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Ras-Association Domain Family 1 Isoform A (RASSF1A) Gene Polymorphism rs1989839 is Associated with Risk and Metastatic Potential of Osteosarcoma in Young Chinese Individuals: A Multi-Center, Case-Control Study

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Background: The ras-association domain family 1 isoform A (RASSF1A) gene serves as a bona fide tumor suppressor gene. The polymorphisms in RASSF1A were previously reported to be associated with the risk of solid malignant tumors. We hypothesized herein that RASSF1A gene polymorphisms are involved in the risk and prognosis of osteosarcoma (OS).

Material/Methods: We recruited 279 young OS cases and 286 tumor-free controls from the east Chinese population. Five tagSNPs of RASSF1A gene (rs2236947A/C, rs2073497A/C, rs1989839C/T, rs72932987C/T, and rs4688728G/T) were genotyped. DNA was isolated from blood samples and then underwent PCR analysis for genotyping.

Results: rs1989839C/T is an important predictor of osteosarcoma risk and outcome. The CT genotype of rs1989839 is highly related to elevated risk of osteosarcoma. Furthermore, rs1989839C/T is also associated with the Enneking stage of osteosarcoma and risk of lung metastasis. One of the other 4 SNPs, rs2236947A/C, shows a borderline significance in predicting osteosarcoma risk.

Conclusions: Our study is the first to prove that RASSF1A gene polymorphisms may potentially be predictive for osteosarcoma risk and prognosis.

MeSH Keywords: Genetic Predisposition to Disease • Osteosarcoma • Polymorphism, Genetic

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Background

Osteosarcoma, the most common malignant bone tumor, has the potential of forming lung metastasis at an early stage, and is characterized by poor prognosis [1]. Surgical resection and multi-agent chemotherapy is essential for a standard strategy, but it is currently limited by multi-drug resistance to chemotherapy. Clinical trials are ongoing to verify whether patients with advanced diseases will benefit from more aggressive therapeutic regimens (such as trial EURAMOS-1). Many attempts, such as trying to add ifosfamide and etoposide or IFN-α-2b to the ordinary regimen, have not revealed promising results yet [2,3]. However, things may change if there are ways to predict prognosis at an early stage. Patients with poor outcomes may benefit from early administration of more aggressive chemotherapy regimens. Thus, biomarkers which can be easily examined will be of great value for predicting outcomes.

Single-nucleotide polymorphisms (SNPs) are believed to be related with malignancy risk and progression. Emerging studies on different kinds of cancers have provided abundant evidence for SNPs serving as predicting roles [4,5]. Further studies on SNPs in osteosarcoma will also be valuable in identifying potential prognostic biomarkers.

The ras-association domain family 1 isoform A (*RASSF1A*) gene, whose locus is located at 3p21.3 chromosome, is a verified tumor suppressor gene. RASSF1A is involved in multiple cellular processes, including microtubule stabilization, apoptosis, and cellular motility [6]. The RASSF1A protein showed ability in inhibiting the accumulation of cyclin D1, and consequently induced cell cycle arrest [7,8]. Although mutations on *RASSF1A* gene are not common, many polymorphisms have been discovered within this region. Several studies reported that different SNPs in RASSF1A are related to risk or outcome of malignancies, such as lung cancer [9], breast cancer [10], and renal cell carcinoma [11]. However, to the best of our knowledge, there is still no report on the relationship between *RASSF1A* polymorphisms and risk or outcome of osteosarcoma. In consideration of the favored role *RASSF1A* plays in osteosarcoma [12], it is rational to build a hypothesis that *RASSF1A* polymorphisms are associated with osteosarcoma.

Herein, we performed a case-control study on *RASSF1A* polymorphisms in osteosarcoma. We recruited 279 young osteosarcoma individuals and 286 cancer-free controls. We analyzed 5 *RASSF1A* tagging SNPs and tried to find evidence that *RASSF1A* polymorphisms were related with osteosarcoma.

Material and Methods

Osteosarcoma cases, controls, and ethics approval

A total of 279 primary osteosarcoma individuals younger than 20 years old and 286 tumor-free healthy controls treated in different institutions were involved in this study. All patients were diagnosed by pathologic examination during the period from February 2007 to November 2012, and blood samples were collected before performing chemotherapy and were consequently preserved. All cases underwent surgical operations by qualified orthopedists and were followed up for at least 36 months. Tumor-free controls were recruited from ordinary fracture cases. All clinical information was obtained from medical records. Signed informed consent to participate in this research was acquired from all participants or their guardians. The Ethics Committees of the 3 participating institutions approved this study.

