

Myeloid zinc finger 1 protein is a key transcription stimulating factor of *PYROXD2* promoter

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Received January 12, 2017; Accepted July 31, 2017

DOI: 10.3892/or.2017.5990

Abstract. Previous studies revealed that PYROXD2 was more highly expressed in normal liver tissue and liver cell lines than in cancer tissue and cancer cell lines, which indicated that decreased PYROXD2 expression may be involved in hepatocarcinogenesis. To identify the mechanisms which regulate *PYROXD2* gene transcription, we constructed a series of luciferase reporter plasmids and used them to perform luciferase-based reporter assays with HepG2, Sk-hep1, L02 and 293T cells with the purpose of characterizing the *PYROXD2* reporter region. Our results revealed that the transcription factor myeloid zinc finger 1 (MZF1) is necessary for *PYROXD2* gene transcription and that it functions as a *trans*-activator. DNA binding assays revealed that the MZF1 protein binds to the *cis*-element TGGGGA located in the -320/-312 region of the *PYROXD2* promoter. This promoter had a significantly enhanced activity when the MZF1 protein was overexpressed and a significantly decreased activity when the MZF1 protein expression was silenced. However, MZF1 gene expression was not significantly correlated with PYROXD2 protein expression in the samples of resected tumor tissues, which revealed that the *PYROXD2* promoter transcription activity was determined by the aggregated effect of numerous transcription factors. This finding may be helpful in understanding the underlying mechanism which regulates the PYROXD2 expression.

Introduction

PYROXD2, also known as YueF, (GenBank accession no. BC006131), was initially identified as a novel hepatitis B virus X-interacting protein (HBx) in studies conducted using a yeast two-hybrid screening system (1,2). As a putative

tumor-suppressor protein, the overexpression of PYROXD2 can cause cell-cycle arrest in the G1 phase, induce cell apoptosis, enhance the expression of p53 and p21WAF1/Cip1, decrease cyclin D1 and pRb expression and suppress the growth of hepatocellular carcinoma (HCC) tumors in nude mice *in vivo* (3). PYROXD2 is highly expressed in the cytoplasm of normal cells and tissues but is expressed at lower levels in corresponding cancer cells, including liver, lung and renal cell carcinoma and bladder cancer cells (1,2). The biological functions of PYROXD2 and the mechanism which regulates its expression remain largely unknown.

Myeloid zinc finger 1 (MZF1) is a member of the SCAN-zinc finger (SCAN-ZF) family of transcription factors, and has finger-like molecular structures that bind in a sequence-specific manner into the groove of the DNA (4). MZF1 has been implicated in tumorigenicity and it is thought to mediate the migration and invasion of cancer cells by suppressing the activity of certain gene promoter regions *in vivo* and *in vitro* (5-8). Moreover, higher levels of MZF1 RNA were revealed in a series of human cancer tissue than in normal tissue (5). MZF1 binds with the proteins found in promyelocytic leukemia nuclear bodies (9). Promyelocytic leukemia nuclear bodies strongly influence gene transcription activity and chromosomal structure through their interaction with other factors and their formation is dependent on the oligomerization of promyelocytic leukemia proteins (10,11). The MZF1 protein is a promoter/enhancer binding-type transcription factor, which functions both as a *trans*-activator and a *trans*-repressor. This observation revealed that the relative oncogenic activity of MZF1 is determined by the aggregated effects produced by the increase and decrease in gene expression (12), phosphorylation modifications, SUMOylation modifications, and co-activating and co-repressing molecules (5). The MZF1 protein must become phosphorylated in order to respond to the stimulating effects of transforming growth factor- β (TGF- β) (13), which is a growth factor known to be important for facilitating the migration and invasion of cancer cells and the development of the epithelial-mesenchymal transition phenotype (14). SUMOylation of transcription factors usually requires the participation of co-repressors and may thus mediate certain suppressive processes orchestrated by MZF1 during cellular differentiation and oncolytic processes (15).

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Key words: PYROXD2, hepatocarcinogenesis, transcription factor myeloid zinc finger 1, *cis*-element, transcription

In the present study, we examined the expression levels of both PYROXD2 and MZF1 using RT-PCR and western blot analysis. We found increased levels of MZF1 mRNA and protein expression and decreased levels of PYROXD2 mRNA and protein expression in cancer cell lines and HCC tissues compared to the expression levels in normal cell lines and liver tissue. We also sought to identify the *cis*-elements and transcription factors which activate *PYROXD2* transcription in liver cancer cells. To accomplish this goal, we performed a deletion analysis of the *PYROXD2* gene promoter region, followed by a mutant analysis of that region to identify transcription factors that may regulate *PYROXD2* transcription. We then evaluated the influence of MZF1 on PYROXD2 protein expression. Our results revealed that MZF1 is a transcription factor crucial in the regulation of *PYROXD2* gene expression. Moreover, an *MZF1* gene binding site (TGGGGA) located in the -320/-312 region was significant for the functioning of the PYROXD2 promoter.

Materials and methods

Ethics statement and human tissue preparation. The experiments involving humans were approved by the Ethics Committee, and each study participant provided a signed written informed consent document. All the tissue samples were obtained from the Department of Surgery, Zhejiang Provincial People's Hospital, Hangzhou, China.

Twelve samples of live human HCC tissues and 12 samples of corresponding adjacent normal liver tissues were obtained from 12 HCC patients (2 females and 10 males) and examined by a pathologist. All 12 HCC samples displayed a distinct cellular subtype and all of the adjacent tissue samples appeared to be normal and did not have fibrosis or other non-neoplastic changes. Four tumors were at stage II and eight were at stage III. All tissue samples were immediately dissected into several sections (~100 mg/section), washed with normal saline, frozen in liquid nitrogen and stored at -80°C.

Cell culture and transfection. Liver carcinoma cell lines HepG2, L02, Sk-hep1 and 293T were obtained from China Center for Type Culture Collection, Wuhan, China, maintained in our lab and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA) at 37°C in a 5% CO₂ atmosphere. Transfections were performed using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions.

