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Investigation of candidate genes of non-syndromic cleft lip with or without cleft palate, using both case–control and family-based association studies

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Abstract

Objective: Non-syndromic cleft of the lip and/or palate (NSCL/P) is one of the most common polygenic diseases. In this study, both case–control and family-based association study were used to confirm whether the Single Nucleotide Polymorphisms (SNPs) were associated with NSCL/P.

Methods: A total of 37 nuclear families and 189 controls were recruited, whose blood DNA was extracted and subjected to genotyping of SNPs of 27 candidate genes by polymerase chain reaction-improved multiple ligase detection reaction technology (PCR-iMLDR). Case–control statistical analysis was performed using the SPSS 19.0. Haplotype Relative Risk (HRR), transmission disequilibrium test (TDT), and Family-Based Association Test (FBAT) were used to test for over-transmission of the target alleles in case-parent trios. The gene–gene interactions on NSCL/P were analyzed by Unphased-3.1.4.

Results: In case–control statistical analysis, only *C14orf49 chr14_95932477* had statistically significant on genotype model ($P = .03$) and allele model ($P = .03$). Seven SNPs had statistically significant on TDT. None of 26 alleles has association with NSCL/P on FBAT. Some SNPs had haplotype-haplotype interactions and genotype-genotype interactions.

Conclusion: *C14orf49 chr14_95932477* was significantly different between cases and controls on genotype model and allele model by case–control design. Seven SNPs were significantly different on HRR. Four SNPs were significantly different on TDT.

Abbreviations: FBAT = Family-Based Association Test, GWAS = genome-wide association studies, HRR = Haplotype Relative Risk, HWE = Hardy–Weinberg equilibrium, NSCL/P = non-syndromic cleft of the lip and/or palate, PCR-iMLDR = polymerase chain reaction-improved multiple ligase detection reaction technology, SNPs = Single Nucleotide Polymorphisms, TDT = transmission disequilibrium test.

Keywords: candidate gene, Family-Based Association Test, NSCL/P, single nucleotide polymorphisms

1. Introduction

Non-syndromic cleft of the lip with or without palate (NSCL/P) is one of the most common birth defects found among live births, about 1.00 to 2.00 per 1000 births in all populations worldwide.[1] In China, the prevalence is about 1.60 per 1000 live births higher than the world's average level.[2] NSCL/P can not only cause facial deformity in patients, but also influence their sucking, swallowing and development of language and hearing, and even result in psychological problems. The anomaly may place a heavy mental and financial burden on the patients and their families to have a direct impact on their quality of life.[3] Therefore, the prevention, treatment and prognosis of NSCL/P now become an important public health issue in world.

Identification of etiology associated with NSCL/P may facilitate efforts at prevention, treatment, and prognosis of the disease. Therefore, the etiology of NSCL/P has been a focus area of research for centuries. Most scientists believe it involves multifactorial contributions including both genetic and environmental factors.[4,5] Due to the complex pathogenesis of NSCL/P, the etiology and mechanism of the disease have not been fully understood.[6–8] Although numerous studies have identified a number of genes as likely to play some roles in the etiology of NSCL/P, it is difficult to achieve consistency across studies.

With the advent of the genomics era, genome-wide association studies (GWAS) have provided insights into the genetic factors of NSCL/P through the identification of several risk loci. Several GWAS have identified and confirmed several significant locus and genes contributing to the etiology of NSCL/P.[9–12] Birnbaum et al confirmed the impact of IRF6 using the GWAS, which had previously been identified in the previous studies.[11] However, some studies had contradictory results about some genes,

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including *TGFβ*, *FOXE1*, *TGFα*, *MTHFR*, and *BMP4*.[13–17] For example, some studies have shown that the *MTHFR C677T* variant is associated with NSCL/P. However, other studies have not found the association between *MTHFR C677T* and NSCL/P.[17]

In our previous research, 12 reported candidate genes and 16 novel Single Nucleotide Polymorphisms (SNPs) were found may make contributions to NSCL/P, by whole-exome sequencing (WES) in 8 fetuses with NSCL/P in China.

Here, we recruited 37 nuclear families and 189 controls and genotyped 27 candidate genes, based on our previous research,[4] by polymerase chain reaction-improved multiple ligase detection reaction technology (PCR-iMLDR), to verify the candidate genes.

