Int J Clin Exp Med 2016;9(2):2937-2944
www.ijcem.com /ISSN:1940-5901/IJCEM0016739

Original Article

Cyclin-dependent Kinase 14 polymorphisms is associated with vascular endothelial growth factor in HBV-related hepatocarcinoma

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Received September 23, 2015; Accepted January 21, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: Background: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and highly invasive and metastatic. The relationship between vascular endothelial growth factor (VEGF) single-nucleotide polymorphisms (SNPs) and HBV-related HCC is still poorly understood. Methods: A total of 242,901 single-nucleotide polymorphisms (SNPs) were determined via the Illumina Infinium Human Exome BeadChip V1.0 in tumor DNA of 478 patients with HBV-related HCC. The odds ratios or adjusted odds ratios were calculated for the evaluation of relative risk of VEGF expression, and the influence of these SNPs on the clinical characteristics of HBV-related HCC was evaluated. Results: CDK14 rs10488004 (MAF = 0.45; $P = 1.48 \times 10^{-6}$) of chromosome 7 was significantly associated with VEGF expression in patients with HBV-relative HCC. After adjusting for other covariants, HBV-related HCC patients who carried G genotype (GG and GA) at rs10488004 had a lower risk for VEGF positive expression as compared to those carrying wild-type genotype (AA) (OR: 0.341, 95% CI: 0.184-0.632). Moreover, patients carrying allele gene G at the same SNP showed a relatively better liver function. Conclusion: Our results indicate that CDK14 rs10488004 is associated with VEGF expression in HBV-related HCC, and selected SNP may be a potential predictor of liver function in HCC patients.

Keywords: Hepatocellular carcinoma, single-nucleotide polymorphisms, vascular endothelial growth factor

Introduction

Hepatocellular carcinoma (HCC), a common malignancy with a poor prognosis, has been the second leading cause of cancer-related death in men worldwide and for both males and females in China [1, 2]. The high prevalence of HCC in China is largely ascribed to the high incidence of chronic hepatitis B virus (HBV) infection and aflatoxin exposure. Other common risk factors of HCC include obesity, type 2 diabetes, alcoholic cirrhosis, nonalcoholic fatty liver disease and smoking [2-4]. The development of HCC emerges through a multi-step and complex process. In recent years, several reports indicate that the progressive accumulation of genetic alterations and diverse single nucleotide polymorphisms (SNPs) contribute to the hepatocarcinogenesis under the stimulation of a variety of factors [5].

Metastasis is the major cause of morbidity and mortality in HCC patients. Moreover, vascular endothelial growth factor (VEGF), as a tumor marker, plays an important role in the angiogenesis which is essential for the tumor growth, invasion, and metastasis [6-8]. The increased VEGF in the serum and tumor tissues has been found to be correlated with a poor prognosis of HCC patients [9]. In addition, the serum VEGF is helpful to predict the response to hepatic arterial infusion chemotherapy as well as the malignant potential including metastasis, pathological type and vascular invasion in HCC patients [10].

Table 1. Clinical characteristics of 100 VEGF negative patients and 330 VEGF positive patients

Variable	VEGF positive	VEGF negative	P
Age (yrs)	46.82 ± 11.11	47.04 ± 10.18	0.859
Gender			
Male	298 (90.3%)	86 (86.0%)	0.223
Female	32 (9.7%)	14 (14.0%)	
Extrahepatic metastasis			
No	302 (91.5%)	89 (89.0%)	0.443
Yes	28 (8.5%)	11 (11.0%)	
BCLC stage			
0 or A	188 (57.0%)	56 (56.0%)	0.598
B	57 (17.2%)	14 (14.0%)	
C	85 (25.8%)	30 (30.0%)	
Child-Pugh grade			
A	267 (86.1%)	78 (83.0%)	0.449
B or C	43 (13.9%)	16 (17.0%)	
Edmondson-Steiner's Grade			
Well differentiated	18 (6.3%)	7 (8.0%)	0.889
Moderately differentiated	260 (90.9%)	78 (89.7%)	
Poorly differentiated	8 (2.8%)	2 (2.3%)	
Undifferentiated			
Tumor size			
< 3 cm	46 (13.9%)	17 (17.0%)	0.448
≥ 3 cm	284 (86.1%)	83 (83.0%)	
Tumor nodules			
Single	238 (72.1%)	77 (77.0%)	0.334
Multiple	92 (27.9%)	23 (23.0%)	
PVTT			
No	275 (84.4%)	80 (80.0%)	0.392
Vp1	10 (3.1%)	1 (1.0%)	
Vp2	11 (3.4%)	6 (6.0%)	
Vp3	24 (7.4%)	11 (11.0%)	
Vp4	6 (1.8%)	2 (2.0%)	

