Functional Genetic Single-Nucleotide Polymorphisms (SNPs) in Cyclin-Dependent Kinase Inhibitor 2A/B (CDKN2A/B) Locus Are Associated with Risk and Prognosis of Osteosarcoma in Chinese Populations

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Background: Cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B) encodes several tumor suppressor proteins. Aberrant genetic alterations in CDKN2A/B were found in some malignancies, which were believed to be associated with tumor originating and progression. We hypothesized that CDKN2A/B genetic polymorphisms might be associated with the risk of poorer prognosis of osteosarcoma in Chinese populations.

Material/Methods: We included 184 validated osteosarcoma cases and 185 cancer-free healthy controls in the study. Five single-nucleotide polymorphisms of CDKN2A/B (rs1063192, rs3218009, rs3217986, rs3217992, and rs3731257) were genotyped and underwent bioinformatic analysis. DNA from osteosarcoma individuals was isolated from frozen peripheral blood and DNA from healthy controls was extracted from fresh prepared peripheral blood.

Results: An allele of the SNP rs3217992 is predictive for susceptibility to osteosarcoma, and it is associated with poorer prognosis of osteosarcoma. The GA and AA genotypes of rs3217992 are related to elevated risk of osteosarcoma. In addition, the GA and AA genotypes of rs3217992 in CDKN2A might indicate higher stage and increased risk of lung metastasis of osteosarcoma, resulting in worse prognosis.

Conclusions: Functional genetic polymorphisms in CDKN2A/B predict the susceptibility and outcome of osteosarcoma in Chinese individuals.

MeSH Keywords: **Genes, p16 • Osteosarcoma • Polymorphism, Genetic**

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Background

Osteosarcoma, the most common primary malignant tumor originating from musculoskeletal tissues, and severely threatens adolescents [1]. About 30% of patients with osteosarcoma have already formed lung metastasis when diagnosed [2]. Although current therapeutic strategies involving surgical excision and chemotherapy have dramatically increased the 5-year overall survival from ~20% to 60–70%, once the tumor becomes chemoresistant or refractory, the prognosis is very poor [1]. Also, controversies on the chemotherapy regimens still exist. It is still difficult for physicians to decide when and how to choose more aggressive regimens, especially regimens including high-dose methotrexate (HD-MTX) and Ifosfamide (CTX). Numerous efforts were taken to discover biomarkers to predict poor prognosis so as to apply more aggressive regimens, but there are still few reliable methods to evaluate the response rate in the stage of neoadjuvant chemotherapy, except for necrosis rate, which is a painstaking pathological assessment. Biomarkers that are easy to evaluate to predict the risk and prognosis of osteosarcoma would allow physicians can be more proactive in applying more aggressive therapeutic regimens to treat osteosarcoma at an early stage.

Cyclin-dependent kinase inhibitor 2A/B (CDKN2A/B), located on human chromosome 9, generates 2 tumor suppressor proteins: p16 and p14 [3]. p14 and p16 proteins play important roles in the G1/S regulation by mediating the interactions between CDK4/6 and cyclin D1 [4,5]. In addition, p14 protein can bind to MDM2 and induce its degradation, stabilizing p53 and consequently controlling the progression of cancers. Somatic alterations of CDKN2A/B locus were found in multiple malignancies [6–8], including osteosarcoma [9], suggesting that the heritable risk alleles in this locus play important roles during tumorigenesis and tumor progression. In consideration of the relatively low morbidity of osteosarcoma, systemic study using large osteosarcoma cohorts might be challenging. Thus, we chose to use frozen blood samples to study the genetic alterations in osteosarcoma patients.

Single-nucleotide polymorphisms (SNPs) are believed to be associated with the risk and progression status of multiple solid malignancies [10]. However, SNPs in osteosarcomas are still elusive because of their rarity. As emerging studies have revealed the prognostic role SNPs play in tumors, it will be valuable to evaluate genetic polymorphisms of specific genes in osteosarcoma. The genetic polymorphisms in CDKN2A/B were discovered to affect the risk and/or the progression of pancreatic cancer, leukemia, and cervical cancer [8,11]. Hence, we hypothesized that genetic polymorphisms in CDKN2A/B are involved in the process of tumorigenesis and progression of osteosarcoma.

