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ORIGINAL RESEARCH

KRAS rs7973450 A>G increases neuroblastoma risk in Chinese children: a four-center case-control study

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Background: Neuroblastoma is one of the most common extracranial solid pediatric tumors. *KRAS* plays an important role in regulating cell proliferation, differentiation, and apoptosis. Single nucleotide polymorphisms (SNPs) in *KRAS* have been shown to modify susceptibility to multiple tumors, but no specific molecular epidemiology study was reported regarding neuroblastoma.

Methods: We conducted a four-center case-control study to explore the association between *KRAS* gene polymorphisms (rs12587 G>T, rs7973450 A>G, rs7312175 G>A) and neuroblastoma susceptibility with 505 Chinese children and 1070 matched controls.

Results: We found that rs7973450 A>G was associated with significantly increased neuroblastoma risk [GG vs. AA: adjusted odds ratio (OR)=4.26, 95% confidence interval (CI) =1.28–14.19, *P*=0.018; GG vs. AA/AG: adjusted OR=4.27, 95% CI=1.28–14.24, *P*=0.018]. The stratified analysis further demonstrated that rs7973450 GG genotype carriers had a higher risk to develop neuroblastoma in the subgroups of males, tumor originated from the adrenal gland and clinical stages III+IV.

Conclusions: Overall, our results suggested that rs7973450 A>G was associated with increased neuroblastoma risk.

Keywords: neuroblastoma, *KRAS*, polymorphism, susceptibility

Introduction

Neuroblastoma is one of the most common extracranial pediatric tumors, which accounts for 7–10% of pediatric tumors worldwide. It has a morbidity of 7.7 per million in China.[1,2] Neuroblastoma often occurs to children younger than 1-year-old, with an average diagnosis age of 17 months.[3] Neuroblastoma has diverse clinical phenotypes and its prognosis varies greatly. For instance, a fraction of neuroblastoma patients regress spontaneously. In contrast, about 50% of patients have high-risk neuroblastoma.[4] Despite the comprehensive treatment including surgery, chemotherapy, radiotherapy, and autologous stem cell transplantation, the five-year survival rate is still lower than 40% in high-risk neuroblastoma which accounts for 15% mortality of early childhood malignant tumor.[5] Moreover, survivors often sustainably suffer from chronic health problems and have a poor life quality.[6]

The effects of environmental exposures on neuroblastoma are not clear, such as medication, infection and parents' living habits. Significantly statistical result for the association between external factors and neuroblastoma is lacking.[7] With the development of genome-wide association studies (GWASs), more and more evidence indicated that genetic factors may predispose to neuroblastoma. Genetic differences in individuals mainly result from single nucleotide polymorphisms (SNPs).[8] *PHOX2B*[9] and *ALK*[10]

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mutations have been shown to contribute to familial neuroblastoma risk. Meanwhile sporadic neuroblastoma susceptibility is related to SNPs in *TP53*,[11,12] *CASC15*,[13] *MYCN*,[14] *BARD1*,[15,16] *LMO1*,[17] *XPG*,[18] *NEFL*,[19] *CDKN1B*,[20] and *HACE1* genes.[21] Although researchers have extensively explored the genetic etiology of neuroblastoma, many susceptibility genes remain to be determined.

RAS gene family consists of several oncogenes, including *KRAS, NRAS*, and *HRAS. KRAS* is the most frequently mutated gene in the *RAS* gene family, mutations in which have been discovered in 1/4 human tumors. K-Ras protein acts as a switch in the RAS-RAF-MEK-MAPK pathway so that regulates cell proliferation, differentiation, and apoptosis by transmitting extracellular signals to the nucleus.[22,23] *KRAS* mutations are considered as the first step in tumorigenesis.[24] Frequent mutations in codon 12 and 13 have been found in a wide spectrum of human tumors,[25] such as pancreatic cancer[24] and non-small cell lung cancer.[26] In recent years, the research focused on *KRAS* has gradually shifted to the regulation sequence. Polymorphisms in 3′ UTRs (rs61764370 T>G, rs712 T>G, rs1137282 A>G) and introns (rs12427141 G>A, rs7315339 T>C) have been observed to significantly modified the susceptibility to lung cancer,[27] ovarian cancer,[28] and triple-negative breast cancer.[29]

