RESEARCH ARTICLE

PIK3CA and AKT Gene Polymorphisms in Susceptibility to Osteosarcoma in a Chinese Population

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Abstract

Purpose: To explore the association between PIK3CA and AKT single nucleotide polymorphisms(SNP) and osteosarcoma susceptibility. Methods: TaqMan polymerase chain reaction(PCR) was used to detect the genotypes of SNPs (rs7646409, rs6973569 and rs9866361) in peripheral blood samples from 59 patients with osteosarcoma and from 63 healthy controls. Unconditional logistic regression was used to analyze the correlation between SNPs and osteosarcoma risk. Results: No statistically significant difference was found between osteosarcoma patients and healthy controls in the genotype of AKT rs6973569 ($P = 0.7$). However, after stratified analysis, the genotype AA of AKT rs6973569 carried a higher risk of osteosarcoma metastasis (OR:2.94, 95%CL:1.00-8.59); the difference of rs7646409 genotype distributions between the case and control groups was statistically significant ($P = 0.032$). Taking genotype TT as a reference, the risk of osteosarcoma increased three fold in patients with genotype CC (OR:3.47, 95%CL:1.26-9.56). A statistically significant difference was found between the alleles C and T ($P=0.005$). Further analysis showed that the risk factor was more pronounced in male patients with Enneking's stage IIB and osteoblastic osteosarcoma. PIK3CA rs9866361 did not fit Hardy-Weinberg equilibrium ($P < 0.05$). Conclusions: Genotype CC in locus PIK3CA rs7646409 may increase the risk of osteosarcoma in the Chinese population.

Keywords: Osteosarcoma - single nucleotide polymorphisms - susceptibility - PIK3CA - AKT

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Introduction

Osteosarcoma is the most common primary malignant bone tumors (Hameed et al., 2011), the incidence is about 4.7 persons per million population per year, of which in men is slightly higher than in women (Linabery et al., 2008). Osteosarcoma occurs in children and adolescents, its favorite locations are the distal femur, proximal tibia and proximal humeral metaphyseal. Osteosarcoma has a high degree of malignancy, early lung metastases and poor prognosis. Although the neoadjuvant chemotherapy and surgical techniques improved the five-year survival of osteosarcoma patients from 20% -30% to 70% (Gorlick et al., 2009; Subbiah et al., 2011; Liu et al., 2012), the level of osteosarcoma treatment has not obtained a significant improvement in recent years, there are still 30% newly diagnosed patients eventually died of lung metastasis (Foster et al., 2007). In order to reduce the morbidity and mortality of osteosarcoma, we should further study its pathogenesis. Therefore, it is very important to study the osteosarcoma susceptibility gene and its signaling pathway for the diagnosis and treatment of the disease.

Osteosarcoma is a complex disease caused by a combined effect of genetic and environmental factors. Studies on tumor susceptibility gene are always hot in the field of molecular oncology, with the application of genome-wide linkage analysis (Savage et al., 2013), many susceptibility genes in monogenic disease were screened out. however, the studies of polygenic diseases such as cancer, have been extremely limited. So people began to turn their attention to single nucleotide polymorphism, which is expected to become the molecular markers as to predict disease susceptibility and guide individual treatment. Most of SNPs have no function, which do not affect the gene product, but those SNPs combined with the coding region may be functional. The single nucleotide polymorphisms of PIK3CA and AKT related to the tumorigenesis (Samuels et al., 2004; XingJC et al., 2012). PIK3CA is a key member of PI3K family members (Vivanco et al., 2002), and it encodes the p110α catalytic subunit of PI3K (Rosty et al., 2013). Akt is a Ser/Thr kinase comprised of Akt 1, 2, and 3 (Mure et al., 2010). PIK3CA and Akt are important components in the PI3K/Akt signaling pathway (Fresno Vara et al., 2004; Liu et al., 2008; Jiang et al., 2009). PI3k/Akt signaling pathway is regulated by multiple factors. The PI3K activation can be induced by dimer conformational change and direct binding between Ras and p110, resulting in the production of phosphatidylinositol 3, 4, 5 - triphosphate (PIP3) on the plasma membrane, the combination of the

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signaling proteins Akt containing PH domain in cells and PIP3, thereby transferring Akt from the cytosol to the membrane and obtaining catalytic activity. The activated Akt could further activate the downstream factors, such as mammalian target of rapamycin (mTOR), bc-2 family, E2F, glycogen synthesis kinase 3 (GSK3), fork-head related transcription factor (FKHR) and S6 protein kinase to regulate cell proliferation, apoptosis and migration.