2. Materials and methods

2.1. Subjects

The participants of this study consisted of consenting, unrelated patients with NSCL/P and their parents, from Xuzhou Maternity and Child Health Care Hospital, located in Jiangsu province. Cases comprised 37 nuclear families, including 37 patients and 66 parents. Controls were 189 healthy Han Chinese born in Jiangsu province with no known history of CL/P in their family members, recruited from staff and students of Xuzhou Medical University. Blood sample of 3 mL was drawn in an ethylenediamine tetraacetic acid collection vacuum tube from each participant. DNA was subsequently extracted by GentraPuregene Blood Kit. Report of DNA sample quality test (Table S1) and electrophoregram (Figure S1, http://links.lww.com/MD/D54) were presented in supplementary materials, http://links.lww.com/MD/D54.

2.2. Genotyping

Peripheral blood was collected and genomic DNA was extracted by a standard procedure. According to our preliminary study,[4] 27 candidate genes were selected, including *ABCA4*, *BMP4*, *C14orf49*, *CENPJ*, *CRISPLD2*, *EIF2B3*, *EPHA3*, *FGFR2*, *HEATR8*, *IRF6*, *KIF20B*, *KRTAP5-4*, *LACTB*, *MEF2A*, *MTHFR*, *MYH9*, *PARVA*, *PAX7*, *PDGFC*, *PKP1*, *RECQL5*, *REG3A*, *SEC16A*, *SPRY2*, *TEX11*, *TTN*, and *YOD1*. Details of the alleles of the 27 candidate genes were presented in Table S2. PCR-iMLDR was used for amplification, connection, and genotyping. PCR primers (Table S3) were designed by Primer premier 5 software on the basis of Genbank. Procedure of PCR-iMLDR found in Supplementary Material File, http://links.lww.com/MD/D54. The genes sequenced by ABI 3730XL DNA sequencer and GeneMapper 4.1 software (AppliedBiosystems, CA).

2.3. Ethics

This study was approved by the local institutional Ethics Committee, and all individuals gave written informed consent for participation at the time of recruitment. Parents or legal guardians provided written consents on behalf of minors.

2.4. Statistical methods

Hardy–Weinberg equilibrium (HWE) was used to assess the genotype distribution of the 27 SNPs. Data in HWE suggest a good homogeneity, which is necessary for Gene Association Analysis. Case–control statistical analysis was performed using the SPSS 19.0 (SPSS, Inc., Chicago, IL). Haplotype Relative Risk (HRR) was used to identify excess transmission of the target alleles from parents to the affected offspring using only complete case-parent trios in analyses. A transmission disequilibrium test (TDT) was used to identify excess transmission of the target alleles from heterozygous parents to the affected offspring using only complete case-parent trios in analyses. The Family-Based Association Test (FBAT), including additive mode, dominant model and recessive model, was used to test for over-transmission of the target alleles in case-parent trios by FBAT package (www.biostat.harvard.edu/fbat/default.html). The gene–gene interations on NSCL/P were analyzed by Unphased-3.1.4. We considered P values $\leq .05$ to be statistically significant.

3. Results

3.1. General conditions of subjects

A total of 37 nuclear families and 189 controls were recruited. The 37 nuclear families included 37 patients and 66 parents (35 mothers and 31 fathers). The 29 nuclear families were case-parents trios (78.38%). The 6 nuclear families were case-mother only (16.22%) and 2 nuclear families were case-father only (5.41%). Controls were normal person. HWE was used to assess all SNPs of 27 candidate genes, *ABCA4*, *BMP4*, *C14orf49*, *CENPJ*, *CRISPLD2*, *EIF2B3*, *EPHA3*, *FGFR2*, *HEATR8*, *IRF6*, *KIF20B*, *KRTAP5-4*, *LACTB*, *MEF2A*, *MTHFR*, *MYH9*, *PARVA*, *PAX7*, *PDGFC*, *PKP1*, *RECQL5*, *REG3A*, *SEC16A*, *SPRY2*, *TEX11*, *TTN* and *YOD1* (Table S4). All SNPs, except *TEX11 chrX_69772000*, were in HWE, suggesting good homogeneity within the study subjects. In that case, *TEX11 chrX_69772000* did not be analyzed by HRR, TDT, or FBAT.

