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Original Article

Association between PKA gene polymorphism and NTDs in high risk Chinese population in Shanxi

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Abstract: Objective: This study aimed to investigate the single nucleotide polymorphisms (SNPs) of PKA and neural tube defects (NTDs) in Chinese population. Method: A total of 183 NTDs cases and 200 healthy controls were used in this study. 7 selected single nucleotide polymorphism (SNP) sites in the PKA gene were analyzed with MassArray high-throughput DNA analyzer with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. A series of statistical methods were carried out to investigate the correlation between the SNPs and the patient susceptibility to NTDs. Results: Statistical analysis showed a significant correlation between the SNP sites rs12132032 in PRKACB and NTDs. The AA genotype, A-allele and dominant AA in rs12132032 significantly increased the incidence of NTDs especially anencephaly (OR=3.87, 95% CI: 1.80-8.34 with genotype; OR=2.08, 95% CI: 1.43-3.04 with allele; OR=3.10, 95% CI: 1.53-6.26 with dominant). The T-allele of rs594631 in PRKACB was correlative with NTDs in male but not in female. Conclusions: The gene polymorphism loci rs12132032 in PRKACB maybe a potential risk factor for anencephaly in Chinese population from Shanxi, while gender susceptibility may influence the correlation.

Keywords: Neural tube defects (NTDs), single nucleotide polymorphisms (SNPs), protein kinase A (PKA), Sonic hedgehog pathway, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

Introduction

Neural tube defects (NTDs) are one of the most common birth defects because neural tube does not close completely early in embryonic development. They mainly include spina bifida, cerebral meningocele and no brains. The incidence of NTDs in Lvliang area in China is the highest in the world. They occur in approximately 199.38 in 10,000 live births [1, 2]. NTDs are multifactorial and arise from interactions between genetic and environmental factors. Environmental factors such as folic acid and vitamin B12 are very important in reducing the occurrences of NTDs. Several studies have evaluated specific genetic NTDs risk factors and identified more than 100 genes. However, the mechanism of NTDs is still not fully understood [3, 4].

The Sonic Hedgehog (Shh) signaling pathway is involved in the development of most vertebrate organs and tissues including nervous system and regulates cellular proliferation and differentiation [5, 6]. Shh is the key regulator of granule cell development and is a critical mitogen for granule cell precursors (GCPs) during normal development [7, 8]. Previous studies indicated that excessive activated Shh signaling pathway could generate NTDs phenotype [9, 10]. The Gli family is the important component of the Shh pathway and there are three members of the Gli family (Gli1, Gli2, Gli3) in vertebrates. Gli1 and Gli2 are primarily as transcriptional activators. Gli3 exists in two forms: full-length activator (Gli3A) and shorter repressor (Gli3R) [11-13]. The repressor formation depends on cAMP-dependent protein kinase A (PKA), which is an important and conserved negative regulatory factor of Shh signaling pathway in vertebrate. It was confirmed that the role of PKA in the Shh pathway is mainly realized through the phosphorylation of Gli family members, PKA activity may influence the outcome of Shh signaling in

normal development [14-16]. Gli family members were hydrolyzed by PKA phosphorylation in the absence of Shh, while the role of phosphorylation was released at the presence of Shh. Complete loss of PKA catalytic activity in *Cα–/–Cβ–/–* double mutant embryos causes a complete ventralization of the neural tube. The Shh pathway is maximally activated in all neural progenitors in the absence of PKA. PKA is important for the formation of Gli3 repressor. The major function of PKA in the neural plate is to prevent Gli2 from activating the targets of the pathway [16-18].

There are two catalytic subunit allele of PKA (Cα and Cβ) which encoded by PRKACA and PRKACB genes. The mutant PRKACA and PRKACB genes could reduced PKA activity and the mutant mice with reduced PKA activity developed localized neural tube defects (NTDs) [19]. However, Zhu et al found that there was no a strong association between these PKA SNPs and spina bifida risk [20]. In this study, we explored the association between the single nucleotide polymorphisms of PRKACA and PRKACB genes and the risk of NTDs in high risk population.

