J. Cell. Mol. Med. Vol 15, No 6, 2011 pp. 1415-1428

Promoter hypomethylation up-regulates CD147 expression through increasing Sp1 binding and associates with poor prognosis in human hepatocellular carcinoma

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Received: February 22, 2010; Accepted: June 8, 2010

Abstract

CD147 is a transmembrane glycoprotein overexpressed in human hepatocellular carcinoma (HCC) which could promote HCC progression and metastasis. Promoter methylation is one of the most important processes in gene regulation. In this study, we aim to investigate CD147 promoter methylation status and the correlation with clinicopathological features and prognosis in HCC. CD147 promoter methylation statuses and expression levels in normal and HCC cell lines and 54 paired HCC and adjacent non-tumour (ANT) tissues were, respectively, examined by bisulphite genomic sequencing, methylation-specific PCR, real-time RT-PCR, Western blot and immunohistochemistry. The correlations of promoter methylation statuses with CD147 expression level and the clinicopathological features were statistically analysed in HCC patients. Significantly higher expression of CD147 and significantly lower promoter methylation level were observed in HCC cell lines compared to normal cell lines and tissues control. *In vivo* and *in vitro* analysis indicated that demethylation with 5-Aza-2′-deoxycytidine led to increased CD147 expression through enhancing Sp1 binding affinity, and methylation with methyltransferase reduced CD147 transcriptional activity through interfering Sp1 binding. CD147 promoter methylation level in HCC tissues (22.22%) was lower than that in ANT tissues (46.30%; $P < 0.05$). Within HCC tissues, a significant inverse correlation was observed between CD147 expression and methylation level ($r = -0.615$). Moreover, HCC patients with unmethylated CD147 promoter had a significantly higher recurrence rate (88.1% *versus* 58.3%; $P < 0.05$) and death rate (83.3% *versus* 50.0%; $P < 0.05$) than patients with methylated CD147 promoter. In conclusions, promoter hypomethylation up-regulates CD147 expression primarily through increasing Sp1 binding and associates with poor prognosis in HCC patients.

Keywords: CD147 • hypomethylation • Sp1 • hepatocellular carcinoma • prognosis

Introduction

Human hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with an annual incidence rate of about 600,000 cases, among whom 55% are in China [1]. HCC is characterized by vascular invasion, rapid progression and poor prognosis. The 5-year overall survival rate of individuals with HCC is only 8.9%, and this has barely improved over the past two decades [2]. New prognostic markers are needed to help identify patients who are likely to have a poor prognosis and benefit from more aggressive treatment approaches.

CD147 is a transmembrane protein with highly glycosylated modifications. It contains two extracellular immunoglobulin domains (C2 and V sets), exhibiting the characteristics of the immunoglobulin superfamily members [3]. Several laboratories

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doi:10.1111/j.1582-4934.2010.01124.x

have subsequently identified the CD147 molecule in different origins of human cells and tissues, designating it the names of extracellular matrix metalloproteinase inducer (EMMPRIN) [4], basigin [5], HAb18G [6] and M6 antigen [7]. Earlier studies demonstrated that CD147 molecule is highly expressed on the surface of various cancer cells, including cancers of liver, lung, breast, kidney, colon, prostate and oesophagus [8]. There is emerging evidence indicating that increased CD147 expression significantly contributes to tumour progression by inducing the expression of matrix metalloproteinases (MMPs; mainly MMP-2 and MMP-9) [9–11] and vascular endothelial growth factor [12], activating the urokinase-type plasminogen activator system, conferring the resistance of tumour cells to anoikis and thus promoting tumour invasion and metastasis [13, 14]. Additionally, CD147 overexpression in tumour cells was significantly associated with a poor prognosis [15, 16]. Nonetheless, although CD147 plays a critical role in tumour progression and prognosis, the mechanisms underlying the up-regulation of this molecule in cancer cells remain largely unknown.

Recently, DNA methylation at CpG dinucleotides has been well documented as a common mechanism for regulation of gene expression in mammalian cells. A series of studies demonstrated that aberrant methylation (hypermethylation and hypomethylation) is one of the most consistent epigenetic hallmarks of human cancer [17–19]. Hypermethylation of normally unmethylated tumour suppressor genes and hypomethylation of normally methylated proto-oncogenes have been identified as crucial events in the process of carcinogenesis [20]. Furthermore, altered DNA methylation patterns of these genes have been reported to be significantly correlated with tumour differentiation, progression and prognosis [21]. Although altered methylation of a series of genes has been to date identified in human cancers, whether the methylation status influence the CD147 expression has not yet to be elucidated.

The most well-defined mechanism involved in the regulation of methylation-mediated gene expression is the direct binding blocking of transcription factors to their CpG-containing binding sites [22]. Sp1 is a well-characterized sequence-specific transcriptional factor that regulates a large number of house-keeping and tissue-specific genes by binding to GC-rich DNA sequences in the promoter region of many human genes [23, 24]. Numerous previous studies have shown that methylation status of the Sp1 consensus binding site influences the Sp1 binding and thus regulates the gene transcription [25, 26]. It has been convincingly reported that CpG islands and potential binding sites of Sp1 exist in the promoter region of CD147 [27, 28]. However, whether methylation status of CD147 gene promoter affects Sp1 binding and thus regulates the expression of CD147 have not been explored.

To investigate the potential mechanism of CD147 up-regulation in HCC, we examined the expression level and methylation status of CD147 gene promoter in three pairs of epithelial cancer cell lines and corresponding transformed normal cell lines. We also explored the potential molecular mechanism underlying the methylation-mediated regulation of CD147 expression. In addition, we analysed the clinical values of CD147 methylation in predicting the prognosis of HCC patients.

Materials and methods

Cell culture and tissues collection

The following cell lines were used in this study: human normal liver cell QZG [29], QSG-7701 [30] and HL-7702 [31]; HCC cell lines: HepG2, BEL-7402, FHCC-98, SMMC-7721, HCC-9724 and HCC-9204; human embryonic kidney cell HEK-293. All cell lines were purchased from Shanghai Institute for Biological Sciences (Shanghai, China). All cell lines were routinely cultured in RPMI-1640 medium (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% foetal calf serum (Gibco BRL, Rockville, MD, USA) at 37°C in a humidified atmosphere of 5% CO_2. Three fresh normal liver tissues (NL1, NL2 and NL3) obtained from Department of Hepatobiliary Surgery in Xijing Hospital were used as normal control. Informed consents were obtained from the patients.

Fifty four pairs of paraffin-embedded tissue specimens of HCC and corresponding adjacent non-tumour (ANT) tissues were obtained from the Department of Pathology, Xijing Hospital affiliated to Fourth Military Medical University (Xi'an, China) with signed informed consent. All histologically confirmed HCC patients had undergone surgical resections at Xijing Hospital. Demographic and clinical data were collected from the patient medical record. All follow-up data were evaluated as of December 2005. Study approval was obtained from the Xijing Hospital Institutional Review Board.

Regular and real-time quantitative RT-PCR

Total RNA was isolated from cultured cells and normal liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out using ReverTra Ace reagents kit (TOYOBO, Osaka, Japan). Regular PCR was used to amplify CD147, SP1 and glyceraldehyde-3-phosphate (GADPH) (internal control). In addition, real-time quantitative PCR was also performed with MiniOpticon real-time PCR detection system (Bio-Rad, Hercules, CA, USA) in SYBR Green mastermix (TaKaRa, Otsu, Japan). The thermal cycling conditions comprised one cycle at 94°C for 4 min. and 40 cycles at 94°C for 15 sec., 60°C for 15 sec. and 72°C for 15 sec. All data were analysed using the Opticon Monitor software (ver3.1, Bio-Rad) and the expression of CD147 was calculated as relative expression level to GAPDH using the δ-Ct method as described previously [32]. The sequences of all PCR primers were listed in Table 1.

