［原著］Association study of folate pathway gene polymorphisms and nonsyndromic cleft lip with/without cleft palate in a Japanese population

Association study of folate pathway gene polymorphisms and nonsyndromic cleft lip with/without cleft palate in a Japanese population

Takahiro Goto, D.D.S.[1], Keiichi Arakaki, D.D.S., PhD.[1]
Nagato Natsume, D.D.S., PhD.[2] and Hajime Sunakawa, D.D.S., PhD.[1]

[1]*Department of Clinical Neuroscience, Oral and Maxillofacial Functional Rehabilitaion, Faculty of Medicine, University of the Ryukyus,* (*Chief: Prof. Hajime SUNAKAWA.*)
[2]*Division of Research and Treatment for Oral and Maxillofacial Cogenital Anomalies, School of Dentistry, Aichi-gakuin University*

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ABSTRACT

Objective: The aim of this study was to verify the association between 4 polymorphisms in the folate pathway genes—5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms (rs1801133 and rs1801131); methionine synthase (*MTR*) gene polymorphism (rs1805087); and reduced folate carrier 1 (*RFC1*) gene polymorphism (rs1051266)—and the development of familial and/or sporadic nonsyndromic cleft lip with/ without cleft palate (NSCL±P) in a Japanese population. Method: Our study to evaluate the distribution of genotype and allele frequencies between patients and controls for the folate pathway gene polymorphisms as well as the relative risk of the interaction among the polymorphisms involved 82 Japanese familial NSCL±P patients and their 52 mothers, 152 sporadic NSCL±P patients and their parents (102 mothers and 82 fathers), and 242 control subjects for comparison. Moreover, we performed the transmission disequilibrium test with 77 sporadic NSCL±P case/parent triads as well as gene-gene interaction studies. Results: The results of our case-control and gene-gene interaction studies did not reveal any significant differences in any categories. Conclusion: Our results revealed that rs1801133 and rs1801131 in the *MTHFR* gene, rs1805087 in the *MTR* gene, and rs1051266 in the *RFC1* gene were not significantly associated with the development of familial and/or sporadic NSCL±P in the Japanese population. *Ryukyu Med. J., 28 (3,4)13～21, 2009*

Key words: *MTHFR, MTR, RFC1,* Nonsydromic cleft lip with/without palate, Folate pathway

INTRODUCTION

Cleft lip and/or cleft palate (CL/P) is among the most common congenital birth defects with the prevalence at birth estimated as 1.44 per 1000 Japanese newborn infants[1]. The incidence is reported to be the highest in Asian and American Indian populations (1/500 or higher), intermediate in European-derived population (1/1100), and the lowest in African-derived population (1/2500)[2]. Most cases of CL/P are of the nonsyndromic form (NSCL/P), although many syndromes with CL/P are known. Evidence for genetic factors involved in the development of NSCL/P was obtained from subsequent studies on familial recurrence[3], concordance in twins[4], and segregation[5]. Evidence for environmental factors involved in the development of NSCL/P also revealed an increased risk for CL/P with smoking[6] and alcohol[7] exposure during pregnancy. Moreover, NSCL/P is thought to occur owing to interaction between genetic and environmental factors[8], and the etiology of NSCL/P may involve heterogeneous and complex factors.

Previous epidemiologic evidence suggests that periconceptional supplementation with multivitamins including folic acid, reduces the risks of

NSCL/P[9,10,11]. However, the underlying process by which folic acid may reduce the risk of CL/P-affected pregnancies still remains poorly understood. Studies of genetic variation in folate metabolism might reveal new information on this unknown mechanism[12].

The rs1801133 in the 5,10-Methylenetetrahydrofolate reductase (*MTHFR*), a common mutation resulting in decreased activity of the enzyme, has been associated with neural tube defect (NTD)[13,14]. Some investigators report a relationship between rs1801133 in the folate pathway and the development of NSCL/P[15,16,17]. However, subsequent studies failed to confirm these findings[18,19,20].

The rs1801131 in the *MTHFR* was also suggested to be associated with decreased enzyme activity, although to a lesser extent than rs1801133[21].

