]Significant P-values (< 0.05) are in bold face.

extracted from whole blood according to standard procedures. Genotyping of the SNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, California, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μl total reaction volume was used, and allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). In addition, we genotyped eight SNPs in 32 randomly selected subjects (64 chromosomes) by a direct DNA sequencing method to check for typing errors by the TaqMan method. We confirmed that all genotypes determined by the direct sequencing method were in agreement with the genotypes of the TaqMan methods for all eight SNPs. Detailed information on the PCR conditions and the primer pairs are shown in Supplementary Methods and Supplementary Table 1.

2.3. Statistical analysis

Statistical analysis was performed using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan). The presence of Hardy–Weinberg equilibrium (HWE) was examined by using the χ^2 test for goodness of fit. The statistical significance of HWE analysis was defined at $P<0.01$. The allelic and genotypic distributions of *G72* polymorphisms between patients and controls were analyzed using χ^2 tests for independence. We performed correction for multiple testing in single marker analysis by using the SNPSpD program (Nyholt, 2004). Pairwise linkage disequilibrium (LD) analysis, expressed by D' values, was applied to detect the intermarker relationship in each group using the Haploview software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated by the method of maximum likelihood from the genotyping data through the use of the Expectation-Maximization algorithm. Rare haplotypes found in less than 3% of both patients and controls were excluded from the association analysis. We performed 10,000 permutations for the most significant test to determine an empirical significance. We used a 2- to 5-window fashion analysis. Bonferroni corrections were applied for multiple comparisons of the haplotype analysis. All P-values reported are two tailed. Statistical significance was defined at $P<0.05$.

2.4. Power analysis

We performed power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/) (Skol et al., 2006). Power estimates were based on allele frequencies of the associated markers ranging from 0.15 (M19) to 0.38 (M20), the odds ratio ranging from 1.33 (M14) to 1.46 (M12) for the markers indicated by Chumakov et al. (2002) and an alpha level of 0.05. Power was calculated under prevalence of 0.01 using an additive or a multiplicative model, assuming various degrees of allele frequencies and the odds ratios of the markers.

3. Results

Our sample size of 1774 cases and 2092 controls had sufficient power (>0.99) to detect an effect of the odds ratio

Table 2
Haplotype analysis of G72 between patients and controls.

		Haplotypic global P value			
		Window size			
LD[a]	SNP IDs[b]	2	3	4	5
block I	M12 (rs3916965)				
		0.93			
	M14 (rs3916967)		0.97		
		0.92		**0.03**	
	M15 (rs2391191)		**0.03**		0.05
		0.03		0.06	
	M19 (rs778294)		0.08		**0.03**
		0.04		0.06	
	M20 (rs3916970)		0.15		0.27
		0.23		0.47	
block II	M22 (rs778293)		0.28		0.25
		0.09		0.32	
	M23 (rs3918342)		0.06		
		0.10			
	M24 (rs1421292)				

LD, linkage disequilibrium.
[a]According to the result of LD analysis, we divided tightly linked SNPs into two LD blocks: block I (M12, M14 and M15), block II (M22, M23 and M24).
[b]The db SNP IDs equivalent to the M–SNP IDs designed by Chumakov et al. (2002) are shown in parentheses.
Haplotypes with frequencies <3% in each group are excluded.
Significant P-values (<0.05) are in bold face.

(1.33 or more) described in the initial report for each SNP (Chumakov et al., 2002). Genotype and allele frequencies of eight SNPs located in the *G72* gene and the flanking regions are shown in Table 1. Genotyping completeness ranged from 98.5% (M15) to 99.5% (M12). No deviation from HWE was detected in cases and controls (data not shown). Significant differences in the genotype frequency of M22 ($\chi^2=6.75$, $P=0.034$) and in the allele frequencies of M22 ($\chi^2=5.48$, $P=0.019$), M23 ($\chi^2=4.72$, $P=0.030$) and M24 ($\chi^2=4.35$, $P=0.037$) between patients and controls were observed. However, the associations did not survive after correction for multiple testing (the effective number of independent marker loci: 6.0, M22 allelic association: $P=0.11$ after SNPSpD correction). There was no allelic or genotypic association of the other five SNPs with schizophrenia.

