Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;42:1342-1357
DOI: 10.1159/000478963
Published online: July 12, 2017
Accepted: May 11, 2017

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Original Paper

Genome-Wide Association Study of MKI67 Expression and its Clinical Implications in HBV-Related Hepatocellular Carcinoma in Southern China

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Key Words
HBV-related HCC • MKI67 • GWAS • Southern China

Abstract
Background/Aims: Hepatocellular carcinoma (HCC) is a common malignant tumor with a high rate of recurrence. Immunohistochemical analysis of the marker of proliferation Ki-67 (MKI67) is used to assess proliferation activity of HCC. The regulation of MKI67 expression remains unclear in HCC. This study aims to explore the association between MKI67 expression and gene variants. ***Methods*:** A total of 195 hepatitis B virus (HBV)-related HCC patients were genotyped using Illumina HumanExome BeadChip-12-1_A (242,901 markers). An independent cohort (97 subjects) validated the association of polymorphism determinants and candidate genes with MKI67 expression. The relationships between MKI67 with p53 and variants of candidate genes in the clinical outcomes of HCC patients were analyzed. ***Results***: We found that MKI67 combined with p53 was associated with a 3-year recurrence-free survival and five variants near TTN and CCDC8 were associated with MKI67 expression. TTN harboring rs2288563-TT and rs2562832-AA+CA indicated a favorable outcome for HCC patients. ***Conclusion***: Variants near TTN and CCDC8 were associated with MKI67 expression, and rs2288563 and rs2562832

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in TTN are potential biomarkers for the prediction of clinical outcomes in HBV-related HCC patients.

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Introduction

Primary liver cancer in males is the fifth most common and second leading cause of cancer death globally. Approximately 782,500 new cases and 745,500 deaths occurred worldwide in 2012, with China accounting for more than half of the total number of cases and deaths [1]. Hepatocellular carcinoma (HCC) comprises 85–90% of all primary liver cancer [2]. Hepatocarcinogenesis is an extremely complex process, and the etiological factors of HCC include the hepatitis B virus (HBV), hepatitis C virus, aflatoxin exposure, excessive alcohol intake, liver flukes, and cirrhosis [3, 4]. Previous studies have identified that aberrantly expressed oncogenes or tumor suppressive genes related to RNA, microRNAs, inflammatory cytokines and gene variants are also increasingly believed to be functionally involved in the control of various biological processes that are involved in cancer development, such as tumorigenesis, metastasis, and proliferation, by regulating the expression of oncogenes or tumor suppressive genes [5–9]. Although diagnoses and treatments have been greatly improved, the outcome of HCC patients remains unfavorable, with an overall 5-year survival rate of approximately 30% after hepatic resection [10]. Despite resection with curative intent, the clinical course is variable and recurrence occurs in a high proportion of cases [11, 12]. Thus, the ability to predict patients at a higher risk of recurrence and with a poor clinical outcome would contribute in guiding surgical and chemotherapeutic treatment according to individual risk. With advances in the application of tumor biology, available molecular biomarkers for carcinogenesis have grown. The prognosis significance of the biomarkers and their potential as therapeutic targets has attracted attention.

The proliferative activity of a tumor provides an indication of its rate of growth and correlates with an aggressive phenotype. Many studies have performed immunohistochemical assessments of the proliferation activity of tumors. The marker of proliferation Ki-67 (MKI67), located on the 10q25 chromosome, expresses in all phases of the cellular cycle other than the G_0 phase [13]. MKI67 protein expression in carcinomas has been intensively investigated, and the MKI67-positive cell rate (called labeling index) has been shown to be associated with clinical-pathological features and even clinical outcome in cases of cancers, including HCC [14]. In a study of patients undergoing surgical resection for HCC, higher levels of MKI67 expression in tumor tissue were associated with a higher tumor grade [15] and early disease recurrence [16]. Further, staining for MKI67 and p53 (encoded by a tumor suppressor gene) are widely used to predict the clinical outcomes of HCC patients after resection, and even liver transplantation [17, 18]. However, despite the significance of MKI67 in estimating the proliferative activity of HCC, the polymorphism determinants associated with MKI67 expression remain unclear.

Guangxi is a district in southern China, with a high prevalence of HCC [19]. We performed a genome-wide association study (GWAS) to detect gene polymorphisms involved in MKI67 expression and to assess the clinical implication with p53. The aim was to reveal the association of p53 with MKI67 and to find potential biomarkers in patients with HBV-related HCC in Guangxi.

Materials and Methods

Ethics approval

This study was approved by the Ethics Committee of the First Affiliated hospital of Guangxi Medical University.

Study subjects

Two independent cohorts of patients with HCC at the First Affiliated Hospital of Guangxi Medical University were enrolled in this study. In the initial GWAS, a total of 195 subjects were enrolled from 2003 July to 2013 November. In the validation study, an independent sample set (in total 97 individuals with HCC) was also enrolled from 2015 October to 2016 September. All subjects were histopathologically confirmed with HBV-related HCC after hepatectomy by the Department of Pathology at the First Affiliated Hospital of Guangxi Medical University. HBV-related HCC were defined as those subjects in which both HBV surface antigen and HBV core antibody were positive serologically over a 6-month period. Informed consent was written and obtained from all patients before collection of the specimens.

Demographic and clinical characteristics were extracted from medical operation records and postoperative pathological reports. The Child-Pugh classification and Barcelona Clinic Liver Cancer (BCLC) staging system [20] were used to measure the liver function and tumor status, respectively. Radical hepatic resection was defined as complete removal of tumor; incision margin of more than 2 cm; no visible tumor thrombus in the portal vein, hepatic vein, and vena cava; no extrahepatic lymph node and distal metastasis [21]. Intrahepatic metastasis was defined as metastases exhibiting similar histological characteristics and cell types but having the same or a worse level of pathological differentiation when HCC cells infringed the portal vein [22]. Distant metastasis was defined as pulmonary metastasis; extrahepatic lymph node metastasis; and implantation metastasis. Portal vein tumor thrombus (PVTT) was classified as follows: vp1 = PVTT in distal to second-order portal branches, vp2 = PVTT in second-order portal branches, vp3 = PVTT in first order branches, vp4 = PVTT in main trunk [23].

Immunohistochemical staining

Immunohistochemical staining for MKI67 and p53 was performed on all samples by eligible full-time pathologists from aforementioned subjects according to previous criteria [24, 25]. To measure the MKI67 labeling index, up to 10 randomly selected ×40 high-power fields were analyzed when at least 1000 tumor cells were counted. Only nuclear staining (plus mitotic figures which were stained by MKI67) were incorporated into the MKI67 score that is defined as the percentage of positively stained cells among the total number of malignant cells scored [26]. Positive scoring for p53 was considered when ≥ 10% tumor cells were stained. Negative and internal positive controls were served for each analyzed sample. Percentage scores for MKI67 and p53 were examined independently and checked collectively by two pathologists who were blinded to the clinicopathologic variables. Whenever possible, a consensus was reached by a joint review in the case of disagreement.

Based on aforementioned features, the representative staining of MKI67 and p53 in HCC tissue is shown in Fig. 1A and 1B. All subjects were divided into high and low MKI67 labeling index groups by the median (25%) (the percentage of MKI67 positive tumor cells was ranged: 1–90%). Accordingly, 79 subjects were allocated to the low (≤ 25%) and 118 subjects to the high MKI67 labeling index group (>25%).

