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ORIGINAL RESEARCH

Associations of polymorphisms in *NAT2* gene with risk and metastasis of osteosarcoma in young Chinese population

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Abstract: Osteosarcoma is the most common primary malignancy of bone in young individuals. Genetic factors may play an important role in the tumorigenesis of osteosarcoma. Here we carried out a case-control study to investigate seven NAT2 single-nucleotide polymorphisms (rs1799929, rs120, rs1041983, rs1801280, rs1799930, rs1799931, and rs1801279) on the risk and prognosis of osteosarcoma. This study included 260 young osteosarcoma cases and 286 controls. The TaqMan method was used to determine genotypes. We found that rs1799931 G>A polymorphisms were associated with a decreased risk of osteosarcoma in young Chinese population, and rs1041983 CT genotype seemed to play a protective role in the risk of osteosarcoma. However, further analysis showed that rs1041983 polymorphisms were associated with an elevated risk of tumor metastasis, predicting poor prognosis. This study provided the first evidence for the associations between NAT2 polymorphisms and osteosarcoma risk and metastasis in Chinese population.

Keywords: osteosarcoma, NAT2, SNP, metastasis, susceptibility

Introduction

Osteosarcoma is the most common malignant tumor of bone in childhood and adolescence and is associated with early metastatic potential and a poor prognosis.[1] Albeit the fact that quite a few studies on osteosarcoma are emerging these days, its tumorigenesis and roles that predict outcomes or malignancy risk are still poorly revealed. Genetic factors, such as single-nucleotide polymorphisms (SNPs), may play a role in the tumorigenesis and progression of osteosarcoma. Better understanding on genes factors is needed to identify the prognostic markers and therapeutic targets.

NAT2 gene encodes a Phase II xenobiotic-metabolizing enzyme.[2] As a Phase II metabolizing enzyme, NAT2 catalyzes the metabolic activation of aromatic and heterocyclic amine carcinogens via O-acetylation and N-acetylation. Given that a single-nucleotide alteration causing substitution of amino acid residues may play a role in affecting the biological activity of the gene product,[3] polymorphism in *NAT2* gene may be correlated with cancer risk and outcome.

A substantial number of researches on different ethnic populations have revealed that *NAT2* alleles have an impact on the risk to a variety of malignancies, including acute lymphoblastic leukemia,[4] lung squamous carcinoma,[5] urinary bladder cancer,[6] gastric cancer,[7] and so on. However, to the best of our knowledge, there is still no study on the correlation between *NAT2* polymorphisms and osteosarcoma incidence. Assuming that osteosarcoma risk can be linked to ionizing radiation exposure, it is plausible that genetic alterations in *NAT2* gene may modulate osteosarcoma incidence.

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Hence, we launched a project on *NAT2* polymorphisms in children and teenaged osteosarcoma patients in four institutions in 2007, in order to unveil the puzzle that whether *NAT2* polymorphisms were associated with osteosarcoma incidence and risk of tumor metastasis in young-aged individuals. In this case-control study, we performed genotyping analyses of seven *NAT2* tagging SNPs in 260 osteosarcoma patients and 286 controls in East China population, who share similar diet customs and living environment.

Materials and methods

Ethics approval

This case-control study was approved by the Ethics Committees of four institutions (Xinhua Hospital, The Second Affiliated Hospital of Zhejiang University School of Medicine, The First People's Hospital of Wenling, and Fudan University) and was performed according to the Declaration of Helsinki. Informed consents were obtained from all participants or their guardians involved in the study.

Study subject

This study included 260 newly diagnosed osteosarcoma cases under the age of 20 years and 280 cancer-free controls, in the period between February 2007 and March 2012. All individuals involved in this study were specified as Chinese Han people. Diagnosis was confirmed by histopathological examination before radiotherapy and chemotherapy. All the included osteosarcoma cases underwent proper surgical operations by experienced surgeons, as well as nonsurgical therapeutic regimens according to the protocol. Detailed information such as tumor location and stage of osteosarcoma was obtained from medical records. All included patients were followed up regularly from the time of diagnosis for at least 36 months. The cancer-free controls were all recruited from trauma-induced fracture cases and were matched to osteosarcoma subjects by age and sex. Samples of 10 mL venous blood were obtained from each individual, and tumor tissues were conserved in liquid nitrogen.

