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Transfection of HCVc Improves hTERT Expression Through STAT3 Pathway by Epigenetic Regulation in Huh7 Cells

Ning Guo,[1] Di Cheng,[2] Zhi Hua Li,[2] Quan Bo Zhou,[3] Jia Jia Zhou,[3] Qing Lin,[3] Bing Zeng,[3] Qiaofang Liao,[2] and Ru Fu Chen[3*]

[1]*Department of Organ Transplant,Qi Lu hosptial of Shan Dong university,Jinan,250012, China*

[2]*Department of Oncology, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou 510120, China*

[3]*Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou 510120, China*

ABSTRACT

Previous studies showed that transient transfection of HCVc improved hTERT expression in hepatoma cell lines and it was noteworthy that phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and DNA methyltransferases (DNMTs) were up regulated simultaneously. This study was designed to investigate the role of epigenetic regulation in the process of hTERT up regulation after HCVc transfection. Q-PCR and Western blot were used to analyze the expression of pSTAT3, DNMT1, and hTERT after the transfection of HCVc in hepatoma cell line Huh7. Proliferation and hTERT activity of Huh7 after HCVc transfection were examined by CCK8 and ELISA, respectively. Then, we blocked the JAK/STAT3 pathway or inhibited DNMT1 expression to investigate the regulation of pSTAT3, DNMT1, and hTERT. Methylation status of the promoter of hTERT gene was monitored by MS-PCR. Cell proliferation, hTERT expression level and activity of hTERT were promoted after HCVc transfection. The expression of pSTAT3 and DNMT1 were up-regulated simultaneously. DNMT1 and hTERT were down-regulated after blocking JAK/STAT3 pathway and the expression of hTERT weakened with DNMT1 inhibition. MS-PCR showed HCVc transfection increased the methylation level of hTERT promoter, and this effect was weakened after blocking the JAK/STAT3 pathway or with the treatment with DNMT1 inhibitor. HCVc transfection improved hTERT expression via epigenetic regulation. JAK/STAT3 pathway could be one of the essential factors in regulating DNMT1 expression during this process. J. Cell. Biochem. 113: 3419–3426, 2012.

KEY WORDS: HEPATIC CARCINOMA; HCV; DNA METHYLTRANSFERASE; TELOMERASE; STAT3

Hepatitis C virus (HCV) infection is a global public health problem leading to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) and the incidence of HCC is increased in HCV related cirrhosis [Heathcote, 2004]. The core protein of HCV (HCVc) is a structural protein encoded by HCV genome conservative region and was considered as an important role in tumor genesis [Yamamoto et al., 2004; Chen et al., 2007]. Although the oncogenicity of HCV core protein has been studied extensively, the molecular mechanisms responsible for this activity appeared complicated. Core protein interacts with numerous enzymatic systems of the hepatocyte chiefly through activation or repression of proteins such as p53, p21, other transcriptional factors, proto-oncogenes, growth factors, and elements of apoptosis [Kasprzak and Adamek, 2008].

Telomeres are complex nucleoprotein structures situated at the ends of chromosomes that protect ends and prevent their being sensed as double strand breaks [Satyanarayana et al., 2004]. The mechanism to restore telomeric sequences was lack in normal cells, therefore, telomeres shorten progressively with each round of mitosis. When telomeres reach the threshold length, cells will stop growing or go apoptosis. Human telomerase reverse transcriptase (hTERT) is a specialized enzyme complex which is capable of maintaining telomere lengths. As we know, the enzyme is not expressed in most somatic cells and progressive shortening of telomeres leads to a finite cellular life span. However, telomerase expression is preserved in germ cells, stem cells, and neoplastic cells. The activation of telomerase expression is a critical step in over 80% of human cancers. It has been reported that the transfection of HCVc

Ning Guo and Di Cheng contributed equally to this work.

