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METHOD

Machine Learning Models for Genetic Risk Assessment of Infants with Non-syndromic Orofacial Cleft

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Abstract The isolated type of **orofacial cleft**, termed non-syndromic cleft lip with or without cleft palate (NSCL/P), is the second most common birth defect in China, with Asians having the highest incidence in the world. NSCL/P involves multiple genes and complex interactions between genetic and environmental factors, imposing difficulty for the genetic assessment of the unborn fetus carrying multiple NSCL/P-susceptible variants. Although genome-wide association studies (GWAS) have uncovered dozens of single nucleotide polymorphism (SNP) loci in different ethnic populations, the genetic diagnostic effectiveness of these SNPs requires further experimental validation in Chinese populations before a diagnostic panel or a predictive model covering multiple SNPs can be built. In this study, we collected blood samples from control and NSCL/P infants in

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Han and Uyghur Chinese populations to validate the diagnostic effectiveness of 43 candidate SNPs previously detected using GWAS. We then built predictive models with the validated SNPs using different machine learning algorithms and evaluated their prediction performance. Our results showed that logistic regression had the best performance for risk assessment according to the area under curve. Notably, defective variants in *MTHFR* and *RBP4*, two genes involved in **folic acid** and **vitamin A** biosynthesis, were found to have high contributions to NSCL/P incidence based on feature importance evaluation with logistic regression. This is consistent with the notion that folic acid and vitamin A are both essential nutritional supplements for pregnant women to reduce the risk of conceiving an NSCL/P baby. Moreover, we observed a lower predictive power in Uyghur than in Han cases, likely due to differences in genetic background between these two ethnic populations. Thus, our study highlights the urgency to generate the HapMap for Uyghur population and perform resequencing-based screening of Uyghur-specific NSCL/P markers.

Introduction

Orofacial cleft is a common birth defect associated with more than 300 recognizable congenital syndromes, and the isolated type of orofacial cleft, termed non-syndromic cleft lip with or without cleft palate (NSCL/P), accounts for ~70% of affected infants based on world-wide statistics [1]. NSCL/P has been reported to occur in 0.5‰–2‰ of newborns world widely, varying by geographical origin and ethnic groups. In general, East Asians and Native Americans have the highest rates, while Caucasians and Africans exhibited the lowest rates [2]. Notably, the second largest Chinese minority population, Uyghurs, who have a mixed genetic background of both Caucasian and East Asian populations [3], show an incidence of NSCL/P (1.96‰) greater than the average national level (1.42‰) in China [4]. Newborn infants affected by congenital NSCL/P impose great economic burden for the family. Serial therapies for NSCL/P children usually span several years, which include orofacial surgery, orthodontic dentistry, speech correction, and psychological treatment, imposing a substantial financial burden on both families and society [5].

NSCL/P is a multifactorial, partially heritable, congenital disease that involves multiple genes and complex interactions between genetic and environmental cues [1]. During the human embryonic development, maxillary and medial nasal processes derived from neural crest cells fuse to form the upper lip around the first 5–7 weeks [6], and two lateral palatal processes fuse subsequently to form the palate around the 12th week [7]. The rapid proliferative expansion and complex morphogenetic events that control facial development are highly sensitive to environmental influences along with defective gene variants. Among the environmental factors, maternal nutrition has been under intensive investigation for its impact on NSCL/P, as nutrition for fetal development fully depends on food intake and the metabolic efficiency of the maternal body [8]. Several nutrients have been proven to be important in preventing NSCL/P during pregnancy, and among them folic acid and vitamin A are particularly essential [1]. Folic acid is required for the one-carbon metabolism pathway as a donor during the biosynthesis of purines and pyrimidines. It is also an indispensable factor for homocysteine re-methylation and DNA methylation, which in turn play an important role in gene expression regulation [9]. Evidence has shown that an appropriate increase in the intake dosage of folic acid for pregnant women with a familial history of NSCL/P can significantly reduce the chance of having a newborn with NSCL/P [10–12]. In addition to folic acid, supplementation with vitamin A during pregnancy is also essential for preventing numerous birth defects [13]. A lower vitamin A intake by pregnant women is associated with an increased rate of newborns with NSCL/P [14]. Along the same line, infants with NSCL/P are found to possess significantly lower levels of serum vitamin A than infants without NSCL/P [15]. However, studies using animal models indicate that an excessive vitamin A supplementation might increase the chance of NSCL/P [16]. Therefore, supplementation of vitamin A during the conceptional and peri-conceptional period at the proper dosage is highly recommended to lower the risk of NSCL/P [15].

To discover the NSCL/P-susceptible loci and genetic variants, genome-wide association studies (GWAS) have been carried out in different ethnic populations, leading to the identification of several single nucleotide polymorphism (SNP) sites showing large genetic effects on NSCL/P. For instance, the risk allele forms of the SNPs rs2235371-T and rs861020-A located within the *IRF6* gene account for 12% of the genetic contribution to NSCL/P [17]. Recently, Yu et al. performed a GWAS in a Chinese population and identified 26 SNP loci associated with high risk of NSCL/P, though these loci collectively account for only 10.94% of the heritability of NSCL/P [18]. Until now, studies on NSCL/P in China have mostly focused on Han populations, and the genetic and genomic characteristics associated with NSCL/P in other populations like Uyghurs remain largely unknown. To develop a model for genetic risk assessment of NSCL/P, we collected 43 SNP markers and validated their diagnostic ability in 587 Han Chinese or Uyghur Chinese infants with or without NSCL/P recruited for the current study. We found that variations in two nutritional genes, which encode methylenetetrahydrofolate reductase (*MTHFR*) and retinol binding protein 4 (*RBP4*), respectively, play important roles in NSCL/P development.

Results

Differential allele frequencies of the 43 SNPs between Caucasian, East Asian, and Uyghur Chinese

In this study, 43 SNPs associated with NSCL/P were extracted from the GWAScatalog database (https://www.ebi.ac.uk/gwas/) which integrates the significant SNPs from GWAS analyses in multiple ethnic groups. These SNPs were used as the initial candidate SNP set to examine their contributions to the risk of NSCL/P in the Chinese population. The

NSCL/P sample set used in this study include 103 Han Chinese infants and 279 Uyghur Chinese infants affected by NSCL/P, with an addition of 205 normal Uyghur infants as control (Figure 1A). First we analyzed their allele frequencies in four ethnic groups, namely, European, East Asian, Han Chinese, and Uyghur Chinese populations. The principal component analysis (PCA) showed nearly identical genotypic frequencies for the 43 SNPs between the 504 East Asian individuals from the 1000-genome database (http://www.internationalgenome.org/) and the 103 Han infants diagnosed with NSCL/P, indicating that the 504 East Asians with normal phenotypes from the public database can be used as background controls for the Han Chinese subjects with NSCL/P (Figure 1B). In contrast, the European population was separated from Asian and Han Chinese populations, indicating allele frequencies of the 43 SNPs are different between European and Asian populations. Interestingly, Uyghur Chinese were present between the Asian and European populations, showing a distribution of the allele frequency between Caucasian and East Asian. Therefore, we use the 504 East Asians with normal phenotypes from the public database as the control group for the Han Chinese cases of NSCL/P, but the normal Uyghur Chinese as the control group for the Uyghur NSCL/P cases in the subsequent analyses.