DNA isolation

DNA was isolated from blood samples, which were collected into EDTA tubes, by using standard phenol–chloroform extraction and ethanol precipitation. DNA Blood Mini Kit (Qiagen, Berlin, Germany) was also used to isolate genomic DNA. As blood samples of 16 osteosarcoma patients were lost in an accident, corresponding tissue samples were used to extract DNA, with proteinase K digestion followed by phenol–chloroform extraction.

SNP selection and genotyping

Seven SNPs were selected in this study, that is, *NAT2* rs1799929, rs120, rs1041983, rs1801280, rs1799930, rs1799931, and rs1801279. Date collection was conducted utilizing the Sequence Detection Software on an ABI StepOnePlus System (Thermo Fisher Scientific, Waltham, MA, USA). TaqMan primers and probes were designed using the ABI Assay-by-Design custom service. Samples were performed in triplicate and averaged. Genotyping results were validated by repeating once more. Amplification conditions on ABI StepOnePlus were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and at 60°C for 60 seconds. The completed PCR plates were read with the ABI software. Laboratory personnel were blinded to the case-control status. NAT2 alleles were identified according to the Human *NAT2* Alleles (Haplotypes) (http://nat.mbg.duth.gr/Human%20NAT2%20alleles_2013.htm).

Statistical analysis

The χ^2 test was used to evaluate the differences in distributions of subject characteristics, selected variables, and genotypes of *NAT2* variants between the osteosarcoma cases and controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to estimate the associations between the seven SNPs and the risk of osteosarcoma. Crude ORs were computed using logistic regression analyses, and adjusted ORs were adjusted for age and sex. The Hardy–Weinberg equilibrium for SNPs was tested with the Pearson's χ^2 test. All statistical analyses were two-sided, and $P<0.05$ was considered as statistically significant. SPSS software (v.21.0; IBM Corporation, Armonk, NY, USA) was used to perform all analyses above.

Results

Clinical features

Clinical features of the included individuals are shown in Table 1. In this study, 260 young osteosarcoma cases and 286 controls were included. The median age of the 260 cases (151 males and 109 females) was 15.90 years (6–20) and that of controls was 16.24 years. All malignancies were graded according to Enneking GTM system.[8] Differences in age and sex did not show statistical significance between two groups (P=0.228 and 0.559, respectively).

Associations between NAT2 tagging polymorphisms and risk of osteosarcoma

The seven candidate SNPs (rs1799929, rs1208, rs1041983, rs1801280, rs1799930, rs1799931, and rs1801279) and the incidence risk of osteosarcoma are shown in Table 2.

Table 1 General characteristics of the subjects

Variables	Osteosarcoma cases [n (%)]	Control [n (%)]	*P*-value
Age			
Mean ± SD (year)	15.90±3.37	16.24±3.30	0.228
Sex			
Male	151 (58.08)	159 (55.59)	0.559
Female	109 (41.92)	127 (44.41)	
Location			
Trunk	33 (12.69)		
Limbs	227 (87.31)		
Enneking stages			
IA or IB	41 (15.76)		
IIA or IIB or III	219 (84.24)		
Operation			
Amputation	53 (20.38)		
Limb salvage	207 (79.62)		
Metastasis			
Yes	47 (18.07)		
No	213 (81.93)		

Genotype distributions of all these SNPs were in the Hardy–Weinberg equilibrium in the control group (*P*=0.295, 0.063, 0.115, 0.205, 0.216, 0.081, and 0.125, respectively). In the single locus analyses, there was no statistically significant difference in genotype frequencies of *NAT2* rs1041983 C>T SNP between the cases and the controls (*P*=0.081). When the CC homozygote genotype was used as the reference group, the CT genotype was associated with a decreased risk of osteosarcoma (CT versus CC: crude OR =0.67, 95% CI =0.47–0.96, *P*=0.029; adjusted OR =0.68, 95% CI =0.47–0.97, *P*=0.033), whereas the TT genotype was not related to the risk of osteosarcoma (TT versus CC: crude OR =0.94, 95% CI =0.54–1.63, *P*=0.816). In the dominant model, a borderline statistical difference was found between CT/TT variants with a decreased risk of osteosarcoma when compared with the rs1041983 CC genotype (CT/TT versus CC: crude OR =0.72, 95% CI =0.51–1.01, *P*=0.057; adjusted OR =0.72, 95% CI =0.51–1.02, *P*=0.062). In the recessive model, when rs1041983 CC/CT genotypes were used as the reference group, no statistical significance was shown between the TT homozygote genotype and the risk of osteosarcoma.

