Int J Clin Exp Pathol 2016;9(7):7592-7599
www.ijcep.com /ISSN:1936-2625/IJCEP0025285

Original Article

Associations of valosin-containing protein gene polymorphisms with hepatocellular carcinoma: a case-control study in a Chinese Han population

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Received January 30, 2016; Accepted May 20, 2016; Epub July 1, 2016; Published July 15, 2016

Abstract: Objective: This article aims to investigate the relationship between the single nucleotide polymorphisms (SNPs) of VCP and the risk and progression of HCC. Methods: Four SNPs in the VCP gene were analyzed by directly genotyping DNA from peripheral blood specimens collected from 122 cases with HCC, 120 non-cancer cases and 111 non-HCC hepatitis B virus (HBV) carriers. Associations between polymorphic genotypes and HCC were analyzed. Results: The distribution of the alleles and genotypes of rs546982 differed significantly ($P < 0.05$) between the HCC and control groups. Individuals with a GG genotype of the rs546982 had a higher risk of HCC than individuals with a AA genotype. However, HCC patients with the GG genotype of the rs546982 had a lower risk of lymph node metastasis and vascular tumor embolism. There was no difference between the HCC and control groups for the rs2074549, rs607671 and rs10972300 polymorphisms. Conclusion: Our results suggest that VCP polymorphisms correlate with HCC. The GG genotype of the rs546982 was associated with a higher risk of HCC occurrence, but it could decrease the risk of lymph node metastasis and vascular tumor embolism among HCC patients.

Keywords: Polymorphism, valosin-containing protein, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. It is also the forth-diagnosed malignancy and second cause of death in China [1, 2]. Surgical resection is the main radical treatment for HCC, and other adjuvant therapies have also been used in recent years. However, the recurrence rate for HCC after surgery remains high at approximately 60%-70% within 2 years after operation.

Valosin-containing protein (VCP) is a member of the ATPase associated with lots of cellular activities (AAA) family. It is a structural protein that is conserved in all eukaryotes. VCP has a significant association with ubiquitin-proteasome-mediated protein degradation, membrane fusion, transcriptional activation, cell cycle control and apoptosis [3], and it takes part in the regulation of these functions as a molecular chaperone via the ubiquitin-proteasome system (UPS) [4-6]. This protein widely expressed in the cytoplasm and accounts for greater than 1% of cellular protein. VCP is involved in numerous different cellular functions; thus, there have been many studies of this protein in different fields.

VCP dysfunction is related to various diseases such as Paget's bone disease, amyotrophic lateral sclerosis (ALS) and other types of cancers including pancreatic cancer, gastric carcinoma, lung cancer, HCC, prostate cancer and colorectal cancer. Moreover, some clinical studies have also demonstrated a correlation between elevated VCP expression and the risk, progression and treatment outcome of some previously

described human cancers [7]. Single nucleotide polymorphisms (SNPs) are DNA-sequence variations that commonly occur within a population (e.g., 1%) in which a single nucleotide i.e., A, T, C or G, in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes [8, 9]. SNPs have become popular tools for genetic mapping. In previous studies, a single nucleotide polymorphism in the VCP gene was suggested to be associated with the pathogenesis of sporadic Paget's disease, ALS and other neurological diseases [10]. However, there have rarely been studies of VCP polymorphisms in cancers, particularly digestive tract cancers. Thus, this study was designed to identify associations between single nucleotide polymorphisms in VCP and the risk and progression of HCC.

Materials and methods

Patient selection

This study consisted of 122 patients with hepatocellular carcinoma (HCC) from Ruijin Hospital, Shanghai Jiaotong University School of Medicine and 120 non-cancer controls. In Asia, particularly in China, hepatitis B virus (HBV) is the primary cause of HCC. To eliminate the effects of HBV, we also selected 111 patients with HBV but not HCC as controls. All patients and controls provided informed consent. All participants provided written informed consent to participate in this study. This study was approved by the Institutional Review Board of Ruijin Hospital, Shanghai Jiao Tong University, China. Consecutive patients were enrolled from Feb 2013 to Feb 2015 at Ruijin Hospital, Shanghai Jiaotong University, School of Medical. HCC was diagnosed by elevations in alpha-fetoprotein (AFP) and the results of imaging tests including B-type ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI). All patients were excluded from having other primary tumors by examinations including CT, MRI or positron emission computed tomography (PET-CT) and other liver diseases such as primary biliary cirrhosis, alcoholic liver disease, Budd-Chiari syndrome, and schistosomal liver fibrosis. The patients were also negative for hepatitis C virus (HCV) and hepatitis D virus (HDV). We clinically classified all of the patients with HCC according to the criteria of International Union Against Cancer (UICC) tumor-node-metastasis staging system using results from imaging tests and the postoperative pathological examinations. The patients underwent suitable treatments including hepatectomy, Transhepatic Arterial Chemotherapy and Embolization (TACE), liver transplantation and other adjuvant therapies according to tumor stage.