DNA specimens and genotyping

HCC specimens (tumor and paracancer tissues) were obtained after surgical resection and stored immediately at −80°C. Tissue DNA was extracted using a TIANamp Genomic DNA Kit (Tiangen Biotech (Beijing) Co, Ltd, China), and DNA yield and purity were measured by a NanoDrop2000 system (Thermo Fisher Scientific, Waltham, MA, USA).

In the GWAS scan procedure, all samples were genotyped using the Illumina HumanExome BeadChip-12-1_A system (242,901 markers). Samples were conducted according to the Illumina HD Assay Ultra manual and imaging of the BeadChip was processed on an iScan system. A total of 20 random samples (over 10%) were sequenced for the candidate loci using ABI Prism 3100 (Applied Biosystems, Shanghai Sangon Biological Engineering Technology & Services Co, Ltd, Shanghai, China), yielding a 100% concordant with the results of genotyping by BeadChip-12-1_A. Genotype calling was carried out using Genotyping Module v1.0 in GenomeStudio version 2011.1.

For the replication study, we selected candidate SNPs located on candidate gene regions after quality control (QC). Genotyping in the validation samples were done by using polymerase chain reaction (PCR) amplification assay implemented in a 50 μl volumes using HotMaster PCR MasterMix (KT208-02, Tiangen Biotech (Beijing) Co. Ltd.), and Sanger DNA sequencing using an ABI Prism 3100 (Applied Biosystems, Shanghai Sangon Biological Engineering Technology and Services).

Quality control

To eliminate interference of multi-ethnic genetic backgrounds, we performed an analysis of population stratification using principal components analysis (PCA) by the EIGENSOFT package (see URLs). Subjects were excluded with (i) genotyping call rate < 95%; (ii) genome-wide identity-by-descent > 0.1875; and (iii) outliers in PCA plot for ancestry and population stratification. For further analysis, candidate SNPs passed the following QC criteria: (i) a genotype call rate > 95%; (ii) a Hardy–Weinberg equilibrium $P > 1\times10^{-6}$; (iii) a minor allele frequencies (MAF) > 0.05. Finally, candidate SNPs with acceptable quality were genotyped and analyzed.

Patients follow-up

All patients were followed up after discharge until death or the last time of follow-up (last follow-up: September 2014) by personal or family contacts. The median follow-up time for 195 patients was 42 months (ranging from 4 to 125 months), and the median survival time (MST) was 23 months. A total of 185 patients received complete follow-up successfully. Overall survival (OS) was defined as the interval from the date of surgery to the date of death. Recurrence-free survival (RFS) was defined as the interval between the date of surgery and the first recurrence or metastasis, or the last follow-up.

Statistical analysis

GWAS. The QC procedure was performed through Plink version 1.07, R 3.0.1 and the EIGENSOFT package (see URLs). The potential impact of population stratification was evaluated by quantile-quantile (Q-Q) plot and PCA, which was implemented in MATLAB 7.0 (see URLs). In this procedure, 11 subjects were excluded. A linear regression model was used to test the association of SNPs in the genome using the PLINK software package [27], adjusting for age, gender, ethnicity, smoking status, drinking status, preoperative transcatheter arterial chemoembolization (TACE), BCLC stage as covariates, and an additive model for allelic effects was assumed. Due to the limited sample size, the *P*-value and MAF threshold for the GWAS were 0.01 and 0.1, respectively.

Differences in the distributions of demographic characteristics were calculated using the chi-squared test and Fisher's exact test. Logistic regression was applied to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the risk factors associated with the MKI67 labeling index in the initial GWAS and replication study. Local linkage disequilibrium (LD) and recombination patterns of nearby candidate SNPs were analyzed using LocusZoom [28] to create regional association plots. The association between candidate genes with MKI67 at the mRNA level was analyzed from the Gene Expression Omnibus (GEO) database. The correlation of metric data was analyzed by Pearson's correlation coefficient. Hardy–Weinberg equilibrium was calculated by the goodness-of-fit χ^2 test.

Bioinformatics analysis. Gene ontology was enriched and annotated via the DAVID database (see URLs). Gene pathway analysis was predicted by GeneMania software (see URLs). The LD values among SNPs were analyzed by the Haploview 4.2 program (see URLs). The function of candidate SNPs was predicted using the Snpfunc website (see URLs).

Survival analysis. The Kaplan–Meier method with log-rank test was used to calculate the MST and median recurrence time (MRT) in different subgroups classified by demographic parameters and genotypes. Univariate and multivariate Cox proportional hazard regression model were performed to calculate hazard ratios (HRs) and 95% CIs. Stratified analysis for candidate SNPs was performed to evaluate the relative HRs and combined analysis was used to assess joint effects on the prognosis of HCC patients. All statistical analyses were two-sided and performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). A P-value of less than 0.05 was considered to be statistically significant.

Results

Baseline characteristics

All 195 subjects' clinicopathologic characteristics are given in Table 1. No statistical difference in the distributions was demonstrated in most characteristics, including age, gender, ethnicities, smoking status, drinking status, preoperative TACE, serum alpha-fetoprotein (AFP) levels, Child-Pugh classification, BCLC stage, tumor size, tumor nodes,

Table 1. Clinicopathological characteristics of HBV-related HCC cases. Note: [a] *P* for Chi-square test or Fisher's exact test. [b] OR and [b] *P* for univariate logistic regression analysis. [d] HR and [c, d] *P* are for univariate survival analysis. Abbreviations: OR, odds ratio; HR, hazard ratio; 95% CI, 95% confidence intervals; OS, overall survival; MST, median survival time; RFS, recurrence free survival; MRT, median recurrence time; Ref., reference; NA, not applicable; TACE, transcatheter arterial chemoembolization; BMI, body mass index; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; PVTT, portal vein tumor thrombus