Western blot analysis

Cell and tissue samples were lysed with RIPA buffer (Beyotime, Inc., NanTong, China). Equal amounts (10 μg) of total protein were loaded, and then subsequently immunoblotted with the primary antibodies, including anti-CD147 monoclonal antibody prepared in our lab [33], anti-Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-tubulin monoclonal antibody (NeoMarkers, Freemont, CA, USA). The proteins were detected using the Amersham enhanced chemiluminescence system (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Table 1 Oligonucleotide sequence of PCR amplification, siRNA fragment and EMSA probes

Primers for real-time quantitative RT-PCR		
CD147	5′-TCGCGCTGCTGGGCACC-3′	5′-TGGCGCTGTCATTCAAGGA -3′
Sp1	5′-AATTTGCCTGCCCTGAGTGC-3′	5′-TTGGACCCATGCTACCTTGC -3′
GAPDH	5′-AGCAATGCCTCCTGCACCACCAAC-3′	5′-CCGGAGGGGCCATCCACAGTCT -3′
Primers for regular RT-PCR		
CD147	5′-GCGGAATTCATATGGATATGGCTGCCGGCACAGTC-3′	5′-CAATACTCGAGTTAATGAGTGCGCACGCGGAGCG-3′
Sp1	5′-TGTGAATGCTGCTCAACTCTCC-3′	5′-CATGTATTCCATCACCACCAG-3′
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′	5′-TCCACCACCCTGTTGCTGTA-3′
Primers for BGS analysis		
CD147	5′-GCGGCTGGGTAATATATTTTTAATGTTTTAATTTT-3′	5′-AACAACACAAACAACACAACCACCATAATT-3′
Primers for vectors construction		
CD147P/pGL3	5′-ATCGGCTAGCCTGGGTAACACACTTTCAACGCTTC-3′ (*Nhe* I)	5′-ATCGAAGCTTGATTCCTATTCCTCGCCGGT-3′ (*Hind* III)
Sp1/pcDNA3.1	5′-ATCGGGTACCATGAGCGACCAAGATCACTCCAT-3′(*Kpn* I)	5′-ATCGCTCGAGTCAGAAGCCATTGCCACTGATAT-3′(*Xho* I)
Primers for MSP*		
CD147-M	5′-AGCGTGTGCGCGCGTG-3′	5′-ACCGCTATAAAAAACGACGAA-3′
CD147-U	5′-AGTGTGTGTGTGTGGAGTTT-3′	5′-ACCACTATAAAAAACAACAAA-3′
Primers for ChIP assay promoter-specific PCR		
CD147	5′-ACATATGAGCTCGAAGCGCCGGAAG-3′	5′-TAATAGCGGCCGCGAGGTGAGAAC-3′
SiRNA designed to target Sp1		
si-Sp1	5′-AAUGAGAACAGCAACAACUtt-3′	5′-AGUUGUUGCUGUUCUCAUU tt-3′
The probes for EMSA†		
SpWT	5′-CCGGCGTCCCCGGCGCTCG<u>CCCCGCC</u>CCCG-3′,	5′-CGGG<u>GGCGGGG</u>CGAGCGCCGGGGACGCCGG-3′
SpMT	5′-CCGGCGTCCCCGGCGCTCG**<u>AGATCTA</u>**CCCG-3′	5′-CGGG**<u>TAGATCT</u>**CGAGCGCCGGGGACGCCGG-3′

*Restriction enzyme cutting sites introduced in the oligonucleotides are shown in italics.
†The core sequence of the Sp1-binding site is underlined; mutations are shown in bold.

Sodium bisulphite genomic sequencing (BGS)

Genomic DNA was extracted from cultured cells and fresh normal liver tissues using QIAamp DNA mini kit (Qiagen, Valencia, CA) and from paraffin slides using EX-WAX Paraffin-Embedded DNA Extraction kit (Chemicon, Temecula, CA) following the manufacturer's instructions. Sodium bisulphite modification of genomic DNA was performed as described previously [31]. CpG island region in CD147 promoter was amplified with sodium bisulphite-treated DNA as template using Advantage GC Genomic PCR kit (Clontech, Palo Alto, CA, USA) under the following PCR conditions: 97°C for 3 min. and then 80°C for 3 min., followed by 35 cycles of 94°C for 30 sec., 54°C for 30 sec. and 72°C for 50 sec. and ended by a final extension step at 72°C for 10 min. The sequences of PCR primers were listed in Table 1. The purified PCR products were cloned into the pMD18-T vector (TaKaRa, Otsu, Japan), and six clones for each sample were randomly selected and sequenced by Shanghai Sangon Co. (Shanghai, China).

Vector constructions

The promoter region (−338 to +37, relative to the translation start site) of CD147 gene was PCR-amplified using Advantage-GC Genomic PCR kit (Clontech) from human genomic DNA isolated from HEK-293 cells and inserted into the pGL3-Basic vector (Promega, Madison, WI, USA) to construct CD147P/pGL3 vector. Then, the CD147P/pGL3 and pGL3-Basic vectors were methylated through incubation with CpG methyltransferase M.SssI (New England BioLabs, Beverly, MA, USA) for 3 hrs at 37°C in the presence of 160 mM S-adenosylmethionine. Methylated vectors were denoted as metCD147P/pGL3 and metpGL3-Basic. The methylation status was verified by digestion with *Hha* I, a methylation sensitive enzyme. The coding region of human Sp1 gene was PCR-amplified using the synthesized cDNA from HEK-293 cells as the template. The PCR products were gel-purified, enzyme-digested and inserted into plasmid pcDNA3.1 (Invitrogen) to construct the Sp1 expression vector Sp1/pcDNA3.1. All constructed vectors were identified by enzyme-digestion and DNA sequencing. DNA sequencing was performed by Shanghai Sangon Co. All PCR primer sequences were listed in Table 1.

Transfection and luciferase assay

For transient transfection, HEK-293 cells were plated onto a 24-well plate 1 day before transfection at 3×10^5 cells/well. Cells were co-transfected with a combination of 0.4 μg expression plasmid (pcDNA3.1 or Sp1/pcDNA3.1) and 0.4 μg luciferase reporter vector (pGL3-Basic or CD147P/pGL3 or metCD147P/pGL3) by using lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. The pRL-TK plasmid (Promega; 0.1 μg/well) containing the Renilla luciferase gene was also co-transfected into cells as an internal control reporter to normalize the luciferase activity. To validate the role of Sp1 in regulation of CD147 transcription, transfected cells were further treated for 24 hrs with mithramycin A (Sigma-Aldrich, St. Louis, MO, USA), a drug known to modify GC-rich regions of the DNA and inhibit Sp1 binding [34, 35], at different concentrations (0, 0.5, 1 and 2.5 μM) 6 hrs after transfection. Cells were harvested to determine luciferase activity 48 hrs after transfection using Dual-Luciferase Reporter Assay System kit (Promega) using a luminometer (Tecan, Männedorf, Switzerland). All experiments were conducted in triplicate.

Sp1 transfection and knockdown

Sp1 eukaryotic expression vector and small interfering RNAs designed to knockdown Sp1 [36] were transfected into HepG2 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions, respectively. Sp1 siRNA duplex at a final concentration of 0.2 μM was transfected into 40% confluent cells, which were harvested 36–44 hrs after transfection.

5-Aza-2′-deoxycytidine treatment

QZG cell was cultured in RPMI-1640 medium supplemented without or with different concentrations (1, 2.5 and 5) of DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-Aza-dC, Sigma) for 3 days. All mediums were daily changed. Finally, RNA and protein were extracted from untreated and treated cells for PCR and Western blot analyses.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out using the ChIP-IT kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, QZG cells were cultured without or with 5-Aza-dC (1.0, 2.5 and 5.0 mM) for 3 days. Immunoprecipitation was carried out with 2 μg of anti-Sp1 antibody (Santa Cruz Biotechnology) or rabbit IgG by incubating with cells overnight at 4°C with rotation. The immunoprecipitated DNA was amplified by the promoter-specific primers (Table 1). The PCR products were analysed on 1% agarose gel. Three independent experiments were performed.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from HepG2 cells as previously described [37]. The wild-type probe of the CD147 promoter (from-105 to −75) was generated by annealing two complementary oligonucleotides (The sequences were listed in Table 1) and the 3′ recessive ends were labelled with biotin. The mutated probe of the CD147 promoter was generated by the same procedure, except that several oligonucleotides were mutated (The sequences were listed in Table 1) in the core sequence of the Sp1-binding site. The methylation of the CD147 promoter probe was obtained through incubation with CpG methyltransferase M.SssI (New England BioLabs) as above-mentioned. The DNA binding reaction was carried out in a 20 μl reaction mixture containing Gel Shift Binding Buffer (Promega), 10 μg nuclear extract and the biotin-labelled probe or methylated biotin-labelled probe, with or without 100-fold molar excess unlabelled competitor. For supershift reactions, extracts were preincubated with 1 μg anti-Sp1 (Santa Cruz Biotechnology) or rabbit IgG (Sigma) antibody for 45 min. on ice. Samples were loaded on a 5% polyacrylamide gel and then transferred to a positively charged nylon membrane. Signals were detected using an ECL kit (Pierce) according to the manufacturer's instructions.

Methylation-specific PCR (MSP) analysis

Genomic DNA (2 μg for each sample) isolated from HCC or ANT tissue was treated with sodium bisulfate and purified using DNA clean-up kit (Promega). The purified DNA was dissolved in 20 μl water and 2 μl was used for MSP analysis. The primers specific for unmethylated DNA were CD147-U and the primers for methylated DNA were CD147-M. All PCR primer sequences were listed in Table 1. These two sets of primers were designed to amplify the same region of promoter from −135 to −18, covering the CpG sites 6–31 and yielding a product of 117 bp. The PCR conditions were as follows: 1 cycle at 94°C for 5 min., then 30 cycles of 94°C for 30 sec., 54°C for 15 sec. for CD147-M or 52°C for 15 sec. for CD147-U, and 72°C for 15 sec., followed by a final elongation at 72°C for 10 min. PCR products were loaded onto 2% agarose gel for analysis.

Immunohistochemical staining

CD147 expressions in HCC tissue specimens were detected by immunohistochemical staining as described previously [8, 33] and then evaluated as described by Lu *et al.* [31]. Briefly, a proportion score was assigned, which represented the estimated proportion of positively stained tumour cells (none, 0; <1%, 1; >1% to <10%, 2; >10% to <33%, 3; >33% to <66%, 4; >66%, 5). An intensity score was also assigned, which represented the average intensity of the positive tumour cells (none, 0; weak, 1; intermediate, 2; strong, 3). The proportion and intensity scores were then multiplied to obtain a total score, which ranged from 0 to 15. A total score greater than 5 was considered positive.

Statistical analysis

All statistical analyses were performed with the SPSS 16.0 statistical software package (SPSS, Chicago, IL). The difference of luciferase activity was assessed by Student's *t*-test. Spearman's ρ was calculated to analyse the correlation of CD147 expression and its promoter metheylation. Pearson chi-square (χ^2) test was used to compare the difference in the distribution of demographic and clinical variables in methylated and unmethylated HCC patients. For all HCC patients, median follow-up period was 12.3 months (Mean = 10.99). The association between the

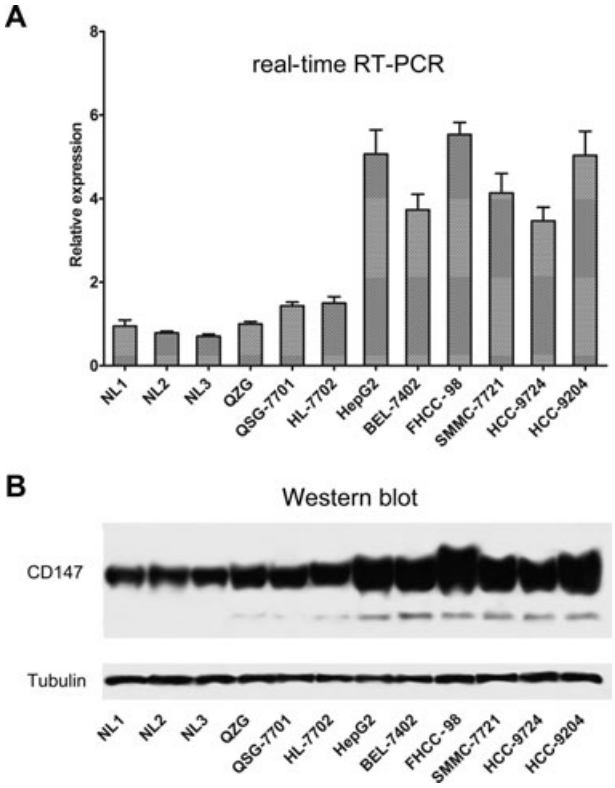

Fig. 1 Up-expression of CD147 in HCC cell lines. (**A**) mRNA expressions of CD147 gene were detected by real-time quantitative RT-PCR in HCC cell lines, normal cell lines and normal liver tissues. GAPDH gene was used as an internal control. (**B**) CD147 protein expression was determined by Western blot with tubulin as an internal control.

recurrence and overall survival time and methylation status of CD147 promoter was evaluated by Kaplan–Meier plots and log-rank test. All the statistical tests were 2-sided and *P* value of <0.05 was considered to be significant.

Results

Up-expression of CD147 in HCC cell lines

The expression levels of CD147 RNA and protein were evaluated in three human normal liver tissues, normal liver cell lines and HCC cell lines. Real-time quantitative RT-PCR analysis showed that there were significantly higher mRNA expression of CD147 in all HCC cell lines (HepG2, BEL-7402, FHCC-98, SMMC-7721, HCC-9724 and HCC-9204), compared with the normal cell lines (QZG, QSG-7701 and HL-7702) and normal tissues (NL1, NL2 and NL3) (Fig. 1A). Similar results were also observed in Western blot analysis, indicating that the protein expression of CD147 was dramatically higher in HCC cell lines (Fig. 1B).

DNA Methylation profile of the CpG island at the CD147 promoter region

We first analysed the DNA sequence of the CD147 promoter region (*NT_011255*) defined by Liang L *et al.* [28] using the CpG Island Searcher program CPGPLOT with the default setting (%GC $>55\%$, ObsCpG/ ExpCpG >0.65) [21]. Our result indicated that one typical CpG island ranging from -340 to $+37$ existed in CD147 promoter region (Fig. 2A). With the assistance of the TRANSFAC database, four potential binding sites of transcription factor Sp1 were found in the region of -340 to $+37$. The typical 46 CpG sites located in this region was also indicated in a simplified schematic overview (Fig. 2B). To examine the correlation between DNA methylation and CD147 expression, we further investigated the methylation status of CD147 promoter region in all cell lines and normal liver tissues by BGS. As shown in Figure 2C, all normal cell lines and normal liver tissues exhibited a denser methylation pattern at CD147 promoter region than corresponding HCC cell lines. Further analysis indicated that the methylation levels of four potential Sp1 binding sites (including CpG sites 3, 4, 19, 20, 26, 39 and 40) were also significantly lower in HCC cell lines than in normal cell lines and tissues (Fig. 2C). These findings suggested that the hypomethylation of CD147 promoter region may contribute to the increased CD147 expression in HCC cell lines.

The involvement of Sp1 in regulating CD147 expression

We first examined the transcription activity of CD147 promoter and the regulation role of Sp1 in HEK-293 cell through the co-transfection of luciferase reporter plasmid and expression vector (Fig. 3A). Our results showed that the relative luciferase activity in HEK-293 cells transfected with CD147P/pGL3 was significantly higher than in those cells transfected with pGL3-Basic ($P < 0.05$), indicating that the CD147 promoter region (-340 bp to $+37$ bp) had obvious transcriptional activation capacity in HEK-293 cell. The transfection of Sp1/pcDNA3.1 notably enhanced the transcription activity of CD147 promoter region ($P < 0.05$). The MitA treatment of transfected HEK-293 cells significantly inhibited CD147 promoter activity in a dose-dependent manner. These results demonstrated that Sp1 was essential in the regulation of CD147 promoter activity.

To illustrate the biological importance of Sp1 in CD147 gene regulation, we knocked down Sp1 expression in HepG2 cell using RNA interference approach. We also up-regulated the Sp1 expression in HepG2 through transient transfection of Sp1 expression vector. Then the CD147 mRNA expression was examined by RT-PCR. As shown in Figure 3B, Sp1 siRNA strongly reduced Sp1 mRNA expression, whereas control siRNA had no effect on Sp1 mRNA level. Meanwhile, the expression of endogenous CD147 RNA was effectively blocked by Sp1 siRNA transfection. In contrast, the expression of Sp1 mRNA was dramatically increased after transfected the Sp1 eukaryotic expression

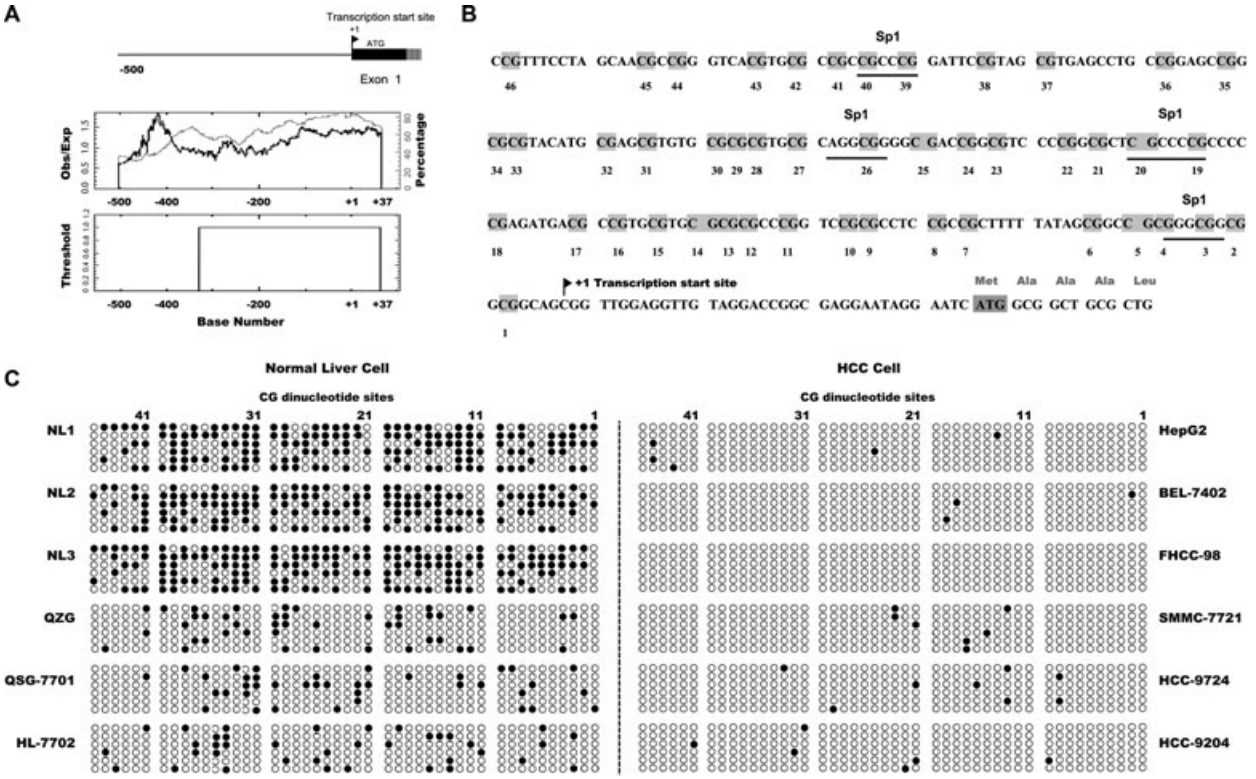

Fig. 2 DNA Methylation profile of the CpG island at the CD147 promoter region. (**A**) Map of predicted CpG islands. One CpG island located at −340 to +37 was identified in CD147 promoter (+1, transcriptional start site; ATG, translational starting site). GC percentage and observed/expected CpG were calculated using the CPGPLOT program. (**B**) Schematic representation of CpG island. The 46 CG dinucleotides were denoted as grey shadow. CpG sites were numbered from 1 to 46 relative to the transcription start site (+1 indicated with an arrow). Underlined region indicated Sp1 binding sites. (**C**) Sequences of the CD147 promoter region after bisulphite modification were analysed in normal and cancer cell lines. Black circle, methylated cytosine; white circle, unmethylated cytosine in the dinucleotide CpG.

plasmid and the up-regulated expression of Sp1 resulted in a significantly improved CD147 mRNA expression. These data demonstrate the involvement of Sp1 in regulating the human CD147 gene expression.

Induction of CD147 gene expression by 5-Aza-dC

To verify the role of DNA methylation in CD147 expression regulation, the QZG cell was treated with DNA-demethylating reagent 5-Aza-dC and then CD147 expression was examined. The mRNA and protein expression of CD147 were significantly increased in a dose-dependent manner after treatment (Fig. 4A–C). The methylation status in treated QZG cell was then determined by BGS. We found that the methylation level of CD147 promoter region was significantly decreased with the increased concentration of 5-Aza-dC in a dose-dependent manner (data not shown). Considering the possible role of Sp1 in the activation of CD147 transcription, to rule out the possibility of Sp1 involvement in 5-Aza-dC-induced CD147 up-regulation, we examined the Sp1 expression level in treated QZG cell. Our data showed that the 5-Aza-dC treatment did not change the mRNA and protein expression level of Sp1 (Fig. 4A–C).

Demethylation increased the binding of Sp1 to the CD147 promoter *in vivo*

To elucidate the effects of CD147 promoter methylation on the binding of Sp1 to the promoter, ChIP assays were performed in CD147-low-expressing QZG cells. Very weak Sp1 binding activity was observed in QZG cells without treatment, whereas markedly enhanced binding activity of Sp1 to the CD147 promoter was found in QZG cells treated with 5-Aza-dC, along with the induction of CD147 expression (Fig. 4D). These findings suggested that methylation of CpG sites in the CD147 promoter interfered with the binding of Sp1. The results in Figure 4 indicate that 5-Aza-dC-induced CD147 up-regulation is mostly due to the demethylation of CD147 promoter region through increasing Sp1 binding rather than the increased Sp1 expression.

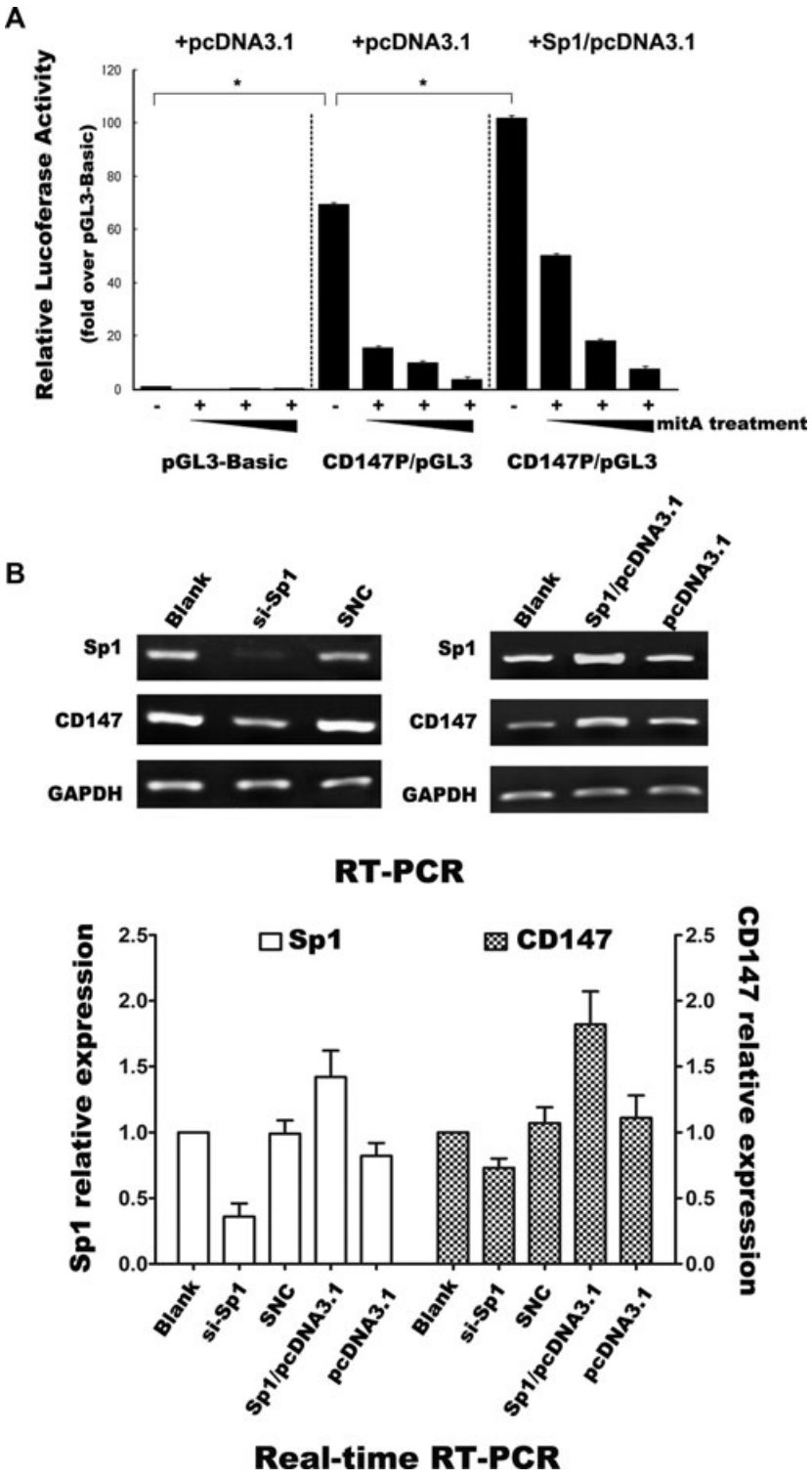

Fig. 3 The involvement of Sp1 in regulating CD147 expression. (**A**) Analysis for transcription activity of CD147 promoter and the regulation role of Sp1 in HEK-293 cell by the co-transfection of luciferase reporter plasmid (CD147P/pGL3 or pGL3-Basic) and expression vector (pcDNA3.1 or Sp1/pcDNA3.1). Transfected cells were either untreated or treated with increasing concentration (0.5, 1, 5 μM) of mithramycin A. The luciferase activity value of each sample was first normalized for transfection efficiency by co-transfection with the pRL-TK plasmid. The transcriptional activity of promoter construct was shown as the luciferase activity relative to that of the pGL3-Basic vector (a promoter-less vector) and shown as the mean ± S.D. for three independent experiments. $^{*}P < 0.05$. (**B**) The involvement of Sp1 in regulating CD147 expression. HepG2 cell was transfected with siRNA targeting Sp1 and Sp1 expression vector, respectively. Upper panel, regular RT-PCR. Lower panel, real-time quantitative RT-PCR. Control siRNA was used as negative control.

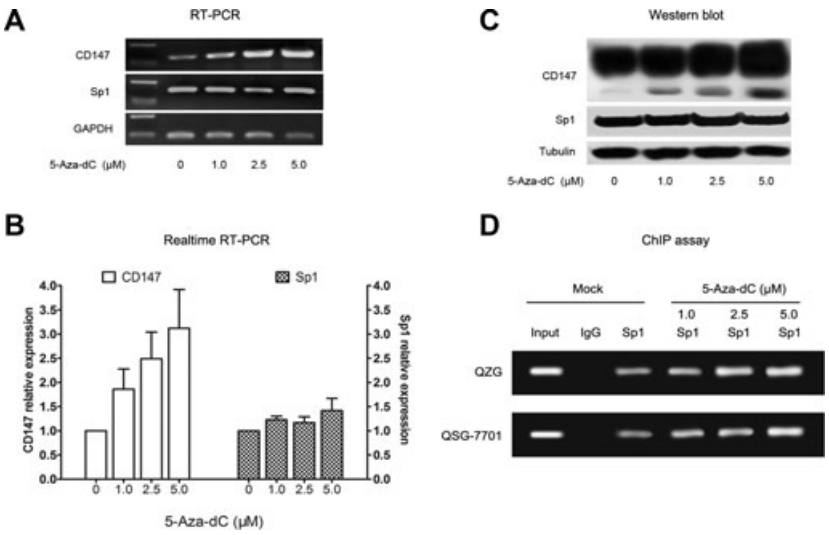

Fig. 4 Promoter hypomethylation up-regulated CD147 expression through increasing Sp1 binding *in vivo*. (**A**, **B** and **C**) The mRNA and protein expression of CD147 and Sp1 detected by regular RT-PCR, real-time quantitative RT-PCR and Western blot in QZG cell treated with different concentration of 5-Aza-dC. (**D**) Demethylation increased the binding of Sp1 to the CD147 promoter *in vivo*. ChIP assay using antibody against Sp1 was performed in before and after 5-Aza-dC treatment QZG cell. The normal rabbit IgG was used as a negative control and Input indicates 5% input DNA, a positive amplification control.

Methylation status interfered the Sp1 binding to the CD147 gene promoter and inhibited CD147 transcriptional activity *in vitro*

To directly examine if Sp1 binds to the putative binding site within the critical CD147 promoter region, EMSA was performed with biotin-labelled oligonucleotides spanning the Sp1-binding sites as probe. Shifted complexes were observed when the labelled wild-type probe was used (Fig. 5A, lane 2), while these complexes disappeared when 100-fold molar excess of unlabelled wild-type probe was added (Fig. 5A, lane 3). EMSA was also performed by using mutant probes in order to understand the contribution of the Sp1-binding sites to the observed binding patterns. The mutation in the Sp1-binding sites dramatically decreased the level of complex formation (Fig. 5A, lanes 4). To further confirm the specific binding of Sp1 to the CD147 minimal promoter, supershift assay was performed with anti-Sp1 antibody. The protein–DNA complex of interest was supershifted by anti-Sp1 antibody (Fig. 5A, lane 6). However, no supershift was observed when control IgG was used (Fig. 5A, lane 5). We also observed that Sp1 could not bind to the methylated CD147 gene promoter probe (Fig. 5A, lane 7). The EMSA results demonstrated that Sp1 specifically bound to the Sp1-binding sites in the CD147 gene promoter and this binding could be interfered by methylation status.

We then analysed the effect of *in vitro* DNA methylation on the CD147 promoter activity through transfecting HEK-293 cells with methylated CpG reporter constructs (Fig. 5B). Our results demonstrated that there was no obvious CD147 promoter activity in HEK-293 cells co-transfected with the metCD147P/pGL3 and an empty expression vector pcDNA3.1 or metCD147P/pGL3 and Sp1/pcDNA3.1, whereas high relative luciferase activity was observed in HEK-293 cells transfected with unmethylated CD147P/pGL3 vector ($P < 0.05$). These results suggest that Sp1-mediated transcriptional activation of CD147 promoter is inhibited by CpG methylation. The results of Figure 3 indicated that promoter hypomethylation up-regulated CD147 expression through increasing Sp1 binding.

Methylation analysis of CD147 promoter in HCC and ANT tissues

BGS was carried out to investigate the methylation level of CD147 promoter in 54 pairs of HCC and ANT tissues. The percentage of methylated CpG sites in all sequenced CpG sites was used to represent the methylation level for each sample as previously described [38]. The representative data from 10 paired tissues were shown in Figure 6A. Our results showed that the methylation level of HCC tissues was significantly lower than that of ANT tissues (45.95 $\pm$ 11.41% *versus* 12.5 $\pm$ 5.44%, $P < 0.05$). Similar results were also obtained from MSP analysis in which methylation of CD147 promoter was observed in 12 of 54 (22.22%) HCC tissues and 25 of 54 (46.3%) ANT tissues ($P < 0.05$). The representative MSP amplifications from 10 paired tissues were shown in Figure 6B. To analyse the correlation between CD147 expression and methylation status of CD147 promoter in HCC tissues, immunohistochemistry analysis was done to detect the CD147 protein expression. Our data showed that 42 of 54 (77.78%) HCC tissues exhibited positive staining. The representative positive and negative staining of CD147 was shown in Figure 6C. Correlation analysis indicated that there was a significant inverse correlation between the CD147 expression level (denoted as total score of immunohistochemistry) and promoter methylation with a correlation coefficient (r) = -0.615 and R^2 linear = 0.378 (Fig. 6D).

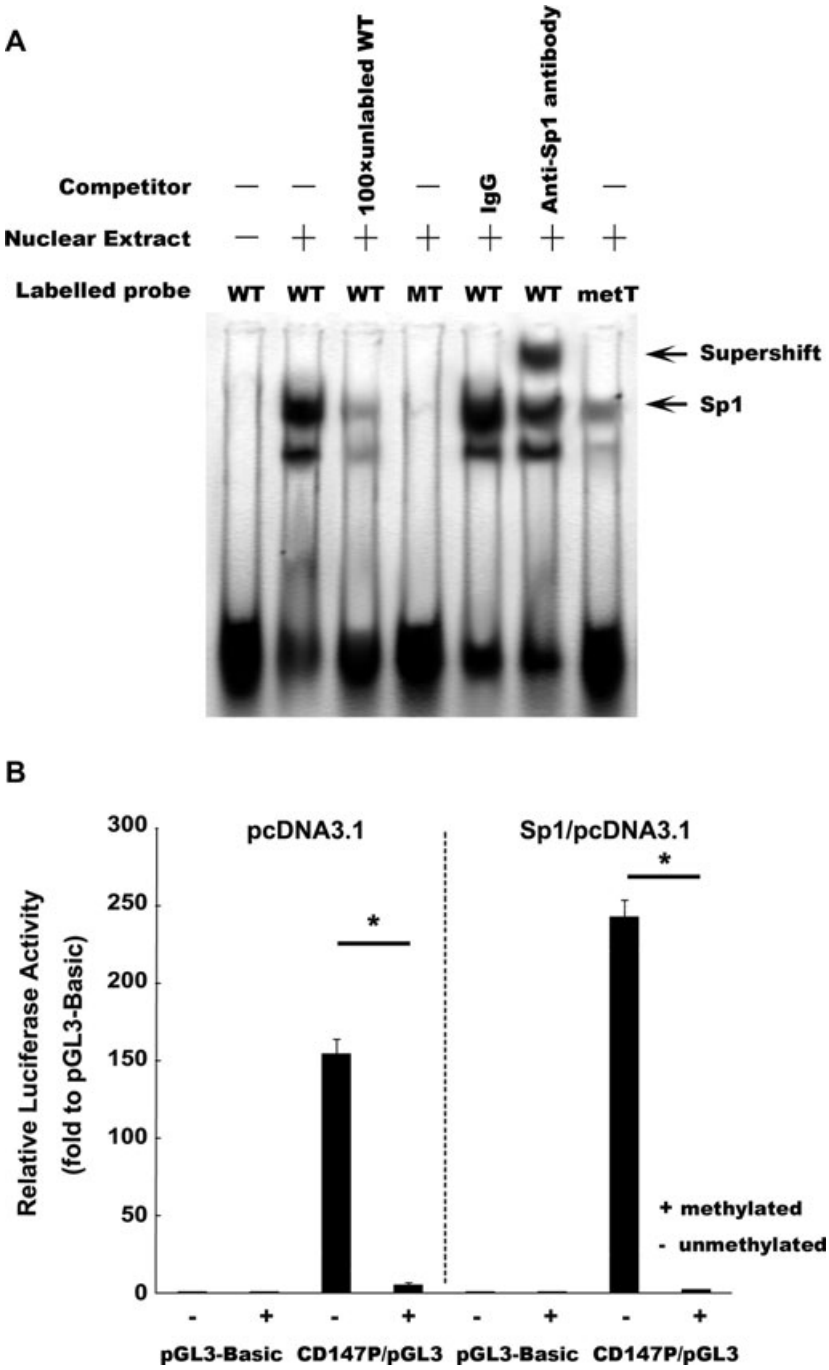

Fig. 5 Methylation status interfered the Sp1 binding to the CD147 gene promoter and inhibited CD147 transcriptional activity *in vitro*. (**A**) Methylation status interfered the Sp1 binding to the CD147 gene promoter *in vitro*. The binding of Sp1 to the CD147 gene promoter was determined by electrophoretic mobility shift analysis (EMSA). By using biotin-labelled 30-bp double-stranded oligonucleotides containing wild, mutated or methylated Sp1-binding sites as probes, EMSAs were performed with the same amount of nuclear extracts from HepG2 cells, and the products were separated on a 5% polyacrylamide gel (lanes 2–7). Lane 1, free probe; lane 2, biotin-labelled wild-type Sp1 consensus oligonucleotides were mixed with nuclear proteins; lane 3, the same reaction was performed as that in lane 2, except for the presence of a 100-fold excess of unlabelled wild-type Sp1 consensus oligonucleotides as a competitor; lanes 4, binding assays of biotin-labelled mutant-type Sp1 consensus oligonucleotides mixed with nuclear proteins; lanes 5–6, 1 μg each of IgG and anti-Sp1 antibody were added to the binding reaction mixtures with biotin-labelled wild-type probe; lane 7, binding assays of biotin-labelled methylated Sp1 consensus oligonucleotides mixed with nuclear proteins. (**B**) Analysis for the effect of *in vitro* DNA methylation on the CD147 promoter activity through the transfection of HEK-293 cells with methylated CpG reporter constructs. CD147P/pGL3 treated or untreated with SssI methylase was co-transfected with the pcDNA3.1 or Sp1/pcDNA3.1 into HEK-293 cells with pGL3-Basic as control. The relative luciferase activity was denoted as above-mentioned method and also expressed as the mean ± S.D. for three independent experiments. $^{*}P < 0.05$.

Hypomethylation of CD147 gene was associated with poor prognosis in HCC patients

Associations of CD147 promoter methylation with clinicopathological characters were analysed in 54 HCC patients (Table 2). Our results showed that there was no obvious association between methylation status of CD147 promoter derived from MSP analysis and age, gender, differentiation or tumour size of HCC patients ($P = 0.964$, $P = 1.000$, $P = 0.798$ and $P = 0.826$, respectively). Methylated CD147 promoter was more frequently observed in HCC patients with low serum level of α-fetoprotein (AFP; <500 ng/ml) than that in patients with high serum level of

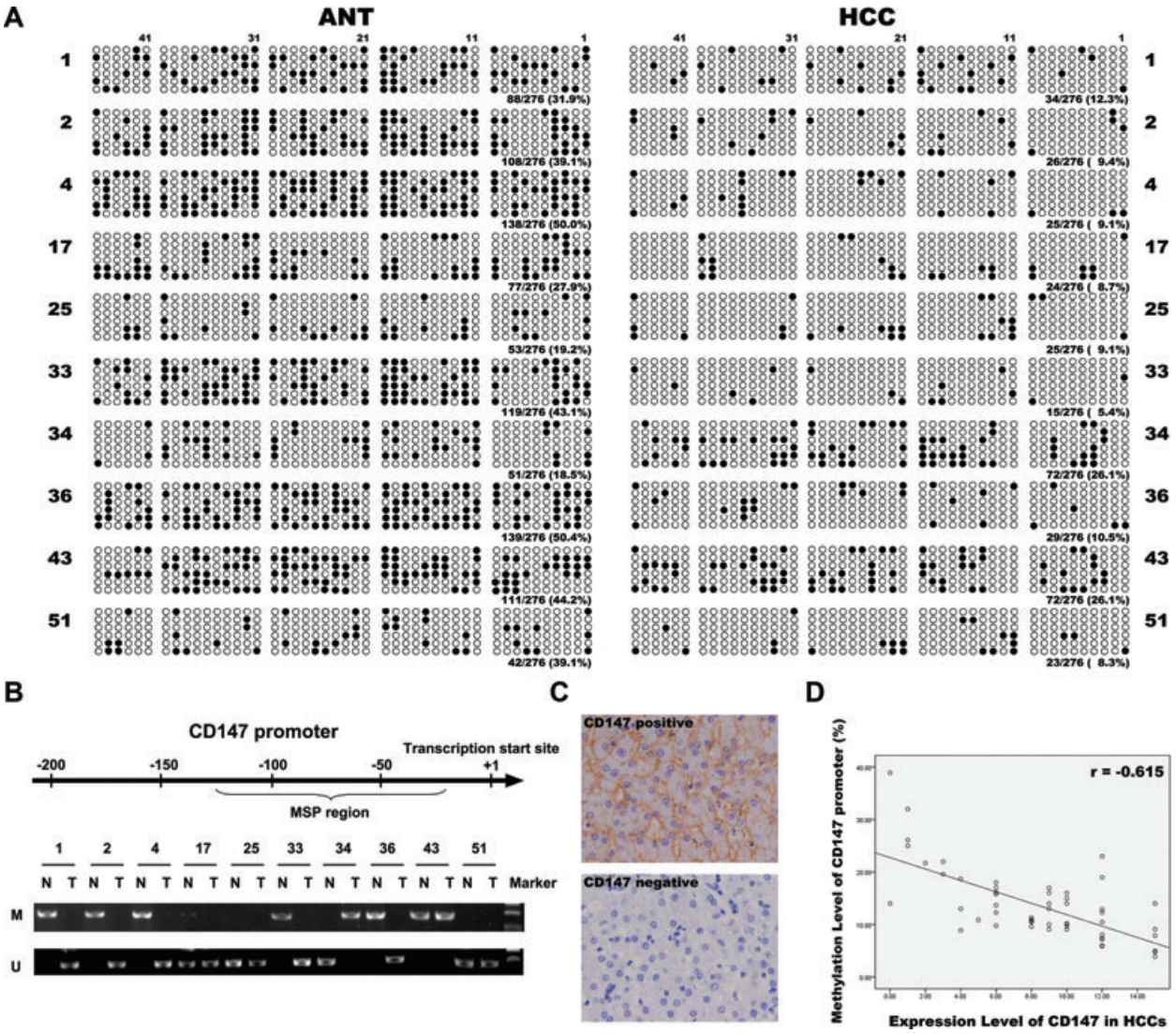

Fig. 6 Analysis of methylation status in CD147 promoter and CD147 expression level in HCC and ANT tissues. (**A**) Representative methylation profiles of CpG island in CD147 promoter in HCC and ANT tissues detected by BGS. Open and closed circles indicate unmethylated and methylated CpG sites, respectively. The percentage of methylated CpG sites in all sequenced CpG sites is shown in parentheses. (**B**) Representative results of MS-PCR analysis in HCC tumour tissues (T) and ANT tissues (N). M: methylation; U: unmethylation. (**C**) Immunohistochemical staining analysis for CD147 expression in HCC tissues. Top, positive CD147 immunostaining. Bottom, negative CD147 immunostaining. Scale bars, 50 μm. (**D**) Analysis for correlation of CD147 expression and its promoter metheylation in HCC tissues by calculating Spearman's ρ method.

AFP (≥500 ng/ml) (66.7% *versus* 33.3%; $P < 0.05$), while unmethylated CD147 promoter was more commonly observed in HCC patients with high serum level of AFP (≥500 ng/ml) than that in patients with low serum level of AFP (<500 ng/ml) (73.8% *versus* 26.2%; $P < 0.05$), indicating a significant association between methylation status of CD147 promoter and serum level of AFP in HCC patients ($P = 0.025$). Prognosis analysis revealed that, compared with patients with ummethylated CD147 promoter, patients with methylated CD147 promoter exhibited a lower recurrence rate (58.3% *versus* 88.1%; $P = 0.019$) and death rate (50% *versus* 83.3%; $P = 0.017$) (Table 2). A Kaplan–Meier analysis showed that HCC patients with methylated CD147 promoter had significantly longer median recurrence duration than patients with unmethylated CD147 promoter (12 months *versus* 4.5 months, $P = 0.022$, log-rank test) (Fig. 7A), and that HCC patients with methylated CD147 promoter had significantly longer median survival duration than patients with unmethylated CD147 promoter (12 months *versus* 7.0 months, $P = 0.023$, log-rank test)

Table 2 Distribution of demographic and clinical variables by methylation status of CD147 promoter in HCC patients

Variable	Methylated ($n = 12$)	Unmethylated ($n = 42$)	*P*-value
	n (%)	*n* (%)	
Age, years			
<60	3 (25)	8 (19)	
≥60	9 (75)	34(81)	0.964*
Sex			
Female	1 (8.3)	3 (7.1)	
Male	11 (91.7)	39 (92.7)	1.000*
AFP level, ng/ml			
<500	8 (66.7)	11 (26.2)	
≥500	4 (33.3)	31 (73.8)	0.025*
Tumour size, cm			
<5	5 (41.7)	19 (45.2)	
≥5	7 (58.3)	23 (44.8)	0.826
Histodifferentiation			
Low	6 (50.0)	23 (54.8)	
Medium	4 (33.3)	15 (35.7)	
High	2 (16.7)	4 (9.5)	0.798†
Recurrence			
Yes	7 (58.3)	37 (88.1)	
No	5 (41.7)	5 (11.9)	0.019
Death			
Yes	6 (50)	35 (83.3)	
No	6 (50)	7 (16.7)	0.017

*Continuity correction, †Fisher's exact test.

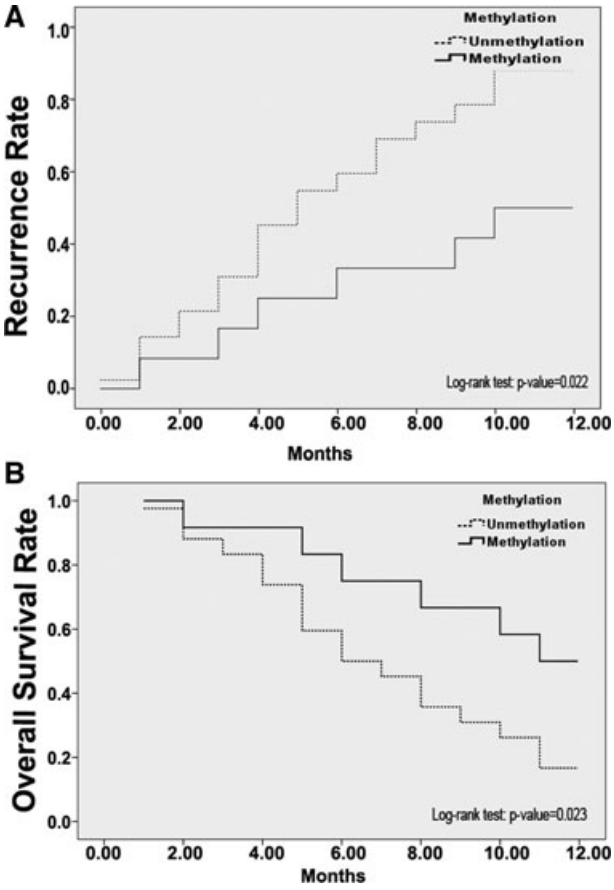

Fig. 7 Kaplan–Meier recurrence and overall survival estimates based on the methylation status of CD147 promoter for all HCC patients. (**A**) Kaplan–Meier recurrence curve. (**B**) Kaplan–Meier overall survival curve.

(Fig. 7B). These results indicated that hypomethylation of CD147 gene was associated with poor prognosis in HCC patients.

Discussion

In our study, we evaluated the effect of methylation status of CD147 promoter on CD147 expression and the possible functional mechanism. Our results demonstrated that the hypomethylation of CD147 promoter significantly up-regulated CD147 expression by increasing the Sp1 binding. In addition, we also observed that low methylation of CD147 promoter was significantly associated with poor prognosis in HCC patients.

A series of previous reports demonstrated that CD147 is overexpressed in various malignant tumour cells and involved in tumour progression [39, 40]. Our previous study systematically evaluated the CD147 expression profile in human normal and tumour tissues from 14 organs and found that epithelium-derived carcinoma exhibited an overall CD147 positivity rate of 67.76%, while sarcomas was 27.34% and normal epithelial tissues was only 5.18% [8]. In the present study, consistent result was obtained in six human HCC cell lines and normal liver cell lines, indicating that the CD147 expression was remarkably up-regulated in HCC cell lines. Our previous and present studies also consistently showed that the up-regulation of CD147 mRNA expression existed in carcinoma tissues and cancer cell lines, suggesting a possibility of regulation at transcriptional level for CD147 overexpression [6, 8]. However, to date, there has been no report to elucidate the mechanism underlying the transcriptional up-regulation of CD147.

Many reports have emphasized that epigenetic modifications play crucial roles in the transcriptional regulation of gene expression [21]. Previous analyses showed that promoter region of CD147 gene contains CpG islands that have numerous potential

DNA methylation sites (CpG motifs) [27, 28], suggesting that methylation of CpG sites in promoter regions might be an important regulatory factor for CD147 expression. Our data showed that both HCC cell lines and tissues examined in our study exhibited a significantly lower methylation level of CD147 promoter region than corresponding controls and there was a significant inverse correlation between the CD147 expression level and promoter methylation in HCC tissues. These results demonstrated that increased expression of CD147 gene in HCC is associated with hypomethylation of CD147 gene promoter region. Our study also demonstrated that CD147 expression in QZG cell was significantly increased in a dose-dependent manner after treatment with 5-Aza-dC and *in vitro* methylation treatment of CD147 promoter resulted in the elimination of transcriptional activity. These results strongly suggest that hypomethylation of CD147 promoter region may be a key contributor to the increased CD147 expression in cancer cell lines and tissues.

Promoter hypomethylation induces histone hyperacetylation and release of other transcriptional repressors such as MeCP2, which can increase Sp1 binding and subsequent transactivation [41]. Our study shows that the specific Sp1 binding sites are located in the CpG region of CD147 promoter. Perhaps in HCC, this hypomethylation of CD147 gene may results in increasing Sp1 binding and subsequent transactivation. Recently, growing number of transcription factor had been identified possessing similar structural and transcriptional properties to Sp1, such as Sp2, Sp3, Sp4 and KLFs, forming Sp/KLF family. We considered that these Sp/KLF family members mentioned above may involve in Sp1 mediated CD147 transcriptional regulation, and we will investigate their detailed regulation mechanisms in the future work.

Previous reports mainly focused on the abnormal methylation of promoter regions in tumour suppressor genes during cancer development and progression. Many potential cancer biomarkers such as methylated p16INK4a and p15INK4b were developed in [42, 43]. Although the global hypomethylation in cancers has been frequently observed, the demethylation of individual genes has not received much attention until recently. Increasing evidence has indicated that DNA demethylation significantly contribute to the transcriptional reactivation of important genes in cancers. Genome-wide hypomethylation in HCC is a continuing process that persists throughout the lifetime of the tumour cells rather than a historical event occurring in pre-cancer stages or in cell origins for HCC [44]. The hypomethylation and overexpression of proto-oncogenes *c-myc* and *c-jun* have been found in chemically induced tumours in mouse liver [45]. Additionally, the correlation of hypomethylation and overexpression of *MAGE-A1*, *MAGE-A3*, insulin-like growth factor (*IGF*)-*II* and trefoil factor (*TFF*)*3* genes were also clearly observed in human HCC tissues [46–48].

Our study also sought to elucidate the molecular mechanisms underlying demethylation-dependent CD147 overexpression in cancer cells. Previous studies have suggested several possible mechanisms for methylation-induced transcriptional inhibition of gene expression. Among these mechanisms, the direct binding block of transcription factors to their CpG-containing binding sites seems to play more important roles in transcriptional inhibition [22]. For example, previous reports have suggested that the methylation of Sp1 consensus site in promoter region might block Sp1 binding and thus inhibit transcription [25, 26]. Our sequence analysis showed that there were four Sp1 binding sites in the CpG island region of CD147 promoter. Therefore, we examined the effect of Sp1 on transcriptional activity of CD147 promoter. Our results demonstrated that Sp1 was an essential factor in the mediation of CD147 promoter activity. We further confirmed the role of Sp1 in regulation of CD147 through down-regulating Sp1 expression by RNA interference or up-regulating Sp1 expression by co-transfected expression plasmid. Consistent with our reporter assays demonstrating a crucial role for Sp1 in CD147 promoter activity, changing Sp1 expression dramatically influenced CD147 expression. BGS analysis also indicated that the methylation level of specific CpG sites in potential Sp1 binding motifs of CD147 promoter was significantly lower in all cancer cell lines than that in corresponding normal cell lines. Further investigation indicated that Sp1-mediated transcriptional activation of CD147 promoter was inhibited by CpG methylation. Demethylation of CD147 could increase the Sp1 binding to the promoter region. Thus, it follows that the up-regulation of CD147 expression induced by promoter demethylation in HCC cells may result from the binding of additional transcription factor Sp1 to the promoter region.

To explore the clinical significance of CD147 promoter hypomethylation, we also analysed the association between methylation status of CD147 promoter and clinicopathological characters and prognosis in 54 HCC patients. Our data showed that methylation status of CD147 promoter was not associated with age, gender and tumour size in HCC patients. Interestingly, methylated CD147 promoter was more frequently observed in HCC patients with low serum level of AFP than that in patients with high serum level of AFP (66.7% *versus* 33.3%; $P < 0.05$). AFP, a tumour marker for HCC, is highly expressed during foetal liver development, but is rapidly repressed within a few weeks after birth. Approximately 70% of HCC are positive for AFP, and levels of AFP increase with tumour progression [49]. Renewed AFP expression occurs only when the differentiated liver exits G_0 and enters a program of resumed cellular proliferation, as a consequence of hepatic tumorigenesis, liver regeneration, or tissue damage due to chronic disease [50, 51]. CD147 is highly expressed on the HCC and the expression level is significantly associated with the serum AFP level [52, 53]. Other research works also reported the correlation of MMPs level and serum AFP level. The regulation of AFP is rather complicated. A sequence of -200bp of the AFP gene is characterized by tissue-specific promoter activity and contains multiple overlapping binding sites for ubiquitous and tissue-specific transcription factors, including activator protein (AP)-1, CCAAT enhancer binding proteins (C/EBP), Hepatic Nuclear Factor (HNF)1, AT-binding transcription factor (ATBF)1, etc. [54]. These TFs may also involve in the promoter methylation mediated expression regulation of CD147. As we shown in our study, the methylated CD147 promoter was more frequently observed in HCC patients with low serum level of AFP than that in patients with high serum level of AFP. The significant association of CD147 methylation and AFP levels suggests that

CD147 methylation status might have potential as a diagnostic marker in HCC. The up-regulation of CD147 and renewed AFP expression in HCC may be the common consequences of tumour genesis and progression. Further study is necessary to clarify the link between AFP expression and methylation of CD147, and global analysis will be of increasing importance in the identification of novel hypomethylated gene in HCC.

Survival analysis revealed that patients with unmethylated CD147 promoter had a significantly higher recurrence rate and death rate than patients with methylated CD147 promoter, suggesting that hypomethylation of CD147 gene was associated with poor prognosis in HCC patients. Our earlier study demonstrated that the CD147 expression at protein level is significantly related to the prognosis of HCC patients [53]. Previous reports show that tumour biomarkers based on the detection of DNA methylation pattern are of higher specificity than the traditional ones based on the test of mRNA and protein expressions [55]. As a result, our results indicated a potential of CD147 methylation status as a prognosis biomarker for HCC management.

In conclusion, our data suggest that demethylation of the CpG island at the promoter of CD147 gene may be a key mechanism for CD147 up-regulation in HCC cells and that the effect of CD147 promoter hypomethylation on CD147 expression is mainly mediated by increasing the binding of transcription factor Sp1 to the Sp1 binding sites in the CD147 promoter region, thus resulting in the up-regulation of the CD147 promoter activity. This is the first demonstration of a methylation-dependent mechanism responsible for regulation of CD147 expression. In addition, we also observed that low methylation of CD147 promoter was significantly associated with poor prognosis in HCC patients, indicating that the methylation status of CD147 promoter may serve as a prognosis biomarker with potential applications in HCC management.

Acknowledgements

This work was supported by National Natural Science Foundation of China (30872927, 30772479 and 30872514) and National Basic Research Program of China (2009CB521704).

Conflict of interest

The authors confirm that there are no conflicts of interest.

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