Original Research

The predictive potential of genetic single nucleotide polymorphisms in *CBX4* for hepatocellular carcinoma survival

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1. Abstract

Background: Our previous studies have reported that polycomb chromobox 4 (*CBX4*) has a potential promoting hepatocellular carcinoma (HCC) angiogenesis and tumor progression. However, it is unclear whether genetic single-nucleotide polymorphisms (SNPs) in this gene are associated with HCC prognosis. **Methods**: We conducted a hospital-based two-phase study, including 598 patients with pathologically diagnosed HCC for the SNPs screening phase and 328 HCC patients for clinic significance validating phase, to elucidate the association between SNPs of *CBX4* and the survival of HCC. The genotypes of *CBX4* were tested using the SNaPshot method and the effects of *CBX4* SNPs on HCC prognosis were analyzed using Kaplan–Meier survival model and Cox regression model. **Results**: A total of 33 SNPs were selected and genotyped in this study. We found the rs77447679 SNP was significantly

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related to survival in individuals with HCC. Specifically, survival was noticeably decreased in HCC patients who have mutant homozygote AA of this SNP (rs77447679-AA) compared with these with wild type (rs77447679-CC). An additive effect of rs77447679 polymorphism and aflatoxin B1 exposure level was also observed in the survival analyses of HCC cases. Furthermore, this SNP was positively correlated not only with tumor size, grade, stage, and microvessel density (correlation coefficient r = 0.17, 0.23, 0.23, and 0.42, respectively), but also with increasing *CBX4* expression (r = 0.57). Interestingly, the mutant genotypes of rs77447679 can significantly improve the therapeutic response of HCC cases on post-operative adjuvant transarterial chemoembolization (pa-TACE), but wild type not. **Conclusions**: These data suggest that genetic polymorphisms in the *CBX4* may be a prognostic biomarker for HCC, and the rs77447679 SNP is such a potential candidate.

2. Introduction

Liver cancer is the second-leading cause of malignancy-associated deaths worldwide, and more than 85% of patients have hepatocellular carcinoma (HCC) [1]. Evidence from clinical and epidemiological studies has shown that the five-year survival rate is about 70%, if disease is diagnosed at early-stage HCC (also called BCLC-0 or A stage) with solitary tumors [1–3]. Therefore, discovery of biomarkers for the early-stage diagnosis and prognostic prediction of this cancer may improve the outcome of patients with HCC. However, in spite of some efforts being made, trustable biomarkers have still been lacking and the prognosis of patients remains poor.

The chromobox 4 (*CBX4*) is a known post-translational regulating gene (GenBank accession NO. 8535) that consists of five introns and six exons, and spans about 6.26 kb on chromosome 17q25.3. The encoding protein of this gene includes 560 amino acids and is one major constituent of polycomb repressive complex 1 (PRC1) [4, 5]. Functionally, *CBX4* mainly involves in PRC1-regulated transcription repression and post-translation modification [6, 7]. Our previous studies have also displayed that the dysregulation of *CBX4* progress hepatocarcinoma angiogenesis and affects the prognosis of patients with HCC [8–10]. Recently, several reports have been indicative of genetic variants in this gene [11, 12]. However, it is not clear whether genetic single nucleotide polymorphisms (SNPs) in *CBX4* modify HCC prognosis. Here, we conducted a hospital-based retrospective study to explore the association between CBX4 SNPs and the outcome of HCC in the Guangxi Region, a high incidence area of this malignancy.

3. Materials and methods

3.1 Study design and subjects

The present study was approved by the Ethics Review Committees of Hospitals involved in this study. This study was a hospital-based two-phase retrospective study (**Supplementary Fig. 1**), including the first stage for screening positive SNPs in *CBX4* gene affecting HCC survival and the second stage for validating clinical significance of positive *CBX4* SNPs. In this study, patients were from the Southwest Guangxi Zhuang Autonomous Regin, a high incident area of HCC. For screen stage, the inclusion criterion cases are as follows: (1) cases with pathologically diagnosed HCC; (2) cases featuring cancer in I-II Tumor, Node, and Metastasis (TNM) stage and receiving curative resection treatment; (3) cases with available biopsy samples and clinic-pathological data for further analyses; and (4) cases understanding the objective of the study and providing informed consent. The exclusion criteria consisted of: (1) cases receiving chemotherapy or radiotherapy treatment before liver tumor resection; and (2) cases rejected, dropped out, or lost information. According to our inclusion and exclusion criterion of cases, a total of 598 patients were selected for the first-phase analyses in the Affiliated Hospitals of Youjiang Medical University for Nationalities and Guangxi Medical University during January 2016 and December 2018. Meanwhile, a total of 328 HCC cases receiving non-curative resection as their primary therapy were selected for the second-phase analyses in the same hospitals according to our previously reported inclusion and exclusion criterion of cases [13]. These cases were divided into two groups: transarterial chemoembolization (TACE) treatment group (n = 163) and non-TACE control group (n = 165), according to whether they accepted postoperative adjuvant TACE (pa-TACE) as their initial treatment.

In this study, each patient provided written informed consent and donated 5 mL peripheral venous blood for the analyses of *CBX4* genotypes. Surgical removed samples with HCC were obtained from each patient for analyzing the amount of *CBX4* protein expression and aflatoxin B1 (AFB1)-DNA adducts. At the same time, the corresponding demographic data and clinicopathological information was also collected using a standard interviewer-administered questionnaire and/or medical records by a Youjiang Cancer Institution staff member. Follow-up was conducted according to our previously described method [14]. In brief, all patients received a series of monitoring for detecting any tumor recurrence. In this study, the last follow-up time was set on May 31, 2021, and final survival status was ascertained using telephone contact and medical records.

3.2 AFB1 exposure data

AFB1 exposure level was elucidated using the amount of AFB1-DNA adducts (AADA) in tissue samples with tumor as previously described [15]. To analyze, AFB1 exposure level was divided into two groups: high exposure (AADA $>$2.87 μmol/mol DNA) and low exposure (AADA $\leq$2.87 μmol/mol DNA), according to the average AADA among all patients with HCC. More detailed AFB1-exposure analyses can be found in the **Supporting Materials and Methods**.

3.3 SNPs selection and genotyping

A total of 1376 SNPs were obtained in the *CBX4* gene from the ENSEMBL database using the Programming Interface Tools. Based on the criteria of minor allele frequency (MAF) more than 0.001, thirty-three of these SNPs were finally chosen for further analysis in this study (**Supplementary Table 1**). Genotyping was performed by using SNaPshot method (Applied Biosystems [ABI], Foster City, CA, USA), according to the manufacturers' instructions. For quality control, controls were included in each run, and repeated genotyping and sequencing of a random 5% subset yielded 100% identical genotypes. More detailed SNP selection and genotyping information analyses can be found in the **Supporting Materials and Methods**.

3.4 Immunochemistry

The expression levels of *CBX4* protein and the elucidation of microvessel density (MVD) were analyzed by immunohistochemistry in tissue slides. In this study, the amount of *CBX4* protein-expressing was calculated according to the value of IRS systems [16]. More detailed corresponding information can be found in the **Supporting Materials and Methods**.