Reporter constructs and expression vectors. Polymerase chain reaction (PCR) was used to amplify the full length of the *PYROXD2* promoter (-1998/-1) present in the genomic DNA of L02 cells. Subsequently a directional PCR cloning strategy was employed to clone the amplified promoter region into pGL3 basic vectors (luciferase reporter plasmids; Promega, Madison, WI, USA) at locations between the *KpnI* and the *XhoI* restriction enzyme sites. The luciferase reporter plasmids were designated as (-1998/-1)-PYROXD2 promoter vectors. Based on the selection made for the starting and ending nucleotide base in the *PYROXD2* promoter sequence, the serial *PYROXD2* promoter deletion mutants were designated

as (-1800/-1)-*PYROXD2* promoter vector, (-1600/-1)-*PYROXD2* promoter vector, (-1200/-1)-*PYROXD2* promoter vector, (-1000/-1)-*PYROXD2* promoter vector, (-800/-1)-*PYROXD2* promoter vector, (-600/-1)-*PYROXD2* promoter vector, (-400/-1)-*PYROXD2* promoter vector, (-200/-1)-*PYROXD2* promoter vector, (-1998/-200)-*PYROXD2* promoter vector, (-1998/-400)-*PYROXD2* promoter vector, (-1998/-600)-*PYROXD2* promoter vector, (-1998/-800)-*PYROXD2* promoter vector, (-1998/-1000)-*PYROXD2* promoter vector and (-1998/-1200)-*PYROXD2* promoter vector, respectively. Two siRNA sequences (GATCCGTACACAAGGGGACCATTC ATTCTTCAAGAGAGAATGAATGGTCCCCTTGTGTATT TTTTACGCGTG and GATCCGGCAGGTCCAGGTAGT GTAATTCAAGAGATTACACTACCTGGACCTGCTTTTT TACGCGTA) were cloned into pLVX-U6 between the *BamHI* and the *EcoRI* restriction enzyme sites with the purpose of silencing the MZF1 protein expression. A control sequence (GATCCGGCAACCTATGGGTGGGTAATTTTCAAGAG AAATTACCCACCCATAGGTTGCTTTTTTACGCGTA) was cloned into the same vector at the same restriction enzyme site. The *MZF1* gene was cloned into N-p3xflag-CMV (then designated as MZF1-Flag) to force the overexpression of the MZF1 protein. All deletion and mutant constructs were checked using DNA sequencing methods prior to being used in any experiments.

Site-directed mutagenesis. Constructs bearing the mutant promoter variants of *PYROXD2* were generated by PCR, with the (-1998/-1)-PYROXD2 promoter vector as a template. Potential transcription factor binding sites were identified using TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and the results are listed in Table I. Site-directed mutagenesis was performed with a KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The mutant primers used for site-directed mutagenesis (information provided upon request) were designed and produced by Generay Biotech Co., Ltd. (Shanghai, China). All mutants were verified by sequencing.

Dual-luciferase reporter gene assay. Cells were seeded into 96-well plates and cultured for 12 h, after which they were transfected with luciferase reporter plasmids which had the selected serial *PYROXD2* promoter. Subsequently, each sample was co-transfected with 20 ng of *Renilla* luciferase control vector pGL4.70 (Promega) to monitor the transfection efficiency. The pGL3 vectors were used as controls. The luciferase activity was assessed at 24 h post-transfection using a Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. The assay results were assessed with a Varioskan Flash Spectra Scanning Multimode Reader (Thermo Fisher 3001; Thermo Fisher Scientific, Waltham, MA, USA).

DNA binding assay. A DNA binding assay was used to detect the interactions between the MZF1 and the putative promoter core binding DNA sequence (AGGGGA, -320/-312). The biotinylated positive DNA sequence was biotin-TCTCC TCCCCTGTGCATCTACCTTC-3'. The putative positive DNA duplexes, MZF1 binding duplexes, were created by annealing biotin-TCTCCTCCCCTGTGCATCTACCTTC-3'

and 5'-GAAGGTAGATGCACAGGGGAGGAGA-3'. The control DNA duplexes were produced by annealing biotin-TCTCCTCCCTGTGCATCTACCTTC-3' and 5'-GAAGGTAGATGCACAGTAGAGGAGA-3'. All oligonucleotides were coupled to M-280 Streptavidin Dynabeads (Milenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. A Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Jiangsu, China) was used to extract total nuclear proteins from the L02 cells, according to the manufacturer's instructions. The DNA binding assays were performed as described by Plotz *et al* (16) and the MZF1 protein antibody (Abcam, Cambridge, UK) was used to immunoprecipitate the protein-DNA complex.

Real-time PCR. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen) and from liver tumor and normal tissue using the PureLink® RNA Mini kit (Thermo Fischer Scientific) according to the manufacturer's instructions. The extracted mRNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Pleasanton, CA, USA). First Universal SYBR-Green Master Reagent (Rox; Roche) and KOD FX enzymes (Toyobo) were used to perform comparative Ct analyses with a CFX96™ Real-Time system (Bio-Rad, Hercules, CA, USA). The sense and antisense primers used to detect PYROXD2 mRNA were: 5-AAGTGCTCCATTGGATCAGC-3' and 5-GAGGCATGGGCATAAGGTCA-3', respectively. The sense and antisense primers used to detect MZF1 mRNA were: 5-GAAACTGAGCCTCCAACTCC-3' and 5-GGGTGGGTACAGACTCCTG-3', respectively. The sense and antisense primers used to detect actin mRNA were: 5-ATCAGCAAGCAGGAGTATGACGAGT-3' and 5-ATGCCAATCTCATCTTGTCTTCTGC-3', respectively.

Western blot analysis. The cells were lysed by incubation in a RIPA buffer (Beyotime Biotechnology) and their total soluble proteins were isolated by centrifugation. The soluble proteins were then separated by electrophoresis on a 12% SDS gel and the individual protein bands were transferred onto nitrocellulose membranes for analysis using western blotting and standard antibody detection procedures. The proteins were extracted from tissue samples using a T-PER® Tissue Protein Extraction kit (Thermo Fisher Scientific). The primary antibodies used for immunostaining were anti-MZF1 (rabbit), anti-PYROXD2 (rabbit) and anti-GAPDH (mouse) (all from Abcam Cambridge, MA, USA). The membranes were incubated with the primary antibodies, washed with TBST buffer and then incubated with anti-mouse IgG (H+L) conjugate (Dylight™ 800) or anti-rabbit IgG (H+L) conjugate (Dylight™ 800) (both from Cell Signaling Technology, Danvers, MA, USA), depending on which primary antibody was used during the first incubation. The LI-COR® Biosciences Odyssey® Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA) was used to detect antibody binding and quantify the individual protein bands.