Han and Uyghur ethnic groups showed different ORs for the 43 SNPs

We defined the minor allele of a SNP with a frequency less than 0.5 according to its frequency among the 504 East Asians in the 1000 Genomes database, and then computed the minor allele frequencies (MAFs) of the 43 SNPs in the NSCL/P cases and control samples from the Han and Uyghur populations. We found that most of these SNPs showed different allele frequencies between the two populations. For instance, SNP rs1801133 in the folic acid-related gene *MTHFR* showed distinct allele frequencies in the control and case groups between Han (44.2%) and Uyghur (32.1%) populations. A same pattern was also seen in rs10882272, a SNP in the vitamin A-related gene *RBP4*, which shows different allele frequencies of 22.4% in Han Chinese and 33.0% in Uyghur Chinese (Table 1). In addition, all four SNPs in the *IRF6* gene showed different frequencies of RAs between the Han and Uyghur populations. For example, the frequency of the RA of rs2235371 was 29.2% in Han Chinese with NSCL/P, while it was only 13.7% in Uyghur Chinese with NSCL/P. Furthermore, some SNPs located in the same genes are highly likely to be linked genetically, such as the rs2235371 and rs10863790 pair, and the rs861020 and rs642961 pair, located in the genic region of *IRF6*, which exhibits almost the same allele frequencies in both Han and Uyghur populations.

Odds ratios (ORs) represent the odds of an incidence in a case/disease group versus that in a control/normal group, with greater OR implicating greater risk of being exposed to the disease. An allele with OR > 1 is defined as a risk allele, whereas an allele with OR < 1 is protective, which may reduce the chance of passing on this disease to an infant. We thus computed the ORs for each SNP site in the two ethnic populations and defined the risk and protective role of the two alleles. Among the 43 SNPs (Table 1), 28 SNPs had ORs consistently above or below one in both the Han and Uyghur populations. For instance, the respective OR values in Han and Uyghur populations was 4.189 and 1.390 for rs10882272, which were 0.545 and 0.718 for rs2235371. Nevertheless, 13 SNPs showed an opposite trend in the two populations. For instance, the OR of rs2294426 was 2.146 in the Han population but 0.764 in the Uyghur population. It is worth noting that the minor allele of SNP rs17085106 was not found in the control Han Chinese group, while it was present in a frequency of 8.0% in Han Chinese with NSCL/P and 3.2% and 4.9% frequencies were observed in Uyghur control and NSCL/P groups, respectively.

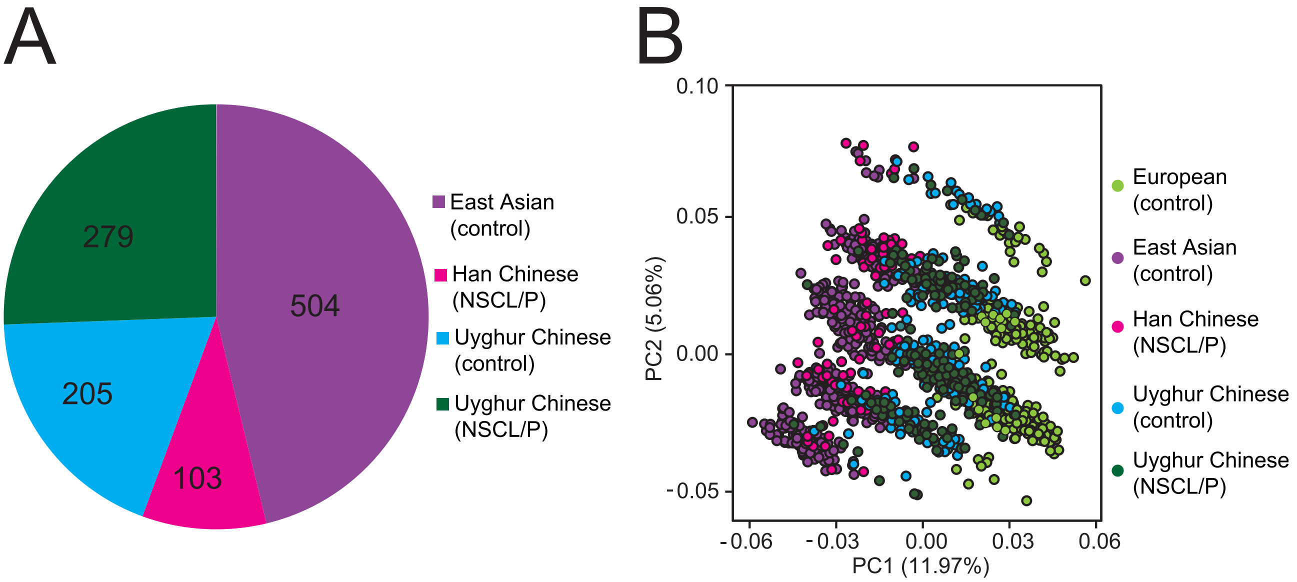


Figure 1 PCA classification of ethnic groups

A. The number of NSCL/P cases and controls included in this study. **B.** PCA classification exhibited different genotypic frequencies based on the 43 SNPs associated with NSCL/P collected from GWAScatalog, among 504 East Asian individuals in 1000 Genomes database, 103 Han Chinese with NSCL/P, 484 Uyghur Chinese, and 503 European individuals in 1 K genome database.

