Downregulation of PLZF in human hepatocellular carcinoma and its clinical significance

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Abstract. Promyelocytic leukemia zinc finger (PLZF) acts as a tumor-suppressor gene in a series of cancers including prostate, melanoma, colon cancer and leukemia. However, its role in hepatocellular carcinoma (HCC) has not yet been illustrated. The present study aimed to investigate the expression and epigenetic regulation of PLZF as well as its clinical significance in HCC. We found that the expression of PLZF was significantly downregulated in HCC samples at both the RNA level ($P<0.001$) and protein level compared with these levels in adjacent normal tissues. The relative expression level of PLZF was also positively correlated with the ALP level ($P=0.026$) noted in the HCC patients. However, hypermethylation was only detected in one out of 5 paired HCC samples, indicating that methylation of the selected promoter region (from -1702 to -1388) may not be the major regulatory mechanism for the downregulation of PLZF in HCC. A receiver operating characteristic (ROC) curve was created to evaluate the diagnostic value for differentiating between HCC and benign diseases. The area under the ROC curve (AUC) for indicating the value of PLZF as an HCC biomarker was 0.794 (95% CI, 0.697-0.892; $P<0.001$). Taken together, our results suggest that PLZF may play an important role in HCC development and may be a potential biomarker for the diagnosis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world and is the third leading cause of cancer-related death due to its aggressive metastasis and poor clinical diagnosis (1). In China, the mortality rate of HCC is the second highest of all cancers, and the five-year survival rate is less than 5% with nearly 60,000 HCC patients succumbing to the disease every year (2). Increasing evidence suggests that multiple genetic and epigenetic alterations contribute to the tumorigenesis of different cancers. Recently, increased attention has been given to the role of epigenetically regulated genes in HCC.

Promyelocytic leukemia zinc finger (PLZF), also known as zinc finger (ZF) and BTB domain containing 16 (ZBTB16), belongs to the POK (POZ and Krüppel ZF) family, which plays an important role in stem cell maintenance and oncogenesis. With DNA binding capability conferred by 9 Krüppel-type zinc-finger motifs at the carboxyl terminus, PLZF can function as a transcriptional factor to regulate various genes (3). In mammals, PLZF is co-expressed with Oct4 in undifferentiated spermatogonia and is essential for stem cell self-renewal (4-6). In acute promyelocytic leukemia, PLZF may function as a chromosomal translocation partner (7). As a transcriptional repressor, PLZF is able to regulate cyclin A2, c-Myc, HoxD11 and other growth-related targets (8).

Downregulation of PLZF has been reported in various solid tumors and malignant cell lines. For example, PLZF expression was found to be downregulated in the majority of pancreatic cancer samples among which 35.2% of the samples presented with hypermethylation in the PLZF promoter (9). PLZF was also found to be downregulated in several malignant mesothelioma cell lines with loss of heterozygosity in the 11q region which encompasses the PLZF gene (10). In addition, PLZF expression was found to be substantially more downregulated in high-risk melanomas (overall survival of less than 5 years) than in low-risk melanomas (overall survival of more than 5 years) (11).

Regarding the functional role of PLZF in tumorigenesis, a recent study suggested that PLZF promoter hypermethylation reduces its expression and subsequently elicits a regulatory effect in non-small cell lung cancers (12). Moreover, overexpression of PLZF in human cervical cell lines inhibits cell growth by inducing apoptosis and suppressing the promoter activity of human cyclin A2 (13). However, little is known concerning PLZF with regard to its function and epigenetic regulation in HCC. Here, we report the differential expression

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of PLZF, its association with HCC traits, the methylation status of the promoter and its potential diagnostic significance as a biomarker in HCC.

Materials and methods

Tissue samples. Forty-one pairs of liver tissue samples including the tumor tissues of HCC patients and their corresponding tumor-adjacent normal tissues were used in this study. The study protocol was approved by the Ethics Committee of the Chinese University of Hong Kong. Written informed consent was obtained from each patient before the sample harvesting at the Prince of Wales Hospital, Hong Kong, China. All of the samples were immediately frozen in liquid nitrogen after surgery and stored at -80˚C before use.