Statistical analysis. Each data point represents the mean \pm SD obtained from at least three independent experiments. The

Student's t-test was used to analyze differences between two independent groups; a two-sided P-value <0.05 was considered to indicate a statistically significant difference.

Results

PYROXD2 expression in liver tumor tissue and liver cancer cell lines compared with its expression in normal liver tissue and normal cell lines. Previous studies had found lower levels of PYROXD2 expression in several carcinoma tissues than in corresponding normal tissues. This finding revealed that PYROXD2 plays an important role in tumor suppression (3). In order to further assess the different expression models of PYROXD2, we collected a sample of hepatic carcinoma tissue and a sample of normal liver tissue from each of the 12 liver carcinoma patients who underwent therapeutic surgery. We then used reverse transcription-polymerase chain reaction (RT-PCR) to determine the relative levels of PYROXD2 and GAPDH mRNA expression and western blotting techniques in order to determine the relative levels of PYROXD2 and GAPDH protein expression in each tissue sample. We also examined these expression levels in several liver cell lines.

We obtained the same results observed in previous studies (3). The PYROXD2 protein was highly expressed in the normal hepatic cell line L02 but it was barely detectable in the HepG2 and Sk-hep1 hepatoma cell lines (Fig. 1A). The level of PYROXD2 mRNA expression in the three cell lines, as assessed by RT-PCR, displayed the same pattern as the level of PYROXD2 protein expression (Fig. 1B). The level of PYROXD2 protein expression in normal tissue samples (Level^{normal}) and the corresponding HCC tissue samples (Level^{HCC}) as detected by western blotting (Fig. 1C) were calculated based on the ratio of the gray value for PYROXD2 and the gray value for GAPDH in the same tissue sample (gray-value^{PYROXD2}/gray-value^{GAPDH}). By establishing the Level^{normal} value as 1, the relative expression levels of PYROXD2 protein in the corresponding HCC tissue could be expressed as the ratio: Level^{HCC}/Level^{normal}. The results of these calculations are illustrated in Fig. 1D. While 9 of the 12 patients had a level of PYROXD2 protein expression in their HCC tissue sample that was 10 to 90% lower than that in their corresponding sample of normal liver tissue, 3 of the 12 HCC patients had a higher level of PYROXD2 expression in their HCC sample than in their normal tissue sample. Furthermore, similar to the trend revealed by the PYROXD2 protein expression, the levels of PYROXD2 mRNA in the samples of normal liver tissue were higher than the levels in the corresponding samples of HCC tissue (Fig. 1E). These results demonstrated that although PYROXD2 was highly expressed in normal liver tissue and cells, its expression was decreased in HCC tissue and certain HCC cell types, a finding which revealed that decreased PYROXD2 expression plays a role in hepatocarcinogenesis.

MZF1 expression in HCC tissue and various liver cell lines. We examined the relative levels of MZF1 protein expression in L02, HepG2 and Sk-hep1 cells using western blotting (Fig. 1A). MZF1 which served as a transcription factor was mainly detected as a component of cell nuclear proteins and exhibited different expression in diverse tissues and cell