Table 1 List of the 43 candidate SNPs examined in the current study

					Han Chinese			Uyghur Chinese		
SNP ID	**Gene**	**Major**	**Minor**	**RA**	**OR**	**RA frequency**		**OR**	**RA frequency**	
						Control	**NSCL/P**		**Control**	**NSCL/P**
rs742071	*PAX7*	G	T	T	1.522	3.3%	5.0%	1.244	17.6%	21.0%
rs560426	*ABCA4*	A	G	G	1.239	24.9%	29.1%	1.132	47.0%	50.1%
rs2235371	*IRF6*	C	T	T	0.545	43.0%	29.2%	0.718	18.1%	13.7%
rs861020	*IRF6*	G	A	A	1.509	19.0%	26.2%	1.024	21.9%	22.3%
rs10863790	*IRF6 – DIEXF*	A	C	C	0.574	42.9%	30.1%	0.682	18.3%	13.3%
rs642961	*IRF6 – DIEXF*	G	A	A	2.003	19.0%	32.0%	1.113	24.1%	25.8%
rs4441471	LOC105373445	G	A	A	0.985	26.4%	26.2%	0.813	55.8%	50.7%
rs7590268	*THADA*	T	G	G	2.853	2.7%	8.3%	0.799	23.5%	19.7%
rs3815854	LOC105373888	A	G	G	0.564	33.7%	22.3%	1.184	39.2%	43.7%
rs7632427	*EPHA3 – PROSP*	T	C	C	1.468	19.8%	26.6%	1.018	26.9%	27.3%
rs3733585	*SLC2A9*	T	C	C	1.198	41.7%	46.2%	1.130	49.3%	52.4%
rs12543318	LOC105375626	C	A	A	0.825	42.0%	37.4%	0.734	51.3%	43.6%
rs987525	*GSDMC – PVT1*	C	A	A	1.364	8.5%	11.2%	1.638	13.9%	20.9%
rs7078160	*SHTN1*	G	A	A	1.423	41.3%	50.0%	1.420	28.1%	35.7%
rs9574565	*SPRY2*	C	T	T	2.126	12.5%	23.3%	0.829	29.3%	25.9%
rs8001641	LOC105370275	G	A	A	1.964	13.4%	23.4%	1.551	24.9%	34.0%
rs1258763	*GREM1 – FMN1*	G	A	A	1.052	7.0%	7.3%	0.962	45.4%	44.5%
rs1873147	*TPM1*	C	T	T	1.302	14.0%	17.5%	0.737	44.7%	37.3%
rs8049367	*CREBBP – ADCY9*	C	T	T	0.98	33.9%	33.4%	0.764	47.0%	40.8%
rs4791774	*NTN1*	A	G	G	1.323	18.3%	22.9%	1.157	35.2%	38.6%
rs17760296	*NOG*	T	G	G	1.847	0.8%	1.5%	1.198	3.2%	3.8%
rs227731	*NOG*	A	C	C	1.358	31.9%	38.9%	1.097	34.0%	36.1%
rs17085106	LOC102724913	G	T	T	NA	0.0%	8.0%	0.624	4.9%	3.2%
rs13041247	LOC102724968	T	C	C	0.899	41.9%	39.4%	0.941	43.2%	41.4%
rs2066836	*PTCH1*	C	T	T	1.248	8.4%	10.2%	0.918	21.5%	20.1%
rs1801133	*MTHFR*	C	T	T	3.391	29.6%	58.8%	1.075	31.3%	32.8%
rs1801131	*MTHFR*	A	C	C	0.967	22.0%	22.4%	0.795	34.1%	28.8%
rs10882272	*FFAR4/RBP4*	T	C	C	4.189	10.6%	34.1%	1.390	29.2%	36.8%
rs1667255	*TTR*	A	C	C	1.14	40.0%	44.4%	1.128	35.9%	38.3%
rs2236225	*MTHFD1*	C	T	T	1.399	19.9%	25.8%	0.941	39.7%	38.2%
rs41268753	*GRHL3*	C	T	T	NA	0.0%	0.5%	2.043	0.8%	1.5%
rs4460498	*FOXE1*	C	T	T	1.022	11.9%	13.1%	1.002	36.0%	36.5%
rs13542	*ZIC2*	G	A	A	1.383	26.7%	33.5%	0.973	30.5%	30.0%
rs1373453	*BEST3*	T	C	C	1.279	22.9%	28.5%	0.884	17.1%	15.8%
rs1536895	*SMC2*	T	C	C	1.165	13.2%	16.1%	1.003	10.0%	10.4%
rs2294426	*ADTRP*	C	T	T	2.146	28.0%	44.6%	0.764	58.6%	51.9%
rs4132699	*SEMA4D*	A	C	C	1.094	39.5%	41.7%	0.997	40.8%	40.4%
rs4703516	*ACOT12*	G	T	T	1.146	48.6%	53.0%	1.126	26.8%	29.6%
rs5765956	*ARHGAP8*	T	C	C	1.011	46.3%	46.6%	0.987	53.9%	53.5%
rs7820074	Intergenic	T	C	C	6.455	37.5%	79.5%	0.797	81.8%	78.5%
rs7950069	*CADM1*	A	G	G	1.196	34.9%	39.1%	1.076	58.9%	60.6%
rs8076457	*NTN1*	C	T	T	3.425	3.3%	11.6%	1.021	15.1%	15.7%
rs813218	*CMSS1*	G	A	A	1.106	32.3%	34.5%	0.738	49.0%	41.6%

Note: Major (≥50%) and minor (< 50%) alleles were defined according to the genotypic frequencies in the 504 east Asian population in the 1000 Genomes database; RA, risk allele; OR, odds ratio.

Effects of key genes implicated by frequencies of risk alleles

The frequencies of the homozygous and heterozygous forms of the risk alleles may implicate a dominant or recessive genetic effect for each variant (Table S1). As shown in Figure S1, higher percentages subjects with homozygous genotypes for genes *IRF6-DIEXF* (rs642961), *SLC2A9* (rs3733585), and *NOG* (rs227731) were found in the NSCL/P group than in controls in both Han and Uyghur populations, indicating that these alleles may be recessive to NSCL/P incidence. Conversely, higher percentage of subjects with heterozygous genotypes for gene *GSDMC-PVT1* (rs987525) was found in the NSCL/P group than in the control, suggesting its potential dominant effect.

In addition, variants of some nutritional genes also showed differential allele frequencies between the NSCL/P and control groups (Figure S2). Among the Han population, the homozygous genotypes of *MTHFR* with the risk allele for rs1801133 (TT) and rs1801131 (CC) accounted for 36.8% and 5.8% in the NSCL/P group, respectively, significantly higher than the frequencies of 10.5% and 3.7% in the control group. Nevertheless, these two alleles in the Uyghur population showed a reversed trend, with the percentages of the homozygous genotypes in NSCL/P being lower than those in the control group (Figure S2A and B). For another folic acid metabolism-related gene, *MTHFD1*, the heterozygous genotypes for the risk allele accounted for 43.8% and 48.5% in the NSCL/P groups of the Han and Uyghur populations, respectively, which was slightly increased compared to 34.7% and 46.6% in the control groups (Figure S2C). Furthermore, the homozygous genotype of the vitamin A-related gene *RBP4* accounted for 20.6% and 15.4% in the NSCL/P groups of the Han and Uyghur populations, respectively, in sharp contrast to the 1.5% and 9.3% in the control Han and Uyghur groups, respectively (Figure S2D). Apart from these functionally-known genes, higher percentages of subjects with genotypes homozygous for the risk alleles in the gene *ADTRP* (rs2294426) and the intergenic SNP rs7820074 were found in the NSCL/P group than in the control group from the Han population but lower in the Uyghur population (Figure S2E and F), similar as shown in *MTHFR* (rs1801133 and rs1801131).