We genotyped 184 frozen blood samples from osteosarcoma patients and 185 healthy controls by extracting the DNA, and investigated whether CDKN2A/B polymorphisms are associated with the risk and prognosis of osteosarcoma. In this laboratory study, we analyzed 5 tagging CDKN2A/B SNPs: rs1063192, rs3218009, rs3217986, rs3217992, and rs3731257.

Material and Methods

Ethics approval

This study used blood samples; thus, before the study was started, we obtained ethics approval from the Ethical Committee of the Fourth Affiliated Hospital of Zhejiang University School of Medicine, the Second Affiliated Hospital of Zhejiang University School of Medicine, and Fudan University. In addition, before collection of blood samples for research, signed informed consent was obtained from all involved individuals or their legal guardians.

Study population

In this study, 184 conventional osteosarcoma patients and 185 cancer-free healthy controls were recruited in this retrospective study. All individuals were identified as Chinese Han ethnicity according to their registered ID information. Osteosarcoma patients were diagnosed by biopsy and pathology validation during the period from Oct 2009 to Jun 2013. In osteosarcoma cases, 5-ml peripheral blood samples were collected before receiving neoadjuvant chemotherapy and were preserved in liquid nitrogen. Surgical excision of the primary tumor was performed by orthopedic oncology specialists; all of the included osteosarcoma patients received follow-up for at least 5 years. Cancer-free healthy controls were mainly trauma or fracture patients or healthy volunteers, and were matched to osteosarcoma patients by ages and sex. All of the clinical information was recorded and saved in the medical information system and database of 2 institutions.

SNP selection

Five tagging SNPs of CDKN2A/B were chosen using the tagger tool of the Haploview software in the HapMap web site (*http://www.hapmap.org and www.broad.mit.edu/mpg/tagger/*) with the discipline of frequency >5% in Chinese Han people. These 5 tagging SNPs include 2 functional SNPs (rs1063192 and rs3217992) predicted to change the binding status and ability of micro-RNAs to their targets.

Table 1. General characteristics.

Variables		Osteosarcoma cases [n (%)]		Cancer-free control n (%)]		P
Age	Mean ±SD (year)	15.44±3.03		15.96±3.24		0.335
Sex	Male	97	(52.72)	99	(53.51)	0.679
	Female	87	(47.28)	86	(46.49)	
Location	Trunk	23	(12.50)			
	Limbs	161	(87.50)			
Enneking stages	IA or IB	25	(13.59)			
	IIA or IIB or III	159	(86.41)			
Operation	Amputation	29	(18.71)			
	Limb salvage	155	(81.29)			
Metastasis	No	88	(47.82)			
	Yes	96	(52.18)			

Sample preparation and SNP genotyping

Total DNA from frozen peripheral blood was extracted following the phenol-chloroform protocol using a Blood DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, frozen blood samples were thawed and then mixed with proteinase K, followed by heated at 55°C for 10 min. Next, ethyl alcohol was added into the mixture and vortexed and then centrifuged at 6000 g for 1 min. To isolate the DNA from the mixture, AW2 Buffer from the kit was added and then centrifuged at 20 000 g. The ABI StepOnePlus system (ThermoFisher, Waltham, MA) was utilized for DNA genotyping and data analyses. TaqMan method was used to perform the genotyping. Primers were designed online using the ABI Assay-by-Design service. The amplification conditions set as follows: 2 min at 50°C at the start, 10 min at 95°C, 40 cycles of amplification at 95°C for 15 s, and then 60°C for 60 s. For quality control, duplicates of 20% of the samples were interspersed throughout the plates.

