Table 2 Comparison of the location of significant increases in BOLD signal during the modified Stroop task performed by TBI patients and healthy subjects

Region	Size of voxels	Coordinates (mm)			Z score
		x	y	z	
Controls					
Right anterior cingulate and medial frontal gyrus (BA 6, 32)	1,904	2	−2	58	6.48
		2	18	46	5.71
Left inferior parietal gyrus and precuneus (BA 7, 19, 40)	4,292	−24	−90	16	6.28
		−32	−62	52	5.93
Right inferior parietal gyrus and precuneus (BA 19, 40)	2,290	34	−76	30	6.06
		46	−54	52	5.74
		34	−68	38	5.62
Left middle and inferior frontal gyrus (BA 44, 46)	865	−44	42	22	5.75
		−44	18	26	4.32
Right inferior parietal and precuneus (BA 19, 37)	2,386	−48	8	34	3.46
		50	−66	−16	5.69
		56	−54	−12	5.40
Right middle frontal gyrus (BA 6)	527	36	−4	52	4.70
Right inferior frontal gyrus (BA 10, 46)	194	48	36	22	3.73
		40	50	22	3.65
Left middle frontal gyrus (BA 6)	117	−28	−10	56	3.63
Patients with TBI					
Left inferior frontal gyrus (BA 45)	93	−56	18	8	5.14
Left middle frontal gyrus (BA 6)	442	−48	4	24	4.67
Right middle and inferior frontal gyrus (BA 6, 9, 44)	227	40	8	26	4.21
		52	8	42	3.81
		44	4	34	3.44
Right inferior parietal gyrus (BA 40)	197	36	−64	42	4.13
Right medial frontal gyrus (BA 6)	77	10	4	54	3.95

The cingulate cortex is comprised of the anterior and posterior cortices, each of which possesses different thalamic and cortical connections [9]. The ACC can be divided into discrete anatomic and behavioral subdivisions: the affective division (ACed) and the cognitive division (ACcd) [14]. The ACed includes areas 25, 33, and rostal area 24 and plays a role in emotion and motivation; the ACcd includes caudal areas 24 and 32 and plays a role in complex cognitive/attentional processing [10, 11, 14]. Neuroimaging studies have shown that the ACcd is activated by numerous cognitive/attentional tasks including Stroop tasks, divided attention tasks, and working memory tasks [12, 24, 25]. The ACcd is vitally important for the proper and efficient functioning of frontostriatal attention networks.

One key region of working memory is the prefrontal cortex, and, in patients with TBI, the frontal cortices tend to be damaged both structurally and functionally

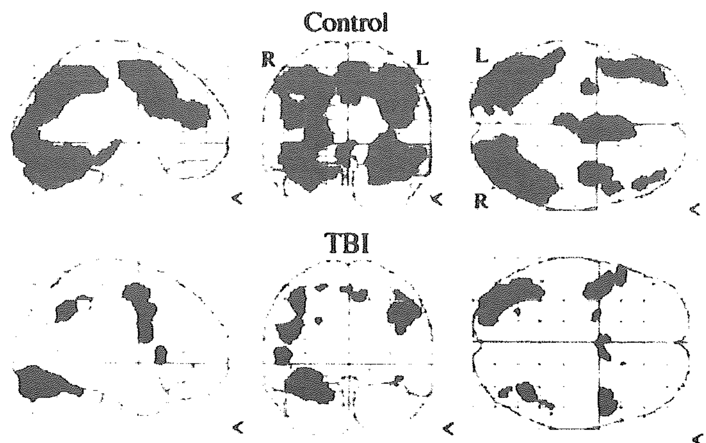

Fig. 1 Maximum-intensity projections of the statistical parametric maps during the Stroop task for TBI patients (*bottom*) and healthy controls (*top*) ($P < 0.001$, uncorrected). For localization of activation see Table 1

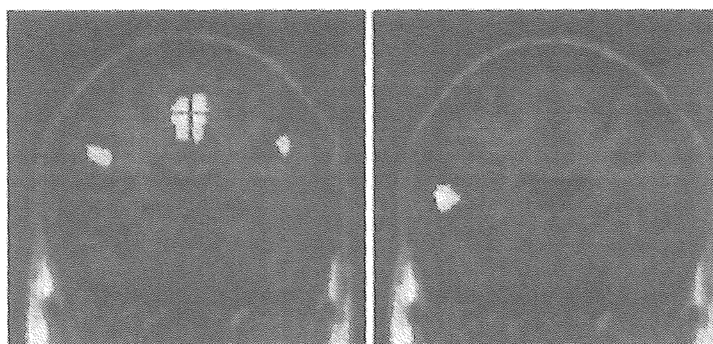

Fig. 2 Location of the cognitive division of the ACC (ACcd) on coronal slices (y = + 18 mm). The absence of activity in TBI patients (*right*) and significant activity in control subjects (*left*) is superimposed on group-averaged Talairach and Tournoux transformed high-resolution structural scans [23]

[5, 25]. In a functional PET study, TBI patients showed increased activity in the ACC and in frontal and occipital lobe activity during working memory tasks [26]. fMRI study of TBI patients showed higher levels of activation under the high processing load condition than under the low condition [27]. This extensive activation under working memory load may represent a response to increased task difficulty [24]. However, our TBI patients showed less ACcd activation than the controls. Decreased activation of the ACcd has been noted in patients with attention-deficit/hyperactivity disorder (ADHD) who manifested performance deficits in the Stroop task [28]. Even patients able to keep up with the processing demands did so less efficiently for a given level of performance accuracy than poorly performing subjects [29]. However, under-activation in a poorly performing subject may reflect failure to fully engage in the task when it exceeds the subject's processing capacity [30].

Brain function is now interpreted on the basis of neural networks rather than separate anatomical structures. In our TBI patients, cerebral activation was more regionally dispersed and diminished in the prefrontal and parietal cortex. This decrease may be the result of a decrease in the functional and structural connectivity of parietal and prefrontal regions [31, 32]. It has been shown that the ACcd keeps up with the lateral prefrontal cortex, parietal cortex, and lower motor area [10]. However, it is not known whether it is the sole dominant region for cognitive function, attentional selection, and processing. ACcd dysfunction in a parallel-distributed network is not eliminated, as apparent frontostriatal deficits may be the downstream effects of ACcd dysfunction [14, 33, 34]. At any rate, these changes are probably the result of diffuse axonal injury and may reflect either cortical disinhibition attributable to disconnection or compensation for an inefficient cognitive process.

Several limitations of this study deserve mention. First, our results may not be applicable to older and more severely disabled patients who cannot undergo fMRI. Second, our participants were not subjected to detailed neuropsychological testing of mental status, visual memory, reaction time, and language function testing. Although psychological assessments can yield important information, they fail to reveal the neural substrates or pathways. fMRI scans make it possible to graphically interrogate the cortical physiology under various stimulation paradigms. Third, we utilized a block design fMRI technique rather than an event-related fMRI approach. Event-related designs allow the extraction of relative timing information on the onset of activity in different neural substrates as well as the duration of cognitive processing during a task, offering new opportunities for cognitive studies [35]. Further investigations utilizing an event-related design are necessary for the full explication of our findings. Lastly, our study population consisted of a relatively small number of subjects, and additional experiments with a larger sample size and a randomized design are necessary.