A relative risk scoring model exhibits better discrimination resolution than an absolute scoring method

An additive linear model is commonly used to assess the genetic risk of a heritable disease by deriving a genetic risk score (GRS) based on the genotypic information of given DNA variants. In our method, the GRS model takes two forms of an OR for each SNP to compute a GRS, with either standardized absolute OR values or relative risk probabilities transformed as the ranks of ORs in the control group.

A comparison of the distributions of GRSs computed in the NSCL/P and control groups is shown in Figure 2. For Han Chinese, both absolute OR-based (Figure 2A) and ranking

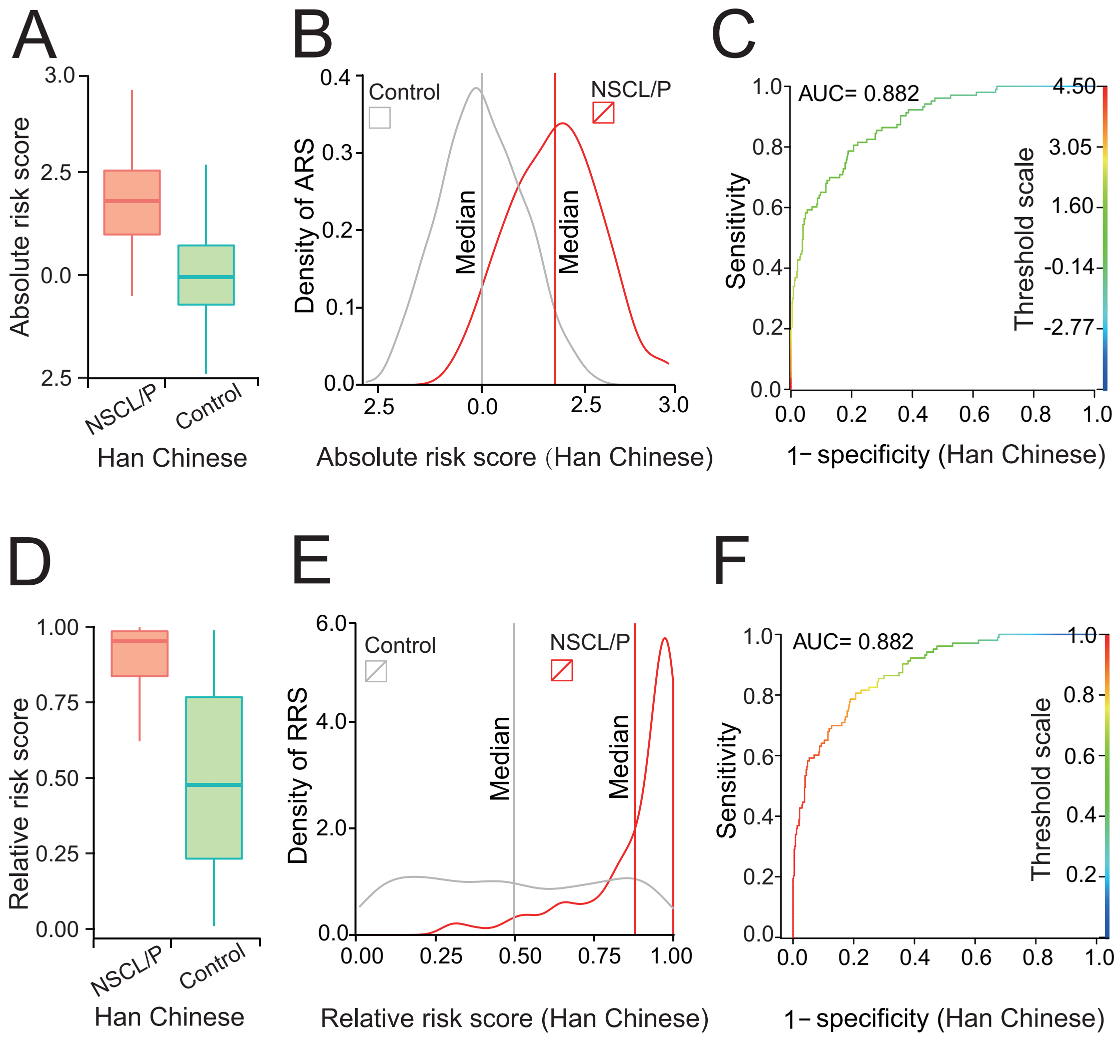


Figure 2 The genetic risk scoring models developed for the Han population

A. Boxplot of the ARS in the NSCL/P and control groups. **B.** Density profile of ARS in the NSCL/P and control groups. The vertical lines represent the median ARS values for the NSCL/P (red) and control (gray) groups, respectively. **C.** ROC curve for the model based on ARS to distinguish the NSCL/P and control groups. **D.** Boxplot of RRS in the NSCL/P and control groups. **E.** Density profile of RRS in the NSCL/P and control groups. The vertical lines represent the median RRS values for the NSCL/P (red) and control (gray) groups, respectively. **F.** ROC curve for the model based on RRS to distinguish the NSCL/P and control groups. ARS, absolute risk score; RRS, relative risk score; ROC, receiver operating characteristic; AUC, area under curve.

probability-based (Figure 2D) GRSs can clearly distinguish the NSCL/P group from controls, indicating that the model may effectively distinguish the individuals carrying multiple risk alleles that additively increase NSCL/P incidence. While the absolute value-based GRSs of the NSCL/P and control groups partially overlap (Figure 2B), the GRSs based on the relative risk probability (Figure 2E) showed a much clearer discrimination, with a median relative risk probability of 0.94 in NSCL/P versus 0.50 in the control group. In another word, if an individual was assessed with GRS > 0.9, he/she has a much higher chance of being NSCL/P than being normal. The GRS models for NSCL/P risk assessment in Han Chinese population were also evaluated by the receiver operating characteristic curve (ROC) analysis, and both had area under curve (AUC) values of 0.882 (Figure 2C and F).

