Tumor Biol. (2016) 37:1105–1112
DOI 10.1007/s13277-015-3904-2

ORIGINAL ARTICLE

Association of *GRM4* gene polymorphisms with susceptibility and clinicopathological characteristics of osteosarcoma in Guangxi Chinese population

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Received: 24 June 2015 / Accepted: 5 August 2015 / Published online: 15 August 2015

Abstract Osteosarcoma is the most frequent malignant primary bone tumor. *GRM4* is expressed in human osteosarcoma cells, and high expression of mGluR4 in osteosarcoma tissues is related to poor prognosis. The aim of this study was to investigate the association between polymorphism of the *GRM4* gene and the susceptibility to osteosarcoma in a Chinese population. In a case–control study, we investigated polymorphisms in the *GRM4* gene (rs2229901, rs733457, and rs1906953) with a real-time quantitative polymerase chain reaction (PCR) assay (TaqMan). The study was conducted with 126 Chinese patients with osteosarcoma and 168 Chinese subjects in a control group. Unconditional logistic regression was used to analyze the correlation between single nucleotide polymorphisms (SNPs) and osteosarcoma risk. Different survival rates of different genotypic patients with osteosarcoma were analyzed through Kaplan–Meier. There were statistically significant differences in the distributions of the rs1906953 genotypes between the cases and control group (P=0.034). However, there was no remarkable difference in the three genotypes of GRM4 gene rs2229901 locus between the patient group and control group (P=0.369). Survival analysis for rs1906953 showed that the median survival time of osteosarcoma patients with the CC genotype was significantly shorter compared to the CT and TT genotypes; patients carrying CC genotype have apparently got a decrease in their recurrence-free survival time in comparison with patients carrying TT genotype. Our data suggest that *GRM4* gene polymorphism is closely related to the morbidity and metastasis of osteosarcoma in a Chinese population.

Keywords Osteosarcoma · *GRM4* · Single nucleotide polymorphism · Clinicopathological characteristics

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Introduction

Osteosarcoma is the most frequent malignant primary bone tumor and a main cause of cancer-related death in children and adolescents [1]. Its etiology is still unknown, but its genesis and progression may be regulated by genetic factors [2]. The osteosarcoma is very likely to happen in the metaphysis of the long bone; the most common bones of involvement include the femur at about 40 %, the tibia at about 20 %, the humerus at 10 %, and the pelvis at 8 % [3]. The average morbidity of osteosarcoma in all races is four cases per million population every year [4]. With the development of new adjuvant chemotherapy and surgical techniques, the 5-year survival rate has been enhanced from 20 to nearly 50 % [5, 6]. However, the prognosis of patients with recurrence and metastasis is still poor. Therefore, it is necessary to understand the natural history and biology of osteosarcoma to improve our therapeutic approaches [7].

As the most vital regulatory factor for human body, mGluRs are G-protein-coupled receptors (GPCRs) that have been subdivided into three groups, group I, II, and III, based on sequence similarity, pharmacology, and intracellular signaling

mechanisms [8]. Group I includes mGluR1 and mGluR5, which activates phospholipase C (PLC) pathway, leading to the hydrolysis of phosphatidylinositol (PI) and mobilization of intracellular Ca^{2+}. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) coupled negatively with adenylyl cyclase (AC) inhibit the formation of cyclic AMP (cAMP) and further suppress protein kinase A (PKA) [9–11].

Human's *GRM4* gene spans 178 Kbp on chromosome 6p21.3 and contains 15 introns and 16 exons (PubMed Databases). *GRM4* is a plausible candidate gene that has been implicated in intracellular signaling and inhibition of the cyclic AMP (cAMP) signaling cascade. The important role of the cAMP pathway in osteosarcoma has been demonstrated in mice, in which a cAMP-dependent protein kinase (Prkar1a) has been shown to suppress osteosarcoma tumor growth [12, 13]. The expression of mGluR4 in osteoblast and osteoclast indicates that glutamate signals are involved in cell differentiation and regulation during bone formation and reabsorption [14]. *GRM4* is expressed in human osteosarcoma cells [15] and high expression of mGluR4 in osteosarcoma tissues is related to poor prognosis [16]. *GRM4* has also been proved to be correlated with poor prognosis of many tumors such as malignant neuroglioma [9], colorectal cancer [17], pediatric CNS tumors [18], rhabdomyosarcoma and multiple myeloma [19], as well as cancer cell proliferation in vitro [20]. Together, these results suggest that *GRM4* could be implicated in osteosarcoma.