Certain PIK3CA and AKT single nucleotide polymorphisms were found to be associated with other human tumors (Xing et al., 2010; Chen et al., 2012), but we want to know whether or not it could be associated with the incidence of osteosarcoma risk. The study was conducted to explore the association between PIK3CA, AKT single nucleotide polymorphisms and osteosarcoma susceptibility by detecting the genotypes of SNPs (rs7646409, rs6973569 and rs9866361) in peripheral blood samples from osteosarcoma patients and healthy controls.

Materials and Methods

59 osteosarcoma patients confirmed by histopathological diagnosis in First Affiliated Hospital of Guangxi Medical University were consided as the case group, which did not receive any treatment before. 63 healthy controls were randomly selected from healthy persons or trauma patients. People in case or control groups were all Chinese Han nationality. Osteosarcoma was staged according to Enneking-Musculoskeletal Tumor Staging System.Osteosarcoma patients received neoadjuvant chemotherapy.All cases were followed up after treatment, the detailed information included date of death and date of last follow-up.The study was agreed by institutional Review Board of Guangxi Medical University. All participants signed written informed consent.

2mL blood was extracted from each participant, anticoagulant was added in each sample. Accordance with the manufacturer's instructions, we used blood genomic DNA extraction kit (DP319-01, Tiangen Biochemical Science and Technology, China) to extract genomic DNA from peripheral blood. The concentration and purity of samples were measured by a spectrophotometer (NanoDrop 2000,USA), and samples finally stored at -20 °C. SNPs (rs7646409, rs6973569 and rs9866361) were selected from genotyped SNPs of Chinese populations in the HapMap database(http://snp.cshl.org/index.html). The genotypes of rs7646409, rs6973569 and rs9866361 were detected with the TaqMan probes C_28954299_10, C_22273625_1 and C_29787425_10 (Applied Biosystems, USA). The genotype results have been recorded and inputted independently by two experiments.

Statistical analysis

Statistical software SPSS19 (SPSS, USA)was used to run the statistical analysis. Two-sample T-test was used for the age difference between osteosarcoma patients and healthy controls. The differences of gender, genotype and allele frequency between the two groups was analyzed by χ^2 test. Goodness-of-fit χ^2 test was used to detect Hardy-Weinberg equilibrium (HWE). Kaplan-Meier method was used to compute the survival analysis.Unconditional logistic regression was used to analyze the correlation between SNPs and osteosarcoma.Statistically significance was set at $P < 0.05$.

Results

Clinical characteristics between cases and controls

This study included 59 osteosarcoma patients and 63 healthy controls, their age, gender, Enneking's stage, metastasis, tumor location, histologic subtype and other information were summarized in Table 1. The age difference was statistically significant between osteosarcoma patients and healthy controls ($P = 0.001$). The average age of the case and control groups were 21.25 ± 11.1 year-old and 31.25 ± 16.57 year-old, respectively. No significant difference was found between the two groups in gender ($P=0.44$), but we found that osteosarcoma was predominant in males, the incidence ratio of men versus women was approximately 1.5:1.

