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Original Article

Association between *NAT2* genetic polymorphism and osteosarcoma susceptibility

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Abstract: Objective: This study was conducted to investigate the association between N-acetyltransferase 2 (*NAT2*) gene polymorphism and the susceptibility to osteosarcoma (OS) and to explore the functional activity significance of *NAT2* gene polymorphism in OS susceptibility. Methods: 283 cases of OS were selected as the OS group, and 264 subjects who received health examination in The Third People's Hospital of Qingdao during January 2012 and September 2014 were selected as the control group. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was applied to identify the *NAT2* gene polymorphism, and SPSS 21.0 software was applied for data analyses. Results: Four types of WT, M1, M2 and M3 *NAT2* alleles were identified among the 283 OS patients, including the rapid acetylators genotype and the slow acetylators genotype. The frequencies of *NAT2* genotypes (homozygous wild-type WT/WT, heterozygous mutant WT/Mx, homozygous mutant Mx/Mx) were 36.36%, 51.14%, and 12.50% in the control group, and 47.35%, 45.23%, and 7.42% in the OS group, and the frequencies of the two groups were significantly different (all $P < 0.05$). The frequencies of the four alleles were not significantly different between the two groups (all $P > 0.05$). The frequency of the rapid acetylators genotype in the OS group was significantly higher than that of the control group (92.58% vs. 87.50%), while the frequency of the slow acetylators genotype in the control group was significantly lower than that of OS group (7.42% vs. 12.50%, $\chi^2 = 3.961$, $P < 0.05$). Patients with the rapid acetylators genotype was 1.782 times higher in risk of OS formation than patients with the slow acetylators genotype (95% CI = 1.003~3.168, $P < 0.05$). The OS patients with the rapid acetylators genotype had bigger tumor size (OR = 3.706, 95% CI = 1.007~9.396, $P = 0.039$), lower tumor differentiation (OR = 3.350, 95% CI =1.192~9.414, $P = 0.016$), and higher transitivity (OR = 5.116, 95% CI = 1.917~13.65, $P < 0.001$) compared with those with the slow acetylators genotype. Age, gender, tumor location were not significantly different (all $P > 0.05$). Conclusion: *NAT2* gene polymorphism may be associated with OS susceptibility, and *NAT2* rapid acetylators genotype may be a risk factor for OS.

Keywords: N-acetyltransferase 2, osteosarcoma, rapid acetylators genotype, slow acetylators genotype

Introduction

Osteosarcoma (OS), which may occur in any bone (the frequent sites are femur, tibia and the humerus), is the most common bone malignancy and most frequently observed in children and young adults [1, 2]. It originates from primitive bone-forming mesenchymal cells and is characterized by complex, unbalanced karyotypes and changes in multiple genes and pathways [3]. Many studies have been conducted on the etiology of OS, but the exact mechanism remains poorly understood. With the rapid development of genetics and molecular biology, more and more researchers have started to investigate the mechanism of OS development at the genetic level, which is significant for developing new clinical methods of detection, diagnosis, and treatment for OS [4, 5].

N-acetyltransferase 2 (*NAT2*), which is known to metabolize the carcinogen arylamine, is based on several point mutations in the coding area [6]. The polymorphism of the *NAT2* gene, known as an important determinant of individual susceptibility to OS, has also been reported to be associated with the susceptibility to various types of cancers and some other diseases [7, 8]. Based on the speed of acetylation, *NAT2* polymorphisms can be divided into two types,

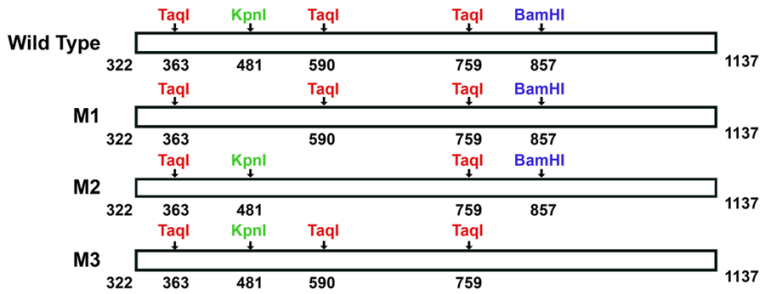

Figure 1. Restriction enzyme sites of four *NAT2* alleles (Wild type: Wild allele; M1, M2, M3: mutant allele; arrow (↓) indicates restriction enzyme sites); Restriction enzyme sites of four *NAT2* alleles. 815 bp (322~1137) for PCR-amplified products, arrow (↓) for restriction enzyme sites.

the rapid acetylators genotype with autosomal dominant and the slow acetylators genotype with autosomal recessive. Notably, there were very few studies describing in detail the association between *NAT2* gene polymorphisms and OS susceptibility [9, 10]. This study focused on the association between *NAT2* gene polymorphisms and OS susceptibility with an expectation of providing a new and effective target for the treatment of OS.