Moreover, Harmon et al. (1999)[22] showed that rs1805087 in the methionine synthase (*MTR*) had a significant but moderate effect on homocysteine (Hcy) concentrations. Mostowska et al. (2006)[12] provided evidence to suggest that maternal rs1805087 plays an important role in the development of NSCL/P. In addition, rs1805087 has been reported to be a significant risk factor for having a child with spina bifida[23] or Down syndrome[24].

In the gene polymorphism rs1051266, previous studies indicated the polymorphisms affects the susceptibility to spina bifida[25][26][27], but not to CL/P[28]. On the other hand, a recent study has indicated that rs1051266 may contribute to cleft lip only[29].

Therefore, we evaluated the influence of 4 polymorphisms (rs1801133, rs1801131, rs1805087 and rs1051266) in the folate pathway genes by the etiological analyses of familial and/or sporadic NSCL±P in a Japanese population.

Materials and Methods

Study population and sample collection

Patients and control subjects were recruited and personal written informed consent was obtained from all participants at the Aichi-gakuin University Dental Hospital, Dokkyo Medical University Hospital, and University of the Ryukyus Hospital. All patients and controls resided in Japan. The sample study included 82 familial NS CL±P patients, their 52 mothers, and 152 sporadic NSCL±P patients, their 102 mothers and 82 fathers, and 242 control subjects for comparison (Table 1). We used triads with 77 sporadic NSC L±P patients and their parents for the transmission disequilibrium test (TDT). A familial case was defined as a case with family history, a sporadic case was defined as a case without family history. No subject had accompanying defects or findings, suggestive of a syndromic diagnosis. The sample number and the relationship between our analyzed familial patients and their affected family members are shown in Table 1. All controls were unrelated people who agreed to participate in the study and were unaffected by any congenital anomalies including other family members after our interview.

The study was approved by the ethics committee of each institution.

Genotype Analyses of rs1801133, rs1801131, rs1805087, and rs1051266 polymorphisms

a) DNA extraction

The genomic DNA was isolated from whole blood or blood spot with a QIAamp DNA Blood Mini or Tissue Kit according to manufacturer's protocol (QIAGEN Inc, Valencia, CA, USA).

Table 1 Sample number and the relationship between our analyzed familial cases and their affected family members

		n	relationship	% (n)
Familial NSCL±P	case	82	father or mother	21% (22)
	mother	52	parents' siblings	12% (12)
Sporadic NSCL±P	case	152	offsprings of parents' siblings	8% (8)
	mother	102	patients' siblings	18% (19)
	father	82	grandparents	9% (9)
Total NSCL±P	case	234	grandparents' siblings	8% (8)
	mother	154	offsprings of grandparents' siblings	19% (20)
Control		242	other relatives	6% (6)

b) Genotype analysis

Genotyping was carried out using TaqMan SNP Genotyping Assays on the ABI Prism 7900HT machine and analyzed using the SDS 2.2 software (Applied Biosystems, Foster City, CA, USA). The genotyping success rate was extremely high (>98%), and the mendelian error rate was less than 2 %.

c) Statistical Analysis

The distributions of the genotype and allele frequencies were compared between the patients and the controls by the x^2 test (SPSS version 13.0), and the relative risk of the interaction between the polymorphisms was evaluated with the odds ratio (95% confidential intervals [95%CI]).

We performed the TDT, which was introduced by Spielman et al. (1993)[30], with sporadic NSCL±P patients and their parents. Alleles at each marker were tested for association with CL±P using the Family Based Association Test program (FBAT version 2.0.2c)[31,32,33].

RESULTS

The genotype and allele frequencies of the 4 gene polymorphisms analyzed in the patient and control groups are summarized in Table 2. In the

Table 2 Distribution of genotypes and allele frequencies relative to polymorphisms of the *MTHFR, MTR, RFC1* on Familial, sporadic and Total NSCL±P cases and their parents