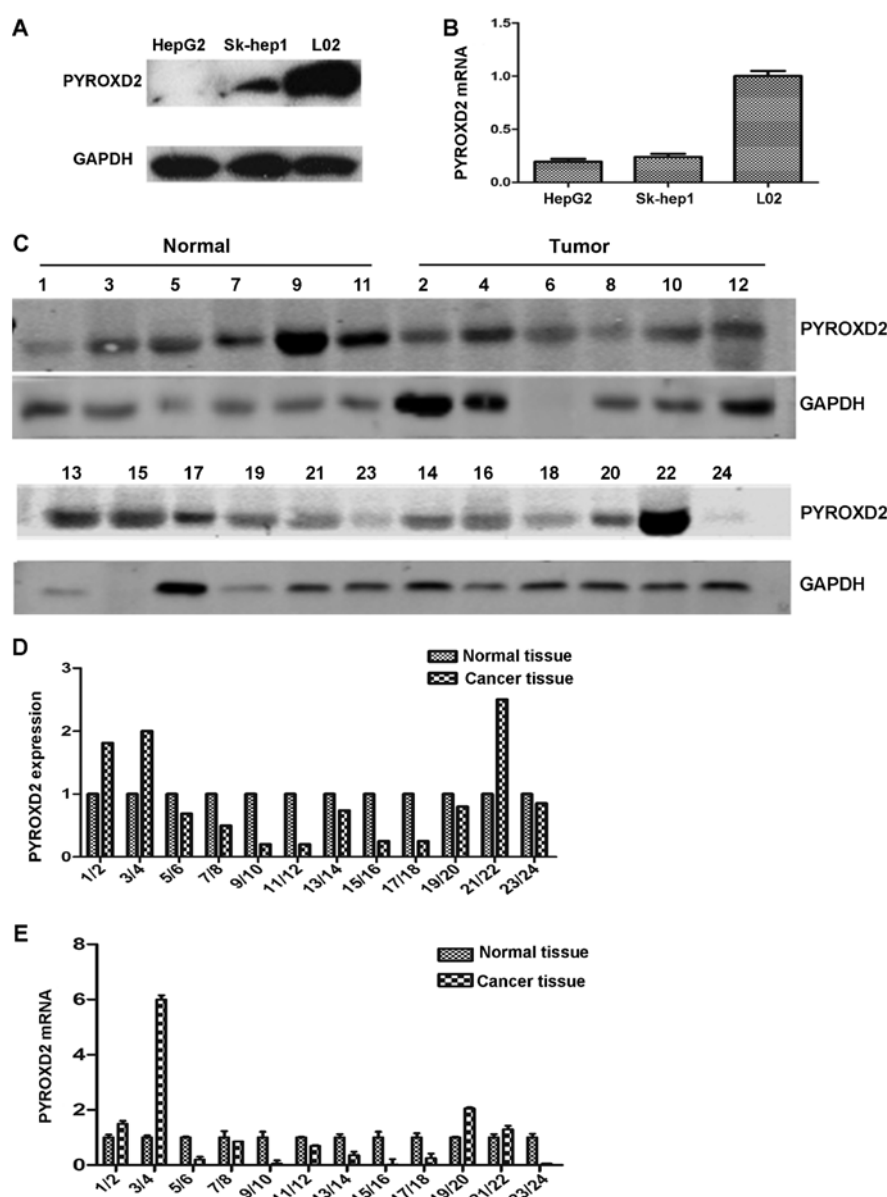


Figure 1. Relative levels of the PYROXD2 protein and mRNA expression in samples of normal tissue, cancer tissue and 3 different cell lines. The samples are numbered as follows: a number x represents the normal tissue and a number (x+1) represents the tumor tissue obtained from the same patient. (A) Relative levels of PYROXD2 protein expression in L02, HepG2 and Sk-hep1 cells as determined with western blotting. (B) Relative levels of PYROXD2 mRNA expression in L02, HepG2 and Sk-hep1 cells as determined with RT-PCR. (C) Relative levels of PYROXD2 protein expression in samples of hepatic carcinoma tissue and the corresponding samples of normal liver tissue as determined with western blotting. (D) Relative expression levels of the PYROXD2 protein in samples of cancer tissue and normal tissue. The results were calculated based on the ratio of the gray values of the western blots shown in C. (E) Expression of PYROXD2 mRNA in samples of hepatic carcinoma tissue and the corresponding samples of normal liver tissue as they were determined using RT-PCR.

lines (5). The same result was obtained by immunofluorescence analysis (data not shown) and MZF1 also displayed different expression levels in HepG2, L02 and Sk-hep1 cells (Fig. 2B), which indicated that MZF1 was less expressed in the HepG2 cells than in the L02 and Sk-hep1 cells as determined by western blotting. We also examined the endogenous levels of the MZF1 protein and mRNA expression in the 12 resected HCC tissue samples and the 12 corresponding normal tissue samples using western blotting and RT-PCR, respectively. The levels of MZF1 protein and mRNA expression were calculated using the same methods as those used to calculate the levels of PYROXD2 protein and mRNA expression; the results are shown in Fig. 2C-E. The tumors in 6 of the 12 patients had a significantly increased level of MZF1 expression compared

with the level in the corresponding normal liver sample; however, the tumors in the other 6 patients had 10-90% lower levels of MZF1 expression than those in the corresponding normal tissue samples.

Sequence AGGGGA (-320/-312) in the PYROXD2 promoter is the main element controlling PYROXD2 expression. We cloned the 1999 base pair (bp) fragment (-1998/-1) of the promoter region of the *PYROXD2* gene located upstream of the ATG transcription initiation codon of exon 1, with the purpose of analyzing the promoter and its regulatory elements. A search for potential regulatory motifs which was performed using the TFSEARCH identified the putative transcription factor binding sites and is listed in Table I. Subsequently,