Materials and methods

Study subject

A total of 283 cases of OS were selected as the OS group from the Third People's Hospital of Qingdao during January 2012 and September 2014. Another 264 subjects who received health examination in the same hospital were selected as the control group during the same period. Inclusion criteria for the OS group: patients should be pathologically diagnosed as OS (including newly diagnosed patients and patients who were already diagnosed with OS) in accordance with the 2002 bone tumor classification standards of WHO [11], and the patients should have no history of other malignancies. There was no restriction on gender, age or pathological type. Inclusion criteria for the control group: patients without tumor history; patients without history of radiation therapy and/or chemotherapy treatment for unknown circumstances.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for identification of NAT2 gene polymorphisms

Peripheral blood sample (3 ml) was collected from each included subject. Furthermore, he extracted blood samples were added into the test tubes containing EDTA anticoagulant. DNA extraction was performed using a rapid extraction kit for whole blood genomic DNA (Aidlab Biotechnologies Co., Ltd, Beijing). The *NAT2* polymorphisms involved four types of gene mutations (M1, M2, M3, M4), but only three gene mutations (M1, M2, M3) were observed in this study, because M4 gene mutation only exists in African people and their descendants [12]. PCR-RFLP was adopted to detect polymorphism for restriction enzyme could identify different loci of NAT structural gene. There were 100 ng of genomic DNA, 200 nmol/L of sense primer and anti-sense primer, 1× PCR of buffer solution (produced by Shanghai Biological Engineering Technology Services Limited) and 1 U of Taq enzyme (produced by Shanghai Biological Engineering Technology Services Limited) in the 50 μl reaction system. Reaction conditions: 5 min of denaturation at 94°C, 35 cycles of 45 s of denaturation, 40 s of annealing at 58°C, and 50 s of extension at 72°C, followed by 7 min of final extension at 72°C. Primers were designed as follows: 5'-CTTCTCCTGCAGGTGACCAT-'3 (sense primer) and 5'-AGCATGAATCACTCTGCTTC-'3 (anti-sense primer). 1 μl PCR products were extracted and added with 10 U of KpnI, TaqI and BamHI endonucleases, 5 μl of 10× endonuclease reaction buffer solution, followed by 3 hours of endonuclease digestion at 37°C and 10 min of endonuclease inactivation at 65°C afterward. Gel electrophoresis stained by 0.1% $AgNO_3$ solution was applied for gene classification after endonuclease reaction.

Analysis of detection results

Restriction enzyme sites of the four *NAT2* alleles are shown in **Figure 1**. *NAT2* gene mutation occurred mainly in three sites (481, 590 or 857), and there were four types of alleles detected in this study: wild type (WT), M1, M2 and M3. M1 allele represented loss of Kpn Ñ restriction site; M2 allele represented loss of Taq Ñ restriction site; M3 allele represented loss of BamH Ñ restriction site. *NAT2* rapid acetylators genotype contained WT genotype (WT/WT, WT/M1, WT/M2, WT/M3), which