KRAS mutations have been found in some cases of primary and relapse neuroblastomas.[30–32] However, there is no orthodox molecular epidemiology study about *KRAS* and neuroblastoma. Considering the universal importance of the *KRAS* gene in tumorigenesis, we intended to explore the association between *KRAS* gene polymorphisms and neuroblastoma susceptibility in Chinese children.

Patients and methods

Study population

We performed a four-center case-control study, which involved 505 patients and 1070 healthy children as described previously (Table S1).[33] Briefly, patients were confirmed as new neuroblastoma cases by histopathological diagnosis. According to the INSS, patients were divided into clinical stages I, II (IIA, IIB), III, IV, and 4S.[34,35] A total of 1070 healthy children were randomly selected as controls from those who visited these four participating hospitals in the same period. Patients and controls were matching by age, gender, and ethnicity. To achieve relevant legal and ethical requirements, our study was approved by the Institutional Review Committee of four hospitals (the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, the First affiliated Hospital of Zhengzhou University, the Second Affiliated Hospital of Xi'an Jiaotong University, Guangzhou Women and Children's Medical Center). Our study was conducted following the Declaration of Helsinki, and participants or guardians were required to sign informed consent forms. Blood samples were obtained from cases before receiving radiotherapy or chemotherapy.

Genotyping

We screened potential function polymorphic sites in the *KRAS* gene by NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) and SNPinfo (http://snpinfo.niehs.nih.gov/snpfunc.htm).[36,37] *KRAS* rs12587 and rs7973450 were predicted to be located in the microRNA binding sites, while rs7312175 in a potential transcription factor binding site. As shown in Figure S1, there exists weak linkage disequilibrium (R^2<0.8) among rs12587, rs7973450 and rs7312175. The R^2=0.349 between rs12587 and rs7973450; R^2=0.447 between rs12587 and rs7312175; and R^2=0.015 between rs7973450 and rs7312175. TIANamp Blood DNA Kit (TianGen Biotech Co., Ltd., Beijing, China) was used to extract genomic DNA and TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) for genotyping.[38–40] To ensure the accuracy, reliability, and repeatability, our study was carried out in strict accordance with the instructions and no false-positive result was found in the negative control. Besides, 10% of samples were randomly selected for repeated experiments and the repeatable rate was 100%.

Statistical analysis

SAS release 9.1 (SAS Institute, Cary, NC, USA) was used for data analysis. Hardy-Weinberg equilibrium (HWE) in controls was estimated by a good-of-fit test. The differences in demographic characteristics and genotype distribution between cases and controls were detected by *t*-test and chi-square test, respectively. For adjusting age and gender, an unconditional multiple logistic regression model was taken to reveal the association between three polymorphisms and neuroblastoma susceptibility. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to be statistical indicators. Stratified analysis was performed by age, gender, tumor origin and clinical stages. All analyses were two-sided. P<0.05 was considered statistically significant.

Results

KRAS gene polymorphisms and neuroblastoma susceptibility

As revealed in Table 1, all of the three polymorphisms in controls conformed to the HWE ($P>0.05$). Moreover, we found that rs7973450 A>G was significantly associated with increased neuroblastoma risk (GG vs. AA: adjusted OR=4.26, 95% CI=1.28–14.19, $P=0.018$; and GG vs. AA/AG: adjusted OR=4.27, 95% CI=1.28–14.24, $P=0.018$) after adjusting for age and gender. Unfortunately, we failed to find a significant association with rs12587 G>T and rs7312175 G>A.