Association between genotypes of SNPs (rs7646409, rs6973569 and rs9866361) and osteosarcoma risk

The genotypes of SNPs (rs7646409, rs6973569 and rs9866361) detected by TaqMan PCR were shown in Figure 1.Genotype frequencies of PIK3CA rs7646409 and AKT rs6973569 in the control group were in accordance with HWE ($P > 0.25$, $P > 0.05$), but PIK3CA rs9866361 did not fit HWE ($P < 0.05$). The analysis showed that the

Table 1. Comparison of the Clinical Features of the Case and Control Groups

Variable	Case	Control	*P* value
Overall	59	63	
Age/year			0.001
Mean ± SD	21.25±11.1	31.25± 16.57	
age < 20	34	24	
age ≥ 20	25	39	
Gender			0.44
Female	24	30	
Male	35	33	
Tumor metastasis			
No	23		
Yes	36		
Enneking stages			
I	4		
IIA	11		
IIB	38		
III	6		
Histologic Subtype			
Mixed	17		
Chondroblastic	20		
Fibroblastic	6		
Osteoblastic	16		
Location			
Femur	28		
Tibia	19		
Humur	8		
Others	4		

The χ^2 test was used to compare gender between the two groups and the two-sample t-test was used to compare age

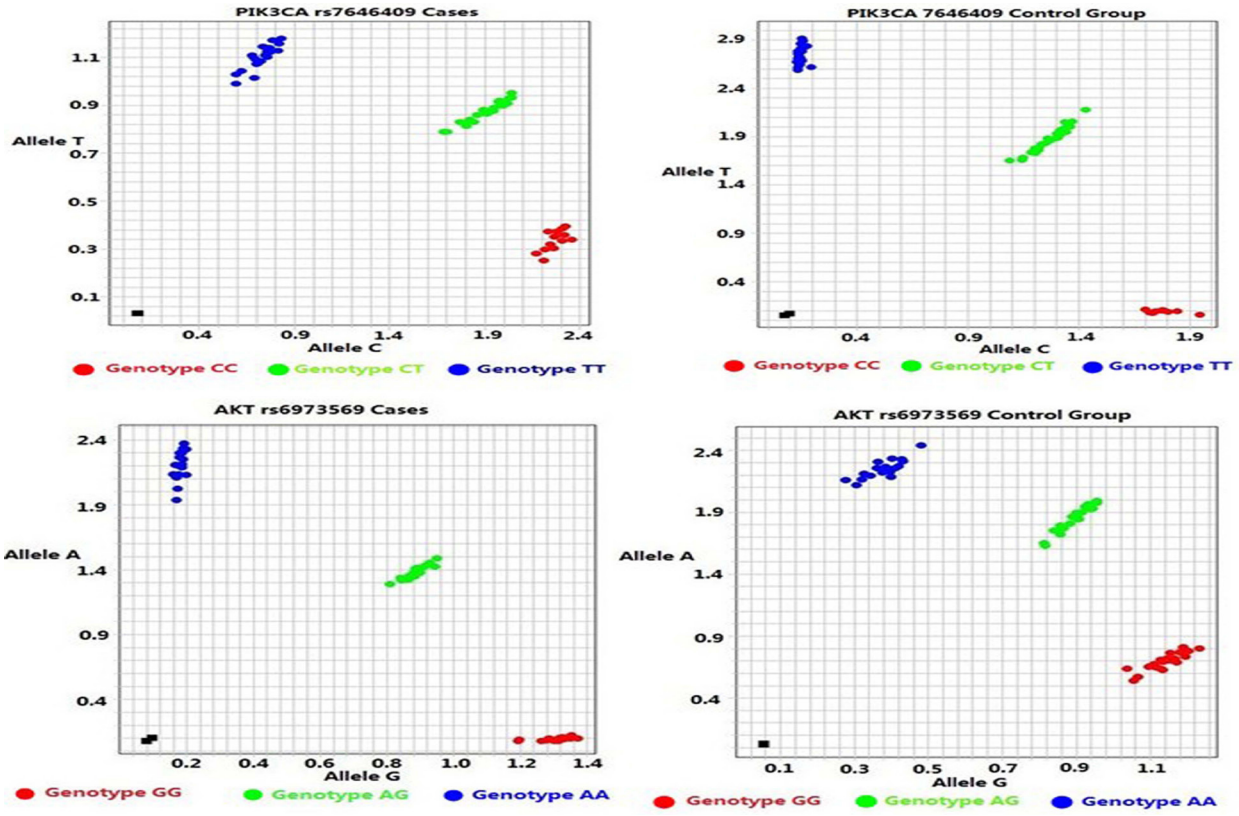

Figure 1. The Genotypes of PIK3CA rs7646409 and AKT rs6973569 Detected by TaqMan Method

