RESEARCH ARTICLE

Genetic variations in the *PRKCG* gene and osteosarcoma risk in a Chinese population: a case-control study

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Abstract Osteosarcoma is a common malignant tumor, which exists widely in the bone of children and adolescents. Protein kinase C gamma (*PRKCG*) gene, which encodes γPKC, plays important roles in tumor promotion, cell proliferation, differentiation, and migration. The objective of the present study was to investigate the relationship between *PRKCG* polymorphisms and the risk of osteosarcoma. Five tag single nucleotide polymorphisms (SNPs) of *PRKCG* were retrieved from the HapMap database and genotyped by the method of SNapShot in a hospital-based study containing 388 patients and 388 healthy individuals. Odds ratios (ORs) and their 95 % confidence intervals (CIs) were used to evaluate the association SPSS 20.0 statistical software package was used to analyze statistical data. Our results suggested that the T/C variant of rs454006 located in the intron 3 region of *PRKCG* gene was significantly associated with an increased risk of osteosarcoma (CC vs. TT, OR=1.91; 95 % CI 1.29–2.85; P=0.001; CC vs. TT+TC, OR=2.14, 95 % CI=1.48–3.09, P=0.001; C vs. T, OR=1.32, 95 % CI=1.08–1.62, P=0.008). Similarly, the rs3745406 T/C variant can also elevate the risk of osteosarcoma in the dominant model (OR=1.45, 95 % CI=1.08–1.96, P=0.014), homozygous model (OR=1.68, 95 % CI=1.10–2.59, P=0.002), and allelic model (OR=1.31, 95 % CI=1.07–1.61, P=0.009). However, there were no significant differences in genotypes and allele frequencies of rs2547362 (T>C), rs8103851 (C>G), and rs2242245 (T>C) SNPs between osteosarcoma patients and healthy controls. The results showed that carrier of rs454006*C allele and rs3745406*C might elevate the risk of osteosarcoma. Further studies are needed to validate the coalition between *PRKCG* gene polymorphisms and risk of osteosarcoma relying on a larger population that included the participants in different ethnicity and hospital.

Keywords Osteosarcoma · *PRKCG* · Single nucleotide polymorphisms · Genetic · Case-control study

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Introduction

Osteosarcoma, one of the most frequent bone tumors in adolescents and children, stems from primitive bone forming mesenchymal cells, and it accounts for 0.2 % of all malignant tumors and 20 % of all primary bone tumors [1, 2]. It occurs at different age but mainly below the age of 20, and the yearly incidence is 2–3 in every 630,000 people. The prevalence ratio is 8–11 in 630,000 people aged from 15 to 19 and the sex ratio is about 1.4:1 (male vs. female) [3]. Because of its poor diagnosis, a 5-year survival rate of osteosarcoma patients after amputation is only 40 % [4]. Following the development of medical technology, a 5-year survival rate improved significantly and it got up from 50–70 % [5]. However, the mortality ratio of osteosarcoma remains very high because of tumors' rapid migration and proliferation [6, 7]. For instance, Yang et al. found that patients of osteosarcoma with the same tumor size and stages showed diverse response to chemotherapy and had different survival time [8]. In addition, cumulative studies revealed that genetic factors may affect clinical outcome and treatment of chemotherapy [9, 10]. Therefore, a better perceive about the relationship between osteosarcoma and gene is needed.

Protein kinase C (PKC) family, which belongs to one subtype of serine/threonine kinase family, is widely found in cells and numerous tissues [11]. According to the structure of regulatory domain and some other classical characteristics, 12 different PKC isozymes are divided into three major subgroups [12]. PKCs, one of the three subgroups, play as an important calcium- and/or phospholipid clinging protein phosphatase in the biological signal transfer, such as from cell membrane to cell nucleus [13]. Meanwhile, it also regulates various cell functions such as cell proliferation, apoptosis, and differentiation [13]. Generally, the biological functions of PKC are mainly divided into two sections: reversible PKC expression can activate mitogen-activated protein kinase to enter cell nucleus to promote DNA replication and transcription with the action of relevant growth factors; the other kind of PKC obstruct cell apoptosis through many pathways such as Bcl-2 [14]. γPKC is one member of typical PKCs, and it mainly has two domains (C1 and C2 domains), which were used to bind diacylglycerol and Ca^{2+}, respectively. In addition, C3 and C4 domains are also its catalytic domain.