Western blot analysis. Total protein was extracted from the tissue by using T-PER tissue protein extraction reagent followed by concentration measurement using the BCA assay kit (both from Thermo Scientific, USA). The proteins were separated on 10% SDS-PAGE and transferred to a polyacrylamide difluo-ride (PVDF) membrane (Immobilon; Millipore, USA). The membrane was blocked with 5% nonfat milk (Blotting-Grade Blocker; Bio-Rad) in TBST (10 mM Tris-HCL, 150 mM NaCl, 0.1% Tween-20) for 1 h, and then incubated with the PLZF antibody (Santa Cruz Biotechnology, Inc.) overnight at 4˚C with gentle agitation. After washing for 3 times in TBST and incubation with the secondary antibody (1:2,000) for 1 h at room temperature, the membrane was developed by Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences) and autographed on X-ray film using a medical X-ray processor (model 102; Kodak). The β-actin antibody (Santa Cruz Biotechnology, Inc.) was used at a dilution ratio of 1:5,000 for normalization.

DNA preparation, RNA extraction, and quantitative real-time PCR. Total RNA was isolated from the clinical tissues using the TRIzol kit (Invitrogen Life Technologies). cDNA was synthesized with a reverse transcription kit (Qiagen, Germany) following the manufacturer's instructions. Quantitative real-time PCR was performed using an Applied Biosystems ViiA™7 Real-Time PCR System with QuantiTect SYBR®-Green PCR kits. PCR reaction contained 2 μl of distilled water, 0.5 μl of 1.5 mM forward and reverse primers, 2 μl of cDNA and 5 μl of 2X SYBR-Green. PLZF was amplified with primers PLZF-F (5'-TCA CAT ACA GGC GAC CAC C-3') paired with PLZF-R (5'-CTT GAG GCT GAA CTT CTT GC-3'). All samples were prepared in triplicate. Comparative Ct method ($2^{-\Delta\Delta Ct}$) was adopted to calculate the relative level of the mRNA normalized to β-actin (ACTB).

Bisulfite-sequencing PCR (BSP) analyses. Genomic DNA samples from HCC tissues and adjacent normal tissues were treated with bisulfite (EZ DNA methylation kit; Zymo Research). A pair of primers including the forward (5'-GGG AGA GAG AAA AGT TTT TTT TA-3') and the reverse (5'-CAA TAT TTA CCC CAA TTC AAT AC-3') were used for PCR amplification under the following condition: 94˚C for 10 min followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec. The PCR products were cloned
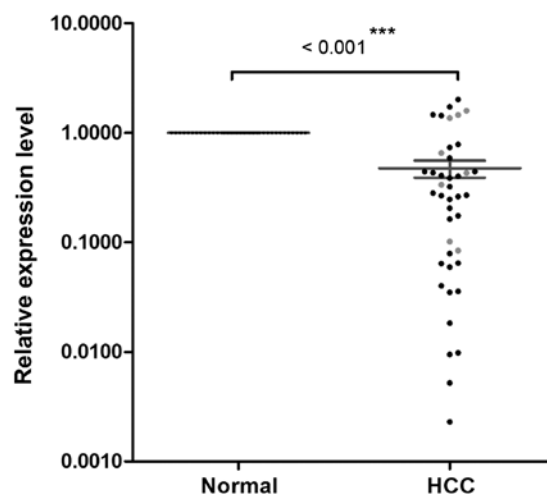

Figure 1. Relative expression level of promyelocytic leukemia zinc finger (PLZF) mRNA in 42 pairs of clinical tissues revealed by quantitative real-time PCR. The horizontal line indicates the mean of the relative expression level. The error bars indicate the standard error of the mean, while the black and gray dots indicate samples from male and female patients, respectively.
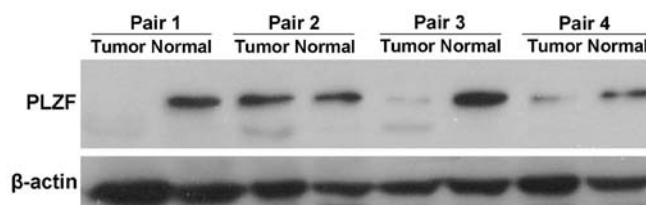

Figure 2. Promyelocytic leukemia zinc finger (PLZF) expression level in 4 pairs of representative clinical tissues revealed by western blot analysis. Each pair includes tumor tissue and the corresponding adjacent normal control.

into the pCR 2.1 vector (Invitrogen Life Technologies). The DNA sequence was confirmed by automatic DNA sequencing (service provided by BGI).

Statistical analysis. Statistical analysis was performed with either GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) or SPSS (version 16.0) using different methods including paired-samples t-test, Fisher's exact test, bivariate correlation statistics and one-way ANOVA. A receiver operating characteristic (ROC) curve was created to evaluate the diagnostic value for differentiating between HCC cancer and benign diseases. The correlation curve and ROC curve were plotted by SPSS. P-value of 0.05 or less was considered to indicate a statistically significant result.