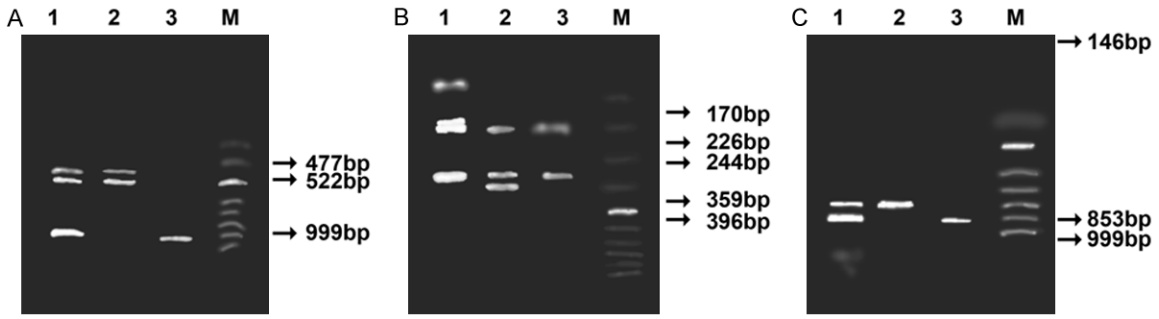

Figure 2. Agarose gel electrophoresis of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) fragments. A. The fragment length of homozygous mutant (M1/M1) was 999 bp; the fragment lengths of heterozygous mutation (WT/M1) were 999 bp, 477 bp and 522 bp. B. The fragment lengths of heterozygous mutation (WT/M2) were 170 bp, 226 bp, 244 bp, 359 bp and 396 bp. C. The fragment lengths of heterozygous mutant (WT/M3) were 146 bp, 853 bp and 999 bp.

Table 1. Frequencies of alleles/genotypes of the *NAT2* SNPs between OSAs group and Control group

Genotypes	Control group (%) (n = 264)	Case group (%) (n = 283)	OR (95% CI)	*P*
WT/WT	96 (36.36%)	134 (47.35%)	Ref.	
WT/Mx	135 (51.14%)	128 (45.23%)	0.679 (0.476~0.970)	0.033
Mx/Mx	33 (12.50%)	21 (7.42%)	0.456 (0.248~0.836)	0.010
WT	327 (61.93%)	396 (69.96%)	Ref.	
M1	34 (6.44%)	25 (4.42%)	0.607 (0.355~1.039)	0.066
M2	94 (17.80%)	83 (14.66%)	0.729 (0.524~1.014)	0.060
M3	73 (13.83%)	62 (10.96%)	0.701 (0.485~1.014)	0.059

Note: Mx = M1+M2+M3; OR, odd ratio; 95% CI, 95% confidence interval; Ref, reference.

belonged to homozygous wild type or heterozygous wild type; *NAT2* slow acetylators genotype didn't contain WT genotype (M1/M1, M1/M2, M1/M3, M2/M2, M2/M3, M3/M3), which were all homozygous mutant.

Statistical analysis

The SPSS 21.0 software (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. The quantitative data were presented as mean ± standard deviation. χ^2 test of goodness-of-fit index (GFI) was applied to determine whether the genotype distributions of the two groups were in accordance with Hardy-Weinberg equilibrium or not. The categorical data were tested by χ^2 test. Comparisons on the distribution frequencies of the alleles and genotypes between the OS group and the control group were performed by χ^2 test. Odds ratios (OR) with 95% of confidence intervals (CI) were calculated to measure the relative risks. $P < 0.05$ was considered statistically significant.

Result

Genotype analysis of NAT2

Three genotypes of *NAT2* gene were obtained after PCR amplification and KpnI enzyme digestion, with the first mutant (M1) generated by the mutation procedure of C→418→T. The fragment lengths of homozygous wild-type (WT/WT) were 477 and 522 bp; the fragment length of homozygous mutant (M1/M1) was 999 bp; the fragment lengths of heterozygous mutation (WT/M1) were 999 bp, 477 bp and 522 bp (**Figure 2A**). *NAT2* gene also presented three genotypes after PCR amplification and TaqI enzyme digestion, with the second mutant (M2) was generated G→590→A. The fragment lengths of homozygous wild-type (WT/WT) were 170 bp, 226 bp, 244 bp and 359 bp; the fragment lengths of homozygous mutant (M2/M2) were 244 bp, 359 bp and 396 bp; the fragment lengths of heterozygous mutation (WT/M2) were 170 bp, 226 bp, 244 bp, 359 bp and 396 bp (**Figure 2B**). *NAT2* gene produced three genotypes after PCR amplification and BamHI enzyme digestion, with the third mutant (M3) generated by G→857→A. The fragment lengths of homozygous wild-type (WT/WT) were 146 and 853 bp; the homozygous mutant (WT/M3) fragment length was 999 bp. The fragment

Table 2. Frequencies of the *NAT2* SNPs of the rapid/slow acetylator between OSAs group and Control group

Groups	n	Rapid acetylator [n (%)]	OR[a] (95% CI)	Slow acetylator [n (%)]	OR[b] (95% CI)
Control group	264	231 (87.50)	1.782 (1.003~3.168)	33 (12.50)	0.561 (0.316~0.997)
OSAs group	283	262 (92.58)	$P < 0.05$	21 (7.42)	$P < 0.05$

Note: OR[a] (95% CI), compared with the Slow acetylator; OR[b] (95% CI), compared with the Rapid acetylator. OR, odd ratio; 95% CI, 95% confidence interval; Ref, reference.