Recently, some studies found that *PRKCG* gene, which encoded γPKC, played important roles in many diseases [15]. Schlaepfer et al. found that rs2242244 and rs307941 variants of *PRKCG* gene could elevate susceptibility of behavioral disinhibition [16]. Missense variants in exon 4 (C114Y/G123R/G123E) of *PRKCG* gene has the relationship with spinocerebellar ataxia type 14 [4, 17, 18]. Additionally, *PRKCG* gene variants can lead to tumors' development and migration. Parsons et al. discovered that gene mutation had the relationship with cells migration of colon carcinoma [19]. It was reported that *PRKCG* gene variations (R659S) could enhance susceptibility of breast cancer [20]. However, the relevance between the *PRKCG* gene and osteosarcoma is still puzzling. Up to now, few publications have revealed the association of *PRKCG* polymorphisms and risk of osteosarcoma to our knowledge. Understanding the relationship between *PRKCG* gene mutation and osteosarcoma could benefit for people to grasp the pathogenesis of osteosarcoma and provide effective treatment for patients.

Considering the important roles of *PRKCG* in cancers, it is possible that single nucleotide polymorphisms (SNPs) in the functional regions of *PRKCG* may elevate risk of osteosarcoma. In order to confirm our speculation, we performed a study on the association between *PRKCG* gene variations and osteosarcoma risk in a Chinese population using the case-control design.

Materials and methods

Study subjects

A case-control study was performed to confirm whether *PRKCG* gene variation has the relationship with the risk of osteosarcoma. Seven hundred seventy-six participants (388 osteosarcoma patients and 388 healthy individuals) in total from the Third Affiliated Hospital of Sun Yat-sen University were enrolled in our research between August 2010 and December 2014. All the subjects recruited were ethnically Han Chinese who lived in Guangzhou or the surrounding provinces permanently. Individuals who participated in our study should fit the following criteria: (1) None of the participants had history of other cancer and received chemotherapy or radiotherapy; (2) All patients have been histopathologically diagnosed; (3) All of the subjects have no genetic relationship with each other. (4) The cases and controls were well matched for age, sex, and body mass index (BMI). Detailed information about the study population is summarized in Table 1. All blood samples from each subject were obtained before surgery. All subjects included in the study accepted the pretested questionnaire, which included the information and the data about demographic data, smoking, drinking, mental history, environmental exposure, and family diseases history. The mission did by experienced and trained interviewers. The subjects involved in the study all gave their written informed consents completely voluntarily. Meanwhile, the present study was approved by the Third Affiliated Hospital of Sun Yat-sen University Ethics Committee for Human Subject including written assent forms.

Table 1 Comparison of patients and controls by selective characteristics

Clinical characteristics	Osteosarcoma patients	Healthy controls	P value
Total	388	388	
Age (years)			
<18	293	282	0.367
≥18	95	106	
Sex			
Male	208	193	0.281
Female	180	195	
BMI (kg/m^2)	20.32±3.31	19.64±3.02	0.879
Tumor size (cm)			
<8	236	–	
≤8	152	–	
Clinical stage			
I/II	188	–	
III/IV	200	–	
Tumor location			
Femur or tibia	346	–	
Others	42	–	
Subtype			
Osteoblastic	316	–	
Others	72	–	

SNPs selection

The SNP selection was executed by fetching the genotype data of ethnically Han Chinese who lived in Beijing (CHB) from the HapMap database (HapMap Data Rel 24/Phase II, Nov08, on NCBI B36 assembly, dbSNP b126). Haplotype block analysis was conducted using Haploview 4.2 software (Cambridge, MA, USA). SNPs with minor allele frequency (MAF) less than 0.05 and r^2 less than 0.8 were excluded.

