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Research Paper

Functional Polymorphisms in *BARD1* Association with Neuroblastoma in a regional Han Chinese Population

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Abstract

Neuroblastoma (NB) is a sympathetic nervous system cancer for children, occupying approximately 15% of pediatric oncology deaths. BARD1, a tumor suppressor, is essential for genome stability by interaction with BRCA1. Here, we performed a systematic investigation for the association between SNPs in *BARD1* and the risk of NB in Chinese population. After SNP screening in *BARD1* gene, we performed case-control study of eleven selected SNPs in *BARD1* with 339 NB patients and 778 cancer-free controls. The OR and 95% CI of these candidate SNPs were computed by logistic regression. After adjusted gender and age, seven out of eleven SNPs in *BARD1* were significant associated with the risk of NB, including one SNP in 5'-UTR (rs17489363 G > A), two SNPs in exon (rs2229571 G > C and rs3738888 C > T), and four SNPs in intron (rs3768716 A > G, rs6435862 T > G, rs3768707 C > T and rs17487792 C > T). When stratified by the INPC, primary tumor site and the INSS, these seven SNPs were significant associated with GNB/NB, stage III/IV and adrenal origin of NB. Dual-luciferase reporter assay showed rs17489363 A allele-containing haplotypes (TAC, CAC, TAG and CAG), composed with rs34732883 T > C, and rs1129804 C > G, dramatically reduced the transcriptional activity of reporter gene. The major of our study showed that seven SNPs of *BARD1* associated with increased NB risk in Chinese population, and four haplotypes could reduce transcription activity of *BARD1*.

Key words: Neuroblastoma; Single nucleotide polymorphism; *BARD1*; Case-control study

Introduction

Neuroblastoma (NB) is the most common type of pediatric extracranial cancer, accounting for approximately 15% of all cancer deaths in children [1, 2]. It could arise in any part of sympathetic nervous system, especially in adrenal medulla and abdomen [3]. The clinical presentation is highly variable, ranging from local invasion and distant metastasis [4]. Although advanced immunotherapy and multiple cytotoxic therapies are applied, the survival rate of high-risk NB remains below 50% [4, 5]. However, the complex pathogenesis of NB is still not well understood. Therefore, it is of great significance to explore the molecular mechanism of NB in order to accelerate the development of novel drugs and therapy methods.

Although only about 1% of NB patients are heredofamilial, accumulating evidences indicate the complicated genomic abnormalities play significant roles in NB heterogeneity [6, 7]. To recognize tumor genetic variations, genome-wide association studies

(GWASs) are considered as a powerful and hypothesis-free method [8]. Based on GWAS results, multiple genetic variations associated with NB risk have been identified, such as *ALK*, *FLJ22536*, *FLJ44180*, *BARD1*, *LMO1*, *HACE1* and *LIN28B*[9-15]. The earliest GWAS on NB was performed by Maris and colleagues in 2008, including 1032 cases and 2043 controls in European American, which validated that chromosome 6p22 locus was associated with clinically aggressive NB[10]. In high-risk NB, they further found six SNPs were associated with NB susceptibility in *the BRCA1 associated RING domain 1 (BARD1)* gene [11]. Moreover, these *BARD1* SNPs has been confirmed in both Italians [16] and African-Americans [17]. In Chinese population, three *BARD1* SNPs have been studied in NB, but only stratified analysis demonstrated that rs3768716 and rs6435862 were risk factors for aggressive NB [18]. As a result, further systematic research and functional assessment of SNPs in *BARD1* gene need to be performed. In addition, identifying SNPs in Chinese population is informative and it can explore potential ethnic differences and strengthen the role of *BARD1* as locus of NB susceptibility.

Herein, we performed a systematic screening of the SNPs in *BARD1* and investigated the function of SNPs located in functional regions such as 5′-UTR and exon. We selected eleven candidate SNPs in *BARD1* gene and carried out a case-control study in a total of 339 NB patients and 778 cancer-free controls. Association analysis indicated that seven SNPs contributed to NB risk. Functional evaluation of SNPs in 5′-UTR and exon region revealed that rs17489363 G > A might influence *BARD1* promoter activity in four haplotypes. The major aim of our study was to discover the association between *BARD1* SNPs and NB susceptibility, and further explore the precise molecular mechanism of the functional SNPs.

Methods and Materials

Ethics statement

This study was approved by the Ethics Committee of the Beijing Children's Hospital, Capital Medical University (Beijing, China). The written informed consents have been obtained from participants and/or their legal guardians involved in this study prior to inclusion in the study.

