Association between *AKT* rs2494752 single nucleotide polymorphism and the risk of metastasis in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common types of human tumors, which is characterized by high morbidity and mortality rates. AKT1 transcriptional activity is implicated in HCC initiation and development. In the present study, the effects of rs2494752 single nucleotide polymorphism (SNP) on AKT1 transcriptional activity in the progression of HCC cells were investigated. A case-control study was analyzed in 1,056 HCC patients and 1,080 healthy individuals using the PCR assay method. Results indicated AKT1 expression levels were up-regulated in HCC tissue compared to adjacent normal tissues. Furthermore, a higher frequency of *AKT* rs2494752 AG and AA genotypes were observed in HCC cases (P=0.0046). Gene polymorphism identified C and T alleles were frequency in HCC patients compared to healthy individuals. Individuals harboring *AKT* rs2494752 AG/AA genotype had a vital increased susceptibility to HCC in the dominant model (P=0.0028). In addition, *AKT1* rs2494752 GG genotype showed an increasing of *AKT1* promoter activity determined by the luciferase assay. Furthermore, it was demonstrated that *AKT1* rs2494752 GG and C polymorphism was more aggressive than other *AKT1* rs2494752 cancer cells. Moreover, *AKT1* rs2494752 GG markedly increased rates of response to NCT chemotherapy. Additionally, results revealed that *AKT1* rs2494752 GG and C increased the risk factors of HCC. In conclusion, these results indicate that *AKT1* rs2494752 polymorphisms may be regarded as a candidate gene in assessing the susceptibility, metastasis and responses to chemotherapy in the progression of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is aggressive hypervascular solid tumor, and the vascularity is significantly different from peripheral parenchyma of liver (1). Advanced stage HCC possesses aggressive potent for adjacent and distant cells and/or organs (2,3). Many systematic review and meta-analysis have indicated that hepatocellular carcinomas presents a high recurrence and the second cancer-death rates even received surgery, radiotherapy, chemotherapy and biotherapy (4,5). Previous reports have indicated that the current therapeutic schemes remain limited, especially for patients with advanced hepatocellular carcinoma, which exhibited poor survival in a 5-year survival statistical survey (6,7). Therefore, it is an urgent need to explore novel therapeutic targets for hepatocellular carcinoma.

Clinical therapies are eagerly needed for inhibiting migration and invasion to maximal prolong survival of patients with hepatocellular carcinoma (8,9). In recent year, various gene polymorphisms have been reported to association with HCC metastasis, which become a potential strategy for determining HCC diagnosis, susceptibility, target, metastasis, apoptotic sensibility and prognosis (10-13). Report has indicated that *AKT* gene polymorphisms may be associated with prostate cancer, gastric cancer and osteosarcoma (14-16). Yin *et al* (17), reported that PI3K/Akt pathway involved in in human HCC cell lines by regulation of metastasis-related gene expression. Additionally, down-regulation of PI3K/Akt-PAK1 signal pathway could inhibit the metastasis properties of hepatocellular carcinoma, which suggested that AKT is a potential target for the treatment of hepatocellular carcinoma (18).

In the present study, we investigated the effects of rs2494752 polymorphism on *AKT1* gene transcriptional activity in 1,056 HCC patients and 1,080 healthy individuals using the TaqMan assay method. Here, we reported that *AKT1* rs2494752 GG and C polymorphism HCC presented more aggressive. We analyzed the association between *AKT1* rs2494752 gene polymorphism and responses to chemotherapy in the progression of hepatocellular carcinoma.

