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Promoter demethylation mediates the expression of *ZNF645*, a novel cancer/testis gene

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Cancer/testis (CT) antigens exhibit highly tissue-restricted expression and are considered promising targets for cancer vaccines. Here we identified a novel CT gene *ZNF645* which restrictively expresses in normal human testes and lung cancer patients (68.3%). To investigate the promoter methylation status of *ZNF645*, we carried out bisulfite genomic sequencing and found that the CpG island in its promoter was heavily methylated in normal lung tissues without the expression of *ZNF645*, whereas there was high demethylation in normal human testes and lung carcinoma tissues with its expression. Also *ZNF645* could be remarkably activated in A549 and HEK293T cells treated by DNA demethylation agent 5'-aza-2'-deoxycytidine. And the dual luciferase assay revealed that the promoter activity of the *ZNF645* was inhibited by methylation of the CpG island region. Therefore, we proposed that *ZNF645* is a CT gene and activated in human testis and lung cancers by demethylation of its promoter region. [BMB reports 2010; 43(6): 400-406]

INTRODUCTION

Cancer/testis (CT) antigens are a group of tumor antigens with normal expression restricted to adult male germ cells, but aberrantly expressed in about 40% types of human cancer including lung cancer, and are ideal targets for cancer vaccine (1, 2). Since the first CT antigen MAGE-1 identified in 1991, more than 130 CT antigens which belong to 83 gene families have been identified. Among them 83 genes are located on X chromosome (3). It is reported that, about 10% of the genes on the X chromosome are CT antigen genes (4), and these CT-X genes are notably more testis-restrictively than those on other chromosomes (5). However, CT proteins expression shows heterogeneous in many cancers including lung carcinoma, therefore more CT antigens need to be identified to develop the polyvalent vaccines to avoid immune escape of tumors (1, 6).

DNA methylation is considered necessary in suppression of gene transcription and the control of tissue-specific gene activation (7, 8). By changing chromosome structure and interfering transcription-factor binding, methylation of CpG islands (CGI) within gene promoter results in gene silencing (9). Epigenetic alterations such as histone deacetylation and DNA methylation are known to play an important role in CT gene expression (10). For examples, MAGE-A1 transcription is correlated with demethylation of CpG sites in its 5' region (11) and the expression of SSX and NY-ESO-1 can be restored in certain cancer cell lines by the DNA hypomethylating agent 5'-Aza-2'-Deoxycytidine (5'-aza-CdR) (12, 13).

In searching for testis-specific genes, we found that one gene named *ZNF645* located at Xp22.11 from NCBI Unigene database (FLJ25735 fis, clone TST05676; Genbank accession No.: AK098601). In this report, we identified that *ZNF645* was specifically expressed in normal human testis and some lung cancer tissues. So it may be a new member of CT gene families. Furthermore, we discovered that the expression of the *ZNF645* was primarily regulated by methylation of CpG sites in its promoter region.

RESULTS

ZNF645 gene is restrictively present in human testis among normal somatic tissues and frequently present in some lung cancer tissues

One of the criteria for identifying CT antigen genes is their specific expression in tumors, but not in normal tissues except testis. Here the expression of *ZNF645* mRNA was first examined by PCR in 16 different normal human tissues including heart, liver, whole brain, spleen, lung, kidney, pancreas, prostate, ovary, small intestine, colon, peripheral blood, placenta, thymus skeletal muscle and testis, and the results showed that *ZNF645* mRNA was only found in normal human testis (Fig. 1A). Meanwhile the RT-PCR analysis of *ZNF645* mRNA expression in paired lung cancer and non-cancerous tissues showed that it was also present in some lung cancer tissues (Fig. 1A). Furthermore the western blot with whole protein ex-