Conclusions

Cognitive impairment in TBI patients appears to be associated with alterations in functional cerebral activity and, especially, with decreased activation of the ACC. Decreased activation in the ACcd regions may represent evidence of cognitive impairment.

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References

1. Levin HS, Gary HE Jr, Eisenberg HM, Ruff HM, Barth JT, Kreutzer J, High WM Jr, Portman S, Foulkes MA, Jane JA, Marmarou A, Marshall LF (1990) Neurobehavioral outcome 1 year after severe head injury. Experience of the traumatic coma bank data. J Neurosurg 73:699–709
2. Levin HS, Williams DH, Eisenberg HM, High WM Jr, Guinto FC Jr (1992) Serial MRI and neurobehavioural findings after mild to moderate closed head injury. J Neurol Neurosurg Psychiatry 55:255–262
3. Stamatakis EA, Wilson JTL, Hadley DM, Wyper DJ (2002) SPECT imaging in head injury interpreted with statistical parametric mapping. J Nucl Med 43:217–226
4. Levin HS, Williams DH, Valastro M, Eisenberg HM, Crofford MJ, Handel SF (1990) Corpus callosal atrophy following closed head injury: detection with magnetic resonance imaging. J Neurosurg 73:77–81
5. Langfitt TW, Obrist WD, Alavi A, Grossman RI, Zimmerman R, Jaggi J, Uzzell B, Reivich M, Patton DR (1986) Computerized tomography, magnetic resonance imaging, and positron emission tomography in the study of brain trauma. Preliminary observations. J Neurosurg 64:760–767
6. D'Esposito M (2000) Functional neuroimaging of cognition. Semin Neurol 4:487–498
7. Christodoulou C, DeLuca J, Ricker JH, Madigan NK, Bly BM, Lange G, Kalnin AJ, Liu WC, Steffener J, Diamond BJ, Ni AC (2001) Functional magnetic resonance imaging of working memory impairment after traumatic brain injury. J Neurol Neurosurg Psychiatry 71:161–168
8. Ricker JH, Muller RA, Zafonte RD, Black KM, Millis SR, Chugani H (2001) Verbal recall and recognition following traumatic brain injury. A [O-15]-water positron emission tomography study. J Clin Exp Neuropsychol 23:196–206
9. Vogt BA, Finch DM, Olson CR (1992) Functional heterogeneity in cingulate cortex. The anterior executive and posterior evaluation regions. Cereb Cortex 2:435–443
10. Devinsky O, Morrell MJ, Vogt BA (1995) Contributions of anterior cingulate cortex to behavior. Brain 118:279–306
11. Whalen PJ, Bush G, McNally RJ, Wilhelm S, McInerney SC, Jenike MA, Rauch SL (1998) The emotional counting Stroop paradigm. A functional magnetic resonance imaging probe of the anterior cingulate affective division. Biol Psychiatry 44:1219–1228
12. Bush G, Whalen PJ, Rosen BR, Jenike MA, McInerney SC, Rauch SL (1998) The counting Stroop. An interference task specialized for functional neuroimaging—validation study with functional MRI. Hum Brain Mapp 6:270–282
13. Leung HC, Skudlarski P, Gatenby JC (2000) An event-related functional MRI study of the Stroop color word interference task. Cereb Cortex 10:552–560
14. Bush G, Luu P, Posner MI (2000) Cognitive and emotional influences in anterior cingulate cortex. Trends Cogn Sci 4:215–222
15. Gruber SA, Rogowska J, Holocomb P, Soraci S, Yurgelun-Todd D (2002) Stroop performance in normal control subjects. An fMRI study. Neuroimage 16:349–360
16. Stroop JR (1935) Studies of interference in serial verbal reactions. J Exp Psychol 18:643–662
17. Bate AJ, Mathias JL, Crawford JR (2001) Performance on the test of every attention and standard tests of attention following severe traumatic brain injury. Clin Neuropsychol 15:405–422
18. Teasdale G, Jannett B (1974) Assessment of coma and impaired consciousness. A practical scale. Lancet:ii:81–84
19. Folstein MF, Folstein SE, McHugh PR (1975) "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 12:189–198
20. Wechsler D (1985) The Wechsler adult intelligence scale-revised. Psychological Corp., New York
21. Wechsler D (1987) The Wechsler memory scale-revised. The Psychological Corp., San Antonio
22. Gur RC, Gur RE, Obrist WD, Hungerbuhler JP, Younkin D, Rosen AD, Skolnick BE, Reivich M (1982) Sex and handedness differences in cerebral blood flow during rest and cognitive activity. Science 217:659–661
23. Talairach J, Tournoux P (1988) A co-planar stereotactic atlas of a human brain. Thieme, Stuttgart
24. Barch DM, Braver TS, Nystrom LE, Forman SD, Noll DC, Cohen JD (1997) Dissociating working memory from task difficulty in human prefrontal cortex. Neuropsychologia 35:1373–1380
25. Braver TS, Cohen JD, Nystrom LE, Jonides J, Smith EE, Noll DC (1997) A parametric study of prefrontal cortex involvement in human working memory. Neuroimage 5:49–62
26. Levine B, Cabeza R, McIntosh AR, Black SE, Grady CL, Stuss DT (2002) Functional reorganization of memory after traumatic brain injury: a study with $H_2^{15}O$ positron emission tomography. J Neurol Neurosurg Psychiatry 73:173–181
27. McAllister TW, Saykin AJ, Flashman LA, Sparling MB, Johnson SC, Guerin SJ, Mamourian AC, Weaver JB, Yanofsky N (1999) Brain activation during working memory 1 month after mild traumatic brain injury. A functional MRI study. Neurology 53:1300–1308
28. Bush G, Frazier JA, Rauch SL, Seidman LJ, Whalen PJ, Jenike MA, Rosen BR, Biederman J (1999) Anterior cingulated cortex dysfunction in attention-deficit/hyperactivity disorder revealed by fMRI and the counting Stroop. Biol Psychiatry 45:1542–1552
29. Weiss EM, Golaszewski S, Mottaghy FM, Hofer A, Hausmann A, Kemmler G, Kremser C, Brinkhoff C, Felber SR, Fleischhacker WW (2003) Brain activation patterns during a selective attention test. A functional MRI study in healthy volunteers and patients with schizophrenia. Psychiatry Res 123:1–15
30. Bullmore E, Brammer M, Williams SC, Curtis V, McGuire P, Morris R, Murray R, Sharma T (1999) Functional MR imaging of confounded hypofrontality. Hum Brain Mapp 8:86–91
31. Cabeza R, McIntosh AR, Tulving E, Nyberg L, Grady CL (1997) Age-related differences in effective neural connectivity during encoding and recall. Neuroreport 8:3479–3483
32. Raz N, Gunning FM, Head D, Dupuis JH, McQuain J, Briggs SD, Loken WJ, Thronton AE, Acker JD (1997) Selective aging of the human cerebral cortex observed in vivo. Differential vulnerability of the prefrontal gray matter. Cereb Cortex 7:268–282
33. Cohen JD, Dunbar K, McClelland JL (1990) On the control of automatic processes. A parallel disturbed processing account of the Stroop effect. Psychol Rev 97:332–361
34. Goldman-Rakic PS (1988) Topography of cognition. Parallel distributed networks in primate association cortex. Ann Rev Neurosci 11:137–156
35. Menon RS, Kim SG (1999) Spatial andtemporal limits in cognitive neuroimaging with fMRI. Trends Cogn Sci 3:207–216