Results

Differential expression of PLZF in the HCC tissues. Result of the real-time PCR revealed that the mRNA expression level of PLZF was downregulated (P<0.0001) by 52.6% when compared with this level in the adjacent normal controls (Fig. 1). Consistence with the real-time PCR results, the PLZF protein level was also decreased in 3 out of 4 paired HCC tumor tissues (Fig. 2).

Table I. Demographic and clinical features of the 41 HCC patients.

Pathological parameter	Total	PLZF expression		P-value
		Reduced	Preserved	
Gender				
Male	33	29	4	0.033[a]
Female	8	4	4	
Age (years)				
<60	20	16	4	0.719
≥60	21	15	6	
AFP level (ng/ml)				
<1,000	28	19	9	0.231
≥1,000	12	11	1	
HBsAg				
Positive	31	23	8	1.000
Negative	9	7	2	
Differentiation				
Well	6	3	3	0.140
Moderate	14	11	3	
Poor	6	6	0	
AJCC staging				
Stage I	31	21	10	0.235
Stage II	6	6	0	
Stage III	2	2	0	
Cirrhosis				
Yes	14	12	2	0.365
No	12	8	4	
Recurrent				
Yes	24	19	5	0.714
No	17	12	5	
Disease-free status				
Censored	9	6	3	0.090
Recurrent/death	17	14	3	

Not all clinical parameters are available for every sample. HCC, hepatocellular carcinoma; PLZF, promyelocytic leukemia zinc finger. [a]Significant association with PLZF expression.
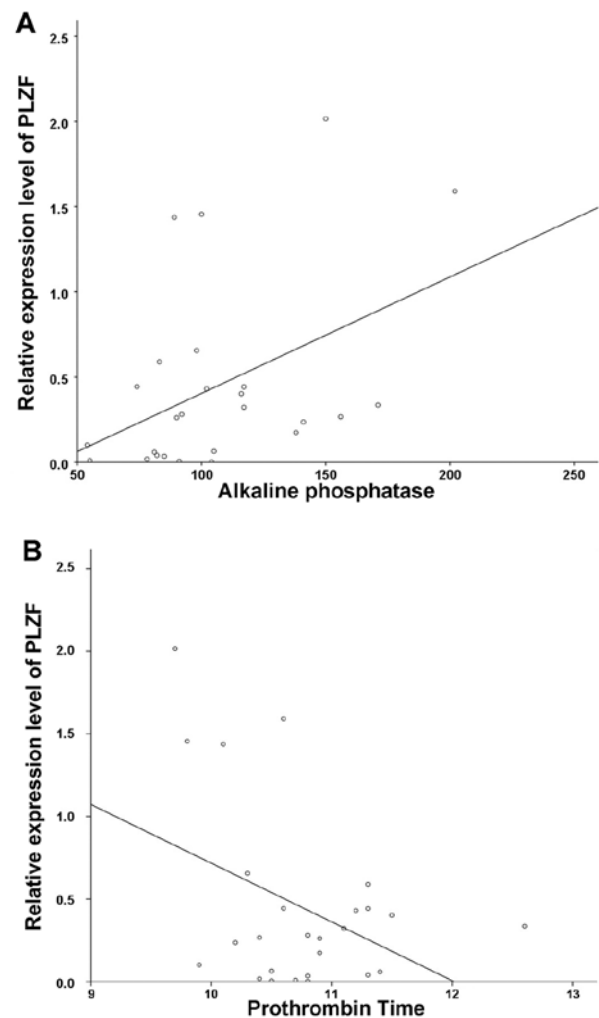
Figure 3. Correlation between the expression levels of promyelocytic leukemia zinc finger (PLZF) and (A) alkaline phosphatase and (B) prothrombin time.

Relationship between PLZF expression and clinical features. To evaluate the clinical significance of PLZF in HCC, association analysis between PLZF expression and the clinical features was performed. We found that the gender of the patients was significantly associated with the relative expression level of PLZF (P=0.033). Moreover, associations between PLZF expression and alkaline phosphatase level (P=0.026) (Fig. 3) and prothrombin time (P=0.043) (Table I) were also observed. However, no significant associations were observed for other parameters (data not shown).

Promoter methylation of PLZF in the HCC samples. To investigate whether the downregulation of PLZF in HCC was induced by hypermethylation of the PLZF promoter, the methylation status of the PLZF promoter CpG island (from -1702 to -1388) was determined by bisulfite sequencing in 6 pairs of randomly selected HCC samples. However, promoter methylation was only observed in one HCC tissue sample (Fig. 4), indicating that promoter methylation was not the major mechanism responsible for the downregulation of PLZF in HCC.