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*Correspondence to: Dr. Ru Fu Chen, Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, 107 Yan Jiangxi Road, Guangzhou 510120, China. E-mail: gnn2000@yahoo.cn

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enhances telomerase activity in hepatoma cell Huh7 [Zhaowen et al., 2010], but the mechanism has not been clarified yet. Gonzalo et al. [2006] reported that TERT could be regulated by DNA methyltransferases (DNMTs) and Zhang et al. [2005a] reported that DNMT1 was a target of signal transducer and activator of transcription 3(STAT3). Given the results of these previous studies, we speculated HCVc transfection may activate STAT pathway and promote hTERT expression through epigenetic regulation by DNMT1. This study was designed to further verify our hypothesis.

MATERIALS AND METHODS

MATERIALS AND REAGENTS

The human hepatoma cell line Huh7 was kindly provided by Professor Zhuang Shimei (Sun Yat-sen University, China). The plasmid pGPU6/GFP/NEO-HCVc and empty pGPU6/GFP/NEO were kindly provided by Professor Wang shangwu (Sun Yat-sen University, China). CCK8 reagent was purchased from Dojindo Biotechnology (Japan), Q-PCR kit was purchased from TaKaRa Biotechnology (Japan). Cell culture medium and fetal bovine serum were purchased from GIBCO (USA). TRAPeze® ELISA Telomerase Detection Kit was purchased from Millipore (USA). RIPA buffer was purchased from Beyotime (China). Supersignal® West Pico Chemiluminescent Substrate was purchased from Thermo (USA). Antibodies against HCVc, STAT3, pSTAT3, DNMT1, hTERT, and Tubulin were purchased from Abcam (UK). The transfection reagent Lipofectamine™ LTX with Plus™ Reagent was purchased from Invitrogen (USA). MethylDetector Kit was purchased from Active-Motif (USA).

ANALYSIS OF hTERT EXPRESSION AFTER HCVc TRANSFECTION

The hepatoma cell line Huh7 were cultured at 37°C in DMEM medium containing 10% FBS. The cells were divided into normal group, empty plasmid transfected group and HCVc transfected group. The plasmid pGPU6/GFP/NEO-HCVc was transiently transfected into Huh7 cell line to make an overexpression of HCVc. Lipofectamine™ LTX with Plus™ Reagent (USA) was used as the transfection reagent. The total RNA of Huh7 was extracted by RNAiso plus (TaKaRa, Japan) and total protein was extracted by RIPA buffer at 24, 48, and 72 h after transfection. Q-PCR and Western blot were performed to check the mRNA and protein level of hTERT and ELISA experiment were performed to exam activity of hTERT.

To perform Q-PCR, RT reaction was performed using PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Japan). The products were run on a Roche LC480 instrument with the SYBR® Premix Ex Taq™ II. The reaction was performed as described in manufacturer's protocol. The Q-PCR reaction proceeded as follows: 95°C for 30 s followed by 40 cycles of extension with each consisting of 90°C for 30 s and 60°C for 30 s. Sequences of Q-PCR primers of hTERT were as following: forward: 5′-TCATCGCCAGCATCATCAAAC-3′; reverse: 5′-ATGTACGGCTGGAGGTCT GTCA-3′. Primers of internal control of beta-actin were as follows: forward: 5′-TGGCACCCAGCACAATGAA-3′; reverse: 5′-CTAAGTCATAGTCCGCCTAGA AGCA-3′. The results were analyzed by LC-480 system.

To perform Western blot, equal amounts of total protein were loaded onto the 8% SDS–polyacrylamide gel and then subsequently transferred to a PVDF membrane via electroblotting. The membranes were blocked with 5% nonfat milk in TBST for 1 h at room temperature and incubated with hTERT antibodies overnight at 4°C. After washing with TBST buffer three times (5 min each), the PVDF membranes were incubated with anti-rabbit IgG–horseradish–peroxidase for 1 h at room temperature. Supersignal® West Pico Chemiluminescent Substrate was used to detect the bands. Tubulin was used as the internal control. The results of Western blot were analyzed by software Quantity One software.