3.2. Case–Control

In case–control statistical analysis, none of 26 SNPs of *ABCA4, BMP4, CENPJ, CRISPLD2, EIF2B3, EPHA3, FGFR2, HEATR8, IRF6, KIF20B, KRTAP5-4, LACTB, MEF2A, MTHFR, MYH9, PARVA, PAX7, PDGFC, PKP1, RECQL5, REG3A, SEC16A, SPRY2, TEX11, TTN,* and *YOD1* were significantly different between cases and controls on genotype model or allele model (Table S5). *C14orf49 chr14_95932477* was significantly different between cases and controls on genotype model and allele model by case–control design (Table 1). The prevalence of the potential risk allele C in

Table 1

The association between SNPs and NSCL/P by case–control design.

		Genotype					Allele				
gene	SNP	genotype	control	case	χ^2	*P*	allele	control	case	χ^2	*P*
C14orf49	*chr14_95932477*	C/T	0	2	–	.03	C	0	2	–	.03
		T/T	189	41			T	378	84		

NSCL/P = non-syndromic cleft of the lip and/or palate, SNPs = Single Nucleotide Polymorphisms

chr14_95932477 among NSCL/P cases was 2.38%, whereas none of the controls had the C allele. It was indicated that C14orf49 might be a susceptibility gene of NSCL/P.

3.3. HRR and TDT

Haplotype Relative Risk (HRR) and transmission disequilibrium test (TDT) were used to identify excess transmission of the target alleles from parents to the affected offspring using only complete case-parent trios in analyses. Seven SNPs including EIF2B3 chr1_207224322, IRF6 chr1_209964080, ABCA4 chr1_94461717, FGFR2 chr10_123310871, CENPJ chr13_25487103, LACTB chr15_63414085 and CRISPLD2 chr16_84906098, were significantly different on HRR (Table 2). Four SNPs including IRF6 chr1_209964080, CENPJ chr13_25487103, LACTB chr15_63414085, and CRISPLD2 chr16_84906098, were significantly different on TDT (Table 2).

3.4. FBAT

Additive mode, dominant model and recessive model were used to test for over-transmission of the target alleles in case-parent trios on the FBAT. None of 26 alleles were associated with NSCL/P on FBAT (Table 3).

3.5. Gene–gene interations

The gene–gene interations on NSCL/P were analyzed by Unphased-3.1.4. The interactions comprised haplotype-haplotype interations (Table S6, http://links.lww.com/MD/D54) and genotype-genotype interactions (Tanble S7). The significant results of haplotype-haplotype interactions and genotype-genotype interactions were presented in Table 4.

4. Discussion

In our previous research, 12 reported candidate genes and 16 novel SNPs have been found to be associated with NSCL/P, by whole-exome sequencing (WES) in 8 fetuses with NSCL/P in China.[4] In the present study, we recruited 37 nuclear families and 189 controls and genotyped 27 candidate genes by PCR-iMLDR to verify the candidate genes, using both case–control and family-based association study methods. All analyzed SNPs were in HWE, suggesting good homogeneity within the study subjects.

C14orf49 chr14_95932477 was significantly different between cases and controls on genotype model or allele model by case–control design. However, on case-parents trios design, HRR, TDT, and FBAT analyses provided no evidence of significant associations of NSCL/P with *C14orf49 chr14_95932477*. *C14orf49*, also known as spectrin repeat containing nuclear envelope family member 3, *SYNE3*, might be associated with epithelial-to-mesenchymal transition, in vitro, and in silico analyses.[18] Recently, *C14orf49* was reported in small-cell lung cancer and epithelial-type cancers studies.[18,19] Furthermore, *C14orf49* was identified as a novel candidate gene of NSCL/P using whole-exome sequencing and Sanger sequencing in 2015.[4] Therefore, the causation of NSCL/P may involve the abnormality of epithelial-to-mesenchymal transition regulated by C14orf49. In this study, we found a positive association of C14orf49 variant in the case–control analysis, which was not

Table 2

Results of HRR and TDT.