DNA extraction and genotyping

Three milliliters of peripheral blood was obtained from patients and healthy controls after written informed consents. Blood samples were stored in aseptic anticoagulants tubes at −70 °C for following use. The genomic DNAs were extracted from blood samples with Qiagen DNA blood kit (Qiagen, Hilden, Germany) according to the producer's instructions. Briefly, the blood samples need digestion and was further purified with proteinase K and phenol-chloroform, respectively. The genotyping of 5 SNPs were analyzed using the method of SNapShot (Applied Biosystems). The primers of PRKCG gene polymorphisms were designed using Premier 5 software, as shown in Table 2. Firstly, 50 μl reaction volumes was performed, which includes 2.5 μl extracted genomic DNA, 1.25 μl 10 mM dNTP, 1.25 μl primer mix, 1.25 μl 50 mM MgCl$_2$, 0.5 μl 5 U Taq DNA polymerase, 5 μl 10x buffer, and add H$_2$O to 50 μl. The tubes were amplified for 45 cycles as follows: 96 °C for 2 min, 45 cycles of 95 °C for 25 s, 57 °C for 25 s, and then 72 °C for 30 s. Secondly, mixed 0.2 μl exonuclease I, 0.3 μl SAP, 2 μl PCR product, and 7.5 μl ddH$_2$O in the tubes and put it at 37 °C for 100 min, 75 °C for 15 min in order to purify the PCR product. Four microliters of reaction volumes for extension was prepared, including 2 μl reaction mix, 1.2 μl PCR product, and 0.8 μl probe mix. The tubes were performed for 40 cycles at 96, 51, and 60 °C for 10, 5, and 30 s subsequently. Finally, mixed the extension products with 0.4 μl SAP and 2 μl ddH$_2$O at 37 °C for 1 h, and then at 75 °C for 15 min in order to terminate SAP reaction. A 96-well PRISM 3730 DNA Sequencer (Applied Biosystems) was used to analyze the SNPs of PRKCG. In order to get the accuracy of the results, we used the double control in the procedure of genotyping. Additionally, selected 5 % of the samples randomly to approve the results were 100 % accordant.

Statistical analysis

Student's t-test or chi-square test was used to analyze demographic characteristics and frequency of genotypes and allele between healthy individuals and osteosarcoma patients. Four genetic models were established to analyze distribution frequency of each genotype and allele, respectively. The referents were the wild genotype in the healthy controls. Chi-square test was used to calculate Hardy-Weinberg equilibrium (HWE) among every five tag SNPs. Odds ratios (ORs) from unconditional logistic regression and 95 % confidence intervals (CIs) from multivariate logistic regression were used to reveal corresponding risks in order to analyze the association between PRKCG gene mutation and osteosarcoma. SPSS 20.0 statistical software package (SPSS Inc, Chicago, USA) was used to analysis statistical data. Normally, the two-sided P value less than 0.05 was defined as statistically significant. GraphPad Prism 6.0 was used to generate the graphs.

Results

Sample characteristics

In this case-control study, clinical characteristics of 388 osteosarcoma patients and 388 healthy controls are showed in Table 1. Comprehensively, they appeared to be matched well on age, sex, and BMI (all P>0.05). The ratio of subjects aged below 18 years is 72.9 % in osteosarcoma patients and 72.7 % in healthy controls. Male accounted for 53.6 % in patients and 49.7 % in healthy controls. The mean of BMI in patients was 20.32 and 19.64 in healthy individuals. Of all the patients, tumor size of 236 subjects was less than 8 cm, 188 patients were in the clinical stage of I/II when being diagnosed, and