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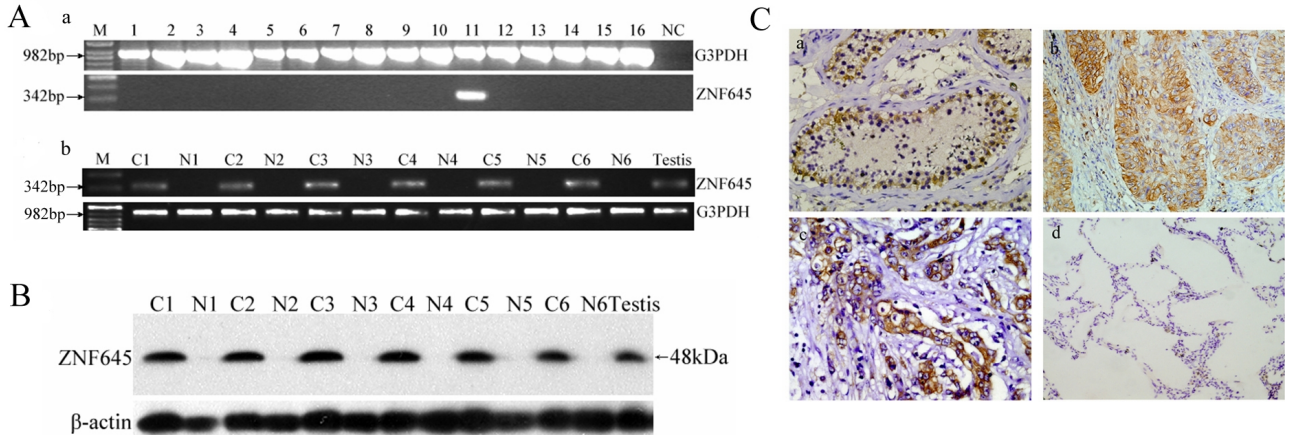

Fig. 1. *ZNF645* mRNA and protein expression in human normal tissues and lung tumors. (A) a: PCR analysis of *ZNF645* mRNA among 16 human normal tissues and cells. Lanes: M, 100 bp DNA ladder; 1, brain; 2, heart; 3, placenta; 4, lung; 5, spleen; 6, kidney; 7, ovary; 8, liver; 9, pancreas; 10, leukocytes; 11, testis; 12, skeletal muscle; 13, small intestine; 14, thymus; 16, prostate; NC, negative control. b: RT-PCR analysis of *ZNF645* mRNA in six paired lung cancer and adjacent non-cancerous tissues. (C) cancer tissues; N, paired non-cancerous tissues. The integrity and quantity of RT-PCR was evaluated by amplification of G3PDH. (B) ZNF645 protein expression analysis in paired lung cancer and adjacent noncancerous tissues by western blot. The molecule weight of ZNF645 protein is 49 kDa. The human testis total protein was used as a positive control and β-actin as an internal reference. The protein of ZNF645 showed the same bands in testis and lung cancer with positive expression. (C) Immunohistochemical analysis of ZNF645 protein expression in adult testis [a] and lung cancers [b, squamous cell carcinoma; c, adenocarcinoma]; Original magnification ×200 and [d] normal lung tissue as the negative control. Original magnification ×100.

Table 1. Summary of ZNF645 mRNA expression in lung carcinoma*

		No. positive†	No. negative‡
Gender	Male	51	30
	Female	31	8
Clinical stage	Stage I and II	69	28
	Stage III and IV	13	10
Histologic type	Adenocarcinoma	34	51
	Squamous cell carcinoma	42	54
	Adenosquamous carcinoma	0	1
	Large cell lung carcinoma	0	1
	Small cell lung carcinoma	0	3
	Other types of lung cancer	1	3
Total		82 (68.3%)	38 (31.7%)

*There was not significant difference for the expression of ZNF645 between genders, age, clinical stages or tumor histologic types in lung cancer patients. †The number of cases with ZNF645 protein expression, ‡The number of cases without ZNF645 protein expression. The mean age of † and ‡is 57.7 and 53.2 respectively

tracted from human testis and lung cancer tissues confirmed that *ZNF645* protein was detected in testis and most of lung cancer tissues (Fig. 1B) as a specific band of 49 kDa. Through screening the *ZNF645* protein expression in 120 lung cancer patients, it was observed in 82 of the cases (68.3%, Table 1). Additionally, there was no correlation between *ZNF645* expression and sex, age, clinical stages or tumor histologic types of the lung cancers (Table 1).

Immunohistochemistry (IHC) was carried out to analyze the location of the *ZNF645* protein by using sections of formalin-fixed, paraffin-embedded sections of lung cancer tissues and paired adjacent non-cancerous tissues. The results showed that *ZNF645* protein was predominantly present in the cytoplasm of lung cancer cells (Fig. 1C).

These results indicated that *ZNF645* may be a novel member of the cancer/testis genes.

