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Original Article

PLZF and PLZF-MAPK10 can predict the prognosis of postoperative patients with hepatocellular carcinoma

Shanshan Hu[1], Yue Chen[1], Liheng Liu[2], Xuedong Yin[3], Yaying Yang[4], Liping Tang[1]

Departments of [1]Gastroenterology, [2]Gastrointestinal Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China; [3]Chongqing Key Laboratory of Molecular Oncology and Epigenetics, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China; [4]Department of Pathology, Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing, China

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Abstract: Objective: The current study aimed to explore the expression level of promyelocytic leukaemia zinc finger (PLZF) in hepatocellular carcinoma tissues and to investigate the value of detecting the expression levels of PLZF and mitogen-activated protein kinase 10 (MAPK10) on predicting prognosis. Methods: This study selected data from 53 patients with HCC who had undergone hepatectomy in our hospital. The expressions of PLZF and MAPK10 in tumor tissues and normal tissues were compared and related clinical factors were analyzed. The clinical data including patient's gender, age, hepatitis B virus infection (HBV), alpha-fetoprotein levels (AFP), tumor size, TNM stage (AJCC), cirrhosis, portal vein tumor thrombus (PVTT), bile duct tumor thrombus (BDTT), and OS (Overall survival) was collected. Results: We found that PLZF expression was significantly down-regulated in HCC samples compared with that in adjacent non-tumor tissues (P=0.001). The expression level of PLZF was correlated with patients' gender (P=0.046), tumor stage (P=0.039), and OS (P=0.015). Moreover, the expression level of PLZF-MAPK10 (P-M protein) was correlated with gender (P=0.000) and tumor stage (P=0.045). Multivariate analyses showed that microsatellite nodules, PLZF, and P-M protein were independent risk factors of HCC prognosis. Postoperative patients with a normal expression level of PLZF and MAPK10 have a longer overall survival than those with abnormal levels (P=0.039). Conclusion: PLZF expression was significantly down-regulated in HCC tissues and itself and PLZF-MAPK10 were both independent prognostic factors for the OS of patients with HCC.

Keywords: Hepatocellular carcinoma, promyelocytic leukaemia zinc finger protein, mitogen-activated protein kinase 10, tumor suppressor genes, prognosis

Introduction

Dysregulation of cancer-related gene expression plays an important role in hepatocarcinogenesis [1]. Hepatocellular carcinoma (HCC) is the second most common cause in cancer-related death globally. HCC patients are often diagnosed at an advanced stage and died within 6-20 months [2-4]. Novel therapy methods and sensitive diagnostic or prognostic markers of HCC are in high request in clinic [5]. Increasing evidence has proved that the identification of oncogene or tumor suppressor genes (TSG) in tumor samples were valuable for early diagnosis, prognosis evaluation, and therapeutic strategy options [6].

Promyelocytic leukaemia zinc finger (PLZF), also named as BTB domain containing 16 (ZBTB16), belongs to the family of Krueppel C2H2 type zinc finger protein and is mainly expressed in brain, lung, endocrine, and testicle tissues [7]. PLZF was initially found in acute promyelocytic leukaemia patients with chromosomal translocation. It acts as an activator or repressor by regulating transcription-mediated effects primarily [8-10]. PLZF is also essential for the differentiation and development of healthy tissues and can maintain the self-renewal of stem cells [11-13]. PLZF can even regulate antimicrobial immunity, tumor rejection, and inflammatory diseases through invariant natural killer T (iNKT) cells [14, 15]. In previous researches, PLZF expression was down-regulated in multifarious solid tumor and malignant cell lines and acted as a TSG in a series of tumors, such as pancreatic cancer, malignant mesothelioma cell lines, high-risk melanomas,

prostate cancer, thyroid cancer, and colorectal cancer [16-18]. However, the role of PLZF in HCC remains unclear. Although its expression was never tested to be down-regulated in some HCC samples, no clinical significance was observed [19].