Statistical analysis

SPSS software (v21.0; IBM Corporation, Armonk, NY) was used to perform statistical analysis. Statistical differences in distributions of given variables, subject characteristics, and genotypes of CDKN2A/B between osteosarcoma individuals and cancer-free controls were evaluated by χ^2 test. Odd ratios (ORs) and 95% confidence intervals (95% CIs) were calculated to estimate the associations between the 5 genotyped SNPs and the risk and prognosis of osteosarcoma. Logistic regression analyses were used to calculate crude ORs, and ORs were consequently adjusted for age and sex. The Hardy-Weinberg equilibrium for SNPs was tested with the Pearson's χ^2 test. $P<0.05$ was regarded as statistically significant, and all analyses were two-sided.

Results

Individual characteristics

The characteristics and clinical information of included osteosarcoma patients and cancer-free healthy controls are shown in Table 1. The medium ages of osteosarcoma patients and cancer-free controls were 15.44±3.03 and 15.96±3.24 years, respectively. Ninety-seven (52.72%) osteosarcoma patients were male and 99 (53.51%) were female. Further analysis on statistical difference of age and sex did not reveal significant differences (P=0.335 for age and 0.679 for sex). The Enneking staging system (GTM grading) was applied to evaluate the stages of osteosarcoma patients [12]. Of all 184 osteosarcoma patients, 25 (13.59%) were identified as early-stage and the remaining 159 (86.41%) were identified as late-stage (stage II or III). Surgical techniques, including amputation or limb salvage, were performed during the therapy; 29 (18.71%) patients underwent amputation, while the other 155 (81.29%) individuals received limb salvage. In addition, 96 (52.18%) cases had detectable lung metastases during the follow-up.

CDKN2A/B tagging SNP rs3217992 was associated with osteosarcoma susceptibility

We selected 5 tagging SNPs (rs1063192, rs3217992, rs3217986, rs3218009, and rs3731257) and genotyped them. The pooled

Table 2. Logistic regression analyses of associations between CDKN2A/B rs1063192, rs3217992, rs3217986, rs3218009, and rs3731257 polymorphisms and susceptibility to osteosarcoma.

CDKN2A/B genotype	Cases (n=184)		Controls (n=185)		Crude OR (95%CI)	P	Adjusted OR (95%CI)	P
	n	%	n	%				
Rs1063192 G/A								
GG	54	29.35	52	28.10	1.00		1.00	
GA	88	48.83	89	48.10	0.83 (0.48–1.22)	0.329	0.85 (0.44–1.21)	0.331
AA	42	22.83	44	23.78	1.31 (0.83–1.43)	0.321	1.33 (0.85–1.41)	0.311
GA+AA	130	71.76	133	71.88	0.88 (0.60–1.32)	0.421	0.87 (0.63–1.40)	0.417
GG+GA	142	77.17	141	76.22	1.00		1.00	
AA	42	22.83	44	23.78	0.92 (0.88–1.21)	0.572	0.91 (0.85–1.39)	0.568
rs3217992 G/A								
GG	114	62.96	142	76.76	1.00		1.00	
GA	63	34.24	42	22.70	1.43 (1.10–1.77)	0.024*	1.44 (1.09–1.88)	0.025*
AA	7	3.80	1	0.54	1.61 (1.15–2.02)	0.006*	1.60 (1.13–2.02)	0.005*
GA+AA	70	38.04	43	23.24	1.59 (1.07–1.96)	0.013*	1.58 (1.08–2.02)	0.014*
GG+GA	177	86.20	184	99.46	1.00		1.00	
AA	7	3.80	1	0.54	1.87 (1.43–2.33)	0.017*	1.85 (1.41–2.35)	0.016*
rs3217986 C/A								
CC	66	35.68	69	37.30	1.00		1.00	
CA	90	48.91	86	46.49	1.15 (0.81–1.35)	0.235	1.12 (0.79–1.36)	0.229
AA	28	15.22	30	16.22	0.83 (0.68–1.09)	0.220	0.85 (0.69–1.12)	0.222
CA+AA	118	64.13	116	62.71	1.12 (0.59–1.25)	0.311	1.13 (0.58–1.29)	0.317
CC+CA	156	84.58	155	83.78	1.00		1.00	
AA	28	15.22	30	16.22	0.98 (0.75–1.11)	0.125	0.96 (0.69–1.07)	0.122
rs3218009 C/G								
CC	78	42.39	86	46.49	1.00		1.00	
CG	83	45.11	79	42.70	1.29 (0.83–1.42)	0.311	1.30 (0.82–1.44)	0.301
GG	23	12.50	20	10.81	1.21 (0.87–1.60)	0.229	1.20 (0.86–1.62)	0.219
CG+GG	106	57.61	99	53.51	1.25(0.87–1.91)	0.320	1.24(0.86–1.94)	0.318
CC+CG	161	87.50	165	89.19	1.00		1.00	
GG	23	12.50	20	10.81	1.15 (0.81–1.35)	0.235	1.12 (0.79–1.36)	0.229
rs3731257 C/T								
CC	115	62.50	119	64.32	1.00		1.00	
CT	57	30.98	53	28.65	1.11 (0.93–1.23)	0.521	1.13 (0.95–1.21)	0.511
TT	12	6.52	13	7.03	0.89 (0.50–1.52)	0.771	0.91 (0.51–1.53)	0.750
CT+TT	69	37.50	66	35.68	1.20 (0.61–1.45)	0.320	1.22 (0.60–1.52)	0.318
CC+CT	172	93.48	172	92.98	1.00		1.00	
TT	12	6.52	13	7.02	0.94 (0.88–1.21)	0.442	0.95 (0.85–1.29)	0.448