For the Uyghur Chinese population, the predictive power was relatively low (**Figure 3**). Although the peak value of GRS was 0.89, the lower boundary was approximately 0.7 (Figure 3E). Although AUC values of 0.716 were obtained using GRSs based on the absolute OR or ranking probability, the discrimination resolution in the Uyghur population was lower than that observed in the Chinese population (Figure 3C and F). These results indicate that if an individual was assessed to have GRS > 0.9, the false positive rate would be below 0.1; however, for a subject with GRS of 0.75, there might be a 25% chance of a false positive diagnosis.

Machine learning methods for genetic risk assessment independent of ORs

The additive models for GRS are dependent on OR values; therefore, a large training set is essential to ensure the accuracy of computed ORs. In addition, when the sampling population changes, the ORs may also change. Moreover, when calculat-

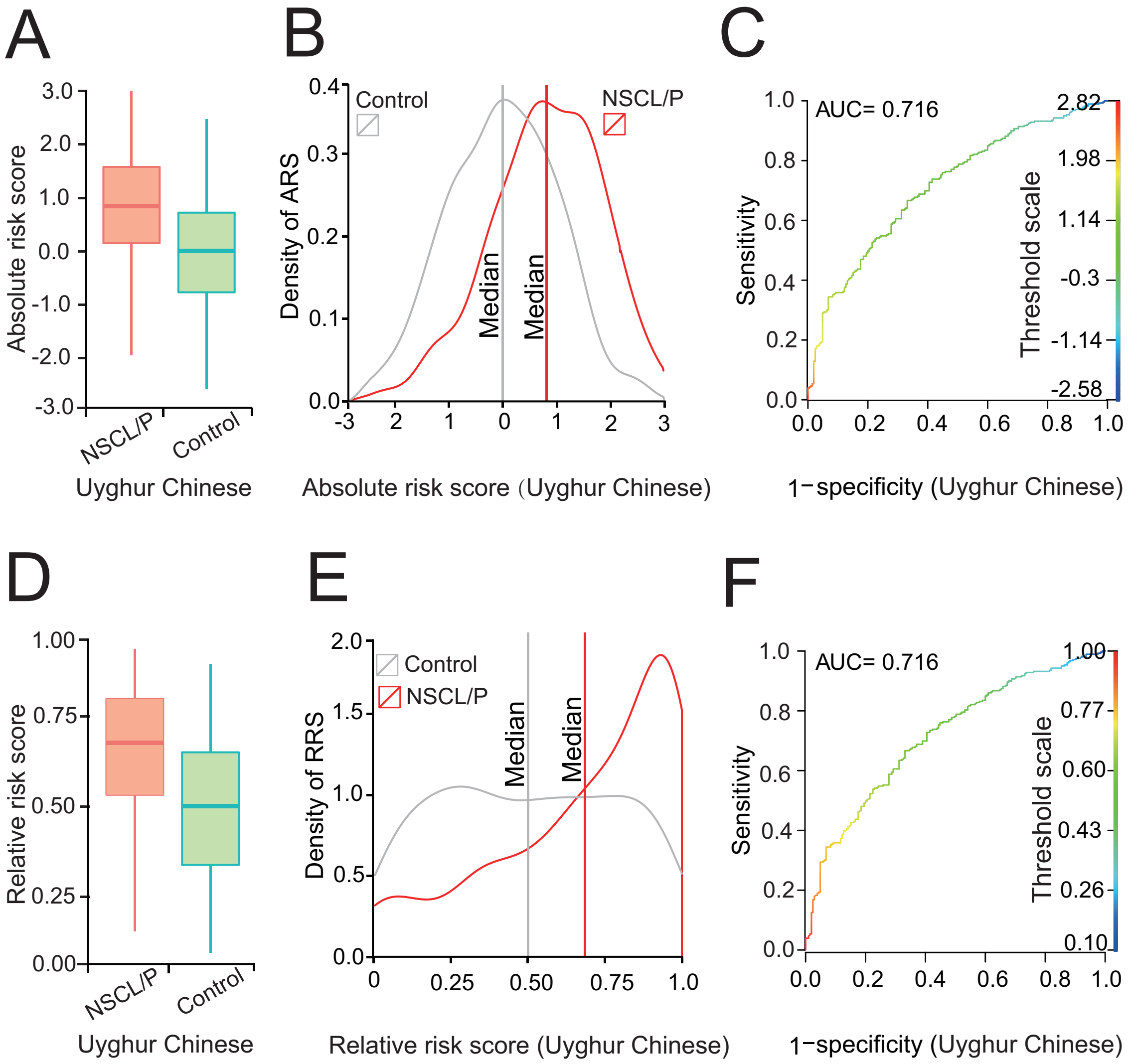


Figure 3 The genetic risk scoring models developed for the Uyghur population
A. Boxplot of the ARS in the NSCL/P and control groups. **B.** Density profile of the ARS in the NSCL/P and control groups. The vertical line represents the median of the ARS. **C.** The ROC for the model based on ARS to distinguish between the NSCL/P and control groups. **D.** Boxplot of the RRS in the NSCL/P and control groups. **E.** Density profile of the RRS in the NSCL/P and control group. The vertical line represents the median of the RRS. **F.** The ROC for the model based on RRS to distinguish between the NSCL/P and control groups.

ing OR value for each SNP site, all the variants are treated as an additive genetic effect by default, without considering the dominant and recessive effects caused by allele zygosity. The machine learning methods may overcome these shortcomings, as a binary classification of susceptibility or non-susceptibility is purely based on genotypes, without the need to pre-calculate the OR value for each SNP. Therefore, we attempted to build machine learning models for genetic risk assessment.

Based on the same panel of 43 SNPs, we used seven types of classical machine learning methods to evaluate their risk assessment accuracy with 10-fold cross-validation in Han and Uyghur populations. These include the support vector