3.5 Statistical analysis

Overall survival (OS) time was calculated from the date of patients receiving curative therapy to the date of death or last follow-up, whereas tumor recurrence-free survival (RFS) time was done from the date of patients accepting curative therapy to the date of tumor recurrence or last follow-up. The association between patient survival, demographic and clinicopathological features, and *CBX4* SNPs were estimated using Kaplan–Meier survival model (with the log-rank test). In this model, median OS time (MST), median RFS time (MRT) and corresponding 95% confidence interval (95% CI) was calculated. Mean survival time or ND (not determined) would be shown if MST or MRT could not be computed. Death risk and tumor recurrence risk (also hazard ratio, HR) for interesting variables was evaluated using univariate or multivariate Cox regression models. In multivariable Cox regression models, significant variables were selected using the stepwise forward selection based on a likelihood ratio test (p-value less than 0.05 for entering and more than 0.10 for removal in models). The relationship between *CBX4* SNPs and the clinicopathological features of patients (including tumor size, grade, stage, MVD, and the amount of *CBX4* protein expression) was analyzed by *Spearman*'s correlation method. All statistical analyses were finished utilizing the SPSS (Version 18, SPSS Institute, IBM Corp., Chicago, IL, USA).

4. Results

4.1 The demographic and clinicopathological features and the survival of HCC patients

A total of 598 patients with HCC were included in the screening stage of our study and all cases' demographic and clinicopathological information was shown in Table 1. To explore whether these factors modified the outcome of HCC patients, we accomplished a series of Kaplan–Meier survival and univariable Cox regression survival analyses. AFB1 exposure level, tumor size, tumor grade, TNM stage, and tumor MVD were found to significantly affect patients' survival. For example, these patients featuring high AFB1 exposure had a shorten MST and MRT compared with those with low AFB1 exposure (MST, 24.00 vs. 41.00 months; MRT, 28.00 vs. 52.27 months). Furthermore, results from Cox regression analyses also displayed that the patients having high AFB1 exposure featured a higher death risk (HR = 2.46, 95% CI = 1.99–3.04) and tumor recurrence risk (HR = 5.15, 95% CI = 3.56–7.45). However, other factors (including age, gender, race, hepatitis B virus (HBV) and hepatitis C virus (HCV) infection status, drinking, smoking, and liver cirrhosis) were not significantly associated with the survival of patients with HCC (Table 1).

One hundred and sixty-three patients receiving pa-TACE treatment (TACE group) and 165 cases not doing this type of treatment (non-TACE group) were included in the validating stage. The baseline demographic and clinicopathological features were well matched between two groups (**Supplementary Table 2**). Results from survival analyses displayed that the pa-TACE treatment can significantly prolong the OS and RFS time of patients with HCC (**Supplementary Fig. 3A**,**B**, left plots) and decrease death risk and tumor reoccurrence risk of cases (**Supplementary Fig. 3A**,**B**, right plots).

4.2 Univariable analyses indicating CBX4 SNPs as significant prognostic biomarker for HCCs' survival

A total of 33 SNPs in the *CBX4* genes were genotyped in this study. The linkage disequilibrium (LD) of these SNPs was analyzed among different individuals with HCC and a weak LD for some SNPs was observed (**Supplementary Fig. 2**). Table 2 summarizes the association between the genotypes of 33 selected SNPs in *CBX4* gene and the outcome of HCC patients. Among of these SNPs, rs528507291 (SNP15) and rs77447679 (SNP20) significantly modified the OS and RFS of HCC patients.

Table 1. Patients' characteristics and clinical features.

		OS				RFS			
Variables	Pa, *n* (%)	De, *n* (%)	MST (95% CI), mo	Log-rank *p*	HR (95% CI/p_{trend})	Re, *n* (%)	MRT (95% CI), mo	Log-rank *p*	HR (95% CI/p_{trend})
Age, yrs				0.18				0.13	
≤49	339 (56.7)	301 (57.6)	30.00 (27.84–31.16)		Reference	188 (60.8)	35.00 (32.06–37.94)		Reference
>49	259 (43.3)	222 (42.4)	24.00 (22.15–25.85)		0.96 (0.76–1.09/0.31)	121 (39.2)	34.00 (19.10–48.90)		0.84 (0.67–1.06/0.14)
Gender				0.22				0.62	
Male	453 (75.8)	393 (75.1)	28.00 (26.07–29.93)		Reference	233 (75.4)	34.00 (26.45–41.55)		Reference
Female	145 (24.2)	130 (24.9)	27.00 (27.73–31.28)		0.89 (0.73–1.08/0.23)	76 (24.6)	35.00 (30.63–39.37)		0.94 (0.72–1.22/0.63)
Race				0.96				0.51	
Han	291 (48.7)	254 (48.6)	28.00 (25.71–30.29)		Reference	145 (46.9)	36.00 (31.11–40.67)		Reference
Zhuang	307 (51.3)	269 (51.4)	27.00 (24.49–29.52)		1.00 (0.84–1.18/0.96)	164 (53.1)	34.00 (29.93–38.07)		1.08 (0.86–1.35/0.52)
Smoking				0.23				0.76	
No	442 (73.9)	382 (73.0)	27.00 (24.95–29.05)		Reference	224 (72.5)	34.00 (29.93–38.07)		Reference
Yes	156 (26.1)	141 (27.0)	30.00 (27.02–32.98)		0.89 (0.73–1.08/0.24)	85 (27.5)	35.00 (28.47–41.53)		0.96 (0.75–1.24/0.76)
Drinking				0.31				0.94	
No	440 (73.6)	380 (72.7)	27.00 (24.92–29.08)		Reference	222 (71.8)	35.00 (30.34–39.67)		Reference
Yes	158 (26.4)	143 (27.3)	29.00 (26.13–31.88)		0.91 (0.75–1.10/0.32)	87 (28.2)	35.00 (28.69–41.33)		0.99 (0.77–1.27/0.94)
HBsAg				0.34				0.99	
Negative	146 (24.4)	130 (24.9)	26.00 (22.42–29.50)		Reference	72 (23.3)	34.00 (28.91–39.09)		Reference
Positive	452 (75.6)	393 (75.1)	28.00 (26.03–29.97)		0.91 (0.75–1.11/0.35)	237 (76.7)	35.00 (30.89–39.11)		1.00 (0.77–1.30/0.99)
Anti-HCV				0.27				0.86	
Negative	498 (83.3)	440 (84.1)	27.00 (24.78–29.23)		Reference	252 (81.6)	36.00 (31.41–40.59)		Reference
Positive	100 (16.7)	83 (15.9)	28.00 (25.49–30.51)		0.88 (0.69–1.11/0.28)	57 (18.4)	33.00 (39.99–36.01)		1.03 (0.77–1.37/0.86)
AFB1 exposure				5.96×10^{-3}				3.15×10^{-22}	
Low	144 (24.1)	118 (22.6)	41.00 (36.13–45.87)		Reference	33 (10.7)	52.27 (49.35–55.19)		Reference
High	454 (75.9)	405 (77.4)	24.00 (22.11–25.89)		2.46 (1.99–3.04/1.64×10^{-16})	276 (89.3)	28.00 (24.94–31.06)		5.15 (3.56–7.45/2.82×10^{-18})
Tumor size				2.14×10^{-16}				7.51×10^{-7}	
≤5 cm	133 (22.2)	98 (18.7)	39.00 (34.93–43.07)		Reference	60 (19.4)	46.00 (36.28–55.72)		Reference
>5 cm	465 (77.8)	425 (81.3)	24.00 (22.03–25.97)		2.44 (1.95–3.05/6.00×10^{-15})	249 (80.6)	30.00 (25.38–34.62)		2.02 (1.51–2.68/2.00×10^{-6})
Tumor grade				2.86×10^{-29}				1.73×10^{-18}	
Low	286 (47.8)	238 (45.5)	37.00 (34.68–39.32)		Reference	124 (40.1)	47.00 (42.39–51.61)		Reference
High	312 (52.2)	285 (54.5)	21.00 (19.45–22.55)		2.72 (2.26–3.26/1.13×10^{-26})	185 (59.9)	24.00 (21.40–26.60)		2.79 (2.19–3.55/5.09×10^{-17})
TNM stage				4.34×10^{-15}				1.71×10^{-8}	
I	102 (17.1)	79 (15.1)	43.00 (37.91–48.09)		Reference	42 (13.6)	48.45 (45.34–51.55)		Reference
II	496 (82.9)	444 (84.9)	24.00 (22.14–25.86)		2.52 (1.97–3.21/1.09×10^{-13})	267 (86.4)	30.00 (25.34–34.66)		2.48 (1.78–3.45/6.76×10^{-8})
MVD				3.43×10^{-52}					
Low	335 (56.0)	277 (53.0)	36.00 (34.26–37.74)		Reference	132 (42.7)	48.00 (42.82–53.18)		Reference
High	263 (44.0)	246 (47.0)	17.00 (15.56–18.44)		3.84 (3.19–4.63/3.32×10^{-45})	177 (57.3)	17.00 (15.60–18.40)		4.88 (3.83–6.22/1.16×10^{-37})
Liver cirrhosis				0.11				0.86	
No	171 (28.6)	154 (29.4)	26.00 (23.23–28.78)		Reference	85 (27.5)	32.00 (27.07–36.93)		Reference
Yes	427 (71.4)	369 (70.6)	28.00 (25.75–30.25)		0.86 (0.71–1.04/0.12)	224 (74.5)	35.00 (31.06–38.94)		0.98 (0.76–1.26/0.86)

