pGL3-TK-La21Fw and pGL3-TK-La21Rv plasmid 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.

Acknowledgements

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Figure legends:

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.

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 La215'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.

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.

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

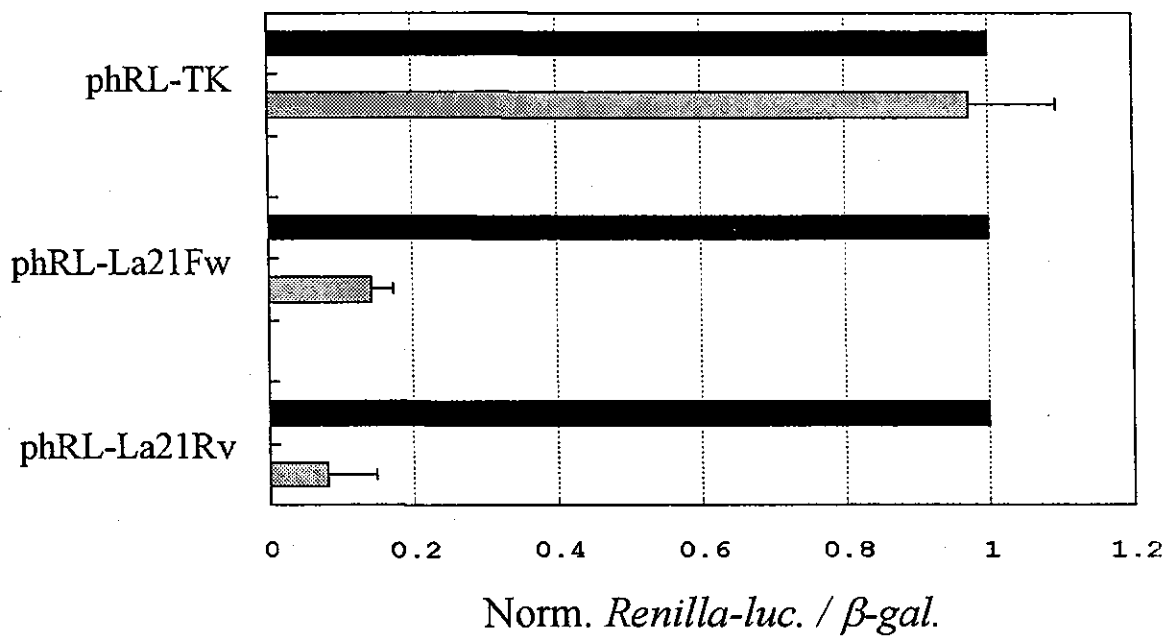
La21-conv.

La21-5'm2

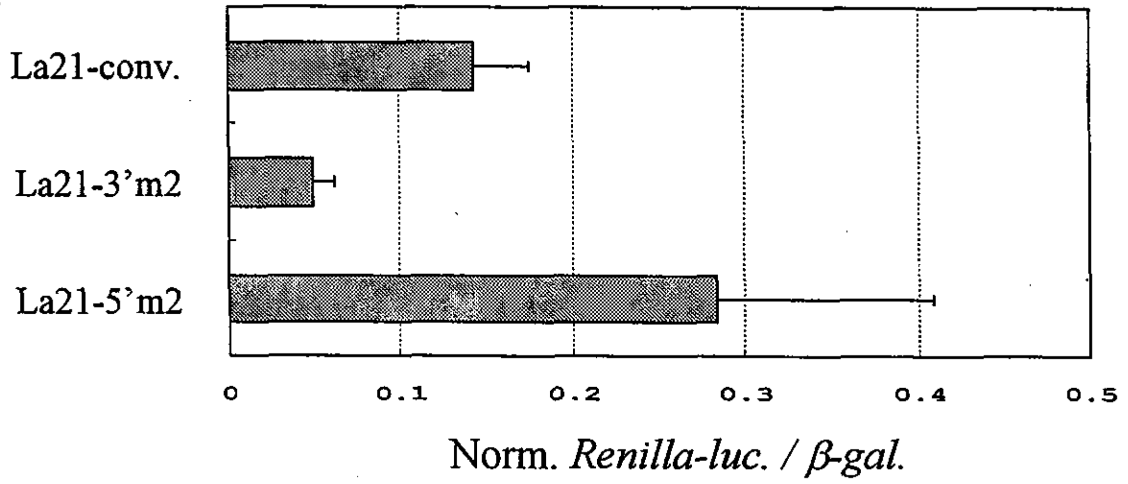
La21-ss19

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

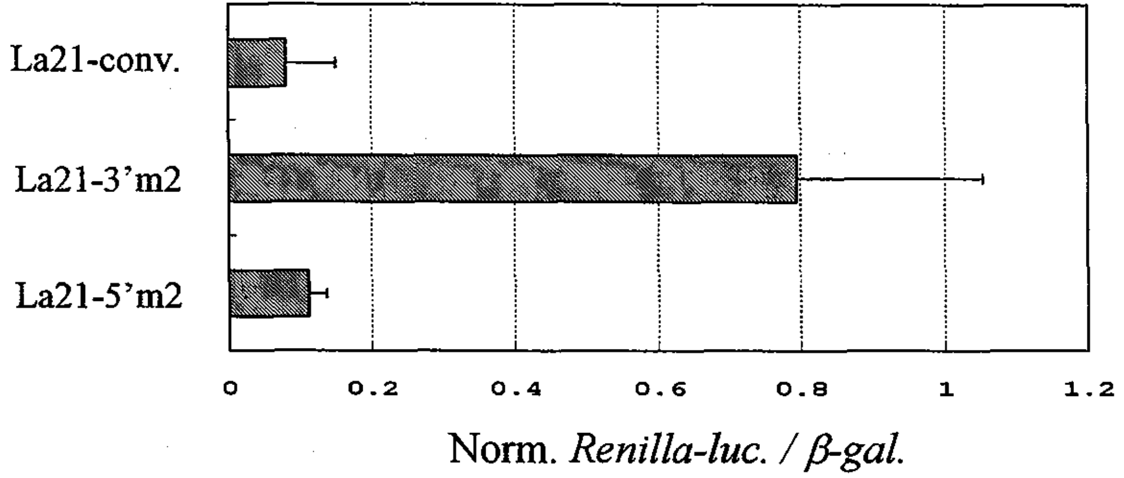
Norm. *Photinus* Luc. / *Renilla* Luc.