Table 2 Primers of PRKCG gene polymorphisms for PCR amplification

Studied SNPs	Gen position	Primers for PCR amplification	P value of HWE	Global MAF
rs2547362	Exon 1	F: 5′- CCCCTGTTTTGCAGAAAG-3′ R: 3′-GTCTTCCACCAGGTGCTT-5′	0.061	C: 0.104
rs454006	Intron 3	F: 5′- TTCTGTGACATTTCCTGA-3′ R: 3′-ACGAGTGATACCTAACAA-5′	0.131	C: 0.401
rs3745406	Exon 6	F: 5′- GTTTCACATAGGGATCAG-3′ R: 3′-CAGGTATCCTTAATCCAA-5′	0.929	C: 0.330
rs8103851	Intron 9	F: 5′- AAGTGCTGGGATTACAGG-3′ R: 3′-CTGTCTTAGAGGAAAACA-5′	0.059	G: 0.459
rs2242245	Intron 13	F: 5′- GCAAGAAGTGTTGAGTTCAG-3′ R: 3′-AAGGTCCCCGCACTAAAG-5′	0.130	C: 0.061

most of the tumors were located on the femur or tibia. Meanwhile, 316 osteosarcoma patients belong to osteoblastic sarcoma.

Tag SNP selection

According to the NCBI database, *PRKCG* gene spanning 25, 373 bp includes 810 SNPs. Based on CHB population of International HapMap databases, we obtained 12 tag SNPs with MAF more than 0.05. Using linkage disequilibrium (LD) coefficient r^2 to examine the pairwise linkage disequilibrium among the 12 tag SNPs, r^2 of 0.8 was selected as the edge for the following analyses. The linkage disequilibrium (LD) of the 12 tag SNPs is illustrated in Fig. 1. Using the Haploview 4.2 software defines haplotype blocks. Finally, five tag SNPs (rs2547362, rs454006, rs3745406, rs8103851, rs2242245) were selected. The genetic locations of five tag SNPs are shown in Fig. 2. These five tag SNPs located from the 5′ un-translated region (UTR) (rs2547362), exon (rs3745406), and introns (rs454006, rs8103851, rs2242245) of *PRKCG* gene.

Association between individual SNP and risk of osteosarcoma

The distribution frequency of genotype and allele of the selected five tag SNPs in osteosarcoma patients and healthy controls were shown in Table 3. There was no significance in genotypes and allele frequencies of rs2547362 (T>C), rs8103851 (C>G), and rs2242245 (T>C). For rs454006 (T>C, in the region of intron3), no apparent difference was

found in dominant model (TC+CC vs. TT). However, compared with wild homozygous TT genotype, those subjects took the homozygous mutation (CC) showed a significant association with risk of osteosarcoma (OR=1.91; 95 % CI 1.29–2.85; $P=0.001$). Similarly, recessive model (CC vs. TT+TC) and allele model (C vs. T) also revealed significance with risk of osteosarcoma, with OR of 2.14 (95 % CI=1.48–3.09, $P=0.001$) and OR of 1.32 (95 % CI=1.08–1.62, $P=0.008$), respectively. Another SNP (rs3745406, 567 T>C), which is located in exon 6, showed no association with susceptibility of osteosarcoma in recessive model (CC vs. TT+TC). But we discovered that the combined TC+CC of rs3745406 could elevate risk of osteosarcoma when compared with the wild genotype TT (OR=1.45, 95 % CI=1.08–1.96, $P=0.014$). Meanwhile, compared with the wild genotype TT, the homozygous model CC showed significantly correlation with the risk of osteosarcoma (OR=1.68, 95 % CI=1.10–2.59, $P=0.016$). Additionally, the distribution frequencies of the C allele of rs3745406 is significantly higher in patients with osteosarcoma than in the healthy controls (OR=1.38, 95 % CI=1.13–1.69, $P=0.002$).