Haplotype analysis showed associations in two-marker haplotypes: M15–M19 (global $P=0.03$) and M19–M20 (global $P=0.04$), three-marker haplotype: M14–M15–M19 (global $P=0.03$), four-marker haplotype: M12–M14–M15–M19 (global $P=0.03$), and five-marker haplotype: M14–M15–M19–M20–M22 (global $P=0.03$) (Table 2). This weak evidence for association became negative after correction for multiple testing (22 independent global test, M15–M19 haplotypic association: $P=0.66$ after Bonferroni correction).

M22 showed a strong LD with M24 and a moderate LD with M23 in controls, and similar LD results were obtained in patients (Supplementary Fig. 1). The LD pattern of our data was similar to that of other ethnic groups in the previous studies. The two strong LD structures (Block 1 and Block 2) observed in the present study were similar to those observed in previous studies (Detera-Wadleigh and McMahon, 2006; Li and He, 2007; Ma et al., 2006).

4. Discussion

The purpose of the present study was to investigate the association between *G72* polymorphisms and schizophrenia in a large Japanese population, comparable to the sample size included in the meta-analysis of Li and He (2007). Eight *G72* SNPs, which have been associated with schizophrenia in previous studies, were examined in case-control subjects (Detera-Wadleigh and McMahon, 2006; Li and He, 2007; Shi et al., 2008). We failed to replicate the association of any *G72* polymorphism (M12, M14, M15, M19, M20, M22, M23 and M24) with schizophrenia after correction for multiple testing. Power analysis showed that our subjects had sufficient power (>0.99) to detect an effect of the odds ratio (1.33 or more) for each SNP shown in the original study. The findings of the power calculation did not support the hypothesis that the eight SNPs in the *G72* gene are associated with schizophrenia in Japanese population.

Nominal associations of the alleles, M22, M23 and M24, and the genotype of M22 with schizophrenia in this study were no longer positive after correction for multiple testing. However, we discuss the direction of the association in the SNPs, as there are considerable discrepancies among studies. These three SNPs are located from 25.8 Kb to 54.8 Kb 3' downstream of the last exon (exon 5) of *G72*. These SNPs form a highly strong LD block whose pattern is similar among different ethnic groups. The first study by Chumakov et al. reported that the minor allele G of M22 was less frequent in Canadian patients with schizophrenia (31%) than in controls (40%). However, the following studies have indicated the reverse direction of the association of M22 compared with the original study (patients vs. controls: Ma et al.; 47.5 vs. 39.1 in Scottish samples, 41.1 vs. 34.6 in Chinese samples). Our results indicated that the minor allele G of M22 was enriched in patients with schizophrenia (26.0) than in control (23.6). Consistent to the discrepancies among studies (different direction in European populations and same direction in Asian population), recent meta-analysis has reported significant evidence for the association of M22 with schizophrenia in Asian population, but not in European population (Ma et al., 2006; Shi et al., 2008). The direction of association of M23 has shown significant heterogeneity between individual studies in European populations (C allele at M23, patients vs. controls: Chumakov et al.,2002; 43 vs. 51 in Canadian samples, 40 vs. 49 in Russian sample, Korostishevsky et al., 2006; 37.7 vs. 56.7 in Ashkenazi Jewish samples, Schumacher et al., 2004; 53 vs. 46 in German samples, Ma et al., 2006; 59.3 vs. 46.4 in Scottish samples) (Shi et al., 2008). Our results (patients vs. controls: 43.6 vs. 41.2) were consistent with the direction of the association of M23 in an Asian population (Shi et al., 2008; 53.3 vs. 52.4), although the statistical significance was not evident. The direction of association of M24 among the previous studies was identical (patients vs. controls: Chumakov et al., 2002; 55 vs. 47 in Canadian samples, Schumacher et al., 2004; 56 vs. 50 in German samples). We firstly examined a possible association of M24 with schizophrenia in an Asian population and did not find association with schizophrenia (the frequency of the T allele at M24 in patients: 49.8 and controls: 47.4). This suggests that M24 is not likely to have ethnic heterogeneity between Asian and European populations.