Subjects and clinical information

Volunteers were enrolled from January 2004 to December 2016 at Beijing Children's Hospital, which is the National Center for Children's Health (NCCH). A total of 339 NB patients and 778 cancer-free controls were recruited. Even the subjects were from all over the country, more than half of them were from Northern of China. To avoid minority differences of genetic background, all the volunteers in our research were Han Chinese children. According to the International Neuroblastoma Pathology Classification (INPC) [19], we stratified cases into three pathological groups, namely Neuroblastoma (NB), Ganglioneuroma (GN) and Ganglioneuroblastoma (GNB). In addition, the tumor stage was classified based on the International Neuroblastoma Staging System (INSS) by two professional pathologists. All controls were health children, admitted by physical examination center of Beijing Children's Hospital. All recruited volunteers were collected EDTA-anticoagulated blood samples for this research, and stored at -80°C.

SNPs screening

We used computer-facilitated strategy to select candidate SNPs in *BARD1* gene. The following steps were operated (Figure 1). Firstly, candidate SNPs were filtered by using the Haploview (http://www.broad.mit.edu/mpg/haploview/) with selection criteria of minor allele frequency (MAF) > 0.05 and r^2 values > 0.8 from Han Chinese in Beijing (CHB) in the 1000 Genomes database (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). The candidate SNPs screening region extended to a 2 kb (1kb upstream and 1kb downstream the gene). Secondly, to identify functional SNPs in *BARD1*, we excluded synonymous mutations and unconfirmed intron SNPs.

Sample preparation and genotyping

DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract Genomic DNA from 300μL peripheral blood of all samples, and then the DNAs were stored at -20°C until use. By using the SequenomMassArray platform, SNP genotyping was performed following the manufacturer's protocol (Sequenom, San Diego, USA) [13, 20].

Cell culture

Human embryonic kidney cell line (293T) and human neuroblastoma cell line (SH-SY5Y) were obtained from Cell Resource Center, Chinese Academy of Medical Sciences (CAMS, Beijing, China). The cells were cultured in DMEM supplemented with 10% fetal bovine serum, in a humidified, 5% CO_2 incubator at 37°C.

Plasmid construction

Three SNPs (rs34732883 T > C, rs17489363 G > A and rs1129804 C > G) located in 5′-UTR of *BARD1*. For these SNPs, a GV238 reporter (Genechem, Shanghai, China) plasmid encompassing 5′-UTR and truncated promoter region of *BARD1* was constructed,

designated as pT-G-C according to sequence analysis. Subsequently, this pT-G-C was used as a template, 5'-UTR site-specific mutagenesis in rs34732883 T > C, rs17489363 G > A and rs1129804 C > G were performed to generate other constructs containing all of the possible haplotypes, namely pC-G-C, pT-G-G, pC-G-G, pT-A-C, pC-A-C, pT-A-G and pC-A-G (Figure 2A).

The exon SNP rs1048108 located near zinc finger ring region, which played a key role in the interaction of BARD1 and Breast cancer 1 (BRCA1). To explore the function of rs1048108, *BARD1* ORF expression plasmid (pCMV3-Myc-*BARD1*) was obtained from Sino Biological lnc (Beijing, China). This plasmid with rs1048108 C allele was named as BARD1-Myc-CC. Then, site-specific mutagenesis was performed to creating single nucleotide mutation in rs1048108 C > T, named as BARD1-Myc-TT accordingly. All constructs were sequenced to confirm their authenticity.

Daul-luciferase reporter assay

Total of 1×10^5 cells were plated in 24-well plates (Corning, NY, USA) and grew to 80-90% confluence for transient transfection with Lipofectamine 2000 Reagent (Life Technologies, Inc., Rockville, USA). Cells were then transfected with 50 ng/well of reporter plasmid. Identically, Renilla luciferase reporter plasmid (10 ng/well) was co-transfected for standardization of the transfection efficiencies. Luciferase activity was measured by the Dual-Luciferase Reporter Assay system (Promega, Madison, USA) on a microplate reader (CLARIOstar, BMG labtech, Germany) according to the manufacturer's protocol. Results were normalized by Renilla activity, and labeled as relative luciferase activity. Three independent transfection experiments were performed, and each was done in triplicate.

Co-immunoprecipitation (Co-IP) and Western blotting

To study the interaction between BARD1 and BRCA1, we obtained the human BRCA1 ORF expression plasmid (pcDNA3-HA-BRCA1) from Sino Biological lnc (Beijing, China). Co-IP was performed as follows. Briefly, 293T cells were co-transfected with plasmid containing *BRCA1* gene (pcDNA3-HA-BRCA1) and plasmid containing different genotype *BARD1* gene (BARD1-Myc-CC or BARD1-Myc-TT) for 48 h. Proteins were then extracted with RIPA cell lysis buffer (Dingguo, Beijing). Total cell protein extracts were incubated with anti-HA antibody (Abcam, UK) overnight at 4°C followed by immunoprecipitation with protein A agarose beads (Roche, Switzerland). The beads were washed, collected and resolved in prechilled RIPA lysis buffer for six times. Finally, the protein-agarose complexes were boiled for SDS-PAGE. Immunoblots were carried out according to the standard operating procedure. The relative densities of the desired protein bands were quantified by Image Lab software (Bio-Rad, USA).