DNA extraction

Whole DNA was isolated from blood. Genomic DNA was extracted using a DNA Blood Mini Kit from Qiagen, Berlin, Germany. The extraction was conducted according to the manufacturer's instructions.

Genotyping

Five SNPs (rs2236947A/C, rs2073497A/C, rs1989839C/T, rs72932987C/T, and rs4688728G/T) were tested in this study. ABI StepOnePlus system and software (Thermo Fisher Scientific, Waltham, MA, USA) were used to perform PCR analysis and to collect data. TaqMan primers and related probes were designed by the custom TaqMan assay design tool. All samples were added to 96-well plates and run in triplicate. The amplification condition was set as: initial denaturing step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min.

Haplotype analysis

Haplotype analysis was performed by computational haplotyping. The 5 candidate SNPs were analyzed on SHEsis to find the most frequent haplotypes (proportions over 3%). SHEsis (*http://analysis.bio-x.cn/myAnalysis.php*) was reported to be a powerful online platform for analyses of genetic correlation at polymorphisms [13,14].

Statistical analysis

The standard χ^2 test was utilized to assess the potential differences in the distributions of subject characteristics, variables, and genotypes of *RASSF1A* alterations between the

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Table 1. General characteristics of subjects.

Variables		Osteosarcoma cases [n (%)]	Control [n (%)]	*P*
Age	Mean±SD (year)	15.92±3.33	16.24±3.30	0.241
Gender	Male	167 (59.86)	159 (55.59)	0.305
	Female	112 (40.14)	12 (44.41)	
Location	Trunk	34 (12.19)		
	Limbs	245 (87.81)		
Enneking stages	IA or IB	43 (15.41)		
	IIA or IIB or III	236 (84.59)		
Operation	Amputation	54 (19.35)		
	Limb salvage	225 (80.65)		
Metastasis	Yes	53 (19.00)		
	No	226 (81.00)		

osteosarcoma cases and tumor-free controls. Odds ratios (ORs) and 95% confidential intervals (95% CIs) were calculated to evaluate the relationship between the 5 selected SNPs and the prognosis and risk of osteosarcoma. To estimated crude ORs, logistic regression analysis was performed and was subsequently adjusted for age and sex. The Hardy-Weinberg equilibrium was assessed using Pearson's χ^2 test. All of the statistical analyses were 2-sided, and $P<0.05$ was regarded as statistically significant. Data analyses were performed by using SPSS (v21.0; IBM, NY, USA).

Results

Clinical characteristics

Clinical features of recruited cases and controls are shown in Table 1. There were 167 male osteosarcoma cases and 112 female cases. The median ages of cases and controls were 15.92 years and 16.24 years, respectively. The Enneking GTM System was used to define the grades of tumors [145]. No statistical significance was found for age or sex between osteosarcoma cases and tumor-free controls (P=0.241 and 0.305, respectively).

RASSF1A SNPs were associated with risk of osteosarcoma

Pooled data of distributions of 5 tested RASSF1A SNPs (rs2236947A/C, rs2073497A/C, rs1989839C/T, rs72932987C/T, and rs4688728G/T) in osteosarcoma cases and tumor-free controls are shown in Table 2. Genotype distributions of the 5 selected SNPs were all in Hardy-Weinberg equilibrium in the control group (P=0.079, 0.730, 0.102, 0.132, and 0.260, respectively).

In rs1989839C/T, the CT genotype showed elevated risk of osteosarcoma (CT *vs.* CC: crude OR=1.69, 95% CI=1.18–2.41, P=0.004; adjusted OR=1.66, 95% CI=1.16–2.38, P=0.006) when the CC homozygote genotype was set as the reference group. In addition, the TT genotype was also associated with increased risk of osteosarcoma (TT *vs.* CC: crude OR=1.77, 95% CI=1.05–2.99, P=0.034; adjusted OR=1.74, 95% CI=1.03–2.95, P=0.039) when compared with CC genotype. Furthermore, a stronger statistical significance was revealed in the dominant model (CT/TT *vs.* CC: crude OR=1.70, 95% CI=1.22–2.38, P=0.002; adjusted OR=1.68, 95% CI=1.20–2.35, P=0.003). No statistically significant differences were found in the recessive model.