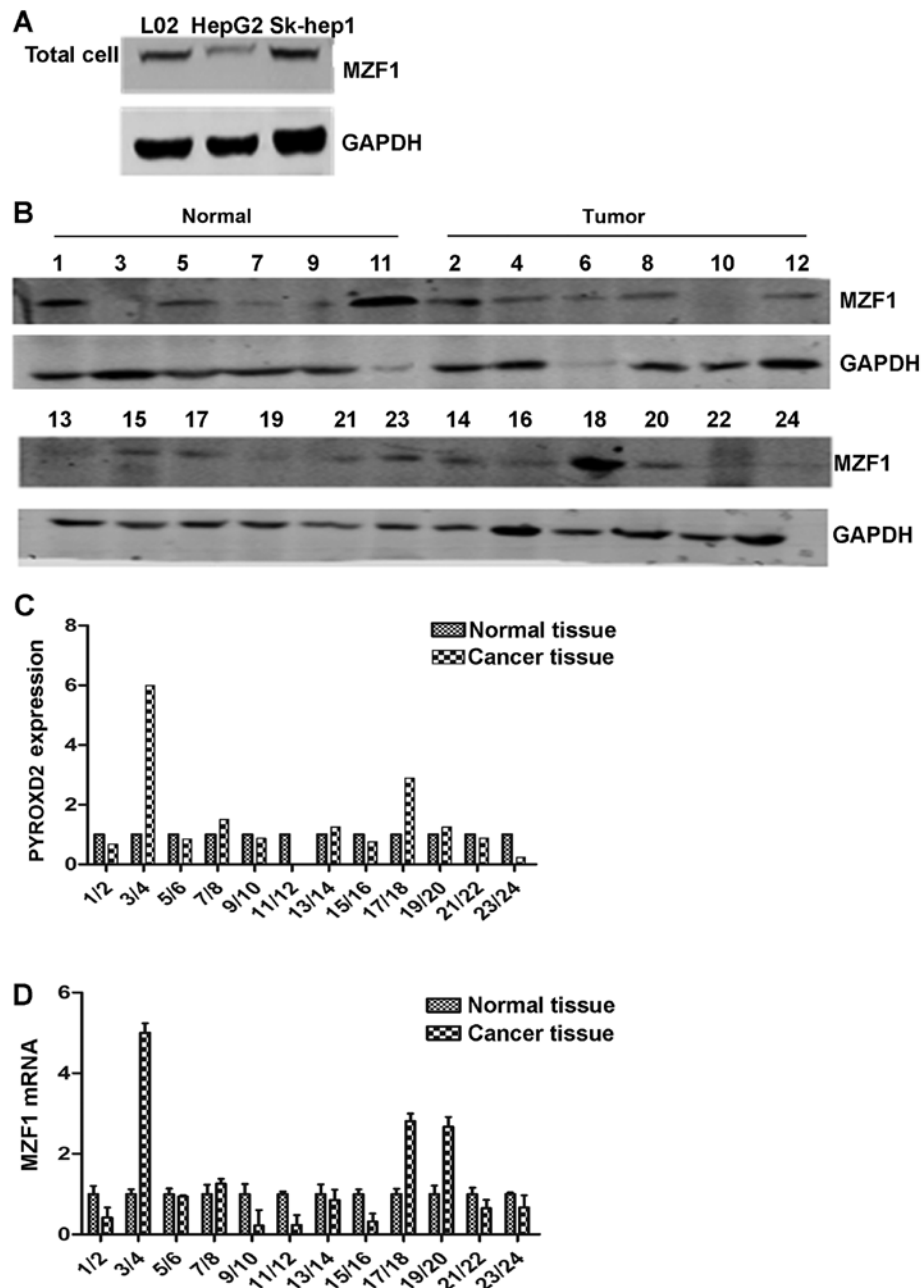


Figure 2. Relative levels of the MZF1 protein and mRNA expression in samples of normal tissue, cancer tissue and 3 different cell lines. The samples are numbered as follows: a number x represents the normal tissue and a number (x+1) represents the tumor tissue obtained from the same patient. (A) The relative levels of MZF1 protein expression in L02, HepG2 and Sk-hep1 cells as determined with western blotting. (B) The relative levels of MZF1 protein expression in samples of hepatic carcinoma tissue and the corresponding samples of normal liver tissue as determined with western blotting. (C) The relative levels of PYROXD2 protein expression in samples of cancer tissue and normal tissue. The results were calculated based on the ratio of the gray values for the western blots shown in Fig. 2B. (D) The expression of MZF1 mRNA in samples of hepatic carcinoma tissue and the corresponding samples of normal liver tissue as determined with RT-PCR. MZF1, myeloid zinc finger 1.

the serial deletion mutants were constructed using strategies and primers (relevant information provided upon request). A nucleotide deletion in the promoter regulatory region could potentially affect the binding of transcription factors and alter the transcription rate of a gene. Promoter activity was determined with a luciferase assay system and the results were normalized by h-galactosidase activity. The absorbance value of a blank control sample was subtracted from each assay result. After the activity of the (-1998/-1)-PYROXD2 promoter was defined as 1, the relative activities of other serial mutant promoters were expressed as the ratio $I^{\text{mutant}}/I^{\text{fulllength}}$, in

which I is the intensity of an absorbance value, the superscript 'mutant' signifies a serial mutant promoter and the superscript 'full length' signifies the (-1998/-1)-PYROXD2 promoter.

To analyze various characteristics of the PYROXD2 promoter and identify the region most commonly activated, we constructed a series of 200 bp fragment deletions which ranged from base -1998 to base -1. As revealed in Fig. 3A, deletion of bases -1998/-1801 produced only a slight decrease in promoter activity. The most remarkable change in promoter activity occurred when the deletions were produced between the -1600/-1201 and -800/-401 regions,