Mitogen-activated protein kinase 10 gene (MAPK10), also known as JNK3, is located in the 4q21.3 fragment of the chromosome region, which mainly expresses in neurons, heart, brain, fat, and other tissues [20]. In recent years, the JNK signaling pathway played a vital role in pathological mechanism for various diseases, including Parkinson and Alzheimer's, ischemic diseases, cardiovascular diseases, and tumors, by regulating cell differentiation, proliferation, apoptosis, protein expression, and inflammation [21-25]. In addition, MAPK10 was reported to be down-regulated in nasopharyngeal cancer, breast cancer, ovarian cancer, myeloma, glioblastoma, and other tumors as a TSG [24, 26-29]. In our previous work, the expression of MAPK10 was significantly down-regulated in HCC patients and appeared to be a TSG frequently methylated in HCC, suggesting its clinical significance (in press).

We detected the PLZF expression in tumor and normal tissues from HCC patients by the immunohistochemical method and investigated its correlation with clinical characteristics. We also analyzed the value of PLZF-MAPK10 in predicting the prognosis of HCC.

Materials and methods

Patients data

This study was approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University and all patients signed the informed consent. This study selected data from 53 patients with HCC who had undergone hepatectomy at The First Affiliated Hospital of Chongqing Medical University from June 2005 to December 2009.

Inclusion criteria: patients who received radical resection and confirmed as HCC by postoperative pathology; patients who had no radio- or chemotherapy treatment history; patients with complete clinical data; patients with 5-year follow-up data; and patients who had paraffin-embedded tumor and normal tissues. The adjacent normal tissues samples were obtained 5 cm outside the tumor margin.

Exclusion criteria: patients with other pathological types such as bile duct cell type and mixed type; patients with preoperative radio/chemotherapy and drug anti-tumor therapy; patients with adjacent normal tissues samples that less than 5 cm outside the tumor margin; patients who had other tumors; and patients who had severe diseases in other organs.

Observation indicators

The clinical data regarding a patient's gender, age, hepatitis B virus infection (HBV), alpha-fetoprotein levels (AFP), tumor size, TNM stage (AJCC), cirrhosis, portal vein tumor thrombus (PVTT), bile duct tumour thrombus (BDTT), and OS (Overall survival) were collected from operation to death or to the last follow-up. OS and quality of life (SF-36) were investigated by telephone follow-up (5 years).

The paraffin-embedded tumor and normal tissues that 5 cm outside the tumor margin were collected for PLZF expression detection. The expression of MAPK10 in the identical set of HCC samples had been detected in our previous work. Therefore, the value of PLZF-MAPK10 in the predicting the prognosis of HCC.

Immunohistochemistry (IHC) staining

IHC staining was carried out by using UltraSensitiveTMS-P (MXB, China). The primary antibody against PLZF was obtained from Santa Cruz and used at a dilution of 1:100. The staining intensity was scored as 0 (absent expression), 1 (weak expression), 2 (moderate expression), and 3 (strong expression). The percentage of positive staining was scored as 0 (0%-9%), 1 (10%-25%), 2 (26%-50%), or 3 (51%-100%). The final score = the staining intensity score × the percentage of positive staining, with a total score of 0 to 9. The expression of PLZF was defined as "-", "+", "++" and "+++" corresponding to the score 0, 1-3, 4-6, and 7-9 respectively. For further clinical analysis, the patients were divided into two groups: the down-regulated group ("-" and "+") and the normal level group ("++" and "+++"). The IHC staining score was performed in a double-blind man-

ner by two pathologists to minimize the observational bias.

Statistical analysis

SPSS 21.0, Graphpad Prism 8 and R 3.6.4 software were used for statistical analysis. Count data were expressed as percentage and analyzed by χ^2-test and Fisher's exact test. The cox regression statistical calculations were performed by the R 3.6.4. Kaplan-Meier and Log-rank tests were used to evaluate the relationship between gene expression and OS. P<0.05 indicated a statistically significant difference.