Abbreviations: CI, confidence interval; De, deaths; HR, hazard ratio; mo, months; MRT, median tumor recurrence-free survival time; MST, median overall survival time; MVD, microvessel density; OS, overall survival; Pa, patients; Re, recurrences; RFS, tumor recurrence-free survival; yrs, years.

Table 2. *CBX4* SNPs and HCC prognosis.

NO.	*CBX4* SNP			OS			RFS		
		Genotypes	Patients	Deaths	$MST_{XX/XY/YY}$ [c]	Log-rank	Recurrences	$MRT_{XX/XY/YY}$ [d]	Log-rank
		(XX/XY/YY[a])	($N_{XX/XY/YY}$ [b])	($N_{XX/XY/YY}$)	(months)	*p*	($N_{XX/XY/YY}$)	(months)	*p*
SNP01	rs200337966	AA/AG/GG	578/16/4	507/12/4	27/30/33	0.86	298/10/1	35/31/33	0.49
SNP02	rs190466399	GG/GA/AA	594/4/0	519/4/0	28/23/ND	0.18	306/3/0	35/24/ND	0.17
SNP03	rs181600574	GG/GT/TT	592/6/0	518/5/0	28/26/ND	0.60	306/3/0	35/24/ND	0.68
SNP04	rs185689265	CC/CG/GG	584/11/3	509/11/3	37/32/29	0.95	301/7/1	34/35/31	0.84
SNP05	rs137865005	CC/CA/AA	580/16/2	507/14/2	27/33/30	0.98	301/6/2	34/35/28	0.41
SNP06	rs1285251	TT/TC/CC	153/327/118	130/290/103	28/27/28	0.80	73/171/65	35/35/34	0.57
SNP07	rs532803018	AA/AG/GG	585/13/0	510/13/0	28/27/ND	0.47	303/6/0	35/26/ND	0.79
SNP08	rs551152388	CC/CG/GG	579/19/0	507/16/0	28/23/ND	0.94	298/11/0	35/30/ND	0.54
SNP09	rs112860646	GG/GC/CC	548/50/0	479/44/0	27/31/ND	0.38	281/28/0	35/34/ND	0.83
SNP10	rs552696007	GG/GT/TT	583/15/0	508/15/0	28/27/ND	0.60	299/10/0	35/28/ND	0.43
SNP11	rs1285249	CC/CG/GG	95/117/386	85/103/335	28/27/28	0.85	57/59/193	31/35/35	0.34
SNP12	rs143907687	CC/CG/GG	569/26/3	495/25/3	28/26/24	0.75	293/15/1	35/34/28	0.90
SNP13	rs116088574	GG/GA/AA	578/20/0	506/17/0	28/27/ND	0.50	299/10/0	35/30/ND	0.79
SNP14	rs144342325	CC/CT/TT	581/17/0	508/15/0	28/25/ND	0.92	301/8/0	35/23/ND	0.70
SNP15	rs528507291	AA/AG/GG	543/44/11	488/28/7	26/47/58	1.19×10^{-11}	297/11/1	32/51/57	1.64×10^{-7}
SNP16	rs115617840	GG/GC/CC	572/26/0	501/22/0	28/25/ND	0.97	292/17/0	35/24/ND	0.21
SNP17	rs73422123	CC/CT/TT	356/146/96	313/128/82	27/29/28	0.53	192/64/53	32/39/30	0.09
SNP18	rs1285248	AA/AG/GG	440/131/27	395/108/20	28/27/28	0.58	231/62/16	35/34/26	0.63
SNP19	rs551216240	TT/TC/CC	576/22/0	503/20/0	27/36/ND	0.73	298/11/0	34/35/ND	0.47
SNP20	rs77447679	CC/CA/AA	359/127/112	297/115/111	35/21/12	1.31×10^{-76}	173/54/82	42/30/12	1.85×10^{-53}
SNP21	rs548628916	GG/GT/TT	583/15/0	509/14/0	28/24/ND	0.26	299/10/0	35/33/ND	0.16
SNP22	rs140936854	TT/TC/CC	584/14/0	514/9/0	28/36/ND	0.30	301/8/0	35/40/ND	0.83
SNP23	rs62075210	CC/CT/TT	479/101/18	422/85/16	28/27/23	0.87	242/56/11	35/34/24	0.50
SNP24	rs577428735	GG/GA/AA	582/16/0	510/13/0	28/23/ND	06.0	303/6/0	35/ND/ND	0.39
SNP25	rs544749108	CC/CT/TT	586/12/0	513/10/0	28/21/ND	0.67	303/6/0	35/24/ND	0.83
SNP26	rs562977478	CC/CA/AA	585/13/0	513/10/0	27/36/ND	0.52	301/8/0	35/35/ND	0.81
SNP27	rs56223906	GG/GA/AA	558/32/8	488/27/8	28/29/15	0.25	290/15/4	34/39/43	0.76
SNP28	rs113588843	TT/TC/CC	580/16/2	508/13/2	27/30/37	0.87	302/6/1	36/42/38	0.63
SNP29	rs2289728	GG/GA/AA	319/171/108	283/147/93	27/29/28	0.85	172/84/53	34/36/34	0.71
SNP30	rs62075211	GG/GC/CC	576/22/0	503/20/0	28/27/ND	0.28	297/12/0	35/26/ND	0.48
SNP31	rs537859290	CC/CA/AA	578/20/0	508/15/0	27/28/ND	0.98	299/10/0	35/27/ND	0.99
SNP32	rs553833239	GG/GC/CC	584/14/0	511/12/0	27/31/ND	0.89	303/6/0	35/37/ND	0.48
SNP33	rs200937251	CC/CA/AA	570/26/2	501/20/2	27/34/31	0.14	301/8/0	ND/ND/ND	0.05

[a] XX/XY/YY represents wild-type homozygote/heterozygote/variant-type homozygote.