Notes: Vp1 (PVTT in distal to second-order portal branches), Vp2 (PVTT in second order branches), Vp3 (PVTT in first-order branches), and Vp4 (PVTT in the main trunk). $P < 0.05$ was considered statistically significant.

Recent years, genome-wide association analysis (GWAS) has provided a platform for the investigation of molecular genetics of HCC development and progression [11-13]. Currently, several studies have shown a content of candidate cancer genes are related to HCC and some genetic variants and single-nucleotide polymorphisms (SNPs) are associated with the risk for HCC [14-17]. Thus, in the present study, 242,901 SNPs were detected by an exome array and the relationships of SNPs with the risk for HCC and clinicopathological characteristics of HCC were investigated in 478 patients with HBV-related HCC from Guangxi China.

Subjects and methods

Subjects

A total of 478 patients diagnosed with HBV-related HCC were recruited into this study between 2005 and 2013 from the First Affiliated Hospital of Guangxi Medical University. All the subjects were diagnosed with HBV-related HCC based on the Chinese Guideline for the Clinical Diagnosis and Treatment of HCC, and the postoperative pathological findings. Of them, tumor tissues undergoing immunohistochemistry were collected from 430 patients. Patients were divided into two groups according to VEGF expression in the tumor tissues (positive expression: VEGF group; negative expression: control group). The clinicopathological characteristics were recorded, including BCLC stage, Child-Pugh grade, tumor size, tumor nodules, extrahepatic metastasis, Edmondson-Steiner's grade and portal vein tumor thrombus (PVTT). The association between distribution of genomic frequency and clinical status was evaluated in 478 patients. This study was approved by the local Ethical Committees, and written informed consent was obtained from each patient before the study.

Sample collection and processing

Tumor tissues were collected from HCC patients during the surgery, stored at -80°C and processed for immunohistochemistry for VEGF in the Department of Pathology of the First Affiliated Hospital of Guangxi Medical University. Genomic DNA was extracted from tumor tissues using TIANamp Genomic DNA Kit (Tiangen Biotech (BEIJING) CO, LTD), according to the manufacturer's instructions, and the DNA was dissolved in TE buffer (10 mM Tris (pH 7.8) and 1 mM EDTA). DNA concentra-

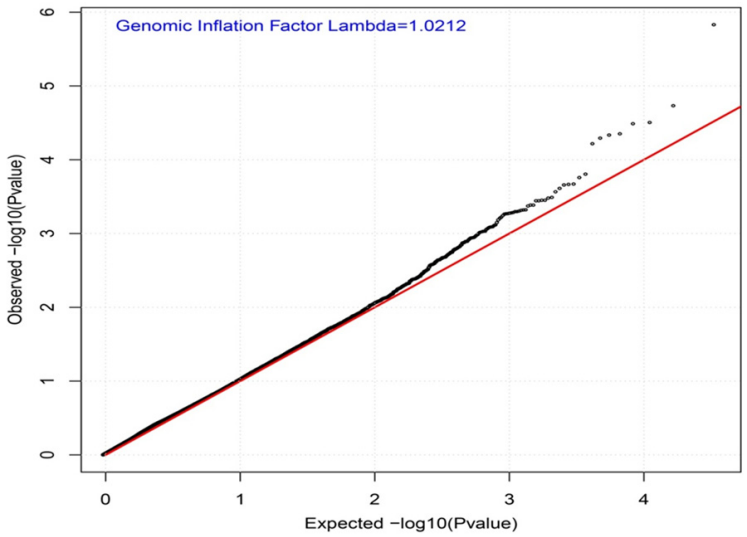

Figure 1. Quantile-quantile (Q-Q) plots for Single Variant Test results. No population stratification was found base on the genomic inflation factor λ (lambda) = 1.0212.

tion and purity were measured with the NanoDrop 2000 system (Thermo Fisher Scientific, Waktham, MA, USA).