Table 3. Associations between Clinical parameters and *NAT2* genotypes in OSAs patients

Clinical parameters		Rapid acetylator (n = 262)		Slow acetylator (n = 21)		OR (95% CI)	*P*
		n	%	n	%		
Age	≤ 25	156	59.54	15	71.43	1	
	> 25	106	40.46	6	28.57	0.589 (0.221~1.566)	0.284
Gender	Male	94	35.88	8	38.10	1	
	Female	168	64.12	13	61.90	0.909 (0.364~2.273)	0.839
Tumor site	Femur	145	55.33	10	47.62	1	
	Tibia	66	25.19	4	19.05	0.879 (0.266~2.905)	0.832
	Humerus	38	14.50	4	19.05	1.526 (0.454~5.137)	0.492
	Other bones	13	4.96	3	14.28	3.346 (0.817~13.70)	0.077
Tumor size	≤ 5 cm	110	41.98	4	19.05	1	
	> 5 cm	152	58.02	17	80.95	3.076 (1.007~9.396)	0.039
Tumor grade	Low	134	51.15	5	23.81	1	
	High	128	48.85	16	76.19	3.350 (1.192~9.414)	0.016
Metastasis	No	176	67.17	6	28.57	1	
	Yes	86	32.83	15	71.43	5.116 (1.917~13.65)	< 0.001

Note: OR, odd ratio; 95% CI, 95% confidence interval; Ref, reference.

lengths of heterozygous mutant (WT/ M3) were 146 bp, 853 bp and 999 bp (**Figure 2C**).

Distribution of NAT2 genotypes and alleles

The frequencies of all *NAT2* genotypes and alleles were in accordance with Hardy-Weinberg genetic equilibrium ($P > 0.05$). As shown in **Table 1**, the frequencies of *NAT2* genotypes (homozygous wild-type WT/WT, heterozygous mutant WT/Mx, homozygous mutant Mx/Mx) were 36.36%, 51.14%, and 12.50% in the control group, and 47.35%, 45.23%, and 7.42% in the OS group; the frequencies of the two groups were significantly different (all $P < 0.05$). But the frequencies of four *NAT2* alleles between the groups were not significantly different (all $P > 0.05$).

Distribution of rapid acetylators genotype and slow acetylators genotype between groups

As shown in **Table 2**, the frequency of the rapid acetylators genotype in the OS group was significantly higher than the control group (92.58% vs. 87.50%, $P < 0.05$), while the frequency of the slow acetylators genotype in the OS group was significantly lower than the control group (7.42% vs. 12.50%, $\chi^2 = 3.961$, $P < 0.05$). The risk of OS for subjects with the rapid acetylators genotype in *NAT2* was 1.782 times than the risk for subjects with the slow acetylators genotype (95% CI = 1.003~3.168, $P < 0.05$). Therefore, the rapid acetylators genotype might be a risk factor of OS.

Relationship of NAT2 gene polymorphism and clinical features of OS

The results in **Table 3** show that the tumor size of the OS patients with the rapid acetylators genotype were larger (OR = 3.706, 95% CI = 1.007~9.396, $P = 0.039$), lower in the degree of tumor differentiation (OR = 3.350, 95% CI = 1.192~9.414, $P = 0.016$) and higher level of metastasis (OR = 5.116, 95% CI = 1.917~13.65, $P < 0.001$) compared with the OS patients with the slow acetylators genotype. Age, gender,

tumor location were not significantly different (all $P > 0.05$).

Discussion

Expression dysregulation of several growth factors, such as transforming growth factor beta (TGF-β), connective tissue growth factor (CTGF), and insulin-like growth factors (IGFs), were suggested to accelerate the occurrence and development of OS [13-15]. In recent years, some studies have also pointed out that genetic polymorphisms of some other genes play important roles in the occurrence and development of OS, including *ARHGAP35* gene, *FGFR3* gene, *MDM 2* gene, *FGFR 3* gene, and *p53* genes [16, 17]. Some genes polymorphisms may be associated with the prognosis of OS patients, such as ERCC1Asn118Asn, ERCC1Gln504Lys, ERCC2-Asp312Asn and ERCC2Lys751Gln [18]. Some gene polymorphisms will affect the treatment of OS, such as *ABCB1* and *ABCC3* [19]. However, the association between *NAT2* gene polymorphisms and OS remains poorly understood.