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Influence of assembly of siRNA elements into RNA-induced silencing complex by fork-siRNA duplex carrying nucleotide mismatches at the 3′- or 5′-end of the sense-stranded siRNA element

Yusuke Ohnishi [a,b], Katsushi Tokunaga [b], Hirohiko Hohjoh [a,*]

[a] *National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[b] *Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

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Abstract

RNA interference (RNAi) is a powerful method for suppressing the expression of a gene of interest, and can be induced by 21–25 nucleotide small interfering RNA (siRNA) duplexes homologous to the silenced gene, which function as sequence-specific RNAi mediators in RNA-induced silencing complexes (RISCs). In the previous study, it was shown that fork-siRNA duplexes, whose sense-stranded siRNA elements carried a few nucleotide mismatches at the 3′-ends against the antisense-stranded siRNA elements, could enhance RNAi activity more than conventional siRNA duplexes in cultured mammalian cells. In this study, we further characterized fork-siRNA duplexes using reporter plasmids carrying target sequences complementary to the sense- or antisense-stranded siRNA elements in the untranslated region of *Renilla* luciferase. The data presented here suggest that nucleotide mismatches at either the 3′- or 5′-end of the sense-stranded siRNA elements in fork-siRNA duplexes could influence assembly of not only the antisense-stranded siRNA elements but also the sense-stranded elements into RISCs. In addition, we further suggest the possibility that there could be a positional effect of siRNA duplex on RNAi activity.
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Keywords: RNA interference; Fork-siRNA; Mismatch; RNAi mediator; RISC

RNA interference (RNAi) is the process of a sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced gene (reviewed in [1–4]). DsRNAs introduced or generated in cells are subjected to digestion with an RNase III enzyme, Dicer, into 21–25 nucleotide (nt) RNA duplexes [5–8], and the resultant RNA duplexes, referred to as small interfering RNA (siRNA) duplexes, can be associated with the RNA-induced silencing complexes (RISCs) and function as sequence-specific RNAi mediators in the complexes [5,7]. In terms of rapid and potent induction of RNAi by exogenous dsRNAs, RNAi has become a powerful reverse genetic tool for suppressing the expression of a gene of interest in various species including mammals.

In mammals, direct introduction of chemically synthesized 21–25 nt siRNA duplexes into cells is often used for induction of RNAi [9–12], although different siRNAs induce different levels of RNAi activities [10,13]. In previous studies, where the effect of various types of synthetic siRNAs on the induction of mammalian RNAi was tested, an improvement of the siRNA duplexes for enhancing RNAi activity was found [14]. The improved siRNA duplexes, named 'fork-siRNA duplexes,' possess mismatched sequences at their termini due to introduction of base substitutions into the

* Corresponding author. Fax: +81 42 346 1748.
E-mail address: hohjohh@ncnp.go.jp (H. Hohjoh).

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3′-ends of the sense-stranded siRNA elements: one of the ends of the fork-siRNA duplex, on the 3′-end of the sense-stranded siRNA element, remains open (unannealed). In addition to the properties of fork-siRNA duplex, recent studies suggested that siRNA duplexes conferring a strong RNAi activity could be characterized by a low base-stability due to AU-rich sequences around the 3′-ends of the sense-stranded siRNA elements, i.e., the functional siRNA duplexes have the asymmetrical features of the AU (or GC) content in their sequences [14–16]. Based on these observations, a model was proposed: the ease of unwinding from one of the ends of the siRNA duplex could determine the orientation of the duplex, and the siRNA element unwound from the 5′-end could be determined and function as a sequence-specific RNAi mediator in RISC [14].

In order to further characterize fork-siRNA duplexes, we report herein the construction of reporter plasmids carrying target sequences complementary to the sense- and antisense-stranded siRNA elements, and examination of the levels of gene silencing depending upon the sense- and antisense-stranded siRNA elements functioning as RNAi mediators in RISCs. The data presented here suggested that the structural features of fork-siRNA duplexes could influence incorporation of their siRNA elements into RISCs.

A
phRL-La21Fw 5′ 3′
phRL-La21Rv 5′ 3′
pGL3-TK 5′ 3′
pGL3-TK-La21Fw 5′ 3′
pGL3-TK-La21Rv 5′ 3′

B
La21-conv.
5′-ACCGCUGGAGAGCAACUGCUU-3′
3′-UUUGGCGACCUCUCGUUGACG-5′

La21-3′m2
5′-ACCGCUGGAGAGCAACUUU-3′
3′-UUUGGCGACCUCUCGUUGACG-5′

La21-5′m2
5′-UACGCUGGAGAGCAACUGCUU-3′
3′-UUUGGCGACCUCUCGUUGACG-5′

La21-ss19
5′-CGCUGGAGAGCAACUGCUU-3′
3′-UUUGGCGACCUCUCGUUGACG-5′

C
La21-conv.
La21-5′m2
La21-ss19
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Norm. *Photinus* Luc. / *Renilla* Luc.

Fig. 1. Schematic drawing of the luciferase transcripts derived from plasmids constructed in this study (A), used synthetic siRNA duplexes (B), and RNAi activities induced by the siRNA duplexes (C). (A) The names of constructed plasmids are indicated. Hatched and open boxes represent the luciferase coding and untranslated regions, respectively. Gray and solid (black) regions indicate target sequences complementary to the antisense- and sense-stranded La21 siRNA elements, respectively. (B) The names of synthetic La21 siRNA duplexes are indicated. Upper and lower sequences in the duplexes represent the sense- and antisense-stranded siRNA elements, respectively. (C) Gene silencing of the *Photinus* luciferase gene. RNAi induction was carried out as described previously [14]. Indicated La21 siRNA duplexes together with pGL3-control and phRL-TK plasmids carrying *Photinus* and *Renilla* luciferase genes, respectively, were transfected into HeLa cells. Twenty-four hours after transfection, cell lysate was prepared and the level of luciferase activity was examined. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are shown: the ratios of luciferase activity determined in the presence of the La21 siRNA duplexes were normalized to the ratio obtained for a control in the presence of a non-silencing siRNA duplex (Qiagen). Data are averages of at least three independent determinations. Error bars represent standard deviations.