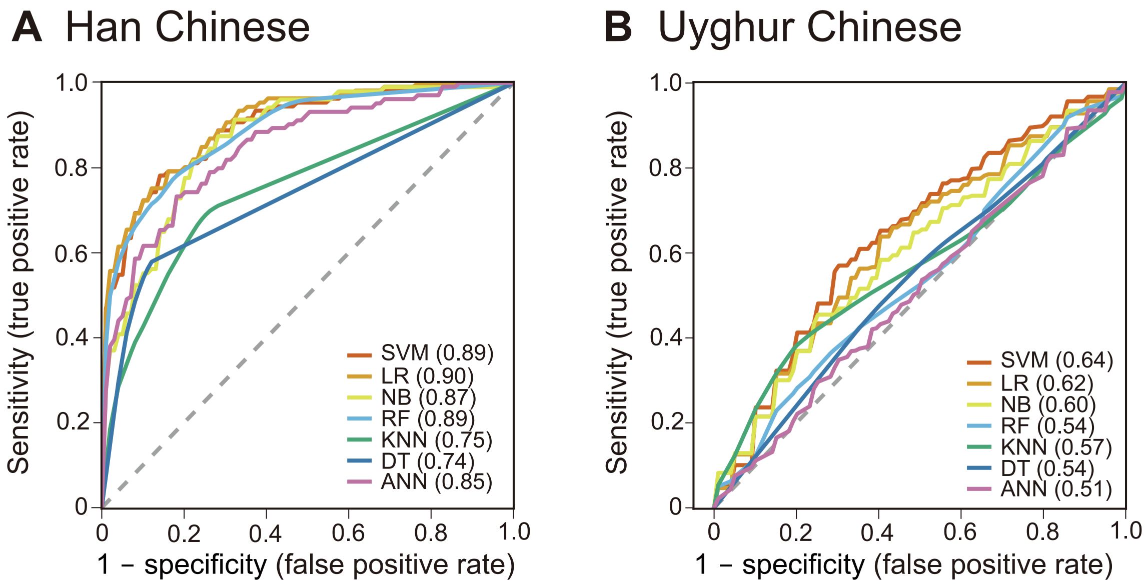


Figure 4 Comparison of predictive power by seven machine learning models

Seven NSCL/P risk assessment models were developed based on machine learning methods for the Han population (**A**) and Uyghur population (**B**), respectively. SVM, support vector machine; LR, logistic regression; NB, naive bayesian; RF, random forest; KNN, k-nearest neighbor; DT, decision tree; ANN, artificial neural network. The dashed line represents AUC of 0.5, and AUC values obtained using the respective method are included in the parenthesis.

Table 2 24 SNP markers for machine learning-based predictive effectiveness evaluation

No.	SNP ID	Gene	OR	Control	NSCL/P	Genotype	Class
1	rs1801133	*MTHFR*	3.391	10.5%	36.8%	Homozygous	Risk
2	rs1801131	*MTHFR*	0.967	3.7%	5.8%	Homozygous	Protective
3	rs2236225	*MTHFD1*	1.399	34.7%	43.8%	Heterozygous	Risk
4	rs10882272	*FFAR4/RBP4*	4.189	1.5%	20.6%	Homozygous	Risk
5	rs2294426	*ADTRP*	2.146	8.1%	36.2%	Homozygous	Risk
6	rs7820074	intergenic	6.455	12.6%	63.7%	Homozygous	Risk
7	rs642961	*IRF6 – DIEXF*	2.003	1.9%	19.4%	Homozygous	Risk
8	rs7632427	*EPHA3 – PROSP*	1.468	5.5%	17.3%	Homozygous	Risk
9	rs3733585	*SLC2A9*	1.198	18.0%	26.2%	Homozygous	Risk
10	rs7950069	*CADM1*	1.196	12.6%	16.8%	Homozygous	Risk
11	rs9574565	*SPRY2*	2.126	21.6%	40.8%	Heterozygous	Risk
12	rs8001641	LOC105370275	1.964	24.9%	39.0%	Heterozygous	Risk
13	rs4791774	*NTN1*	1.323	29.1%	36.0%	Heterozygous	Risk
14	rs227731	*NOG*	1.847	43.5%	52.5%	Heterozygous	Risk
15	rs17085106	LOC102724913	2.146	0.0%	10.0%	Heterozygous	Risk
16	rs1667255	*TTR*	1.145	42.8%	52.4%	Heterozygous	Risk
17	rs4703516	*ACOT12*	1.146	50.1%	61.7%	Heterozygous	Risk
18	rs7590268	*THADA*	2.853	5.4%	10.7%	Heterozygous	Risk
19	rs987525	*GSDMC – PVT1*	1.364	15.0%	20.5%	Heterozygous	Risk
20	rs7078160	*SHTN1*	1.423	48.1%	53.4%	Heterozygous	Risk
21	rs10863790	*IRF6 – DIEXF*	0.574	19.0%	9.7%	Homozygous	Protective
22	rs3815854	LOC105373888	0.564	47.3%	35.1%	Heterozygous	Protective
23	rs2235371	*IRF6*	0.545	47.9%	38.9%	Heterozygous	Protective
24	rs12543318	LOC105375626	0.825	46.7%	41.8%	Heterozygous	Protective

Note: The 24 SNPs were manually selected according to a series of criteria, including OR, allele frequency, previous studies, and detection quality of SNP genotyping. An allele with OR > 1 is defined as a risk allele, whereas an allele with OR ≤ 1 is considered protective.

machine (SVM), logistic regression (LR), naive Bayesian (NB), random forest (RF), k-nearest neighbor (KNN), decision tree (DT), and artificial neural network (ANN). For the Han population, the best performance was obtained using LR (AUC = 0.90), followed by SVM (AUC = 0.89), and random forest (AUC = 0.89; **Figure 4**A), which were all slightly higher than the GRS additive model (AUC = 0.88; Figure 2). Similarly, we applied the same seven methods to the Uyghur population, but noticed a poorer performance than that observed in the Han population, with the best method (SVM) only having an AUC of 0.64 (Figure 4B). Although the GRS model for the Uyghur population (AUC = 0.716; Figure 3) showed a higher AUC than the best of the machine learning models.

Compilation of a minimum SNP set based on marker effectiveness evaluation

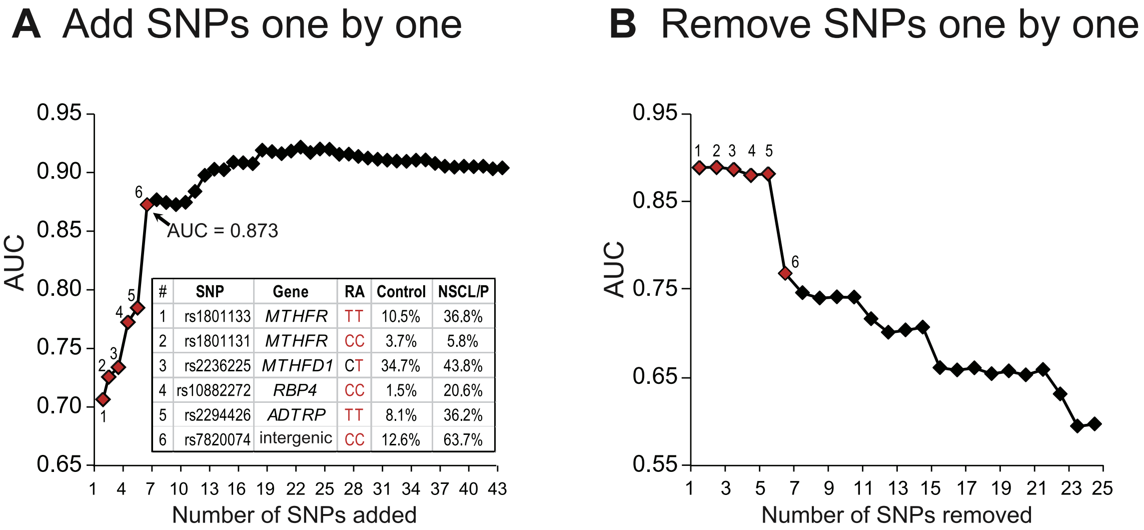
Based on MAFs, ORs, zygosity, discrimination ability and detection quality, we manually selected a panel of 24 SNPs in the Han population (**Table 2**). We scrutinized SNPs in the genes related to folic acid and vitamin A biosynthesis and metabolism, considering their potential application in nutritional intervention for lowering the risk of NSCL/P incidence.