Recently, a large scale genome-wide association study (GWAS) using 479 cases and 2937 controls reported that 12 SNPs (odd ratios ranging 1.27–2.06, $P<1\times10^{-5}$) were associated with schizophrenia in the first analysis (O'Donovan et al., 2008). The subsequent replication studies using 16,726 of total subjects showed the evidence for association with three of the 12 SNPs ($P<1\times10^{-5}$) (O'Donovan et al., 2008). They reported the odd ratios 1.12–1.16 in the three positive SNPs in the overall analysis (O'Donovan et al., 2008). The sample size in the first analysis of the GWAS had power (>0.85) to detect an effect of the odd ratio (1.33 or more) for each SNP in the original *G72* study. However, association between SNPs in *G72* and schizophrenia was not detected in the study. Failure to detect association signal does not provide conclusive exclusion of any given gene identified so far. The discrepancies among previous studies and the present study might be explained by differences in ethnic heterogeneity, phenotypic heterogeneity or study designs, such as sample size and case-control versus family-based association study. Factors like incomplete coverage of common variants, inadequate power, allelic and locus heterogeneity could all affect our ability to detect genetic association.

In conclusion, the present study did not support a strong association of the *G72* gene with schizophrenia in a Japanese population. Three SNPs and several haplotypes gave nominal evidence for association and this did not survive correction for multiple testing. *G72* is not likely to be a major susceptibility gene for schizophrenia in this Japanese population.

Role of funding source

This work was supported in part by Grants-in-Aid from the Japanese Ministry of Health, Labor and Welfare (H18-kokoro-005, H19-kokoro-002), the Japanese Ministry of Education, Culture, Sports, Science and Technology (18689030), CREST of JST, Grant-aid for Scientific Research on Priority Areas — Research on Pathomechanisms of Brain Disorders — from the MEXT (18023045) and Japan Foundation for Neuroscience and Mental Health. The study sponsors had no further role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

R. Hashimoto supervised the entire project, collected the data, wrote the manuscript, was critically involved in the design, analysis and interpretation of the data and was responsible for performing the literature review. K. Ohi was critically involved in the collection and analysis of the data, and contributed to the editing of the final manuscript and contributed intellectually to the interpretation of the data. Y. Yasuda, T. Yoshida, H. Takahashi, N. Iike, M. Fukumoto, H. Takamura, M. Iwase, K. Kamino, R. Ishii, H. Kazui, R. Sekiyama, Y. Kitamura, M. Azechi, K. Ikezawa, R. Kurimoto, E. Kamagata, H. Tanimukai, S. Tagami, T. Morihara, M. Ogasawara, M. Okochi, H. Tokunaga, S. Numata, M. Ikeda, T. Ohnuma, T. Fukunaga, T. Tanaka, T. Kudo, S. Ueno, H. Arai, T. Ohmori, N. Iwata, N. Ozaki and M. Takeda were heavily involved in the collection of the majority of the data and contributed intellectually to the interpretation of the data. All authors contributed to and have approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

Acknowledgments

We thank all subjects who participated in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.01.019.

References

Addington, A.M., Gornick, M., Sporn, A.L., Gogtay, N., Greenstein, D., Lenane, M., Gochman, P., Baker, N., Balkissoon, R., Vakkalanka, R.K., Weinberger, D.R., Straub, R.E., Rapoport, J.L., 2004. Polymorphisms in the 13q33.2 gene G72/G30 are associated with childhood-onset schizophrenia and psychosis not otherwise specified. Biol. Psychiatry 55, 976–980.

Bakker, S.C., Hoogendoorn, M.L., Hendriks, J., Verzijlbergen, K., Caron, S., Verduijn, W., Selten, J.P., Pearson, P.L., Kahn, R.S., Sinke, R.J., 2007. The *PIP5K2A* and *RGS4* genes are differentially associated with deficit and non-deficit schizophrenia. Genes Brain Behav. 6, 113–119.