Methylation of *ZNF645* gene promoter is correlated with its expression in cell lines and primary lung tumors

To understand the mechanism governing the specificity of the *ZNF645* gene expression, we investigated the methylation situation of its CpG sites. Firstly we analyzed 5' upstream region of *ZNF645* gene by using Methyl Primer Express (Applied Biosystems, USA) and found a typical CpG island (CGI) including 13 CpG sites from −300 bp to −50 bp (length > 300 bp, C+Gs/total bases > 50%, CpG observed/CpG expected > 0.6), which could be potential targets for DNA methylation (Fig. 2A) (14, 15). Then we performed bisulfite genomic sequencing to examine the methylation status of selected *ZNF645* CGI in genomic DNA extracted from normal human testis and lung cancer tumors respectively. It was found that the 13 CpGs are highly unmethylated in the normal human testis tissue (Fig. 2B) and in two lung neoplasms tissues (Fig. 2C). In contrast, there was strong methylation in the 13 CpGs in two paired non-cancerous tissue samples. Hence, these methylation data revealed that the expression of *ZNF645* was mediated by the methylation status of these CpGs in the 5' upstream region.

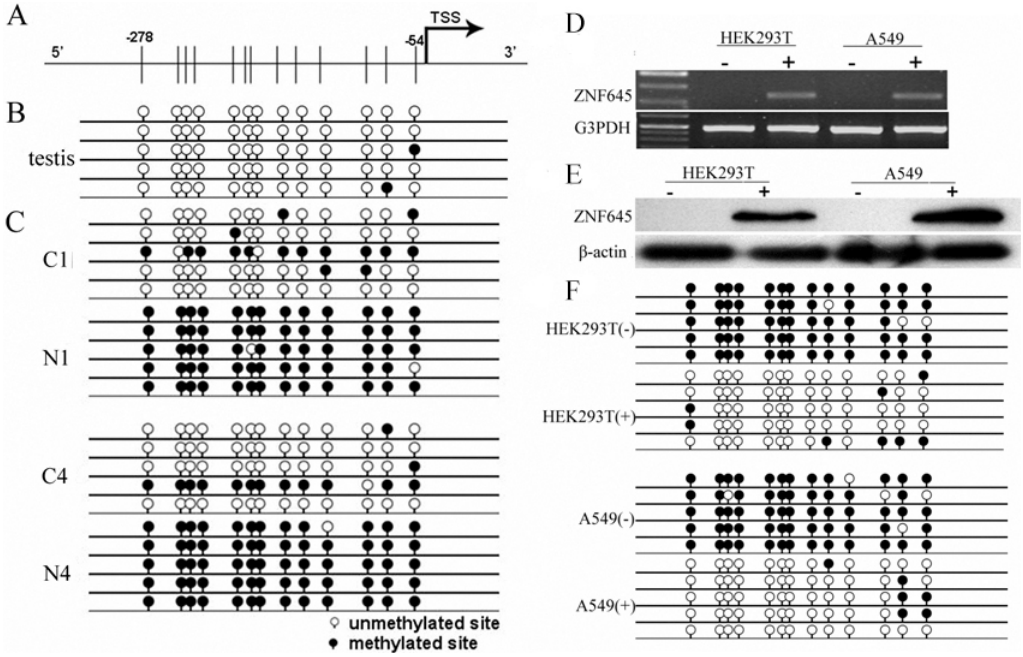

Fig. 2. Methylation status of the CpG island in 5'-region of *ZNF645*. (A) The location of 13 CpG sites in 5'-region of *ZNF645*. Each vertical bar indicates a CpG site. TSS means the transcription start site of *ZNF645*. (B) Bisulfite genomic sequencing of the *ZNF645* CpG island in normal testis. (C) Bisulfite genomic sequencing of the *ZNF645* CpG island in two lung cancer tissues from previously characterized patients. Strong demethylation was observed in testis and two cancer tissues while heavy methylation was shown in paired non-cancerous tissues. Five clones of each sample were sequenced. Solid circle: methylated CpG site; hollowed circle: unmethylated CpG site. (D) Expression of *ZNF645* mRNA in HEK293T and A549 cells treated with 5'-aza-CdR. The mRNA of *ZNF645* was detected in treated (+) cells and not in untreated (−) cells. (E) The protein expression of ZNF645 in HEK293T and A549 cells with 5'-aza-CdR treatment. The untreated (−) cell lysates showed absent protein expression in both two cell lines and the treated (+) cells showed the activation of ZNF645 by using western blot. (F) The comparison of CpG methylation status in treated and untreated HEK293T and A549 cells. Bisulfite genomic sequencing displayed great demethylation in treated cells.