		Genotype frequencies					Allele frequencies			
rs1801133(*MTHFR*)		CC n(ratio)	CT	TT	x^2	p-value	C n(ratio)	T	x^2	p-value
	control(n=242)	94(0.39)	107(0.44)	41(0.17)		ref	295(0.61)	189(0.39)		ref
Familial	case(n=82)	26(0.32)	39(0.48)	17(0.21)	1.4855	0.4816	91(0.55)	73(0.45)	1.5277	0.2320
	mother(n=52)	19(0.37)	22(0.42)	11(0.21)	0.5239	0.7580	60(0.58)	44(0.42)	0.3798	0.5810
Sporadic	case(n=152)	49(0.32)	73(0.48)	30(0.20)	1.8241	0.3936	171(0.56)	133(0.44)	1.7072	0.2059
	mother(n=102)	36(0.35)	46(0.45)	20(0.20)	0.5393	0.7453	118(0.58)	86(0.42)	0.5775	0.4955
	father(n=82)	28(0.34)	41(0.50)	13(0.16)	0.8511	0.6639	97(0.59)	67(0.41)	0.1668	0.7120
Total	case(n=234)	75(0.32)	112(0.48)	47(0.20)	2.5256	0.2886	262(0.56)	206(0.44)	2.4185	0.1303
	mother(n=154)	55(0.36)	68(0.44)	31(0.20)	0.7709	0.6599	178(0.58)	130(0.42)	0.7805	0.4137
rs1801131(*MTHFR*)		AA	AC	CC	x^2	p-value	A	C	x^2	p-value
	control(n=242)	167(0.69)	66(0.27)	9(0.04)		ref	400(0.83)	84(0.17)		ref
Familial	case(n=82)	62(0.76)	18(0.22)	2(0.02)	1.3422	0.5967	142(0.87)	22(0.13)	1.3903	0.2724
	mother(52)	30(0.58)	19(0.37)	3(0.06)	2.5298	0.2452	79(0.76)	25(0.24)	2.5319	0.1259
Sporadic	case(n=152)	102(0.67)	42(0.28)	8(0.05)	0.5698	0.7433	246(0.81)	58(0.19)	0.3755	0.5683
	mother(n=102)	66(0.65)	29(0.28)	7(0.07)	1.7557	0.3995	161(0.79)	43(0.21)	1.3216	0.2819
	father(n=82)	53(0.65)	26(0.32)	3(0.04)	0.5974	0.7187	132(0.80)	32(0.20)	0.3877	0.5562
Total	case(n=234)	164(0.70)	60(0.27)	10(0.04)	0.2311	0.8839	388(0.83)	80(0.17)	0.0114	0.9318
	mother(n=154)	96(0.62)	48(0.31)	10(0.06)	2.6367	0.2689	240(0.78)	68(0.22)	2.7068	0.1155
rs1805087(*MTR*)		AA	AG	GG	x^2	p-value	A	G	x^2	p-value
	control(n=242)	142(0.59)	90(0.37)	10(0.04)		ref	374(0.77)	110(0.23)		ref
Familial	case (n=82)	50(0.61)	30(0.37)	2(0.02)	0.5347	0.8687	130(0.79)	34(0.21)	0.2822	0.6641
	mother(n=52)	34(0.65)	17(0.33)	1(0.02)	1.1179	0.6923	85(0.82)	19(0.18)	0.9934	0.3620
Sporadic	case(n=152)	100(0.66)	48(0.32)	4(0.03)	2.1997	0.3544	248(0.82)	56(0.18)	2.0823	0.1524
	mother(n=102)	68(0.67)	31(0.30)	3(0.03)	1.9623	0.4169	167(0.82)	37(0.18)	1.7996	0.1873
	father(n=82)	56(0.68)	25(0.30)	1(0.01)	3.2322	0.2259	137(0.84)	27(0.16)	2.8828	0.0975
Total	case(n=234)	150(0.64)	78(0.33)	6(0.03)	1.9424	0.3989	378(0.81)	90(0.19)	1.7529	0.2031
	mother(n=154)	102(0.66)	48(0.31)	4(0.03)	2.4782	0.2864	252(0.82)	56(0.18)	2.3474	0.1291
rs1051266(*RFC1*)		AA	AG	GG	x^2	p-value	A	G	x^2	p-value
	control(n=242)	75(0.31)	116(0.48)	51(0.21)		ref	266(0.55)	218(0.45)		ref
Familial	case(n=82)	33(0.40)	37(0.45)	12(0.15)	2.9819	0.2312	103(0.63)	61(0.37)	3.0758	0.0836
	mother(n=52)	18(0.35)	25(0.48)	9(0.17)	0.4754	0.7954	61(0.59)	43(0.41)	0.4735	0.5515
Sporadic	case(n=152)	52(0.34)	76(0.50)	24(0.16)	1.7517	0.4304	180(0.59)	124(0.41)	1.3742	0.2681
	mother(n=102)	23(0.23)	53(0.52)	26(0.25)	2.6573	0.2664	99(0.49)	105(0.51)	2.3817	0.1325
	father(n=82)	29(0.35)	37(0.45)	16(0.20)	0.5399	0.7584	95(0.58)	69(0.42)	0.4374	0.5255
Total	case(n=234)	85(0.36)	113(0.48)	36(0.15)	3.1169	0.2118	283(0.60)	185(0.40)	2.9606	0.0884
	mother(n=154)	41(0.27)	78(0.51)	35(0.23)	0.8731	0.6581	160(0.52)	148(0.48)	0.6863	0.4219