ANALYSIS OF CELL PROLIFERATION AND ACTIVITY OF hTERT AFTER TRANSFECTION OF HCVc

The cell proliferation status was examined by CCK8 assay following the protocol as below. The cells were divided into normal group, empty plasmid transfected group and HCVc transfected group and cultured in 96-well plates for an initial density of 1×10^3 cells/well in 200 μl of growth medium (3 wells for each group) at 37°C. Once the cells grew into the logarithmic growth phase (8 h after planting), 20 μl of CCK8 was added into each well. After incubating for 4 h, the absorbance at 630 nm was detected with SpectraMax M5 ELISA reader every 12 h. SPSS 16.0 was used for variance analysis.

The telomerase activity was examined by TRAP–ELISA analysis. Cells were collected by trypsinization and washed twice with ice-cold PBS solution. HCVc transient transfected cells (1×10^5) were for TRAP–ELISA assay and the untreated cells were used as the control group, and the process was performed following the protocol of Trapeze ELISA telomerase detection Kit. The cells were collected and TRAP–ELISA was performed at 24, 48, and 72 h after transfection and was detected by SpectraMax M5 ELISA reader under reading OD value at 450/690 nm.

ANALYSIS OF STAT3, pSTAT3, AND DNMT1 EXPRESSION AFTER HCVc TRANSFECTION

This step was performed to observe the changes of STAT3, pSTAT3, and DNMT1 after HCVc transfection in Huh7 for 24, 48, and 72 h, respectively. The cells were divided into three groups: normal group, empty plasmid transfected group, and HCVc transfected group. Total RNA and total protein were extracted as described above. The expression of STAT3 and pSTAT3 were monitored in protein level by Western blot and the expression of DNMT1 was monitored in both RNA and protein level via realtime PCR and Western blot. The protocol of Q-PCR and Western blot were as described above. Sequences of Q-PCR primers of DNMT1 were as follows: forward: 5′-GCCAACGAGTCTGGCTTTGAG-3′ reverse: 5′-GTGTCGATGGGACACAGG TGA-3′. The results of Q-PCR were analyzed using LC-480 system and the results of Western blot were analyzed using software Quantity One.

ANALYSIS OF THE CHANGES OF DNMT1 AND hTERT FOLLOWING INHIBITION OF JAK/STAT3 PATHWAY BY TYRPHOSTIN AG 490(AG490)

The hepatoma cell line Huh7 was cultured and transfected with HCVc plasmid as described in step 1. After 12 h of transfection, the culture medium was changed to DMEM medium containing 10%

FBS, and 100 μmol/L of AG490 reagent was added to the culture medium after 42 h of transfection. Total protein and RNA of the cell lines were extracted after 48 h of culture. Q-PCR and Western blot were performed to compare the RNA and protein level of DNMT1 and hTERT between the groups with or without AG490 treatment. The primers of DNMT1 and hTERT were the same as described above.

TREATMENT OF THE CELLS BY DNMTs INHIBITOR 5-AZA-2-DEOXY-CYTIDINE (AZA)

Huh7 was cultured and transfected with HCVc plasmid as step1 described and cultured for 36 h. DNMTs inhibitor reagent 5-aza-2-deoxy-cytidine (AZA) was added to the culture medium to achieve a concentration of 12 μmol/L and the cells were cultured for another 12 h. Total protein and RNA of the cell line was extracted after 48 h of culture. Q-PCR and Western blot were performed to compare the RNA and protein level of DNMT1 and hTERT between the groups with or without AZA treatment.

METHYLATION-SPECIFIC POLYMERASE CHAIN REACTION (MS-PCR) OF hTERT PROMOTER

The methylation status in *hTERT* promoter region was monitored via MS-PCR using MethylDetector Kit (Active-Motif, Carlsbad) according to manufacturer's protocol. To amplify the bisulfate-converted hTERT promoter sequences, MS-PCR primers were designed for an upstream (−625 bp from transcription start site) and downstream region (encompassing the transcription and translation start sites) of the hTERT promoter according a formerly designed protocol [Zinn et al., 2007]. The primer sequences could be found in Table I. We analyzed the methylation status of hTERT promoter region in the following conditions: (1) Normal condition without any treatment; (2) HCVc transfection for 48 h; (3) HCVc transfection and AG490 treatment; and (4) HCVc transfection and AZA treatment.