In control groups, we select 120 non-cancer patients and 111 HBV carriers. HBV carriers were defined as positive for both hepatitis B surface antigen and immunoglobulin G against hepatitis B core antigen. Controls were selected from individuals who underwent hepatitis examinations in our hospital during the study period. Therefore, we could reduce the confounding effects of HBV infection in research of genetic susceptibility to HCC. All controls had no diagnoses of cancer or other liver disease at the time of sample collection.

SNP site selection

The data for total VCP SNPs genotyped in the Chinese people population were from the International HapMap project database (http://hapmap.ncbi.nlm.nih.gov/index.html.zh). We downloaded SNP genotype data for the VCP gene (including 20 kb up-and downstream), which we analyzed using Haploview software (http://www.broadinstitute.org/haploview). Four SNPs were selected using the criteria of minor allele frequencies ≥ 10% in the Chinese population for rs2074549, rs607671, rs10972300 and rs546982. These SNPs can cover greater than 80% of the SNP sites for minor allele frequencies that are greater than 10%. The others were eliminated.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes. Genomic regions of interest were amplified by multiplex polymerase chain reaction (PCR). The PCR reactions were performed, and all four SNPs were genotyped by the Sanger method using an ABI3100® automated sequencer.

We used a blinded approach for genotype analysis in which the staff was unaware of the case or control status. A 10% random sample of cases and controls was tested by different investigators for quality control.

Table 1. Baseline characteristics for the hepatocellular carcinoma cases and controls

	HCC cases *n* (%)	Non-cancer controls *n* (%)	HBV carriers *n* (%)	*P* value
Number	122	120	111	
Age (y)				
<55	59 (48.4)	57 (47.5)	54 (46.7)	0.983
≥55	63 (51.6)	63 (52.5)	57 (53.3)	
Gender				
Female	102 (83.6)	90 (75.0)	84 (76.6)	0.199
Male	20 (16.4)	30 (25.0)	27 (23.4)	
α-FP level				
>400 ng/ml	40 (32.7)	NA	NA	
≤400 ng/ml	82 (67.3)			
Stage				
I-II	67 (54.9)			
III-IV	55 (45.1)			

α-FP: Alpha-fetoprotein; NA: No data.

Table 2. Genotype frequencies of the VCP single nucleotide polymorphisms

SNP	Location	Genotype	HCC cases *n* (%)	Non-cancer controls *n* (%)	*P* value
rs2074549	35051503	TT	55 (45.1)	61 (50.8)	
		CT	55 (45.1)	47 (39.2)	0.631
		CC	12 (9.8)	12 (10.0)	
rs607671	35052613	GG	24 (19.7)	25 (20.8)	
		AG	60 (49.2)	58 (48.3)	0.975
		AA	38 (31.1)	37 (30.8)	
rs10972300	35058201	TT	2 (1.6)	0 (0.0)	
		CT	15 (12.3)	19 (15.8)	0.282
		CC	105 (86.1)	101 (84.2)	
rs546982	35075146	AA	17 (13.9)	39 (32.5)	
		AG	62 (50.8)	53 (44.2)	0.002
		GG	43 (35.2)	28 (23.3)	

P value was calculated by a χ^2-test 3*2 contingency table (df = 2).

Statistical analysis

Data are presented as proportions and means and standard deviations (SDs). We performed all tests using SPSS version 19.0 for Windows. *P* values were all two sided.

The Hardy-Weinberg equilibrium (HWE) test was performed using Chi-squareanalysis for each SNP for all participants. Genotype frequency differences were tested between HCC patients and control subjects for each SNP using the Chi-square test.