To evaluate the risk assessment efficiency of the 24 selected SNPs, we adopted a strategy of sequentially removing or adding one SNP for each instance of training or prediction using a LR model based on the order shown in Table 2. With the one-by-one adding process, we obtained AUC values of 0.778 and 0.873 using 4 and 6 SNPs, respectively. The highest AUC was obtained (0.925) when 18 SNPs were used to build the model where it then plateaued regardless of the addition of more SNPs (**Figure 5**A). In the same order, using a one-by-one removal approach, a remarkable decline in the AUC values from 0.87 to 0.76 was observed when the first six SNPs were removed (Figure 5B). Therefore, it appears that the first four SNPs in three genes involved in the folic acid and vitamin A biosynthesis, *MTHFR*, *MTHFD1,* and *RBP4*, play important roles in NSCL/P incidence.

Discussion

In this study, we first examined the distribution of 43 SNPs in Han and Uyghur populations, which were previously discovered in association with NSCL/P according to GWAScatalog database collection. Then, we developed genetic risk assessment models for NSCL/P in Han Chinese population using traditional risk scoring method and machine learning methods. Compared to scoring-based method, one of the advantages of machine learning method is that it can be directly applied to the genotypes of SNPs without pre-calculation of ORs [19]. This merit of machine learning predictive model may firstly reduce the bias caused by inconsistent OR values of SNPs when populations and samples are changed, and secondly take homozygous and heterozygous forms of risk alleles into account. Among the seven models tested in our study, logistic regression model (LR) showed the best predictive performance in Han population (AUC = 0.903). However, the prediction power of LR was much less robust for the Uyghur population (AUC = 0.627), likely due to the genetic difference between Han and Uyghur Chinese. Therefore, GWAS analyses in Uyghur Chinese population and identification of NSCL/P related variants is highly demanded for risk assessment model development for Uyghur infants.

Among the 43 SNPs, four SNPs are associated with folic acid and vitamin A metabolism-related genes. These include rs1801133 (*MTHFR*) and rs10882272 (*RBP4*) whose homozygous risk alleles showing significantly higher enrichment in NSCL/P group compared to control in Han Chinese population. *MTHFR* encodes an enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the predominant circulatory form of folate and the methyl donor for the



#	SNP	Gene	RA	Control	NSCL/P
1	rs1801133	*MTHFR*	TT	10.5%	36.8%
2	rs1801131	*MTHFR*	CC	3.7%	5.8%
3	rs2236225	*MTHFD1*	CT	34.7%	43.8%
4	rs10882272	*RBP4*	CC	1.5%	20.6%
5	rs2294426	*ADTRP*	TT	8.1%	36.2%
6	rs7820074	intergenic	CC	12.6%	63.7%

Figure 5 Evaluation of predictive effectiveness of the 43 SNPs using one-by-one adding and removing SNPs method in the Han population
Alterations in AUC when adding (**A**) or removing (**B**) these 24 SNP markers one by one. Numbers above the red dots indicate the ID of the SNPs associated with nutritional genes in the table. Nucleotides marked in red represent the forms of the risk alleles. The genotypic frequencies of the six SNPs are also shown in the table. RA, risk allele.

re-methylation of homocysteine to regenerate methionine [20]. Previous reports show that TT homozygous genotype of rs1801133, a missense variant causing amino acid alteration (Ala222Val), may reduce folate concentration in serum, plasma, and red blood cells [21–25]. Variant of rs1801133-T appears to be a risk allele specific to East Asian population, since among the Caucasian infants, the homozygous TT genotypes showed no correlation with NSCL/P [26,27]. It is of note that homozygous TT genotype showed no correlation with NSCL/P in Uyghur Chinese population. Another missense variant of *MTHFR*, homozygous CC genotypes of rs1801131 causing amino acid alteration (Glu470Gly), may also reduce enzyme activity to a lesser extent than rs1801133, although it is not associated with lower plasma folate or higher homocysteine levels [28,29]. This is consistent with our validation result that the OR value of rs1801131 is 0.967 (P = 0.858) and 0.794 (P = 0.103) in Han Chinese and Uyghur Chinese populations.

Another SNP showing high effective discrimination ability is rs10882272, which is located upstream *RBP4*. *RBP4* encodes a plasma transport protein, which acts as a specific carrier of vitamin A and is responsible for transporting retinol from hepatic stores to peripheral target tissues in the body, including the placenta [30]. Previous proteomic studies also show that the RBP4 protein level in serum is significantly lower in infants with NSCL/P than control infants [15]. Our analysis on rs10882272 supports these previous studies, as higher percentage of subjects with homozygous risk genotype CC at rs10882272 are found in the NSCL/P group than in the control group in both Han and Uyghur Chinese populations. Therefore, both proteomics and genomics analyses suggest that appropriate vitamin A supplementation may be helpful to prevent the birth of NSCL/P infants for pregnant women who are carriers of rs10882272 risk alleles.

Addition of two functionally unknown SNPs (rs2294426 and rs7820074) to the four SNPs above can increase the prediction power from AUC = 0.761 to AUC = 0.873. The rs2294426 (homozygous risk genotype TT) and rs7820074 (homozygous risk genotype CC) with OR values of 2.146 and 6.455 indicate that both of them are risk allele in Han Chinese population. However, these two SNPs show opposite trend in Uyghur Chinese population, as the OR values (0.764 for rs2294426 and 0.797 for rs7820074) indicate that both of them are protective alleles in Uyghur population. The SNP rs2294426 at 6p24.1 is located within *ADTRP*. *ADTRP* encodes an androgen-dependent tissue factor pathway inhibitor (TFPI) regulating protein, which regulates the expression and function of TFPIs in human endothelial cells under normal conditions and in response to androgens [31,32]. *ADTRP* has been reported to be associated with early-onset coronary artery disease in southern Han Chinese populations [31]. Another SNP at 8q21.3, rs7820074, was mapped to an intergenic region, without known function or overlap with any other adjacent genes. Whether this genomic locus harbors any regulatory *cis*-elements or encodes a noncoding RNA gene related to orofacial cleft development also requires further investigation. More lines of evidence are needed to determine whether these two functionally unknown variants are correlated with the occurrence of NSCL/P.