Statistical Analysis

A two-sided $\chi2$ test was performed to describe the distribution of the age and gender between cases and controls, as well as the deviation of genotypes. All samples were detected for Hardy-Weinberg equilibrium by Goodness-of-fit $\chi2$ test. In order to get an exact result, we analyzed the association between the polymorphisms and NB susceptibility by using the multivariate logistic regression analysis to assess Odds ratios (ORs) and 95% Confidence intervals (95%CIs) with age and gander as covariates. The *P* value below 0.05 from either model was considered statistically significant. Bonferroni test was carried out to adjust for multiple comparisons in the single SNP analysis ($P < 0.05/n$, n=11). All statistical analyses described above were computed using SAS software (version 9.1; SAS Institute, Cary, NC). Haploview 4.2[21] was used to measure the LD and the haplotype.

Results

Clinical characteristics

To analyze the association between NB risk and *BARD1* SNPs, 339 cases and 778 controls were recruited in Beijing Children's Hospital from January 2004 to December 2016 (Table 1). Two groups were matched with gender and age ($P > 0.05$). Pathological types classified by INPC were categorized into NB (240, 70.80%), GN (33, 9.73%), and GNB (59, 17.40%). According to INSS, the case group was divided into stage I (45, 13.27%), II (59, 17.40%), III (99, 29.20%), IV (109, 32.15%) and IVs (24, 7.08%). Besides, among these 339 cases, 152 (44.84%) of the primary tumors located in adrenal gland and 171 (51.33%) developed from non-adrenal regions including abdominal/ retroperitoneal region (137, 40.41%), pelvic cavity (20, 5.90%), and other sites (14, 4.13%). The remaining 13 (3.83 %) cases were not classified into a specific region.

Identification of SNPs

Based on the screening strategy (Figure 1), eleven candidate SNPs were selected for further study, including three SNPs in 5'-UTR (rs34732883, rs17489363 and rs1129804), three SNPs in exon (rs2229571, rs3738888 and rs1048108), and five SNPs

in intron (rs3768716, rs6435862, rs3768707, rs17487792 and rs7587476) (Table 2).

Table 1. Distribution of Subject Characteristics

	Case (N=339)		Controls (N=778)		*P**
	No.	(%)	No.	(%)	
Sex					
Male	194	57.23	453	58.23	0.76
Female	144	42.48	325	41.77	
Unknown	1	0.29	0	0.00	
Age					
≤12months	64	18.87	144	18.51	0.91
>12months	275	81.13	633	81.36	
Unknown	0	0.00	1	0.13	
Tumor stage[a]					
I	45	13.27			
II	59	17.40			
III	99	29.20			
IV	109	32.15			
IVs	24	7.08			
Unknown	3	0.88			
Categories[b]					
GN	33	9.73			
GNB	59	17.4			
NB	240	70.80			
Unknown	7	2.06			
Primary tumor sites					
Adrenal	152	44.84			
Abdominal	137	40.41			
Pelvic	20	5.90			
Others	14	4.13			
Unknown	13	3.83			

*Two-sided χ^2 test. [a] According to INSS. [b] According to INPC.

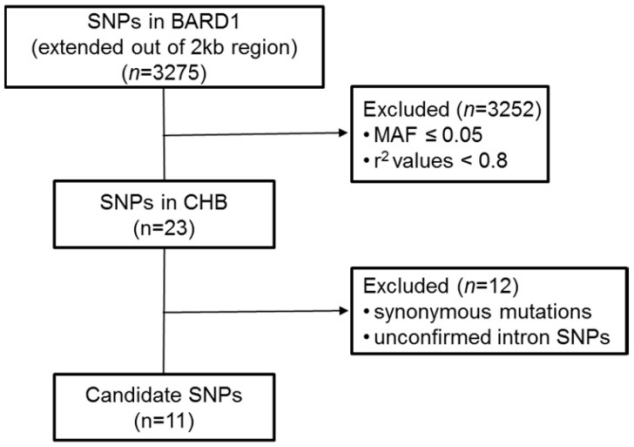

Figure 1. The schematic for the strategy of SNP selection.

Since linkage disequilibrium (LD) represented correlating genotypes between SNPs at different loci [22], the pair-wise LD between these eleven SNPs were measured by Haploview software. The disequilibrium coefficient D' and r^2 were represented of the proportion or the maximum possible disequilibrium. The results showed that three SNPs in 5′-UTR were in strong LD in our study population, with a D' of 0.99 (r^2 = 0.97) for rs34732883 and rs1129804 (P < 0.001), a D' of 1.00 (r^2 = 0.98) for rs34732883 and rs17489363 (P < 0.001), and a D' of 1.00 (r^2 = 0.99) for rs17489363 and rs1129804 (P < 0.001). In exon, rs2229571 and rs1048108 also showed strong LD (D' = 0.88, r^2 = 0.7, P < 0.001) (Supplement Figure 1).