Discussion

Osteosarcoma is a common malignant tumor, which exists widely in the bone of children and adolescents [21]. It aroused people's concern universally owing to its highly malignant, facilely reversion, and readily metastases [22, 23]. Up to now, inaugural mechanism of osteosarcoma was considered

Fig. 1 Linkage disequilibrium of the 12 tag SNPs in *PRKCG* gene
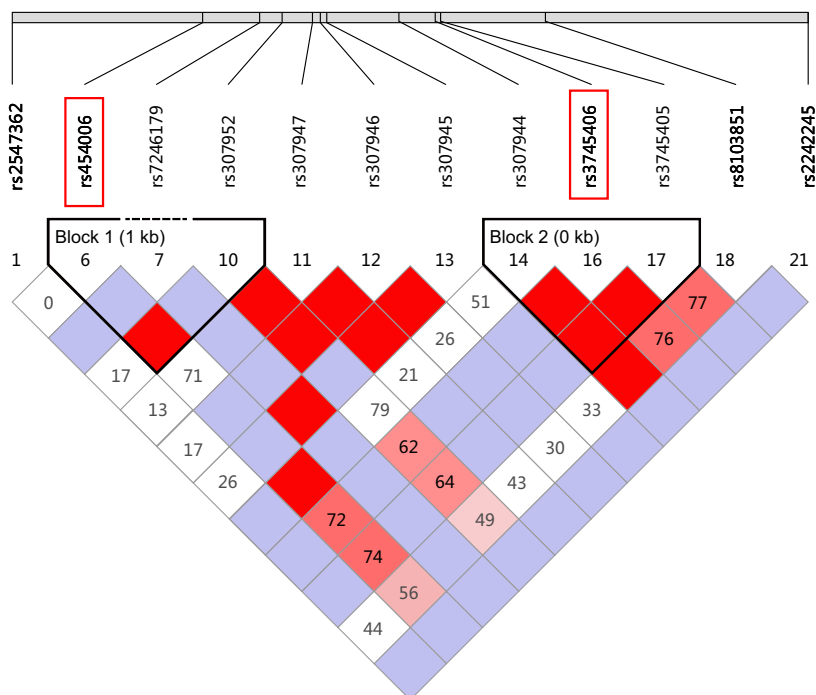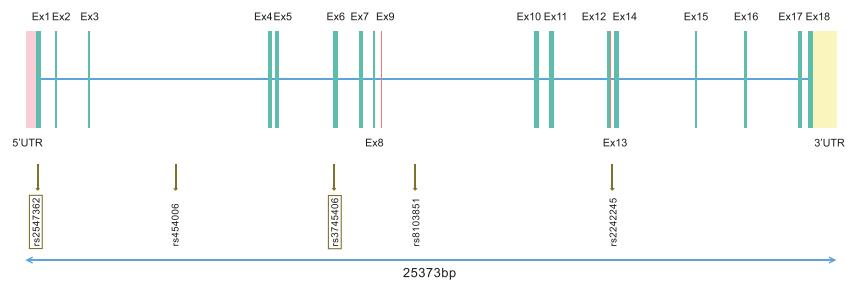
Fig. 2 Genetic location of the selected five tag SNPs in *PRKCG* gene

as a complex process and was not clear, but it was universally acknowledged that environment carcinogens could induce genomic polymorphism, such as oxidative stress, drinking, smoking, and ionizing radiation [9, 10]. Previous research found that genetic variants of *PRKCG* can initiate the onset of homologous diseases, such as behavioral disinhibition, major depressive disorder, and myotonic dystrophy [16, 17, 24]. Therefore, in the present study, we aimed to study the involvement between five SNPs of *PRKCG* (rs2547362, rs3745406, rs454006, rs8103851, rs2242245) and risk of osteosarcoma in the ethnically Han Chinese. We found that polymorphisms of rs454006 and rs3745406, which are located in intron 3 and

exon 6 of *PRKCG*, respectively, could significantly elevate susceptibility of osteosarcoma. However, the other three SNPs (rs2547362, T/C; rs8103851, C/G; and rs2242245, T/C) showed no correlation with the risk of osteosarcoma.