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Materials and methods

Study design, subjects and sampling. 1056 HCC patients and 1,080 healthy individuals were included in this retrospective cohort in Chinese Han population collected form archives department of Qingdao Sixth People's Hospital from May 2008 to July 2015. The numbers of male and female HCC patients and healthy individuals were approximate equal. Patients with cancer history were excluded. This study was approved by the ethics committee of Qingdao Sixth People's Hospital (ethics code: QDPHCAN132X1P). All patients were asked to provide 5 ml venous blood and were required to write informed consent with signature.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HCC tissues and adjacent normal tissues using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturers' protocol. RNA was reversed transcribed using a PrimeScript RT Master Mix kit (Takara Bio, Inc., Otsu, Japan). All forward and reverse primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sequences were as follow: AKT1, Forward: 5'-CTTCCTCACAGCCCTGAA GTAC-3', Reverse: 5'-GCATGAGGTTCTCCAGCTTGAG-3'; GAPDH, Forward: 5'-CCAGGGCTGCTTTTAACTCTG-3', Reverse: 5'-CGCTCCTGGAAGATGGTGATG-3'. For amplification diluted cDNA was combined with a reaction mixture containing SYBR-Green PCR core reagents (cat. no. 4304886; Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (19). PCR cycling was performed under the following conditions: 94°C for 30 sec and 45 cycles of 95°C for 5 sec, 56°C for 10 sec and 72°C for 10 sec. The results were expressed as the n-fold of the control.

Migration assay. The migration of HCC cells were evaluated using transwell plates. The isolated HCC cells were directly seeded on the upper chamber (8 mm pore size, 6.5 mm diameter; Corning Incorporated, Corning, NY, USA) with DMEM (Thermo Fisher Scientific, Inc.). The lower chamber was filled with DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.). Cells were then incubated at 37°C for 48 h. The migrated cells from at least six random microscopic fields (x200) were counted under a light microscope (Olympus Corporation, Tokyo, Japan).

Western blot analysis. HCC tissues and adjacent normal tissues were homogenized in a lysate buffer containing protease-inhibitor (P3480; Merck KGaA, Darmstadt, Germany) and were centrifuged at 6,000 x g at room tempreture for 10 min. Western blot analysis was subsequently performed as previously described (20). Protein concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Protein samples (10 μg/lane) were resolved by 12.5% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Merck KGaA). After blocking with 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA), rabbit anti-human AKT1 (ab81283) or GAPDH (ab9485) antibodies (all, 1:2,000; Abcam, Shanghai, China) were incubated with protein samples for 2 h at room temperature. Membranes were washed with

PBS for 15 min at room temperature and then incubation with horseradish peroxidase-conjugated polyclonal anti-rabbit immunoglobulin G antibodies (1:10,000; PV-6001; OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature. Signals were visualized by chemiluminescence detection (Z370398; Merck KGaA). Densitometric quantification of the immunoblot data was performed using Quantity-One software (v3.24; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunohistochemistry. Immunohistochemical procedures were performed as described previously (21). HCC tissues and adjacent normal tissues were frozen and coronal sections were cut in a cryostat. The tissues were cut into 4-μm thick sections and mounted on glass slides. The paraffinized sections were heated in an oven at 65° for 24 h, dewaxed to water and rinsed with PBS three times. The washed sections were placed in EDTA buffer (Beinuo Bioscience Inc., Shanghai, China), and then boiled at a low heat following an interval of 10 min at 65°C for a total of 3 intervals. Following natural cooling, the sections were washed with PBS three times, and were placed into 3% hydrogen peroxide solution (Beina Bioscience Inc.), for incubation at room temperature for 10 min, to block endogenous peroxidase. Free-floating sections were rinsed with PBS and placed in a solution containing primary mouse monoclonal antibodies directed against AKT1 (ab81283, 1:2,000; Abcam) at 4°C overnight. After rinsing with PBS, sections were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG mAb (1:5,000 dilution; PV-6001, OriGene Technologies, Inc., Beijing, China). The sections were then washed with PBS and observed by fluorescent video microscopy (BZ-9000; Keyence Corporation, Osaka, Japan).