		MKI67 index					OS					RFS			
		Low	High	P^a	OR(95%CI)[b]	P^b	number	MST	P^c	HR(95%CI)[d]	P^d	MRT	P^c	HR(95%CI)[d]	P^d
Gender	-	69	100	0.819	Ref.		169	96	0.899	Ref.		13	0.968	Ref.	
	Female	10	16		1.10(0.17–2.58)	0.819	26	82		1.03(0.50–2.22)	0.900	11		0.99(0.61–1.62)	0.970
Age(year)	≤46	36	59	0.468	Ref.		95	123	0.585	Ref.		11	0.768	Ref.	
	>46	43	57		0.81(0.46–1.44)	0.468	100	82		1.16(0.69–1.95)	0.587	14		0.95(0.67–1.35)	0.779
Ethnicity	Han	39	49	0.326	Ref.		88	123	0.863	Ref.		14	0.708	Ref.	
	Minority	40	67		1.33(0.75–2.37)	0.327	107	82		1.05(0.62–1.77)	0.864	11		1.07(0.75–1.53)	0.719
Preoperation TACE	No	62	93	0.774	Ref.		155	96	0.667	Ref.		14	0.952	Ref.	
	Yes	17	23		0.90(0.15–1.82)	0.774	40	82		1.14(0.62–2.09)	0.669	11		0.99(0.64–1.52)	0.955
Postoperation TACE	No	39	56	0.881	Ref.		95	NA(>100)	0.311	Ref.		14	0.618	Ref.	
	Yes	40	60		1.05(0.59–1.85)	0.881	100	82		0.76(0.45–1.29)	0.315	11		0.92(0.64–1.32)	0.635
Smoking status	None	51	66	0.284	Ref.		117	96	**0.042**	Ref.		14	0.295	Ref.	
	Ever	28	50		1.38(0.77–2.49)	0.284	78	NA(>50)		**1.71(1.01–2.91)**	**0.046**	5		1.21(0.83–1.76)	0.322
Drinking status	None	48	65	0.512	Ref.		113	82	**0.029**	Ref.		14	0.259	Ref.	
	Ever	31	51		1.22(0.68 2.17)	0.512	82	43		**1.77(1.05 2.98)**	**0.032**	6		1.22(0.85 1.75)	0.280
BMI	≤25	65	91	0.512	Ref.		156	96	0.780	Ref.		14	0.705	Ref.	
	>25	14	25		1.28(0.62–2.64)	0.512	39	82		1.10(0.58–2.08)	0.781	3		1.08(0.70–1.68)	0.721
AFP(ng/ml)	≤400	45	51	0.051	Ref.		96	NA(>100)	0.055	Ref.		16	0.093	Ref.	
	>100	31	63		1.79(1.00–3.23)	0.052	94	82		1.67(0.98–2.85)	0.059	7		1.34(0.94–1.93)	0.110
	miss	3	2		NA		5			NA				NA	
Childpugh	A	71	106	0.721	Ref.		177	82	**0.042**	Ref.		13	0.557	Ref.	
	B	8	10		0.84(0.32–2.22)	0.722	18	NA(>36)		**2.23(1.00–4.95)**	**0.049**	12		1.23(0.66–2.53)	0.572
BCLC stage	A	49	64	0.607	Ref.		113	96	**1.35×10^{-4}**	Ref.	**8.92×10^{-4}**	17	**0.015**	Ref.	**0.023**
	B	12	19		1.21(0.54–2.73)	0.643	31	NA(>47)		**3.19(1.48–6.88)**	**0.003**	6		1.71(0.94–3.10)	0.078
	C	18	33		1.40(0.71–2.78)	0.331	51	27		**6.12(3.28–11.41)**	**1.22×10^{-6}**	5		**1.66(1.11–2.50)**	**0.014**
Number of tumors	Single(n=1)	58	81	0.586	Ref.		139	96	**8.34×10^{-4}**	Ref.		14	**0.033**	Ref.	
	Multiple(n>1)	21	35		1.19(0.63 3.02)	0.587	56	28		**2.76(1.62 4.68)**	**1.78×10^{-4}**	4		**1.54(1.01 2.33)**	**0.043**
Tumor size(cm)	≤10	68	95	0.439	Ref.		163	96	**0.015**	Ref.		14	**0.003**	Ref.	
	>10	11	21		1.37(0.62–3.02)	0.440	32	35		2.08(1.13–3.82)	0.019	5		**1.98(1.23–3.17)**	**0.005**
Intrahepatic metastasis	Absence	41	56	0.558	Ref.		97	96	**0.001**	Ref.		14	**0.038**	Ref.	
	Presence	37	60		1.19(0.67–2.11)	0.558	97	41		**2.47(1.42–4.31)**	**0.001**	5		**1.44(1.00–2.06)**	**0.048**
Distant metastasis	Absence	73	104	0.515	Ref.		177	96	**0.001**	Ref.		14	**0.016**	Ref.	
	Presence	6	12		1.40(0.50 1.85)	0.516	18	18		**2.93(1.47 5.82)**	**0.002**	2		**1.96(1.09 3.50)**	**0.024**
Cirrhosis	Absence	20	31	0.876	Ref.		51	82	0.463	Ref.		14	0.586	Ref.	
	Presence	49	72		0.95(0.49–1.85)	0.876	121	96		1.24(0.69–2.24)	0.467	11		1.12(0.74–1.68)	0.602
	miss	10	13		NA		23			NA				NA	
Pathological grade	Well	9	4	**0.026**	Ref.		13	41	0.894	Ref.		19	0.397	Ref.	
	Moderately and poorly	62	102		**3.70(1.09–12.53)**	**0.035**	164	82		0.93(0.34–2.59)	0.895	12		1.37(0.64–2.97)	0.418
	miss	8	10		NA		18			NA				NA	
Regional invasion	No	70	91	0.066	Ref.		161	82	0.879	Ref.		14	0.291	Ref.	
	Yes	9	25		2.14(0.94–4.87)	0.071	34	43		0.95(0.48–1.88)	0.879	7		1.28(0.80–2.04)	0.313
PVTT	None	68	94	0.881	Ref.		162	96	**5.26×10^{-4}**	Ref.	**3.26×10^{-4}**	14	0.052	Ref.	0.069
	VP1	2	4		1.45(0.26–8.13)	0.675	6	NA(>37)		1.34(0.18–9.79)	0.776	15		0.58(0.21–1.60)	0.297
	VP2	4	6		1.09(0.30–3.99)	0.902	10	17		**6.58(2.84–15.26)**	**1.13×10^{-4}**	1		1.90(0.88–4.13)	0.105
	VP3	4	9		1.63(0.48 5.50)	0.433	13	28		**3.66(1.68–7.95)**	**0.001**	6		1.69(0.85 3.37)	0.136
	VP4	1	3		2.17(0.22–21.32)	0.506	4	6		**6.89(2.09 22.70)**	**0.002**	2		3.19(0.77–13.21)	0.110
Radical resection	No	24	50	0.086	Ref.		74	96	0.206	Ref.		14	0.111	Ref.	
	Yes	53	65		1.70(0.93–3.12)	0.087	118	41		1.40(0.83–2.38)	0.210	7		1.33(0.92–1.93)	0.128
	miss	2	1		NA		3			NA				NA	
Antiviral therapies	No	33	58	0.292	Ref.		91	NA(>49)	**0.001**	Ref.		14	0.22	Ref.	
	Yes	45	58		0.73(0.41 1.31)	0.293	103	41		**2.57(1.42–4.65)**	**0.002**	9		1.24(0.87 1.78)	0.241
MKI67 index	low	79	NA		NA		79	123	0.698	Ref.		16	0.518	Ref.	
	high	NA	116		NA		116	82		1.11(0.65–1.90)	0.700	11		1.18(0.82–1.70)	0.372
TP53	Negative	37	37	**0.007**	Ref.		74	NA(>100)	0.294	Ref.		16	**0.043**	Ref.	
	Positive	52	74		**2.31(1.25–4.28)**	**0.008**	106	82		1.35(0.77–2.37)	0.298	6		1.45(0.99–2.11)	0.054
	miss	10	5		NA		15			NA				NA	