Materials and methods

Subjects

A case-control approach was carried out to investigate the relationship between PKA polymorphisms and NTDs occurrences in Lvliang mountain area located in Shanxi of China, which is a high prevalence in NTDs. From May 2006 to August 2010, pregnant women who were adopting prenatal health care or had delivered were recruited according to the diagnosis from 9 country hospitals as NTD-affected pregnancy group and normal pregnancy group. All the pregnant women had submitted their informed consent, and the study protocol was reviewed and approved by the Institutional Review Board of the Capital Institute of Pediatrics in Beijing, China. Clinical information such as pregnant week, gender of embryo was collected by the local trained doctors. The subjects were unanimous Han nationality.

Embryo samples for DNA extraction were conserved at -20°C until autopsy by experienced pathologists. Embryos affected with NTDs were Medical termination of pregnancy based on the B-mode ultrasound screen and made a definite diagnosis by the postmortem according to the International Classification of Disease, Tenth Revision, codes Q00 anencephaly, Q05 spina bifida, and Q01 encephalocele. The control samples were from non-pathological induced labor and confirmed had no deformity by the postmortem.

Genomic DNA extraction

Genomic DNA was extracted from frozen shin tissue for genotyping using a standard phenol/chloroform extraction procedures, followed by ethanol precipitation. Qualified DNA were subsequently used for the genotype, determining by absorbance OD260/OD280 in 1.8-1.9.

SNP selection and genotyping

Tag SNPs were selected from HapMap database in promoter and full-length region of PRKACA gene and PRKACB gene, exon SNPs were obtained from NCBI SNP database. The primers designed through AssayDesigner 3.0 software for the SNPs which had a minor allele frequency of no less than 0.10 in Han nationality and in the strong linkage disequilibrium with other SNPs, r^2 was set to be no more than 0.8. Polymerase chain reaction (PCR) were used to amplify the polymorphic region. Genotyping was conducted by an experienced technician who was blinded to the diagnosis using MassArray high-throughput DNA analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom, San Diego, CA). To insure the results of genotyping, the negative controls were set and 60% of samples were re-genotyped. Subsequent analyses about frequency of genotype/allele were conducted in 7 tag SNPs of PRKACB gene, for more than 90% of samples were successfully genotyped, information and primer of these SNPs conducted analyses were listed in **Table 1**.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Science (SPSS, version 13.0). All Statistical tests were 2-sided, and $p < 0.05$ was considered to be significant. Hardy-Weinberg equilibrium was assessed by Chi-square test, meanwhile Chi-square and Fisher's exact tested were executed to compare frequencies of pregnant week, gender and genotype/allele. Adjusted odds ratios with 95% confidence interval (CI) were performed by mul-

Table 1. Details of SNPs and primers used in this study

rank	SNP	pHWE*	MAF#	Wild type	allele	location	Forward primer	Reverse primer	Genotype ratio
1	rs594631	0.711	0.476	A	A/T	intron	ACGTTGGATGGGATTTTTGTTAGCTAGTG	ACGTTGGATGACCAGACAAGCACACAACA	96.08%
2	rs970318	0.901	0.233	A	A/G	intron	ACGTTGGATGAGCCTATCTCACAAGAGGGA	ACGTTGGATGCTATTTGCGATAATAATGCTG	95.56%
3	rs2642186	0.987	0.439	G	C/G	intron	ACGTTGGATGCTCTAGGTCAAAAGTCGTGG	ACGTTGGATGTCCAATTCATTTCCACCCCC	96.61%
4	rs2792975	0.995	0.578	C	C/G	intron	ACGTTGGATGAATCAACAGGGTGAAGAGAC	ACGTTGGATGGGATATTAGTCCCCTGTTGC	92.43%
5	rs6696125	0.974	0.244	T	C/T	intron	ACGTTGGATGCAAATATTTCCTTCTAGTC	ACGTTGGATGACGATAAATTGGACTTCAC	93.21%
6	rs12039822	1.000	0.293	C	C/T	intron	ACGTTGGATGGCTGTAATTAGCCTCAGTGG	ACGTTGGATGAAAGCCTCACCACAGGCTAC	97.65%
7	rs12132032	0.160	0.167	A	A/G	intron	ACGTTGGATGAGAGGTAACTGAGGCATAG	ACGTTGGATGCTCTAGAGTCACTGTTTGGG	95.56%

*p-value for deviation from Hardy-Weinberg equilibrium. #Minor allele frequency in Han Chinese.