To further confirm that hypermethylation is responsible for the silencing of the *ZNF645*, a DNA methyl-transferase inhibitor 5'-aza-CdR was used to treat human embryo kidney cell line 293T and lung cancer cell line A549 cells in which *ZNF645* expression is negative. We observed that the expression of *ZNF645* was restored on mRNA and protein levels in both cell lines after treatment (Fig. 2D, E). Then bisulfite sequencing was also used to analyze the DNA methylation status in both cell lines with and without 5'-aza-CdR treatment, and the results showed that the above 13 CpGs was demethylated in those treated cells (Fig. 2F). All of these results indicated that the expression of *ZNF645* in the two cell lines was regulated by the methylation of CGI in the 5' upstream region.

The methylation status of 5' upstream CGI region of *ZNF645* mediate the expression of reporter gene luciferase

To examine the effect of methylation status in 5' upstream CGI region of *ZNF645* gene in the regulation of gene expression *in vitro*, we cloned several 5' upstream sequences of *ZNF645* and subcloned them into the pGL3 basic reporter vector (Fig. 3A). Then by transiently transfecting these constructs into HepG2 cells, we observed that the largest construct P1 (−1,140/+115) displayed the highest promoter activity, the transcriptional activity of P4 (−164/+115) was significantly decreased, and P5 (−87/+115) even had no transcriptional activity (Fig. 3A). These results suggested that the basal promoter of *ZNF645* gene was located in the region from −314nt to −87nt. It was especially worth noting that the CGI (−290 to −40) of *ZNF645* gene was also mainly located in the region of putative basal promoter.

Then we performed *in vitro* methylation analysis to confirm whether the promoter activities of *ZNF645* gene were inhibited by CGI methylation. The HepG2 cells were transiently transfected with the P1 and P3 promoter constructs methylated with SssI (CpG) methylase and the untreated P1 and P3 mock construct vectors, and the following luciferase activities were measured respectively. As shown in Fig. 3B, the luciferase activities of the methylated *ZNF645* promoter constructs were about eight-fold lower than that of mock constructs. And the similar results were also found when transfecting A549 cells (data not shown). These results further confirmed the hypoth-

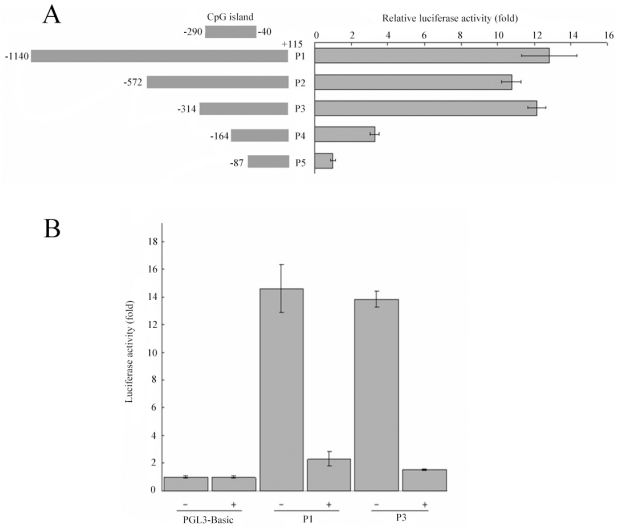

Fig. 3. *In vitro* experiment analysis of the methylation status in 5' upstream CGI region of *ZNF645* regulating the expression of reporter gene luciferase. (A) Left panel, constructs of functional deletion of the *ZNF645* gene 5'-region. Right panel, the luciferase activity of each deletion construct of *ZNF645* gene5'-region. All the luciferase activity was normalized by *Renilla* luciferase activity. The P1, P2 and P3 showed similar activity and all of them contained the whole CpG island. The activity of P4 construct decreased rapidly compared with P3. The activity of P5 was set as 1-fold. (B) The luciferase activity of methylated P1 and P3 promoter constructs following transient transfection into HepG2 cells. The activity of constructs treated with SssI methylase (+) decreased remarkably compared with those untreated (−).

esis that the promoter activity of *ZNF645* could be repressed by DNA methylation and the expression of *ZNF645* was mediated by CGI methylation.

DISCUSSION

Characterization of novel CT antigens is considered promising for cancer immunotherapy. In this study, we identified a novel potential human CT gene *ZNF645* which is abundantly expressed in most human lung cancers (68.3%). *ZNF645* contains a ring finger C3HC4 domain, a C2H2 domain and a praline-rich region, and is a new member of zinc finger protein family. It is known that there are several zinc finger protein members, such as ZNF165, BORIS, SSX, *et al*. (16-18), which play an important role in carcinomagenesis as CT antigen. We will carry out further investigation on the biological function of *ZNF645* in tumor and testis.