difference of rs6973569 genotype distribution between the case and control groups was not statistically significant (*P*= 0.7, Table 2), while the difference of rs7646409 genotype distributions between the case and control groups was statistically significant (*P* = 0.032). Adjusted for age, gender and other relevant factors and taken genotype TT as a reference, the data showed that genotype CC increased the risk of osteosarcoma three folds (OR:3.47, 95%CL:1.26-9.56). Statistically significance was found between alleles in frequency distribution (*P*=0.005).

Association between genotype frequencies and clinicopathological features

In order to further investigate the PIK3CA gene rs7646409 polymorphism versus osteosarcoma risk, we run the stratified analysis in the clinicopathological features and genotype frequencies (Table 3). The results indicated that, no significance was found in people older than 20 years old. Genotype CC increased osteosarcoma risk by four-fold in men (OR:4.71, 95%CI:1.06-20.94). For PIK3CA rs7646409, the risk of suffering osteoblastic osteosarcoma in patients with genotype CC was five times than patients with genotype TT (OR:5.07, 95%CL:1.08-23.70). While, there was no statistically significance between mixed cell type and fibroblast.Compared with genotype TT, patients with genotype CC had more risk of suffering Enneking's stage II B (OR:3.52, 95%CL:1.11-11.17). Patients with genotype CC had a higher risk of osteosarcoma metastasis than those with genotype TT (OR:3.32, 95%CL:1.05-10.56). But there was no statistically significance between the tumor location and genotype.

For AKT rs6973569,after stratified analysis, patients with genotype AA had a higher risk of suffering chondroblastic osteosarcoma (OR:4.27, 95%CL:1.05-17.41). Furthermore, genotype AA of AKT rs6973569 carried the higher risk of osteosarcoma metastasis (OR:2.94, 95% CL:1.00-8.59).

Table 2. Association Between Genotypes of SNPs (rs7646409, rs6973569 and rs9866361) and Osteosarcoma Risk

Genotype	Case	Control	*P* value	OR(95%CL)
rs7646409			**0.032**	
CC	21	11		3.47(1.26-9.56)
CT	25	27		1.61(0.67-3.88)
TT	13	25		
			0.005	
C	67(56.8%)	49(46.2%)		
T	51(43.2%)	77(53.8%)		
rs6973569			0.7	
AA	20	17		1.60(0.64-4.05)
AG	21	24		1.19(0.49-2.86)
GG	18	22		
			0.376	
A	61(51.7%)	58(46.0%)		
G	57(48.3%)	68(54.0%)		
rs9866361			**0.025**	
AA	19	24		1.48(0.60-3.63)
AG	23	11		5.22(1.86-14.69)
GG	17	28		
			0.45	
A	61(50.8%)	59(46%)		
G	57(49.2%)	67(54%)		

P value was calculated by χ^2 test; OR(95%CL) was adjusted for age, gender and other relevant factors

Table 3. Association Between Genotype Frequencies and Clinicopathological Features