DNA genotyping. All candidates' loci of *AKT1* gene for tag gene were based on NCBI dbSNP database and SNP info. The genomic DNA was 10 μg of genomic DNA were isolated from extracted by the method of buffy-coat fractions with TIANamp blood DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) (50 ng of genomic DNA, 200 μM dNTP, 2.5 units of Taq DNA polymerase, and 200 μM primers) and used for PCR amplification followed preliminary denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, annealing temperature reduced to 64°C for 30 sec, and 72°C for 10 min by volume of 20 μl containing 50 ng of genomic DNA, 200 μM dNTP, 2.5 units of Taq DNA polymerase, and 200 μM primers. PCR primers (Forward: 5'-CTTCCTCACAGCCCT GAAGTAC-3', Reverse: 5'-GCATGAGGTTCTCCAGCT TGAG-3') were designed using Sequenom Assay Design v3.1 software (Sequenom, San Diego, CA, USA). Genotyping of *AKT1* gene was conducted by PCR-restriction fragment length polymorphisms (RFLP) as described previously (22,23).

Trans-well invasion assay. HCC tissues were obtained from Qingdao Sixth People's Hospital. Transwell invasion assays were carried out in 24-well plates. In brief, HCC cells in serum-free medium contained Matrigel insert filters at 1:6 dilutions. The lower chamber was filled with culture medium with 10% FBS+DMEM. After incubation for 48 h at 37°C, cells were fixed and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA). The cells that invaded through the Matrigel membrane were quantified.

Luciferase assays. HCC cells were cultured in a 24-well culture plates for 24 h. Each well was transfected with 1.0 μg of each AKT1-reporter plasmid (pRL-SV40) with the allele C or T using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The control-reporter vector was used as a negative control. HCC cells were transfected with 1.0 μg pRL-SV40 (containing the Renilla luciferase gene) plasmids per well. HCC cells were then lysed with the passive lysis buffer (Promega Corporation, Madison, WI, USA) and for luciferase expression activity analysis using the Dual-Luciferase Reporter Assay System (Promega Corporation) after 48-hour transfection. Three independent transfection experiments were performed in this experiment.

Statistical analysis. Continuous variables were shown as mean ± SD and analyzed by student t test. All data were analyzed using SPSS Statistics 19.0 and Graphpad Prism v5.0 with the help of Microsoft Excel. Allele and genotype frequencies were calculated by using direct counting. Hardy-Weinberg equilibrium (HWE) and the differences between allele and genotype frequencies were calculated using Fisher's exact test. Results of allele and genotype frequencies were determined by STATA SE 12.1 software. The risk of HCC was analyzed by regression analysis. *P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of study population. This study included 1056 HCC with pathologically confirmed and a group of 1080 age- and gender- matched healthy individuals. The age between HCC patients and healthy individuals 42.6±10.7 and was 50.4±12.8 (median: 50.4 years) and 50.4±14.6 (median: 50.4 years), respectively. There were no significant differences in the distributions of age between patients and healthy individuals (P=0.726). In this cohort, 348 (32.8%) patients underwent preoperative NCT (CE(A)F regimen), and 636 (60.2%) of the patients received postoperative anthracycline-based chemotherapy. The characteristics of patients were summarized in Table I.

Analysis of AKT1 expression in hepatocellular carcinoma. AKT1 gene and protein expression levels were higher in HCC tissues and adjacent normal tissues from the same patients. We showed that AKT1 gene levels were up-regulated in HCC tissues compared to adjacent normal tissues (Fig. 1A). Western blot demonstrated that AKT1 protein levels were higher in HCC tissues than adjacent normal tissues (Fig. 1B). Immunohistochemistry found that AKT1 expression was markedly increased in HCC tissues compared to adjacent normal tissues (Fig. 1C). These data suggest that AKT1 may be associated with the progression of hepatocellular carcinoma.

Meta-analysis AKT1 rs2494752 gene polymorphism between HCC patients and healthy individuals. We analyzed AKT1 rs2494752 gene polymorphism allele and genotype polymorphism in hepatocellular carcinomamigraine patients and healthy individuals. We showed that genotypes of AKT1 rs2494752 were AG, AA, C and T in HCC patients and healthy individuals. We demonstrated that a higher frequency of *AKT* rs2494752 AG and AA genotypes were observed

Table I. The characteristics of patients.