Genome-wide DNA demethylation is often observed in tumor and male germ cells (19-21), and known to be an important mechanism involved in human carcinogenesis and germ-cell development (22-24). Expression of CT-X genes always related with globe DNA hypomethylation during tumorigenesis (25). In this study, we examined the correlation between DNA methylation and the expression of *ZNF645*. It was observed that there existed DNA hypomethylation of the 5' flanking CGI region of *ZNF645* in testis and some lung cancer cases whereas heavy DNA methylation was observed in the same region of the corresponding adjacent non-cancer tissue without *ZNF645* expression. Growing evidence suggested that many transcription factors including SP1, CRE, and et al have methylation sensitivity (26). By using TRANSFAC 4.0 program (http://www.gene-regulation.com) we found that there were several potential binding sites for transcription factors such as GATA-1, C/EBP, SP1 and OCT1 in this CGI region of *ZNF645* gene (Data no shown). Hence, the hypermethylation of these CGI sequences may interfere with the activity of these proteins binding in this region, leading to the silence of *ZNF645* gene expression.

The demethylation agent 5'-aza-CdR could restore the expression of *ZNF645* in HEK293T and A549 cells. Similar results were also described in other CT-X antigens such as MAGE-1 and GAGE (25, 27). One of the possible mechanisms is that 5'-aza-CdR can inhibit the activity of mammalian DNA methyltransferases (DNMT) (28). Recent studies showed that the inactivation of two DNA methyltransferases, DNMT1 and DNMT3b, could also induce the abundant expression of CT-X antigen genes in human cancer cells (29). Hence we propose that the reversible DNA methylation status is necessary to regulate the expression of *ZNF645* in cancer cells as well as in normal cells.

In summary, present study first demonstrates that *ZNF645* is a novel CT gene and its promoter demethylation is correlated with its expression. Further functional study on the gene would provide evidences for its role in carcinogenesis and male gametogenesis, as well as a novel target for immunotherapeutic vaccination and a biomarker for lung cancer.

MATERIALS AND METHODS

Human specimens

Normal human testes were obtained from a body donor who had died in a car accident. Lung cancer and paired adjacent noncancerous tissue specimens were obtained from West China Hospital, Sichuan University, P.R. China. All tumor and paired noncancerous tissue samples were pathologically confirmed, and informed consent was signed by each donor. This study was approved by the Ethics Committee of West China Hospital, Sichuan University.

RNA isolation and reverse transcription PCR

Normal tissue cDNA panels (multiple tissue cDNA panels I and II) composed of human brain, colon, heart, kidney, leukocytes, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, thymus, and testis were purchased from Clontech (Palo Alto, USA). Total RNA was isolated from cell lines and tissues using Trizol reagent (Invitrogen, CA) according to the manufacturer's protocols and 2 μg

RNA was reverse transcribed into single-strand cDNA in 20 μl of reaction buffer using Molony murine leukemia virus reverse transcriptase (Promega, USA) and oligo (dT)15 (Promega, USA) as a primer. Then RT-PCR was performed as follows: 94°C, 15 s; 60°C, 30 s; and 72°C, 30 s; 30 cycles, followed by 5 min at 72°C. Visualization of target bands on a 1.2% agarose gel with ethidium bromide staining was performed to determine the expression of *ZNF645* mRNA in different types of tissues. The sequences of paired primers for the amplification of *ZNF645* gene are: forward 5'-GTATGTCCGCGCTGTCGTTAT-3'; reverse 5'-TGTGGCTGATTATGTTGCTCTTGT-3'. The integrity and quantity of the cDNA were evaluated by amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

Western blot

Total protein extracted from tissues and cells was separated in 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking with phosphate-buffered saline (PBS) solution containing 10% dry milk, the membranes were sequentially incubated with anti-ZNF645 rabbit polyclonal antibody (Aviva systems biology, USA) at a dilution of 1 : 1,000 overnight, then, incubated with horse radish peroxidase (HRP) conjugated rabbit anti-human IgG for 1 h, and immunoreactive bands were identified in X-ray films (Kodak, Japan) using chemiluminescent HRP substrate kit (Millipore, USA) with the β-actin as an internal reference.