Genotyping and quality control

Illumina Infinium Human Exome BeadChip 12-v1-1 system (Illumina Inc. USA) was used for genome scanning, in which 242,901 markers of protein-altering variants were detected. Genotyping was performed by using Illumina's Genotyping Module v1.0 clustering algorithm with the Genome Studio software (V2011.1). Polymorphisms showing genotyping with call rates less than 95% in either cases or controls, no Hardy-Weinberg equilibrium (HWE) ($P < 1.0 \times 10^{-6}$), or the allele frequency of less than 5% in both cases and controls were excluded. Genotyping rates less than 95% or evidence of relatedness with other subjects were also excluded. Kinship between studied subjects was estimated by PLINK version 1.07. And study specimens which were outlier in principal components analysis (PCA) for ancestry and population stratification were also eliminated. Quantile-quantile plot (QQ-plot) was used to assess the population stratification of the study.

Statistical analysis

Single Variant Test (chose the Logistic Grade Test) [18] was used for the evaluation of the association of variants with VEGF expression after adjustment for age, gender, BCLC stage, PVTT and extrahepatic metastasis with the EPACTS package version 3.2.6. The clinical characteristics at baseline were compared with chi square test or Fisher's exact test between VEGF group and control group. The odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated in the evaluation of association between genotype frequency and VEGF expression and analyzed using multiple logistic regression model after adjustment for covariates including age, gender, BCLC stage, extrahepatic metastasis and PVTT. A value of $P < 0.05$ was considered statistically significant. The SPSS version 20.0 statistical software was used for statistical analysis. Linkage disequilibrium (LD) between SNPs in the Illumina Infinium Human Exome Bead-Chip was assessed based on the genotypes. LD and recombination pattern nearby CDK14 gene were analyzed using LocusZoom [19].

Results

The clinical characteristics of patients are summarized in **Table 1**. Of 430 HCC patients, 330 were positive for VEGF expression, and 100 negative for VEGF expression. In addition, 15.45% of patients (51/330) in VEGF group and 20.0% of patients (20/100) in control group suffered from PVTT; 8.5% of patients in VEGF group (28/330) and 11.0% of patients in control group (11/100) developed extrahepatic metastasis. No significant differences were found in the gender (P = 0.223), age (P = 0.859), tumor size (P = 0.448), tumor nodules (P = 0.334), BCLC stage (P = 0.598), Child-Pugh grade (P = 0.449), Edmondson-Steiner's grade (P = 0.889), PVTT (P = 0.392), and extrahepatic metastasis (P = 0.443) between VEGF group and control group.

After quality control, total genotyping rate in remaining patients was 99.62%. DNA specimens from 330 VEGF positive patients and 100 controls were subjected to genome scanning with the Illumina Human-Exome BeadChip