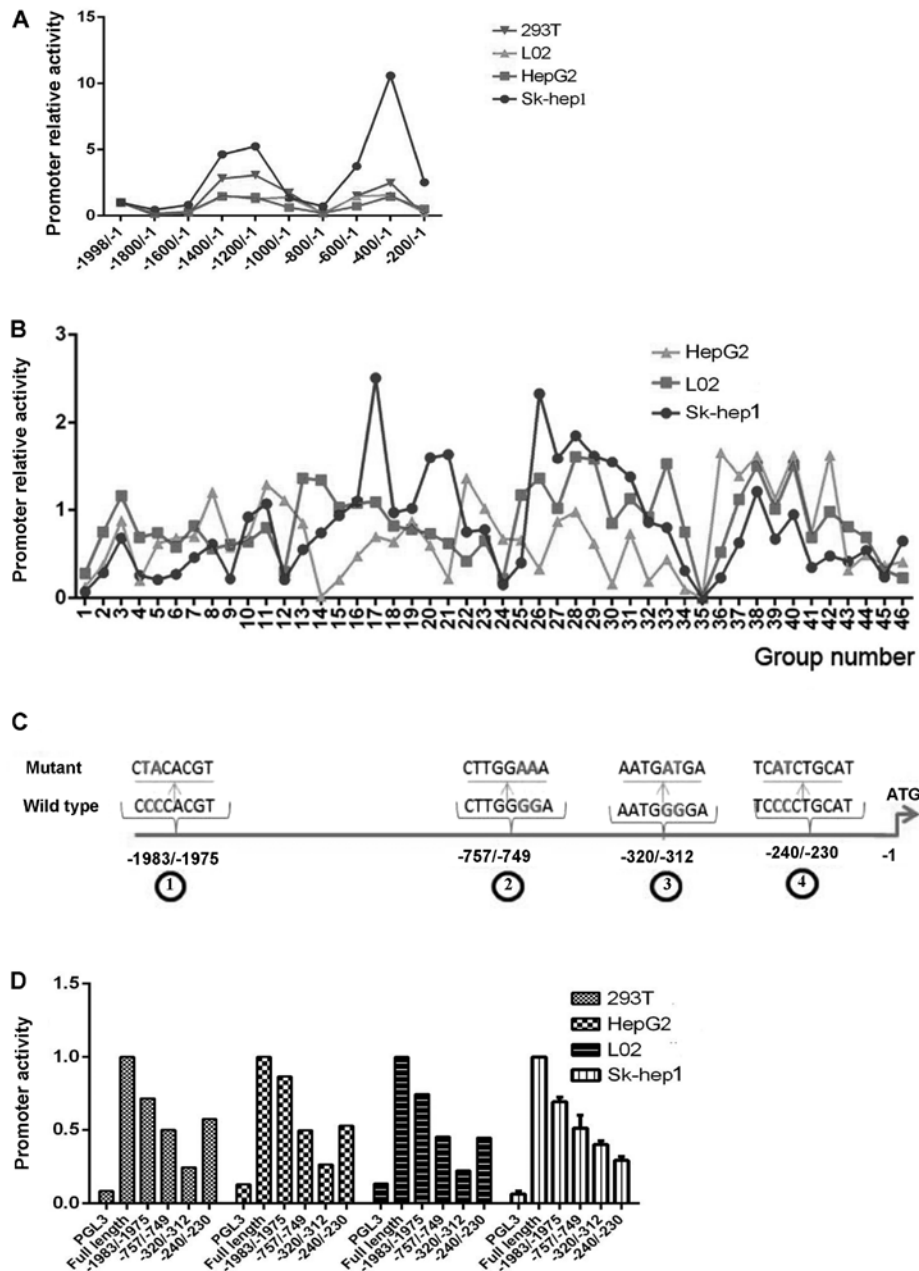


Figure 3. Mutant analysis of the *PYROXD2* promoter. The activities of the various mutant promoters were assessed using the dual-luciferase reporter gene assay. The relative promoter activity of each mutant was calculated using the formula: $A_{mutant} = I_{mutant}/I_{fulllength}$, in which I indicates the intensity of the absorbance value, the superscript 'mutant' signifies a serial mutant promoter and the superscript 'full length' signifies the (-1998/-1)-*PYROXD2* promoter. (A) Promoter activity of the (-1998/-1)-*PYROXD2* promoter with serial 200 bp fragment deletions. (B) Site-directed mutagenesis promoter activities, based on the (-1998/-1)-*PYROXD2* promoter sequence. All potential transcription factors and their corresponding binding sites on the *PYROXD2* promoter were predicated through the TFSEARCH software online (<http://www.cbrc.jp/research/db/TFSEARCH.html>). Site-directed mutagenesis was performed based on (-1998/-1)-*PYROXD2* promoter vector. Mutant sequences with corresponding primers are available upon request. The vectors which were cloned with the mutant or wild-type promoter of *PYROXD2* were transfected into the Sk-hep1, HepG2 and L02 cell lines, respectively. Relative promoter activities were detected at 24 h after transfection using a Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. Each group demonstrated one or more mutants of the transcription factor binding site in *PYROXD2* promoter which is listed in Table I. (C) The mutation strategies were used for four putative MZF1 binding sites in the (-1998/-1)-*PYROXD2* promoter sequence. (D) The relative transcription activities of four promoters with mutated MZF1 binding sites.

in which case the promoter exhibited increased activity. In contrast, the (-1998/-1)-*PYROXD2* promoter displayed decreased activity when the deletions were produced in both the -1200/-801 and -400/-201 regions. It should be emphasized that the *PYROXD2* promoter lost all its activity when the deletions were produced in either the -400/-201 or -200/-1 regions. Our results indicated that *cis*-elements located in the -400/-1 region constitute the core promoter

responsible for basal transcription of the *PYROXD2* gene. Next, we used site-directed mutagenesis to generate a series of mutant reporters based on the (-1998/-1)-*PYROXD2* promoter vector, with the purpose of identifying critical *cis*-elements in the promoter region. As revealed in Fig. 3B and Table I, the different mutants produced different effects on the promoter activity in the three cell lines. Notably, insertion of a mutation into the -320/-312 region

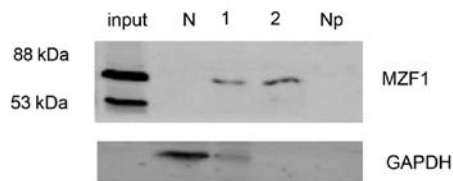


Figure 4. DNA binding assay between MZF1 and the putative promoter core binding DNA sequence (AGGGGA, -320/-312) was performed. The putative positive DNA duplexes or the control DNA duplexes were coupled to magnetic beads and incubated with 100 μ g nuclear extract of L02 cells. The remaining protein on beads after the elution were detected by western blotting. The sample input was nuclear extract proteins. Sample N was the remaining protein of the putative positive DNA duplexes incubated with total cytoplasmic proteins. Samples 1 and 2, two duplicate samples, were the remaining protein of the putative positive DNA duplexes incubated with nuclear extract. Sample Np was prepared in the same way as samples 1 and 2 except for the replacement of the putative positive DNA duplexes by the control DNA duplexes.

produced a complete loss of promoter activity in all three cell lines (Fig. 3B and Table I). These results indicate that the putative MZF1 binding site (TGGGGA) located in the -320/-312 region of the *PYROXD2* promoter may be crucial for the transcription activity of the promoter.

A preliminary sequence analysis of the -1998/-1 domain revealed the presence of four *cis*-elements that may bind with MZF1. Among them, the -1983/-1975 and -320/-312 regions were non-overlapping, whereas the -757/-749 and -240/-230 regions overlapped with other transcription factors. To assess how the four MZF1 binding *cis*-elements of the *PYROXD2* promoter may affect *PYROXD2* gene transcription, we constructed mutant promoters in which only two nucleotides were substituted in the core MZF1 binding sequence. As revealed in Fig. 3C, the substitutions did not affect the binding ability of other overlapping transcriptional effectors. When compared with the effects produced by other mutations, a mutation in the -320/-312 region appeared to produce the largest decrease in luciferase activity (Fig. 3D).

MZF1 is a key trans-acting factor controlling PYROXD2 expression. A separate set of experiments was conducted to elucidate how the -320/-312 region of the *PYROXD2* promoter interacts with MZF1 and to determine whether endogenous MZF1 binds to the -320/-312 region of the *PYROXD2* promoter. To accomplish these goals, we performed DNA binding assays using primers that spanned the putative MZF1-binding site of the region (Fig. 4). The specific sequence within the -320/-312 region was precipitated from cell lysates by the addition of the anti-MZF1 antibody but not by the addition of the control IgG. Our data markedly indicated that MZF1 binds to the TGGGGA domain in the proximal promoter of *PYROXD2*.

To further ascertain the involvement of MZF1 in *PYROXD2* transcription, we assessed the levels of endogenous *PYROXD2* expression in the HepG2 and L02 cells after transfecting them with MZF1 expressing plasmids, or by silencing their endogenous MZF1 expression with siRNA (Fig. 5). Increased endogenous *PYROXD2* expression was observed in MZF1 overexpressing the HepG2 and L02 cells, and decreased endogenous *PYROXD2* expression was observed in the L02 cells transfected with the MZF1 siRNA (Fig. 5). These findings indicate that MZF1 functions as a key regulator of the *PYROXD2* transcription.

Table I. TFs in each group whose binding site was mutated in Fig. 3B are listed.