Single nucleotide polymorphism (SNP) in the *GRM4* gene has been detected in the coding region of the *GRM4* gene and its promoter region. Several single nucleotide polymorphisms in coding regions or noncoding regions have been identified such as rs9380405, rs2029461, and rs2229901 [21–23]. Shi has found that *GRM4* gene rs733457 polymorphism relates to neurotransmission systems with bipolar disorder [24]. To our knowledge, only one single nucleotide polymorphism (rs1906953) of *GRM4* relating to osteosarcoma has been found [25, 26]. Savage et al. [25] performed a multi-stage genome-wide association study (GWAS) consisting of 941 cases and 3291 cancer-free adult controls of European ancestry and concluded that rs1906953 is associated with susceptibility to osteosarcoma. Jiang found that rs1906953TT genotype carriers have shorter median survival time of osteosarcoma [26]. Therefore, we conducted a case–control study to examine whether mutations in the *GRM4* gene (rs2229901, rs733457, and rs1906953) are associated with the risk and prognosis of osteosarcoma in Guangxi Chinese population.

Material and methods

Subject selection

This study includes 126 patients who had been diagnosed with osteosarcoma at the First Affiliated Hospital of Guangxi Medical University; the 168 cancer-free control subjects were chosen from healthy physical examinees. All the cases of the study are Chinese population, and the follow-up period ranged from 1.0 to 36.0 months with a median follow-up of 20.0 months. This research was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University, and an informed consent form was signed by each case. Then, demographic information and clinical pathological data (including age, gender, race, histology, tumor location, tumor size, Enneking stages, therapy, and metastasis) were collected using a standard interviewer-administered questionnaire and/or medical records. For survival analysis, we followed up on all the patients. All osteosarcoma cases underwent serial monitoring every 2 months for the first 2 years and semiannually thereafter for detection of any recurrence. The survival status of the patients was confirmed by clinical records and either patient or family contact. The duration of overall survival (OS) was

Table 1 The characteristics of the subjects

Characteristics	Osteosarcoma, *n* (%)	Controls, *n* (%)	*P* value
Overall	126	168	
Age			0.009
<20	78 (61.9)	78 (46.4)	
≥20	48 (38.1)	90 (53.6)	
Mean age (years)	24.6±14.4	34.1±24.1	
Gender			0.589
Male	70 (55.6)	88 (52.4)	
Female	56 (44.4)	80 (47.6)	
Tumor location			
Femur	51 (40.5)		
Tibia	40 (31.7)		
Humerus	23 (18.3)		
Others	12 (9.5)		
Enneking stages			
I	19 (15.1)		
IIA	23 (18.2)		
IIB	67 (53.2)		
III	17 (13.5)		
Histologic type			
Osteoblastic	37 (29.4)		
Chondroblastic	47 (37.3)		
Fibroblastic	15 (11.9)		
Mixed	27 (21.4)		
Therapy			
Amputation	54 (42.9)		
Limb salvage	72 (57.1)		
Metastasis			
Yes	51 (40.5)		
No	75 (59.5)		

The chi-square test was used to compare age and gender between the two groups

defined as the date from the start of curative treatment to the date of death or last known date alive, whereas the recurrence-free survival (RFS) was defined as the date from the start of curative treatment to the date of tumor recurrence or last known date alive.

Genomic DNA extraction and genotyping

For all subjects, 2-ml samples of venous blood were placed in tubes with ethylenediaminetetraacetic acid (EDTA) anticoagulant for cryopreservation at −70 °C. The DNA extraction was performed using the TIANamp Blood DNA Kit (DP319-01, Tiangen Biochemical Science and Technology, China) according to the manufacturer's instructions. SNP information of *GRM4* gene was obtained from the HapMap project (http://snp.cshl.org/index.html). We choose the TagSNPs that accord with the threshold value of r^2=0.8 and MAF>0.05 by Haploview 4.0.