rs1801133 in the *MTHFR* gene, the genotype frequencies of CC, CT, TT were 32%, 48%, and 21% in familial cases, and were 37%, 42%, and 21% in familial mothers, and were 39%, 44%, and 17% in controls, respectively. The allele frequencies of C and T were 55% and 45% in familial cases, and were 58% and 42%, and were 61% and 39% in controls, respectively. The genotype and allele frequency of rs1801133 in the *MTHFR* gene among familial cases and mothers did not show any significant differences. The genotype frequencies of CC, CT, TT were 32%, 48%, and 20% in sporadic cases, and were 35%, 45% and 20% in sporadic mothers, and were 34% and 50% and 16% in sporadic fathers, and were 39%, 44% and 17% in controls, respectively. The allele frequencies of C and T were 56% and 44% in sporadic cases, and were 58% and 42% in sporadic mothers, and were 59% and 41%, and were 61% and 39% in controls, respectively. The genotype and allele frequency of rs1801133 in the *MTHFR* gene among sporadic cases and their parents did not show any significant differences nor did cases and their mothers in the total group. We also analyzed the genotype and allele frequencies of rs1801131, rs1805087, and rs1051266 of familial and/or sporadic NSCL±P patients and their parents, however we did not find significantly from those of the control subjects in any categories. Additionally, the results of the TDT involving sporadic NSCL±P case/parent triads were shown in Table 3. However, there were no significant differences in our analyzed polymorphisms (p=0.5472, 1.0000, 0.4560 and 0.6547, respectively).

We examined the interaction of 2 common polymorphisms in the *MTHFR* gene with the odds ratios and 95%CI, as summarized (Table 4). The frequencies of CT-AC and TT-AA of combination of rs1801133 and rs1801131 in the *MTHFR* gene were 15% and 21% in familial cases, and were 19% and 21% in familial mothers, and were 12% and

Table 3 Results for Association analysis (TDT) using FBAT

		A freq[α]	IF[β]	p-value
rs1801133	C	0.575	49	0.5472
	T	0.425		
rs1801131	A	0.790	40	1.0000
	C	0.210		
rs1805087	A	0.809	37	0.4560
	G	0.191		
rs1051266	A	0.602	60	0.6547
	G	0.398		

[α]Allele frequency of sporadic NSCL±P cases, [β]Number of informative family

Table 4 The interaction of polymorphisms among rs1801133 and rs1801131 in the *MTHFR* gene