Materials and methods

Preparation of oligonucleotides. RNA and DNA synthetic oligonucleotides were obtained from PROLIGO and INVITROGEN, respectively. For preparation of duplexes, sense- and antisense-stranded oligonucleotides (20 μM each) were mixed in an annealing buffer (30 μM Hepes, pH 7.4, 100 μM potassium acetate, and 2 μM magnesium acetate), heat-denatured at 90 °C for 3 min, and annealed at 37 °C overnight. The siRNA duplexes used in this study were as follows: the La21-conv. siRNA duplex (a conventional siRNA duplex), La21-3′m2 siRNA duplex (a fork-siRNA duplex carrying two-base mismatches at the 3′-end of the sense-stranded siRNA element), La21-5′m2 siRNA duplex (a fork-siRNA duplex carrying two-base mismatches at the 5′-end of the sense-stranded siRNA element), and La21-ss19 siRNA duplex (a siRNA duplex containing 19 nt sense-stranded siRNA element) (Fig. 1B) [14]. Note that these La21 siRNA duplexes possess the same antisense-stranded siRNA elements. Non-silencing siRNA duplex (Qiagen) was used as a negative control.

Construction of reporter plasmids. In order to insert target sequence complementary to the sense- or antisense-stranded La21 siRNA element [10] against *Photinus* luciferase in the 3′ untranslated region (UTR) of the *Renilla* luciferase transcripts, the phRL-TK plasmid (Promega) carrying the *Renilla* luciferase gene was digested with *Xba*I and *Not*I, and subjected to ligation with synthetic oligonucleotide duplexes carrying the La21 siRNA target sequences. The sequences of the synthesized oligonucleotides were as follows:

sLa21Fw: 5′-CTAGCATGCAACCGCTGGAGAGCAACTGCA-3′
asLa21Fw: 5′-GGCCTGCAGTTGCTCTCCAGCGGTTGCATG-3′
sLa21Rv: 5′-CTAGCATGCAGCAGTTGCTCTCCAGCGGTA-3′
asLa21Rv: 5′-GGCCTACCGCTGGAGAGCAACTGCTGCATG-3′

The resultant plasmids possessing the target sequences for the antisense- and sense-stranded La21 siRNA elements were named ‘phRL-La21Fw’ and ‘phRL-La21Rv,’ respectively.

We further constructed reporter plasmids carrying two target sites of the La21 siRNA duplex. The *Hin*dIII–*Xba*I fragment encoding the *Photinus* luciferase gene was isolated from the pGL3-control plasmid

(Promega), and substituted for the *Hin*dIII–*Xba*I regions carrying *Renilla* luciferase in the phRL-La21Fw and phRL-La21Rv plasmids. The resultant plasmids derived from the phRL-La21Fw and phRL-La21Rv plasmids were named 'pGL3-TK-La21Fw' and 'pGL3-TK-La21Rv,' respectively, and possessed two La21 siRNA duplex target sites, one in the *Photinus* luciferase coding region and the other in the 3′ UTR. We also constructed 'pGL3-TK' plasmid by substitution of *Photinus* luciferase for *Renilla* luciferase in the phRL-TK plasmid using the same procedure described above.

Cell culture, transfection, and luciferase and β-galactosidase assays. HeLa cells were grown as described previously [10]. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 24-well culture plates (approximately 0.5×10^5 cells/well). Cotransfection of synthetic siRNA duplexes with reporter plasmids was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions, and to each well, 0.24 μg siRNA duplexes, 0.05 μg phRL-La21Fw or phRL-La21Rv plasmid, and 0.1 μg pSV-β-galactosidase control vector (Promega) as a control were applied. Twenty-four hours after transfection, cell lysate was prepared and the expression levels of luciferase and β-galactosidase were examined by a Dual-Luciferase reporter assay system (Promega) and a Beta-Glo assay system (Promega), respectively, according to the manufacturer's instructions. In the case of transfection with pGL3-TK, pGL3-Tk-La21Fw or pGL3-TK-La21Rv, 0.24 μg siRNA duplexes, 0.1 μg of any one of the pGL3-TK, pGL3-Tk-La21Fw, and pGL3-TK-La21Rv plasmids, and 0.05 μg of the phRL-TK plasmid as a control were applied into HeLa cells. Twenty-four hours after transfection, a Dual-Luciferase reporter assay was conducted.

Results and discussion

Influence of assembly of siRNA elements into RISCs by fork-siRNA duplexes

In the previous study, it was shown that fork-siRNA duplexes carrying nucleotide mismatches at the 3′-ends of the sense-stranded siRNA elements could enhance RNAi activity more than conventional siRNA duplexes [14]. This suggests the possibility of greater occurrence of assembly of the antisense-stranded siRNA elements rather than the sense-stranded elements into RISCs in fork-siRNA duplexes over that in conventional duplexes. Accordingly, we attempted to examine whether fork-siRNA duplexes could influence incorporation of their siRNA elements into RISCs. To address this, we constructed two reporter plasmids, phRL-La21Rv and phRL-La21Fw, carrying the target sequences for the sense- and antisense-stranded La21 siRNA elements, respectively, in the 3′ untranslated region (UTR) of *Renilla* luciferase (Fig. 1A). This is because the previous study showed that the La21-conv., La21-3′m2, and La21-5′m2 siRNA duplexes (Fig. 1B) could confer different levels of RNAi activities, although they possessed the same antisense-stranded siRNA element [14]. In addition to the previous results, the result with a newly designed siRNA duplex, the La21-ss19 siRNA duplex (Figs. 1B and C), also supported the idea that the forked terminus of siRNA duplex could influence RNAi activity. Accordingly, we decided to use the sequences of the sense- and antisense-stranded La21 siRNA elements as targets in this study. Using the phRL-La21Fw and -La21Rv plasmids, and a series of the La21 siRNA duplexes, the levels of gene silencing depending upon the sense- and antisense-stranded La21 siRNA elements were investigated.

First we examined if the sense-stranded siRNA elements, like the antisense-stranded siRNA elements, could have potential for functioning as sequence-specific RNAi mediators in RISCs. To see this, the La21-conv. siRNA duplex together with phRL-La21Rv, phRL-La21Fw or phRL-TK and pSV-β-galactosidase control vector as a control were cotransfected into HeLa cells, and the levels of the expression of *Renilla* luciferase were examined. As a result, significant suppression of the expression of *Renilla* luciferase was detectable in the presence of either phRL-La21Rv or phRL-La21Fw, whereas little or no suppression was seen in the presence of phRL-TK as a negative control (Fig. 2). Therefore, these results strongly suggest that either the sense- or antisense-stranded La21 siRNA element can be incorporated into RISC and function as a sequence-specific RNAi mediator in the complex.