The *GRM4* (rs2229901, rs733457, and rs1906953) genotypes were analyzed by TaqMan polymerase chain reaction (PCR) on the Applied Biosystems Step One System (ABI, Foster City, USA). The PCR was performed in a total volume of 20 μL solution containing 10 μL TaqMan Universal PCR Master Mix (2×), 0.5 μL TaqMan SNP assay (40×), 2 μL (20 ng) template DNA, and 7.5 μL H_2O. The PCR cycle conditions were as follows: an initial step at 95 °C for 10 min followed by 40 cycles of 15 s at 92 °C, 30 cycles of 60 s at 60 °C, and holding at 4 °C. We also randomly selected 5 % of the cases and control subjects to repeat genotyping the three SNPs, and the results were confirmed with the previous results.

Statistical analysis

Data analysis was performed using SPSS 19.0 software (SPSS, Inc.). The differences of gender, age, allele, and genotype frequencies between patients and controls were tested by chi-square test. In addition, the chi-square test was used to verify that the observed allele distribution in the control group was in Hardy–Weinberg equilibrium. Odds ratio (OR) values and 95 % confidence interval (95 % CI) for each genotype frequencies and clinicopathological characteristics of osteosarcoma were estimated by unconditional logistic regression and adjusted for patients' age (as a continuous variable) and sex. Survival analysis was calculated by Kaplan–Meier method. In the present study, a P value <0.05 was considered statistically significant.