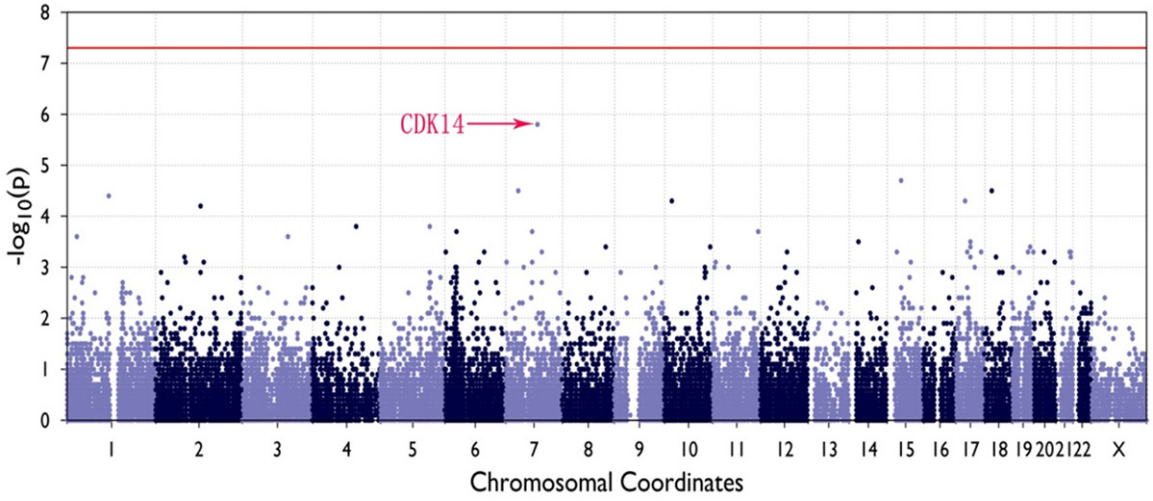

Figure 2. Manhattan plot for the association analysis of VEGF expression and SNP in 430 HBV-related HCC patients who received detection of 238,850 SNPs by using Human Exome Bead Chip v1.0 (Illumina Inc., San Diego, CA, USA).

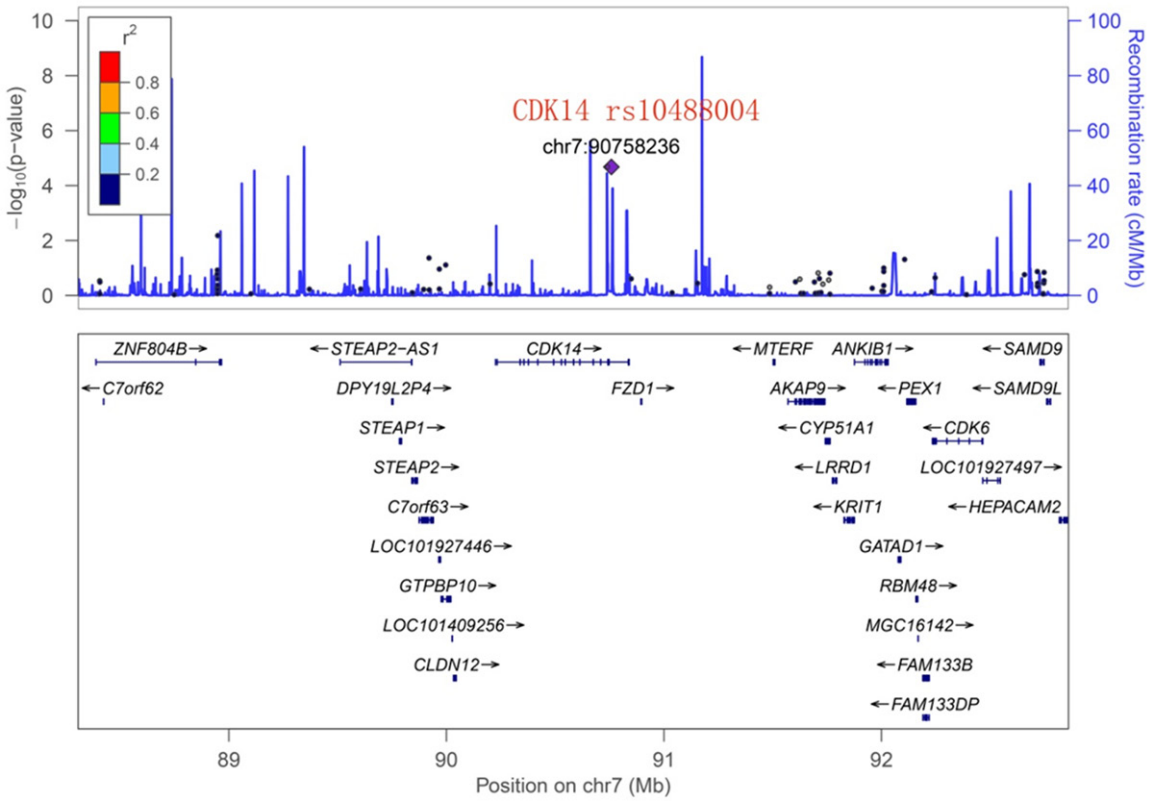

Figure 3. LocusZoom plot for the localization of rs10488004 and associated regions near the CDK14 gene.