Results

The clinical characters in HCC patients

This research collected 53 paraffin specimens from HCC patients. There were 10 females and 43 males with a median age of 49 years (range, 21-77). Chronic HBV carriers accounted for the majority (69.8%). The median AFP (α-fetoprotein) value was 258 ng/mL (range 2.8-96401), and the median tumor size was 7.0 cm (range, 1.5-20). All of the 53 patients with clinical data were well qualified for the clinical correlation analysis (**Table 1**). As for OS analysis, the median follow-up time was 6 months (range 0-60).

The protein expression of PLZF and MAPK10 in clinical samples of HCC

In **Figure 1A**, HE staining figures showed that the non-tumor tissue had regular and normal cell morphology. However, the tumor tissue had irregular cell morphology and the nuclear had a darker color. IHC results showed that there are few cells with positive expression of PLZF or MAPK10, conversely, which in the HCC tissue were numerous.

Among all the specimens, only samples of 40 cases were stained clearly. For PLZF detection, 28 of 40 showed the down-regulated expression in HCC tissues, which could be found in only 12 of 40 in the adjacent non-tumor tissues (P=0.001). For MAPK10 detection, 24 of 39 showed the down-regulated expression in HCC tissues, and 12 of 39 in the adjacent non-tumor tissues (P=0.012). The down-regulated expression rate of the two markers between the HCC and the adjacent non-tumor tissues were significantly different (**Figure 1B** and **1C**).

The relationship between PLZF or MAPK10 expression level and clinical characters

In the current clinical study, HCC patients were separated into the down-regulated group and the normal level groups according to the expression level of PLZF and MAPK10 in HCC tissue. The relationship between protein level and clinical characters were analyzed (**Table 1**). It showed that all female patients (10/10) had decreased or silent PLZF expression and most of the patients (24/38) with down-regulated PLZF expression tended to have advanced stages. There was a statistical difference between these two groups concerning gender (P=0.046) and tumor stage (P=0.039). As for MAPK10, its protein expression level was also closely related to the tumor stage (P=0.013). There were no statistically significant differences with age, hepatitis B virus infection, AFP level, tumor size, cirrhosis, or portal vein tumor thrombus (P>0.05).

The relationship between P-M protein expression level and clinical characters

Our previous work has found that MAPK10 was significantly down-regulated and appeared to be a TSG frequently methylated in HCC. In this study, we also detected the expression of PLZF protein in the identical set of samples, so we combined PLZF with MAPK10 for analysis. We regrouped HCC patients into two sub-groups according to the level of P-M protein: patients in the normal level group had both PLZF and MAPK10 in "++" or "+++"; patients in the down-regulated showed low expression of PLZF or MAPK10 ("-", "++").

Finally, only 38 of 53 cases' data could used for analysis. Results showed that 27 of 38 showed the down-regulated expression in HCC tissues, and 13 of 38 in the adjacent non-tumor tissues (P=0.003, **Figure 1D**). For clinical characteristics analysis, we found that P-M expression was closely associated with gender (P=0.000, **Table 1**) and tumor stage (P=0.045, **Table 1**).

Univariate and multivariate analyses of prognostic factors for postoperative overall survival

Those variables with P<0.2 in univariate analyses (**Table 1**) for OS were included in the multivariate analyses. The multivariate Cox proportional hazards model was used to estimate

Table 1. The relationship between PLZF, MAPK10, or PLZF-MAPK10 expression and clinical pathology characteristics in HCC