Table 2. General information of subjects

	control n=190 (%)*	NTDs n=176 (%)*	P	anencephaly n=83 (%)*	spina bifida n=69 (%)*	encephalocele n=24 (%)*
Gender#						
male	87 (45.8%)	82 (46.9%)	0.838	33 (39.8%)	31 (44.9%)	18 (75.0%)
female	103 (54.2%)	93 (53.1%)		50 (60.2%)	37 (53.6%)	6 (25.0%)
missing	-	1 (0.6%)		-	1 (1.4%)	-
Gestational week#						
≤ 16	19 (10.0%)	14 (8.0%)	0.012	6 (7.2%)	6 (8.7%)	2 (8.3%)
17~24	159 (83.7%)	134 (76.1%)		68 (81.9%)	48 (69.6%)	18 (75.0%)
≥ 25	12 (6.0%)	28 (15.9%)		9 (10.8%)	15 (21.7%)	4 (16.7%)

*Percentages may not equal 100 because of rounding. #Chi-square test was used to calculate the p-values.

Table 3. The distribution of genotypes and alleles of rs12132032 in control and NTDs groups and in each subtype

	control	NTDs			anencephaly			spina bifida			encephalocele		
	n=190 n (%)	n=176 n (%)	P*	OR	n=83 n (%)	P*	OR	n=69 n (%)	P*	OR	n=24 n (%)	P*	OR
GG	61 (32.1%)	38 (21.6%)		1	11 (13.3%)		1	19 (27.5%)		1	8 (33.3%)		1
AG	76 (40.0%)	71 (40.3%)	0.125	1.5 (0.89-2.52)	35 (42.2%)	0.015	2.55 (1.20-5.44)	27 (39.1%)	0.703	1.14 (0.58-2.24)	9 (37.5%)	0.843	0.903 (0.33-2.48)
AA	53 (27.9%)	67 (38.1%)	0.011	2.03 (1.18-3.49)	37 (44.6%)	0.001	3.87 (1.80-8.34)	23 (33.3%)	0.360	1.39 (0.69-2.84)	7 (29.2%)	0.990	1.01 (0.34-2.96)
G	98 (52.1%)	147 (41.8%)	0.005	1	57 (34.3%)	0.000	1	65 (47.1%)	0.314	1	25 (52.1%)	0.998	1
A	182 (47.9%)	205 (58.2%)		1.52 (1.13-2.03)	109 (65.7%)		2.08 (1.43-3.04)	73 (52.9%)		1.22 (0.83-1.81)	23 (47.9%)		1.00 (0.55-1.83)
GG+GA	61 (32.1%)	38 (21.6%)	0.024	1	11 (13.3%)	0.001	1	19 (27.5%)	0.482	1	8 (33.3%)	0.903	1
AA	129 (67.9%)	138 (78.4)		1.72 (1.07-2.75)	72 (86.7%)		3.10 (1.53-6.26)	50 (72.5%)		1.24 (0.68-2.29)	16 (66.7%)		0.95 (0.38-2.33)

*Chi-square test was used to calculate the *p*-values. Fisher's exact test was used when the sample size is less than 5.