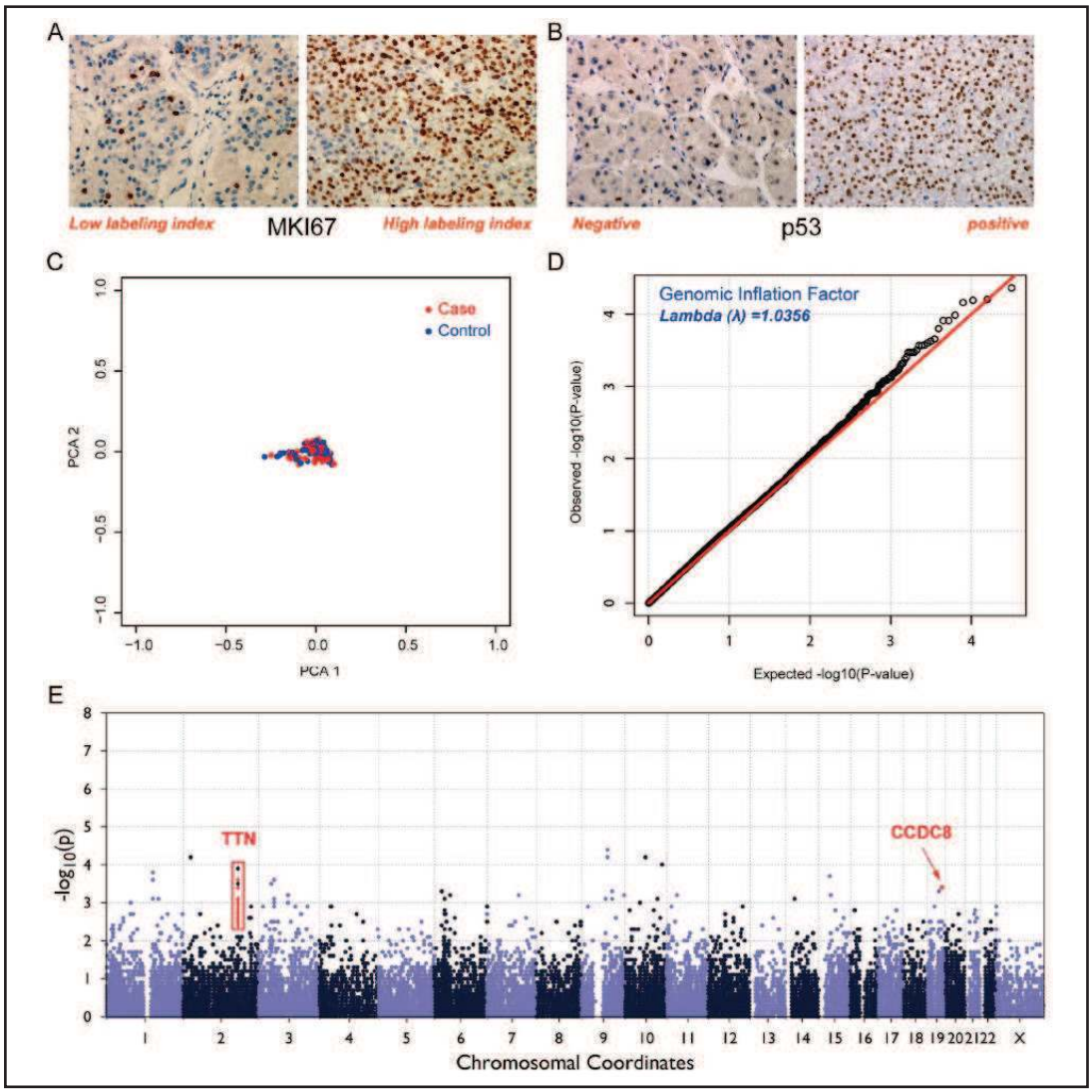

Fig. 1. Representative immunohistochemical staining of MKI67 and p53 and the results of initial quality control in the genome-wide association study. Representative staining of MKI67(A) and p53 (B) in HCC tissue. Original magnification was ×400. (C) principal components analysis plot for ancestry and population stratification implemented in the EIGENSOFT package. The blue dots represent a low MKI67 index and the red dots represent a high MKI67 index. (D) quantile-quantile plot for the genomic control inflation factor (λ). The value of λ is 1.0356 when calculated according to the *P*-value using MATLAB 7.0. (E) Manhattan plot for the association analysis.

cirrhosis status, intrahepatic metastasis, distant metastasis, PVTT, radical resection, and antiviral therapies. However, the group of moderate and low pathological differentiation grades was different from the high differentiation grade in the distribution of the MKI67 labeling index (P = 0.035). Moreover, compared with the high-grade group and negative p53 expression, the group of moderate and low pathological differentiation grades and positive p53 expression have a higher risk for positive MKI67 labeling index (OR=2.31, 95%CI=1.25–4.28) in the univariate regression, while most other characteristics remain insignificant.

QC

After QC filtering, a total number of 184 subjects with 22,428 SNPs were submitted for further analysis. The genomic inflation factor (λ) in this study was 1.0356 (Fig. 1D), which

Table 2. Association between candidate SNPs and clinical outcomes of HBV-related HCC patients in initial GWAS. Note: [a] *P*-value is from a genome-wide association study. [b, c] HR and [b, c] *P*-value are adjusted for age, gender, ethnicity, smoking status, drinking status, Barcelona Clinic Liver Cancer stage, Child-Pugh stage, preoperative serum AFP level, transcatheter arterial chemoembolization status before hepatectomy, pathological grade, cirrhosis, intrahepatic metastasis, portal vein tumor thrombus and use of antiviral therapies. Abbreviations: SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; OS, overall survival; RFS, recurrence-free survival; HR, hazard ratio; 95% CI, 95% confidence intervals

SNPs	Chr	Position	Gene	Allele	Function	MAF	HWE	[a]*P*-value	MKI67 index		OS		RFS	
									Low group	High group	[b]HR (95%CI)	[b]*P*-value	[c]HR (95%CI)	[c]*P*-value
rs16866378	2	179393111	TTN	A/G	Nonsynonymous	0.18	0.89	3.48×10^{-3}	42/27/6	80/29/0	0.79(0.54–1.16)	0.231	1.05(0.83–1.34)	0.671
rs3731752	2	179398509	TTN	C/A	Nonsynonymous	0.17	0.71	1.16×10^{-3}	42/28/5	82/27/0	1.21(0.82–1.78)	0.335	0.95(0.75–1.21)	0.671
rs2278196	2	179400895	TTN	C/T	Nonsynonymous	0.15	0.74	1.62×10^{-3}	46/24/5	86/23/0	0.81(0.36–1.82)	0.614	0.86(0.67–1.12)	0.260
rs72648270	2	179404628	TTN	T/A	Nonsynonymous	0.15	0.88	2.59×10^{-3}	46/24/5	84/25/0	0.79(0.35–1.75)	0.554	0.86(0.67–1.12)	0.260
rs3813243	2	179434516	TTN	T/C	Nonsynonymous	0.20	0.98	1.04×10^{-2}	40/28/7	79/30/0	1.21(0.84–1.75)	0.301	0.92(0.73–1.17)	0.509
rs2288563	2	179499530	TTN	T/C	Nonsynonymous	0.10	0.96	1.34×10^{-3}	53/20/2	94/15/0	1.72(0.92–3.24)	0.092	1.24(0.93–1.67)	0.146
rs2562832	2	179581835	TTN	C/A	Nonsynonymous	0.16	0.98	1.58×10^{-3}	44/27/4	84/24/1	0.83(0.59–1.18)	0.305	1.06(0.83–1.35)	0.657
rs2742347	2	179600648	TTN	C/T	Nonsynonymous	0.15	0.88	1.83×10^{-3}	45/26/4	85/23/1	0.81(0.57–1.15)	0.242	1.02(0.79–1.30)	0.901
rs1883085	2	179604160	TTN	T/G	Nonsynonymous	0.16	0.55	4.40×10^{-3}	45/26/4	85/23/1	1.23(0.87–1.75)	0.242	0.99(0.77–1.26)	0.901
rs2562829	2	179604366	TTN	T/G	Nonsynonymous	0.16	0.88	4.40×10^{-3}	45/25/5	85/23/1	0.87(1.75–0.90)	0.242	0.99(0.77–1.26)	0.901
rs66677602	2	179611711	TTN	C/A	Nonsynonymous	0.17	0.12	2.72×10^{-4}	42/25/8	85/23/1	0.64(0.40–1.04)	0.074	1.00(0.68–1.46)	0.979
rs2306636	2	179634936	TTN	C/T	Nonsynonymous	0.15	0.00	2.72×10^{-4}	44/27/4	00/21/0	0.50(0.33–1.00)	0.051	0.90(0.63–1.54)	0.935
rs56142888	2	179637861	TTN	C/G	Nonsynonymous	0.16	0.30	8.38×10^{-4}	43/26/6	87/21/1	0.65(0.40–1.07)	0.089	0.92(0.60–1.40)	0.694
rs12476289	2	179641975	TTN	C/T	Nonsynonymous	0.15	0.37	3.91×10^{-4}	44/26/5	89/19/1	0.62(0.40–1.06)	0.080	0.96(0.62–1.46)	0.831
rs16866538	2	179659912	TTN	G/A	Nonsynonymous	0.42	0.14	3.33×10^{-3}	35/30/10	31/50/28	0.94(0.59–1.48)	0.782	0.90(0.67–1.21)	0.486
rs34186470	6	46914921	CCDC8	G/A	Nonsynonymous	0.34	0.80	4.20×10^{-4}	44/26/5	37/55/17	1.40(0.88–2.23)	0.162	1.21(0.87–1.69)	0.253