Borderline significant differences were found in the research of rs2236947A/C. The AC genotype displayed a tendency toward elevated risk of osteosarcoma (P=0.080), as did CC genotype (P=0.054) and dominant model (P=0.055).

The other 3 SNPs (rs2073497A/C, rs72932987C/T, and rs4688728G/T) did not show any association for osteosarcoma risk in young Chinese individuals.

RASSF1A SNPs were associated with the stage and metastatic potential of osteosarcoma

Data on clinical features of osteosarcoma cases were collected to investigate the associations between the examined SNPs and prognosis of osteosarcoma. Tumor location, Enneking stage, operation method, and metastasis was compared, and rs1989839C/T was found to be correlated with some of these prognostic factors (Table 3). In rs1989839C/T, the frequencies of the CT and TT genotypes at Enneking stage II or III (50.00%

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Table 2. Logistic regression analyses of correlations between *RASSF1A* rs2236947A/C, rs2073497A/C, rs1989839C/T, rs72932987C/T, and rs4688728G/T polymorphisms and risk of osteosarcoma.

RASSF1A genotype	Cases (n=279)		Controls (n=286)		Crude OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
	n	%	n	%				
rs2236947A/C								
AA	16	5.73	7	2.45	1.00		1.00	
AC	51	18.28	53	18.53	0.42 (0.16–1.11)	0.080	0.43 (0.16–1.13)	0.086
CC	212	75.99	226	79.02	0.41 (0.17–1.02)	0.054	0.43 (0.17–1.06)	0.067
AC+CC	263	94.27	279	97.55	0.41 (0.17–1.02)	0.055	0.43 (0.17–1.06)	0.066
AA+AC	67	24.01	60	20.98	1.00		1.00	
CC	212	75.99	226	79.02	0.84 (0.57–1.25)	0.388	0.86 (0.58–1.29)	0.470
rs2073497A/C								
AA	34	12.19	37	12.94	1.00		1.00	
AC	113	40.5	128	44.76	0.96 (0.57–1.63)	0.882	0.98 (0.58–1.67)	0.935
CC	132	47.31	121	42.31	1.19 (0.70–2.01)	0.523	1.18 (0.70–2.01)	0.532
AC+CC	245	87.81	249	87.06	1.07 (0.65–1.76)	0.788	1.08 (0.65–1.78)	0.768
AA+AC	147	52.69	165	57.69	1.00		1.00	
CC	132	47.31	121	42.31	1.22 (0.88–1.71)	0.232	1.20 (0.86–1.69)	0.278
rs1989839C/T								
CC	105	37.63	145	50.70	1.00		1.00	
CT	133	47.67	109	38.11	1.69 (1.18–2.41)	0.004*	1.66 (1.16–2.38)	0.006*
TT	41	14.7	32	11.19	1.77 (1.05–2.99)	0.034*	1.74 (1.03–2.95)	0.039*
CT+TT	174	62.37	141	49.30	1.70 (1.22–2.38)	0.002*	1.68 (1.20–2.35)	0.003*
CC+CT	238	85.3	254	88.81	1.00		1.00	
TT	41	14.7	32	11.19	1.37 (0.83–2.24)	0.215	1.36 (0.83–2.23)	0.228
rs72932987C/T								
CC	121	43.37	118	41.26	1.00		1.00	
CT	104	37.28	122	42.66	0.83 (0.58–1.20)	0.320	0.84 (0.58–1.21)	0.341
TT	54	19.35	46	16.08	1.15 (0.72–1.83)	0.571	1.15 (0.72–1.84)	0.557
CT+TT	158	56.63	168	58.74	0.92 (0.66–1.28)	0.612	0.92 (0.66–1.29)	0.640
CC+CT	225	80.65	240	83.92	1.00		1.00	
TT	54	19.35	46	16.08	1.25 (0.81–1.93)	0.309	1.26 (0.81–1.94)	0.307
rs4688728G/T								
GG	38	13.62	33	11.54	1.00		1.00	
GT	94	33.69	116	40.56	0.70 (0.41–1.21)	0.202	0.70 (0.41–1.20)	0.193
TT	147	52.69	137	47.90	0.93 (0.55–1.57)	0.791	0.92 (0.55–1.55)	0.753
GT+TT	241	86.38	253	88.46	0.83 (0.50–1.36)	0.456	0.82 (0.50–1.35)	0.433
GG+GT	132	47.31	149	52.10	1.00		1.00	
TT	147	52.69	137	47.90	1.21 (0.87–1.69)	0.255	1.20 (0.86–1.68)	0.276

* Statistically significant ($P<0.05$).