Blouin, J.L., Dombroski, B.A., Nath, S.K., Lasseter, V.K., Wolyniec, P.S., Nestadt, G., Thornquist, M., Ullrich, G., McGrath, J., Kasch, L., Lamacz, M., Thomas, M.G., Gehrig, C., Radhakrishna, U., Snyder, S.E., Balk, K.G., Neufeld, K., Swartz, K.L., DeMarchi, N., Papadimitriou, G.N., Dikeos, D.G., Stefanis, C.N., Chakravarti, A., Childs, B., Housman, D.E., Kazazian, H.H., Antonarakis, S., Pulver, A.E., 1998. Schizophrenia susceptibility loci on chromosomes 13q32 and 8p21. Nat. Genet. 20, 70–73.

Boks, M.P., Rietkerk, T., van de Beek, M.H., Sommer, I.E., de Koning, T.J., Kahn, R.S., 2007. Reviewing the role of the genes G72 and DAAO in glutamate neurotransmission in schizophrenia. Eur. Neuropsychopharmacol. 17, 567–572.

Brzustowicz, L.M., Honer, W.G., Chow, E.W., Little, D., Hogan, J., Hodgkinson, K., Bassett, A.S., 1999. Linkage of familial schizophrenia to chromosome 13q32. Am. J. Hum. Genet. 65, 1096–1103.

Cardno, A.G., Gottesman, 2000. Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. Am. J. Med. Genet. 97, 12–17.

Chen, Y.S., Akula, N., Detera-Wadleigh, S.D., Schulze, T.G., Thomas, J., Potash, J.B., DePaulo, J.R., McInnis, M.G., Cox, N.J., McMahon, F.J., 2004. Findings in an independent sample support an association between bipolar affective disorder and the G72/G30 locus on chromosome 13q33. Mol. Psychiatry 9, 87–92.

Chumakov, I., Blumenfeld, M., Guerassimenko, O., Cavarec, L., Palicio, M., Abderrahim, H., Bougueleret, L., Barry, C., Tanaka, H., La Rosa, P., Puech, A., Tahri, N., Cohen-Akenine, A., Delabrosse, S., Lissarrague, S., Picard, F.P., Maurice, K., Essioux, L., Millasseau, P., Grel, P., Debailleul, V., Simon, A.M., Caterina, D., Dufaure, I., Malekzadeh, K., Belova, M., Luan, J.J., Bouillot, M., Sambucy, J.L., Primas, G., Saumier, M., Boubkiri, N., Martin-Saumier, S., Nasroune, M., Peixoto, H., Delaye, A., Pinchot, V., Bastucci, M., Guillou, S., Chevillon, M., Sainz-Fuertes, R., Meguenni, S., Aurich-Costa, J., Cherif, D., Gimalac, A., Van Duijn, C., Gauvreau, D., Ouellette, G., Fortier, I., Raelson, J., Sherbatich, T., Riazanskaia, N., Rogaev, E., Raeymaekers, P., Aerssens, J., Konings, F., Luyten, W., Macciardi, F., Sham, P.C., Straub, R.E., Weinberger, D.R., Cohen, N., Cohen, D., 2002. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc. Natl. Acad. Sci. U. S. A. 99, 13675–13680.

Corvin, A., McGhee, K.A., Murphy, K., Donohoe, G., Nangle, J.M., Schwaiger, S., Kenny, N., Clarke, S., Meagher, D., Quinn, J., Scully, P., Baldwin, P., Browne, D., Walsh, C., Waddington, J.L., Morris, D.W., Gill, M., 2007. Evidence for association and epistasis at the DAOA/G30 and D-amino acid oxidase loci in an Irish schizophrenia sample. Am. J. Med. Genet. B Neuropsychiatr. Genet. 144, 949–953.

Detera-Wadleigh, S.D., McMahon, F.J., 2006. G72/G30 in schizophrenia and bipolar disorder: review and meta-analysis. Biol. Psychiatry 60, 106–114.

Fallin, M.D., Lasseter, V.K., Avramopoulos, D., Nicodemus, K.K., Wolyniec, P.S., McGrath, J.A., Steel, G., Nestadt, G., Liang, K.Y., Huganir, R.L., Valle, D., Pulver, A.E., 2005. Bipolar I disorder and schizophrenia: a 440-single-nucleotide polymorphism screen of 64 candidate genes among Ashkenazi Jewish case-parent trios. Am. J. Hum. Genet. 77, 918–936.