[b] $N_{XX/XY/YY}$ represents number of patients with XX genotype/number of subjects with XY genotype/number of subjects with YY genotype.

[c] $MST_{XX/XY/YY}$ represents MST of patients with XX genotype/MST of patients with XY genotype/MST of patients with YY genotype.

[d] $MRT_{XX/XY/YY}$ represents MRT of patients with XX genotype/MRT of patients with XY genotype/MRT of patients with YY genotype.

Abbreviations: MRT, median tumor reoccurrence-free survival time; MST, median overall survival time; ND, not determined; OS, overall survival; RFS, tumor reoccurrence-free survival; SNP, single nucleotide polymorphism.

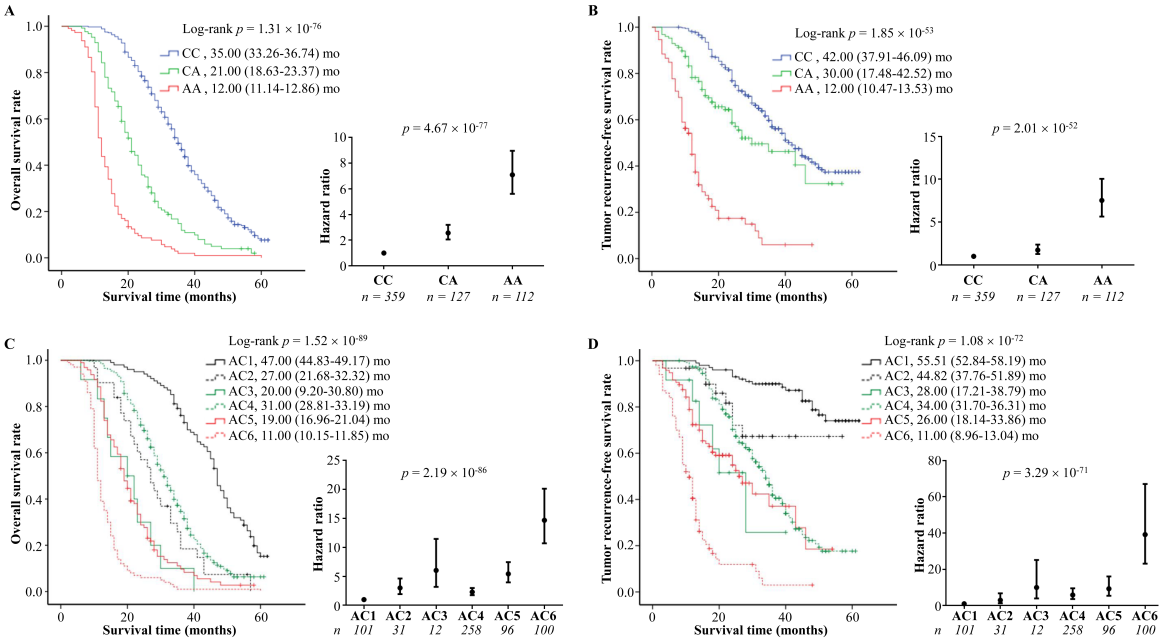

Fig. 1. Association between survival and *CBX4* rs77447679 polymorphism in 598 patients with HCC. (A,B) The effects of *CBX4* rs77447679 genotypes on OS (A) and RFS (B) of HCC. (C–D) The joint effects of aflatoxin B1 exposure combining with *CBX4* rs77447679 genotypes on OS (C) and RFS (D). Cumulative hazard function was plotted by the Kaplan–Meier methodology and the *p* value was calculated with two-sided log-rank tests. MST and MRT was also indicated. Right plots show hazard ratio (dot) and corresponding 95% confidential interval (vertical line segment). Abbreviations: AC1, patients with low AFB1 exposure and rs77447679-CC; AC2, patients with low AFB1 exposure and rs77447679-CA; AC3, patients with low AFB1 exposure and rs77447679-AA; AC4, patients with high AFB1 exposure and rs77447679-CC; AC5, patients with high AFB1 exposure and rs77447679-CA; AC6, patients with high AFB1 exposure and rs77447679-AA; mo, months; MRT, median tumor recurrence-free survival time; MST, median overall survival time; OS, overall survival; RFS, tumor recurrence-free survival.

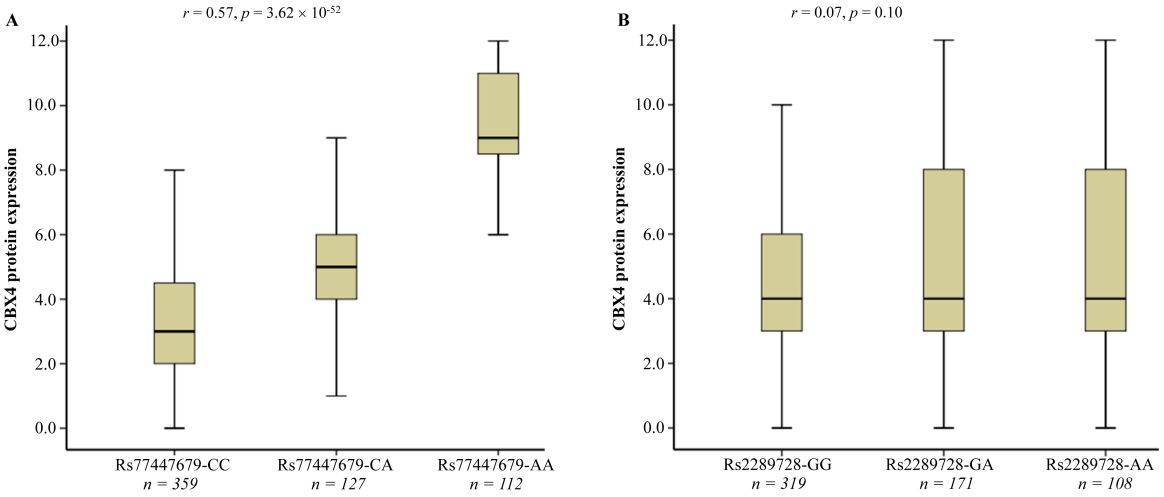

Fig. 2. *CBX4* SNPs correlating with *CBX4* expression. CBX4 protein expression was evaluated using the immunohistochemistry scores of IRS system (*to see Material and methods*) and expression scores are shown as box plots, with horizontal lines representing the median, the bottom and the top of the boxes representing the 25th and 75th percentiles, respectively, and vertical bars representing the range of data. Rs77447679 SNP is positively associated with *CBX4* expression (A), but Rs2289728 SNP not (B).