Table 2. Basic Information of Potential *BARD1* SNPs

#	Identity	Position(GRCh37)	Position	Base change	MAF[a]
1	rs17489363	Ch2:214809617	5′-UTR	G>A	0.18
2	rs34732883	Ch2:214809647	5′-UTR	T>C	0.18
3	rs1129804	Ch2:214809599	5′-UTR	C>G	0.18
4	rs2229571	Ch2:214780740	exon	G>C	0.34
5	rs3738888	Ch2:214730440	exon	C>T	0.04
6	rs1048108	Ch2:214809500	exon	C>T	0.37
7	rs3768716	Ch2:214771070	intron	A>G	0.15
8	rs6435862	Ch2:214807822	intron	T>G	0.09
9	rs3768707	Ch2:214780411	intron	C>T	0.23
10	rs17487792	Ch2:214778776	intron	C>T	0.14
11	rs7587476	Ch2:214789163	intron	C>T	0.23

Abbreviation: MAF, minor allele frequency.
[a] Data from CHB population in 1000 Genomes project

Table 3. Associations between *BARD1* SNPs and NB Risk

	Cases(N=339)		Controls(N=778)		*P*	Adjusted OR[a] (95%CI)
	No.	(%)	No.	(%)		
rs17489363						
GG	206	61.0	529	68.9		1.00
GA	114	33.7	217	28.3	0.03	1.26 (0.95-1.68)
AA	18	5.3	22	2.9	0.035	2.04 (1.06-3.92)
rs34732883						
TT	197	61.2	504	68.3		1.00
CT	109	33.9	212	28.7	0.082	1.24(0.93-1.66)
CC	16	5.0	22	3.0	0.088	1.81(0.93-3.56)
rs1129804						
CC	135	39.8	302	39.8		1.00
CG	163	48.1	254	46.6	0.84	1.08 (0.81-1.43)
GG	42	12.1	103	13.6	0.78	0.98(0.64-1.51)
rs2229571						
CC	67	20.0	232	30.1		1.00
CG	164	49.0	370	48	<0.0001	1.74 (1.24-2.44)
GG	104	31.0	169	21.9	<0.0001	1.84 (1.37-2.49)
rs3738888						
CC	323	95.3	749	97.7		1.00
CT	16	4.7	18	2.4	0.041	2.17 (1.04-4.50)
TT	-	-	-	-	-	-
rs1048108						
CC	147	43.6	307	39.8		1.00
CT	154	45.7	369	47.9	0.60	0.93(0.70-1.23)
TT	36	10.7	95	12.3	0.39	0.83(0.55-1.27)
rs3768716						
AA	211	63.0	555	71.8		1.00
AG	111	33.1	200	25.9	0.014	1.49 (1.12-1.99)
GG	13	3.9	18	2.3	0.26	1.53 (0.73-3.21)
rs6435862						
TT	232	68.8	594	76.9		1.00
GT	96	28.5	168	21.8	0.029	1.40(1.04-1.90)
GG	9	2.7	10	1.3	0.13	2.05(0.81-5.18)
rs3768707						
CC	183	54.6	499	64.4		1.00
CT	132	39.4	242	31.4	0.013	1.45(1.10-1.92)
TT	20	6.0	31	4.0	0.18	1.73(0.95-3.15)
rs17487792						
CC	209	64.7	545	72.4		1.00
CT	104	32.2	192	25.5	0.04	1.45(1.08-1.95)
TT	10	3.1	16	2.1	0.52	1.31(0.58-2.95)
rs7587476						
CC	179	54.9	343	59.5		1.00
CT	126	38.6	206	35.8	0.59	1.04(0.77-1.41)
TT	21	6.4	27	4.7	0.32	1.37(0.74-2.56)

[a]Data were calculated by logistic regression analysis with adjustment for sex and age as covariate