RESULTS

HCV TRANSFECTION PROMOTED hTERT EXPRESSION

Fluorescence microscopy showed that the cells were in good condition after transfection of HCVc plasmid and demonstrated the strongest green fluorescence at 48 h after transfection (Fig. 1A). The results of Q-PCR and Western blot results for hTERT expression were shown in Figure 1B,C. It was shown that the expression of hTERT was higher in the HCVc transfected groups than that in the empty plasmid transfected group and untreated group. There is no significant difference between the empty plasmid transfected group and untreated group. The highest expression of hTERT occurred at 48 h after transfection. This figure indicated that hTERT expression could be regulated by HCVc transfection in hepatoma cells. It can be deduced that HCVc could improve the expression of hTERT by an unknown mechanism.

THE TRANSFECTION OF HCVc UP-REGULATED PROLIFERATION ABILITY AND hTERT ACTIVITY OF HUH7 CELLS

The growth curve of Huh7 cells transfected with HCV plasmid, empty plasmid, and the untransfected cells were shown in Figure 2A. The results suggested that the HCVc transfected Huh7 cell lines grew faster than the nontransfected cell lines, but no significant difference for the growth was observed between empty plasmid transfected group and untransfected group in Huh7 cells. The hTERT activity was detected by ELISA as shown in Figure 2B. The hTERT activity was increased along with the expression of hTERT and reached its maximum at 48 h after HCVc transfection.

pSTAT3, DNMT1 WERE UP REGULATED AS WELL AS hTERT AFTER HCVc TRANSFECTION

STAT3 and pSTAT3 expression were detected by Western blot analysis. It was shown that the expression of pSTAT3 was higher in the HCVc transfected groups than that in empty plasmid transfected group and untreated group (Fig. 3A). DNMT1 showed the same trend as did pSTAT3 and the Q-PCR result confirmed that DNMT1 could be up regulated after the transfection of HCVc (Fig. 3B,C). However, there was no significant change of STAT3 expression after HCVc transfection, indicating that pSTAT3 was the functional form rather than STAT3.

THE UP REGULATION OF DNMT1 AND hTERT BY HCVc TRANSFECTION COULD BE BLOCKED BY AG490

Western blot results showed that pSTAT3 was down regulated by 48.7 ± 3.6% in comparison with the control group after AG490 treatment (Fig. 4A). It was suggested that DNMT1 and hTERT were also down-regulated by 30.2 ± 4.5% and 37.3 ± 5.6% in protein level (Fig. 4B), and 50.1 ± 6.5% and 63.3 ± 0.6% in RNA level (Fig. 4C) after blocking the JAK/STAT3 pathway by AG490 treatment.

hTERT WAS DOWN-REGULATED AFTER THE INHIBITION OF DNMTs BY AZA

AZA reagent successfully reduced the expression of DNMT 1 in both mRNA and protein level. The results of Q-PCR showed that both DNMT1 and hTERT were down-regulated after the treatment with

TABLE I. Primers Sequence Information of MS-PCR for hTERT Promoter

Primer name	Sequence (5′ to 3′)	Product size (bp)	T_m (°C)
TERT MSP-upU	F: GGGAGGTATTTTGGGAGGTTTTGT R: CAAACTCCAAACACCACAAATACCA	126	58
TERT MSP-upM	F: GAGGTATTTCGGGAGGTTTCGC R: ACTCCGAACACCACGAATACCG	121	58
TERT MSP-downU	F: TTGTGGTTTTGTTTTTTTTTTGTGGT R: ACACACAACTCAACAACAAAAAACACA	125	60
TERT MSP-downM	F: GGTTTCGTTTTTTTTTTGCGGC R: GACTCGACAACGAAAAACGCG	115	60