SNPs	Alelles	HRR				TDT			
		Transmited	Untransmitted	χ^2	*P*	Transmited	Untransmitted	χ^2	*P*
chr1_11856378	G	42	9	0.7	.4	27	9	0.76	.38
	A	45	6			30	6		
chr1_207224322	G	66	0	–	.043	3	0	–	1
	T	2	1			2	1		
chr1_209964080	C	59	3	9.32	<.01	27	3	4.81	.03
	T	24	10			20	10		
chr1_55136529	C	28	6	2.41	.12	19	6	0.64	.42
	T	54	3			23	3		
chr1_94461717	C	66	0	–	<.01	3	0	–	.4
	A	1	2			1	2		
chr10_123310871	G	2	3	–	<.01	2	3	–	.17
	A	66	0			5	0		
chr11_1642959	C	66	0	–	.08	6	0	–	1
	T	5	1			5	1		
chr13_25487103	C	23	14	16.94	<.01	13	11	8.55	<.01
	T	50	2			22	2		
chr13_80911525	G	41	9	2.32	.13	26	9	2.36	.12
	A	47	4			31	4		
chr14_54417522	G	27	7	2.94	.09	22	7	1.01	.31
	A	57	4			25	4		
chr15_63414085	G	65	1	–	<.01	7	0	–	.02
	A	2	5			2	5		
chr16_84906098	G	57	2	20.4	<.01	29	2	13.7	<.01
	C	23	15			16	15		
chr22_36684354	C	49	8	0.38	.54	26	8	0	1
	T	35	8			26	8		

HRR = Haplotype Relative Risk, TDT = transmission disequilibrium test.

Table 3

Results of FBAT.

Gene	SNPs	Allele	Additive model			Dominant model			Recessive model		
			fam#	*Z*	*P*	fam#	*Z*	*P*	fam#	*Z*	*P*
MTHFR	chr1_11856378	A	20.00	−0.19	.85	15.00	1.26	.21	14.00	−1.61	.11
		G	20.00	0.19	.85	14.00	1.61	.11	15.00	−1.26	.21
IRF6	chr1_209964080	C	19.00	−0.82	.41	7.00	1.04	.30	–	–	–
		T	19.00	0.82	.41	17.00	1.64	.10	7.00	−1.04	.30
HEATR8	chr1_55136529	T	21.00	−0.82	.41	7.00	−0.20	.84	17.00	−0.87	.39
		C	21.00	0.82	.41	17.00	0.87	.39	7.00	0.20	.84
FGFR2	chr10_123310871	A	5.00	−0.45	.65	–	–	–	5.00	−0.45	.65
		G	5.00	0.45	.65	5.00	0.45	.65	–	–	–
KRTAP5-4	chr11_1642959	C	6.00	−1.63	.10	–	–	–	6.00	−1.63	.10
		T	6.00	1.63	.10	6.00	1.63	.10	–	–	–
CENPJ	chr13_25487103	C	18.00	−0.45	.65	16.00	−0.25	.80	–	–	–
		T	18.00	0.45	.65	–	–	–	16.00	0.25	.80
SPRY2	chr13_80911525	A	23.00	0.52	.60	15.00	1.13	.26	18.00	−0.25	.80
		G	23.00	−0.52	.60	18.00	0.25	.80	15.00	−1.13	.26
BMP4	chr14_54417522	A	18.00	0.00	1.00	7.00	0.85	.39	17.00	−0.51	.61
		G	18.00	0.00	1.00	17.00	0.51	.61	7.00	−0.85	.39
LACTB	chr15_63414085	G	7.00	1.13	.26	–	–	–	7.00	1.13	.26
		A	7.00	−1.13	.26	7.00	−1.13	.26	–	–	–
CRISPLD2	chr16_84906098	G	22.00	1.35	.18	8.00	0.58	.56	19.00	1.31	.19
		C	22.00	−1.35	.18	19.00	−1.31	.19	8.00	−0.58	.56
MYH9	chr22_36684354	C	23.00	−1.46	.14	13.00	−0.75	.46	17.00	−1.41	.16
		T	23.00	1.46	.14	17.00	1.41	.16	13.00	0.75	.46

FBAT = Family-Based Association Test.

confirmed in the trios. This may be an indication of low power. Because of the number of "C" or "C/T" in control group is zero, the power of Fisher exact χ^2 test cannot be calculated. In this case, the difference results between case–control analysis and FBAT may be due to the small sample size, which indicate that we should increase the sample size in our future research.