Variables	Patients	Healthy	P-value
No.	1056	1080	>0.05
Man	536	538	>0.05
Woman	520	542	>0.05
Age	50.4±12.8	50.4±14.6	>0.05
Treatment			
Preoperative NCT	348	-	-
Chemotherapy	636	-	-

in HCC cases (P=0.0046 and P=0.0040; Fig. 2A and B). TaqMan assay method revealed that C and T alleles in *AKT1* rs2494752 gene polymorphism were frequency in HCC patients compared to healthy individuals (P=0.0078 and P=0.0063; Fig. 2C and D). These results suggest that *AKT1* rs2494752 gene polymorphism is higher frequencies in HCC patients compared to healthy individuals.

Effects of the rs2494752 polymorphism on AKT1 transcriptional activity and progression of hepatocellular carcinoma. The effects of the rs2494752 polymorphism on AKT1 transcriptional activity and progression of HCC were analyzed in human tissues. The luciferase assay showed that patients harboring *AKT1* rs2494752 GG genotype promoted *AKT1* promoter activity (P=0.068; Fig. 3A). Aggressiveness assay revealed that *AKT1* rs2494752 GG and C polymorphism HCC cells presented more aggressive than other *AKT1* rs2494752 cancer cells (P=0.026 and P=0.032; Fig. 3B and C). These results suggest that the *AKT1* rs2494752 polymorphism promotes AKT1 transcriptional activity and aggressiveness of hepatocellular carcinoma.

Association of AKT1 rs2494752 gene polymorphism on response to NCT and the survival of HCC patients. Of the HCC patients, 348 (32.8%) patients underwent preoperative NCT (CE(A)F regimen), and 636 (60.2%) of the patients received postoperative anthracycline-based chemotherapy. The the percentages of *AKT1* rs2494752 AA and AG genotypes were 38.6 and 61.4%, respectively. The percentages of *AKT1* rs2494752 C and T alleles were 45.2 and 54.8% respectively. *AKT1* rs2494752 GG significantly increased rates of response to NCT chemotherapy compared to the AA genotype (adjusted OR=0.325, 95% CI=0.107-0.992, P=0.048; Fig. 4A). We showed that there were no significant differences between *AKT1* rs2494752 C and T alleles in responding to NCT chemotherapy (Fig. 4B). As shown in Fig. 4C and D, we found *AKT1* rs2494752 GG patients had a long-term survival, while no significant differences between *AKT1* rs2494752 C and T alleles for HCC patients. These results suggest *AKT1* rs2494752 gene polymorphism is response to NCT and the survival of HCC patients.