		Familial				Sporadic						Total			
		observed frequency		Odds ratio(95%CI)		observed frequency			Odds ratio(95%CI)			observed frequency		Odds ratio(95%CI)	
	control n=242 (ratio)	case n=82 (ratio)	mother n=52 (ratio)	case	mother	case n=152 (ratio)	mother n=102 (ratio)	father n=82 (ratio)	case	mother	father	case n=234 (ratio)	mother n=154 (ratio)	case	mother
CC-AA	48(0.20)	16(0.20)	5(0.10)	1.02 (0.54-1.92)	0.42 (0.16-1.14)	23(0.15)	14(0.14)	15(0.18)	0.72 (0.41-1.24)	0.64 (0.33-1.23)	0.90 (0.47-1.72)	49(0.21)	19(0.12)	1.07 (0.68-1.67)	0.56 (0.32-1.01)
CC-AC	38(0.16)	7(0.09)	9(0.17)	0.50 (0.21-1.17)	1.12 (0.50-2.49)	18(0.12)	15(0.15)	10(0.12)	0.72 (0.39-1.32)	0.92 (0.48-1.77)	0.74 (0.35-1.57)	25(0.11)	24(0.16)	0.64 (0.37-1.10)	0.99 (0.56-1.73)
CC-CC	9(0.04)	2(0.02)	4(0.08)	0.64 (0.13-3.06)	2.16 (0.63-7.29)	8(0.05)	7(0.07)	3(0.04)	1.44 (0.54-3.81)	1.91 (0.69-5.27)	0.98 (0.25-3.72)	10(0.04)	11(0.07)	1.16 (0.46-2.90)	1.99 (0.80-4.92)
CT-AA	78(0.32)	28(0.34)	13(0.25)	1.09 (0.64-1.85)	0.70 (0.35-1.39)	49(0.32)	32(0.31)	24(0.29)	1.00 (0.64-1.54)	0.96 (0.58-1.58)	0.87 (0.50-1.50)	77(0.33)	45(0.29)	1.03 (0.70-1.51)	0.86 (0.55-1.35)
CT-AC	28(0.12)	12(0.15)	10(0.19)	1.31 (0.63-2.71)	1.82 (0.82-4.03)	24(0.16)	14(0.14)	16(0.20)	1.43 (0.79-2.58)	1.22 (0.61-2.42)	1.85 (0.94-3.63)	36(0.15)	24(0.16)	1.39 (0.81-2.36)	1.41 (0.78-2.54)
CT-CC	NO	NO	NO	ND	ND	NO	NO	NO	ND	ND	ND	NO	NO	ND	ND
TT-AA	41(0.17)	17(0.21)	11(0.21)	1.28 (0.68-2.41)	1.32 (0.62-2.77)	30(0.20)	20(0.20)	14(0.17)	1.21 (0.71-2.03)	1.20 (0.66-2.16)	1.01 (0.51-1.96)	47(0.20)	31(0.20)	1.23 (0.77-1.96)	1.24 (0.73-2.07)
TT-AC	NO	NO	NO	ND	ND	NO	NO	NO	ND	ND	ND	NO	NO	ND	ND
TT-CC	NO	NO	NO	ND	ND	NO	NO	NO	ND	ND	ND	NO	NO	ND	ND

NO=not observed. ND=not determined. 95%CI=95% confidence intervals.

17% in controls, respectively (OR=1.31, 1.28; 95%CI, 0.63-2.71, 0.68-2.41 in familial cases, OR=1.82, 1.32; 95%CI, 0.82-4.03, 0.62-2.77 in familial mothers). The frequencies of CT-AC and TT-AA of combination of rs1801133 and rs1801131 in the *MTHFR* gene were 16% and 20% in sporadic cases, and were 14% and 20% in sporadic mothers, and were 20% and 17% in sporadic fathers, and were 12% and 17% in controls, respectively (OR=1.43, 1.21; 95%CI, 0.79-2.58, 0.71-2.03 in sporadic cases, OR=1.22, 1.20; 95%CI, 0.61-2.42, 0.66-2.16 in sporadic mothers, OR=1.85, 1.01; 95%CI, 0.94-3.63, 0.51-1.96 in sporadic fathers). The CT-CC, TT-AC, TT-CC were not shown in any categories. We also analyzed the combination of rs1801133 and rs1801131 in the *MTHFR* gene among the total group, however the result for 2 common polymorphisms in the *MTHFR* gene did not reveal significant differences nor did the combination of other pairs of our analyzed polymorphisms in the folate pathway genes in any categories (data not shown).

DISCUSSION

There may be a preventive effect on the development of CL/P when multivitamins including folic acid are taken during early pregnancy[9,10,11]. The substrate in the folate and methionine metabolism is crucial for the metabolism of nucleic acids and amino acids including those requirement for the synthesis of nucleotides, and consequently, cell division, a fundamental process in development[34]. Those suggestions that folic acid might be involved in the etiology of CL/P has provided encouragement for studies of the hypomorphic polymorphisms of *MTHFR, MTR, RFC 1* genes that encode a key enzyme in the folate and methionine metabolism.