Table 3. Association results of candidate SNPs in the GWAS and replication study. Note: ORs and 95% CIs were calculated by considering the allele which allele frequency of the cases is lower (the posterior allele in the table) as a reference. Abbreviations: OR, odds ratio; 95% CI, 95% confidence intervals

SNPs	Gene	Allele	Study	Genotype		OR(95%CI)	*P*-value
				case	control		
rs3813243	TTN	T/C	GWAS	40/28/7	79/30/0	2.30(1.24–4.28)	8.16×10^{-3}
			Replication	25/13/3	45/10/1	3.20(1.30–7.90)	1.20×10^{-2}
			Combined analysis	65/41/10	124/40/1	2.54(1.53–4.23)	3.15×10^{-4}
rs2288563	TTN	T/C	GWAS	53/20/2	94/15/0	2.60(1.24–5.44)	1.10×10^{-2}
			Replication	29/9/3	50/6/0	3.45(1.17–10.17)	2.50×10^{-2}
			Combined analysis	82/29/5	144/21/0	2.84(1.55–5.22)	7.53×10^{-4}
rs2562832	TTN	A/C	GWAS	44/27/4	84/24/1	2.37(1.25–4.49)	8.40×10^{-3}
			Replication	19/17/5	44/10/2	4.25(1.75–10.29)	1.37×10^{-3}
			Combined analysis	63/44/9	128/34/3	2.91(1.74–4.88)	5.13×10^{-5}
rs56142888	TTN	C/G	GWAS	43/26/6	87/21/1	2.94(1.53–5.66)	1.22×10^{-3}
			Replication	21/16/4	42/13/1	2.86(1.21–6.76)	1.68×10^{-2}
			Combined analysis	64/42/10	129/34/2	2.91(1.73–4.90)	5.62×10^{-5}
rs34186470	CCDC8	G/A	GWAS	44/26/5	37/55/17	0.36(0.20–0.66)	1.04×10^{-3}
			Replication	24/11/6	20/24/12	0.39(0.17–0.90)	2.70×10^{-2}
			Combined analysis	66/37/11	57/79/29	0.37(0.23–0.61)	7.67×10^{-5}

indicates that a minimal population stratification was presented. The PCA plot (Fig. 1C) demonstrates that there were no outliers in this study population.

Table 4. Primers used for polymerase chain reaction with candidate SNPs. Note: *for CCDC8

SNPs	Primers	Sequences (5' to 3')	Annealing temperature (°C)	Amplification length (bp)
rs16866378	Forward	TGGCTGTCCCTCTTTCACATGG	62	585
	Reverse	GGAATCCTCACCTGCATAAGCAAA		
rs3731752	Forward	TTACTGCCGTAGAACCATGAAGAAA	60	591
	Reverse	CCCGTGTCTTCAGGCAAAGTG		
rs2278196	Forward	GCACAGACCACATAGAAACCAGCA	64	600
	Reverse	GGTCTGCAAAGTGACTGGTCATCC		
rs72648270	Forward	CCTGGCCTTCCTTGGTCCAT	62	584
	Reverse	TCAACTTTGCAGCAACTTTAACCA		
rs3813243	Forward	TAGCTGCATCTCGGATTTCACCAT	63	508
	Reverse	CTTAGTGAACCAAGCCCTCCTT		
rs2288563	Forward	TCACCAGCTTGGTCCAGTTTGA	61	586
	Reverse	GCAAAGCATCGTTGGGTTGTCT		
rs2562832	Forward	TCCATTTGGAAGGTCCTTGATCC	62	597
	Reverse	TGCCAAGCTCATTCTCTCAGGTG		
rs2742347	Forward	CAGATGTCTGCTCCAAACTTGTT	60	502
	Reverse	AGGAACGCTTGTCTTTTCTTTGAT		
rs1883085	Forward	ACATTCTGGAAGTGGCAGATAAGTG	60	533
	Reverse	GGGTTGCTTCAGCTGTTGTCTCT		
rs2562829	Forward	TCACCTTCCTCAGAAACAGTGTCCA	62	568
	Reverse	TCACCCAAGAGCCCAGACACA		
rs66677602	Forward	TGCTCATTTGGTGTACCGTCTTCC	62	569
	Reverse	TCACTAGGTCGTCCACTTTCTCCTGA		
rs2306636	Forward	GCTTGGTAAAATCAAAGAGCACTTC	61	531
	Reverse	TCTGTTTATGGCTTCAGGCTTGGA		
rs56142888	Forward	TGCAAGTACTATTGGCTTTGGGAACA	64	591
	Reverse	CAGCGACTAGTCATTAACCGAACTCA		
rs12476289	Forward	TGGCCATATCGCAAATGGAGG	62	550
	Reverse	CAGCCTTACTCCCTCTAAATCATGACC		
rs16866538	Forward	CTGAATTTGGTCTTCAGTTGCTGCT	63	571
	Reverse	TCTGCCTTAAAGCACTTCCAGCTT		
rs34186470*	Forward	CTCACAGCTGGTCTTCTTGCTCT	58	503
	Reverse	AGCTGACCAGAGGTCACAGGG		

Association analysis

Candidate genes containing SNPs following QC were selected if they harbored the following criteria: (i) reached the initial threshold (*P*-value < 0.01 and MAF > 0.1); (ii) correlated statistically in mRNA level; (iii) were predicted by pathway analysis; (iv) were associated with the prognosis of patients or enriched in gene ontology. After filtering, titin (TTN) and coiled-coil domain containing 8 (CCDC8) were considered the candidate genes associated with the MKI67 labeling index in HBV-related HCC patients. In the initial GWAS, a total of 16 candidate SNPs near candidate genes (Table 2) were selected to undertake the replication study via Sanger DNA sequencing. Based on the result of the validation study, a total of five SNPs were identified in accordance with the result of initial GWAS (Table 3, all primers used in sequencing are displayed in Table 4). Further, data from GEO (accession:

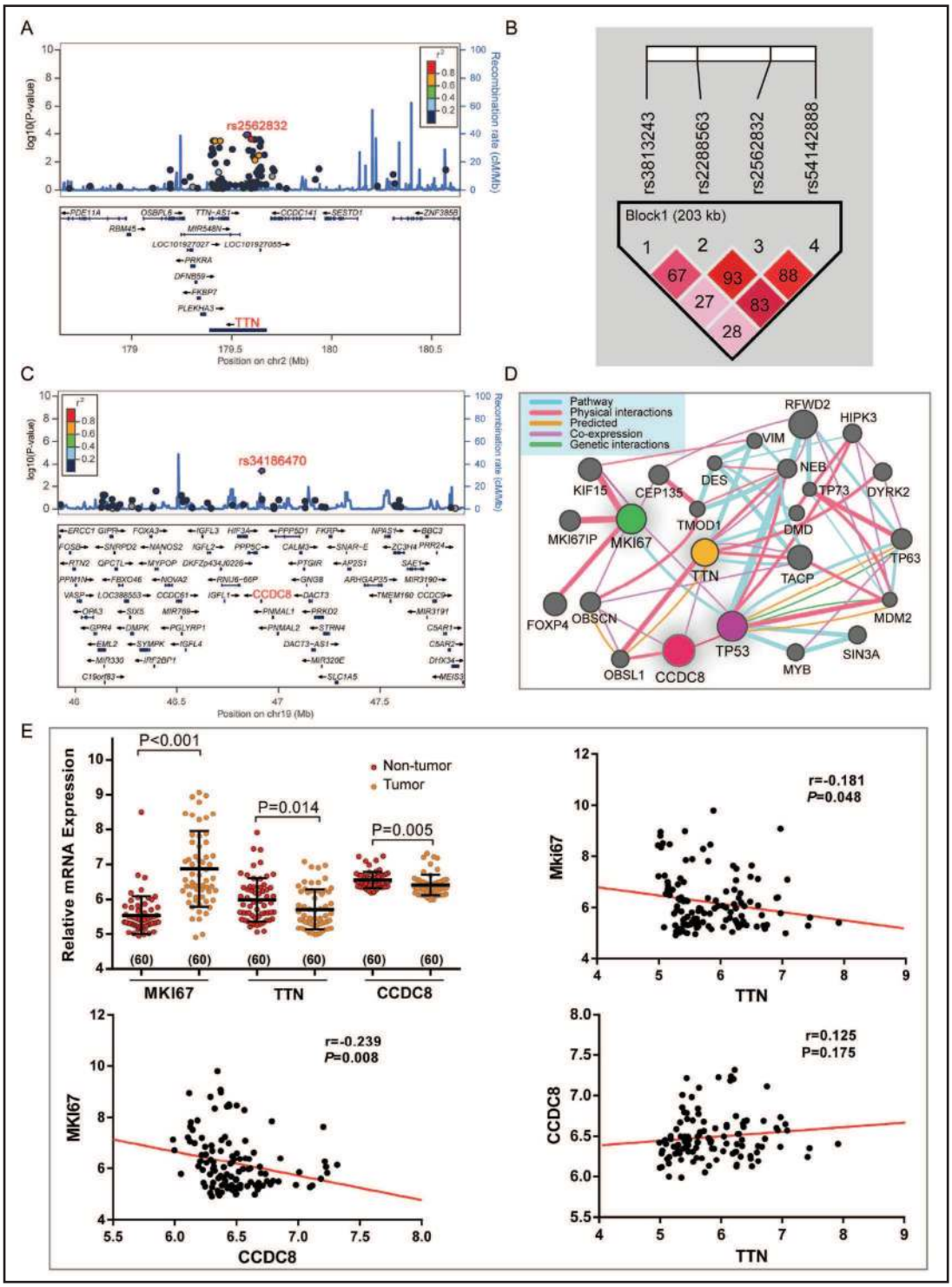

Fig. 2. Variant loci information near TTN and CCDC8 and associations between TTN and CCDC8 with MKI67. (A) and (C) LocusZoom plots for the analysis of local linkage disequilibrium (LD) and recombination patterns near TTN (A) and CCDC8 (C) about 1.5Mb. LD (r^2) and recombination rates are evaluated from the 1000 Genomes Project ASN data (March 2012, Build GRCh37/hg19). (B) Haploview LD plot of variants near TTN. Red color intensity and value inside each cell corresponds to the relative level of LD. (D) Pathway analysis predicted in GeneMania software between TTN, CCDC8, and MKI67. (E) The mRNA expression and correlation of TTN, CCDC8, and MKI67 in HCC tissues (tumor paired paracancer tissues). Data are from the GEO database (accession: GSE64041).

Table 5. Results for the genetic model of candidate SNPs and the association for MKI67 index. Note: [a] Crude OR and [a] *P*-value is for univariate logistic regression. [b] Adjusted OR and [b] *P*-value are adjusted for age, gender, ethnicity, smoking status, drinking status, body mass index, Barcelona Clinic Liver Cancer stage, Child-Pugh stage, preoperative serum AFP level, transcatheter arterial chemoembolization status before hepatectomy, pathological grade, cirrhosis, portal vein tumor thrombus and use of antiviral therapies. Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; HR, hazard ratio; 95% CI, 95% confidence intervals; Ref, reference

SNPs model	MKI67 index		[a]Crude OR(95%CI)	[a]*P*-value	[b]Adjusted OR(95%CI)	[b]*P*-value
	Low	High				
rs3813243 Dominant						
CC	40	79	Ref.	0.173	Ref.	0.035
CT+TT	35	30	0.43(0.23–0.81)	0.008	0.26(0.12–0.57)	0.001
rs2288563 Dominant						
TT	53	94	Ref.	0.079	Ref.	0.034
TC+CC	22	15	0.38(0.18–0.80)	0.011	0.26(0.11–0.65)	0.003
rs2562832 Dominant						
CC	44	84	Ref.	0.021	Ref.	0.052
CA+AA	31	25	0.42(0.22–0.80)	0.008	0.28(0.12–0.64)	0.003
rs56142888 Dominant						
CC	43	87	Ref.	0.004	Ref.	0.052
CG+GG	32	22	0.34(0.18–0.65)	0.001	0.26(0.11–0.60)	0.001
rs34186470 Additive						
GG	44	37	Ref.	0.004	Ref.	0.002
AA	5	17	4.04(1.36–12.01)	0.012	5.68(1.56–20.72)	0.009
AG	26	55	2.52(1.33–4.77)	0.005	3.14(1.47–6.69)	0.003

GSE64041) demonstrated that MKI67, TTN, and CCDC8 are different between tumor and paracancer tissues and associated with mRNA level (Fig. 2E). The relationship of all candidate SNPs and MKI67 labeling index are shown in Table 5. SNPs near TTN were a dominant model and all non-dominant genotypes have a low risk in the MKI67 labeling index. Meanwhile, rs34186470 in CCDC8 is an additive model, and rs34186470-AA possesses a lower risk compared with AG and GG.

Bioinformatics analysis

The pathway network (Fig. 2D) compiled in GeneMania software showed that MKI67 may interact with TTN and CCDC8 though the TP53 pathway. Moreover, CCDC8 and MKI67 in the DAVID database were in the category for regulation of mitotic nuclear division (P=0.025). Additionally, the Snpfunc website predicted that rs2288563 and rs2562832 could be exonic splicing enhancers or silencers.