	rs7646409					rs6973569				
Characteristic	Genotype	Control	Case	*P*	OR(95%CL)	Genotype	Control	Case	*P*	OR(95%CL)
Age										
age < 20	CC	5	12	0.07	4.11(0.90-18.82)	AA	5	11	0.37	1.87(0.48-7.30)
	CT	10	17	0.13	2.87(0.74-11.16)	AG	9	11	0.95	1.04(0.30-3.56)
	TT	9	5			GG	10	12		
age ≥ 20	CC	6	9	0.11	3.04(0.78-11.89)	AA	12	9	0.53	1.52(0.41-5.62)
	CT	17	8	0.93	0.94(0.29-3.12)	AG	15	10	0.67	1.32(0.37-4.70)
	TT	16	8			GG	12	6		
Gender										
Female	CC	7	9	0.22	2.42(0.59-9.91)	AA	6	9	0.15	2.87(0.67-12.20)
	CT	12	9	0.64	1.37(0.36-5.15)	AG	13	9	0.67	1.33(0.36-5.00)
	TT	11	6			GG	11	6		
Male	CC	4	12	0.04	4.71(1.06-20.94)	AA	11	11	0.89	1.09(0.32-3.69)
	CT	15	16	0.35	1.77(0.54-5.81)	AG	11	12	0.87	1.11(0.33-3.68)
	TT	14	7			GG	11	12		
Tumor metastasis										
NO	CC	11	8	0.053	3.83(0.98-14.91)	AA	17	3	0.26	0.43(0.10-1.90)
	CT	27	10	0.4	1.70(0.50-5.78)	AG	24	11	0.77	1.18(0.40-3.45)
	TT	25	5			GG	22	9		
YES	CC	11	13	0.042	3.32(1.05-10.56)	AA	17	17	0.049	2.94(1.00-8.59)
	CT	27	15	0.347	1.64(0.59-4.60)	AG	24	10	0.77	1.18(0.39-3.56)
	TT	25	8			GG	22	9		
Enneking stages										
I	CC	11	2	0.4	3.04(0.23-40.55)	AA	17	0	0.99 0	0
	CT	27	1	0.92	0.87(0.05-15.84)	AG	24	3	0.51	2.29(0.20-26.18)
	TT	25	1			GG	22	1		
IIA	CC	11	2	0.68	1.52(0.21-11.25)	AA	17	2	0.48	0.51(0.08-3.20)
	CT	27	6	0.58	1.55(0.33-7.21)	AG	24	4	0.76	0.79(0.18-3.50)
	TT	25	3			GG	22	5		
IIB	CC	11	13	0.03	3.52(1.11-11.17)	AA	17	16	0.07	2.68(0.92-7.82)
	CT	27	17	0.21	1.91 (0.69-5.27)	AG	24	13	0.46	1.49(0.52-4.30)
	TT	25	8			GG	22	9		
III	CC	11	4	0.08	8.26(0.77-89.13)	AA	25	2	0.91	1.13(0.15-8.72)
	CT	27	1	0.95	0.91(0.05-16.09)	AG	11	1	0.38	0.34(0.03-3.77)
	TT	25	1			GG	27	3		
Histologic Subtype										
Mixed	CC	11	7	0.07	3.84(0.90-16.26)	AA	17	5	0.3	2.32(0.47-11.48)
	CT	27	6	0.71	1.30(0.32-5.27)	AG	24	9	0.13	3.08(0.71-13.25)
	TT	25	4			GG	22	3		
Chondroblastic	CC	11	5	0.13	3.52(0.69-18.07)	AA	17	10	0.04	4.27(1.05-17.41)
	CT	27	12	0.09	3.35(0.82-13.61)	AG	24	6	0.59	1.48(0.35-6.20)
	TT	25	3			GG	22	4		
Fibroblastic	CC	11	2	0.49	2.09(0.26-16.63)	AA	17	3	0.88	1.14(0.19-6.93)
	CT	27	1	0.33	0.31(0.03-3.23)	AG	24	0	0.99 0	0
	TT	25	3			GG	22	3		
Osteoblastic	CC	11	7	0.04	5.07(1.08-23.70)	AA	17	2	0.22	0.35(0.06-1.91)
	CT	27	6	0.43	1.83(0.41-8.15)	AG	24	6	0.6	0.72(0.21-2.45)
	TT	25	3			GG	22	8		
Location										
Femur	CC	11	9	0.07	3.34(0.93 -12.04)	AA	17	8	0.6	1.37(0.42 -4.51)
	CT	27	13	0.25	1.95(0.63-6.07)	AG	24	11	0.67	1.27(0.43-3.76)
	TT	25	6			GG	22	9		
Tibia	CC	11	7	0.08	3.60(0.88-14.75)	AA	17	8	0.29	2.03(0.55-7.45)
	CT	27	7	0.7	1.28(0.36-4.64)	AG	24	6	0.85	1.14(0.30-4.31)
	TT	25	5			GG	22	5		
Humurs	CC	11	3	0.16	5.82 (0.51-66.12)	AA	17	3	0.49	1.93(0.30-12.41)
	CT	27	4	0.32	3.25(0.33-32.58)	AG	24	2	0.62	0.61(0.09-4.32)
	TT	25	1			GG	22	3		
Others	CC	11	2	0.19	5.84(0.42-80.63)	AA	17	1	0.9	1.22(0.07-21.94)
	CT	27	1	0.95	0.91(0.05-15.73)	AG	24	2	0.59	1.98(0.16-23.89)
	TT	25	1			GG	22	1		