Tolarova et al. (1998)[15] and Mills et al. (1999)[16] suggested that rs1801133 homozygotes (TT) were significantly more common in the group of CL/P patients than the control group in an Argentina population. Martinelli et al. (Italian) (2001)[17], Pezzetti et al. (Italian) (2004)[35], and Van Rooij et al. (Dutch) (2003)[36] reported a significantly higher frequency of mutated allele in the mothers of CL/P patients than in those of the controls. In addition, Wong et al. (1999)[37] suggested that Hcy concentrations in the plasma are significantly higher in the mothers of CL/P patients when the folate status is lower than that in controls in a Dutch population. Frosst et al. (1995)[38] suggested that the rs1801133 form encodes for a thermolabile enzyme with reduced activity, which is responsible for elevated Hcy level and lowered folate level in the plasma because of reduced *MTHFR* activity. These studies suggested that higher Hcy level and lower folate level in the plasma might be possibility of involving the risk factor of CL/P though the etiology was unclear. On the other hand, Fohr et al. (2002)[39] suggested that subjects with the genotype TT for rs1801133 in the *MTHFR* gene may be able to compensate for the effects of rs1801133 on the folate pathway if their folate status is adequate.

In our study we separated into 3 categories: cases with family history (familial), cases without family history (sporadic), and both of them (Total), because there might be potential of the genetic and distinct contribution to the etiology of NSCL/P.

Our result did not show the association between the increased NSCL±P risk and rs1801133 in any categories nor did the results of Shaw et al. (1998)[18] and Blanton et al. (2000, 2002)[19,20].

The polymorphism rs1801131 in the *MTHFR* gene was also suggested to be associated with decreased enzyme activity, although to a lesser extent than rs1801133[21]. We evaluated the influence of rs1801131 on the development of CL±P. However, the genotype and allele frequencies did not differ significantly between the patients and the controls in any categories; moreover, the TDT involving the families of sporadic NSCL±P patients also did not reveal significant differences. Shotelesuk et al. (2003)[40] demonstrated a significantly increased risk of having a child with CL/P if the mother was heterozygous for both rs1801133 and rs1801131; we thought that the interaction for both rs1801133 and rs1801131 in the *MTHFR* gene, implying that the combined heterozygous was an important risk factor for CL/P. However, our result for the gene-gene interaction between rs1801133 and rs1801131 in the *MTHFR* gene showed no significant differences in any categories.

Harmon et al. (1999)[22] showed that the polymorphism rs1805087 in the *MTR* gene had a significant but moderate effect on Hcy concentrations in the plasma. Mostowska et al. (2006)[12] suggested that in a Polish population, mothers with rs1805087

AG or GG exhibited a risk that was 2.195 times higher than the risk for those who had AA (95%CI: 1.189-4.050, $p=0.011$). Our findings of no association between the maternal rs1805087 polymorphisms and the occurrence of NSCL±P in any categories was in agreement with the findings of other studies that did not report a significant role for this polymorphism in the etiology of CL/P[41].

Previous studies indicated that the polymorphism rs1051266 in the *RFC1* gene affects the susceptibility to spina bifida[25,26,27], but not that to CL/P[28]. However, the results of a recent study indicated that variations in rs1051266 may contribute to cleft lip only in the South American populations[29]. Our results did not provide any evidence to suggest that rs1051266 in the *RFC1* gene plays a major role in the development of NSCL±P in any categories.

Chango et al. (2000)[42] demonstrated a moderate but significant increase in Hcy levels in doubly homozygous GG (rs1051266)/TT (rs1801133) subjects as compared to the levels in GG/CC or GG/CT subjects, and AA/CT subjects had higher plasma folate levels than GG/CT subjects. In addition, De Marco et al. (2003)[43] suggested that the interaction between rs1801131 and rs1051266 might be a risk factor for NTD. However, although we investigated the interaction between rs1051266 and other analyzed polymorphisms, there were no statistical differences between this interaction and the development of NSCL±P in a Japanese population; additionally, significant differences were also not found for the combination of other pairs of our analyzed gene polymorphisms in any categories.

Taken together, the inconsistence between our results and others on the involvement of folate pathway genes in the development of CL/P may be due to the differences of races, sampling sizes, and unrecognized subphenotypic heterogeneity. Meta-analysis and very large population-based samples with more detailed phenotyping may provide more definitive answers in the future. In the interim, the utility of using folic acid to prevent occurrence or recurrence of NSCL/P remains unknown.

CONCLUSION

In this study we investigated 4 polymorphisms in the folate pathway genes, which are suggested to be associated with the development of NSCL/P. However, our results did not reveal significant differences between the development of familial and/or sporadic NSCL ± P and the analyzed polymorphisms in the folate pathway genes in the Japanese population.

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