Table 3. The effects of *CBX4* rs77447679 SNP on survival stratified by the clinicopathological features of HCC cases.

Variables	Rs77447679-CC			Rs77447679-CA/AA			Log-rank *p* value
	P/D/R[a]	MST (95% CI), mo	MRT (95% CI), mo	P/D/R	MST (95% CI), mo	MRT (95% CI), mo	(MST/MRT)
Age, yrs							
≤49	201/170/107	37.00 (35.53–38.78)	40.00 (36.00–44.00)	138/131/81	14.00 (11.87–16.12)	15.00 (4.75–25.25)	4.75×10^{-30}/1.17×10^{-13}
>49	158/127/66	33.00 (29.65–36.36)	44.26 (41.01–47.52)	101/95/55	18.00 (15.92–20.08)	18.00 (14.88–21.12)	2.78×10^{-23}/6.55×10^{-10}
Gender							
Male	268/218/127	36.00 (34.11–37.90)	43.00 (37.01–48.99)	185/175/106	16.00 (14.10–17.90)	18.00 (13.02–22.98)	2.51×10^{-37}/1.66×10^{-17}
Female	91/79/46	33.00 (30.02–35.98)	40.00 (33.14–46.86)	54/51/30	13.00 (10.30–15.70)	16.00 (11.52–20.48)	5.90×10^{-16}/6.94×10^{-7}
Race							
Han	175/143/79	36.00 (33.75–38.25)	45.00 (38.34–51.66)	116/111/66	15.00 (12.74–17.26)	19.00 (11.51–26.49)	4.38×10^{-33}/3.98×10^{-15}
Zhuang	184/154/94	34.00 (30.65–37.35)	40.00 (34.95–45.05)	123/115/70	16.00 (14.11–17.88)	17.00 (13.26–20.74)	5.10×10^{-21}/5.72×10^{-10}
Smoking							
No	246/198/121	36.00 (33.95–38.05)	42.00 (35.95–48.05)	196/184/103	16.00 (14.04–17.96)	22.00 (17.15–26.85)	5.34×10^{-38}/2.55×10^{-13}
Yes	113/99/52	34.00 (30.47–37.53)	43.00 (37.50–48.50)	43/42/33	15.00 (12.25–17.75)	20.00 (17.57–22.43)	7.98×10^{-13}/2.57×10^{-15}
Drinking							
No	245/197/119	36.00 (33.95–38.05)	42.00 (35.98–48.02)	195/183/103	16.00 (14.05–17.95)	20.00 (15.19–24.81)	3.29×10^{-38}/7.61×10^{-14}
Yes	114/100/54	34.00 (30.28–37.72)	40.00 (33.58–46.43)	44/43/33	15.00 (12.21–17.79)	14.00 (11.53–16.47)	6.05×10^{-13}/3.16×10^{-14}
HBsAg							
Negative	92/77/38	34.00 (31.11–36.89)	42.00 (33.31–50.69)	54/53/34	16.00 (13.31–18.69)	16.00 (10.26–21.74)	6.37×10^{-20}/8.50×10^{-13}
Positive	267/220/135	36.00 (33.98–38.02)	41.00 (36.35–45.65)	185/173/102	16.00 (14.10–17.90)	18.00 (11.53–24.47)	2.35×10^{-34}/3.75×10^{-14}
Anti-HCV							
Negative	291/244/132	36.00 (34.26–37.74)	45.00 (40.31–49.69)	207/196/120	15.00 (13.49–16.51)	16.00 (12.06–19.94)	3.19×10^{-48}/9.68×10^{-23}
Positive	68/53/41	32.00 (28.16–35.84)	34.00 (31.43–36.57)	32/30/16	18.00 (13.57–22.44)	22.00 (14.62–29.37)	4.16×10^{-5}/8.74×10^{-3}
AFB1 exposure							
Low	101/79/19	47.00 (44.83–49.17)	55.51 (52.84–58.19)	43/39/14	24.00 (19.62–28.38)	40.96 (34.35–47.58)	4.56×10^{-15}/5.80×10^{-5}
High	258/218/154	31.00 (28.81–33.19)	34.00 (31.70–36.31)	196/187/122	14.00 (12.67–15.33)	14.00 (11.62–16.38)	2.25×10^{-37}/1.68×10^{-18}
Tumor size							
≤5 cm	100/67/44	43.00 (38.17–47.83)	51.00 (38.07–64.12)	33/31/16	23.00 (18.50–27.50)	33.00 (11.64–54.36)	2.63×10^{-10}/9.48×10^{-4}
>5 cm	259/230/129	33.00 (30.79–35.21)	40.00 (35.42–44.58)	206/195/120	15.00 (13.55–16.45)	16.00 (12.30–19.70)	6.18×10^{-38}/1.15×10^{-17}
Tumor grade							
Low	208/164/82	41.00 (38.24–43.76)	51.00 (47.47–56.43)	78/74/42	17.00 (13.75–20.25)	20.00 (8.21–31.79)	6.38×10^{-22}/1.88×10^{-8}
High	151/133/91	27.00 (24.51–29.49)	29.00 (24.36–33.65)	161/152/94	15.00 (13.22–16.78)	17.00 (13.51–20.49)	1.89×10^{-18}/7.41×10^{-8}
TNM stage							
I	87/65/37	46.00 (39.41–52.59)	48.71 (45.45–51.57)	15/14/5	33.00 (24.16–4184)	44.38 (35.93–52.83)	6.90×10^{-3}/0.74
II	272/232/136	34.00 (31.56–36.24)	40.00 (36.14–43.87)	224/212/131	15.00 (13.63–16.37)	16.00 (13.16–18.84)	2.01×10^{-41}/2.10×10^{-41}
MVD							
Low	259/208/103	38.00 (35.98–40.02)	47.00 (44.85–49.16)	76/69/29	25.00 (22.22–27.78)	43.00 (29.99–56.01)	1.65×10^{-11}/3.31×10^{-3}
High	100/89/70	26.00 (22.56–29.44)	24.00 (22.35–25.65)	163/157/107	1300 (11.96–14.04)	13.00 (11.74–14.26)	7.88×10^{-21}/1.50×10^{-9}
Liver cirrhosis							
No	104/88/43	34.00 (30.92–37.08)	41.00 (36.72–45.28)	67/66/42	17.00 (14.32–19.67)	18.00 (14.29–21.71)	1.33×10^{-24}/3.41×10^{-15}
Yes	255/209/130	36.00 (33.84–38.16)	42.00 (37.09–46.91)	172/160/94	15.00 (13.32–16.78)	18.00 (9.62–26.38)	4.76×10^{-31}/1.82×10^{-12}

[a] P/D/R represents number of cases with hepatocellular carcinoma/number of death cases/number of cases with tumor recurrence.