We estimated odds ratios (ORs) for HCC for variant-allele carriers (homozygous and heterozygous) versus homozygous wild-type allele carriers using unconditional logistic regression.

Results

The baseline characteristics of the HCC and control subjects (including non-cancer controls and HBV carriers) are summarized in **Table 1**. There were no significant differences between the HCC cases and controls in terms of age and sex. Among the HCC cases, 32.7% had an AFP level higher than 400 ng/ml, and the others had an AFP level lower than 400 ng/ml. In addition, 54.9% of the patients with HCC werein UICC classification tumor stages I and II and 45.1% in stages III and IV (**Table 1**).

The D' values between rs607671 and rs2074549, between rs2074549 and rs10972300, and between rs10972300 and rs546982, were 0.79, 0.05, and 0.14, respectively (data not shown). Therefore, each of the four SNPs may be an independent site for investigating associations with HCC. The genotype frequencies of all 4 polymorphisms in the patients and controls (including non-cancer controls and HBV carriers) conformed to the Hardy-Weinberg equilibrium. The distributions of the genotypes for the VCP SNPs are shown in **Table 2**. Chi-square analysis of the genotypes reflected significantly different distributions for one of the VCP SNPs (rs546982) between the HCC and non-cancer groups ($P < 0.05$). To eliminate the effects of HBV, we compared the distributions of the genotypes for the VCP SNPs between non-cancer controls and HBV carriers, and there were no differences (**Table 3**). Also we compared the distributions of the geno-

Table 3. Genotype frequencies of VCP single nucleotide polymorphisms in non-cancer controls and HBV carriers

SNP	Location	Genotype	HBV carriers *n* (%)	Non-cancer controls *n* (%)	*P* value
rs2074549	35051503	TT	39 (35.1)	61 (50.8)	
		CT	57 (51.4)	47 (39.2)	0.055
		CC	15 (13.5)	12 (10.0)	
rs607671	35052613	GG	33 (29.7)	25 (20.8)	
		AG	50 (45.1)	58 (48.3)	0.273
		AA	28 (25.2)	37 (30.8)	
rs10972300	35058201	TT	2 (1.8)	0 (0.0)	
		CT	22 (19.8)	19 (15.8)	0.233
		CC	87 (78.4)	101 (84.2)	
rs546982	35075146	AA	29 (26.1)	39 (32.5)	
		AG	56 (50.4)	53 (44.2)	0.308
		GG	26 (23.4)	28 (23.3)	

P values were calculated by a χ^2-test 3*2 contingency table (df = 2).

Table 4. Genotype frequencies of VCP single nucleotide polymorphisms in HCC cases and HBV carriers

SNP	Location	Genotype	HBV carriers *n* (%)	HCC cases *n* (%)	*P* value
rs2074549	35051503	TT	39 (35.1)	55 (45.1)	
		CT	57 (51.4)	55 (45.1)	0.055
		CC	15 (13.5)	12 (9.8)	
rs607671	35052613	GG	33 (29.7)	24 (19.7)	
		AG	50 (45.1)	60 (49.2)	0.273
		AA	28 (25.2)	38 (31.1)	
rs10972300	35058201	TT	2 (1.8)	2 (1.6)	
		CT	22 (19.8)	15 (12.3)	0.233
		CC	87 (78.4)	105 (86.1)	
rs546982	35075146	AA	29 (26.1)	17 (13.9)	
		AG	56 (50.4)	62 (50.8)	0.028
		GG	26 (23.4)	43 (35.2)	

P values were calculated by a χ^2-test 3*2 contingency table (df = 2).

types for the VCP SNPs between HCC cases and HBV carriers, and there was a significant different distribution for rs546982 (**Table 4**). Thus we eliminate the effections of HBV.

Table 5 shows associations between VCP-SNP-allele variants and HCC risk. Individuals with the GG homozygous genotype for rs546982 were significantly associated with increased risk for HCC compared with that of patients with the wild-type AA homozygous genotype and the AG heterozygous genotype (odds ratio [OR] = 3.523, from 1.677 to 7.401, $P < 0.001$).

In addition, we analyzed associations between rs546982 genotypes and tumor progression. We found that patients with the GG homozygous genotype had decreased risk for lymph node metastasis and intravascular cancer emboli (**Tables 6** and **7**). However, there were no significant differences between all of the genotypes for rs546982 (AA, AG, GG) and the AFP level, tumor size, number of tumors and TNM stage (data not shown).