PRKCG, which is expressed widely in the central nervous system such as in the brain and spinal cord, can encode 697 amino acids containing a Ca^{2+}-sensitive region (C2 catalytic domain) and two cysteine-rich regions (Cys1 and Cys2) [25]. Cys1 and Cys2 can bind diacylglycerol (DAG) and phorbol ester closely with two zinc ions [15]. *PRKCG* gene located at 19q13.4 in chromosomes in human. Owing to its particularly high expression in the cerebellar cortex, most of the study

Table 3 Associations between five SNPs of *PRCKG* gene and the risk of osteosarcoma

| Studied SNPs/genotype	Patients	Controls	Comparative modes	OR (95 % CI)	*P* value
rs2547362 (T>C)					
TT	283	287	Dominant: TC+CC vs. TT	1.05 (0.77–1.45)	0.745
TC	81	88	Recessive: CC vs. TT+TC	1.90 (0.95–3.79)	0.064
CC	24	13	Homozygous: CC vs. TT	1.87 (0.93–3.75)	0.073
C allele	129	114	Allelic: C allele vs. T allele	1.16 (0.88–1.52)	0.295
rs454006 (T>C)					
TT	167	173	Dominant: TC+CC vs. TT	1.06 (0.80–1.51)	0.664
TC	123	162	Recessive: CC vs. TT+TC	2.14 (1.48–3.09)	*0.001*
CC	98	53	Homozygous: CC vs. TT	1.91 (1.29–2.85)	*0.001*
C allele	319	268	Allelic: C allele vs. T allele	1.32 (1.08–1.62)	*0.008*
rs3745406 (T>C)					
TT	120	149	Dominant: TC+CC vs. TT	1.45 (1.08–1.96)	*0.014*
TC	196	176	Recessive: CC vs. TT+TC	1.39 (0.95–2.06)	0.089
CC	72	53	Homozygous: CC vs. TT	1.68 (1.10–2.59)	*0.016*
C allele	340	282	Allelic: C allele vs. T allele	1.31 (1.07–1.61)	*0.009*
rs8103851 (C>G)					
CC	132	135	Dominant: GG+GC vs. CC	1.03 (0.77–1.39)	0.821
CG	169	172	Recessive: GG vs. CC+CG	1.09 (0.78–1.54)	0.601
GG	87	81	Homozygous: GG vs. CC	1.09 (0.75–1.62)	0.634
G allele	343	334	Allelic: G allele vs. C allele	1.05 (0.86–1.28)	0.645
rs2242245 (T>C)					
TT	285	282	Dominant: TC+CC vs. TT	0.96 (0.70–1.32)	0.808
TC	84	93	Recessive: CC vs. TT+TC	1.48 (0.72–3.05)	0.279
CC	19	13	Homozygous: CC vs. TT	1.45 (0.70–2.98)	0.316
C allele	122	119	Allelic: C allele vs. T allele	1.03 (0.78–1.36)	0.833

The significant values are italicized

focused on investigating the relationship between *PRKCG* mutation and SCA14. They found that missense mutation in the exon area of *PRKCG* can enhance risk of SCA14 [26, 27]. Recently, it is reported that *PRKCG* gene played an important role in tumor promotion, cell proliferation, differentiation, and migration [25]. For example, Parsons et al. found that the dual bundling of γPKC and fascin, which were activated by Rac GTPase, could induce cell migration in patients with colon cancer [19, 28]. Overexpression of γPKC in mammary cells can induce tumorigenesis and transition of tumor cells from the epithelium to mesenchyme by activating the mitogenic ERK pathway [29]. However, there is no clear pathway on the relationship between risk of osteosarcoma and gene mutation of *PRKCG*. According to previous studies, we can speculate the mechanism as follows (Fig. 3): Hsp90α and inactive γPKC bind closely in normal situation. Membrane translocation of intracellular Ca^{2+} and DAG induce phosphorylation of Hsp90α and γPKC, and isolate γPKC from Hsp90α. Activated γPKC in the cytoplasm release autoinhibition and involves in cell proliferation, tumors migration and invasion, and survival of cancer cells [30, 31].