Seven SNPs including *ABCA4 chr1_94461717, IRF6 chr1_209964080, EIF2B3 chr1_207224322, FGFR2 chr10_123310871, CENPJ chr13_25487103, LACTB chr15_63414085,* and *CRISPLD2 chr16_84906098* were significantly different between cases and controls on HRR. There were no significant difference of 4 SNPs including *IRF6 chr1_209964080, CENPJ chr13_25487103, LACTB chr15_63414085,* and *CRISPLD2 chr16_84906098* on TDT between cases and controls. The membrane-associated protein encoded by ABCA4 is a member of the superfamily of ATP-binding cassette transporters. Mutations in ABCA4 have been found in patients diagnosed with NSCL/P.[20–23]

Interferon regulatory factor 6, IRF6, encodes a member of the interferon regulatory transcription factor (IRF) family. Mutations in this gene are associated with NSCL/P[24,25] and oral-epithelium development.[26]*IRF6 rs642961* G >A is a dosage allele in cleft lip but not cleft palate.[27] However, the results of some studies are different.[28,29] No statistical difference of *rs2235371* polymorphisms was found between the patients and the control group on Brazilian population.[30] However, our study revealed *IRF6 rs2235371* was a susceptible loci of the NSCL/P. The conflicting results may be due to racial difference and the clinical classification of NSCL/P.

Eukaryotic translation initiation factor 2B subunit gamma, EIF2B3 protein is one of the subunits of initiation factor eIF2B, which catalyzes the exchange of eukaryotic initiation factor 2-bound GDP for GTP. Mutations in *EIF2B3* gene have been shown to be associated with leukodystrophy with vanishing white matter.[31–33] Researches on association between *EIF2B3* and Parkinson disease were different.[34,35] It was also been found to function as target for the rapid molecular diagnosis of CACH/VWM syndrome.[33,36] However, no studies have reported the association between EIF2B3 and NSCL/P except our studies.[4]

The protein encoded by fibroblast growth factor receptor 2, FGFR2, is a member of the fibroblast growth factor receptor family, where amino acid sequence is highly conserved between members and throughout evolution.[37] FGF pathway plays an essential role in craniofacial development. Most FGF ligands and FGF R1 and R2 are expressed during the initial phase of facial development.[38] Mutations in FGFR2 are associated with Crouzon syndrome, Pfeiffer syndrome, Craniosynostosis, Apert syndrome, Jackson–Weiss syndrome, Beare–Stevenson cutis gyrata syndrome, Saethre–Chotzen syndrome, and syndromic craniosynostosis. Furthermore, impaired FGF signaling contributes NSCL/P,[39,40] but not are conformed in a large Irish study population.[41]

Centromere protein J, CENPJ, encodes a protein that belongs to the centromere protein family. Mutations in this gene were associated with primary autosomal recessive microcephaly, a disorder characterized by severely reduced brain size and mental retardation.[42–44] The association between CENPJ and NSCL/P has not been reported now.

Lactamase beta, LACTB, encodes a mitochondrially-localized protein that localized in mitochondria as part of the mitochondrial ribosomal complex.[45,46] Mouse LACTB could be expressed as a GST fusion protein in Escherichia *coli.*[47] A mutation in the S26 subunit of the mitochondrial ribosome in a strain of rat exhibits late-onset obesity, which indicates that LACTB is validated as an obesity gene.[48] LACTB was reported marginally to be associated with penicillin allergy.[49] However, the association between LACTB and NSCL/P is not clear now.

Table 4

Significant results of gene–gene interations.