Stratification analysis

Compared with AA/AG genotypes carriers, the stratified analysis (Table 2) further revealed that rs7973450 GG genotype carriers had a higher risk to develop neuroblastoma in the strata of males (adjusted OR=10.75, 95% CI=1.25–92.61, $P=0.031$), tumor originated from adrenal gland (adjusted OR=6.16, 95% CI=1.52–24.94, $P=0.011$), and clinical stages III+IV (adjusted OR=4.19, 95% CI=1.03–17.02, $P=0.045$).

Discussion

KRAS is located in chromosome 12, coding a KRAS protein with GTPase activity. KRAS protein is activated by attaching to GTP and turned off right after converting the GTP to GDP. As a result, it transmits extracellular signals into the nucleus and regulates the cellular lifecycle of cells. According to previous reports, KRAS not only took part in the RAS-RAF-MEK-MAPK pathway,[41] but also unit PI3K to jointly activate mTOR. It indicated that KRAS is a key regulatory molecule to cellular growth and proliferation.[42] What's more, *KRAS* was associated with

Table 1 Association between *KRAS* gene polymorphisms and neuroblastoma risk

Genotype	Cases (N=505)	Controls (N=1070)	P^a	Crude OR (95% CI)	P	Adjusted OR (95% CI)[b]	P^b
rs12587 G>T (HWE=0.287)							
GG	330 (65.35)	688 (64.30)		1.00		1.00	
GT	146 (28.91)	333 (31.12)		0.95 (0.77–1.18)	0.653	0.94 (0.76–1.17)	0.591
TT	29 (5.74)	49 (4.58)		1.29 (0.80–2.05)	0.295	1.27 (0.79–2.02)	0.326
Additive			0.971	1.00 (0.84–1.20)	0.971	1.00 (0.83–1.20)	0.998
Dominant	175 (34.65)	382 (35.70)	0.685	0.96 (0.77–1.19)	0.686	0.95 (0.76–1.19)	0.661
Recessive	476 (94.26)	1021 (95.42)	0.321	1.27 (0.79–2.04)	0.322	1.26 (0.79–2.03)	0.331
rs7973450 A>G (HWE=0.080)							
AA	422 (83.56)	881 (82.34)		1.00		1.00	
AG	75 (14.85)	185 (17.29)		0.88 (0.66–1.16)	0.359	0.87 (0.66–1.16)	0.336
GG	8 (1.58)	4 (0.37)		**4.32 (1.30–14.40)**	**0.017**	**4.26 (1.28–14.19)**	**0.018**
Additive			0.994	1.00 (0.77–1.30)	0.994	1.00 (0.77–1.30)	0.983
Dominant	83 (16.44)	189 (17.66)	0.547	0.92 (0.69–1.22)	0.547	0.92 (0.69–1.22)	0.540
Recessive	497 (98.42)	1066 (99.63)	0.010	**4.29 (1.29–14.31)**	**0.018**	**4.27 (1.28–14.24)**	**0.018**
rs7312175 G>A (HWE=0.130)							
GG	395 (78.22)	851 (79.53)		1.00		1.00	
GA	102 (20.20)	201 (18.79)		1.12 (0.87–1.44)	0.393	1.11 (0.86–1.43)	0.431
AA	8 (1.58)	18 (1.68)		0.98 (0.42–2.26)	0.960	0.96 (0.41–2.22)	0.921
Additive			0.621	1.06 (0.84–1.33)	0.621	1.06 (0.84–1.33)	0.639
Dominant	110 (21.78)	219 (20.47)	0.549	1.08 (0.84–1.40)	0.549	1.08 (0.83–1.40)	0.564
Recessive	497 (98.42)	1052 (98.32)	0.887	0.94 (0.41–2.18)	0.888	0.93 (0.40–2.16)	0.871
Combined effect of risk genotypes[c]							
0	385 (76.24)	841 (78.60)		1.00		1.00	
1–2	120 (23.76)	229 (21.40)	0.293	1.15 (0.89–1.47)	0.293	1.14 (0.89–1.47)	0.303

Notes: The results were in bold, if the 95% CI excluded 1 or *P*-values less than 0.05. [a]χ^2 test for genotype distributions between neuroblastoma patients and cancer-free controls. [b]Adjusted for age and gender. [c]Risk genotypes were rs12587 TT, rs7973450 GG and rs7312175 GA/AA.
Abbreviations: OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium.