We next examined the RNAi activities directed by the sense- and antisense-stranded siRNA elements derived from the La21-3′m2 and La21-5′m2 siRNA duplexes (fork-siRNA duplexes) as well as the La21-conv. siRNA duplex. As shown in Fig. 3A, when the phRL-La21Fw plasmid was used, ~86%, 95%, and 72% gene silencing mediated by the antisense-stranded La21 siRNA elements derived from the La21-conv., La21-3′m2, and

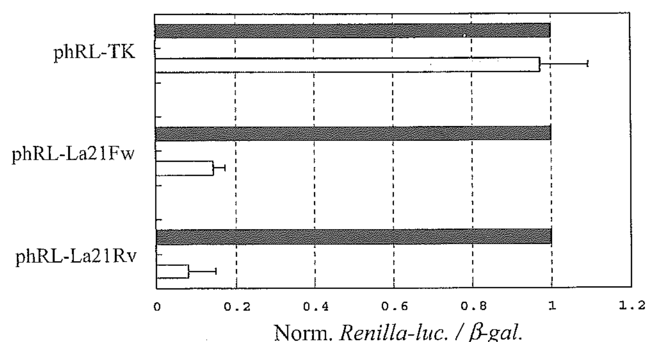

Fig. 2. Gene silencing of exogenous reporter gene with conventional siRNA duplexes. The conventional La21 (La21-conv.) siRNA duplex against the *Photinus* luciferase gene together with phRL-TK, phRL-La21Fw, or phRL-La21Rv plasmid carrying the *Renilla* luciferase reporter gene, and pSV-β-galactosidase control vector as a control were cotransfected into HeLa cells. Twenty-four hours after transfection, cell lysate was prepared, and the levels of the luciferase and β-galactosidase activities were examined. Ratios of normalized target (*Renilla*) luciferase activity to control β-galactosidase activity are shown: the ratios of luciferase activity determined in the presence of the La21-conv. siRNA duplex (gray bars) are normalized to the ratio obtained for a control in the presence of a non-silencing siRNA duplex (Qiagen) (solid bars). Data are averages of at least four independent experiments. Error bars represent standard deviations.

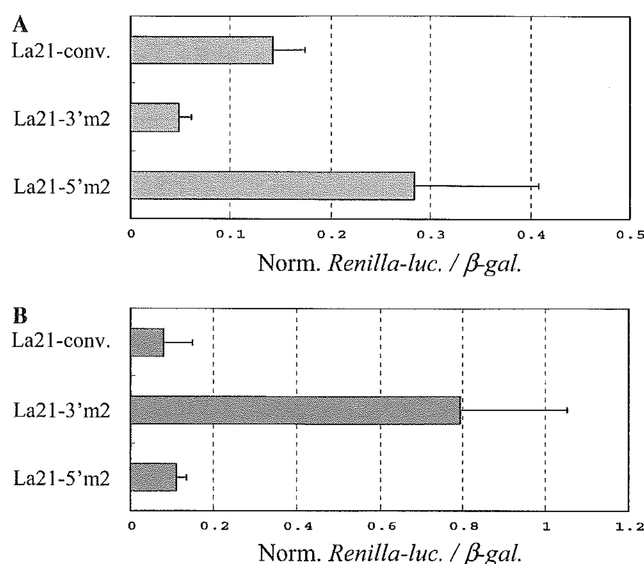

Fig. 3. Silencing of the expression of exogenous reporter gene with various types of siRNA duplexes. The conventional La21 (La21-conv.) or fork-La21 (La21-3′m2 and La21-5′m2) siRNA duplexes together with phRL-La21Fw (A) or phRL-La21Rv (B) reporter plasmid and pSV-β-galactosidase control vector as a control were cotransfected into HeLa cells, and the expression levels of luciferase and β-galactosidase were examined as in Fig. 2. Ratios of normalized target (*Renilla*) luciferase activity to control β-galactosidase activity are shown as in Fig. 2. Data are averages of at least four independent determinations. Error bars indicate standard deviations.

La21-5′m2 siRNA duplexes, respectively, was observed. Although the levels of the gene silencing with phRL-La21Fw as a reporter plasmid increased further than those with the pGL3-control plasmid carrying *Photinus* luciferase in the previous study [14] (further discussion below), the effects of mismatches at the 3′- and 5′-ends of the sense-stranded elements in the La21 fork-siRNA duplexes on RNAi activity appeared to remain unchanged in the experiments using either phRL-La21Fw or pGL3-control.

When the phRL-La21Rv plasmid was used, i.e., when the levels of the RNAi activity directed by the sense-stranded La21 siRNA elements were examined, significant differences in the level of RNAi activity among the La21 siRNA duplexes used were observed: while ∼92% and 89% suppression of the expression of *Renilla* luciferase was detectable in the presence of the La21-conv., and La21-5′m2 siRNA duplexes, respectively, the gene silencing mediated by the sense-stranded element derived from the La21-3′m2 duplex appeared to confer ∼20% inhibition of the expression of *Renilla* luciferase (Fig. 3B), suggesting that the degree of assembly of the sense-stranded siRNA element into RISC in the La21-3′m2 siRNA duplex could be much lower than those in the La21-conv., and La21-5′m2 siRNA duplexes. Taking all the data together, these observations suggest that nucleotide mismatches at the ends of fork-siRNA duplexes can influence assembly of not only the antisense-stranded siRNA elements but also the sense-stranded siRNA elements into RISCs.

The previous in vitro RNAi reaction with *Drosophila* embryo lysate has demonstrated that single nucleotide mismatch around the termini of siRNA duplex can affect target-RNA cleavages directed by the sense- and antisense-stranded siRNA elements [15]. The results of our present study using cultured human cells consistently agree with those in the previous study. Therefore, it appears that the effect of low base-pairing stabilities due to either AU-rich or nucleotide mismatches around the termini of siRNA duplexes on RNAi activity is likely common among various species. In addition, such low base-pairing stability could contribute to ready unwinding of the duplex from that end by a possible helicase activity in RISCs.

Another important point to note in this study is that the sense-stranded siRNA elements have potential for functioning as sequence-specific RNAi mediators in RISCs. As previously suggested [14], this indicates that off-target gene silencing mediated by the sense-stranded siRNA elements could occur in RNAi induction by siRNA duplexes. Our present data also indicated a possible avoidance of such off-target gene silencing: fork-siRNA duplexes carrying nucleotide mismatches at the 3′-end of the sense-stranded elements could reduce such off-target silencing. Therefore, fork-siRNA duplexes may provide us with not only an increase in RNAi activity but also decrease in off-target gene silencing directed by the sense-stranded siRNA elements.

Positional effect of siRNA target site on RNAi activity

The results shown in Fig. 3A led us to the possibility that the position of an siRNA target site on a silenced gene transcript could influence its RNAi activity, i.e., there could be a positional effect of the siRNA target site on RNAi activity. To examine this possibility, we constructed two reporter plasmids carrying *Photinus* luciferase, pGL3-TK-La21Rv and pGL3-TK-La21Fw, whose 3′ UTRs contained the target sequences complementary to the sense- and antisense-stranded La21 siRNA elements, respectively (Fig. 1A). Thus, the resultant *Photinus* luciferase transcripts derived from pGL3-TK-La21Fw and pGL3-TK-La21Rv possess two target sites: one site complementary to the antisense-stranded La21 siRNA element is in the luciferase coding region, and the other complementary to the sense- or antisense-stranded siRNA element is in its 3′ UTR.