OR(95%CL) was adjusted for age, gender and other relevant factors

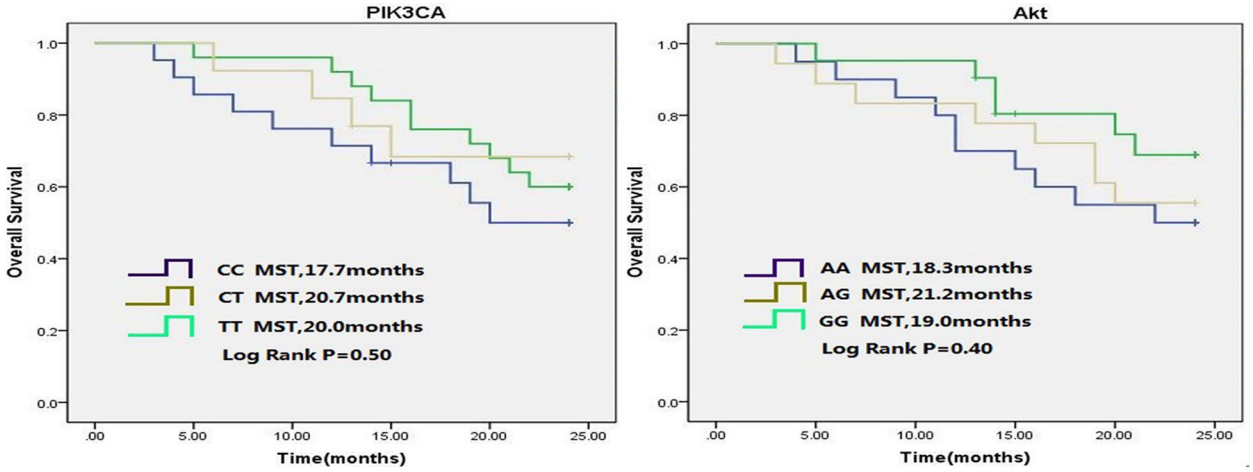

Figure 2. Analysis for Effects of SNPs on Survival of Case Group. MST: median survival time

Analysis for effects of SNPs on survival of case group

Kaplan–Meier curves of survival in osteosarcoma patients by genotypes were shown in Figure 2.The 24-month survival rate for the osteosarcoma patients was 59.3%.Survival analysis showed the Kaplan-Meier curves of survival in osteosarcoma patients by PIK3CA rs7646409 genotypes (CC vs CT vs TT: log-rank P = 0.50) and by Akt rs6973569 genotypes (AA vs AG vs GG: log-rank $P = 0.40$).

Discussion

So far, more and more evidence confirms that polymorphisms in PI3K/Akt signaling pathway play an important role in proliferation, apoptosis and metastasis (Liu et al., 2009; Courtney et al., 2010; Wang et al., 2012). It has been found that the PIK3CA polymorphism reduced the risk of follicular thyroid cancer in a case-control study of 433 cases and 530 controls (Xing et al., 2012). Hildebrandt et al. (2009)found that Akt polymorphism was associated with survival. Since the PI3K/Akt signaling pathway is related to the regulation of cell survival and death, PIK3CA and AKT as the core components of this signaling pathway, and their polymorphisms may change the regulation and lead to the change of incidence risk, so we selected the PIK3CA rs7646409 and AKT rs6973569 to investigate whether or not these SNPs were associated with the incidence of osteosarcoma risk in 59 cases and 63 controls.