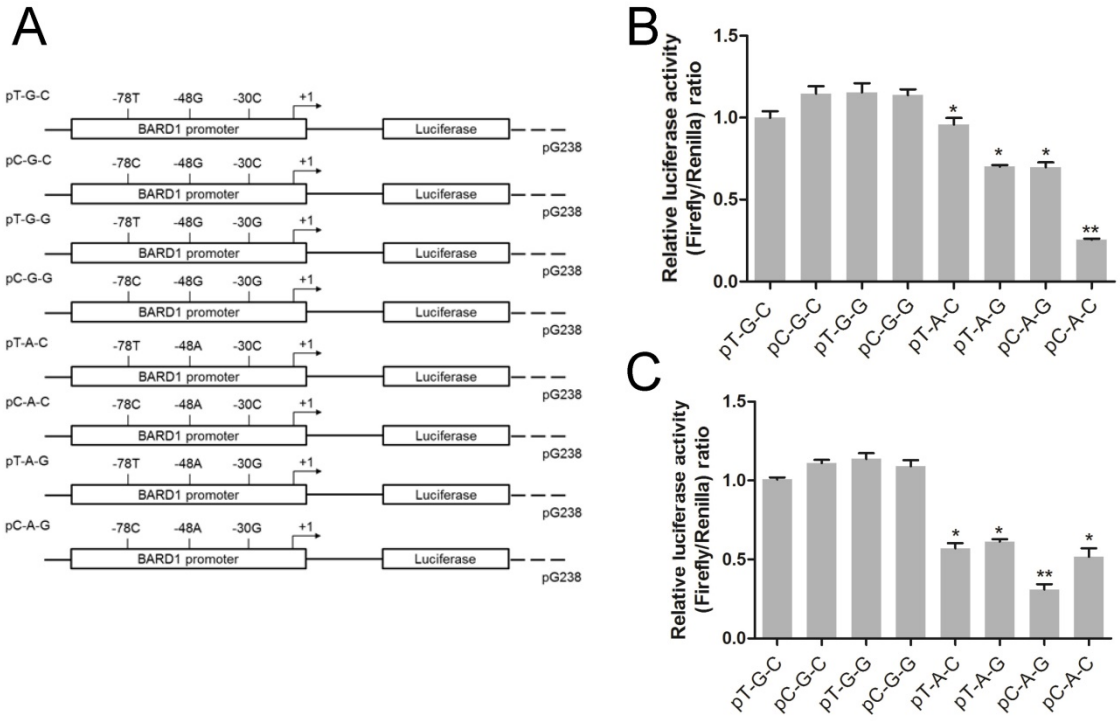

Figure 2. Dual-luciferase reporter assay with constructs containing *BARD1* promoter. (A) Schematic of reporter gene constructs having the *BARD1* promoter, with different haplotypes containing three SNPs in *BARD1* 5'-UTR (rs34732883, rs17489363 and 1129804). 293T cells (B) and SH-SY5Y (C) were co-transfected with 50 ng of the reporter plasmid and 10 ng of Renilla luciferase reporter plasmid for standardization of the transfection efficiencies. The luciferase activity was expressed as Mean ± S.E. of the ratio of firefly expression of *BARD1* promoters. (*) indicate $P < 0.05$ with respect to the general (without stimulation). Abbreviation: NC, negative control.

Effect of *BARD1* SNPs on NB Risk

To explore the effect of eleven candidate SNPs on NB risk, the genotype differences were compared between 339 NB cases and 778 controls. By multivariate logistic regression analysis, we found that seven SNPs were significantly associated with NB risk (Table 3), with one in 5′-UTR (rs17489363 G > A), two in exon (rs2229571 G > C and rs3738888 C > T), and four in intron (rs3768716 A > G, rs6435862 T > G, rs3768707 C > T and rs17487792 C > T). However, the relationship between different haplotypes and NB risk were not detected (data not shown).

Stratification analysis of identified SNPs

For further estimating the association between seven identified SNPs and NB, we stratified the cases according to tumor categories, tumor stages and primary tumor sites. As shown in Supplementary Table 1, six SNPs were associated with malignant type GNB/NB rather than mature type GN. With regards to tumor stages, NB in stage III was related to rs3768707 C > T and rs3768716 A > G, while NB in stage IV was related to rs2229571 C > G, rs6435862 T > G, rs17487792 C > T and rs3768716 A > G. Based on tumor location, all the SNPs showed significant association with primary NB located in adrenal. These results indicated that the seven SNPs in *BARD1* might contribute to NB aggressiveness.

Effect of identified SNPs on *BARD1* transcriptional activity

We have confirmed that rs17489363 G > A in 5′-UTR was a risk SNP and it had strong LD with rs34732883 T > C and rs1129804 C > G, therefore the potential haplotypes should be considered in *BARD1* transcriptional activity. Thus, the Dual-luciferase reporter assay was performed. Compared to reporter gene expressions driven by the rs17489363 G allele *BARD1* promoters, all of rs17489363 A allele-containing counterparts showed 1.2- to 4- fold lower transcriptional activity both in 293T and SH-SY5Y cells ($P < 0.05$, Figure 2B & 2C). The results suggested that rs17489363 G > A may play a major function on *BARD1* promoter activity in these haplotypes.