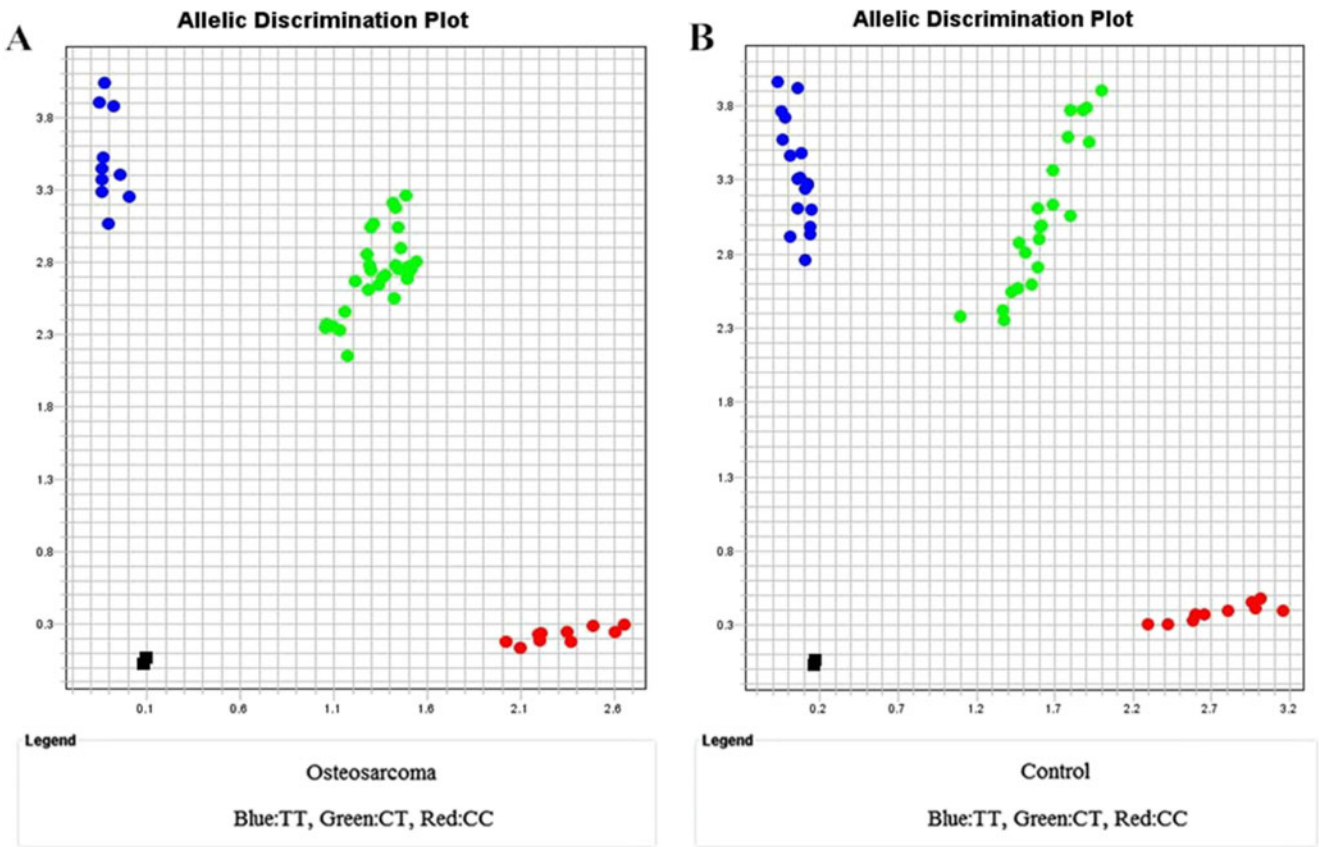

Fig. 1 The genotype of *GRM4* at rs11906953 detected by TaqMan method. **a** Osteosarcoma and **b** control allelic discrimination plots

Results

Patient characteristics

The clinical characteristics of the 126 osteosarcoma patients and the 168 controls are listed in Table 1. All subjects were of ethnic Chinese origin. Among the 126 osteosarcoma patients, 44.4 % (56/126) were female and 55.6 % (70/126) were male. The median age at diagnosis was 24.6 years (range, 6–72 years). The statistical analysis of age distribution between control and cases showed significant differences (P=0.009). Regarding sex distribution, no significant differences were found between controls and cases (P=0.589).

Distributions of alleles and genotypes

The associations of the three variants (rs2229901, rs733457, and rs1906953) in the 126 patients and 168 controls were analyzed (Fig. 1, Table 2). The genotype distributions of *GRM4* gene rs1906953 and rs2229901 showed no deviation from the expected Hardy–Weinberg equilibrium among the controls (P>0.05, P>0.05); however, the distribution of the rs733457 genotype was not in accordance with HWE (P<0.05).

As with rs2229901 and rs733457 genotype frequency distribution, there were no statistically significant differences (P=0.369 and 0.835, respectively) between the cases and control group. However, with respect to the rs1906953 allele frequency distribution, a significant difference of P=0.010 was observed between the cases and the control group. The analysis showed that patients carrying the CC genotype had a higher risk of osteosarcoma (OR=2.17, 95 % CI=1.10–4.30) than those with the TT genotype.

Association between GRM4 polymorphisms and clinicopathological characteristics in osteosarcoma patients

We further evaluated the associations of *GRM4* polymorphisms at rs1906953 and rs2229901 with clinical pathological factors in osteosarcoma patients. The results of stratification analysis with parameters of age, gender, tumor location, histology, Enneking stages, tumor size, therapy, and metastasis are shown in Table 3.

As for rs1906953, we found out that patients carrying the CC genotype have a higher risk of tumor metastasis than patients carrying the TT genotype (OR=3.09, 95 % CI=1.27–7.53). Patients carrying CC genotype with osteosarcoma are mostly in the period of Enneking IIB (OR=2.63, 95 % CI=1.14–6.08). There are no obvious differences in the age, gender, tumor location, histology type, therapy, and the rs1906953 genotype. As for rs2229901, there was no difference in frequency distribution between genotypes (Table 3).

Association between GRM4 gene polymorphisms and survival rate of patients with osteosarcoma

Results from the analysis of overall survival are presented in Figs. 2 and 3 and Table 3. *GRM4* polymorphism at rs1906953 is associated with overall survival time (P=0.021). We found that patients carrying CC genotype (17.14 months) and CT genotype (20.88 months) had shorter survival time when compared with subjects carrying TT genotype (22.74 months). We also found that there is an obvious difference in recurrence-free survival time (P=0.035), which shows that patients carrying the rs1906953CC genotype have shorter recurrence-free survival time, which is 15.52 months, than the TT genotype carriers, which is 20.45 months. As for rs2229901, there are no differences in the survival time among the three different genotypes of patients (P_{OS}=0.696; P_{RFS}=0.667).