* Statistically significant (P<0.05)

Table 3. Correlations between genotype frequencies of CDKN2A/B rs3217992 G>A and clinical features in osteosarcoma individuals.

Variables	n	GG n (%)	GA n (%)	AA n (%)	P
Location					
Trunk	23	14 (60.87)	8 (34.78)	1 (4.35)	0.708
Limbs	161	100 (62.11)	55 (34.16)	6 (3.73)	
Enneking stages					
IA or IB	25	21 (84.00)	4 (16.00)	2 (8.00)	0.034*
IIA or IIB or III	159	93 (58.49)	54 (33.96)	5 (3.14)	
Operation					
Amputation	29	16 (55.17)	11 (37.93)	2 (6.90)	0.182
Limb salvage	155	98 (63.23)	52 (33.55)	5 (3.23)	
Metastasis					
No	88	64 (72.73)	23 (26.14)	1 (1.14)	0.009*
Yes	96	50 (52.08)	40 (41.67)	6 (6.25)	

* 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)	15.57±3.16	15.39±2.86	0.568
Sex	Male	12 (48.00)	85 (53.46)	0.102
	Female	13 (52.00)	74 (46.54)	

Table 5. Confounding variables (metastasis).

Confounding variables		Metastasis cases [n (%)]	Non-metastasis cases [n (%)]	P
Age	Mean ±SD (year)	15.49±2.91	15.41±3.12	0.381
Sex	Male	47 (53.41)	50 (52.08)	0.712
	Female	41 (46.59)	46 (47.92)	

data in this study are displayed in Table 2. In cancer-free healthy control individuals, the genotype distributions of the 5 selected SNPs were validated to be all within HWE (P=0.175, 0.228, 0.331, 0.434, and 0.550, respectively). Further logistic regression analyses revealed that CDKN2A/B polymorphism is associated with susceptibility to osteosarcoma. In rs3217992 G>A, 7 out of 184 osteosarcoma cases were AA genotype, while only 1 case in healthy controls showed this genotype. When the AA homozygote genotype was chosen as the reference, the GA genotype displayed significantly higher risk of osteosarcoma (crude OR=1.43, 95% CI=1.10–1.77, P=0.024; adjusted OR=1.44, 95% CI=1.09–1.88, P=0.025). The AA homozygote genotype was

also shown to be significantly associated with susceptibility to osteosarcoma (crude OR=1.61, 95% CI=1.15–2.02, P=0.006; adjusted OR=1.60, 95% CI=1.13–2.02, P=0.005). Analyses with the G dominant model (GA+AA) also showed a significant difference (GG/GA *vs.* AA: crude OR=1.70, 95% CI=1.29–2.31, P=0.013; adjusted OR=1.72, 95% CI=1.30–2.32, P=0.014).