It's commonly acknowledged that appropriate supplementation with folic acid and vitamin A during the women's conception and peri-conception periods is important to reduce the risk of conceiving a NSCL/P baby. Our analysis demonstrates that four SNPs located in the genes involved in folic acid (rs1801133 and rs1801131 in *MTHFR*, rs2236225 in *MTHFD1*) and vitamin A (rs10882272 in *RBP4*) metabolism, account for about 76.1% (AUC = 0.761) prediction ability of the contribution to NSCL/P incidence, indicating high contribution of the variants of *MTHFR* and *RBP4* to NSCL/P occurrence. Thus, our study provides supporting evidence that genetic diagnosis of *MTHFR* and *RBP4* variants may help design nutritional intervention plan to minimize the occurring chance of congenital NSCL/P, especially when both parents carry the same risk alleles. Therefore, these nutritional markers have the application potential to be used as guides for nutritional intervention to reduce NSCL/P incidence in future.

Materials and methods

Subject recruitment

Patients with NSCL/P were recruited from unrelated Chinese infants attending the "Smile Train" in Yantai, Shandong (Han population) and Kashi, Xinjiang (Uyghur population) during 2015–2016. All subjects were interviewed and clinically assessed by at least two experienced clinicians. A full clinical checkup was completed to identify any further anomalies, such as congenital heart disease, hypospadias, or accessory auricles, which would suggest an underlying syndrome. Additional demographic information was obtained through a detailed questionnaire, including gender, age, nationality, and maternal lifestyle during the first trimester of pregnancy. After genetic and phenotypic quality control, 103 and 279 patients with NSCL/P from Han and Uyghur populations, respectively, were included for subsequent analysis.

Healthy individuals without an orofacial cleft or a family history of orofacial cleft were included as controls, comprising 205 Uyghurs and 504 Han Chinese. The 205 Uyghurs were recruited from the local hospitals in Urumqi, China, while the 504 controls for Han population were selected from East Asians (most participants from Beijing, China) in the 1000 Genomes database (http://www.internationalgenome.org).

This study was approved by the Ethics Committee of the Peking University, School and Hospital of Stomatology (approval No. PKUSSIRB-201520012) and was conducted according to the Helsinki Declaration of ethical principles. Written informed consent was obtained from the participants or their guardians for NSCL/P affected and normal individuals in Han and Uyghur Chinese populations.

SNP genotyping

Peripheral blood (3 ml for each person) samples were collected from all subjects. Genomic DNA was extracted from the samples using a standard DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The DNA concentrations were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and normalized to 5 ng/μl for each sample. A total of 43 SNPs (Table 1) that had been previously reported in five well-designed GWAS analyses [18,33–36] were selected for subsequent analysis. Genotyping was performed using KASPar chemistry (KBioscience, Hoddesdon, UK), a competitive allele-specific PCR-SNP genotyping system that use ≤ s FRET quencher cassette oligos [37,38]. The resulting allele call data were viewed graphically as a scatterplot for each assayed marker using SNPViewer (http://www.lgcgenomics.com).

Allele definition

We first re-defined the major allele (≥ 0.5) and minor allele (< 0.5) based on the allele frequencies in the control groups consisting 504 East Asians and 205 Uyghurs, respectively. Then, the frequencies of major and minor alleles were computed in the groups of patients with NSCL/P including 103 Han Chinese and 279 Uyghurs. OR for the two ethnic populations in each groups was calculated as shown below.

$$\mathrm{Odds}_{(\mathrm{NSCL/P})} = \mathrm{Major\,allele}_{(\mathrm{NSCL/P})}/\mathrm{Minor\,allele}_{(\mathrm{NSCL/P})} \quad (1)$$

$$\mathrm{Odds}_{(\mathrm{control})} = \mathrm{Major\,allele}_{(\mathrm{control})}/\mathrm{Minor\,allele}_{(\mathrm{control})} \quad (2)$$

$$\mathrm{OR} = \mathrm{Odds}_{(\mathrm{NSCL/P})}/\mathrm{Odds}_{(\mathrm{control})} \quad (3)$$

An allele is considered as a risk allele, if OR is greater than 1 (OR > 1), and otherwise a protective allele (OR $\leq$ 1).

Risk scoring

We used two methods to calculate genetic risk scores for a given genotype with an additive model. First, we computed an absolute risk score (ARS) by summing the OR values of SNPs (n = 43) as shown in Equation (4). Each OR of the SNP is multiplied by the assigned digital value of genotypes (0 for the genotype comprising 2 major alleles). Then, the same equation was used to calculate the ARS in the control groups consisting of 504 Asians in order to obtain a distribution of ARS in the control group. Third, we ranked the ARS and assigned the corresponding percentile rank of the ARS value in the control group as the relative risk score (RRS). Finally, the RRS for a given NSCL/P genotype, namely, the percentile rank of the corresponding ARS value in the control group, was used to represent the probability of genetic risk of being an infant with NSCL/P.

$$ARS = \sum_{i=1}^{n=43} Genotype_n \times ln\,(OR_{\mathrm{n}})Genotype \begin{cases} 0, & Major : Major \\ 1, & Major : Minor \\ 2, & Minor : Minor \end{cases} \quad (4)$$

Classification accuracy evaluation

We used seven commonly used machine learning models in the Python Scikit-learn package to build the models, including SVM, LR, NB, RF, KNN, DT, and ANN. The ten-fold cross validation method was used during the training process, and then an AUC value was computed to represent the overall performance for each model. 43 risk and protective alleles were collected from GWAScatalog database to evaluate the predictive effectiveness of the 43 SNPs Alleles with OR < 1 were defined as protective alleles, while alleles with OR > 1 were defined as risk alleles. Then, a one-by-one removal strategy and a one-by-one adding strategy were used to test the model performance in which a marker was removed or added from the marker set, respectively.

Authors' contributions

XXW, FC, XFW, and JL conceived the project; PM, JZ, and PJ collected the samples; XXW and PM conducted the experiments; SZ and XFW analyzed the data. PM, XXW, and XFW wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary material

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