Discussion

Osteosarcoma is the most common malignancy in clinical orthopedics, caused by the combined effects of genetic and

Table 2 Distribution of alleles and genotype of GRM4 gene

SNPs	Case	Control	*P* value	OR (95 % CI)
rs1906953				
Genotype			0.034	
TT	29	60		
TC	65	80		1.70 (0.98–2.98)
CC	32	28		2.17 (1.10–4.30)
Allele			0.010	
T	123	200		
C	129	136		
rs2229901				
Genotype			0.369	
GG	33	33		
GA	62	94		0.69 (0.38–1.24)
AA	31	41		0.84 (0.43–1.67)
Allele			0.446	
G	128	160		
A	124	176		
rs733457				
Genotype			0.835	
TT	60	79		
TG	46	58		1.26 (0.56–1.85)
GG	20	31		1.20 (0.63–2.29)
Allele			0.690	
T	166	216		
G	86	120		

P value was calculated by chi-square test; OR (95 % CI) was adjusted for age and gender

Table 3 Association between genotype frequencies and clinicopathological features

	rs1906953					rs2229901				
Features	Genotype	Control	Case	*P*	OR (95 % CI)	Genotype	Control	Case	*P*	OR (95 % CI)
Age										
<20	TT	15	18			GG	19	22		
	TC	35	37	0.276	1.53 (0.71–3.26)	GA	45	38	0.411	0.73 (0.34–1.55)
	CC	17	23	0.128	1.96 (0.82–4.68)	AA	14	18	0.828	1.11 (0.44–2.82)
≥20	TT	34	11			GG	14	11		
	TC	45	28	0.122	1.92 (0.84–4.40)	GA	49	24	0.308	0.62 (0.24–1.56)
	CC	11	9	0.101	2.54 (0.83–7.74)	AA	27	13	0.349	0.61 (0.22–1.71)
Gender										
Male	TT	31	16			GG	16	19		
	TC	41	35	0.182	1.68 (0.79–3.58)	GA	50	39	0.342	0.68 (0.31–1.50)
	CC	16	19	0.103	2.13 (0.86–5.27)	AA	22	12	0.168	0.50 (0.19–1.34)
Female	TT	29	13			GG	17	14		
	TC	39	30	0.196	1.72 (0.76–3.90)	GA	44	23	0.386	0.68 (0.28–1.64)
	CC	12	13	0.125	2.26 (0.80–6.37)	AA	19	19	0.545	1.35 (0.51–3.58)
Enneking stages										
I	TT	60	4			GG	33	4		
	TC	80	11	0.245	2.03 (0.62–6.71)	GA	94	10	0.878	0.91 (0.26–3.11)
	CC	28	4	0.366	1.97 (0.45–8.58)	AA	41	5	0.874	1.12 (0.27–4.60)
IIA	TT	60	8			GG	33	5		
	TC	80	9	0.889	0.93 (0.35–2.51)	GA	94	11	0.701	0.80 (0.26–2.49)
	CC	28	5	0.739	1.23 (0.36–4.19)	AA	41	7	0.713	1.27 (0.36–4.47)
IIB	TT	60	14			GG	33	18		
	TC	80	35	0.066	1.95 (0.96–3.96)	GA	94	33	0.266	0.67 (0.33–1.36)
	CC	28	18	0.023	2.63 (1.14–6.08)	AA	41	16	0.542	0.77 (0.34–1.77)
III	TT	60	3			GG	33	6		
	TC	80	9	0.234	2.30 (0.58–9.07)	GA	94	8	0.292	0.54 (0.17–1.71)
	CC	28	5	0.158	3.02 (0.65–14.01)	AA	41	3	0.436	0.55 (0.12–2.48)
Histologic type										
Osteoblastic	TT	60	8			GG	33	12		
	TC	80	17	0.340	1.56 (0.63–3.90)	GA	94	19	0.271	0.62 (0.27–1.45)
	CC	28	12	0.060	2.67 (0.96–7.41)	AA	41	6	0.202	0.49 (0.16–1.47)
Chondroblastic	TT	60	10			GG	33	9		
	TC	80	25	0.117	1.92 (0.85–4.33)	GA	94	27	0.850	1.09 (0.46–2.58)
	CC	28	12	0.063	2.49 (0.95–6.50)	AA	41	11	0.860	1.10 (0.40–3.01)
Fibroblastic	TT	60	4			GG	33	5		
	TC	80	7	0.670	1.32 (0.37–4.73)	GA	94	8	0.367	0.58 (0.17–1.91)
	CC	28	4	0.367	1.97 (0.45–8.57)	AA	41	2	0.239	0.35 (0.06–2.00)
Mixed	TT	60	7			GG	33	7		
	TC	80	16	0.290	1.67 (0.65–4.34)	GA	94	8	0.111	0.41 (0.14–1.23)
	CC	28	4	0.798	1.20 (0.32–4.46)	AA	41	12	0.463	1.48 (0.52–4.35)
Location										
Femur	TT	60	12			GG	33	13		
	TC	80	25	0.250	1.58 (0.73–3.43)	GA	94	27	0.518	0.77 (0.35–1.70)
	CC	28	14	0.082	2.24 (0.90–5.56)	AA	41	11	0.701	0.83 (0.32–2.16)
Tibia	TT	60	10			GG	33	11		
	TC	80	22	0.234	1.65 (0.72–3.78)	GA	94	17	0.162	0.54 (0.23–1.28)
	CC	28	8	0.396	1.57 (0.55–4.48)	AA	41	12	0.900	0.94 (0.36–2.44)