Group no.	TFs	Group no.	TFs	Group no.	TFs
1	MZF1	14	GATA-2	29	MZF1
	GATA-1		MZF1		STRE
2	Dfd		STRE	30	SRY
	AML-1a		deltaE		CdxA
	RORalp	15	Skn-1		Lyf-1
	CdxA		CRE-BP	31	AML-1a
	Sox-5	16	cap	32	CRE-BP
3	HSF		C/EBPb		GATA-1
	HSF	17	d1		CREB
	HSF		CdxA	33	GATA-1
4	cap	18	CF1		CRE-BP
	GATA-X		ADR1		GATA-2
	NIT2		SRY	34	NF-E2
5	Sp1	19	GATA-1		p300
	ADR1		NIT2	35	Nkx-2
6	ADR1		BR-C Z		GATA-2
	cap		SRY	36	AP-1
7	GATA-3		cap	37	MZF1
8	MATa1	20	cap	38	E2F
9	ADR1		c-Ets		C/EBPb
	HSF		AP-1		Ik-2
	c-Ets-		GCM	39	deltaE
	HSF	21	c-Myb		TATA
10	p300	22	NF-Y		MZF1
	cap		CREB	40	AML-1a
11	GATA-2		GATA-2	41	GATA-2
	AhR/Ar		GATA-2		GATA-1
	Ttk 69	23	Ttk 69		GATA-3
12	cap		dl		CdxA
	E2F		Hb		c-Ets-
	C/EBPa		Dfd		MZF1
	cap	24	STRE	42	CdxA
13	E2F		Nkx-2	43	MZF1
	ADR1	25	ADR1	44	USF
	SP1		CdxA	45	GATA-1
	ADR1	26	RORalp	46	p300
	MZF1		MZF1		
	HSF	27	NF-1		
14	USF		E2F		
	MZF1	28	c-Myb		
	HSF2		BR-C Z		
	GATA-1		Dfd		

TFs, transcription factors.

Discussion

The tumor-suppressive activity of *PYROXD2* and its different expression levels in normal tissues and several corresponding

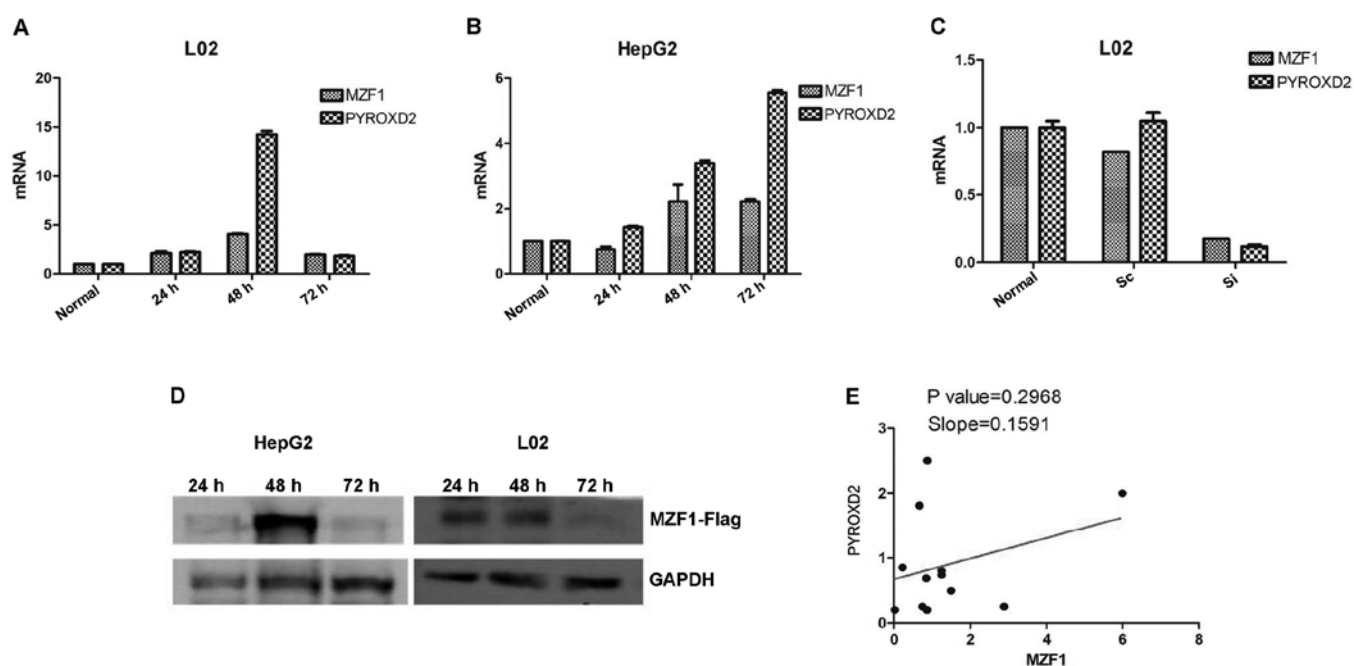


Figure 5. Effects of MZF1 on PYROXD2 protein and mRNA expression. (A and B) Overexpression of MZF1 was associated with enhanced PYROXD2 transcription in the L02 and HepG2 cells. (C) Silencing of MZF1 inhibited PYROXD2 transcription in L02 cells. (D) Identification of the MZF1 protein in MZF1-transfected L02 and HepG2 cells as determined by western blotting. (E) A correlation analysis between the MZF1 and expression of PYROXD2 in tissue samples was accomplished using the data from Figs. 1D and 2C through Prism 5.

tumor tissues have been reported (1,2). In the present study, we collected specimens of cancerous liver tissue and adjacent normal liver tissue from 12 patients, and quantitatively assessed the endogenous levels of MZF1 and PYROXD2 expression in those tissues by western blotting and RT-PCR. We found that the PYROXD2 protein was highly expressed in normal liver tissue and in a normal human liver cell line; however, its expression was either absent or decreased in a large proportion of HCC tissue and hepatocarcinoma cell lines, indicating that decreasing PYROXD2 expression may be involved in hepatocarcinogenesis.