which contains 238,850 of 242,901 SNPs. Population stratification was evaluated by QQ plot. Results indicated a lambda value of 1.0212 in the QQ plot (**Figure 1**), suggesting no excess population stratification in this study. Manhattan plot showed a SNP rs10488004 (MAF = 0.45; P = 1.48 × 10^{-6}) of chromosome 7 as well as CDK14 was significantly associated with VEGF expression in HBV-related HCC patients (**Figure 2**). The frequency of rs10488004 was in the Hardy-Weinberg equilibrium (P = 0.34). rs10488004 locates in an intron of CDK14 showed a loose LD with other SNPs in this region (r^2 < 0.4) (**Figure 3**).

Table 2. Frequency of CDK14 genotypes in 100 VEGF negative patients and 330 VEGF positive patients

Variable	VEGF positive	VEGF negative	OR (95% CI)	AOR (95% CI)	P
Rs10488004					
AA	104	14	1.00	1.00	
GA	168	52	0.435 (0.230-0.824)	0.428 (0.225-0.817)	0.01
GG	53	32	0.223 (0.110-0.453)	0.201 (0.098-0.414)	< 0.01
GA+GG	221	84	0.354 (0.192-0.653)	0.341 (0.184-0.632)	< 0.01

Notes: The odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using logistic regression models. The adjusted odds ratios (AORs) with 95% CIs were estimated using multiple logistic regression models after adjustment for age, gender, PVTT, BCLC stage, and extrahepatic metastasis. P < 0.05 was considered statistically significant.

Table 3. Association between clinical characteristics and CDK14 genotype in 478 patients with HCC

Variables	CDK14 rs10488004			
	AA (n = 133)	GA+GG (n = 345)	OR (95% CI)	P
Child-Pugh grade				
A	91	249	0.556 (0.312-0.992)	0.047*
B or C	23	35		
Extrahepatic metastasis				
Yes	12	27	0.856 (0.420-1.744)	0.669
No	121	318		
PVTT				
Yes	27	56	0.761 (0.457-1.267)	0.294
No	106	289		
Tumor nodules				
Single	102	248	1.287 (0.808-2.050)	0.288
Multiple	31	97		
Tumor size				
≤ 3 cm	20	54	0.954 (0.546-1.665)	0.868
> 3 cm	113	291		

Notes: *P < 0.05 was considered statistically significant.

To decrease the influence of confounding variables, adjusted odds ratios (AORs) with 95% CIs were calculated using the multiple logistic regression model after adjustment for age, gender, PVTT, BCLC stage and extrahepatic metastasis. **Table 2** displays the CDK14 rs10488004 genotype distribution and results showed a significant association between VEGF expression and CDK14 polymorphism. The ancestral allele at rs10488004 was A, and the highest distribution frequency was heterozygous G/A. Patients with heterozygous G/A and homozygous G/G genotype had a lower risk for high VEGF expression of HCC patients (OR: 0.435, 95% CI: 0.230-0.824) (P = 0.01), (OR: 0.223, 95% CI: 0.110-0.453) (P < 0.01) as compared to those with homozygous A/A genotype. This indicates allele G has a protective effect.

The distribution of CDK14 rs10488004 genotype and the correlation between CDK14 genotypes and clinical characteristics were further determined to investigate the effect of CDK14 polymorphism on the clinical characteristics of HBV-related HCC patients. As shown in **Table 3**, for CDK14 rs10488004, heterozygous G/A and homozygous G/G genotypes showed a significant association with Child-Pugh grade and a preferable liver function (GG+GA vs AA, OR: 0.556, 95% CI: 0.312-0.992) (P < 0.05). There were no significant correlations between rs10488004 genotype frequency and other clinical variables.