Diagnostic value of using PLZF as a cancer biomarker. The differences between the HCC tissues and matched adjacent normal tissues were compared based on a cutoff value (0.778) from the ROC curve. The area under the ROC curve (AUC) reached 0.794 (95% CI, 0.697-0.892; P<0.001) (Fig. 5). The sensitivity was 0.895 and specificity was 0.474.

Discussion

As a tumor-suppressor gene, downregulation of PLZF has been involved in the tumorigenesis of various types of cancers. In the present study, PLZF expression was found to be significantly reduced at both the mRNA and protein levels in HCC clinical samples compared with these levels in
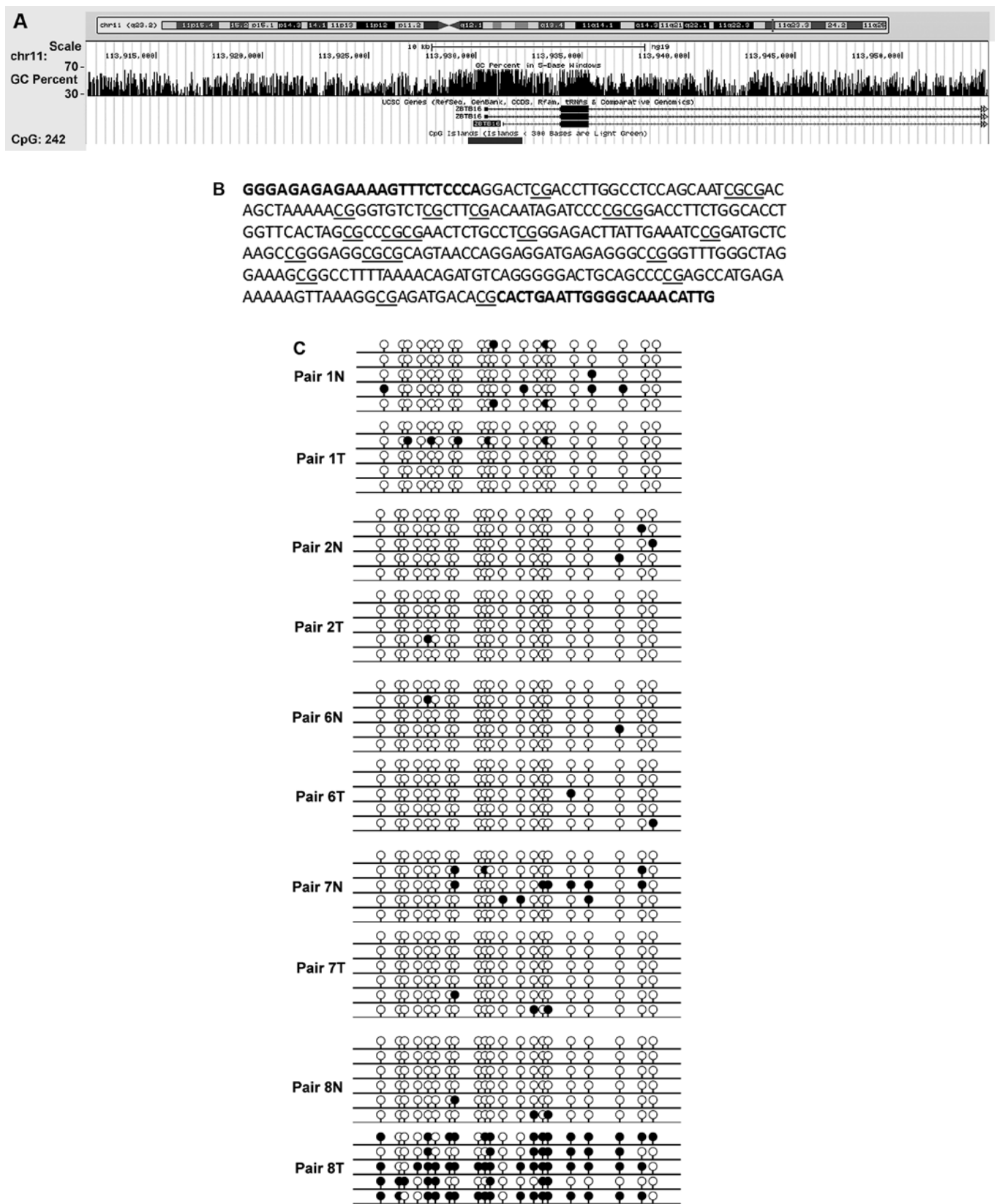
Figure 4. Promoter methylation of promyelocytic leukemia zinc finger (PLZF). (A) The CpG island in the promoter of PLZF (ZBTB16) revealed by UCSC Browser (http://genome.ucsc.edu/). (B) The target sequence in PLZF promoter for bisulfite sequencing. Twenty-one CpG sites were observed in this fragment. (C) Methylation status of the PLZF promoter revealed by bisulfite sequencing. Hypermethylation was noted in 1 out of 5 pairs of clinical samples. N and T represent the samples from the normal and tumor tissues, respectively.