The function of SNPs in *BARD1* exon

To explore the function of SNPs in BARD1 exon, we first overviewed the protein structure of BARD1. According to UniProt database (http://www.uniprot.org/), BARD1 had a BRCA1-interaction region (M26-E119), three ANK repeats (R427-P546) and two BRCT1 domains (M560-I653 and L667 -S777) (Figure 3A). The main function of BARD1 was to interact with BRCA1 via the zinc finger ring region and rs1048108 C > T (P24S) located near the domain. Moreover, rs1048108 had prominent LD with

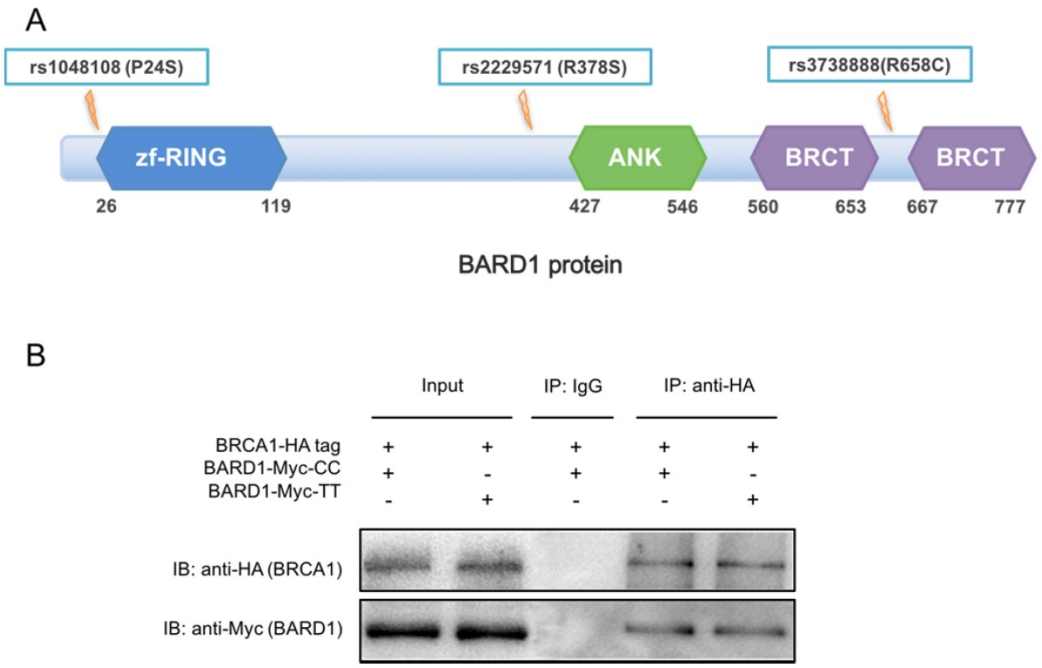

Figure 3. (A) The BARD1 structure. The amino acid mutation caused by rs1048108, rs2229571 and rs3738888 in BARD1 were indicated. Rs1048108 (P24S) located near zinc finger ring region, which played a key role in the interaction of BARD1 and BRCA1. (B) Effect of the rs1048108 C or T allele on BARD1-BRCA1 interaction. Co-IP result showed no difference in BARD1-BRCA1 interaction between wild type and mutant type of rs1048108 C > T (P24S).

rs2229571 (D′=0.9, r^2=0.7), suggesting potential correlating between these two SNPs (Supplementary Figure 1). Even rs2229571 (R378S) and rs3738888 (R658C) were risk SNPs, their sites were away from the functional domain. Thus, we chose rs1048108 C > T as the representative SNP for functional study of BARD1 protein. Therefore, we performed Co-IP to assess if rs1048108 could directly affect BARD1-BRCA1 interaction. As shown in Figure 3B, BARD1 protein containing rs1048108 T allele did not show any difference in BRCA1 binding capacity compared to the protein containing the rs1048108 C allele. This Co-IP result suggested that rs1048108 C > T (P24S) has no detectable influence on the BARD1/BRCA1 heterodimer.

Discussion

Neuroblastoma is the most common pediatric extracranial solid tumor and causes increasing concern on pediatrics due to its malignancy. Several studies have revealed that genetic factors could raise NB risk [9-15]. *BARD1*, as an important tumor suppressor gene [23], has been verified significantly associated with NB susceptibility in different ethnicities [11, 16, 17, 24]. However, the significance of *BARD1* SNPs in Chinese population is far from clear. Thus, the present study was performed to screen significant SNPs in the whole length of *BARD1* in Chinese population. Herein, seven risk SNPs for NB were identified and four rs17489363 A allele-containing haplotypes (composed with rs34732883 T > C and rs1129804 C > G) were demonstrated to affect transcriptional activity of *BARD1* gene.