LD analysis

Haplotype analysis revealed that candidate SNPs near TTN were in an LD block (Fig. 2B), namely Block 1(rs3813243, rs2288563, rs2562832, rs56142888, 203 kb). The LD pattern harbored high r^2 values, which was evidenced by HapMap data. The regional plot (Fig. 2A) shows a combined effect for candidate SNPs in TTN.

Survival analysis

Distribution of clinicopathologic characteristics and clinical analysis. The association of baseline characteristics and clinical outcomes in HBV-related HCC patients is shown in Table 1. We observed that the characteristics of oncological behavior were strongly associated with OS. BCLC stage B (HR=3.19, 95%CI=1.48–6.88, P=0.003) and C (HR=6.12, 95%CI = 3.28–11.41, P=1.22×10^{-8}), Child-Pugh B (HR=2.23, 95%CI=1.00–4.95, P=0.049), multiple

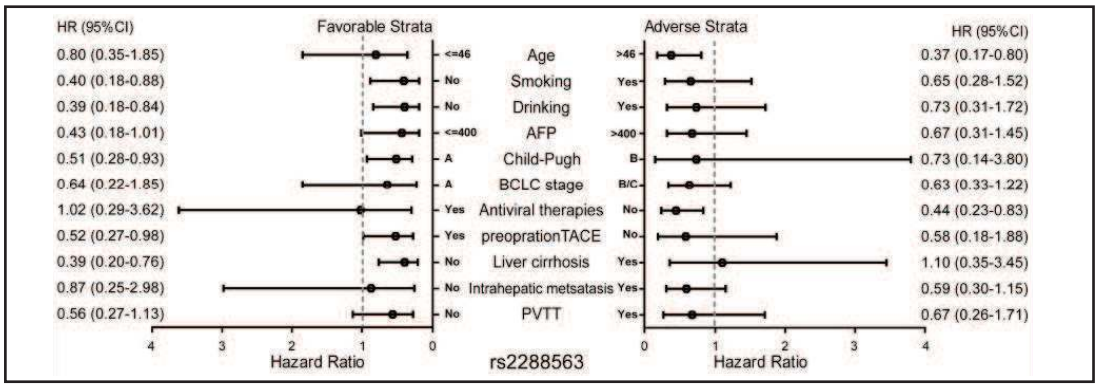

Fig. 3. Overall survival stratified analysis in rs2288563-TT. The clinical characteristics are stratified by favorable and adverse strata.

tumor nodes (HR=2.76, 95%CI=1.62–4.68, P=1.78×10^{-4}), tumor size (>10 cm) (HR=2.08, 95%CI=1.13–3.82, P=0.019), intrahepatic metastasis (HR=2.47, 95%CI=1.42–4.31, P=0.001), distant metastasis (HR=2.93, 95%CI=1.47–5.82, P=0.002), and PVTT (HR_{VP2}=6.58, 95%CI=2.84–15.26, P=1.13×10^{-5}; HR_{VP3}=3.66, 95%CI=1.68–7.95, P=0.001; HR_{VP4}=6.89, 95%CI=2.09–22.70, P=0.002) were the risk factors for mortality. Moreover, patients who were positive for smoking, drinking and antiviral therapy had a poor outcome. For RFS, the characteristics of oncological behavior had the same impact. BCLC stage C, Child-Pugh B, multiple tumor nodes, tumor size (>10 cm), intrahepatic metastasis, distant metastasis and positive p53 expression were the risk factors for recurrence. Although we did not find that MKI67 labeling index was associated directly with clinical outcomes of HCC patients, combined analysis provided a view to explore the association between them. We identified that a positive p53 expression combined with higher MKI67 labeling index was likely to be associated with an adverse outcome for 3-year RFS (Table 5).

TTN and CCDC8 SNPs and clinical analysis. We found rs2288563 in *TTN* was associated with the OS of HCC patients after resection (Table 6). Patients who harbored rs2288563-TC (HR=1.85, 95%=1.04–3.29) and rs2288563-CC (HR=2.59, 95%CI=0.35–18.98) had a poor clinical outcome. Further, rs2562832-AA+CA in TTN was a protective factor for recurrence (Table 6). Nevertheless, no statistical difference was found in other SNPs and haplotypes of TTN in clinical outcomes of HCC patients, as well as in rs34186470. To eliminate the disturbance of clinicopathologic characteristics, stratified analysis was performed to evaluate the association of rs2288563 and OS. We observed that rs2288563-TT is a favorable factor for OS in most characteristics (Fig. 3).

Discussion

In this study, we used GWAS to describe the association between the MKI67 labeling index and its clinical implications with the prognosis of HBV-related HCC after resection in patients of Guangxi Province in China. We observed that the worse pathologic grade was the risk factor for a high MKI67 labeling index. No statistical difference was found with the MKI67 labeling index and clinical outcomes of HCC patients, but the characteristics of oncological behavior were strongly associated with clinical outcomes. Further, combined analysis of MKI67 and p53 was associated with a 3-year recurrence. Moreover, we identified an association in variations of TTN (rs3813243, rs2288563, rs2562832, rs56142888) as well as *CCDC8* (rs34186470) and the MKI67 labeling index. In addition, rs2288563 in TTN was verified to be relevant in relation to the OS of HCC patients, while rs2562832 was associated with 3-year RFS.

A number of studies suggest that the MKI67 labeling index could be useful in assessing the prognosis of HCC patients after resection [24, 29]. In the present study, we do not observe

Table 6. Association between combined effects on clinical outcomes of patients with HBV-related HCC after hepatic resection. Note: [a] HR and [a] *P*-value are adjusted for age, gender, ethnicity, smoking status, drinking status, Barcelona Clinic Liver Cancer stage, Child-Pugh stage, preoperative serum AFP level, transcatheter arterial chemoembolization status before hepatectomy, pathological grade, cirrhosis, intrahepatic metastasis, portal vein tumor thrombus and use of antiviral therapies. Abbreviations: MT, median time; OS, overall survival; RFS, recurrence-free survival; HR, hazard ratio; 95% CI, 95% confidence intervals; Ref, reference

		number	MT	*P*-value	Crude HR(95%CI)	*P*-value	Adjusted [a]HR(95%CI)	[a]*P*-value
			3-year RFS					
p53(-) +Low MKI67		22	20	**0.007**	Ref.	**0.015**	Ref.	**0.023**
p53(-) +High MKI67		18	3		2.84(1.47–5.47)	**0.002**	3.18(1.49–6.78)	**0.003**
p53(+) +Low MKI67		21	9		2.00(1.08–3.70)	**0.028**	1.80(0.86–3.78)	0.121
p53(+) +High MKI67		47	6		1.93(1.14–3.25)	**0.014**	2.02(1.10–3.70)	**0.024**
			OS					
rs2288563	AA	147	96	0.072	Ref.	0.082	Ref.	0.191
	AB	35	35		1.85(1.04–3.29)	**0.035**	1.55(0.80–3.03)	0.197
	BB	2	1		2.59(0.35–18.98)	0.348	4.32(0.54–34.61)	0.168
	AB+BB	37	35	**0.024**	1.88(1.07–3.31)	**0.028**	1.66(0.84–3.29)	0.143
			3-year RFS					
rs2562832	AA+AB	29	18	**0.033**	Ref.		Ref.	
	BB	78	9		1.56(1.01–2.41)	**0.045**	1.44(0.86–2.40)	0.167

that the MKI67 labeling index is associated directly with outcomes of HCC patients, which is in accordance with a previous study [30]. Combined analysis reveals the surprising result that negative p53 expression and a low MKI67 labeling index (<25%) have a favorable outcome in 3-year RFS, which is helpful in a prognosis assessment. There is a growing interest to detect potential genetic factors, which are associated with the MKI67 labeling index. Using GWAS, we found TTN and CCDC8 are associated with the MKI67 labeling index.