Table 4. Genotype and allele frequencies of rs594631 in control and NTD groups based on gender

	Male			Female		
	control n=86	NTDs n=84	OR	control n=103	NTDs n=94	OR
AA	21 (24.4%)	14 (16.7%)	1	25 (24.3%)	23 (24.5%)	1
AT	45 (52.3%)	39 (46.4%)	1.30 (0.58-2.90)	42 (40.8%)	48 (51.1%)	1.24 (0.62-2.51)
TT	20 (23.3%)	31 (36.9%)	2.33 (0.97-5.60)	36 (35.0%)	23 (24.5%)	0.69 (0.32-1.50)
A	87 (70.5%)	67 (39.9%)	1	92 (44.7%)	94 (50.0%)	1
T	85 (49.4%)	101 (60.1%)	1.54* (1.00-2.37)	114 (55.3%)	94 (50.0%)	0.81 (0.54-1.20)
AA+AT	66 (76.7%)	53 (63.1%)	1	67 (65.0%)	71 (75.5%)	1
TT	20 (23.3%)	31 (36.9%)	1.93 (0.99-3.77)	36 (35.0%)	23 (24.5%)	0.60 (0.32-1.12)

*means the *p* value < 0.05 (p=0.048).

Table 5. Distribution of PTCH1 haplotype frequencies

Haplotype	Frequency in control group	Frequency in NTDs group	OR (95% CI)	p
GC	0.4306	0.4232	1.00	---
CG	0.3816	0.3864	0.98 (0.71-1.35)	0.9
GG	0.1765	0.1735	1.01 (0.65-1.56)	0.96
CC	0.0112	0.0169	0.66 (0.15-2.92)	0.59

tiple logistic regression to estimate the risk of NTDs related to the polymorphism.

Results

In all 200 controls and 183 NTDs including 85 anencephaly, 73 spina bifida and 25 encephalocele were recruited in this study. Seven SNPs in Hardy-Weinberg equilibrium of PRKACB gene were executed the statistical analysis for they had a ratio more than 90% of success genotyped. For the polymorphism rs12132032, Males and females in the case group and the control group are both almost half of proportion, the distribution of two groups was not significant. The ratio of gestational week for no less than 25 weeks in control group was much lower than that of case group, for some controls is from the non - pathological abortion (**Table 2**). For the rs594631, the genotyping call rate was 96.08%, and had similar proportion of sex and gestational week between the case group and the control group to rs12132032 (data not shown). As for the rest of the polymorphisms, there was no significant difference of the distribution of genotype and allele was found between the case group and the control group, even stratification analysis was processed according to the gender (data not shown).

A total of 95.56% of total samples were successfully genotyped for rs12132032, including 190 controls and 176 NTDs. Re-genotype results showed 100% concordance. Genotype and allele frequencies for SNP rs12132032 in each of the study groups are displayed in **Table 3**. The portion of homozygous mutation AA, allele A and dominant "AA" in NTDs group were significantly higher than those in the control group (p=0.011, 0.005, 0.024, respectively), meanwhile Case/control analyses also showed that all three were significantly related to NTDs (OR=2.03, 95% CI: 1.18-3.49 with genotype AA; OR=1.52, 95% CI: 1.13-2.03 with allele A; OR=1.72, 95% CI: 1.07-2.75 with dominant AA compared with the combination of "GG+GA"), similar results could be seen in anencephaly subgroup (OR=3.87, 95% CI: 1.80-8.34 with genotype; OR=2.08, 95% CI: 1.43-3.04 with allele; OR=3.10, 95% CI: 1.53-6.26 with dominant), which was deemed to be most serious type of NTDs, these revealed that the polymorphism rs12132032 maybe positively associated with NTDs risk in this population.

Genotype information of rs594631 was successfully obtained from 367 samples (189 controls and 178 NTDs). Although no significant differences in genotype and allele frequencies were found between the NTDs group and the normal controls, stratification analysis according to the gender found that the portion of allele T in NTDs group is significantly higher than that in the control group in male embryos (p=0.048), as well as the risk of NTDs was significantly increased for allele T compared with allele A (OR=1.54, 95% CI: 1.00-2.37), on the contrary this trend were not be seen in female samples (**Table 4**).

All 7 SNPs involved with multiple associations were considered with linkage disequilibrium. Evidence of linkage disequilibrium was observed of rs2642186 and rs2792975. Four common haplotypes were generated, although none of them showed a significantly association with increased risk of NTDs (**Table 5**).