The seven identified SNPs included one in 5′-UTR (rs17489363 G > A), two in exon (rs2229571 G > C and rs3738888 C > T), and four in intron (rs3768716 A > G, rs6435862 T > G, rs3768707 C > T and rs17487792 C > T). Previous studies have demonstrated that rs17489363, rs3768716 and rs6435862 contributed to NB risk [11, 16, 17, 24]. The study in South Chinese population reported that subjects carrying risk alleles of rs3768716 or rs6435862 had a significant trend on NB developing in clinical stage III/IV patients [18]. However, the significant association was failed to be found in total subjects. In our study, significant association appeared in both total subjects and stratification analysis, the larger sample size might contribute to the difference (339 cases and 778 controls in our study vs 201 cases and 501 controls in the study on South Chinese population). Consistent with our results, Capasso et al. also elicited that rs3768716 and rs6435862 polymorphisms were the most significant variants that associated with NB risk in European ancestry [11]. What's important is that a novel SNP rs3738888 was identified associated with NB risk in our study, which was not reported before in European, American African, Italian or Chinese [11, 16, 17, 24].

Functional studies of identified SNPs were further analyzed according to their locations. Among the seven identified SNPs, SNP rs17489363 G > A was

located in 5' -UTR of *BARD1*. By using *in silico* approach, previous study found that rs17489363 might alter the transcriptional binding site of *BARD1* [25], and the latest study clarified this perspective *in vitro* [24]. In the present study, we further confirmed the influence of rs17489363 on *BARD1* transcriptional activity. Due to rs17489363 was in strong LD with rs34732883 and rs1129804 and LD represented correlating genotypes between these SNPs, the functions of different genotypes should be concerned. Compared to rs17489363 G allele-containing counterparts, the four rs17489363 A allele-containing haplotypes (composed with rs34732883 T > C and rs1129804 C > G) were shown to reduce the transcriptional activity of *BARD1* gene. As a result, haplotypes containing rs17489363 A might play a key role in *BARD1* promoter activity.

The BARD1's function is also related to protein structure. BARD1 interacts with BRCA1 via the zinc finger ring region [26-28] and to enhance the stability of BRCA1 [29]. According to UniProt database, rs1048108 C > T (P24S) located near the domain. Even rs2229571 (R378S) and rs3738888 (R658C) were found associated with NB susceptibility, their sites were away from the interaction region. Moreover, rs1048108 C > T is in prominent LD with rs2229571 G > C, which suggested potential correlating between these two SNPs. However, our result suggested that rs1048108 C > T (P24S) has no detectable influence on the BARD1/BRCA1 heterodimer.

For SNPs located in intron region, four SNPs (rs3768716 A > G, rs6435862 T > G, rs3768707 C > T and rs17487792 C > T) were significantly associated with NB susceptibility. The same results were also obtained in European ancestry [11]. Moreover, previous study has reported that NB cells with the rs6435862 G risk allele could significantly increase the expression of one of *BARD1* splice variant, *BARD1*β [30]. Since the splice variant *BARD1*β was an oncogenic driver of high-risk NB tumorigenesis, the G risk allele may participant in the development of NB risk through increasing *BARD1*β expression [30].

Several limitations of this study should be mentioned. Firstly, all of the participants were recruited from Beijing Children's Hospital, including 339 cases and 778 controls. However, most of our patients were lived in the North of China. Although we have adjusted age and gender as the concomitant variable, we couldn't ignore the existent bias of participants' born places. Secondly, as there were eight haplotypes composed with rs34732883, rs17489363 and 1129804, the sample size of per group was small. Thus, the statistical power maybe limited. Therefore, we observed strong effects on *BARD1* transcriptional activity *in vitro*, but found weak relationship between haplotypes and NB risk in SNP analysis. Thirdly, we did not complete 3-years' follow-up for monitoring far-reaching influence. In our future researches, both biological and clinical approaches would be considered and the sample size would be enlarged.

Conclusion

In conclusion, our study revealed the relationship between identified SNPs in *BARD1* and NB susceptibility by systematically whole genetic scanning in Chinese population. Seven identified SNPs in *BARD1* significantly contributed to NB risk. In addition, four rs17489363 A allele-containing haplotypes in 5'-UTR could reduce the promoter activity of *BARD1 in vitro.* These findings have provided novel clues to investigate the genetic mechanism of NB.