Meta-analysis between AKT1 rs2494752 gene polymorphism and risk of hepatocellular carcinoma. To further evaluate the association between *AKT1* rs2494752 gene polymorphism and risk of hepatocellular carcinoma, multiple
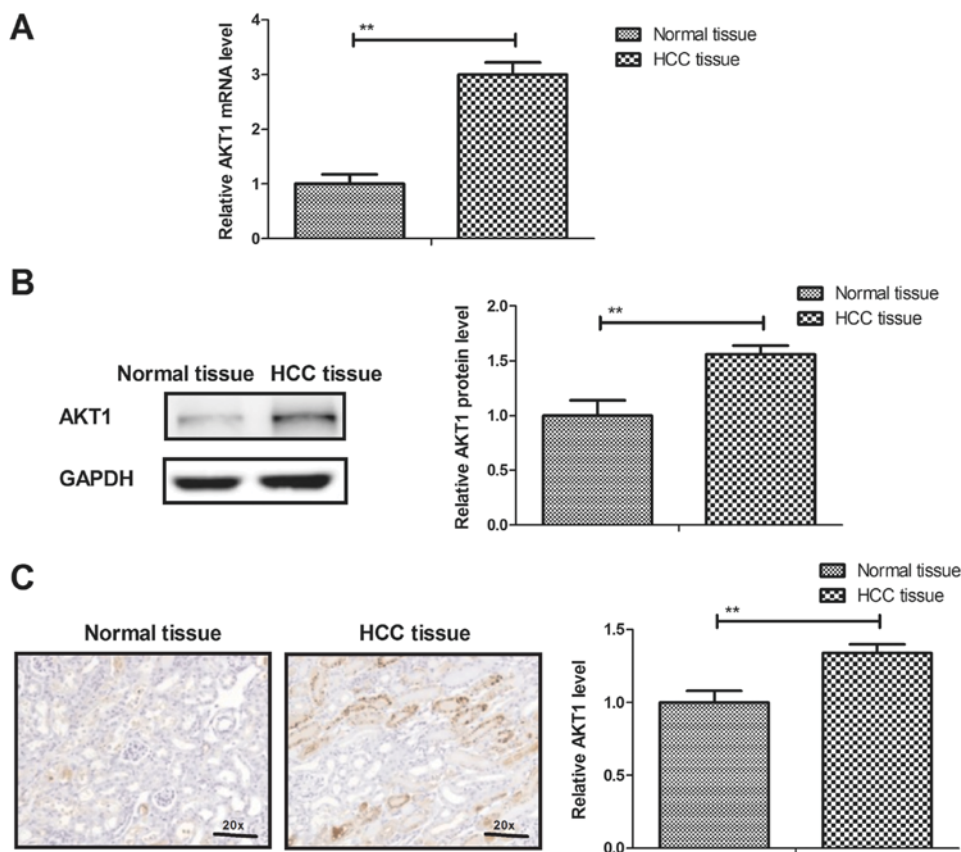
Figure 1. Expression levels of AKT1 between HCC tissues and adjacent normal tissues. AKT1 gene (A) and protein (B) expression levels were significantly higher in HCC tissues than adjacent normal tissues. (C) The AKT1-positive cells in HCC tissues were higher than adjacent normal tissues. Magnification, x20. **P<0.01. HCC, hepatocellular carcinoma.
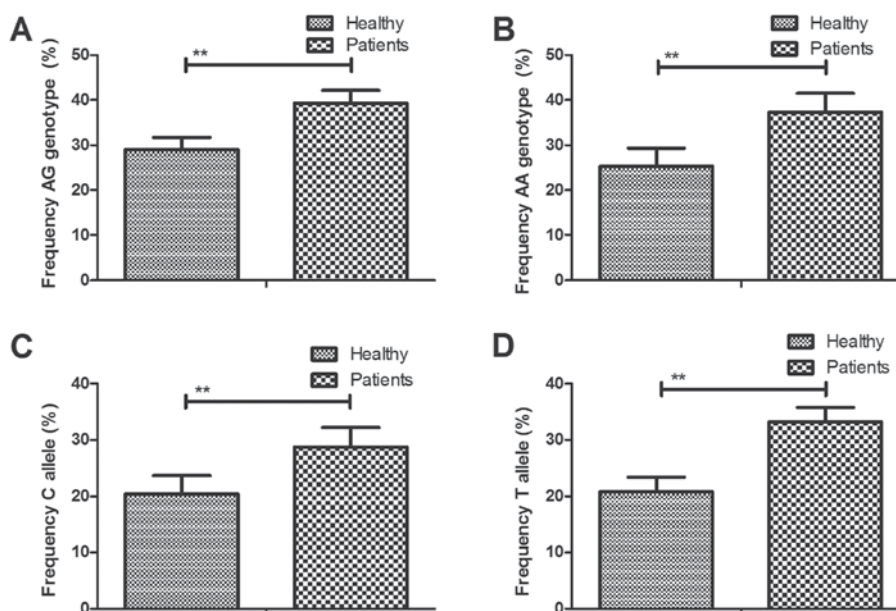