Gene1	Gene2	Haplotype/Genotype	Count	Frequency	AddVal	95% Lo	95% Hi	χ^2	P
chr14_54417522	*chr14_95932477*	A–T	57	0.75	0.00	0.00	0.00	2.45E+03	<.01
chr14_54417522	*chr3_89391019*	A–T	57	0.75	0.00	0.00	0.00	2.45E+03	<.01
chr14_54417522	*chr4_157891963*	A–C	57	0.75	0.00	0.00	0.00	2.45E+03	<.01
chr1_11856378	*chr1_201286752*	AG–AA	23	0.52	0.50	−0.30	1.30	5.35E+03	<.01
		GG–AA	11	0.25	0.37	−0.63	1.37	1.73E+03	<.01
chr1_11856378	*chr13_25487103*	AA–TT	1	0.03	0.64	−1.00	2.29	1.19E+07	<.01
		AG–CT	12	0.35	0.32	−0.76	1.41	5.16E+05	<.01
		AG–TT	6	0.18	0.37	−0.78	1.52	2.48E+06	<.01
		GG–CC	1	0.03	−0.51	−4.58	3.56	5.89E+06	<.01
chr1_11856378	*chr14_54417522*	AA–AA	4	0.09	0.00	0.00	0.00	5.24	.02
		AG–AG	13	0.30	4.29	−29.07	37.65	17.38	<.01
		GG–AG	2	0.05	3.83	−29.55	37.20	6.39	.01
chr1_11856378	*chr17_73627656*	GG–CC	11	0.25	0.37	−0.63	1.37	2.16E+03	<.01
chr1_11856378	*chr22_36684354*	AA–CC	2	0.05	0.00	0.00	0.00	1.77E+07	<.01
		AA–TT	2	0.05	−0.60	−3.27	2.08	2.73E+07	<.01
		AG–CT	8	0.19	0.53	−0.70	1.76	6.46E+05	<.01
chr1_207224322	*chr1_209964080*	GG–CT	13	0.30	0.57	−0.13	1.26	4.48E+04	<.01
chr1_207224322	*chr1_94461717*	GT–CA	1	0.02	10.13	10.13	10.13	4.87E+16	<.01
chr1_207224322	*chr11_12539997*	GT–GC	1	0.02	−3.47	−34.94	28.00	2.09E+05	<.01
chr1_209964080	*chr9_139358155*	CT–CC	15	0.34	0.55	−0.10	1.20	3.49E+05	<.01
		TT–CC	3	0.07	0.17	−1.09	1.43	3.32E+04	<.01
chr11_12539997	*chr13_25487103*	GG–CC	5	0.14	0.00	0.00	0.00	4.03E+04	<.01
		GG–CT	20	0.56	0.05	−1.20	1.29	1.62E+07	<.01
chr13_25487103	*chr9_139358155*	CC–CC	5	0.14	0.00	0.00	0.00	4.03E+04	<.01
		CT–CC	20	0.56	0.05	−1.20	1.29	1.62E+07	<.01
chr13_80911525	*chr9_139358155*	AG–CT	26	0.62	0.47	−0.19	1.12	7.97E+05	<.01
chr14_54417522	*chr22_36684354*	AA–CT	13	0.31	0.62	−0.41	1.65	7.37E+05	<.01
		AA–TT	3	0.07	0.18	−1.28	1.64	1.11E+05	<.01
		GG–CC	3	0.07	0.34	−1.17	1.84	7.01E+06	<.01
chr15_63414085	*chr16_84906098*	GG–GC	16	0.40	0.04	−0.50	0.58	386.30	<.01
		GA–CC	1	0.03	0.26	−1.23	1.76	4.49E+04	<.01
chr16_84906098	*chr17_73627656*	CC–CC	6	0.14	−0.61	−1.81	0.59	3.11E+03	<.01
chr16_84906098	*chr9_139358155*	GG–CC	19	0.43	0.00	0.00	0.00	121.70	<.01
chr22_36684354	*chr9_139358155*	CC–CC	18	0.41	0.00	0.00	0.00	545.50	<.01
		TT–CT	8	0.18	0.35	−0.52	1.23	1.01E+05	<.01

The cysteine-rich secretory protein LCCL domain containing 2, CRISPLD2, was firstly reported as a novel NSCL/P candidate gene in 2007 by Cancasian and Hispanic NSCL/P multiplex families and simplex parent-child trios.[50] More epidemiological investigations have studied the association between CRISPLD2 polymorphisms and NSCL/P in northern Chinese, Irish, Northwestern Chinese, Xinjiang Uyghur, and Brazilian population.[41,51–54] Knocking down CRISPLD2 gene caused zebrafish craniofacial abnormalities.[55] CRISPLD2 was required for neural crest cell migration and cell viability during zebrafish craniofacial development.[56] However, some scholars believed that there was no evidence for a role of CRISPLD2 in NSCL/P among Italian population.[57] It need more evidences, cellular level, and protein level, to verify the associations between candidate genes and SNCL/P by vivo or vitro experiments, in our future research.

5. Conclusions

In summary, C*14orf49 chr14_95932477* was significantly different between cases and controls on genotype model and allele model by case–control design. Seven SNPs including *chr1_207224322*, *chr1_209964080*, *chr1_94461717*, *chr10_123310871*, *chr13_25487103*, *chr15_63414085*, and *chr16_84906098*, were significantly different on NSCL/P on HRR. Four SNPs including *chr1_209964080*, *chr13_25487103*, *chr15_63414085*, and *chr16_84906098*, were significantly different on TDT. None of 26 alleles was associated with NSCL/P on FBAT.

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