Goldberg, T.E., Straub, R.E., Callicott, J.H., Hariri, A., Mattay, V.S., Bigelow, L., Coppola, R., Egan, M.F., Weinberger, D.R., 2006. The G72/G30 gene complex and cognitive abnormalities in schizophrenia. Neuropsychopharmacology 31, 2022–2032.

Hall, D., Gogos, J.A., Karayiorgou, M., 2004. The contribution of three strong candidate schizophrenia susceptibility genes in demographically distinct populations. Genes Brain Behav. 3, 240–248.

Hattori, E., Liu, C., Badner, J.A., Bonner, T.I., Christian, S.L., Maheshwari, M., Detera-Wadleigh, S.D., Gibbs, R.A., Gershon, E.S., 2003. Polymorphisms at the G72/G30 gene locus, on 13q33, are associated with bipolar disorder in two independent pedigree series. Am. J. Hum. Genet. 72, 1131–1140.

Hong, C.J., Hou, S.J., Yen, F.C., Liou, Y.J., Tsai, S.J., 2006. Family-based association study between G72/G30 genetic polymorphism and schizophrenia. Neuroreport 17, 1067–1069.

Korostishevsky, M., Kaganovich, M., Cholostoy, A., Ashkenazi, M., Ratner, Y., Dahary, D., Bernstein, J., Bening-Abu-Shach, U., Ben-Asher, E., Lancet, D., Ritsner, M., Navon, R., 2004. Is the G72/G30 locus associated with schizophrenia? Single nucleotide polymorphisms, haplotypes, and gene expression analysis. Biol. Psychiatry 56, 169–176.

Korostishevsky, M., Kremer, I., Kaganovich, M., Cholostoy, A., Murad, I., Muhaheed, M., Bannoura, I., Rietschel, M., Dobrusin, M., Bening-Abu-Shach, U., Belmaker, R.H., Maier, W., Ebstein, R.P., Navon, R., 2006. Transmission disequilibrium and haplotype analyses of the G72/G30 locus: suggestive linkage to schizophrenia in Palestinian Arabs living in the North of Israel. Am. J. Med. Genet. B Neuropsychiatr. Genet. 141, 91–95.

Li, D., He, L., 2007. G72/G30 genes and schizophrenia: a systematic meta-analysis of association studies. Genetics 175, 917–922.

Lin, M.W., Curtis, D., Williams, N., Arranz, M., Nanko, S., Collier, D., McGuffin, P., Murray, R., Owen, M., Gill, M., Powell, J., 1995. Suggestive evidence for linkage of schizophrenia to markers on chromosome 13q14.1–q32. Psychiatr. Genet. 5, 117–126.

Liu, Y.L., Fann, C.S., Liu, C.M., Chang, C.C., Wu, J.Y., Hung, S.I., Liu, S.K., Hsieh, M.H., Hwang, T.J., Chan, H.Y., Chen, J.J., Faraone, S.V., Tsuang, M.T., Chen, W.J., Hwu, H.G., 2006. No association of G72 and D-amino acid oxidase genes with schizophrenia. Schizophr. Res. 87, 15–20.

Ma, J., Qin, W., Wang, X.Y., Guo, T.W., Bian, L., Duan, S.W., Li, X.W., Zou, F.G., Fang, Y.R., Fang, J.X., Feng, G.Y., Gu, N.F., St Clair, D., He, L., 2006. Further evidence for the association between G72/G30 genes and schizophrenia in two ethnically distinct populations. Mol. Psychiatry 11, 479–487.

Madeira, C., Freitas, M.E., Vargas-Lopes, C., Wolosker, H., Panizzutti, R., 2008. Increased brain D-amino acid oxidase (DAAO) activity in schizophrenia. Schizophr. Res. 101, 76–83.

Mulle, J.G., Chowdari, K.V., Nimgaonkar, V., Chakravarti, A., 2005. No evidence for association to the G72/G30 locus in an independent sample of schizophrenia families. Mol. Psychiatry 10, 431–433.

Nyholt, D.R., 2004. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am. J. Hum. Genet. 74, 765–769.