The La21-conv., La21-3′m2, or La21-5′m2 siRNA duplexes together with the pGL3-TK (carrying one target site in the luciferase coding region), pGL3-TK-La21Rv or pGL3-TK-La21Fw plasmid (Fig. 1A) and the phRL-TK plasmid as a control were cotransfected into HeLa cells, and the levels of RNAi activity were examined by a dual-luciferase assay. When the

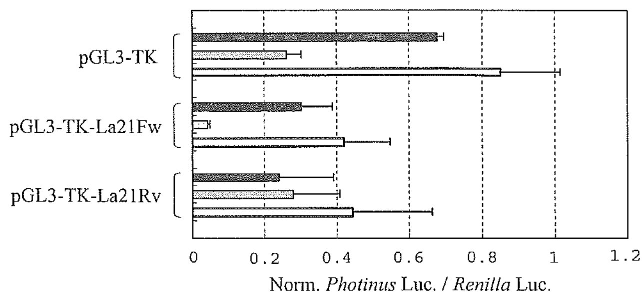

Fig. 4. Gene silencing of *Photinus* luciferase carrying two target sites of the La21 siRNA duplex. The La21-conv., La21-3′m2 or La21-5′m2 siRNA duplex together with pGL3-TK, pGL3-TK-La21Fw or pGL3-TK-La21Rv reporter plasmid carrying *Photinus* luciferase and phRL-TK plasmid carrying *Renilla* luciferase as a control were cotransfected into HeLa cells, and the expression levels of luciferase were examined as in Fig. 1. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are shown as in Fig. 1. Solid, gray, and open bars indicate the data in the presence of the La21-conv., La21-3′m2, and La215′m2 siRNA duplexes, respectively. Data are averages of at least three independent determinations. Error bars represent standard deviations.

pGL3-TK plasmid was used, results similar to those in the previous study using the pGL3-control plasmid encoding *Photinus* luciferase driven by the SV40 promoter [14] were observed (Fig. 4). When the pGL3-TK-La21Fw and pGL3-TK-La21Rv plasmids were used, the levels of RNAi activity, other than those in the presence of the pGL3-TK-La21Rv plasmid and the La21-3′m2 siRNA duplex, appeared to increase more greatly in both the pGL3-TK-La21Fw and pGL3-TK-La21Rv plasmids than in the pGL3-TK plasmid used, suggesting that two target sites of the La21 siRNA duplex on the silenced *Photinus* luciferase transcript probably contributed to the enhancement of RNAi activity. As for the RNAi activity in the presence of pGL3-TK-La21Rv and the La21-3′m2 siRNA duplex, it may be that the target site in the luciferase coding region, not in the 3′ UTR, is only recognizable for active RISCs, since a rather weak RNAi activity mediated by the sense-stranded siRNA element in the La21-3′m2 siRNA duplex was detected in the presence of phRL-La21Rv (Fig. 3B). This may account for the lack of significant difference in the level of RNAi activity between pGL3-TK and pGL3-TK-La21Rv in the presence of the La21-3′m2 siRNA duplex.

It should be noted that the La21-5′m2 siRNA duplex was able to confer ~60% suppression of the expression of *Photinus* luciferase in pGL3-TK-La21Fw, although the duplex was able to induce ~15% inhibition of the *Photinus* luciferase expression in pGL3-TK. Since the *Photinus* luciferase transcripts derived from pGL3-TK-La21Fw carry two identical target sites complementary to the antisense-stranded La21 siRNA element, and since the target site in the luciferase coding region appeared not to contribute much to gene silencing when using the La21-5′m2 siRNA duplex (Fig. 4), it is conceivable that the target site in the 3′ UTR could be more sensitive to cleavage by RISCs than that in the luciferase coding region on the *Photinus* luciferase transcripts derived from pGL3-TK-La21Fw in the presence of the La21-5′m2 siRNA duplex. These observations thus suggest a possible positional effect of target site of siRNA duplex on RNAi activity.

Finally, we add that a difference in RNAi activity between phRL-La21Rv (Fig. 3) and pGL3-TK-La21Rv in the presence of the La21-5′m2 siRNA duplex was observed, although the *Renilla* and *Photinus* luciferase transcripts derived from phRL-La21Rv and pGL3-TK-La21Rv, respectively, which carried the same target sites complementary to the sense-stranded La21 siRNA element in their 3′ UTRs, could be subjected to gene silencing chiefly mediated by the sense-stranded siRNA element. The difference might be attributable to possibly different stabilities between the *Renilla* and *Photinus* luciferase gene products in cells. To further evaluate such a possible difference and also the positional effect of siRNA target site on RNAi activity, more extensive studies need to be carried out.

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References

[1] P.A. Sharp, RNAi and double-strand RNA, Genes Dev. 13 (1999) 139–141.
[2] J.M. Bosher, M. Labouesse, RNA interference: genetic wand and genetic watchdog, Nat. Cell. Biol. 2 (2000) E31–E36.
[3] H. Vaucheret, C. Beclin, M. Fagard, Post-transcriptional gene silencing in plants, J. Cell Sci. 114 (2001) 3083–3091.
[4] H. Cerutti, RNA interference: traveling in the cell and gaining functions? Trends Genet. 19 (2003) 39–46.
[5] S.M. Hammond, E. Bernstein, D. Beach, G.J. Hannon, An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells, Nature 404 (2000) 293–296.
[6] P.D. Zamore, T. Tuschl, P.A. Sharp, D.P. Bartel, RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals, Cell 101 (2000) 25–33.
[7] E. Bernstein, A.A. Caudy, S.M. Hammond, G.J. Hannon, Role for a bidentate ribonuclease in the initiation step of RNA interference, Nature 409 (2001) 363–366.
[8] S.M. Elbashir, W. Lendeckel, T. Tuschl, RNA interference is mediated by 21- and 22-nucleotide RNAs, Genes Dev. 15 (2001) 188–200.
[9] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA

interference in cultured mammalian cells, Nature 411 (2001) 494–498.

[10] H. Hohjoh, RNA interference (RNAi) induction with various types of synthetic oligonucleotide duplexes in cultured human cells, FEBS Lett. 521 (2002) 195–199.

[11] K. Omi, K. Tokunaga, H. Hohjoh, Long-lasting RNAi activity in mammalian neurons, FEBS Lett. 558 (2004) 89–95.

[12] N. Sago, K. Omi, Y. Tamura, H. Kunugi, T. Toyo-oka, K. Tokunaga, H. Hohjoh, RNAi induction and activation in mammalian muscle cells where Dicer and eIF2C translation initiation factors are barely expressed, Biochem. Biophys. Res. Commun. 319 (2004) 50–57.

[13] T. Holen, M. Amarzguioui, M.T. Wiiger, E. Babaie, H. Prydz, Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor, Nucleic Acids Res. 30 (2002) 1757–1766.

[14] H. Hohjoh, Enhancement of RNAi activity by improved siRNA duplexes, FEBS Lett. 557 (2004) 193–198.