Characteristics	PLZF		MAPK10		P-M	
	Normal	Down-regulated	Normal	Down-regulated	Normal	Down-regulated
Age (Year)						
≤ge	13	29	15	27	12	30
>60	2	9	4	7	2	9
Sex		*				**
Male	15	28	18	25	14	29
Female	0	10	1	9	0	10
Serum AFP (ng/ml)						
≤eru	11	20	14	17	11	20
>400	4	18	5	17	3	19
HBsAg						
Positive	12	25	14	23	10	27
Negative	3	13	5	11	4	12
Tumor Size (cm^3)#						
≤i	9	16	8	17	8	17
>5	6	22	11	17	6	22
Microsatellite nodules						
Absent	13	34	17	30	12	35
Present	2	4	2	4	2	4
Cirhosis						
Absent	8	23	12	19	8	23
Present	7	15	7	15	6	16
PVTT						
Absent	14	33	16	31	13	34
Present	1	5	3	3	1	5
BDTT						
Absent	13	37	17	33	12	38
Present	2	1	2	1	2	1
Tumor stage (AJCC)		*		*		*
Stage I	9	14	10	13	9	14
Stage II	0	11	0	11	0	11
Stage III	6	13	9	10	5	14

Note: There was significant difference between the normal level group and the down-regulated group, *P<0.05, **P<0.01; #Tumor size was measured by the length of the largest tumor nodule. PVTT: portal vein tumor thrombus; BDTT: bile duct tumor thrombus; AJCC: American Joint Committee on Cancer.

adjusted hazard ratios (HR) and 95% CIs as well as to detect independent prognostic factors. Moreover, it should be noted that gender was not included in multivariate analyses as PLZF was reported to closely relate with gender by many studies. In multivariate analysis, we performed two statistical calculations (Model I and Model II) to clarify the significance of PLZF and P-M, respectively. In detail, microsatellite nodules, PVTT, PLZF, and MAPK10 expression were incorporated into Model I, while microsatellite nodules, PVTT, P-M expression were incorporated into Model II.

The univariate Cox proportional hazards regression analysis showed that down-regulated of PLZF expression was significantly associated with the worse OS (P=0.029). The other clinicpathologic variables showed no significant impact on the OS. Multivariate Cox proportional hazards regression analysis further determined that presence of microsatellite nodules (Model I: HR=3.88; 95% CI 1.19 to 12.6; P=0.024; Model II: HR=3.9; 95% CI 1.19 to 12.73; P=0.024), normal level of PLZF expression (HR=0.14; 95% CI 0.02 to 0.96; P=0.045), and normal level of P-M expression (HR=0.19; 95%