Statistical significance was revealed in genotype frequencies of *NAT2* rs1799931 G>A SNP between the cases and the controls (*P*=0.035). The GA genotype was found to be associated with a decreased risk of osteosarcoma when GG homozygote genotype was used as the reference group (GA versus GG: crude OR =0.64, 95% CI =0.45–0.91, *P*=0.014; adjusted OR =0.65, 95% CI =0.45–0.93, *P*=0.017), whereas the AA homozygote genotype was not associated with osteosarcoma risk. In the dominant model, statistical difference was found between GA/AA variants with the risk of osteosarcoma when compared with the rs1799931 GG genotype (GA/AA versus GG: crude OR =0.63, 95% CI =0.44–0.90, *P*=0.010; adjusted OR =0.64, 95% CI =0.45–0.90, *P*=0.012). In the recessive model, no statistical significance was found between the AA homozygote genotype and the risk of osteosarcoma when GG/GA genotypes were used as the reference group.

The rest five SNPs (rs1799929, rs1208, rs1801280, rs199930, and rs1801279) were not associated with osteosarcoma susceptibility in young Chinese population.

NAT2 rs1041983 C>T SNP was associated with metastasis of osteosarcoma

Clinical characteristics (location, stage, operation, and metastasis) of osteosarcoma were used to further investigate the relationship between SNPs and the incidence of osteosarcoma (Table 3). The genotype CT frequency of rs1041983 in metastasis cases (59.57%) was greater when compared to cases without tumor metastasis (37.09%), and the difference in frequency distribution showed statistical significance (*P*=0.010). With respect to location, stage, and operation, no statistical difference was revealed. The rest six SNPs showed no statistical significance with clinical factors of osteosarcoma. The confounding variables are listed in Table 4.

Discussion

Basic researches on new preventive and therapeutic strategies, such as immunotherapy[9,10] for osteosarcomas, are showing promising targets. Moreover, expanded studies on risk factors of osteosarcoma will enrich our knowledge. It is widely accepted that both genetic and environmental factors contribute to the tumorigenesis of malignancies, including osteosarcoma. The emerging findings of susceptibility loci and genes correlated with osteosarcoma in the past years have provided insight into the diagnosis of this disease. NAT2 is an important xenobiotic-metabolizing enzyme, and *NAT2* gene polymorphisms may be associated with the risk of malignancies as xenobiotics such as radiation, smoking, and alcohol use may induce carcinogenesis.[11,12]

In this case-control study of osteosarcoma, we found that *NAT2* rs1799931 G>A SNP was associated with a decreased risk of osteosarcoma. A borderline statistical difference was found in genotype frequencies of *NAT2* rs1041983 C>T SNP between the cases and the controls. Furthermore, we analyzed the association of SNPs with some clinical parameters, including age, sex, tumor location, enneking grade, operation, and metastasis. We demonstrated that albeit the result that rs1041983 had a trend to be a protective factor

Table 2 Logistic regression analyses of correlations between *NAT2* rs1799929 C>T, rs1208 A>G, rs1041983 C>T, rs1801280 T>C, rs1799930 G>A, rs1799931 G>A, and rs1801279 G>A polymorphisms and risk of osteosarcoma