Supplementary Material

Supplementary figures and tables.
http://www.jcancer.org/v10p2153s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

References

1. Maris J M, Hogarty M D, Bagatell R, et al. Neuroblastoma. Lancet. 2007; 369:2106-20.
2. London W B, Castleberry R P, Matthay K K, et al. Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. J Clin Oncol. 2005; 23:6459-65.
3. Deyell R J and Attiyeh E F. Advances in the understanding of constitutional and somatic genomic alterations in neuroblastoma. Cancer Genet. 2011; 204:113-21.
4. Maris J M. Recent advances in neuroblastoma. N Engl J Med. 2010; 362:2202-11.
5. Yu A L, Gilman A L, Ozkaynak M F, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med. 2010; 363:1324-34.
6. Matthay K K, Maris J M, Schleiermacher G, et al. Neuroblastoma. Nat Rev Dis Primers. 2016; 2:16078.
7. Capasso M and Diskin S J. Genetics and genomics of neuroblastoma. Cancer Treat Res. 2010; 155:65-84.
8. Pandey J P. Genomewide association studies and assessment of risk of disease. N Engl J Med. 2010; 363:2076-7; author reply 2077.
9. Mosse Y P, Laudenslager M, Longo L, et al. Identification of ALK as a major familial neuroblastoma predisposition gene. Nature. 2008; 455: 930-5.
10. Maris J M, Mosse Y P, Bradfield J P, et al. Chromosome 6p22 locus associated with clinically aggressive neuroblastoma. N Engl J Med. 2008; 358:2585-93.
11. Capasso M, Devoto M, Hou C, et al. Common variations in BARD1 influence susceptibility to high-risk neuroblastoma. Nat Genet. 2009; 41:718-23.
12. Nguyen le B, Diskin S J, Capasso M, et al. Phenotype restricted genome-wide association study using a gene-centric approach identifies three low-risk neuroblastoma susceptibility Loci. PLoS Genet. 2011; 7(e):1002026.
13. Wang K, Diskin S J, Zhang H, et al. Integrative genomics identifies LMO1 as a neuroblastoma oncogene. Nature. 2011; 469:216-20.

14. Diskin S J, Capasso M, Schnepp R W, et al. Common variation at 6q16 within HACE1 and LIN28B influences susceptibility to neuroblastoma. Nat Genet. 2012; 44:1126-30.
15. Capasso M, Diskin S, Cimmino F, et al. Common genetic variants in NEFL influence gene expression and neuroblastoma risk. Cancer Res. 2014; 74:6913-24.
16. Capasso M, Diskin S J, Totaro F, et al. Replication of GWAS-identified neuroblastoma risk loci strengthens the role of BARD1 and affirms the cumulative effect of genetic variations on disease susceptibility. Carcinogenesis. 2013; 34:605-11.
17. Latorre V, Diskin S J, Diamond M A, et al. Replication of neuroblastoma SNP association at the BARD1 locus in African-Americans. Cancer Epidemiol Biomarkers Prev. 2012; 21:658-63.
18. Zhang R, Zou Y, Zhu J, et al. The Association between GWAS-identified BARD1 Gene SNPs and Neuroblastoma Susceptibility in a Southern Chinese Population. Int J Med Sci. 2016; 13:133-8.
19. Shimada H, Ambros I M, Dehner L P, et al. Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. Cancer. 1999; 86:349-63.
20. Gabriel S, Ziaugra L, and Tabbaa D. SNP genotyping using the Sequenom MassARRAY iPLEX platform. Curr Protoc Hum Genet. 2009; Chapter 2:Unit 2.12.
21. Barrett J C, Fry B, Maller J, et al. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21:263-5.
22. Al-Chalabi A. Genome-wide association studies. Cold Spring Harb Protoc. 2009; 2009:pdb.top66.
23. Cimmino F, Formicola D, and Capasso M. Dualistic Role of BARD1 in Cancer. Genes (Basel). 2017; 8.
24. M A, SJ D, Z V, et al. Fine mapping of 2q35 high-risk neuroblastoma locus reveals independent functional risk variants and suggests full-length BARD1 as tumor-suppressor.%A Cimmino F. International journal of cancer. 2018.
25. Alshatwi A A, Hasan T N, Syed N A, et al. Identification of functional SNPs in BARD1 gene and in silico analysis of damaging SNPs: based on data procured from dbSNP database. PLoS One. 2012; 7(e):43939.
26. Wu L C, Wang Z W, Tsan J T, et al. Identification of a RING protein that can interact in vivo with the BRCA1 gene product. Nat Genet. 1996; 14: 430-40.
27. Thai T H, Du F, Tsan J T, et al. Mutations in the BRCA1-associated RING domain (BARD1) gene in primary breast, ovarian and uterine cancers. Hum Mol Genet. 1998; 7:195-202.
28. Fabbro M, Savage K, Hobson K, et al. BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage. J Biol Chem. 2004; 279:31251-8.
29. Fox D, 3rd, Le Trong I, Rajagopal P, et al. Crystal structure of the BARD1 ankyrin repeat domain and its functional consequences. J Biol Chem. 2008; 283: 21179-86.
30. Bosse K R, Diskin S J, Cole K A, et al. Common variation at BARD1 results in the expression of an oncogenic isoform that influences neuroblastoma susceptibility and oncogenicity. Cancer Res. 2012; 72:2068-78.