O'Donovan, M.C., Craddock, N., Norton, N., Williams, H., Peirce, T., Moskvina, V., Nikolov, I., Hamshere, M., Carroll, L., Georgieva, L., Dwyer, S., Holmans, P., Marchini, J.L., Spencer, C.C., Howie, B., Leung, H.T., Hartmann, A.M., Moller, H.J., Morris, D.W., Shi, Y., Feng, G., Hoffmann, P., Propping, P., Vasilescu, C., Maier, W., Rietschel, M., Zammit, S., Schumacher, J., Quinn, E.M., Schulze, T.G., Williams, N.M., Giegling, I., Iwata, N., Ikeda, M., Darvasi, A., Shifman, S., He, L., Duan, J., Sanders, A.R., Levinson, D.F., Gejman, P.V., Buccola, N.G.,

Mowry, B.J., Freedman, R., Amin, F., Black, D.W., Silverman, J.M., Byerley, W.F., Cloninger, C.R., Cichon, S., Nothen, M.M., Gill, M., Corvin, A., Rujescu, D., Kirov, G., Owen, M.J., 2008. Identification of loci associated with schizophrenia by genome-wide association and follow-up. Nat. Genet. 40, 1053–1055.

Owen, M.J., Williams, N.M., O'Donovan, M.C., 2004. The molecular genetics of schizophrenia: new findings promise new insights. Mol. Psychiatry 9, 14–27.

Prata, D., Breen, G., Osborne, S., Munro, J., St Clair, D., Collier, D., 2008. Association of DAO and *G72(DAOA)/G30* genes with bipolar affective disorder. Am. J. Med. Genet. B Neuropsychiatr. Genet. 147B, 914–917.

Rietschel, M., Beckmann, L., Strohmaier, J., Georgi, A., Karpushova, A., Schirmbeck, F., Boesshenz, K.V., Schmal, C., Burger, C., Jamra, R.A., Schumacher, J., Hofels, S., Kumsta, R., Entringer, S., Krug, A., Markov, V., Maier, W., Propping, P., Wust, S., Kircher, T., Nothen, M.M., Cichon, S., Schulze, T.G., 2008. G72 and its association with major depression and neuroticism in large population-based groups from Germany. Am. J. Psychiatry 165, 753–762.

Sanders, A.R., Duan, J., Levinson, D.F., Shi, J., He, D., Hou, C., Burrell, G.J., Rice, J.P., Nertney, D.A., Olincy, A., Rozic, P., Vinogradov, S., Buccola, N.G., Mowry, B.J., Freedman, R., Amin, F., Black, D.W., Silverman, J.M., Byerley, W.F., Crowe, R.R., Cloninger, C.R., Martinez, M., Gejman, P.V., 2008. No significant association of 14 candidate genes with schizophrenia in a large European ancestry sample: implications for psychiatric genetics. Am. J. Psychiatry 165, 497–506.

Schumacher, J., Jamra, R.A., Freudenberg, J., Becker, T., Ohlraun, S., Otte, A.C., Tullius, M., Kovalenko, S., Bogaert, A.V., Maier, W., Rietschel, M., Propping, P., Nothen, M.M., Cichon, S., 2004. Examination of G72 and D-amino-acid oxidase as genetic risk factors for schizophrenia and bipolar affective disorder. Mol. Psychiatry 9, 203–207.

Schumacher, J., Abou Jamra, R., Becker, T., Klopp, N., Franke, P., Jacob, C., Sand, P., Fritze, J., Ohlraun, S., Schulze, T.G., Rietschel, M., Illig, T., Propping, P., Cichon, S., Deckert, J., Nothen, M.M., 2005. Investigation of the DAOA/G30 locus in panic disorder. Mol. Psychiatry 10, 428–429.

Scolari, M.J., Acosta, G.B., 2007. D-serine: a new word in the glutamatergic neuro-glial language. Amino Acids 33, 563–574.

Shi, J., Badner, J.A., Gershon, E.S., Liu, C., 2008. Allelic association of G72/G30 with schizophrenia and bipolar disorder: a comprehensive meta-analysis. Schizophr. Res. 98, 89–97.

Shin, H.D., Park, B.L., Kim, E.M., Lee, S.O., Cheong, H.S., Lee, C.H., Kim, S.G., Sohn, J.W., Park, C.S., Kim, J.W., Kim, B.H., Kim, I.Y., Choi, I.G., Woo, S.I., 2007. Association analysis of G72/G30 polymorphisms with schizophrenia in the Korean population. Schizophr. Res. 96, 119–124.