Figure 2. Meta-analysis *AKT1* rs2494752 gene polymorphism between hepatocellular carcinoma patients and healthy individuals. HCC patients had a higher frequency of *AKT* rs2494752 AG (A) and AA (B) genotypes than healthy individuals. HCC patients had a higher frequency of *AKT* rs2494752 C (C) and T (D) alleles than healthy individuals. **P<0.01. HCC, hepatocellular carcinoma.

dimension reduction was conducted in HCC patients. We showed that *AKT* rs2494752 AA genotype had a vital increased susceptibility to HCC in the dominant model (P=0.028; Fig. 5A). We observed significant differences between rs2494752 C and T alleles for susceptibility HCC patients (P=0.0384, Fig. 5B). These results suggest that *AKT1* rs2494752 AA genotype is associated with the risk of hepatocellular carcinoma.
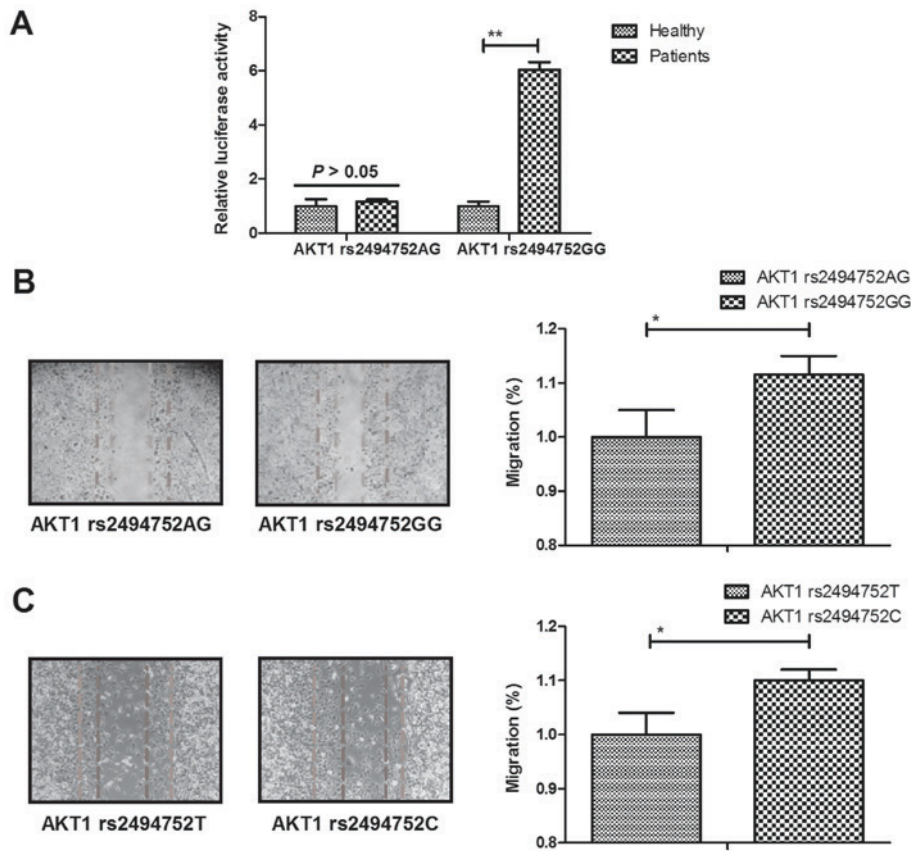
Figure 3. Effects of the *AKT1* rs2494752 on *AKT1* transcriptional activity and progression of HCC. (A) HCC patients harboring *AKT1* rs2494752 GG genotype promoted *AKT1* promoter activity. HCC patients harboring *AKT1* rs2494752 GG (B) and C (C) presented more aggressive than other *AKT1* rs2494752 cancer cells. *P<0.05, **P<0.01. HCC, hepatocellular carcinoma.
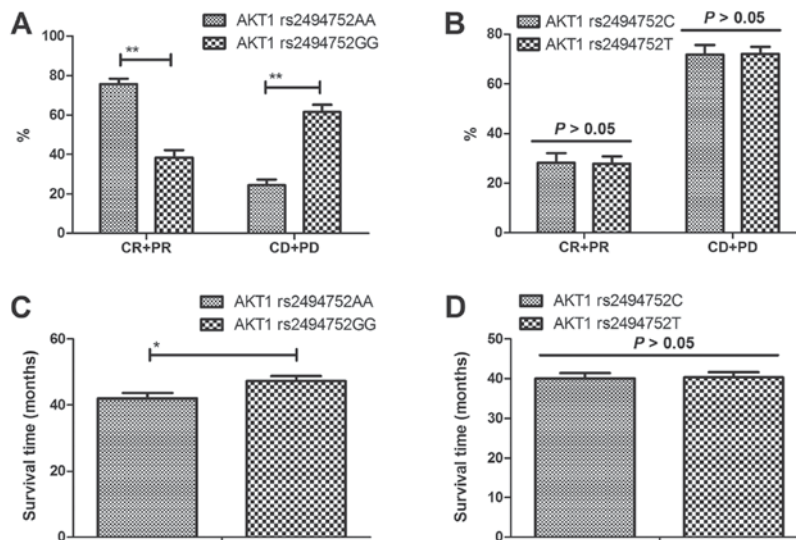