Table 3 (continued)

Features	rs1906953					rs2229901				
	Genotype	Control	Case	*P*	OR (95 % CI)	Genotype	Control	Case	*P*	OR (95 % CI)
Humerus	TT	60	5			GG	33	6		
	TC	80	11	0.488	1.49 (0.48–4.59)	GA	94	11	0.483	0.68 (0.23–2.00)
	CC	28	7	0.055	3.31 (0.98–11.20)	AA	41	6	0.867	0.90 (0.26–3.12)
Others	TT	60	2			GG	33	3		
	TC	80	8	0.170	3.04 (0.62–14.86)	GA	94	7	0.772	0.81 (0.19–3.38)
	CC	28	2	0.435	2.24 (0.30–17.07)	AA	41	2	0.486	0.51 (0.08–3.36)
Therapy										
Amputation	TT	60	13			GG	33	13		
	TC	80	26	0.284	1.51 (0.71–3.21)	GA	94	27	0.511	0.77 (0.35–1.68)
	CC	28	15	0.071	2.25 (0.93–5.42)	AA	41	14	0.950	0.97 (0.40–2.39)
Limb salvage	TT	60	16			GG	33	20		
	TC	80	39	0.083	1.82 (0.92–3.57)	GA	94	35	0.198	0.64 (0.32–1.27)
	CC	28	17	0.081	2.09 (0.91–4.77)	AA	41	17	0.537	0.78 (0.35–1.74)
Tumor metastasis										
No	TT	60	18			GG	33	19		
	TC	80	43	0.077	1.79 (0.94–3.43)	AG	94	34	0.254	0.67 (0.33–1.34)
	CC	28	14	0.290	1.57 (0.68–3.64)	AA	41	22	0.971	1.02 (0.47–2.21)
Yes	TT	60	11			GG	33	14		
	TC	80	22	0.322	1.51 (0.67–3.38)	AG	94	28	0.448	0.74 (0.34–1.60)
	CC	28	18	0.013	3.09 (1.27–7.53)	AA	41	9	0.320	0.6 1 (0.23–1.62)

environmental factors, with the genetic background having the most important role. In recent years, mounting evidence have shown that SNP is associated with the susceptibility and survival of osteosarcoma. Also, an increasing number of studies have revealed that the polymorphisms of many genes are associated with osteosarcoma susceptibility and/or survival. Liu found that −22G/C polymorphism reduced LOX expression and that −22G/C and 473G/A polymorphisms may be new risk factors for incidence of osteosarcoma [7]. *FGFR3* and *MDM2* polymorphisms may play a role in the formation of predisposition to osteosarcoma [27]. The rs8103851 polymorphism of the *PRKCG* gene is correlated to metastatic osteosarcoma and could be the risk factors for metastatic osteosarcoma [28]. In addition, *ABCB1*, *ABCC3*, and *GSTP1* polymorphisms may affect osteosarcoma treatment efficacy [29, 30]. However, since the functional genes for osteosarcoma are not very clear, much research is still being done in this field.