The genetic polymorphisms in *NAT2* expression are due to four mutant alleles (M1, M2, M3 and M4) and the NAT structural genes that modify recognition sites for restriction enzymes. The M1, M2, M3 and M4 mutant *NAT2* alleles can be identified by RFLP analysis, followed by *NAT2* amplification by PCR. *NAT2* activity can be identified through the combination of these *NAT2* alleles. According to several previous studies, the presence of at least one wild type (WT) allele results in a rapid acetylator phenotype, whereas carrying two mutant alleles results in a slow acetylator phenotype [6, 20, 21]. There are significant interethnic differences in certain *NAT2* allele frequencies [22, 23]. Thus, it is necessary to investigate the frequencies of all major polymorphic loci in the gene and the roles they might play in OS susceptibility.

Recently, a practical genotyping method has been developed to predict the acetylator genotype and to distinguish *NAT2* alleles by using three sets of PCR primers [6, 22, 24]. The results of this study indicate that the four *NAT2* alleles are not significantly different. However, the rapid genotype frequencies in the OS group were significantly higher than the control group, while the slow phenotype frequencies in the OS group were significantly lower than the control group. The rapid acetylators genotype not only can catalyze the N-acetylation of aromatic hydroxylation to achieve the effect of detoxification, but also catalyze O-acetylation of aromatic hydroxylation which can activate the performance of carcinogenicity, thus contributing to the occurrence of cancer cells. Therefore, patients with *NAT2* rapid acetylators genotype have a higher risk of OS than those with the slow acetylators genotype. All of the above indicates that *NAT2* gene polymorphism is associated with the occurrence and development of OS, and the rapid acetylators genotype may be a risk factor for OS.

In summary, this study documented that the rapid acetylators genotype in *NAT2* gene might be a risk factor for OS, which could be potentially used for the treatment and prognosis of OS. Since the development mechanism of *NAT2* gene polymorphism for the treatment of OS is still unclear, future studies are expected to further investigate the mechanisms.

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Disclosure of conflict of interest

None.

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References

[1] Schwab JH, Lozano-Calderon SA, Mankin HJ, Raskin KA, Hornicek FJ. What's new in primary bone tumors. J Bone Joint Surg Am 2014; 96: 2099-104.

[2] Dong YZ, Huang YX, Lu T. Single nucleotide polymorphism in the RECQL5 gene increased osteosarcoma susceptibility in a Chinese Han population. Genet Mol Res 2015; 14: 1899-902.

[3] Hattinger CM, Pasello M, Ferrari S, Picci P, Serra M. Emerging drugs for high-grade osteosarcoma. Expert Opin Emerg Drugs 2010; 15: 615-34.

[4] Wang J, Liu H, Liu X, Qi X. Effect of variation of FGF2 genotypes on the risk of osteosarcoma