Table 2 Stratification analysis for association between *KRAS* gene genotypes and neuroblastoma susceptibility

Variables	rs12587 (case/control)		Adjusted OR[a]	*P*[a]	rs7973450 (case/control)		Adjusted OR[a]	*P*[a]	rs7312175 (case/control)		Adjusted OR[a]	*P*[a]	Risk genotypes (case/control)		Adjusted OR[a]	*P*[a]
	GG	GT/TT	(95% CI)		AA/AG	GG	(95% CI)		GG	GA/AA	(95% CI)		0	1–2	(95% CI)	
Age, month																
≤18	131/272	58/153	0.79 (0.55–1.14)	0.210	185/425	4/0	/	/	148/345	41/80	1.20 (0.78–1.82)	0.409	144/343	45/82	1.31 (0.87–1.98)	0.201
>18	199/416	117/229	1.06 (0.80–1.41)	0.665	312/641	4/4	2.06 (0.51–8.29)	0.310	247/506	69/139	1.01 (0.73–1.41)	0.933	241/498	75/147	1.05 (0.77–1.45)	0.755
Gender																
Female	129/283	84/165	1.12 (0.80–1.56)	0.515	208/447	5/1	**10.75 (1.25–92.61)**	**0.031**	165/354	48/94	1.10 (0.74–1.63)	0.647	160/350	53/98	1.18 (0.81–1.74)	0.387
Male	201/405	91/217	0.84 (0.62–1.13)	0.241	289/619	3/3	2.10 (0.42–10.49)	0.365	230/497	62/125	1.26 (0.76–1.50)	0.724	225/491	67/131	1.11 (0.79–1.55)	0.549
Sites of origin																
Adrenal gland	104/688	69/382	1.17 (0.84–1.62)	0.364	169/1066	4/4	**6.16 (1.52–24.94)**	**0.011**	135/851	38/219	1.06 (0.72–1.57)	0.757	131/841	42/229	1.15 (0.79–1.68)	0.477
Retroperitoneal	104/688	43/382	0.76 (0.52–1.10)	0.147	146/1066	1/4	1.99 (0.22–18.18)	0.541	113/851	34/219	1.18 (0.78–1.79)	0.425	111/841	36/229	1.21 (0.81–1.81)	0.362
Mediastinum	90/688	45/382	0.90 (0.61–1.31)	0.577	133/1066	2/4	4.23 (0.76–23.52)	0.099	108/851	27/219	0.98 (0.62–1.53)	0.920	105/841	30/229	1.06 (0.69–1.63)	0.803
Others	26/688	16/382	1.12 (0.60–2.12)	0.718	41/1066	1/4	7.05 (0.77–65.05)	0.085	33/851	9/219	1.08 (0.51–2.30)	0.838	32/841	10/229	1.17 (0.57–2.43)	0.667
Clinical stages																
I+II+4s	166/688	84/382	0.91 (0.68–1.22)	0.533	247/1066	3/4	3.30 (0.73–14.88)	0.121	194/851	56/219	1.13 (0.81–1.57)	0.484	190/841	60/229	1.16 (0.84–1.61)	0.359
III+IV	147/688	85/382	1.01 (0.75–1.37)	0.928	228/1066	4/4	**4.19 (1.03–17.02)**	**0.045**	182/851	50/219	1.04 (0.73–1.47)	0.840	177/841	55/229	1.11 (0.79–1.55)	0.554

Notes: The results were in bold, if the 95% CI excluded 1 or *P*-values less than 0.05. [a]Adjusted for age and gender, omitting the corresponding stratify factor.
Abbreviations: OR, odds ratio; CI, confidence interval.