Immunohistochemistry (IHC)

For detection of ZNF645 protein, 5 μm sections from formalin-fixed, paraffin-embedded tissues were used. After deparaffinized, rehydrated and treated in H_2O_2 to quench the endogenous peroxidase, the sections were placed in 10 mM citrate buffer (pH 6.0) for antigen retrieval by microwave boiling. After 30 min blocking with 10% normal goat serum to prevent nonspecific antibody binding, sections were incubated with anti-ZNF645 rabbit polyclonal antibody at a dilution of 1 : 700 overnight at 4°C. Following further washes in PBS, horseradish peroxidase-conjugated goat anti-rabbit IgG was applied and incubated for 1 h at 37°C. Sections were incubated with 3, 3'-diaminobenzidine (DAB) substrate for 2 min and counterstained with hematoxylin solution. Negative control sections were prepared by replacing the primary antibody with non-immune serum from goat.

5'-aza-2'-deoxycytidine (5'-aza-CdR) treatment

To determine the effect of demethylation on the expression of *ZNF645* gene, HEK293T and A549 cells were treated with 5'-aza-CdR as previously described (30). Cells were seeded at low density in six-well plates and treated with 10 μM of 5-aza-CdR for 60 h (Sigma, USA). At the end of treatment, DNA, RNA and total protein were harvested and analyzed as described above.

Bisulfite genomic sequencing

Genomic DNA was extracted from the tissues and cell lines by standard phenol-chloroform methods (31). Bisulfite treatment was carried out by using the DNA Methylation-Gold kit (Zymo Research, USA) according to the manufacturer's instructions. The PCR amplification was carried out with the following modifications: Semi-nested PCR amplified the CpG island region of the *ZNF645* gene promoter; the first PCR primer set was NESTF: 5′-TAGGGTTTGTTGGGAATTTATT-3′ and NESTR: 5′-CACATTCTTATTCACCAACAAAC-3′; then 1 μl of the PCR product was used for the second PCR amplification. The second PCR primer set was BSPF: 5′-ATTTTTTTATTAGAGTGGT GGGA-3′ and NESTR. PCR conditions were 5 minutes at 94°C; 34 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 20 seconds at 72°C; with a final 10-minute extension at 72°C. PCR products were separated on a 1.5% agarose gel, excised, and gel-purified. TA cloning was used to insert the PCR products into pCRII Vector (Invitrogen, US). The insert was confirmed by restriction digestion and sequenced with the T7/M13 universal primer. About five subclones were picked for each DNA sample.

Plasmid construction of *ZNF645* gene promoters

A pair of primers corresponding to the 5' upstream region of *ZNF645* gene was used to amplify the putative promoter regions from the genomic DNA. The forward primers are listed as follows: P1(−1,140/+115), 5'-GGGGTACCATGTGAGGA AATGTGGGCTAA-3'; P2(−572/+115), 5'-GGGGTACCACAT GCACACACACAGACAT-3'; P3(−314/+115), 5'-GGGGTACC GGACAGGGTAGGTGAGGAG-3'; P4(−164/+115), 5'-TCCCC CGGGTCCCTTGTGAGCCGAAAC-3'; P5(−87/+115), 5'-TCCC CCGGGTGGCCTTCACGTTAGCAG-3'. All forward primers carry KpnI restriction sites. And the reverse primer is 5'-CCGCTCGA GCCCAACGGTAACCAGGAAT-3', carrying a restriction site of XhoI. Five fragments were amplified and cut by double enzymes and inserted into pGL3-Basic vectors (Promega, USA) respectively. All above constructs were sequenced.

In vitro methylation

The *ZNF645* gene promoter constructs P1 (−1,140/+115) and P3 (−314/+115) were incubated overnight with three units of CpG methyltransferase (M.SssI, New England Biolabs, UK) in presence (methylated) or absence (mock-methylated) of 1mM S-adenosylmethionine, according to the manufacturer's recommendation. After the DNA isolation, 2 μg of the methylated or the mock-methylated reporter constructs were transiently transfected into HepG2 cells and the luciferase activities were measured by the dual-luciferase reporter assay system (Promega, USA).

Transfection and luciferase assay

HepG2 cells were seeded in 24-well plates, cultured in Dulbecco modified Eagle medium (DMEM) overnight and co-transfected with plasmid constructs and pRL-CMV Vector (Pro-

mega, USA) for 6 hr with Lipofectamine 2000 (Invitrogen, US). Cells were harvested at 48 h after transfection for luciferase activity assay according to the protocol for the dual-luciferase reporter assay system (Promega, USA). The luciferase activity was normalized according to the Renilla luciferase activity.

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