susceptibly: a case control study. Int J Clin Exp Med 2015; 8: 6114-8.
[5] Wang K, Zhao J, He M, Fowdur M, Jiang T, Luo S. Association of GRM4 gene polymorphisms with susceptibility and clinicopathological characteristics of osteosarcoma in Guangxi Chinese population. Tumour Biol 2016; 37: 1105-12.
[6] Blum M, Demierre A, Grant DM, Heim M, Meyer UA. Molecular mechanism of slow acetylation of drugs and carcinogens in humans. Proc Natl Acad Sci U S A 1991; 88: 5237-41.
[7] Zhou W, Liu G, Thurston SW, Xu LL, Miller DP, Wain JC, Lynch TJ, Su L, Christiani DC. Genetic polymorphisms in N-acetyltransferase-2 and microsomal epoxide hydrolase, cumulative cigarette smoking, and lung cancer. Cancer Epidemiol Biomarkers Prev 2002; 11: 15-21.
[8] Rocha L, Garcia C, de Mendonca A, Gil JP, Bishop DT, Lechner MC. N-acetyltransferase (NAT2) genotype and susceptibility of sporadic Alzheimer's disease. Pharmacogenetics 1999; 9: 9-15.
[9] Zhang JW, Yu WJ, Sheng XM, Chang FH, Bai TY, Lv XL, Wang G, Liu SZ. Association of CYP2E1 and NAT2 polymorphisms with lung cancer susceptibility among Mongolian and Han populations in the Inner Mongolian region. Asian Pac J Cancer Prev 2014; 15: 9203-10.
[10] Tian FS, Shen L, Ren YW, Zhang Y, Yin ZH, Zhou BS. N-acetyltransferase 2 gene polymorphisms are associated with susceptibility to cancer: a meta-analysis. Asian Pac J Cancer Prev 2014; 15: 5621-6.
[11] Carvallo PI, Griffin AM, Ferguson PC, Wunder JS. Salvage of the proximal femur following pathological fractures involving benign bone tumors. J Surg Oncol 2015; 112: 846-52.
[12] Inatomi H, Katoh T, Kawamoto T, Matsumoto T. NAT2 gene polymorphism as a possible marker for susceptibility to bladder cancer in Japanese. Int J Urol 1999; 6: 446-54.
[13] Dong J, Demarest SJ, Sereno A, Tamraz S, Langley E, Doern A, Snipas T, Perron K, Joseph I, Glaser SM, Ho SN, Reff ME, Hariharan K. Combination of two insulin-like growth factor-I receptor inhibitory antibodies targeting distinct epitopes leads to an enhanced antitumor response. Mol Cancer Ther 2010; 9: 2593-604.
[14] Wang YH, Xiong J, Wang SF, Yu Y, Wang B, Chen YX, Shi HF, Qiu Y. Lentivirus-mediated shRNA targeting insulin-like growth factor-1 receptor (IGF-1R) enhances chemosensitivity of osteosarcoma cells in vitro and in vivo. Mol Cell Biochem 2010; 341: 225-33.
[15] Kolb EA, Kamara D, Zhang W, Lin J, Hingorani P, Baker L, Houghton P, Gorlick R. R1507, a fully human monoclonal antibody targeting IGF-1R, is effective alone and in combination with rapamycin in inhibiting growth of osteosarcoma xenografts. Pediatr Blood Cancer 2010; 55: 67-75.
[16] Hu X, Yu AX, Qi BW, Fu T, Wu G, Zhou M, Luo J, Xu JH. The expression and significance of IDH1 and p53 in osteosarcoma. J Exp Clin Cancer Res 2010; 29: 43.
[17] Tamura H, Fukada M, Fujikawa A, Noda M. Protein tyrosine phosphatase receptor type Z is involved in hippocampus-dependent memory formation through dephosphorylation at Y1105 on p190 RhoGAP. Neurosci Lett 2006; 399: 33-8.
[18] Hao T, Feng W, Zhang J, Sun YJ, Wang G. Association of four ERCC1 and ERCC2 SNPs with survival of bone tumour patients. Asian Pac J Cancer Prev 2012; 13: 3821-4.
[19] Caronia D, Patino-Garcia A, Perez-Martinez A, Pita G, Moreno LT, Zalacain-Diez M, Molina B, Colmenero I, Sierrasesúmaga L, Benítez J, Gonzalez-Neira A. Effect of ABCB1 and ABCC3 polymorphisms on osteosarcoma survival after chemotherapy: a pharmacogenetic study. PLoS One 2011; 6: e26091.
[20] Deguchi T, Mashimo M, Suzuki T. Correlation between acetylator phenotypes and genotypes of polymorphic arylamine N-acetyltransferase in human liver. J Biol Chem 1990; 265: 12757-60.
[21] Vatsis KP, Martell KJ, Weber WW. Diverse point mutations in the human gene for polymorphic N-acetyltransferase. Proc Natl Acad Sci U S A 1991; 88: 6333-7.
[22] Bell DA, Taylor JA, Butler MA, Stephens EA, Wiest J, Brubaker LH, Kadlubar FF, Lucier GW. Genotype/phenotype discordance for human arylamine N-acetyltransferase (NAT2) reveals a new slow-acetylator allele common in African-Americans. Carcinogenesis 1993; 14: 1689-92.
[23] Delomenie C, Sica L, Grant DM, Krishnamoorthy R, Dupret JM. Genotyping of the polymorphic N-acetyltransferase (NAT2*) gene locus in two native African populations. Pharmacogenetics 1996; 6: 177-85.
[24] Hickman D, Sim E. N-acetyltransferase polymorphism. Comparison of phenotype and genotype in humans. Biochem Pharmacol 1991; 42: 1007-14.