The molecular mechanism which regulates *PYROXD2* transcription was not elucidated in previous studies. We sought to examine various characteristics of the *PYROXD2* promoter and identify the most commonly activated *PYROXD2* promoter region in liver cells. To accomplish this goal, we constructed a series of luciferase reporter plasmids that contained 5' and 3'-deletions in the *PYROXD2* promoter, and then used them to perform luciferase-based reporter assays in HepG2 and Sk-hep1 liver cancer cell lines as well as in two normal control cell lines (L02 and 293T). The most remarkable change in the promoter activity occurred when the deletions were produced in both the -400/-199 and -200/-1 regions in which case, there was an almost complete loss of *PYROXD2* promoter activity. Our site-directed mutagenesis studies conducted with three different cell lines demonstrated that the putative MZF1 binding site (TGGGGA) located in the -320/-312 region of the *PYROXD2* promoter was largely responsible for the loss of *PYROXD2* promoter activity. The results of the DNA binding assays also indicated the occurrence of interactions between MZF1 and *cis*-elements located in the -317/-313 region of the *PYROXD2* promoter. Ectopic expression of

MZF1 induced an increased expression of PYROXD2; accordingly, silencing of MZF1 inhibited PYROXD2 expression in the same cell lines. These findings suggest that MZF1 is critical for the transcription activity of the *PYROXD2* promoter and functions as a *trans*-activator in regulating PYROXD2 expression. In addition these findings provided novel insights into the mechanism underlying the tumorigenic effect of PYROXD2.

Our results indicated that MZF1 was expressed at higher levels in samples of human cancer tissue than in samples of normal tissue, which were consistent with previous studies (5). Based on the previous cellular experiment results, we analyzed the activating function of MZF1 on the PYROXD2 promoter in tissues by comparing the expression level of MZF1 with the PYROXD2 expression level. Notably *MZF1* gene expression was not significantly correlated with PYROXD2 expression in samples of resected tumor tissues. The protein expression in human cells is regulated at several levels such as in transcription factors and mRNA stability. Promoter transcription activities were affected by the amount and type of the transcription factor family members and their function antagonism associations. Additively, the untranslated regions (UTR) and the AU-rich elements (ARE) render mRNA unstable in cells and tissues leading to a gradual decrease in protein production (17). Although the present study revealed that the MZF1 gene expression was not significantly correlated with the PYROXD2 expression in samples of resected tumor tissues, we speculated that the main mechanisms were relative to the diversity of the transcription factors involved in the regulation of the PYROXD2 promoter activity and MZF1 is only one of these factors. It should be pointed out that MZF1 is an activating factor of the PYROXD2 promoter, and decreased PYROXD2 expression may contribute to

cancer progression. Therefore, it is important to understand the underlying mechanism that regulates the expression level of PYROXD2 by other transcription factor members and their interaction relationships.

Acknowledgements

The present study was supported by the Natural Science Foundation of Zhejiang province (LQ12C03003), the Natural Science Foundation of China (project nos. 31260621 and 31160240) and the Hangzhou Normal University supporting project (no. PE13002004042).

References

1. Zhang JL, Zhao WG, Wu KL, Wang K, Zhang X, Gu CF, Li Y, Zhu Y and Wu JG: Human hepatitis B virus X protein promotes cell proliferation and inhibits cell apoptosis through interacting with a serine protease Hepsin. *Arch Virol* 150: 721-741, 2005.
2. Huang J, Wu K, Zhang J, Si W, Zhu Y and Wu J: Putative tumor suppressor YueF affects the functions of hepatitis B virus X protein in hepatoma cell apoptosis and p53 expression. *Biotechnol Lett* 30: 235-242, 2008.
3. Huang HW, Peng JP and Zhang J: YueF overexpression inhibits cell proliferation partly through p21 upregulation in renal cell carcinoma. *Int J Mol Sci* 12: 2477-2487, 2011.
4. Deng Y, Wang J, Wang G, Jin Y, Luo X, Xia X, Gong J and Hu J: p55PIK transcriptionally activated by MZF1 promotes colorectal cancer cell proliferation. *Biomed Res Int* 2013: 868131, 2013.
5. Eguchi T, Prince T, Wegiel B and Calderwood SK: Role and regulation of myeloid zinc finger protein 1 in cancer. *J Cell Biochem* 116: 2146-2154, 2015.
6. Hsieh YH, Wu TT, Huang CY, Hsieh YS and Liu JY: Suppression of tumorigenicity of human hepatocellular carcinoma cells by antisense oligonucleotide MZF-1. *Chin J Physiol* 50: 9-15, 2007.
7. Asiedu MK, Beauchamp-Perez FD, Ingle JN, Behrens MD, Radisky DC and Knutson KL: AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells. *Oncogene* 33: 1316-1324, 2014.
8. Mudduluru G, Vajkoczy P and Allgayer H: Myeloid zinc finger 1 induces migration, invasion, and in vivo metastasis through Axl gene expression in solid cancer. *Mol Cancer Res* 8: 159-169, 2010.
9. Jensen K, Shiels C and Freemont PS: PML protein isoforms and the RBCC/TRIM motif. *Oncogene* 20: 7223-7233, 2001.
10. Dellaire G and Bazett-Jones DP: PML nuclear bodies: Dynamic sensors of DNA damage and cellular stress. *BioEssays* 26: 963-977, 2004.
11. Van Damme E, Laukens K, Dang TH and Van Ostade X: A manually curated network of the PML nuclear body interactome reveals an important role for PML-NBs in SUMOylation dynamics. *Int J Biol Sci* 6: 51-67, 2010.
12. Edelstein LC and Collins T: The SCAN domain family of zinc finger transcription factors. *Gene* 359: 1-17, 2005.
13. Driver J, Weber CE, Callaci JJ, Kothari AN, Zapf MA, Roper PM, Borys D, Franzen CA, Gupta GN, Wai PY, *et al*: Alcohol inhibits osteopontin-dependent transforming growth factor- β 1 expression in human mesenchymal stem cells. *J Biol Chem* 290: 9959-9973, 2015.
14. Massagué J: A very private TGF- β receptor embrace. *Mol Cell* 29: 149-150, 2008.
15. Stielow B, Krüger I, Diezko R, Finkernagel F, Gillemans N, Kong-a-San J, Philipsen S and Suske G: Epigenetic silencing of spermatocyte-specific and neuronal genes by SUMO modification of the transcription factor Sp3. *PLoS Genet* 6: e1001203, 2010.
16. Plotz G, Raedle J, Brieger A, Trojan J and Zeuzem S: hMutSalpha forms an ATP-dependent complex with hMutLalpha and hMutLbeta on DNA. *Nucleic Acids Res* 30: 711-718, 2002.
17. Sahoo A and Im SH: Molecular mechanisms governing IL-24 gene expression. *Immune Netw* 12: 1-7, 2012.