In the case-control study, we found that rs454006 and rs3745406 mutation had significant association with susceptibility of osteosarcoma. Interestingly, we discovered that rs454006 (T>C), which is located in the downstream of 3 splice site of exon 3, has positively correlated with osteosarcoma in homozygous, dominant, and allele model. It is predicted that missense mutation of rs454006 could provoke a new splice donor site instead of the natural splice site and lead to translation of the nuclear cancer proteins incorrectly [32]. These proteins may regulate oncogene products at the level of transcription and result in dysregulation in cell division and aroused pathogenesis of osteosarcoma. Zhang et al. also found mutation of rs454006 could alter the frequencies of genotype and

allele and might increase the risk of osteosarcoma significantly, which is consistent with our results [33]. On the other hand, non-synonymous variant of rs3745406 (stationed in exon6, T > C) can also elevate risk of osteosarcoma significantly. The result is consistent with the conventionally view that variants in the functional region can effect expression of γPKC normally. For example, the study by Schlaepfer et al. discovered that rs3745406 polymorphisms of *PRKCG* was a susceptibility locus for human disease in samples of Caucasian, which also supports the relationship between gene variation and neuropathic disease [16]. Meanwhile, our results demonstrated that γPKC, which encodes by *PRKCG* gene, played an important role in the central nervous system's transmembrane signal transduction, and the results was accordant with the previous studies [24]. Through the previous and current studies we can get the conclusion that gene variations of *PRKCG* could arouse many diseases of nervous system. Several limitations are also existed in our study. Firstly, the subjects were selected come from the same hospital, and it cannot represent the general individuals. Secondly, our study was limited by a relatively small sample size, which may not fully represent the genotype distribution in entire population. In addition, only Chinese population was recruited in our study. Therefore, multichannel studies containing different hospital, larger samples, and various ethnicities should be performed in further study.

In conclusion, our results suggest that genetic mutants of *PRKCG* (rs454006 and rs3745406) were correlated with osteosarcoma risk in Chinese population. Our findings may provide the possibilities of *PRKCG* as marker to determinate the people with high risk of osteosarcoma. Further studies are needed to validate our results relying on a larger population that included the participants in different ethnicity and hospital. Furthermore, pathological mechanism of *PRKCG* and osteosarcoma is also needed to investigate.

Fig. 3 Potential mechanism of *PRKCG* gene in tumorigenesis of osteosarcoma
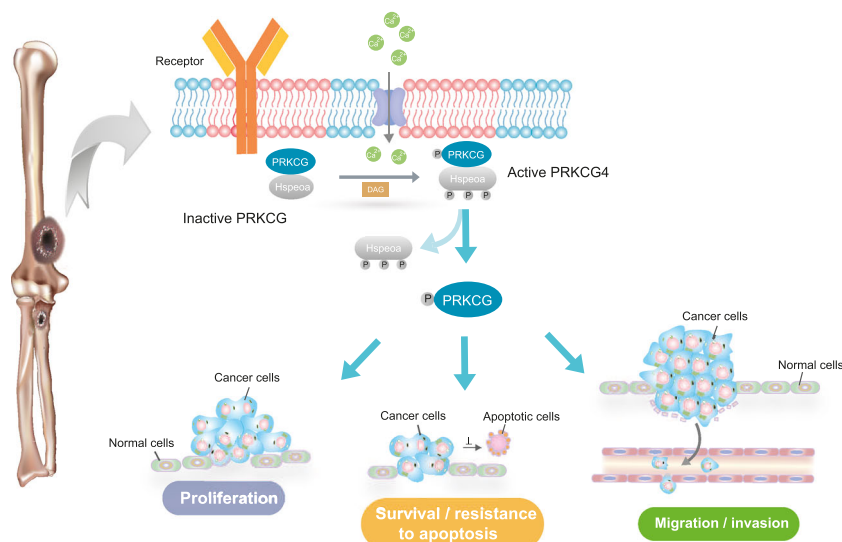
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