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Table 3. Association between genotype frequencies of *RASSF1A* rs1989839C/T and clinical features in osteosarcoma cases.

Variables	n	CC n (%)	CT n (%)	TT n (%)	*P*
Location					
Trunk	34	15 (44.12)	15 (44.12)	4 (11.76)	0.683
Limbs	245	90 (36.73)	118 (48.16)	37 (15.10)	
Enneking stages					
IA or IB	43	24 (55.81)	15 (34.88)	4 (9.30)	0.027*
IIA or IIB or III	236	81 (34.32)	118 (50.00)	37 (15.68)	
Operation					
Amputation	54	17 (31.48)	30 (55.56)	7 (12.96)	0.431
Limb salvage	225	88 (39.11)	103 (45.78)	34 (15.11)	
Metastasis					
Yes	53	15 (28.30)	23 (43.40)	15 (28.30)	0.027*
No	226	90 (39.82)	110 (48.67)	26 (11.50)	

* Statistically significant ($P<0.05$).

Table 4. Confounding variables (Enneking stages).

Confounding variables		IA or IB cases [n (%)]	IIA or IIB or III cases [n (%)]	*P*
Age	Mean ±SD (year)	15.37±3.66	16.02±3.26	0.243
Gender	Male	22 (51.16)	145 (61.44)	0.206
	Female	21 (48.84)	91 (38.56)	

Table 5. Confounding variables (metastasis).

Confounding variables		Metastasis cases [n (%)]	Non-metastasis cases [n (%)]	*P*
Age	Mean ±SD (year)	15.19±3.65	16.09±3.23	0.076
Gender	Male	29 (54.72)	138 (61.06)	0.396
	Female	24 (45.28)	88 (38.94)	

and 15.68%, respectively) were greater when compared with IA or IB stage cases (34.88% and 9.30%, respectively), and a significant difference was found ($P=0.027$). Furthermore, rs1989839C/T was correlated with the risk of metastasis. The genotype TT displayed a much higher frequency (28.30%) in metastatic cases compared with metastasis-free cases (11.50%), and a significant significance was found in frequency distribution ($P=0.007$). These data indicated that rs1989839C/T was closely correlated with prognosis of osteosarcoma.

The confounding variables, which were not statistically significant, are shown in Tables 4 and 5.

Haplotype analyses displayed a significant difference between osteosarcoma cases and tumor-free controls

Analyses of the 5 candidate SNPs picked out 11 frequent (frequency over 3%) haplotypes: CACCG, CACCT, CACTT, CATTT, CCCCG, CCCCT, CCCTG, CCCTT, CCTCG, CCTCT, and CCTTT (Table 6). Among them, CCCCG, CCCTG, CCTCT, and CCTTT showed statistically significant differences between osteosarcoma cases and tumor-free controls (P<0.001, 95% CI=0.18–0.80, P<0.001, 95%=1.49–4.85, P=0.03, 95% CI=1.048–3.648 and P=0.02, 95% CI=1.076–2.726, respectively).

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Table 6. Haplotype analysis.

