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 chemotherapy resistant 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.
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 χ² 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 χ² 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 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) | 161 (87.50) | |
|                    | Limbs 161 (87.50) | | |
| Enneking stages    | IA or IB 25 (13.59) | 159 (86.41) | |
|                    | IIA or IB or III 159 (86.41) | | |
| Operation          | Amputation 29 (18.71) | | |
|                    | Limb salvage 155 (81.29) | | |
| Metastasis         | No 88 (47.82) | | |
|                    | Yes 96 (52.18) | | |
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 |
| 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* |
| 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 |
| AA               | 23 | 12.50        | 20  | 10.81        | 1.21 (0.87–1.60) | 0.229 | 1.20 (0.86–1.62) | 0.219 |
| CC+CG            | 106| 57.61        | 99  | 57.31        | 1.25 (0.87–1.91) | 0.320 | 1.24 (0.86–1.68) | 0.318 |
| GG               | 161| 87.50        | 165 | 89.19        | 1.00           | 1.00 |                    |    |
| 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 |
| 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)
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.

| Variables | n | GG n (%) | GA n (%) | AA n (%) | P |
|-----------|---|----------|----------|----------|---|
| Location  |   | GG       | GA       | AA       |   |
| 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 |   | GG       | GA       | AA       |   |
| 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 |   | GG       | GA       | AA       |   |
| 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 |   | GG       | GA       | AA       |   |
| 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 3. Correlations between genotype frequencies of CDKN2A/B rs3217992 G>A and clinical features in osteosarcoma individuals.

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) |   |
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.
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