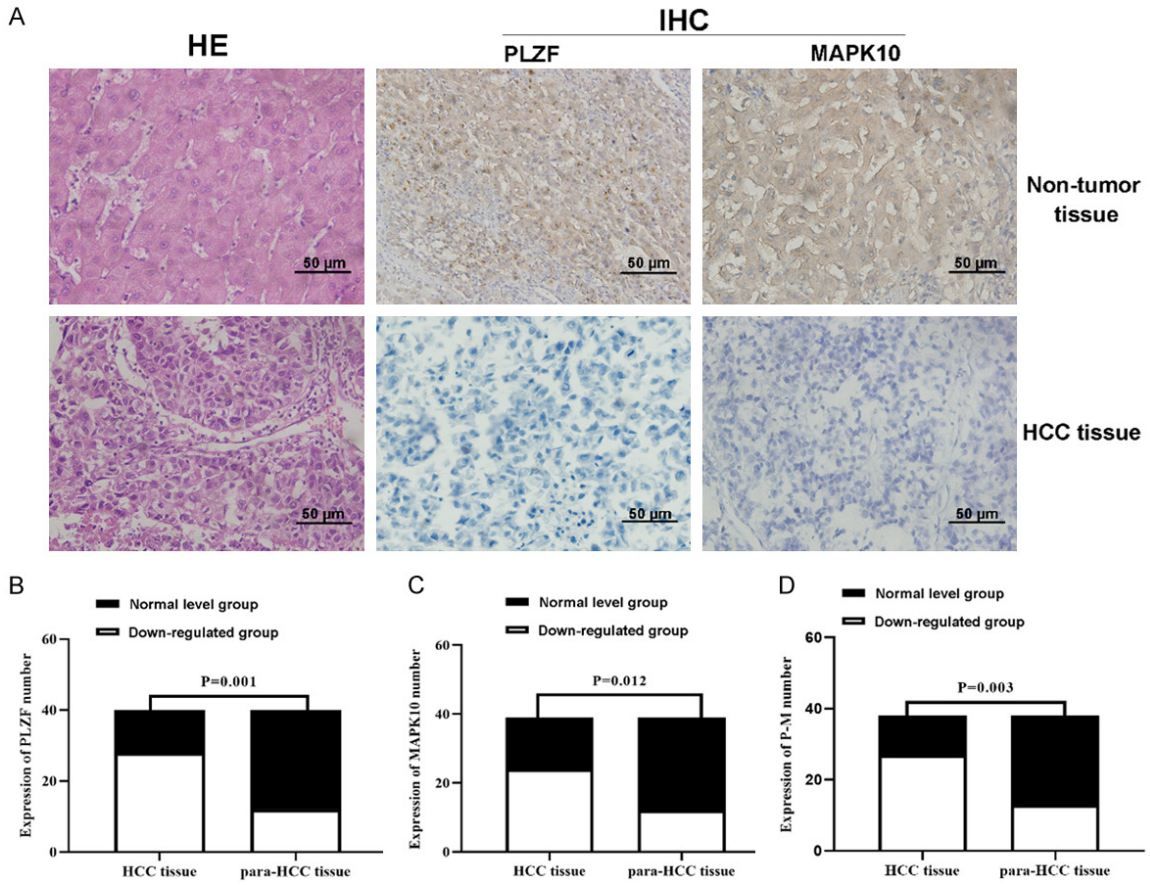

Figure 1. Expression of PLZF and MAPK10 genes in HCC and adjacent non-tumor liver tissues. A: Representative IHC (400×) images of PLZF and MAPK10 expression in HCC and adjacent non-tumor tissues as well as the H&E staining images as a reference; B-D: The expression of PLZF, MAPK10, and PLZF-MAPK10 in HCC tissue and adjacent non-tumor liver tissues were compared. PLZF: promyelocytic leukaemia zinc finger; MAPK10: mitogen-activated protein kinase 10; HCC: hepatocellular carcinoma.

CI 0.04 to 0.87; P=0.032) were independent prognostic factors for the OS of HCC patients who received surgery resection (**Table 2**).

Down-regulated of PLZF/P-M expression indicated a worse prognosis in postoperative patients with HCC

Prognostic significance of PLZF in HCC was studied in 40 patients with follow-up data, which indicated that the expression of PLZF was correlative with survival (Log-rank =5.976, P=0.015): the mean OS time in the down-regulated group and normal group was 26.95 months (95% CI 17.648 to 36.258) and 45.86 months (95% CI 32.224 to 59.498) respectively (**Figure 2A**). Moreover, when it comes to the prognostic analysis of P-M expression, the statistically difference still existed (Log-rank =4.255, P=0.039): the mean OS (overall survival) time in the down-regulated group and normal level group was 28.27 months (95% CI 18.974 to 37.556) and 49.13 months (95% CI 36.278 to 61.972) respectively (**Figure 2B**). Postoperative patients with normal expression of P-M proteins have a longer survival than those with single or without. All these data suggested that PLZF and P-M could be independent predictors for the prognosis of HCC patients.