In this study, we explored the relationship between the three SNPs (rs2229901, rs733457, and rs1906953) in the *GRM4* gene and osteosarcoma risk. The results showed that only rs1906953 polymorphisms of *GRM4* gene were associated with the incidence risk of osteosarcoma, and those carrying CC genotype have two times risk of metastasis than patients carrying TT genotype (OR=2.17, 95 % CI=1.10–4.30). Savage and other researchers indicated that rs1906953 is associated with susceptibility to osteosarcoma by genome-wide association study [25]. In another research including 168 patients with osteosarcoma and 216 healthy controls, Jiang confirmed that rs1906953 in the glutamate receptor metabotropic 4 (*GRM4*) gene is associated with osteosarcoma in the Chinese Han population [26], which is in conformity with our research results. However, our data suggest that patients carrying the rs1906953CC genotype had a higher risk of osteosarcoma, but Jiang's results showed that those carrying the rs1906953TT genotype had a higher risk of osteosarcoma. This difference may be attributed to the relatively small number of cases and different population. Moreover, all our patients and control subjects are Chinese population (including Chinese Han) in the south of China.

Metabotropic glutamate receptors mainly involve in maintaining the stability of the internal environment of cells in the central nervous system. In pediatric CNS tumors, the metabotropic glutamate receptor 4 was expressed at higher levels in the malignant tumors than in low-grade astrocytomas. But, Kalariti found that MG-63 cells express the *GRM4*, which suggest that the Glu system has a potential role in bone pathophysiology [15]. Wu detected protein and mRNA expression level of mGluR4 in 40 osteosarcoma tissues and the corresponding adjacent normal tissues by Western blot and RT-PCR accordingly, which concluded that high mGluR4 expression is correlated with poor prognosis of osteosarcoma [16]. Moreover, most of the analyzed medulloblastoma tissue

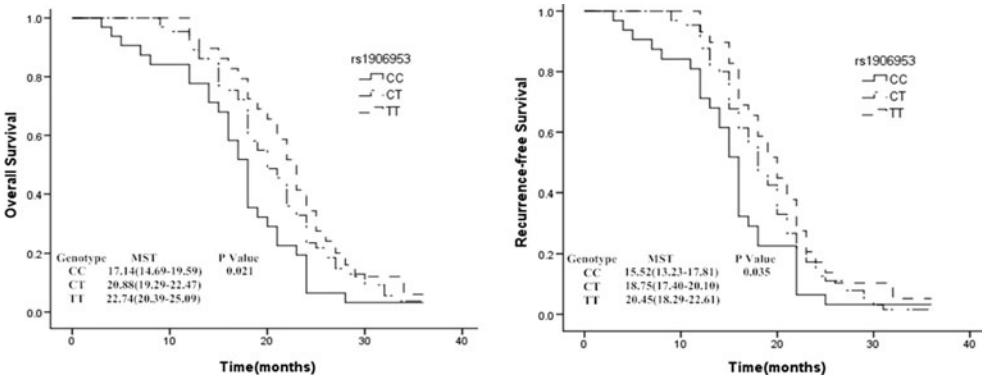

Fig. 2 *GRM4* rs1906953 polymorphism was correlated with the overall survival and the recurrence-free survival of osteosarcoma (P=0.021, P=0.035). *MST* median survival time

samples and medulloblastoma cell lines displayed the presence of mGluR4 receptors, which is inversely correlated with tumor growth [31].