Abbreviations: CI, confidence interval; mo, months; MRT, median tumor recurrence-free survival time; MST, median overall survival time; MVD, microvessel density; ND, not

Given that number of cases having *CBX4* genotypes with rs528507291 G allele is very small, only rs77447679 was further investigated in this study. As shown in Fig. 1, HCC patients carrying genotypes with rs77447679 A alleles (also rs77447679-CA and -AA), compared to those without A alleles (rs77447679-CC), had a lower MST (35 months, 21 months, and 12 months for CC, CA and AA genotype respectively) (Fig. 1A, left plot) and MRT (42 months for CC, 30 months for CA and 12 months for AA respectively) (Fig. 1B, left plot). Results from univariable Cox regression analyses further exhibited that compared to those without A alleles (HR = 1), these patients having rs77447679 A alleles featured an increasing death risk [HR (95% CI/*p*), 2.56 (2.05–3.19/6.67 $\times$ 10^{-17}) for rs77447679-CA and 7.09 (5.61–8.97/4.59 $\times$ 10^{-60}) for rs77447679-AA respectively] (Fig. 1A, right plot). This is indicative of the death risk of HCC patients gradually increasing with number of A alleles. A similar trend was also shown in the analysis of RFS [corresponding tumor recurrence risk value: 1.73 (1.27–2.37/0.001) and 7.53 (5.64–10.06/9.98 $\times$ 10^{-43})] (Fig. 1B, right plot).

4.3 The effects of CBX4 rs77447679 SNP on survival stratified by the clinicopathological features of HCC patients

To explore whether the effects of *CBX4* rs77447679 SNP on survival were modified by the clinicopathological features of HCC patients, we accomplished a succession of stratified analyses on basis of the clinicopathological feature variables (Table 3). In these analyses, risk genotypes (HR >1) of *CBX4* rs77447679 SNP were combined into a variable. Similar MST and MRT were observed in the analyses of some stratified variables, including age, gender, race, smoking and drinking history, HBV and HCV status, and liver cirrhosis. However, other stratified variables such as AFB1 exposure level, tumor size, MVD, and tumor grade and stage were found different MST and MRT (Table 3).

In view of AFB1 exposure as a main environmental cause of HCC among Guangxi population, we questioned whether there was a genotypes-environment interactive role in HCC progression. Therefore, we finished a joint analysis. In this analysis, the combination of AFB1 exposure levels and *CBX4* rs77447679 genotypes were into six groups: AC1, AC2, AC3, AC4, AC5 and AC6 (Fig. 1C,D). Results showed that the effects of *CBX4* rs77447679 on HCC survival would increase under the conditions of increasing AFB1 exposure level. For example, MST (95% CI) for patients with rs77447679-AA would shorten from 20.00 (9.20–30.80) months to 11.00 (10.15–11.85) months when AFB1 exposure level was from low to high (Fig. 1C, left plot). Corresponding death risk was from 9.94 (3.93–25.14) to 39.14 (23.14–67.03) (Fig. 1C, right plot). Further analyses prove this joint effect between *CBX4* rs77447679 genotypes and AFB1 exposure levels was not a kind of multiplicative but additive interaction.

Next, we investigated the association between these different stratified clinicopathological features and *CBX4* rs77447679 SNP (Table 4). Results from *Spearman* correlation analyses displayed that *CBX4* rs77447679 SNP was significantly positively linked with tumor size (r = 0.17), tumor grade (r = 0.23), tumor stage (r = 0.23), and tumor MVD (r = 0.42).

4.4 Multivariable analyses indicating CBX4 rs77447679 SNP as an independent prognostic factor for patients with HCC

Because clinicopathological features such as bigger tumor size, higher MVD, and lower tumor grade are correlated with poor HCC survival, we aimed to pinpoint whether reduced survival among patients with risk genotypes of rs77447679 was an indirect reflection of relationship between the *CBX4* rs77447679 SNP and these clinicopathological features or, alternately, whether *CBX4* rs77447679 SNP could act as an independent biomarker for HCC survival. To answer it, we finished a multivariable analysis based on Cox regression model (Table 5). Results exhibited that *CBX4* rs77447679 SNP was identified as an independent biomarker for OS [HR (95% CI) = 2.06 (1.65–2.59) and 4.64 (3.63–5.93) for rs77447679-CA and -AA, respectively], as well as tumor size, grade and MVD. For RFS, HR (95% CI) adjusted by AFB1 exposure, tumor size, tumor grade and MVD was 1.73 (1.27–2.37) and 7.53 (5.64–10.06) for rs77447679-CA and -AA, respectively (Table 5).

4.5 CBX4 rs77447679 but not rs2289728 SNP significantly linking with CBX4 protein expression

Because our previous studies have shown that increasing *CBX4* expression progressed HCC angiogenesis and shorten HCC survival [8, 9], we explored whether *CBX4* rs77447679 SNP is associated with CBX4 protein expression by immunohistochemistry. Results from *Spearman* correlation analyses showed that this SNP was positively related to the amount of CBX4 protein in tissues with HCC (r = 0.57, p = 3.62 $\times$ 10^{-52}) (Fig. 2A).

Because a recent molecular epidemiological study has shown that *CBX4* rs2289728 SNP (G to A mutation) may increase HCC risk [11] and results from eQTL database (Blood eQTL) (https://www.genenetwork.nl/bloodeqtlbrowser) display that this SNP is associated with decreased expression of *CBX4* in blood cells, we also analyzed the association between *CBX4* rs2289728 SNP and *CBX4* expression in tissue samples with HCC. Results showed that this SNP was not significantly correlated with *CBX4* expression (r = 0.07, p = 0.10) (Fig. 2B).

4.6 CBX4 rs77447679 SNP differently regulating the therapeutic effects of pa-TACE on HCC

To further validate the clinic significance of *CBX4* rs77447679 SNP, we investigated the effects of this SNP on pa-TACE treatment improving HCC prognosis in the

Table 4. ***CBX4*** **rs77447679 polymorphism positively correlates with clinicopathological features of HCC.**

Variable	Rs77447679 genotype			*r*	*p*
	CC	CA	AA		
	(n = 359)	(n = 127)	(n = 112)		
Tumor size				0.17	5.10×10^{-5}
≤5 cm, n (%)	100 (27.9)	19 (15.0)	14 (12.5)		
>5 cm, n (%)	259 (72.1)	108 (85.0)	98 (87.5)		
Tumor grade				0.23	7.21×10^{-9}
Low, n (%)	208 (57.9)	39 (30.7)	39 (34.8)		
High, n (%)	151 (42.1)	88 (69.3)	73 (65.2)		
TNM stage				0.23	1.26×10^{-8}
I, n (%)	87 (24.2)	9 (7.1)	6 (5.4)		
II, n (%)	272 (75.8)	118 (92.9)	106 (94.6)		
MVD				0.42	1.48×10^{-27}
Low, n (%)	259 (72.1)	55 (43.3)	21 (18.8)		
High, n (%)	100 (27.9)	72 (56.7)	91 (81.2)		

Table 5. Multivariate Cox regression analyses on HCC-related survival.