NAT2 genotype	Cases (n=266)		Controls (n=280)		Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
	n	%	n	%				
NAT2 rs1799929 C>T								
CC	236	90.77	254	88.81	1.00		1.00	
CT	20	7.69	30	10.49	0.72 (0.40–1.30)	0.272	0.72 (0.40–1.31)	0.283
TT	4	1.54	2	0.70	2.15 (0.39–11.86)	0.379	2.21 (0.40–12.22)	0.363
CT+TT	24	9.23	32	11.19	0.81 (0.46–1.41)	0.452	0.81 (0.46–1.43)	0.470
CC+CT	256	98.46	284	99.30	1.00		1.00	
TT	4	1.54	2	0.70	2.22 (0.40–12.22)	0.360	2.29 (0.41–12.62)	0.343
NAT2 rs1208 A>G								
AA	248	95.38	272	95.10	1.00		1.00	
AG	9	3.46	13	4.55	0.76 (0.32–1.81)	0.534	0.71 (0.29–1.69)	0.434
GG	3	1.15	1	0.35	3.29 (0.34–31.84)	0.304	3.08 (0.32–29.94)	0.333
AG+GG	12	4.62	14	4.90	0.94 (0.43–2.07)	0.878	0.87 (0.39–1.94)	0.741
AA+AG	257	98.85	285	99.65	1.00		1.00	
GG	3	1.15	1	0.35	3.33 (0.34–32.18)	0.299	3.13 (0.32–30.47)	0.325
NAT2 rs1041983 C>T								
CC	120	46.15	109	38.11	1.00		1.00	
CT	107	41.15	145	50.77	0.67 (0.47–0.96)*	0.029*	0.68 (0.47–0.97)*	0.033*
TT	33	12.69	32	11.19	0.94 (0.54–1.63)	0.816	0.93 (0.54–1.62)	0.803
CT+TT	140	53.85	177	61.89	0.72 (0.51–1.01)	0.057	0.72 (0.51–1.02)	0.062
CC+CT	227	87.31	254	88.81	1.00		1.00	
TT	33	12.69	32	11.19	1.15 (0.69–1.94)	0.588	1.15 (0.68–1.93)	0.610
NAT2 rs1801280 T>C								
TT	238	91.54	247	86.36	1.00		1.00	
TC	20	7.69	36	12.59	0.58 (0.32–1.02)	0.060	0.59 (0.33–1.05)	0.070
CC	2	0.77	3	1.05	0.69 (0.12–4.18)	0.688	0.72 (0.12–4.35)	0.717
TC+CC	22	8.46	39	13.64	0.59 (0.34–1.02)	0.057	0.60 (0.34–1.04)	0.068
TT+TC	258	99.23	283	98.95	1.00		1.00	
CC	2	0.77	3	1.05	0.73 (0.12–4.41)	0.733	0.76 (0.12–4.61)	0.763
NAT2 rs1799930 G>A								
GG	158	60.77	167	58.39	1.00		1.00	
GA	73	28.08	98	34.27	0.79 (0.54–1.14)	0.209	0.79 (0.54–1.14)	0.210
AA	29	11.15	21	7.34	1.46 (0.80–2.67)	0.218	1.50 (0.82–2.74)	0.191
GA+AA	102	36.15	119	41.61	0.91 (0.64–1.28)	0.572	0.91 (0.65–1.28)	0.593
GG+GA	231	88.85	265	92.66	1.00		1.00	
AA	29	11.15	21	7.34	1.58 (0.88–2.85)	0.126	1.62 (0.90–2.94)	0.109
NAT2 rs1799931 G>A								
GG	177	68.08	164	57.34	1.00		1.00	
GA	77	29.62	112	39.16	0.64 (0.45–0.91)*	0.014*	0.65 (0.45–0.93)*	0.017*
AA	6	2.31	10	3.50	0.56 (0.20–1.56)	0.266	0.54 (0.19–1.52)	0.242
GA+AA	83	31.92	122	42.66	0.63 (0.44–0.90)*	0.010*	0.64 (0.45–0.90)*	0.012*
GG+GA	254	97.69	276	96.50	1.00		1.00	
AA	6	2.31	10	3.50	0.65 (0.23–1.82)	0.414	0.63 (0.22–1.75)	0.373
NAT2 rs1801279 G>A								
GG	251	96.54	270	94.41	1.00		1.00	
GA	8	3.08	15	5.24	0.57 (0.24–1.38)	0.213	0.59 (0.24–1.41)	0.236
AA	1	0.38	1	0.35	1.08 (0.07–17.29)	0.959	1.21 (0.08–19.61)	0.892
GA+AA	9	3.46	16	5.59	0.61 (0.26–1.40)	0.238	0.63 (0.27–1.44)	0.271
GG+GA	259	99.62	285	99.65	1.00		1.00	
AA	1	0.38	1	0.35	1.10 (0.07–17.68)	0.946	1.24 (0.08–20.10)	0.878

Notes: ORs were adjusted for age and sex. *Statistically significant ($P<0.05$).
Abbreviations: CI, confidence interval; OR, odds ratio.