Discussion

In this study, the association between PKA polymorphisms and risk of NTDs was analyzed for the Lvliang region, which has the highest prevalence of NTDs in China [1]. The results showed that there was a significant correlation between the SNP sites rs12132032 in PRKACB and NTDs. The AA genotype, A-allele and dominant AA in rs12132032 significantly increased the incidence of NTDs especially anencephaly. The T-allele of rs594631 in PRKACB was correlative with NTDs in male but not in female.

cAMP is a second messenger that acts through PKA to modulate the Shh signaling pathway. It is possible that PKA gene polymorphisms could result in ectopic Shh signaling and excessive ventralization of the neural tube, resulting in NTDs. PKA is a serine/threonine kinase which

is composed of 2 catalytic (C) subunits and 2 regulatory (R) subunits. *PRKACA* and *PRCAKB* genes encode the 2 catalytic subunits α and β respectively. The human *PRKACA* gene maps to chromosome 19p13.1 and *PRKACB* gene maps to chromosome 1p36.1. PKA is known to down-regulate the Hedgehog (Hh) signaling pathway, which is critical to normal pattern formation and morphogenesisSkalhegg, et al. [21]. The rationale for exploring genetic variation within the PRKACA and PRKACB genes as possible risk factors governing abnormal neural tube closure was based on the knowledge that cAMP-dependent PKA plays an important role in embryonic development via its negative regulation of the Hh signaling pathway. PKA-deficient mice were generated by Huang et al through intercrossing double heterozygotes of Cα and Cβ (Cα+/−Cβ+/−). Mice with only 1 allele of each catalytic subunit (Cα+/−Cβ−/− or Cα−/−Cβ+/−) developed spinal neural tube defects (NTDs) with 100% penetrance [21]. However, Zhu et al found that there was no apparent increase in spina bifida risk for infants who carried uncommon alleles for PRKACA and PRKACB SNPs they genotyped [20]. They thought it did not rule out potential contributions of other genetic variations of PKA because they only searched sequence variants in coding regions but not all the noncoding regions. In this study we found that there was a significant correlation between the SNP sites rs12132032 in PRKACB and NTDs. To our knowledge, this is the first report about the association between PRKACB genes and NTDs.

According to the international classification standard, NTDs can be divided into three subtypes. There were 73 patients with spina bifida, 85 with anencephaly and 25 with encephalocele in our study. We found that the gene polymorphism loci rs12132032 in PRKACB maybe a potential risk factor for anencephaly in Chinese population. Anencephaly occurs when the rostral (head) end of the neural tube fails to close and the causes are disputed. Our results suggested that the polymorphisms of PRKACB gene could affected the activity of the Shh pathway and resulted in neural tube closure obstacles, and then leaded to anencephaly.

There was gender difference in the incidence of NTDs [23]. There were no statistically significant differences of the distribution of genotype and allele between the case and control groups when they were stratified by gender in this study. This may be due to small sample size. However, in rs594631 analysis, when the samples were stratified by gender we found that the portion of allele T in NTDs group is significantly higher than that in the control group in male embryos, as well as the risk of NTDs was significantly increased for allele T compared with allele A, on the contrary this trend were not be seen in female samples.

At the same time, we also compared the distribution of genotype in different population based on the HapMap data. We found that there were differences in the distribution of genotype and allele of polymorphic rs12132032 between our populations and two other Asian populations. So we analyzed the correlation between polymorphisms and NTDs according to our population distribution and took GG genotypes and G allele as a reference for analysis. However, we found no differences in the distribution of genotype and allele of polymorphic rs594631 between our populations and two other Asian populations. This further confirmed our typing results.

In conclusion, the gene polymorphism loci rs12132032 in PRKACB maybe a potential risk factor for anencephaly in Chinese population from Shanxi and the T-allele of rs594631 in PRKACB was correlative with NTDs in male but not in female. Gender susceptibility may influence the correlation.

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Disclosure of conflict of interest

None.

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