Discussion

PLZF can regulate genes to achieve stem cell maintenance and oncogenesis by DNA binding capability given by 9 Kruppel-type zinc-finger motifs at the carboxyl terminus. The previous reports have shown that PLZF is down-regulated in multiple tumors, including HCC. However, no evidence shows the correlation of PLZF with

Table 2. Prognostic significance of clinicopathological parameters for HCC

Characteristics	Overall Survival					
	Univariate analysis		Multivariate analysis-model I		Multivariate analysis-model-II	
	HR with 95% CI	P	HR with 95% CI	P	HR with 95% CI	P
Age (Year)						
≤ge	Reference					
>60	0.68 (0.23-2.04)	0.490				
Sex						
Male	Reference					
Female	0.41 (0.13-1.26)	0.118				
Serum AFP (ng/ml)						
≤eru	Reference					
>400	1.27 (0.54-2.99)	0.581				
HBsAg						
Positive	Reference					
Negative	0.72 (0.28-1.86)	0.503				
Tumor Size (cm^3)#						
≤5	Reference					
>5	0.88 (0.38-2.04)	0.760				
Microsatellite nodules						
Absent	Reference					
Present	2.13 (0.71-6.33)	0.175	3.88 (1.19-12.6)	0.024	3.9 (1.19-12.73)	0.024
Cirhosis						
Absent	Reference					
Present	0.66 (0.27-1.65)	0.377				
PVTT						
Absent	Reference					
Present	3.13 (0.89-11.03)	0.077	2.47 (0.69-8.93)	0.167	2.64 (0.73-9.51)	0.137
BDTT						
Absent	Reference					
Present	0.95 (0.12-7.25)	0.962				
Tumor stage (AJCC)						
Stage I	Reference					
Stage II-III	1.07 (0.44-2.64)	0.880				
PLZF expression						
Normal level	Reference					
Down-regulated	0.20 (0.05-0.85)	0.029	0.14 (0.02-0.96)	0.045		
MAPK10 expression						
Normal level	Reference					
Down-regulated	0.39 (0.13-1.17)	0.092	1.13 (0.28-4.56)	0.864		
P-M expression						
Normal level	Reference					
Down-regulated	0.25 (0.06-1.05)	0.059			0.19 (0.04-0.87)	0.032

Note: #Tumor size was measured by the length of the largest tumor nodule. PVTT: Portal vein tumor thrombus; BDTT: Bile duct tumor thrombus; AJCC: American Joint Committee on Cancer; CI: confidence interval; HR: hazard ratio.

the clinical parameters and if it is a TSG in HCC [19]. In our current study, the protein level of PLZF was down-regulated in most of the HCC tissues when compared with the adjacent non-

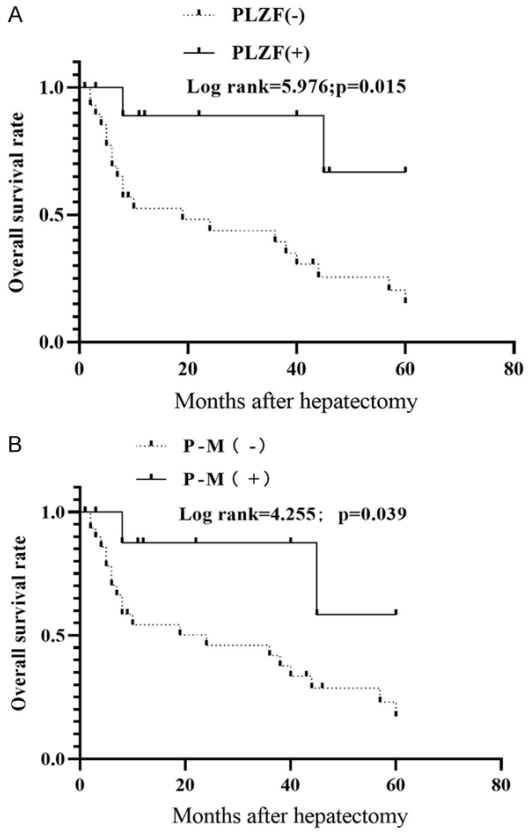

Figure 2. Kaplan-Meier overall survival (OS) curve of HCC patients in correlation with PLZF and P-M expression. A: OS rate of HCC patients with PLZF (+) and PLZF (-); B: OS rate of HCC patients with P-M (+) and P-M (-). PLZF: promyelocytic leukaemia zinc finger; HCC: hepatocellular carcinoma; OS: Overall survival; P-M: PLZF-MAPK10.