The remaining 4 evaluated SNPs (rs1063192, rs3217986, rs3218009, and rs3731257) did not display potential associations between CDKN2A/B polymorphisms and susceptibility to osteosarcoma in Chinese populations.

rs3217992 G>A was associated with stage and metastatic risk of osteosarcoma

Selected clinical features (location, stage, operation technique, and metastasis status) of osteosarcoma individuals were analyzed to determine if rs3217992 SNP was associated with the stage and metastasis risk, which directly affect the prognosis, of osteosarcoma. As shown in Table 3, the frequency of genotype GA in late stages (II or III, 33.96%) was significantly higher when compared with early-stage (stage I) individuals (16.00%), showing a statistically significant difference (P=0.034). In addition, analyses of metastasis status revealed similar results. The genotype GA has higher frequency (41.67%) in cases with metastasis when compared with individuals without remote metastasis (26.14%). Furthermore, in AA genotype, there was only 1 case in metastasis-free individuals, while there were 6 in metastasized ones. After statistical analyses, a significant difference in frequency distribution was revealed (P=0.009).

The possible confounding variables (Enneking stages and metastasis status) are shown in Tables 4 and 5, respectively. No significant difference was found.

Discussion

Emerging studies have focused on the tumorigenesis and progression of osteosarcoma. However, as a highly heterogenous malignancy, osteosarcoma is genetically unstable, complicating the mechanism research. Also, the low morbidity (around 2–3 per million population) severely restricts the number of clinical samples, and consequently reduces the power of data interpretation. Thus, we launched a series of studies using frozen blood samples from individuals with osteosarcoma. We used high-profile gene array to select possible genetic variations and functional alterations, and then further assessed the alteration in a large number of tumor cases. In our previous study, we reported that insulin-like growth factor 1 (IGF-1) genetic polymorphisms might be associated with osteosarcoma risk and prognosis [13]. However, using a single factor to predict the risk and outcome of a cancer is still far from accurate, especially when the given SNP is rare. Thus, finding some more predictors to prepare a combination evaluation system would be of great value [14]. In osteosarcoma, some studies have already emphasized the value of genetic polymorphisms in demonstrating promising prognostic roles, including RASSF1A and TCF21 [15,16]. Our present study further enriches the database of possible candidates for predictors.

Several SNPs in CDKN2A/B have been suggested to be associated with the development of cancer and a variety of other diseases, including heart events, stroke, and glaucoma [17–19]. In addition, the levels of CDKN2A/B encoded proteins p16 and p14 can be altered in malignancies and consequently result in poor outcome. In this study, we provide evidence based on a relatively large number of osteosarcoma cases to demonstrate that genetic polymorphisms in functional SNPs in CDKN2A/B are associated with the susceptibility to and prognosis of osteosarcoma. Our data illustrate that rs3217992 polymorphisms predicts the susceptibility to osteosarcoma, and show that the GA and AA genotype increase the risk of osteosarcoma. Moreover, rs3217992 polymorphisms were also associated with the clinical stage and the metastasis potential of osteosarcoma, affecting the survival. In our data interpretation, an individual carrying "worse" phenotypes of rs3217992 has relatively higher risk of osteosarcoma. More importantly, once an osteosarcoma patient is found to have GA or AA genotypes in rs3217992 in CDKN2A/B, physicians can be aware that the patient has significantly higher risk of metastasis, and more aggressive therapeutic strategy and more frequent follow-ups may be appropriate. Besides, although we cannot make strong conclusions about the possible associations between the other 4 SNPs and osteosarcoma, some unevaluated SNPs may also be of interest.

There are still some limitations to our study. Inherent bias was not avoided, as all the samples were collected from hospitals. Furthermore, some homozygotic types only had very small numbers of cases, which reduced the power to reach firm conclusions. There was only 1 case of AA genotype in rs3217992 in healthy control individuals. Further studies involving more osteosarcoma individuals and healthy controls will help improve the efficacy of CDKN2A/B genetic polymorphisms in predicting osteosarcoma risk and prognosis.