Discussion

The abundant AAA protein VCP (also called p97) is an essential ATPase present in the cytoplasm, on membranes and in the nucleus of eukaryotes ranging from yeast to man [11-13]. VCP is involved in a wide variety of functions and has attracted much attention in different fields. VCP expression is elevated in a number of solid tumors, including prostate cancer, pancreatic cancer, lung cancer, gastric cancer, esophageal cancer and colorectal carcinoma [14]. Yamamoto reported that VCP was overexpressed in HCC and had an association with tumor progression. This protein may also be a prognosis factor for HCC [15]. Yi and colleagues found that in patients with HCC, VCP tyrosine phosphorylation is prevented by sorafenib, which could lead to the disruption of the secretory pathway and cell death mediated by endoplasmic reticulum stress. This result suggested that VCP is a potential therapeutic target for this disease [16].

Until now, the regulatory mechanism for VCP expression in solid tumors was largely unknown. Liu et al. once reported that miR-129-5p could down-regulate the expression of VCP via interaction with two sites located in its 3' untranslated region (UTR). This group also found that miR-129-5p could inhibit the degradation of

Table 5. Association between hepatocellular carcinoma and VCP single nucleotide polymorphisms

SNP	Genotype	*P* value	OR (95% CI)
rs2074549	TT		1
	CT	0.338	1.298 (0.761-2.212)
	CC	0.817	1.109 (0.460-2.672)
rs607671	GG		1
	AG	0.826	1.078 (0.553-2.098)
	AA	0.854	1.070 (0.521-2.198)
rs10972300	TT		1
	CT	0.999	0.000 (0.000)
	CC	0.999	0.000 (0.000)
rs546982	AA		1
	AG	0.375	0.762 (0.418-1.389)
	GG	<0.001	3.523 (1.677-7.401)

OR: Odds ratio; 95% CI: 95% confidence interval. *P* values were calculated by a χ^2-test 2*2 contingency table (df = 1).

Table 6. Association between the rs546982 genotype and risk for intravascular emboli in patients with HCC

	IV emboli *n* (%)	Non-IV emboli *n* (%)	*P* value	OR (95% CI)
rs546982				
AA	5 (21.7)	12 (12.1)		1
AG	14 (60.9)	48 (48.5)	0.559	0.700 (0.211-2.327)
GG	4 (17.4)	39 (39.4)	0.047	0.246 (0.057-1.065)

IV, Intravascular; *P* values were calculated by a χ^2-test 3*2 contingency table (df = 2).

Table 7. Association between the rs546982 genotype and risk for lymph node metastasis in patients with HCC

	LN metastasis *n* (%)	Non-LN metastasis *n* (%)	*P* value	OR (95% CI)
rs546982				
AA	6 (27.3)	11 (11.0)		1
AG	11 (50.0)	51 (51.0)	0.119	0.395 (0.120-1.300)
GG	5 (22.7)	38 (38.0)	0.033	0.241 (0.061-0.943)

LN, Lymph node; *P* value was calculated by a χ^2-test 3*2 contingency table (df = 2).

IkBa [17]. The activation of NF-kB was also investigated together with tumor progression. IkBa is an inhibitor of NF-kB; thus, VCP can affect the cell growth, apoptosis and migration of tumor cells via the NF-kB pathway. Yu et al. proposed a novel mechanism linking VCP phosphorylation and the degradation of ubiquitinated aggregates. This group hypothesized that cross-regulation between phosphorylation and ubiquitination may be a key mechanism resulting in an anti-apoptotic effect [11].

Evidence had supported the idea that VCP was overexpressed in HCC; however, its genetic changes have not been studied. The incidence and progression of cancer represent the accumulation of mutants and have a strong association with gene polymorphisms. The study of SNPs can help explain phenotypic differences, disease susceptibility, drug tolerance and the response to environment factors for different individuals. To our knowledge, this is the first attempt to investigate a few common SNPs located in the VCP gene in patients with hepatocellular carcinoma.