Shinkai, T., De Luca, V., Hwang, R., Muller, D.J., Lanktree, M., Zai, G., Shaikh, S., Wong, G., Sicard, T., Potapova, N., Trakalo, J., King, N., Matsumoto, C., Hori, H., Wong, A.H., Ohmori, O., Macciardi, F., Nakamura, J., Kennedy, J.L., 2007. Association analyses of the DAOA/G30 and D-amino-acid oxidase genes in schizophrenia: further evidence for a role in schizophrenia. Neuromolecular Med. 9, 169–177.

Skol, A.D., Scott, L.J., Abecasis, G.R., Boehnke, M., 2006. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat. Genet. 38, 209–213.

Vilella, E., Costas, J., Sanjuan, J., Guitart, M., De Diego, Y., Carracedo, A., Martorell, L., Valero, J., Labad, A., De Frutos, R., Najera, C., Molto, M.D., Toirac, I., Guillamat, R., Brunet, A., Valles, V., Perez, L., Leon, M., de Fonseca, F.R., Phillips, C., Torres, M., 2008. Association of schizophrenia with DTNBP1 but not with DAO, DAOA, NRG1 and RGS4 nor their genetic interaction. J. Psychiatr. Res. 42, 278–288.

Wang, X., He, G., Gu, N., Yang, J., Tang, J., Chen, Q., Liu, X., Shen, Y., Qian, X., Lin, W., Duan, Y., Feng, G., He, L., 2004. Association of G72/G30 with schizophrenia in the Chinese population. Biochem. Biophys. Res. Commun. 319, 1281–1286.

Williams, N.M., Green, E.K., Macgregor, S., Dwyer, S., Norton, N., Williams, H., Raybould, R., Grozeva, D., Hamshere, M., Zammit, S., Jones, L., Cardno, A., Kirov, G., Jones, I., O'Donovan, M.C., Owen, M.J., Craddock, N., 2006. Variation at the DAOA/G30 locus influences susceptibility to major mood episodes but not psychosis in schizophrenia and bipolar disorder. Arch. Gen. Psychiatry 63, 366–373.

Wood, L.S., Pickering, E.H., Dechairo, B.M., 2007. Significant support for DAO as a schizophrenia susceptibility locus: examination of five genes putatively associated with schizophrenia. Biol. Psychiatry 61, 1195–1199.

Yamaguchi-Kabata, Y., Nakazono, K., Takahashi, A., Saito, S., Hosono, N., Kubo, M., Nakamura, Y., Kamatani, N., 2008. Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effects on population-based association studies. Am. J. Hum. Genet. 83, 445–456.

Yue, W., Liu, Z., Kang, G., Yan, J., Tang, F., Ruan, Y., Zhang, J., Zhang, D., 2006. Association of G72/G30 polymorphisms with early-onset and male schizophrenia. Neuroreport 17, 1899–1902.

Yue, W., Kang, G., Zhang, Y., Qu, M., Tang, F., Han, Y., Ruan, Y., Lu, T., Zhang, J., Zhang, D., 2007. Association of DAOA polymorphisms with schizophrenia and clinical symptoms or therapeutic effects. Neurosci. Lett. 416, 96–100.

Zou, F., Li, C., Duan, S., Zheng, Y., Gu, N., Feng, G., Xing, Y., Shi, J., He, L., 2005. A family-based study of the association between the G72/G30 genes and schizophrenia in the Chinese population. Schizophr. Res. 73, 257–261.