Figure 4. Effects of *AKT1* rs2494752 gene polymorphism on response to NCT and the survival of HCC patients. (A) *AKT1* rs2494752 GG increased rates of response to NCT chemotherapy compared to the *AKT1* rs2494752 AA genotype. (B) No significant differences between *AKT1* rs2494752 C and T alleles in responding to NCT chemotherapy. (C) *AKT1* rs2494752 GG patients show a long-term survival. (D) No significant differences were observed between *AKT1* rs2494752 C and T alleles for HCC patients. *P<0.05, **P<0.01. HCC, hepatocellular carcinoma; CR, complete response, PR, partial response, PD, progressive disease, SD, stable disease.

Discussion

HCC is the most common primary liver malignancy and it is a leading cause of cancer-related death worldwide (24).

Researches have suggested that gene polymorphism is associated with the risk and poor survival in a 5-year survival statistical survey for HCC patients (10,11,25). Importantly, a meta-analysis has indicated that the rs2494752 polymorphism of
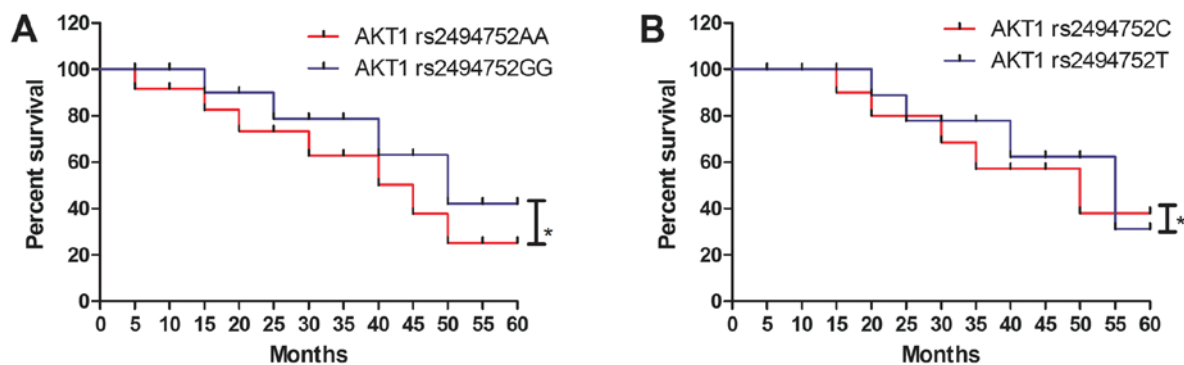
Figure 5. Meta-analysis between *AKT1* rs2494752 gene polymorphism and risk of HCC. (A) *AKT* rs2494752 AA genotype presents a vital increased suscepti-bility to HCC. (B) Significant differences between rs2494752 C and T alleles for susceptibility HCC patients. *P<0.05. HCC, hepatocellular carcinoma.