Peng et al. reported that VCP polymorphisms are associated with severe chemotherapy-related adverse outcomes in platinum-treated patients with advanced non-small-cell lung cancer [18]. Chung PY found a genetic association between a VCP polymorphism and the pathogenesis of sporadic Paget's disease of bone [11]. However, until now, there are no reports of the association between VCP polymorphisms and digestive tract carcinoma. In our study, we selected four SNP sites including rs2074549, rs607671, rs10972300 and rs546982. In Peng's study, the SNP site rs2074549 had an association with side effects for platinum-based chemotherapy for advanced-stage patients with non-small-cell lung cancer. This site might work by influencing the degradation of IkB [19]. However, there was no significant difference in this SNP site between HCC cases and controls in our study. Chung's study revealed an association between the rs565070 SNP and the Paget's disease in the Belgian population. Our data demonstrated that the rs546982 site had the strongest relationship to the risk and progression of HCC. This SNP is located approximately 13 kb upstream of the VCP gene. We analyzed the rs546982 site with Haploview 4.2. The

results demonstrated that the SNP site was relatively far from the VCP gene, but it still had a powerful linkage and high degree of association. This SNP site affects the expression and function of the VCP gene and influence the incidence and progression of HCC. Schatzberg et al. reported that the functional SNP rs10245483, which had a significant effect on remission and side effects, is located upstream from ABCB1 [20]. Hoffjan et al. also found a significant association between rs10499194, which is located in the intergenic region upstream of TNFAIP3, and multiple sclerosis [21]. Agrawal et al. evaluated the effects of an HLA-G 5'-upstream regulatory region (URR) SNP in idiopathic recurrent spontaneous abortion (RSA) and found that HLA-G expression was down-regulated at the transcription level for -1179G>A and -725C>G/T SNPs in patients with idiopathic RSA. These data suggested that the transmission of a mutant allele from single-carrier parents may have an effect on pregnancy outcome [22]. These findings support the idea that SNPs that are not locate in the genomic sequence may continue to play an important role in the function of genes.

Linkage disequilibrium (LD) analysis showed that individuals with the GG haplotype were significantly associated with an increase in risk for HCC (OR = 3.523, from 1.677 to 7.401, $P < 0.001$) compared with others with the GG and AG haplotypes. Although limited by a small sample size, one potentially interesting observation of our study was that patients with HCC had a decreased risk of getting lymph node metastasis and intravascular emboli (OR: 0.241 and 0.246, respectively, $P = 0.033$ and 0.047, respectively) if they carried the haplotype GG. These data indicated an interest phenomenon that although the GG haplotype is a risk factor for HCC, but it decreased the malignancy grade of HCC when tumorigenesis occurred. The other three SNPs are located in introns and have no association with HCC risk. Although SNPs located in introns, it is possible that they also have significance for the incidence and progression of diseases. Wiśniewsk reported five SNPs in intron 14 of LILRB that influenced the risk of non-small-cell lung cancer [23]. Eun et al. found that an intron SNP (rs1571013) of Fas was significantly associated with the development of papillary thyroid cancer by genotyping analysis [24].

Our study has some limitations. 1) We performed a cohort-study analysis using data from a single institution. The sample size was relatively small, which may have weakened our test power for detected the association between SNPs and HCC risk and progression [25]. 2) We investigated only a series of four SNPs associated with VCP. It is known that tumorigenesis is associated with different genes, and additional candidate genes related to HCC also need to be investigated to fully integrate these associations so that we can focus on the interactions of the different genes. 3) Not all diseases have associated SNPs, and not all SNPs will result in the incidence of diseases [26]. 4) VCP levels were not quantified in this cohort. It was not clear that how the expression of VCP correlated with SNP genotypes, specifically SNP rs546982. 5) We may not simply investigate SNPs but also need study the pathogenesis caused by them.

Conclusion

Our study provides evidence of a close association between VCP SNP mutations and hepatocellular carcinoma. The SNP site rs546982 correlates with risk and progression for HCC. Individuals who have the GG haplotype have increased risk for HCC, but patients with HCC who have the GG haplotype have a decreased risk for lymph node metastasis and intravascular emboli. These findings may define a new genetic susceptibility background for hepatocellular carcinoma suggesting the risk for disease and prognosis. More work is needed to understand the mechanism by which VCP SNPs affect the pathological role of VCP in signaling pathways that control tumorigenesis.

Disclosure of conflict of interest

None.

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