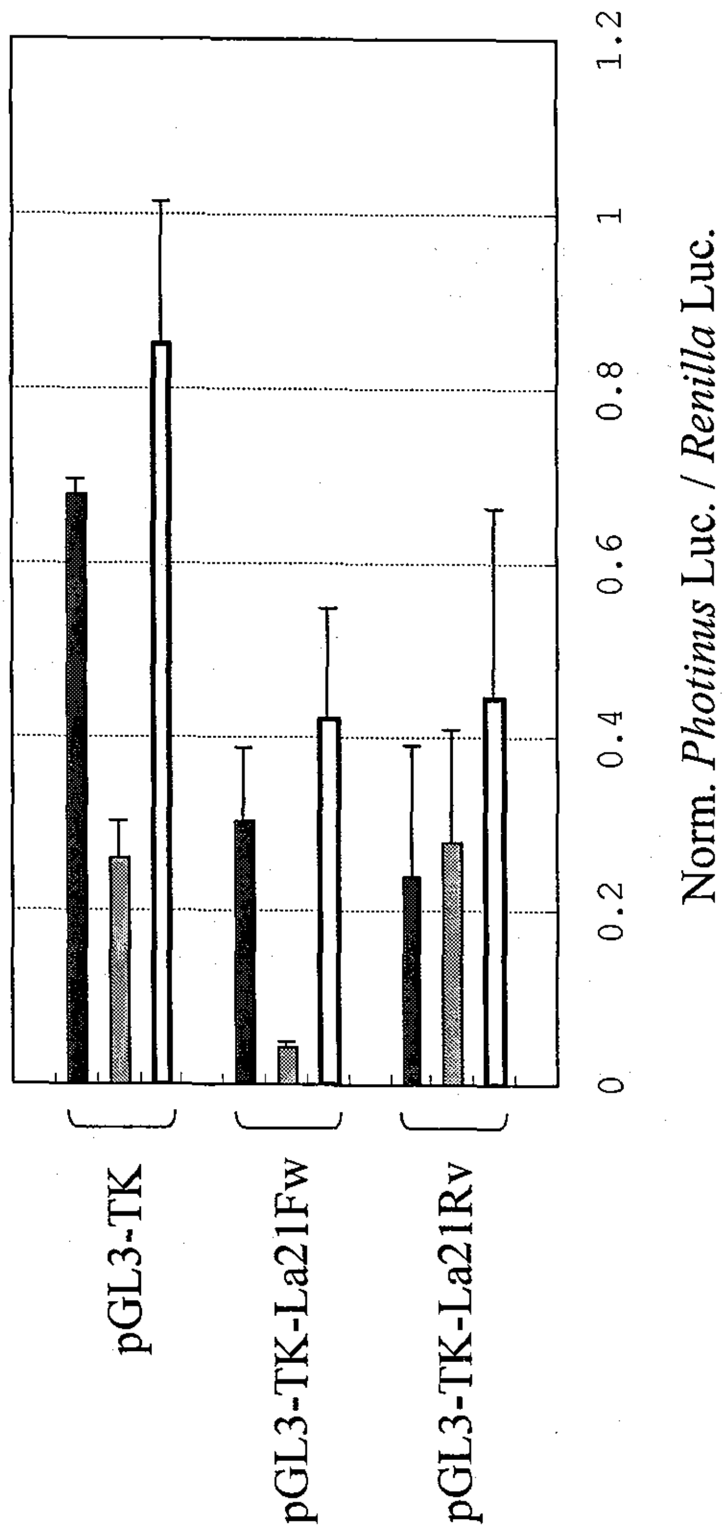

phRL-TK
phRL-La21Fw
phRL-La21Rv
0
0.2
0.4
0.6
0.8
1
1.2
Norm. Renilla-luc. / β-gal.

A



B





pGL3-TK
pGL3-TK-La21Fw
pGL3-TK-La21Rv
0
0.2
0.4
0.6
0.8
1
1.2
Norm. Photinus Luc. / Renilla Luc.

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

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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.

Key words: 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]. 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 x TaqMan Universal PCR Master Mix (Applied Biosystems) containing ~10ng 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 kb 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 df. *P* values were estimated on the basis of 10000 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 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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Table 1. Genotype and allele frequencies of the SNPs in the *CXCL1* locus

SNP name (position*)		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%		

* The nucleotide positions are based on the numbering used in the NCBI public location.

Table 2. Estimated haplotypes and their frequencies

Haplotypes*	Patients (n = 103)	Controls (n = 130)	*P*
	HF (%)	HF (%)	
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

*Estimated haplotypes with the rs3117602, rs4074 and rs1429638 SNPs are indicated and the haplotypes with 5% or more of their frequencies are shown.

HF: haplotype frequency