[15] D.S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin, P.D. Zamore, Asymmetry in the assembly of the RNAi enzyme complex, Cell 115 (2003) 199–208.

[16] A. Khvorova, A. Reynolds, S.D. Jayasena, Functional siRNAs and miRNAs exhibit strand bias, Cell 115 (2003) 209–216.

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Association study of the chemokine, CXC motif, ligand 1 (CXCL1) gene with sporadic Alzheimer's disease in a Japanese population

Yoshiko Tamura[a,1], Yuji Sakasegawa[a,1], Kazuya Omi[a,b], Hitaru Kishida[a,c], Takashi Asada[d], Hideo Kimura[a], Katsushi Tokunaga[b], Naomi S. Hachiya[a], Kiyotoshi Kaneko[a], Hirohiko Hohjoh[a,*]

[a] *National Center of Neurology and Psychiatry, National Institute of Neuroscience, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[b] *Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[c] *Department of Neurology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan*
[d] *Department of Neuropsychiatry, Institute of Clinical Medicine, University of Tsukuba, Tsukuba 305-8577, Japan*

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Abstract

Inflammation is profoundly involved in the development of Alzheimer's disease (AD) and other neurodegenerative diseases. Chemokine, CXC motif, ligand 1 (CXCL1; or GRO1) is an inflammatory cytokine and appears to be implicated in the pathogenesis of AD. It is of interest and importance to see if the *CXCL1* gene, mapped on chromosome 4q12–q13, has potential for conferring the predisposition to AD. Here we report on an association study of the *CXCL1* gene with sporadic AD patients in a Japanese population; three single nucleotide polymorphisms (SNPs) in the *CXCL1* locus were investigated in 103 AD patients and 130 healthy individuals. The results indicate that neither genotype frequencies nor allele frequencies of the examined SNPs attained statistical significance even after being stratified by the presence or absence of the *Apolipoprotein E* ε4 allele. Therefore, the data presented here suggests that the *CXCL1* gene could not be associated with the susceptibility to AD in a Japanese population.
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Keywords: Alzheimer' disease; Chemokine; CXC motif, ligand 1 (CXCL1); Single nucleotide polymorphisms (SNPs); Association study

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the elderly, and characterized by accumulation of neurofibrillary tangles and amyloid deposition resulting in the formation of senile plaques in the brain. Sporadic AD other than familial AD appears to be a multifactorial disorder in which both genetic and environmental factors are involved [2]. A genetic factor strongly associated with sporadic AD has been found in the *Apolipoprotein E* (*APOE*) gene: the *APOE ε4* allele increases the predisposition to AD [10,12,13]. It is likely that other genetic factors besides *APOE ε4* could participate in developing AD, and it is of importance and necessary to determine such genetic factors conferring the predisposition to AD.

Chemokines are inflammatory cytokines which have multiple functions in the immune system, and also have effects on cells of the central nervous system [1,3,4,7–9,15–17]. It appears that inflammation is implicated in the pathogeneses of various neurodegenerative disorders including AD [9,14–17]. Previous study suggested that chemokine, CXC motif, ligand 1 (CXCL1; or GRO1) could work as a potent trigger for the ERK1/2 and PI-3 kinase pathway and induce hypermethylation of the tau protein in mouse primary cortical neurons, and also that the immunoreactivity for CXCL1 increased in a subpopulation of neurons in some AD brains [14]. It was further suggested that a chemokine receptor for CXCL1, CXCR2, was expressed on neurons and was strongly upregulated in a subpopulation of senile plaques in AD [9,15].

* Corresponding author. Tel.: +81 42 342 2711x5176; fax: +81 42 346 1748.
E-mail address: hohjohh@ncnp.go.jp (H. Hohjoh).
[1] These authors contributed equally to this work.

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Table 1
Genotype and allele frequencies of the SNPs in the *CXCL1* locus

SNP name (position[a])		Patients ($n = 103$)	Controls ($n = 130$)	P	OR (95% CI)
rs3117602	Genotype frequency				
(75,199,137)	C/C	90 (87.4%)	107 (82.3%)	0.43	1.0
	C/A	13 (12.6%)	22 (16.9%)		0.7 (0.3–1.5)
	A/A	0 (0%)	1 (0.8%)		–
	Allele frequency				
	C allele	93.7%	90.7%	0.25	
	A allele	6.3%	9.3%		
rs4074	Genotype frequency				
(75,202,395)	G/G	26 (25.2%)	31 (23.8%)	0.95	1.0
	G/A	55 (53.4%)	72 (55.4%)		0.9 (0.45–1.7)
	A/A	22 (21.4%)	27 (20.8%)		1.0 (0.4–2.0)
	Allele frequency				
	G allele	51.9%	51.6%	0.93	
	A allele	48.1%	48.4%		
rs1429638	Genotype frequency				
(75,204,181)	C/C	46 (44.7%)	59 (45.4%)	0.92	1.0
	C/A	51 (49.5%)	65 (50.0%)		1.0 (0.6–1.7)
	A/A	6 (5.8%)	6 (4.6%)		1.3 (0.4–4.2)
	Allele frequency				
	C allele	69.4%	70.2%	0.82	
	A allele	30.6%	29.8%		

[a] The nucleotide positions are based on the numbering used in the NCBI public location.

These observations lead to the possibility that the *CXCL1* gene could confer the predisposition to sporadic AD, i.e., it may be a genetic risk factor for AD, and stimulate our interest in studying if there is any association between the *CXCL1* gene and AD.

In this study, we investigated three single nucleotide polymorphisms (SNPs) around the *CXCL1* locus mapped on 4q12–q13 in sporadic AD patients and healthy individuals. The subjects were all Japanese: 103 patients with AD (47 men and 56 women; mean age of onset, 70.7 years old) were diagnosed by meeting the National Institute of Neurological and communicative Disorders and Stroke and The Alzheimer's Disease and Related Dementias Association criteria (NINCDS-ADRDA) [11], and 130 unrelated healthy individuals (57 men and 73 women; mean age, 70.9 years old) were examined as controls. Peripheral blood samples were obtained and subjected to isolation of genomic DNA with standard protocols. For a high-throughput analysis, allelic discrimination assay with commercially available Assays-on-Demand SNP Genotyping products (Applied Biosystems) was carried out in 25 μl of 1 × TaqMan Universal PCR Master Mix (Applied Biosystems) containing ~10 ng of genomic DNA and 1.25 μl of an Assays-on-Demand SNP Genotyping product (Applied Biosystems) by using the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems) according the manufacture's instructions. The Assays-on-Demand SNP Genotyping products used (the Assay ID numbers; public ID numbers) were as follows: C_9761059_10; rs3117602 (intergenic SNP), C_11820472_1; rs4074 (intron3 SNP), C_2042711_10; rs1429638 (intergenic SNP). The SNPs cover the *CXCL1* gene and the physical distances between rs3117602 and rs4074 SNPs and between rs4074 and rs1429638 SNPs are approximately 3.3 and 1.8 kb long, respectively. After SNP typing, statistical analyses of the data were carried out using SNPAlyse (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium was examined by χ^2-test for goodness of fit. Allele distributions between the patients and controls were examined by χ^2-test for independence. As for haplotype analysis, haplotype frequencies and linkage disequilibrium parameters were estimated on the basis of an expectation-maximization algorithm [5]. Case–control haplotype analyses were carried out by using the permutation method to obtain the empirical significance [6]. Each haplotype was tested for association by grouping all other haplotypes together and applying χ^2-test with 1 d.f. P values were estimated on the basis of 10,000 replications.