Our observation and analysis showed that, compared with the TT genotype, genotype CC in rs7646409 could significantly increase the risk of osteosarcoma. Biologically, we speculate it is possible that the polymorphism of PIK3CA signal pathway will affect the core gene expression and lead to osteosarcoma. SNP rs7646409 is in the intron of PIK3CA gene. Although we did not find any evidence of feature of rs7646409, given the location of this SNP, with bioinformatics analysis, SNP may affect gene expression by transcription and regulatory factors to change the binding properties of the regulatory regions, finally interrupt the process of translation and splicing, then change the transcription of PIK3CA gene. The PIK3CA polymorphism increases the expression of p110α catalytic subunit of PI3K and then further triggers the activation of Akt through the PI3K/Akt signaling pathway, the activation of Akt further activate downstream factor to promote tumor cell proliferation and inhibit apoptosis. Choy 's study showed that PI3K mutations increased risk of osteosarcoma (Choy et al., 2012), this provides strong evidence for our speculation.

Furthermore, stratified analysis showed that the genotype CC had a greater risk of tumor metastasis for osteosarcoma patients, which may also associated with PI3K/Akt signaling transduction pathway for tumor metastasis. By the PI3K/Akt/mTOR/p70s6k pathway (p70S6K, ribosomal protein), the activation of p70s6K improved actin filaments reconstruction, increased cell motility, which conducive to tumor metastasis (Qian et al., 2004); Activity increased Akt1 also has an effect on enhancing cell motility by increasing the transcription of NF-ϰB; In addition, the PI3K/Akt/mTOR pathway could enhance degradation of extracellular matrix and promote tumor metastasis by up-regulating the protein expression of matrix metalloproteinase -2 (MMP-2) .Fendri's study found that PIK3CA amplification was associated with tumor metastasis, high degree of malignancy, low survival rate (Fendri et al., 2009), which is consistent with our findings.

Addition to those related discoveries of rs7646409, this study failed to demonstrate the association between rs6973569 polymorphism and osteosarcoma risk. However, the relationship between rs6973569 genotype AA and tumor metastasis was significance. Patients with genotype AA had a higher risk of osteosarcoma metastasis than those with genotype GG.This could be explained, Akt is a serine/threonine protein kinase expressed and coded by proto-oncogene c-akt, it has a molecular weighting about 60KD. Akt is one of the important components of PI3K/Akt signaling pathway. Its continued activation is closely related to occurrence and development of tumor. Akt family has 3 subtypes including Akt1, Akt2, Akt3(Nakatani K et al., 1999; Zhang L et al., 2011; Sung JS et al., 2012). And all 3 subunits have a similar protein structure and are coded and regulated by 3 different genes, the PH domain, kinase/catalytic domain and the regulatory domain are located from N to C terminal. PH domain of Akt mediates membrane translocation in Akt activation, whose mutations or deletions can lead to reduction or loss of Akt

activity, indicating that the PH domain has an important role. Akt activated by PI3K can activate downstream target proteins Bad, Caspase9, NF-κB, Forkhead and mTOR by phosphorylation, thereby regulating cell motility, proliferation and apoptosis. Statistical analysis showed that the difference of rs6973569 genotype distribution between the case and control groups was not significant, we estimate that the significant was masked because of a limited number of samples. Therefore, further studies will be needed to prove it.

Overall, our study indicated that the rs7646409 polymorphism increased the incidence of osteosarcoma risk in Chinese population, especially in males . However, this study is still imperfect. On one hand, all cases of this case-control research were from one hospital, we couldn't rule out inherent selection bias. On the other hand, sample quantity which we collected is small because of the low incidence of osteosarcoma; some findings were obtained based on the stratified analysis. The small sample quantity was even smaller after stratification, which reduced our statistical convincing. Therefore, if PIK3CA rs7646409 wants to become a genetic marker for the prediction of osteosarcoma incidence in Chinese population, a better and larger designed study will be needed to further confirm our conclusions.

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