persistent inflammatory responses.[43] Point mutations in *KARS* coding regions (such as codon 12, 13) constitutively activated K-Ras protein by increasing GDP/GTP exchange rate or decreasing the GTPase activity.[44] It continuously activating the relevant pathways and providing a favorable tumor microenvironment for tumor cell growth, survival, invasion, and spread. Instead of altering amino acids or structures, SNPs in *KRAS* regulatory sequences tended to change *KRAS* expression. Most of them were highly conserved in evolution. This portended that they would play an indispensable regulating effect on different gene expression processes.[45,46] A study involving 77 samples have reported that *NARS* point mutations were found in 3 samples and no point mutations were found in *HRAS* and *KRAS*.[32] Recently, *KRAS* pGly13Asp and missense mutation were observed in some cases of primary and relapse neuroblastomas.[30,31]

In this study, we draw a conclusion that rs7973450 A>G significantly increased neuroblastoma risk. Unfortunately, we failed to find rs12587 G>T and rs7312175 G>A modified neuroblastoma susceptibility. Interestingly, in our previous study, rs12587 G>T was associated with increased nephroblastoma risk, but rs7973450 A>G and rs7312175 G>A did not modify nephroblastoma susceptibility.[36] Beside, Dai et al did not observe any association between rs12587 G>T and colorectal cancer,[47] but Wang et al presented that rs7312175 G>A was associated with the recurrence and local dose combination therapy of endometrial cancer.[48]

The rs7973450 was predicted to serve as an important microRNA binding site. Its polymorphism would induce neuroblastoma by abnormally regulating the expression of *KRAS* and related microRNA.[49] Moreover, whether the rs12587 and rs7312175 polymorphisms would be the risk factors for neuroblastoma need further validate.

Take *Let-7* complementary sites 6 (LCS6) for example. Chin et al found that *KRAS* rs61764370 T>G increased non-small cell lung cancer risk.[27] Further, a double luciferase reporting experiment revealed that rs61764370 T>G weakened the inhibitory effect of *KRAS* 3′ UTR by destroying its affinity with Let-7 microRNAs, which resulted in increased K-Ras portent and decreased Let-7 microRNAs. Smits et al indicated that the *LCS6* variant seemed to be a primary protective factor for early-stage colorectal cancer susceptibility and prognosis, but it did not associate with advanced colorectal cancer.[50] In metastatic colorectal cancer, although American patients treated with cetuximab had a better response rate and prognosis when carried G allele,[51] an opposite result was observed in the Italians.[52] In recently, G allele carriers were shown to increase the risk of chronic myeloid leukemia[53] and triple-negative breast cancer.[29] However, whether the *LCS6* polymorphism was related to ovarian cancer was uncertain.[28,54]

Based on the appeal examples, there are several reasons why rs7973450 A>G and rs7312175 G>A could not significantly affect neuroblastoma susceptibility in this research. First, the same polymorphism may play different roles in different tumor types, ethnicities and clinical characteristics. Second, one SNP alone was not strong enough to cause tumor, the combination of multiple SNPs might play a significant role in carcinogenesis.[55] Tonini et al summarized the neuroblastoma susceptibility alleles reported lately, and found that most of neuroblastoma-associated SNPs are located in the genes that were involved in maintaining the chromatin and mitosis integrity. They speculated that the cumulative effect of SNPs could lead to chromosome instability and structural damages during the early embryonic life.[56] Moreover, environmental exposure and parents' poor living habits might be confusing factors in the association analyze. Finally, the varying incidence rate of the same polymorphism locus in different populations would change the requirement of sample size to detect the real associattion.[27] It suggests that we should expand sample size, combine other SNPs and control confounders to explore the association between *KRAS* polymorphisms and tumors in multiple ethnicities. As a case-control study basing hospital, this study inevitably had hospitalization bias.

Conclusion

In conclusion, with a four-center case-control study in Chinese children, we found the association between neuroblastoma susceptibility and *KRAS* polymorphisms. Our study suggested that *KRAS* rs7973450 A>G significantly increased neuroblastoma risk. The results needed further investigation.

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Disclosure

The authors report no conflicts of interest in this work.

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