Table 1 shows the results of the SNP typing in the AD patients and healthy controls. The SNPs examined in this study revealed no significant differences in their genotype frequencies, allele frequencies and allele carrier frequencies between the patients and healthy controls. In addition, none of the polymorphisms in each group deviated from expectations based on Hardy-Weinberg equilibrium at a significance level of 0.01. Accordingly, although there was a limitation in the number of the subjects used in this study, i.e., the numbers of the patients and controls used were small; the typing data suggested that the *CXCL1* gene could not be a major risk factor conferring the susceptibility to AD at least. We further examined allelic associations (haplotypes) among the rs3117602, rs4074 and rs1429638 SNPs. As a result, strong

Table 2
Estimated haplotypes and their frequencies

Haplotypes[a]	Patients ($n = 103$), HF (%)	Controls ($n = 130$), HF (%)	P
C–G–C	51.9	50.5	0.75
C–A–A	29.4	28.9	0.66
C–A–C	12.3	11.4	0.75
A–A–C	5.2	7.5	0.32

HF: haplotype frequency.
[a] Estimated haplotypes with the rs3117602, rs4074 and rs1429638 SNPs are indicated and the haplotypes with 5% or more of their frequencies are shown.

allelic associations (haplotypes) among the SNPs were detectable in either the healthy controls or AD patients (Table 2); but, the estimated haplotype frequencies resulted in no significant difference between the patients and controls. We must add that further analyses stratified by either the presence or absence of the *APOE ε4* allele resulted in no statistical significance, although the difference in the frequency of the *APOE ε4* allele alone between the patients and controls attained statistical significance ($P = 0.0079$). Taking all the data together, it is suggested that the *CXCL1* gene is not associated with the susceptibility to sporadic AD. Since inflammation appears to be implicated in the development of AD, it is conceivable that the *CXCL1* gene could contribute to only inflammatory response in the course of the development of AD, but not participate in the pathogenesis of AD as a genetic factor conferring the predisposition to AD.

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References

[1] A. Bajetto, R. Bonavia, S. Barbero, T. Florio, G. Schettini, Chemokines and their receptors in the central nervous system, Front Neuroendocrinol. 22 (2001) 147–184.

[2] D. Blacker, L. Bertram, A.J. Saunders, T.J. Moscarillo, M.S. Albert, H. Wiener, R.T. Perry, J.S. Collins, L.E. Harrell, R.C. Go, A. Mahoney, T. Beaty, M.D. Fallin, D. Avramopoulos, G.A. Chase, M.F. Folstein, M.G. McInnis, S.S. Bassett, K.J. Doheny, E.W. Pugh, R.E. Tanzi, Results of a high-resolution genome screen of 437 Alzheimer's disease families, Hum. Mol. Genet. 12 (2003) 23–32.

[3] R. Bonavia, A. Bajetto, S. Barbero, P. Pirani, T. Florio, G. Schettini, Chemokines and their receptors in the CNS: expression of CXCL12/SDF-1 and CXCR4 and their role in astrocyte proliferation, Toxicol. Lett. 139 (2003) 181–189.

[4] C.M. Coughlan, C.M. McManus, M. Sharron, Z. Gao, D. Murphy, S. Jaffer, W. Choe, W. Chen, J. Hesselgesser, H. Gaylord, A. Kalyuzhny, V.M. Lee, B. Wolf, R.W. Doms, D.L. Kolson, Expression of multiple functional chemokine receptors and monocyte chemoattractant protein-1 in human neurons, Neuroscience 97 (2000) 591–600.

[5] L. Excoffier, M. Slatkin, Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population, Mol. Biol. Evol. 12 (1995) 921–927.

[6] D. Fallin, A. Cohen, L. Essioux, I. Chumakov, M. Blumenfeld, D. Cohen, N.J. Schork, Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease, Genome Res. 11 (2001) 143–151.

[7] J.K. Harrison, C.M. Barber, K.R. Lynch, cDNA cloning of a G-protein-coupled receptor expressed in rat spinal cord and brain related to chemokine receptors, Neurosci. Lett. 169 (1994) 85–89.

[8] J.K. Harrison, Y. Jiang, S. Chen, Y. Xia, D. Maciejewski, R.K. McNamara, W.J. Streit, M.N. Salafranca, S. Adhikari, D.A. Thompson, P. Botti, K.B. Bacon, L. Feng, Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 10896–10901.

[9] R. Horuk, A.W. Martin, Z. Wang, L. Schweitzer, A. Gerassimides, H. Guo, Z. Lu, J. Hesselgesser, H.D. Perez, J. Kim, J. Parker, T.J. Hadley, S.C. Peiper, Expression of chemokine receptors by subsets of neurons in the central nervous system, J. Immunol. 158 (1997) 2882–2890.

[10] S.M. Laws, E. Hone, S. Gandy, R.N. Martins, Expanding the association between the APOE gene and the risk of Alzheimer's disease: possible roles for APOE promoter polymorphisms and alterations in APOE transcription, J. Neurochem. 84 (2003) 1215–1236.

[11] 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.

[12] M. Michikawa, K. Yanagisawa, Apolipoprotein E4 induces neuronal cell death under conditions of suppressed de novo cholesterol synthesis, J. Neurosci. Res. 54 (1998) 58–67.

[13] A.M. Saunders, W.J. Strittmatter, D. Schmechel, P.H. George-Hyslop, M.A. Pericak-Vance, S.H. Joo, B.L. Rosi, J.F. Gusella, D.R. Crapper-MacLachlan, M.J. Alberts, et al., Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease, Neurology 43 (1993) 1467–1472.

[14] M. Xia, B.T. Hyman, GROalpha/KC a chemokine receptor CXCR2 ligand, can be a potent trigger for neuronal ERK1/2 and PI-3 kinase pathways and for tau hyperphosphorylation—a role in Alzheimer's disease? J. Neuroimmunol. 122 (2002) 55–64.

[15] M. Xia, S. Qin, M. McNamara, C. Mackay, B.T. Hyman, Interleukin-8 receptor B immunoreactivity in brain and neuritic plaques of Alzheimer's disease, Am. J. Pathol. 150 (1997) 1267–1274.

[16] M.Q. Xia, B.J. Bacskai, R.B. Knowles, S.X. Qin, B.T. Hyman, Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease, J. Neuroimmunol. 108 (2000) 227–235.

[17] M.Q. Xia, B.T. Hyman, Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease, J. Neurovirol. 5 (1999) 32–41.