Discussion

HCC is one of the most common malignancies worldwide and usually has a poor prognosis. At initial hospital visit, most patients are often diagnosed with HCC at advanced stage (metastasis and vascular invasion), and thus lose the chance for surgical treatment. VEGF is a crucial factor involved in the physiological and pathological angiogenesis, and overexpression of VEGF has been found in HCC [20, 21]*. The circulating VEGF is also reported to be related with the stage of HCC and the highest VEGF expression is identified in patients with metastasis [22, 23]. Previous studies also reported that the vascular endothelial cells in tumor tissues were strong posi-

tive for VEGF in immunohistochemistry, but normal cells were negative for it. Vascular endothelial cells are the main target of VEGF released from HCC cells [24, 25].

According to above-mentioned, this study was conducted to investigate the association of genetic variants and genotypes with VEGF expression in HBV-related HCC patients, in which exome array was used for genome scanning of DNA extracted from tumor tissues. Results showed rs10488004 locating in an intron of CDK14 had significant association with VEGF expression. CDK14, also known as PFTK1 locating in chromosome 7q21-q22, encodes a cyclin-dependent kinase, and has been shown to promote the migration of HCC cells, facilitate the cell cycle progression and regulate several pathways and cellular mechanisms as an oncogene. PFTK1 is expressed in HCC, where its over-expression is usually related more aggressive phenotype and poor prognosis. Studies also report that the PFTK1 expression is upregulated in tumor tissues, and it is able to phosphorylate an intermediate substrate TAGLN2 to regulate the invasiveness and motility of HCC cells [26-29]. Given that rs10488004 localizes in an intron of CDK14 gene, it may affect gene expression and influence the invasiveness and motility of HCC cells. Consequently, it may also indirectly affect the release VEGF from tumor cells. The multiple logistic regression analysis suggested that rs10488004 genotype was significantly associated with the VEGF expression. Patients with allele G had a lower risk for high VEGF expression as compared to those with allele A (GG+GA vs AA, OR: 0.354, 95% CI: 0.192-0.653) ($P < 0.01$). After adjustment for factors related to invasiveness and metastasis of HCC cells (PVTT and extrahepatic metastasis), the AOR was 0.341 (95% CI: 0.184-0.632; $P < 0.01$). This indicates that allele G of CDK14 rs10488004 is beneficial, and the VEGF expression in patients with rs10488004 is different from that in patients carrying the ancestral genotype.

Moreover, CDK14 is a member of the CDK family. CDKs may regulate cell cycle progression, transcription and differentiation [30-32]. Moreover, some CDKs have been implicated in the prognosis as well as the sensitivity to chemotherapy in human esophageal squamous cell carcinoma (ESCC) [33]. For HCC, CDK14 promotes the invasiveness of tumor cells. The degree of malignancy of HCC cells significantly influences the development stages of HCC and the liver function of patients. BCLC stage and Child-Pugh grade are common indicators used for the clinic evaluation of HCC status [34, 35]. In our study, results showed CDK14 rs10488004 (G/G and G/A) was associated with a lower Child-Pugh grade (grade A) (OR: 0.556, 95% CI: 0.312-0.992) in HCC patients. However, the association was not observed between the genetic polymorphism of CDK14 and other clinical characteristics related to development of HCC. This might be ascribed to a small sample size.

In conclusion, our results show a significant correlation between CDK14 polymorphisms and VEGF expression in HBV related HCC patients. A variant CDK14 allele may be beneficial and has a protective effect on HCC in expression of VEGF. We conclude that rs10488004 of CDK14 may be used to predict the liver function of HBV-relative HCC patients. Further studies are needed to confirm our findings in more studies with large sample size and to investigate the potential mechanisms underlying the influence of CDK14 polymorphisms on the VEGF expression.

Acknowledgements

This work was supported in part by the National Nature Science Foundation of China (NSFC 81072321 and 81560535), and 2009 Program for New Century Excellent Talents in University (NCET).

Disclosure of conflict of interest

None.

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