tumor tissues, indicating that PLZF may play the TSG role in HCC. The exact mechanisms of its down-expression regulation and its particular function in HCC are still unclear. Promoter hypermethylation has been put into first consideration, drawing on the experience in non-small cell lung cancer and pancreatic adenocarcinoma [30]. Unfortunately, we failed to detect the structure of promoter methylation in the experimental samples, speculating that the down-regulated expression may not be the cause of methylation mechanism, or, due to the difficulties of the promoter region selection, which delayed publication of our work for many years.

The function of PLZF in carcinogenesis has been widely reported. Many previous studies have confirmed that PLZF can inhibit tumor formation by regulating cell growth, differentiation, apoptosis, as well as ameliorate disease progression in the immune system by control cytokine production in T-cells and innate immune cell development. For example, PLZF suppresses the prostate tumorigenesis by mediating the PTEN/AKT/FOXO3a Signaling, inhibits occurrence and development of gallbladder cancer by controlling genes expression (p21, E-cadherin and N-cadherin genes) and increases the mRNA transcription of interferon-induced protein, and restrains proliferation and invasion in PC by regulating VEGF-mediated angiogenesis [31-33]. In our experiment, the down-regulated expression of PLZF in HCC samples was closely associated with patients' gender, advanced tumor stage, and the worse survival of HCC patients, suggesting PLZF could act as a TSG, which inhibits or affects the development steps of HCC.

In our study, all female HCC specimens showed low/no expression of PLZF. The protein expression of PLZF was closely related to patients' gender. It has been demonstrated that PLZF expression is higher in male-specific tissues such as prostate and testis than any other tissues. PLZF mRNA was reported to be transiently up-regulated by androgens but inhibited by androgen receptor antagonist bicalutamide in the regressed ventral prostate of castrated adult rats [34]. In 2006, Kikugawa T et al. proposed the expression of PLZF is rare in the androgens receptor-deficient PCa cell lines and ectopic expression when androgens receptor restored in DU145 cells [35]. In 2019, Stopsack et al. confirmed that there was a positive correlation between intratumoral androgen receptor (AR) signaling and the expression of PLZF [36]. All of these studies demonstrated that PLZF is an androgen activated gene, which can be used to partly explain why PLZF expression is lower in almost all female HCC patients.

Dis-regulation of cancer-related genes expression is one of the characters of carcinogenesis. In the clinic, gene expression assay has been widely used for tumors' diagnosis and therapy. MAPK10, a member of the c-jun N-terminal kinases (jnk) subgroup in the MAPK superfamily, proposed as a TSG inactivated epigenetically. In this study, the predictive effect of the combination of PLZF and MAPK10 was also detected. As a result, a more significant correlation with gender, advanced tumor stage, and worse OS were found when both PLZF and MAPK10

expression were down-regulated concurrently, indicating that the co-downregulated expression of these two genes could be a potential biomarker of tumor development.

There were also some shortcomings and limitations. For example, the small sample size and lack of data of long follow-up time may increase statistical errors. In addition, other epigenetic mechanisms like histone modifications and ncRNA, may also lead to the down-regulation of PLZF in HCC. The exact research routes are complicated and need more clarification in the future. More studies are still needed to clarify this phenomenon and intrinsic mechanisms.

In conclusion, PLZF expression was significantly down-regulated in HCC tissues and itself and PLZF-MAPK10 were both independent prognostic factors for the OS of patients with HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Liping Tang, Department of Gastroenterology, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Yuanjiagang, Chongqing 400016, China. Tel: +86-023-89012964; E-mail: tanglipingu1cy@163.com

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