Haplotype	Cases (n=279)		Controls (n=286)		p	OR (95% CI)
	n (frequency)		n (frequency)			
CACCG	33.19	(0.059)	30.28	(0.053)	0.583	1.154 (0.692–1.922)
CACCT	25.48	(0.046)	30.45	(0.053)	0.598	0.864 (0.502–1.488)
CACTT	24.87	(0.045)	39.91	(0.070)	0.077	0.629 (0.375–1.056)
CATCT	29.49	(0.053)	25.92	(0.045)	0.514	1.199 (0.696–2.066)
CATTT	20.16	(0.036)	29.36	(0.051)	0.232	0.702 (0.392–1.257)
CCCCG	9.65	(0.017)	25.88	(0.045)	0.008*	0.375 (0.177–0.795)
CCCCT	123.39	(0.221)	149.76	(0.262)	0.135	0.805 (0.606–1.070)
CCCTG	39.73	(0.071)	16.34	(0.029)	<0.001*	2.682 (1.485–4.845)
CCCTT	40.85	(0.073)	46.19	(0.081)	0.690	0.914 (0.587–1.422)
CCTCG	24.73	(0.044)	27.62	(0.048)	0.790	0.929 (0.532–1.625)
CCTCT	29.12	(0.052)	16.03	(0.028)	0.032*	1.955 (1.048–3.648)
CCTTT	49.92	(0.089)	31.84	(0.056)	0.022*	1.713 (1.076–2.726)

* Statistically significant ($P<0.05$).

Discussion

Osteosarcoma is a relatively rare malignancy, with the morbidity rate of about 3–5 per million people every year [1]. However, most osteosarcoma patients are children or adolescents, and this malignancy profoundly harms the whole family, especially in China, where most parents have only 1 child. Accumulating studies have shown some promising alternative none-surgical therapeutic strategies against osteosarcoma. Nevertheless, none of these new strategies, including immunotherapy, have become a standard method in clinical treatment so far. Thus, exploring evidence relevant to traditional chemotherapy is still of great value.

Emerging reports about genomic factors correlated with osteosarcoma tumorigenesis and/or progress have provided some chances to predict osteosarcoma risk and outcome, especially studies on gene polymorphisms, such as *HER2* [16], , *ERCC* [17], and *GRM4* [18]. However, we believe that in predicting cancer risk and outcome, referring to only a few predicting roles is far from satisfactory. Given that osteosarcoma is not common, we launched a series of projects involving over 10 high-rank hospitals in the eastern coastal area of China, which has a population of over 100 million, to profile possible genomic predictors. In this series of projects, we discovered that *WWOX* and *NAT2* polymorphisms may be possible predictors for risk of osteosarcoma [19,20]. Herein, we provide evidence that *RASSF1A* polymorphisms are related to the risk and metastatic potential of osteosarcoma. We analyzed 5 candidate SNPs with recorded clinical data, and discovered that rs1989839C/T was associated with elevated osteosarcoma risk. Furthermore, this SNP was related to high-grade osteosarcoma and elevated risk of forming metastasis. Another SNP, rs2236947A/C, also showed value in predicting risk and outcome of osteosarcoma. We believe that including more cases will help to further analyze the role of this SNP.

RASSF1A has been widely studied in different kinds of tumors. As a tumor suppressor gene, its abnormal expression was believed to be related with tumorigenesis. *RASSF1A* gene promoter methylation was found to be related with carcinogenesis of non-small-cell lung cancer [21] and breast cancer [22]. Furthermore, its genotypes have been revealed to be associated with cancers in Japanese and Korean populations [11,23]. In these studies, similarly, rs1989839C/T was also found to be correlated with worse survival and/or higher risk of cancer. rs1989839C/T may play as a "vicious" role in East Asia. Although we were not able to perform some molecular biological experiments to validate the mechanism underlying this phenomenon, it is reasonable to reach this conjecture according to the role *RASSF1A* plays in controlling cancer. In addition, haplotype analyses showed that the haplotype CCCGG was much more frequent in tumor-free individuals, while CCCTG, CCTCT, and CCTTT were more common in osteosarcoma patients.

However, there are some limitations to our current study. We were not able to avoid inherent bias in our study, as all the blood samples were collected in hospitals. The sample size was

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limited due to the low morbidity of osteosarcoma, and results for homozygotic cases might not be as reliable. We are trying to recruit more osteosarcoma cases from additional institutions and plan to publish updated data.

Conclusions

This is the first study to disclose the correlation between RASSF1A polymorphism and risk and/or outcome of osteosarcoma. This multi-center study recruited a relatively large number of blood samples from young osteosarcoma patients. Our results show that *RASSF1A* rs1989839C/T SNP indicates an elevated risk of osteosarcoma (*P*=0.004 for CT genotype, and *P*=0.034 for TT genotype) and lung metastasis (TT genotype, *P*=0.007) in young Chinese individuals. Our finding provides good evidence for use of more powerful therapeutic regimens.

Conflicts of interest

None.

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