Biochemical and Biophysical Research Communications 379 (2009) 691–695

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Decrease of dynamin 2 levels in late-onset Alzheimer's disease alters Aβ metabolism

Eiichiro Kamagata [a], Takashi Kudo [a,*], Ryo Kimura [b], Hitoshi Tanimukai [a], Takashi Morihara [a], Md.Golam Sadik [a], Kouzin Kamino [c], Masatoshi Takeda [a]

[a] Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Osaka General Medical Center, 3-1-56 Bandai-higashi, Sumiyoshi-ku, Osaka 558-8558, Japan
[c] Shoraiso National Hospital, 2815 Koizumi-cho, Yamatokoriyama, Nara 639-1042, Japan

ARTICLE INFO

Article history:
Received 1 December 2008
Available online 4 January 2009

Keywords:
Alzheimer's disease
Dynamin
Amyloid-β peptide
Amyloid precursor protein
Lipid raft

ABSTRACT

Late-onset Alzheimer's disease (LOAD) is significantly associated with a single nucleotide polymorphism located in the dynamin (DNM) 2 gene, especially in non-carriers of the apolipoprotein E-ε4 allele. In this study we used real-time PCR to show that *DNM2* mRNA is significantly reduced in the cortex of AD brains and in the peripheral blood of dementia patients. Neuroblastoma cells transfected with a dominant negative *DNM2* had increased amyloid beta protein (Aβ) secretion and most of the amyloid precursor protein (APP) in these cells was localized to the plasma membrane. In addition, these cells were rich in flotillin, which is a component of lipid rafts. These data suggest that *DNM2* expression is reduced in LOAD, which results in the accumulation of APP in lipid raft-rich plasma membranes. Consequently, Aβ secretion may increase in LOAD neurons.

© 2008 Elsevier Inc. All rights reserved.

Alzheimer's disease (AD) is the most common form of dementia in the elderly, characterized by cognitive decline and progressive neurodegeneration in the brain. In AD brains, two types of abnormal protein deposits are observed pathologically. One is neurofibrillary tangles (NFTs), which consist mainly of tau protein, forming paired helical filament (PHFs). The other is extracellular amyloid plaques, which are composed of amyloid beta protein (Aβ). The current consensus is that Aβ is a causative molecule in AD by disturbing synaptic function, which leads to neuronal death (for review, see [1,2]). Early-onset AD (EOAD) and late-onset AD (LOAD) exhibit the same neuropathology in the brain, however, LOAD, which represents the majority of AD cases, is genetically classified as a polygenetic disease and is characterized by more heterogeneous conditions compared with EOAD, some of which are autosomal dominant.

Aβ interacts with dynamin1 (DNM1) [3] and *DNM2*, a homologue of *DNM1*, is encoded on chromosome 19p13.2 where a susceptibility locus has been detected by linkage analysis. We previously examined the genetic association of LOAD with the *DNM2* gene and showed a significant association of LOAD with a single nucleotide polymorphism (SNP) marker of the *DNM2* gene, especially in non-carriers of the apolipoprotein E-ε4 allele [4]. Furthermore, the dynamin-binding protein gene on chromosome 10 has also been associated with LOAD [5].

* Corresponding author. Fax: +81 6 6879 3052.
E-mail address: kudo@psy.med.osaka-u.ac.jp (T. Kudo).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.147

The relationship between the function of DNM2 and amyloid pathology is largely unknown. To address this issue, we measured the level of *DNM2* mRNA in LOAD brains and in peripheral blood. Also, using *DNM1* or *DNM2* dominant negative neuronal cells we investigated the influence of DNM2 dysfunction on the metabolism of Aβ and on the localization of amyloid precursor protein (APP).

Materials and methods

Subjects. Postmortem temporal cortex tissues were obtained from the Brain and Tissue Bank for Developmental Disorders of the National Institute of Child Health and Human Development. The samples were from 7 histopathologically confirmed AD patients (age 82.6 ± 3.4) and 7 aged individuals without neurological symptoms (age 65.0 ± 11.5). After obtaining written informed consent to participate in this study, peripheral blood was drawn from 82 dementia patients (49 AD, 14 mild cognitive impairment, 4 vascular dementia, 2 front temporal dementia, 13 other dementia; age 72.0 ± 8.1) and from 11 aged controls (age 63.7 ± 9.6). Total RNA was isolated from frozen temporal cortex tissues and from blood using Isogen (Nippon Gene), an acid guanidine–phenol–chloroform RNA extraction method, and purified using an RNeasy Mini kit (Qiagen). RNA samples with an A_{260}/A_{280} absorption ratio over 1.9 were subjected to cDNA synthesis using a High-Capacity cDNA Archive kit (Applied Biosystems). The procedure to obtain the specimens was approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine.