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Research Article

Depletion of *DNMT3A* Suppressed Cell Proliferation and Restored *PTEN* in Hepatocellular Carcinoma Cell

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Promoter hypermethylation mediated by DNA methyltransferases (DNMTs) is the main reason for epigenetic inactivation of tumor suppressor genes (TSGs). Previous studies showed that *DNMT1* and *DNMT3B* play an important role in CpG island methylation in tumorigenesis. Little is known about the role of *DNMT3A* in this process, especially in hepatocellular carcinoma (HCC). In the present study, increased *DNMT3A* expression in 3 out of 6 HCC cell lines and 16/25 (64%) HCC tissues implied that *DNMT3A* is involved in hepatocellular carcinogenesis. Depletion of *DNMT3A* in HCC cell line SMMC-7721 inhibited cell proliferation and decreased the colony formation (about 65%). Microarray data revealed that 153 genes were upregulated in DNMT3A knockdown cells and that almost 71% (109/153) of them contain CpG islands in their 5′ region. 13 of them including *PTEN*, a crucial tumor suppressor gene in HCC, are genes involved in cell cycle and cell proliferation. Demethylation of *PTEN* promoter was observed in *DNMT3A*-depleted cells implying that DNMT3A silenced *PTEN* via DNA methylation. These results provide insights into the mechanisms of *DNMT3A* to regulate TSGs by an epigenetic approach in HCC.

1. Introduction

Aberrant methylation of tumor suppressor genes (TSGs), which result in their silencing, is a common phenomenon in tumorigenesis [1, 2]. DNA methyltransferases (DNMTs) DNMT1, DNMT3A, and DNMT3B have been identified as functional DNA methylation enzymes in eukaryotic cells [3, 4]. DNMT3A and DNMT3B function as de novo methyltransferases that are responsible for de novo methylation, whereas DNMT1 functions as a maintenance enzyme for retaining methylation patterns. CpG islands residing within the promoter or the 5′ end of genes are normally unmethylated in normal cells, and abnormal methylation of these CpG islands can efficiently repress transcription of the associated gene [5]. Silencing of TSGs by epigenetic alterations at promoter CpG islands is a common event found in many types of human cancers, including hepatocellular carcinoma (HCC). Overexpression of DNMTs has been reported in numerous studies on various malignancies including prostate, colorectal, and breast tumors. These findings suggest that DNMTs may contribute to tumorigenesis through epigenetic silencing of genes [6–11]. The targeting of individual DNMTs to a gene is believed to play a critical role in determining its state of methylation. RNA interference-mediated knockdown or specific inhibition by antisense oligo-nucleotides of *DNMT1* resulted in the reduction of promoter methylation in TSGs, such as *RASSF1A*, *p16INK4a*, and *CDH1* [12, 13]. Satellite DNA is the target of methylation by *DNMT3B*. Interestingly, *DNMT3B* is also responsible for immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome [14, 15]. Moreover, recent studies have shown that *DNMT3B* expression contributes to a CpG island methylator phenotype and that it epigenetically silences *CXCL12* [16], *RECK* [17], and *RASSF1A* [18] through *DNMT3B*-mediated promoter methylation.

The function of *DNMT3A* is obscure and has not been associated with disease. *DNMT3A* is ubiquitously overexpressed in various types of tumors [19–22] implying

that it may be involved in and play an important role in tumorigenesis. However, very little is known about *DNMT3A*'s ability to regulate gene expression, especially in regulating tumor suppressor genes. Recent results showed that *DNMT3A* expression mediates the repression of *SFRP5* [23] and *plakoglobin* transcription [24] by direct hypermethylation of their promoter, indicating that *DNMT3A*'s methylation activities are gene specific. However, the target genes of DNMT3A remain unknown. In this study, we attempted to identify tumor-related genes that are potentially regulated by *DNMT3A* and further discuss the prospect of gene expression regulation by *DNMT3A*. In this report, we also investigated the role of *DNMT3A* in the aberrant methylation and inactivation of genes in human tumor cells as well as its role in the maintenance of the transformed phenotype.

2. Materials and Methods

2.1. HCC Cell Lines

2.1.1. HCC Cell Lines and Tissue Samples from Patients. HCC-derived cell lines (QGY-7701, Bel-7402, Bel-7404, Bel-7405, SMMC-7721) para-carcinoma cell line QSG-7701, and immortalized human normal hepatocyte cell line HL-7702 were obtained from TCC Cell Bank (Shanghai, China) and cultured with standard medium: RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GIBCO-BRL) in a 5% CO_2 humidified chamber at 37°C. Tumor tissues and adjacent noncancerous tissue from 25 HCCs were obtained from the First Affiliated Hospital, Nanjing Medical University, from May 2008 to December 2009. All patients were confirmed histopathologically and samples were obtained with informed consent from patients.

2.2. DNMT3A RNA Interference Constructs and Transfection. Three candidate siRNAs against human DNMT3A were designed for targeting different coding regions of the DNMT3A isoforms (Genbank No. NM_175629.1, NM_153759.2, NM_022552.3). Synthesized 64nt siRNA template oligonucleotides were constructed into *Bgl*II and *Hind*III (TAKARA) sites of pSUPER-EGFP (gift from Professor Dianqing Wu at UCHC, USA), which has a green fluorescent protein marker and resistance to the G418, and named as pMT3A. The scramble sequences of DNMT3A siRNA were used as control and inserted into pSUPER-EGFP to form sMT3A. SMMC-7721 cells were transfected with recombinant plasmids using Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen Corp., Carlsbad, CA) and were selected with medium containing 200 mg/mL G418 (Gibco, Grand Island, NY) for 2 months after the initial transfection. The cells stably harboring the targeting vector were monitored with green fluorescent protein (GFP) expression. SMMC-7721 cells were transfected with pMT3A and labeled as 7721–pMT3A cell lines; these were transfected with sMT3A and were designated as 7721–sMT3A cell lines or control.

2.3. Western Blot Analysis. Cells (5×10^6) were harvested and the total proteins were prepared. Cell precipitate was resuspedded in 200 *u*l ice-cold lysis buffer (10 mmol/L Tris-Hcl (pH 8.0), containing 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% Triton X-100). An equal amount of cellular proteins was loaded on 7% polyacrylamide gels (PAGE) in a 1×SDS-PAGE buffer (1 g/L SDS, 3 g/L Tris-base, and 14.4 g/L glycine). Proteins were transferred to a polyvinylpyrrolidine difluoride membrane electrophoretically and incubated overnight at 4 in block buffer (5% nonfat milk in Tris-Base Tween-20 (TBST). Membranes were incubated for 1 hour at room temperature with DNMT3A-specific monoclonal antibodies (1 : 5000, Imagenex) followed by six five-minute washes with TBST. After washing, the membrane was incubated with anti-mouse horseradish peroxidase-conjugated secondary antibodies (1 : 80000, Sigma), and the immune complexes were detected with enhanced chemiluminescence (Pierce). The membranes were probed with an antiactin antibody (Biomedical Technologies, Stoughton, MA) to confirm equal loading of each protein samples. β-actin protein levels were used as a control to ensure equal protein loading (β-actin, 1 : 8000, Sigma). Each experiment was repeated at least 3 times. The intensity ratios of the 2 bands from the same sample were determined and calculated by *Gel-Pro Analyzer 3.0*.

2.4. Microarray Hybridizations and Induced Genes Analysis. Affymetrix Human Genome U133A 2.0 Array (Affymetrix, Santa Clara, CA), encompassing 18,400 transcripts and variants, including 14,500 well-characterized human genes on one array was processed. Microarray hybridization was performed in the SBC (Shanghai, China) using standard affymetrix procedures. Raw microarray data were acquired using *GCOS1.2* software of affymetrix. The data were preprocessed using robust multiarray analysis (RMA) with a log base 2 (log2) transformation. Genes identified as differentially expressed genes, which is exhibiting, upregulated more than 2-fold to the control. Gene ontology of the differentially expressed genes was performed through the GO Mining Tool (http://www.affymetrix.com/analysis/query/go_analysis.affx). The potential promoter regions spanning 2000 bp upstream and 5'UTR around the transcription start site of 153 upregulated genes. Subsequently, CpG islands were identified within the core promoter region by CpG Island Searcher program (http://www.methdb.de/) in DNA Methylation Database (http://www.uscnorris.com/cpgisland2).

2.5. Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real-Time Quantitative RT-PCR (qPCR) Analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, NY). cDNA was synthesized from 1 μg of total RNA using Oligo$(dT)_{18}$ as primers and reverse transcriptase following reverse-transcribed with Reverse Transcription System (Promega,) according to the kit's instructions. The expression levels of differentially expressed genes of microarray after DNMT3A RNAi were quantified using qRT-PCR analysis. PCR reactions containing 10 ng cDNA, SYBR green

(1 : 20,000 dilutions) were included in each reaction to allow quantification of RNA levels using the ABI 7300 detection system (Applied Biosystems). To normalize the input load of cDNA among samples, either *β-actin* was quantified and used as an endogenous standard. The relative levels of expression of each target genes among different samples were calculated accordingly *(ABI PRISM 7300 Detection System, USA)*. The conditions and primers designed for detected genes were listed in Table 1. Each duplex PCR was repeated with at least 3 different cDNA preparations and 2 independent qPCR reactions for each cDNA.

2.6. Methylation-Specific PCR (MSP), Bisulfite Sequencing, and Treatment of 5-aza-dC. Genomic DNA was extracted according to standard method [25]. Bisulfite treatment was performed by the Methylamp DNA Modification Kit (Epigentek) following the manufacturer's instruction. Modified DNA was amplified to determine the methylation status of the promoter region of gene by MSP. The primer sequences and PCR conditions for amplification above were shown in Table 2. PCR products for the bisulfite sequencing were gel-purified, subcloned into a pEASY-T3 vector system (TransGen Biotech). At least ten colonies were sequenced to assess the degree of methylation in each CpG site. For demethylation analysis, cells were plated and treated with 100 μmol/L 5-aza-dC (Sigma) for up to 4 days. The total RNA was extracted from exponentially growing cultures on the 3rd day after treatment.

2.7. Cell Proliferation and Colony Formation Assay. Cell proliferation of transfected stable SMMC-7721 cells with construct pMT3A and its control sMT3A were measured by Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). The absorbance at 450 nm was measured to represent the cell viability, which was measured to assess the cell proliferation of those cell lines. All experiments were independently repeated at least three times.

1×10^3 cells were suspended in 1.5 ml of 0.3%-melted agar in RPMI 1640 medium (Invitrogen, Carlsbad, CA. USA) with 5% FCS, and plated in a standard 6-well cell culture plate containing a solidified layer of 3 ml 0.6% agarose gel in the same medium. After 14 days of incubation at 37°C in 5% CO_2, colony growth in soft agar was assayed by visually counting the colonies. All evaluations were performed in a blinded manner. The experiments were independently performed three times.

2.8. Statistical Analysis. Data were presented as means ± standard error (SE). Data were analyzed using *Graphpad Prism 5 software* (GraphPad Software, San Diego, CA). Mean difference was determined by Student *t*-tests. Statistical significant differences were defined as $P < .05$. Standard statistical tests were performed using *SPSS* (version 13.0).

3. Results

3.1. Increased Expression of DNMT3A in HCC Cell Lines and HCC Tissues. The expression level of DNMT3A was measured in HCC cell lines by western blot and was normalized to β-actin. There was lower expressed DNMT3A in human normal liver cells (HL-7702) and differentially expressed in HCC cell lines (Figure 1(a)). Increased expression of DNMT3A was found in 3 out of 6 HCC cell lines, especially in SMMC-7721 and BEL-7402. Specifically, there was a 6.1- and 5.8-fold increase in DNMT3A expression in SMMC-7721 and BEL-7402, compared to HL-7702, respectively. The mRNA expression level of the DNMT3A was detected by qPCR in tumor tissues and noncancerous liver tissues from 25 HCC patients. Increased expression of DNMT3A was detected in 16 out of 25 cases (64%) of HCCs (Figure 1(b)). These data suggested that DNMT3A may play a role in the progression of HCC tumorigenesis, and this finding needs to be confirmed by a larger study.

3.2. DNMT3A siRNA Construct Suppresses DNMT3A Specifically and Stably in HCC Cell Line. Knockdown of transcripts using siRNA is a powerful tool for studying gene function. We designed three target siRNAs (Figure 2(a)), which can suppress all of the different *DNMT3A* transcripts, and one nonspecific scramble siRNA, which was used as control (see Table 1 in Supplementary Material available online at doi:10.1155/2010/737535). The candidate siRNA-2 effectively suppressed *DNMT3A* expression in levels of protein ($P = .001$) and encoding mRNA ($P = .011$) (Figures 2(b), 2(c), 2(d)). No significant inhibition on *DNMT1* and *DNMT3B* was detected in *DNMT3A* RNAi treatment cells (Figure 2(d)). Expression of endogenous *β-actin* was used as a control to normalize the expression level of *DNMT1*, *DNMT3A*, and *DNMT3B*.

3.3. Depletion of DNMT3A Inhibits Cell Proliferation and Colony Formation in SMMC-7721 Cells. To explore the functional consequences of *DNMT3A* depletion in HCC, we evaluated the role of *DNMT3A*-specific siRNA treatment on cell proliferation and colony formation in HCC cells. Transfected clones were picked up and expanded for an additional 2 months and analyzed for DNMT3A in protein levels. *DNMT3A* expression was stably suppressed in SMMC-7721 (Figure 3(a)). Compared to control 7721-sMT3A cells, 7721-pMT3A formed significantly fewer colonies (about 65%) in soft agar assay (Figure 3(b)) and with lower cell proliferation ability in CCK-8 cell assay (Figure 3(c)).

3.4. Gene Expression Profiling Induced by DNMT3A Knockdown. To elucidate the effects of increased *DNMT3A* expression in HCC tumorigenesis, Affymetrix HG U133A oligonucleotide microarray was used to evaluate induced gene expression profiling in DNMT3A depletion cells compared with control. The data revealed that 153 genes were upregulated in DNMT3A knockdown cell (Supplementary Table 2). These genes were categorized to numerous biological processes by using the GO Mining Tool [26]. All genes were combined into 17 superordinate categories (Figure 4). Three categories contained the largest number of affected genes: molecular transport (26 genes), transcription regulation (14 genes), and cell cycle (13 genes). Since a CpG island (CGI) near the transcription initiation site is usually important

Table 1: Primers sequences and related amplicons for quantitative real-time RT-PCR or RT-PCR.

Genes	Primers (5′–3′)	Anneal temperature (°C)	Amplicon (bp)
β-actin	Forward: AAAGACCTGTACGCCAACAC Reverse: GTCATACTCCTGCTTGCTGAT	61	220
DNMT3A	Forward: TATTGATGAGCGCACAAGAGAGC Reverse: GGGTGTTCCAGGGTAACATTGAG	65	111
DNMT3B	Forward: GACTTGGTGATTGGCGGAA Reverse: GGCCCTGTGAGCAGCAGA	64	271
DNMT1	Forward: CCGAGTTGGTGATGGTGTGTAC Reverse: AGGTTGATGTCTGCGTGGTAGC	61	325
PTEN-q	Forward: CCGTTACCTGTGTGTGGTGATATC Reverse: AATGTATTTACCCAAAAGTGAAACATT	60	100

Table 2: Primers sequences for MSP and bisulfite sequencing analysis.

Gene	Primers (5′–3′)	Anneal temperature (°C)
PTEN-M[a]	Forward: TTTTTTTTCGGTTTTTCGAGGC Reverse: CAATCGCGTCCCAACGCCG	59
PTEN-UM[b]	Forward: TTTTGAGGTGTTTGGGTTTTTGGT Reverse: ACACAATCACATCCCAACACCA	59
PTEN-BGS[c]	Forward: ATTGGGTATGTTTAGTAGAGTT Reverse: CAACTCTCAAACTTCCATCATA	60

a: Methylated primer sequences used for MSP. b: Unmethylated primer sequences used for MSP. c: Primer sequences used for bisulfite genomic sequencing.

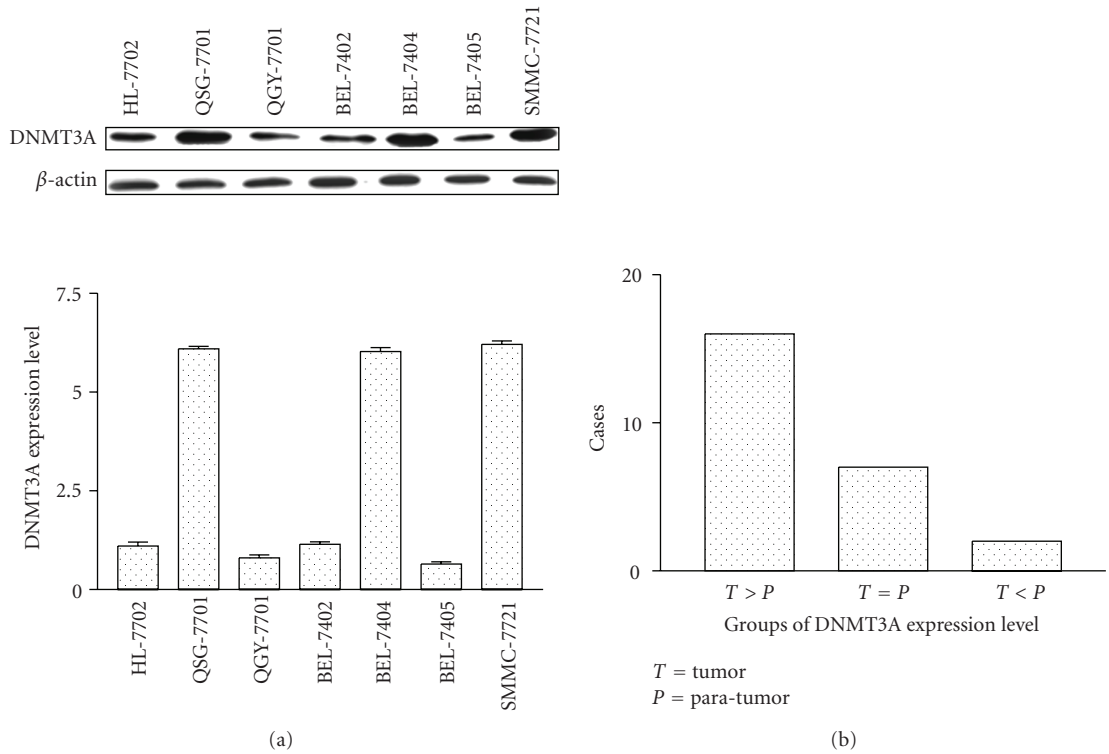

Figure 1: *DNMT3A* expression is analyzed in HCC cell lines, HCC tumor tissue, and adjacent nontumor samples. (a) DNMT3A protein expression level was detected in HCC cell lines by western blot. Upper panel, DNMT3A was examined by western blot in HCC cell lines. Bottom panel, the intensity ratios of both target gene band and control from the same sample were determined and calculated by *Gel-Pro Analyzer 3.0*. DNMT3A was increased more in 3 HCC cell lines than in immortalized normal liver cell line HL-7702. (b) Real-time quantitative RT-PCR analysis of *DNMT3A* expression in 25 pairs of HCC specimens and adjacent noncancerous liver tissues, normalized to *β-actin*.

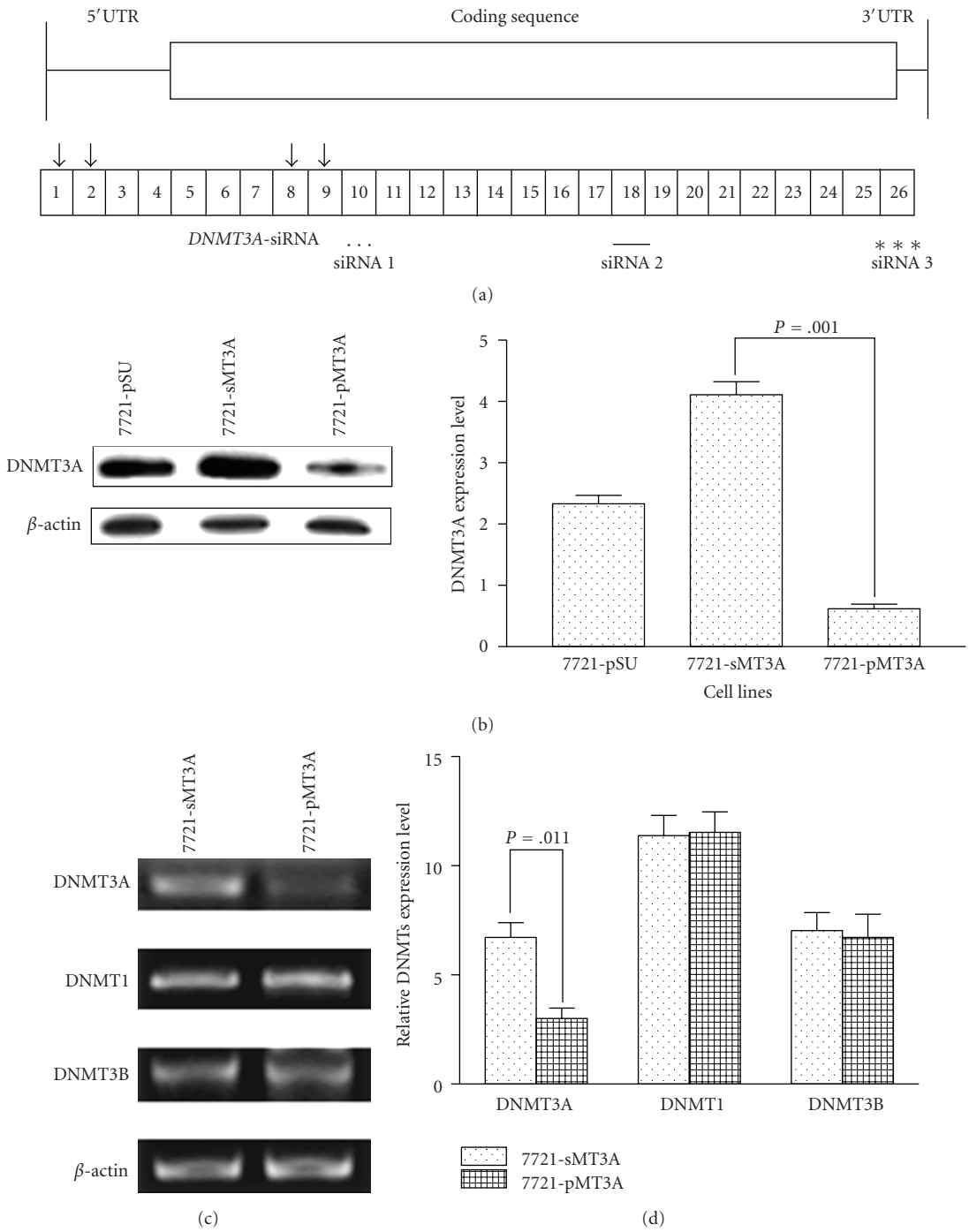

Figure 2: *DNMT3A* gene expression is inhibited by siRNA of *DNMT3A* in SMMC-7721 cells. (a) Schematic representation of *DNMT3A* mRNA structure and siRNA positions is showed. (b) Western blot analysis efficiency of *DNMT3A* siRNA, β-actin was used as reference control. Lanes 1–3 indicated the cell line transfected with plasmid pSUPER-EGFP, sMT3A, and pMT3A, respectively. Right: the intensity ratios of both target gene band and control from the same sample were determined and calculated by *Gel-Pro Analyzer 3.0*. (c) RT-PCR evaluated the affect of *DNMT3A*-specific siRNA on other DNMTs expression. (d) Real-time quantitative RT-PCR confirmed the efficiency of *DNMT3A*-specific siRNA and the affect on other *DNMTs* mRNA expression.

for gene repression by DNA methylation, we searched for the presence of CGIs in each gene by using *CpG Island Searcher* in the *DNA Methylation Database*, referring to the criteria established by Takai and Jones [27]. We found that almost 71% (109/153) of genes contain CpG islands in their 5′ region. This indicated that these genes might be regulated by DNA methylation. In an effort to provide further experimental evidence that the gene expression

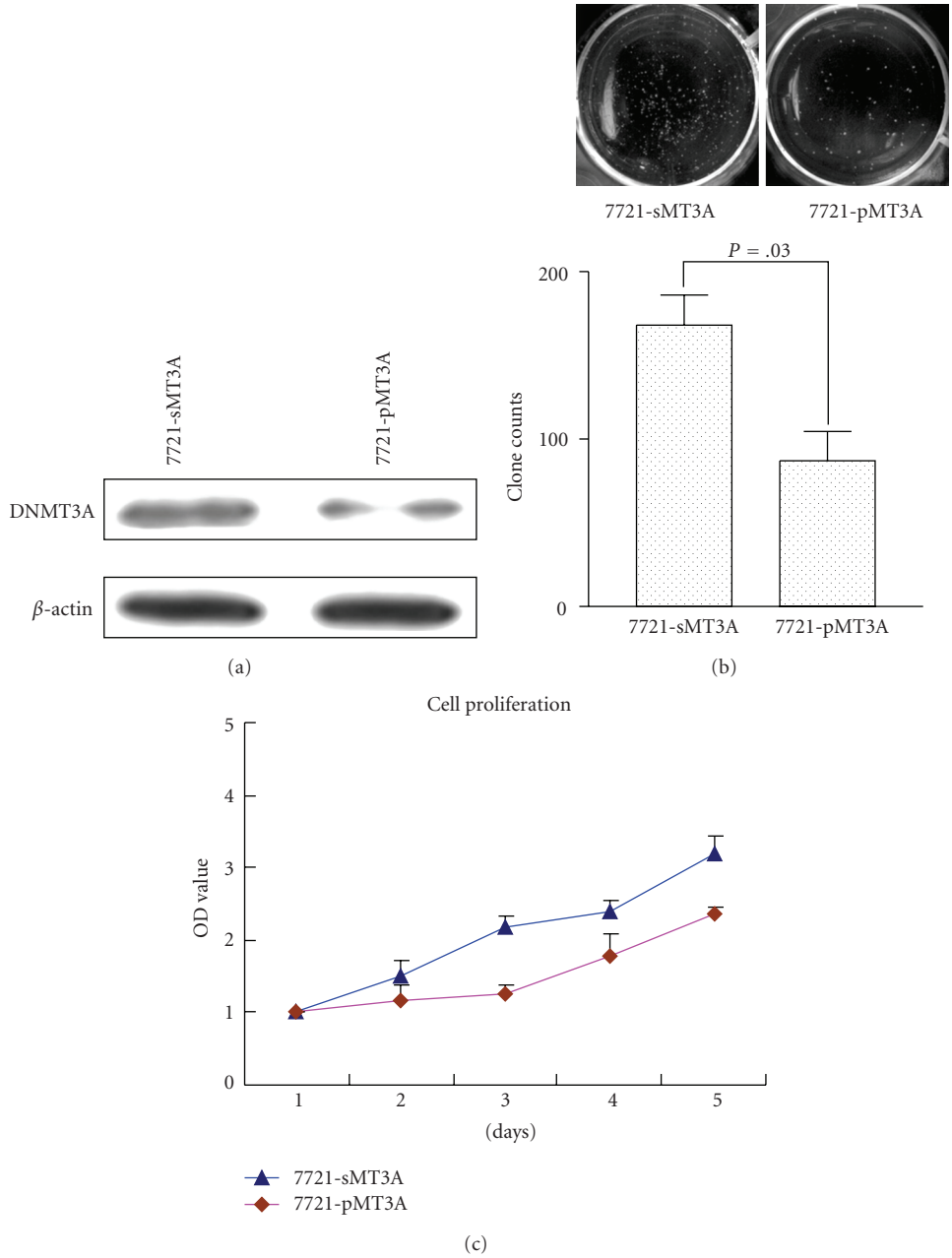

Figure 3: Depletion of *DNMT3A* suppressed HCC cell growth and colony formation ability. (a) Expression of *DNMT3A* was stably suppressed in SMMC-7721. 7721-sMT3A, which is transfected with scramble vector sMT3A, 7721-pMT3A, which is transfected with candidate DNMT3A siRNA constructs. (b) Colony formation of SMMC-7721 cells was inhibited when transfected with *DNMT3A* siRNA. The scramble vector served as a control. The number of colonies was counted from three independent experiments. The histogram shows the colony formation efficiency, where the numbers represent the average value of three independent experiments.(c) Cell growth curve of SMMC-7721 cells treated with the *DNMT3A* siRNA. The cells transfected with the scramble vector served as control. The experiments were repeated at least three times. The result represents the average value of triplicate wells +/- the standard deviation.

changes observed on arrays were valid, quantitative real-time PCR was used to analyze several cancer-related genes and confirmed the data from the array.

3.5. Depletion of DNMT3A Induced PTEN via Demethylation of CpG Sites in Its Promoter Region. In *DNMT3A* depletion cells, tumor suppressor gene *PTEN* was activated (Figure 5(a)). As 5-Aza-2′deoxycytidine, a demethylation agent could restore *PTEN* expression (Figure 5(b)); we supposed that activated *PTEN* in *DNMT3A* depletion cells is maybe involved in demethylation. To explore whether the repression of *PTEN* expression is due to DNA methylation;

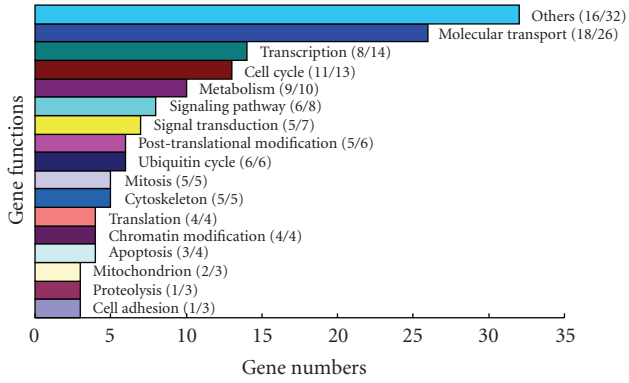

Figure 4: Superordinate categories of biological processes identified by gene ontology (GO) mapping in depletion DNMT3A HCC cell lines. All genes induced by inhibiting DNMT3A were combined into 17 superordinate categories. Three categories contained the largest number of affected genes: molecular transport, transcription regulation, and cell cycle.

we examined the status of methylation of its promoter in *DNMT3A* depletion cells 7721-pMT3A and control by MSP method. The result showed that restoring of the PTEN was significantly associated with its promoter demethylation in the 7721-pMT3A cell line (Figure (5(c))). Subsequently, a 331 bp (−1128/−798) fragment that contained part of the *PTEN* promoter and its 5′ untranslated region (5′ UTR) (Figure 5(d)) is chosen for further methyaltion analysis by bisulfite sequencing. Ten individual colonies were sequenced to identify methylated cytosine residues. We found that all six CpG sites were unmethylated in 7721-pMT3A. In contrast, different CpG sites (S1, S2, S4, or S6) were methylated in the 7721-sMT3A cell line. The results indicated that *DNMT3A* knockdown changed the methylation status of certain CpG sites within the 5′ UTR and promoter of the *PTEN* gene. These data suggested that *PTEN* expression is regulated in a methylation-dependent manner and that *PTEN* may be preferentially methylated by *DNMT3A*.

4. Discussion

HCC is a severe disease with a very poor prognosis and is the fourth largest cause of cancer-related deaths worldwide [26, 28]. DNA hypermethylation of tumor suppressor genes is frequently observed in HCCs [25, 29–31]. The process of aberrant DNA methylation is catalyzed by DNA methyltransferases (DNMTs). Moreover, DNMTs expression is increased during early tumorigenesis. In hepatocarcinogenesis, increased expression of DNMTs mRNA is correlated with a progressive increase in the number of methylated genes from normal liver, chronic hepatitis/cirrhosis to HCC [32]. One hypothesis is that abnormal expression of the three functional DNMTs may be important in regulating tumor suppressor gene expression during hepatocellular tumorigenesis. *DNMT1* and *DNMT3B* were both shown to be important for cancer cell survival and tumorigenesis [33]. However, the relationship between *DNMT3A* and tumorigenesis is still uncertain. Previous studies suggested that *DNMT3A* is developmentally regulated and was shown to be important in establishing maternal imprints in mice [34, 35]. Recently, researchers found that elevated *DNMT3A* activity promotes polyposis in Apc (Min) mice [23] and that knockdown of *DNMT3A* dramatically inhibits melanoma growth and metastasis in mouse melanoma models [36]. Several studies showed that increased expression of *DNMT3A* in HCC implied that *DNMT3A* was involved in hepatocarcinogenesis. In order to evaluate the effect of DNMT3A depletion, we examined the characteristics of SMMC-7721 cells in vitro, using proliferation and colony-forming. The results showed that cell proliferation and colony-forming ability did differ between the WT and DNMT3A-depleted SMMC-7721 cells. *DNMT3A* depletion produced primarily antiproliferative effects. These results were observed in another HCC cell line QSG-7701 (Supplementary). Microarray analysis revealed that inhibition of *DNMT3A* induced expression of various genes that are involved in cell cycle regulation, transcription regulation, and signal transduction and are thus implicated in the inhibition of HCC cell growth. Whether depletion of DNMT3A is capable of inhibiting the growth of hepatocellular carcinoma in vivo, mice malignant hepatocellular carcinoma models should be established in the further study.

Our previous studies showed that depleting *DNMT1* and *DNMT3B* induced expression of different sets of TSGs in HCC cell lines [37, 38]. Here, *DNMT3A* depletion significantly altered the expression of tumor-related genes on the array. However, the regulation of these genes by *DNMT3A*-mediated methylation or other epigenetic mechanisms is unknown. In addition to analyzing the influence of *DNMT3A* depletion on the broad changes in gene expression by cDNA arrays, we sought to elucidate the potential mechanism by which *DNMT3A* may regulate TSGs. To this end, we began an analysis of CGI sites contained within the promoter regions of altered genes by using *CpG Island Searcher*. Almost 71% (109/153) of induced genes contain CpG islands in their 5′ region, suggesting that DNA methylation plays an important role in the regulation of gene expression by *DNMT3A*. Compared with control, phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene in HCC, is significantly induced by *DNMT3A* knockdown. *PTEN* is silenced by DNA methylation at high frequencies in HCC, and promoter methylation and silencing of *PTEN* is reported to be associated with HCC [39, 40]. One of the mechanisms of *PTEN* inactivation is epigenetic silencing, which has been reported in various tumors including hematologic malignancies and some solid tumors [41–44]. In this study we found that *PTEN* can be restored due to demethylation of its promoter by demethylating agents in SMMC-7721 cells. *PTEN* is known for its essential role in regulating the PI3K/AKT signaling pathway [45]. *PTEN* loss leads to activation of AKT kinases, which contributes to cell survival, growth, proliferation, and invasion [46, 47]. The methylation of the *PTEN* promoter and its associated loss in activity is observed in HCC. These results demonstrate that depletion of *DNMT3A* affects cell proliferation and that this effect may be a result of the reexpression of the formerly silenced *PTEN*. In the present study, we found that *DNMT3A* may

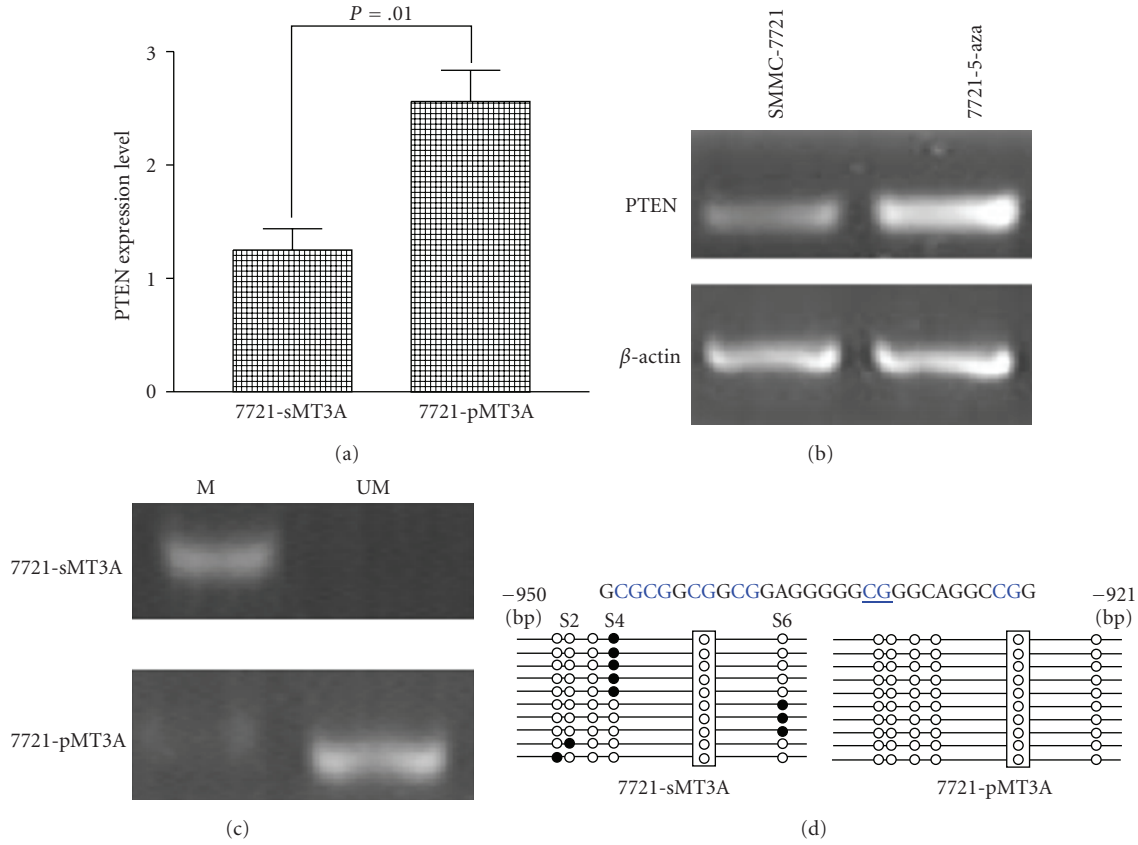

Figure 5: Restoring of *PTEN* expression by depletion of *DNMT3A* in HCC cells is partly due to methylation of its promoter. (a) Quantitative-PCR analysis of *PTEN* expression level in *DNMT3A*-depleted cell line. (b) 5-Aza-2′deoxycytidine restored endogenous *PTEN* mRNA expression in SMMC-7721 cells. (c) Methylated status evaluation of *PTEN* promoter region determined by MSP. The presence of visible PCR product in lanes marked "U" indicates the unmethylated gene, whereas the presence of product in lanes marked "M" indicates the methylated gene. (d) Bisulfite sequencing of the selected region of the *PTEN* promoter in *DNMT3A*-depleted SMMC-7721 cells. Top, sequences within the fragment include 6 CpG sites (S1, S2, S3, S4, S5, S6). Bottom, the methylation pattern of 6 CpG sites in *PTEN* promoter region is shown. Ten random clones from *DNMT3A*-depleted SMMC-7721cell line and control were sequenced to examine the methylated status. Open and closed circles indicate unmethylated and methylated sites, respectively.

play a role in hepatocellular carcinogenesis by its role in regulating the expression of some tumor suppressor genes such as *PTEN*. However, it is necessary to further study additional *DNMT3A* mechanisms of regulation of TSGs and its function in tumorigenesis.

5. Conclusion

Our results provide evidence that *DNMT3A* depletion significantly inhibited cell growth and colony formation in HCC cells. The most likely mechanism for this inhibition of tumorigenesis includes the reactivation of epigenetically silenced tumor suppressor genes, such as the *PTEN* gene.

Acknowledgments

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