Final variables	OS			RFS		
	HR	95% CI	*p*	HR	95% CI	*p*
AFB1 (high vs. low)	1.66	1.33–2.07	7.00×10^{-7}	3.73	2.56–5.43	6.45×10^{-12}
Tumor size (>5 vs. ≤5 cm)	1.71	1.36–2.16	6.00×10^{-7}	1.38	1.02–1.87	0.03
Tumor grade (high vs. low)	1.66	1.35–2.00	5.98×10^{-7}	1.87	1.45–2.41	1.00×10^{-7}
MVD (high vs. low)	2.58	2.11–3.14	8.16×10^{-21}	3.55	2.78–4.53	4.39×10^{-24}
Rs77447679 (CA vs. CC)	2.06	1.65–2.59	3.72×10^{-10}	1.73	1.27–2.37	5.23×10^{-4}
Rs77447679 (AA vs. CC)	4.64	3.63–5.93	1.98×10^{-34}	7.53	5.64–10.06	9.98×10^{-43}

Abbreviations: CI, confidence interval; MVD, microvessel density; OS, overall survival; RFS, tumor recurrence-free survivals.

second stage analyses. In this stage, the association between *CBX4* rs77447679 SNP and HCC prognosis was first analyzed and results like screening stage were observed (**Supplementary Fig. 3C**,**D**). Next, we analyzed possible different effects of pa-TACE on HCC patients with different genotypes of *CBX4* rs77447679 SNP (Fig. 3A–F). Among these patients having rs77447679-CA or -AA genotypes, pa-TACE treatment significantly prolonged their OS (Fig. 3C,E, left plots) and RFS time (Fig. 3D,F, left plots). Also, this kind of treatment significantly decreased death risk [HR (95% CI), 0.26 (0.18–0.38) for patients with rs77447679-CA and 0.18 (0.06–0.48) for patients with rs77447679-AA, respectively] and tumor reoccurrence risk [HR (95% CI), 0.33 (0.23–0.47) for patients with rs77447679-CA and 0.18 (0.07–0.49) for patients with rs77447679-AA, respectively] of patients with HCC (Fig. 3C–F, right plots). However, similar effects were not observed among those cases having rs77447679-CC genotype. Altogether, these results imply that different genotypes of *CBX4* rs77447679 SNP may differentially change therapeutic role of pa-TACE treatment on HCC.

5. Discussion

To our best knowledge, no studies have investigated the association between common SNPs in *CBX4* and HCC survival. In this hospital-based retrospective study, we explored the effects of *CBX4* SNPs on the outcome of HCCs among Guangxiese population. Results showed that the genotypes of rs77447679 A alleles had a substantial correlation with poor OS (adjusted HR 2.06 for rs77447679-CA; 4.64 for rs77447679-AA) and RFS (adjusted HR 1.73 for rs77447679-CA; 7.53 for rs77447679-AA) of patients with HCC.

In Guangxi area of China, HCC is the most common malignant tumor [17]. In the past several decades, the annual incidence rate (AIR) and the annual death rate (ADR) of this cancer in this area has been gradually increasing. AIR is up to about 100–200 per 10,000 from 30–50 per 10,000; whereas ADR is from about 10 per 10,000 to about 50 per 10,000 [18]. Growing evidence has exhibited that the infection of HBV and HCV and the exposure of AFB1 are two major causes for this high ADR and AIR among Guangxi population [19]. For AFB1 exposure, lots of epidemiological studies have proved that this carcinogen plays its carcinogenicity via binding to DNA and resulting in the formation of AFB1-DNA adducts. During the carcinogen-

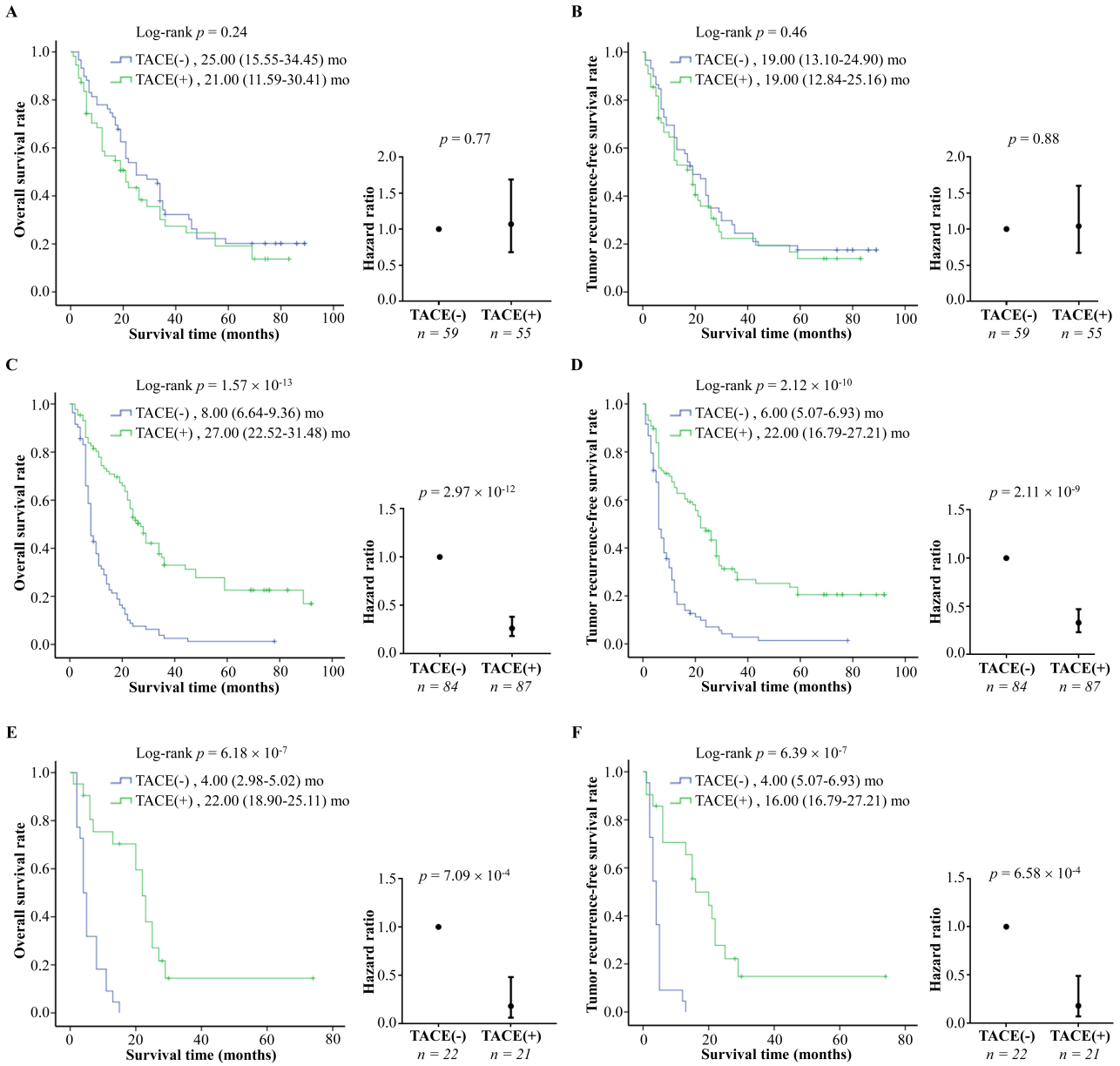

Fig. 3. *CBX4* rs77447679 polymorphism modifying the therapeutic effects of pa-TACE treatment on the survival of patients with HCC. (A,B) The effects of pa-TACE treatment on OS (A) and RFS (B) of HCC cases featuring rs77447679-CC genotype. (C,D) The effects of pa-TACE treatment on OS (A) and RFS (B) of HCC cases featuring rs77447679-CA genotype. (E,F) The effects of pa-TACE treatment on OS (A) and RFS (B) of HCC cases featuring rs77447679-AA genotype. Cumulative hazard function was plotted by the Kaplan–Meier methodology and the *p* value was calculated with two-sided log-rank tests. MST and MRT was also indicated. Right plots show hazard ratio (dot) and corresponding 95% confidential interval (vertical line segment). Abbreviations: mo, months; MRT, median tumor recurrence-free survival time; MST, median overall survival time; OS, overall survival; RFS, tumor recurrence-free survival; pa-TACE, post-operative adjuvant transarterial chemoembolization; TACE, transarterial chemoembolization.