the adjacent normal tissues. However, promoter methylation was not detected in the examined samples, indicating that the reduced PLZF expression may not be caused by the promoter methylation in the studied region.
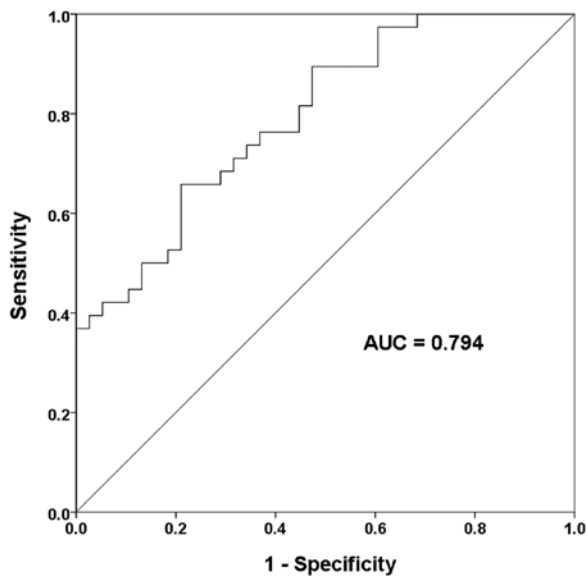
Figure 5. Receiver operation characteristics (ROC) curve of using PLZF as a marker. P<0.001.

Promoter hypermethylation is one of the major factors which contribute to the aberrant expression of certain genes by affecting its transcription factor binding profile. PLZF promoter hypermethylation has been found to be responsible for the downregulation in non-small cell lung cancer as well as in pancreatic adenocarcinoma (9). The promoter region (from -1702 to -1388) we selected was exactly the same as a previous study in lung cancer (9). However, since this promoter region does not cover the entire CpG island, our results do not exclude the possibility that there are other hypermethylated CpG sites outside the selected target region that regulate PLZF expression. Of course, there remain other epigenetic mechanisms such as histone modifications and ncRNA that may contribute to the downregulation of PLZF in HCC (14). Moreover, loss of heterozygosity of PLZF has been found to account for the downregulation of PLZF in malignant mesothelioma cells (10). Further investigation on the hemizygous deletion of PLZF by PCR amplification with genomic DNA may shed light on the downregulation of PLZF in HCC.

In the present study, PLZF expression was found to be positively correlated with the alkaline phosphatase level in the HCC patients. Such a result was consistent with a previous observation in osteoblastic differentiation. Alkaline phosphatase (ALP) is a hydrolase enzyme, which is present in all tissues throughout the entire body, but is particularly concentrated in the liver, bile duct, kidney, bone and placenta. It is notable that an increased ALP level in plasma has been associated with large bile duct obstruction, intrahepatic cholestasis, or infiltrative diseases of the liver (15). In addition, PLZF plays an important role in osteoblastic differentiation and matrix mineralization by regulating ALP expression. Previous studies have shown that siRNA-mediated gene-silencing of PLZF suppressed the expression of the ALP gene in human mesenchymal stem cells (MSCs) resulting in the suppression of matrix mineralization (16,17). It is likely that PLZF suppresses the ALP expression in HCC by sharing a similar

mechanism as that in MSCs. However, the exact mechanism of ALP regulation mediated by PLZF is still unclear.

The present study for the first time revealed the downregulation of PLZF in human HCC samples compared with the that in adjacent tissues. Moreover, PLZF was positively correlated with the ALP level in HCC patients. It has been reported that overexpression of PLZF suppresses the promoter activity of human cyclin A2 and induces cell apoptosis in human cervical cell lines (13). Together with our results, it is reasonable to believe that reduced PLZF may play an important role in HCC development and may serve as a potential biomarker for the diagnosis of HCC.

Acknowledgements

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