Conclusions

The present study is the first to report that functional genetic SNP polymorphisms in CDKN2A/B are associated with susceptibility to and progression of osteosarcoma. CDKN2A/B rs3217992 SNP polymorphisms are valuable prognostic biomarkers for osteosarcoma.

Conflicts of interest

None.

References:

1. Kansara M, Teng MW, Smyth MJ et al: Translational biology of osteosarcoma. Nat Rev Cancer, 2014; 14(11): 722–35

2. Biermann JS, Adkins DR, Agulnik M et al: Bone cancer. J Natl Compr Canc Netw, 2013; 11(6): 688–723

3. Kumar R, Smeds J, Berggren P et al: A single nucleotide polymorphism in the 3'untranslated region of the CDKN2A gene is common in sporadic primary melanomas but mutations in the CDKN2B, CDKN2C, CDK4 and p53 genes are rare. Int J Cancer, 2001; 95(6): 388–93

4. Biankin AV, Waddell N, Kassahn KS et al: Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature, 2012; 491(7424): 399–405

5. De Rienzo A, Archer MA, Yeap BY et al: Gender-specific molecular and clinical features underlie malignant pleural mesothelioma. Cancer Res, 2016; 76(2): 319–28

6. Wrensch M, Jenkins RB, Chang JS et al: Variants in the CDKN2B and RTEL1 regions are associated with high-grade glioma susceptibility. Nat Genet, 2009; 41(8): 905–8

7. Bishop DT, Demenais F, Iles MM et al: Genome-wide association study identifies three *loci* associated with melanoma risk. Nat Genet, 2009; 41(8): 920–25

8. Walsh KM, de Smith AJ, Hansen HM et al: A heritable missense polymorphism in CDKN2A confers strong risk of childhood acute lymphoblastic leukemia and is preferentially selected during clonal evolution. Cancer Res, 2015; 75(22): 4884–94

9. Karlsson EK, Sigurdsson S, Ivansson E et al: Genome-wide analyses implicate 33 *loci* in heritable dog osteosarcoma, including regulatory variants near CDKN2A/B. Genome Biol, 2013; 14(12): R132

10. Xin DJ, Shen GD, Song J: Single-nucleotide polymorphisms of HER2 related to osteosarcoma susceptibility. Int J Clin Exp Pathol, 2015; 8(8): 9494–99

11. Campa D, Pastore M, Gentiluomo M et al: Functional single nucleotide polymorphisms within the cyclin-dependent kinase inhibitor 2A/2B region affect pancreatic cancer risk. Oncotarget, 2016; 7(35): 57011–20

12. Enneking WF: A system of staging musculoskeletal neoplasms. Clin Orthop Relat Res, 1986; (204): 9–24

13. Mao J, Zhuang G, Chen Z: Genetic polymorphisms of insulin-like growth factor 1 are associated with osteosarcoma risk and prognosis. Med Sci Monit, 2017; 23: 5892–98

14. Gianferante DM, Mirabello L, Savage SA: Germline and somatic genetics of osteosarcoma – connecting aetiology, biology and therapy. Nat Rev Endocrinol, 2017; 13: 480–91

15. Xu H, Zhan W, Chen Z: 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. Med Sci Monit, 2016; 22: 4529–35

16. Jiang Z, Zhang W, Chen Z et al: Transcription factor 21 (TCF21) rs12190287 polymorphism is associated with osteosarcoma risk and outcomes in East Chinese population. Med Sci Monit, 2017; 23: 3185–91

17. Burdon KP, Macgregor S, Hewitt AW et al: Genome-wide association study identifies susceptibility *loci* for open angle glaucoma at TMCO1 and CDKN2B-AS1. Nat Genet, 2011; 43(6): 574–78

18. Helgadottir A, Thorleifsson G, Manolescu A et al: A common variant on chromosome 9p21 affects the risk of myocardial infarction. Science, 2007; 316(5830): 1491–93

19. Yasuno K, Bilguvar K, Bijlenga P et al: Genome-wide association study of intracranial aneurysm identifies three new risk *loci*. Nat Genet, 2010; 42(5): 420–25