esis of HCC induced by AFB1, AFB1-DNA adducts are vital. This is because AFB1-DNA adducts are usually characterized by non-enzyme, time-dependence, and apparent persistence and can result in the activation of oncogenes (such as Ras) and the inactivation of tumor suppressor genes (such as TP53) [20, 21]. Furthermore, amount of AFB1-DNA adducts in liver tissues are proved to positively correlate with tumor MVD [10]. Here, our data also showed that HCC patients with increasing amount of AFB1-DNA adducts would feature a shorten OS and RFS and an increased death risk and tumor recurrence risk. Altogether, these findings are indicative of AFB1 involving in HCC startup and progression.

Recently, several studies have displayed that the dysregulation of *CBX4* expression can promote tumorigenesis through several signal pathways, consisting of CBX4/TP53 [22], CBX4/HIF-1α/VEGF [9], CBX4/miR-195 [23], CBX4/HDAC3/Runx2 [6], CBX4/BMI-1 [24],

CBX4/P63 [7], and CBX4/CtIP [25]. A strong relationship between the structure alteration in CBX4 and the biology of cancers has also been reported [11, 12, 26]. Postnatal-acquired or genetic mutations may be pleiotropic and are properly to alter the susceptibility and progression of cancers. In a large-scale case-control study of gastric cancer (including 1021 cases and 1304 controls), it was demonstrated that rs77447679 SNP in the *CBX4* gene had a substantial effect on the risk of gastric cancer (adjusted odds ratio, 1.24; 95% CI, 1.04–1.49; and *p* value, 0.017) [12]. In this study, we not only found that *CBX4* rs77447679 SNP is an independent biomarker for HCC, but this SNP is related to clinicopathological features of HCC such as tumor size, tumor grade and stage, and tumor angiogenesis. Furthermore, our findings also showed that the different genotypes of *CBX4* rs77447679 SNP differently modified the therapeutic effects of pa-TACE intervention on HCC and affected HCC prognosis. These results suggest that *CBX4* rs77447679 SNP may involve in tumorigenesis and be a potential biomarker for cancer survival and formation of therapeutic strategy.

Mechanically, it is associated with *CBX4* rs77447679 SNP regulating *CBX4* expression, because results from our immunohistochemistry analyses of tissue samples with HCC exhibited this SNP was significantly linked with the amount of CBX4 protein. Supporting our findings, several recent reports further prove that increasing expression of *CBX4* can promote the several biofunctions of HCC, including proliferation, invasion and metastasis, angiogenesis, and metastasis [4, 8, 9, 27, 28].

Remarkably, we also observed some evidence of the joint effects of *CBX4* rs77447679 SNP and AFB1 exposure on HCC. Our results showed that risk genotypes significantly interacted with high AFB1 exposure, and that their interaction noticeably enhanced the risk of patient death and tumor recurrence. It was worth noting that the interaction of *CBX4* rs77447679 SNP and AFB1 exposure in the HCC progression was a kind of additive but not multiplicative interaction. This may be because of rs77447679 SNP may regulate *CBX4* expression and the dysregulation of *CBX4* expression could promote tumor angiogenesis, increase tumor MVD, and affect tumor sensitivity on anti-cancer drugs [8, 9]. An our recent study from high AFB1 exposure areas has displayed that increasing CBX4 protein expression in the tissues with HCC is significantly associated with poor survival of patients with AFB1-related HCC [10]. Taken together, these results suggest that *CBX4* rs77447679 SNP may involve in the progression of HCC, especially cancer caused by AFB1 exposure.

In a hospital-based case-control study, Tan *et al.* [11] explored the effects of *CBX4* rs2289728 SNP on HCC risk among Guilin population (from a null AFB1 exposure area) and found this SNP can increase HCC risk among Guilin population. However, our present study showed that this SNP did not affect HCC survival or alter *CBX4* expression in tumor tissues with HCC. Similar to our findings, eQTL analyses based on HapMap 3 database (https://www.sanger.ac.uk/resources/downloads/human/hapmap3.html) also proved that *CBX4* rs2289728 SNP has no effect on the expression of *CBX4* expression in the blood cell samples from Han Chinese in Beijing (HCB), China, although significant correlation is among other non-Chinese population. These results suggest that this kind of different effects of *CBX4* rs2289728 SNP may be associated with different population, different tissues, and different causes.

Although our present study is the first report to our knowledge to propose *CBX4* SNPs and HCC survival with a relatively large study size, several limitations should be taken into consideration. First, although more than 70% of patients received about five-years follow-up, the follow-up period for part cases (especially for cases with I-stage HCC) was relatively short. Second, selective bias from the hospital-based retrospective design may take. Third, joint effects of *CBX4* rs77447679 SNP and AFB1 exposure on the outcome of HCC may be underestimated, because liver injury (from viral hepatitis or other toxins) will result in abnormal metabolite of AFB1 and ultimately argument the amount of AFB1-DNA adducts in hepatic tissues. Fourth, although a relatively large-size subjects were included in this study, the effects of some low-frequent SNPs such as rs528507291 on HCC survival may be neglected. Fifth, notwithstanding clinical value of *CBX4* rs77447679 SNP improving the therapeutic role of pa-TACE invention on HCC, other clinical applications have not been elucidated. Finally, the mechanisms by which *CBX4* rs77447679 SNP affects *CBX4* expression and modifies HCC progression are unknown. Therefore, detailed functional and mechanical analyses deserve further evaluation on the basis of larger sample-size foresighted design and *in vitro* and *in vivo* studies.

6. Conclusions

To conclude, our results provide a new insight into the *CBX4* SNPs as genetic determinants of HCC prognosis, and this kind of determinants may be able to add significant predictive value for the outcome of this malignant tumor except the known traditional predictors such as tumor size, stage, grade, and so on. These findings imply that these HCC patients with the risk genotypes of *CBX4* rs77447679 SNP should accept pa-TACE treatment and/or more severe surveillance to obtain better prognosis. Large well-designed studies with diverse populations (including clinical trial with a potential target drug to HCC based on these findings) and functional evaluations are warranted to confirm and extend our findings.

7. Author contributions

XDL and JGY contributed to the conception of the work, interpretation of the data and critical revision of the manuscript. XYZ, MJH, QYS, XZW, JW, and QQL contributed to the data acquisition. XMW and XYH contributed to IRS evaluation of IHC. XDL drafted and finalized the manuscript. All authors contributed to the final approval of the manuscript.

8. Ethics approval and consent to participate

The study was approved by the Ethics Review Committees (No. 20041225) from the Affiliated Hospitals of Youjiang Medical University for Nationalities.

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11. Conflict of interest

The authors declare no conflict of interest. XDL is serving as one of the Guest editors of this journal. We declare that XDL had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to JZ.

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