The possibility of using *GRM4* SNPs as predictive biomarkers in other kinds of diseases has been studied. Muhle tested 17 SNPs spanning the *GRM4* gene and found that five of them showed significant association with IGE. The most significant SNP in the IGE cohort was rs9380405 located in the first intron of the *GRM4* gene [22]. Parihar concluded that *GRM4* rs2029461 polymorphism has significant association with the JME phenotype, which is predicted to gain MTE [23]. Fallin et al. [32] have reported positive association of *GRM4* with schizophrenia in the Ashkenazi Jewish population. But Shibata discovered that the *GRM7* gene rs12491620 and rs1450099 polymorphisms were associated with schizophrenia, whereas the *GRM4* gene rs2229901 polymorphism was unlikely to be associated with schizophrenia in the Japanese population [21]. What is more, the *GRM4* gene rs733457 polymorphism was found to be associated with neurotransmission systems with bipolar disorder [24].

Survival analysis for rs1906953 showed that the median survival time of osteosarcoma patients with the CC genotype was significantly shorter compared to the CT and TT genotypes. But this result is not consistent with Jiang's finding. Moreover, we also found that patients carrying rs1906953CC genotype have apparently got a decrease in their recurrence-free survival time in comparison with patients carrying rs1906953TT genotype. This may be correlated with the high possibility of patients carrying CC genotype getting metastasis and recurrence.

However, there were several limitations to our study. Firstly, because of the rarity of osteosarcoma, the relatively small number of patients will overestimate the OR value and 95 % CI. Secondly, we did not perform the functional study in these three SNPs. Finally, cases were selected from one hospital, which may not be representative of the general population. Therefore, the gene deserves further elucidation based on a large sample or multicenter collaboration research and the combination of genes.

In summary, our data suggest a potential association between *GRM4* gene rs1906953 polymorphisms and osteosarcoma incidence in Chinese people and the distribution frequency of CC genotype in patients with osteosarcoma has a correlation with the morbidity and metastasis of osteosarcoma. When compared with TT genotype carriers, most of the patients with osteosarcoma carrying CC genotype are in the period of Enneking IIB with lower overall survival rate and recurrence-free survival rate. Thus, CC genotype is one of the most significant genetic factors in influencing the morbidity of osteosarcoma. We expected to find a relationship between rs2229901 and the morbidity of osteosarcoma, but we have not found any relevance between rs2229901 polymorphism and the morbidity of osteosarcoma. It may be that the limited sample size and unclear function of *GRM4* gene in

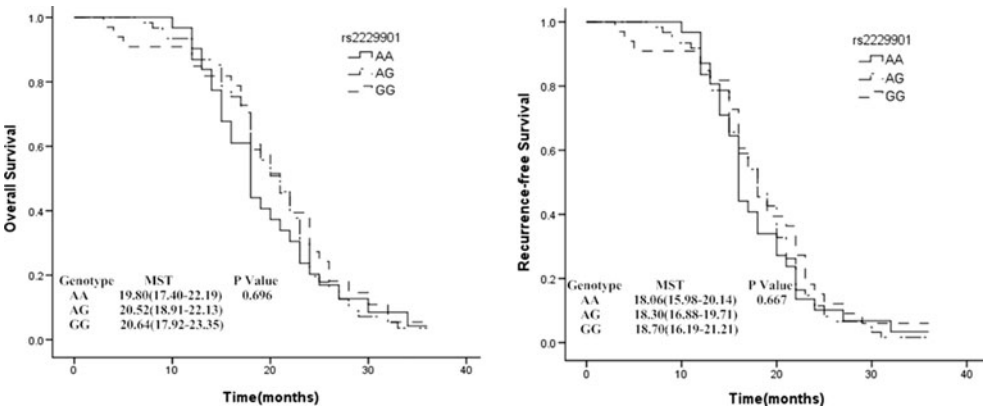

Fig. 3 *GRM4* rs2229901 polymorphism was not correlated with the overall survival and the recurrence-free survival of osteosarcoma (P=0.696, P=0.667). *MST* median survival time

osteosarcoma restricted us. Therefore, rs1906953 can be used as a predictive marker of improved survival, and verification by further large sample studies is needed.

Acknowledgments This research was supported by the National Natural Science Foundation of China (Grant No. 81460407) and the Guangxi Science Funds of China (Grant No. 2012GXNSFAA053087).

Conflicts of interest None

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