TTN is located on chromosome 2q31.2 and encodes a large abundant protein of striated muscle. The product of this gene is divided into two regions, an N-terminal I-band and a C-terminal A-band [31]. The A-band, which is thought to act as a protein-ruler, contains a mixture of immunoglobulin and fibronectin repeats, and possesses kinase activity [32–34]. Mayans et al. [35] explored the role of the TTN kinase domain and further revealed its activation by unlocking a phosphate-binding site and phosphorylation of a tyrosine to activate the substrate-binding site. TTN also contains a catalytic serine-threonine kinase domain, which is inhibited by a specific dual mechanism [36]. Additionally, TTN has also been identified as a structural protein for chromosomes [37]. An antigen in mitotic chromosomes of human epithelial cells and nonmuscle cells from Drosophila was identified as TTN. Chromosome condensation and integrity during mitosis is significant for separation and compaction of chromosomes, and is thought to be a highly specified process [38]. Machado et al. [37] further suggested a role of TTN was not only in myofibrillar assembly and muscle elasticity but also in controlling the axial diameter of mitotic chromosomes. Alternative splicing of the gene results in multiple transcript variants. The mechanisms controlling TTN splicing remain unknown, but the ability to manipulate the splicing of the TTN protein has great potential for affecting human health [39].

Recently, it was verified that MKI67 is a protein that binds to the perichromosomal layer in mitosis [40]. The perichromosomal layer represents 1.4% of the chromosome proteome [41]. High grafting densities of MKI67 molecules at the chromosome surface might be consistent with a brush-like structure formatted for non-biological surface-attached polymers [42]. Cuylen et al. [43] reported that the MKI67 protein enables independent chromosome motility and efficient interaction with the mitotic spindle, through preventing

the chromosome from collapsing into a single chromatin mass after nuclear envelope disassembly with a surfactant-like function maintaining the same tension. In the present study, we have identified an association of variants in TTN with MKI67 labeling index via GWAS and a replication study. TTN has an interaction with MKI67, as predicted by GeneMania software. In addition, the predicted results using the Snpfunc website showed that variants in TTN can change splicing. Moreover, an LD block presented as four variants of *TTN*. Thus, we consider a hypothesis that these variants might change the function or structure of TTN. The change in structure of TTN may result in confusing the axial diameter of mitotic chromosomes or the reversal of transcription factor binding efficiency. Following cell proliferation, MKI67 expression increases sharply to maintain the same tensile force of the chromosome surface. Moreover, recent reports demonstrate that the N terminus of MKI67 contains a forkhead-associated phosphopeptide-binding domain [44] and a protein phosphatase 1-binding site [45]. Despite this, there is little known of its function beyond it being a protein phosphorylated via serine and threonine [46] with a critical role in cell proliferation. Evidence suggests the arrest of cell proliferation when MKI67 is blocked either by inhibition of dephosphorylation [47] or by microinjecting with blocking antibodies [46]. The change in kinase domains of TTN might lead to MKI67 phosphorylation, in order to activate the capability of cell proliferation. Nonetheless, further study needs an exact experiment to prove this assumption.

Another gene, CCDC8, which is mapped to 19q13.32, encodes a coiled-coil domain-containing protein called p90. The encoded protein functions as a cofactor required for P53-mediated apoptosis following DNA damage [48], and may also play a role in growth through interaction with the cytoskeletal adaptor protein obscurin-like-1 [49]. TP53, as a key player in the stress response, demands an exquisitely complicated network of control and fine-tuning mechanisms to ensure correct, differentiated responses to the various stress signals encountered by cells [48]. A study has demonstrated that TP53 inhibits MKI67 promoter activity via a p53 and Sp1-dependent pathway, which is likely to involve transcriptional regulatory mechanisms [50]. In our data, CCDC8 was associated with the MKI67 labeling index and enriched gene ontology terms for regulation of mitotic nuclear division with MKI67 in the DAVID database. We identified that the mRNA level of CCDC8 was associated with MKI67 in the GEO database. Moreover, down-regulated CCDC8 expression was found in tumor tissues, which was in accordance with previous studies [51, 52]. Thus, we suspect that a variant in CCDC8 might result in a weaker function of p90 binding to p53, the effect on p53-mediated MKI67 promoter activity is decreased and the MKI67 labeling index increases naturally. This association needs further experiments to validate assumptions and clarify the underlying mechanism.

Several limitations of this study warrant discussion. First, our sample size is limited and a small fraction of the clinical data is missing, additional studies with larger sample sizes and multiple centers are needed to clarify our results. In addition, because the subjects evaluated in this study include minorities, racial heterogeneity may also represent a major limitation of the study. However, we accounted for this by including ethnicity, age, and gender as covariates in our GWAS model, and based on the low genomic inflation factor and the Q-Q plot, there is no evidence of population stratification. Finally, our research is preliminary, and further mechanistic and functional studies should be undertaken to discern the potential role of variants near TTN and CCDC8.

Conclusions

Our study demonstrated that the MKI67 labeling index can be combined with p53 expression to estimate the postoperative clinical outcomes of HBV-related HCC patients in the Guangxi Province, of southern China. Moreover, variants near TTN and CCDC8 were associated with the MKI67 labeling index, but the association and molecular mechanisms in MKI67 labeling index modulation need further research. Additionally, rs2288563 and

rs2562832 in TTN are potential biomarkers for the prediction of clinical outcomes in HBV-related HCC patients.

Abbreviations

HCC (hepatocellular carcinoma); HBV (hepatitis B virus); MKI67 (Marker of proliferation Ki-67); GWAS (genome-wide association study); TACE (transcatheter arterial chemoembolization); BMI (body mass index); AFP (alpha-fetoprotein); BCLC (Barcelona Clinic Liver Cancer); PVTT (portal vein tumor thrombus); QC (Quality Control); PCA (principal components analysis); MAF (Minor allele frequencies); MST (median survival time); OS (Overall survival); RFS (Recurrence free survival); Q-Q (quantile-quantile); ORs (odds ratios); CIs (confidence intervals); LD (local linkage disequilibrium); GEO (Gene Expression Omnibus); HRs (hazard ratios); TTN (titin); CCDC8 (coiled-coil domain containing 8).

Acknowledgements

The authors thank the researcher Jiaquan Li, Ying Gui for their contribution in specimen management; and Prof. Xigang Chen, Zhixiong Su, Ming Su, Bin Chen, Xinping Ye, Liming Shang, Zhang Wen, Ya Guo, Jingning Lu, Ning Peng, Hai Zhu to provide part of hepatocellular carcinoma samples for this study.

Funding

This work was supported in part by the National Nature Science Foundation of China (No: 81560535, 81072321, 30760243, 30460143 and 30560133), 2009 Program for New Century Excellent Talents in University (NCET), Guangxi Nature Sciences Foundation (No: GuiKeGong 1104003A-7), and Guangxi Health Ministry Medicine Grant (Key-Scientific Research-Grant Z201018).

Disclosure Statement

The authors declare no conflict of interest.

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