AKT1 can be used to predict susceptibility and CE(A)F chemo-therapy response to breast cancer and clinical outcomes (26). In this study, we investigated the association between *AKT1* rs2494752 gene polymorphism and risk of hepatocellular carci-noma, as well as metastasis of HCC cells. Here, we showed a higher frequency of *AKT* rs2494752 AG/AA genotypes and C/T alleles in HCC cases. Outcomes found that *AKT1* rs2494752 GG genotype showed an increasing of *AKT1* promoter activity determined by the luciferase assay. Results demonstrated that *AKT1* rs2494752 GG genotype is more aggressive than other *AKT1* rs2494752 cancer cells. We also indicated that *AKT1* rs2494752 GG significantly increased rates of response to NCT chemotherapy and *AKT1* rs2494752 GG and C increased the risk factors of hepatocellular carcinoma.

Currently, gene polymorphism is associated with tumor diagnosis, target therapy and prognosis (27-29). Previous study has showed the functional polymorphism (rs2494752) in the AKT1 promoter region and gastric adenocarcinoma risk in an eastern Chinese population, which suggested that the potentially functional *AKT1* rs2494752 single nucleotide poly-morphism (SNP) may affect GCa susceptibility by modulating the AKT1 promoter transcriptional activity (30). Wu *et al* (31), have suggested that Pre-miR-149 rs71428439 polymorphism is associated with the increased cancer risk and AKT1/cyclinD1 signaling in hepatocellular carcinoma. In this study, we observed that *AKT1* rs2494752 gene polymorphism AG/AA genotype and C/T allele in *AKT1* is higher frequencies in HCC patients compared to healthy individuals. Our outcomes revealed that *AKT1* rs2494752 polymorphism GG and C promoted AKT1 transcriptional activity and aggressiveness of hepatocellular carcinoma. However, we only chose SNP allele (AG, AA, C, and T) in HCC and healthy hepatic tissue. The effect of AKT rs2494752 SNP in AG, C, T, and GG allele on AKT1 transcrip-tional activity need further investigated in future work.

Previous study has revealed that HCC is genetically complex, multifactorial and heterogeneous tumor (32). AKT mediates various signal pathways, which may provide a novel strategy to improve the therapeutic efficiency of HCC via regu-lation of apoptosis sensitivity induced by chemotherapy (33,34). Wang *et al* (35), have suggested that molecularly targeting the PI3K pathway can sensitize cancer cells to radiotherapy and chemotherapy. We reported that *AKT1* rs2494752 *AKT1* rs2494752 GG genotype increased response to NCT and the survival of HCC patients. We also indicated that *AKT1*

rs2494752 AA genotype is associated with the higher risk of HCC than *AKT1* rs2494752 GG genotype. However, this study did not report the associations between cancer staging or metastasis status and AKT rs2494752 SNP. Further study should be performing to analyze the AKT rs2494752 SNP with other clinical data such as hepatocellular cancer staging or metastasis status in a large population.

In conclusion, the present study provided evidences that *AKT1* rs2494752 polymorphism is associated with susceptibility, metastasis, chemotherapy sensitivity and prognosis in HCC patients. Our results indicate that *AKT1* rs2494752 AA genotype is associated with the risk of hepatocellular carcinoma, which may be a vital response to chemotherapy and prognostic indicator for HCC patients. Findings also suggest that *AKT1* rs2494752 may be a candidate biomarker for the prediction of susceptibility and prognosis in HCC patients after chemotherapy.

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contribution

ZHW and HLF designed the study. WL and HLF performed the experiments. WL and ZHW analyzed the data.

Ethics approval and consent to participate

This study was approved by the ethics committee of Qingdao Sixth People's Hospital (ethics code: QDPHCAN132X1P). All patients provided informed consent.

Consent for publication

Not applicable.

Competing interests

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