Table 3 Association between genotype frequencies of rs1041983 and clinical features in osteosarcoma cases

Variables	n	CC	CT	TT	*P*-value
Location					
Trunk	33	13 (39.39)	16 (48.48)	4 (12.12)	0.645
Limbs	227	107 (47.14)	91 (40.09)	29 (12.78)	
Enneking stages					
IA or IB	41	21 (51.22)	13 (31.71)	7 (17.07)	0.357
IIA or IIB or III	219	99 (45.21)	94 (42.92)	26 (11.87)	
Operation					
Amputation	53	30 (56.60)	19 (35.85)	4 (7.55)	0.181
Limb salvage	207	90 (43.48)	88 (42.51)	29 (14.01)	
Metastasis					
Yes	47	17 (36.2)	28 (59.57)	2 (4.26)	0.010*
No	213	103 (48.36)	79 (37.09)	31 (14.55)	

Notes: *Statistically significant ($P<0.05$). Data presented as n (%).

in the risk of osteosarcoma, it was related to metastasis, predicting poor prognosis. Meanwhile, although rs1799931 played a protective role in carcinogenesis of osteosarcoma, it was not effective specifically to cases with different ages, sex, locations, grades, operations, and metastasis. These findings indicated that NAT2 was playing multifaceted roles in carcinogenesis and progression of osteosarcoma. This is the first research on associations between *NAT2* polymorphisms and osteosarcoma in young Chinese population. Although the possible mechanisms of NAT2 affecting the tumorigenesis of osteosarcoma have barely been studied, we provided the evidence that *NAT2* gene was related to osteosarcoma.

There are several studies on *NAT2* SNPs and malignancy risk, providing different results. In researches on lung cancer, *NAT2* rs1799930 G>A SNP was revealed to have a protective role in resisting lung squamous carcinoma in Chinese smokers.[5] *NAT2* gene polymorphism was also found to be associated with the risk of development of acute lymphocytic leukemia in Egyptian children.[4] A meta-analysis on rs1799930 and rs1799931 polymorphisms with the risk of multiple types of cancer demonstrated that *NAT2* rs1799930 was associated with an elevated risk of cancer in Asian, whereas *NAT2* rs1799931 polymorphism was a protective factor against cancer development.[13] There were also negative results in studies on prostate cancer in Slovak population,[14] esophageal cancer in Chinese population,[15] and breast cancer in Lebanese women.[16] Varied results in different ethnic populations call for further studies to investigate *NAT2* polymorphisms in different malignancies.

Table 4 Confounding variables

Confounding variables	Metastasis cases (n [%])	Nonmetastasis cases (n [%])	*P*-value
Age			
Mean ± SD (year)	15.68±3.48	15.95±3.35	0.623
Sex			
Male	29 (61.70)	122 (57.28)	
Female	18 (38.30)	91 (42.72)	0.578

Gene factors, such as epigenetic mechanisms and recurrent genomic rearrangements, play important roles in carcinogenesis and progression of osteosarcoma.[17,18] SNPs were also revealed to be associated with susceptibility and prognosis of osteosarcoma in different ethnic populations, such as *GRM4* gene,[19] *COL1A1*,[20] and *BMP-2*.[21] Albeit a rare malignancy, whose incidence is approximately 3–4 per million people, osteosarcoma is threatening the health of children and teenagers worldwide. More in-depth researches are in urgent need to unveil the elusive mechanisms of the development of osteosarcoma.

There are still several limitations in this case-control study. First, all the patients and controls were enrolled from three hospitals, and related inherent bias may have already affected our results. Second, the sample size in our study was moderate, which may limit the statistical power. Further replication studies are needed. We are collecting data from newly diagnosed cases to strengthen this project.

In summary, this study demonstrated that *NAT2* rs1799931 polymorphism played a role in osteosarcoma resistance in young Chinese individuals. *NAT2* rs1041983 polymorphism was related to metastasis of osteosarcoma, predicting poor prognosis.

Disclosure

The authors report no conflicts of interest in this work.

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