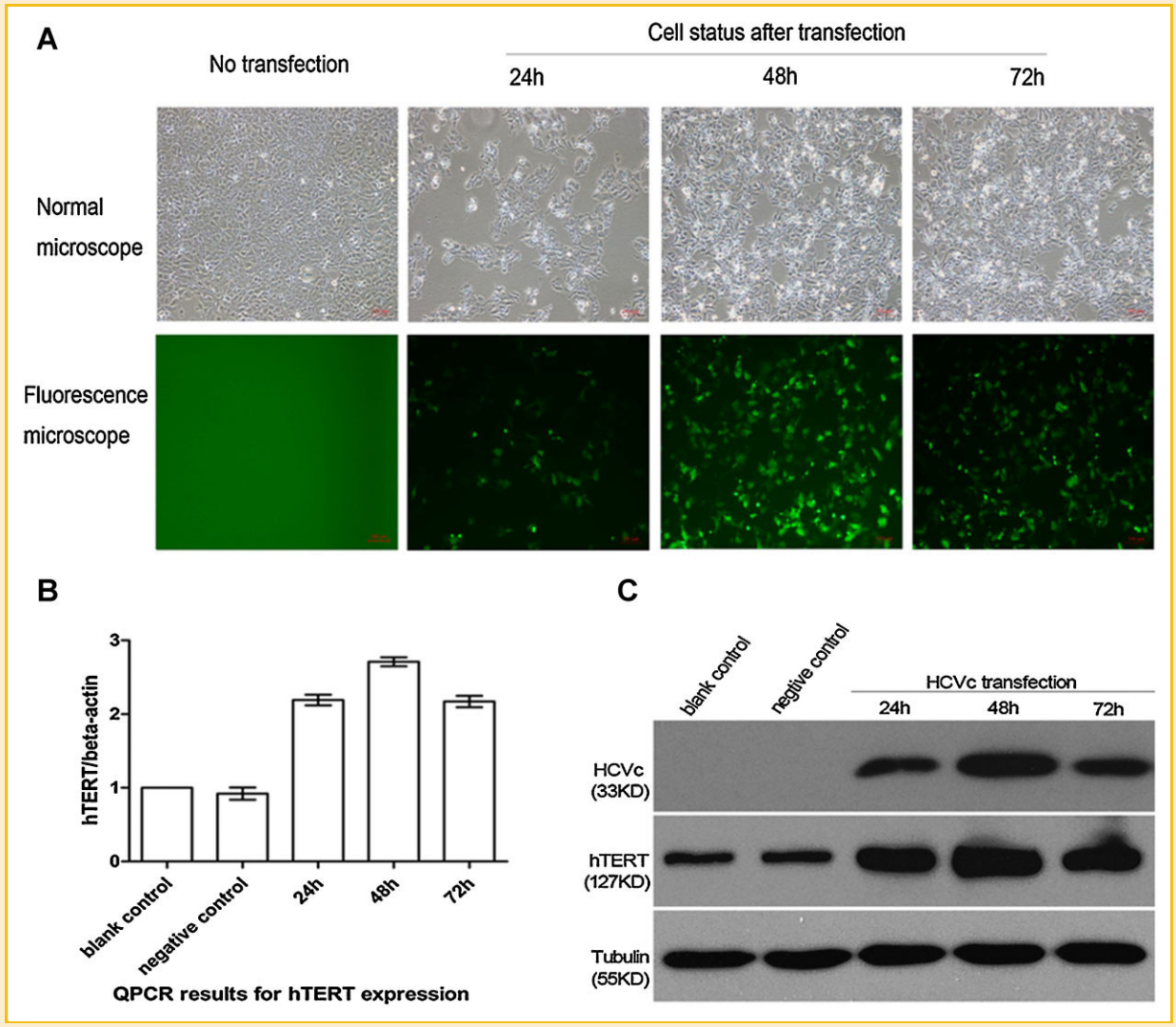

Fig. 1. Cell status and expression of hTERT after HCVc transfection. A: The cells showed green fluorescence after being transfected with pGPU6/GFP/NEO-HCVc plasmid and the fluorescence was strongest at 48 h after transfection. B: The result of Q-PCR showed hTERT expression was up regulated by 59.3 ± 9.0%, 149.4 ± 15.1%, and 83.3 ± 8.1% respectively (at 24, 48, and 72 h) after transient transfection of HCVc. C: Western blot showed Huh7 cells started to express HCVc protein after transfection and hTERT expression followed the same trend as HCVc. The highest expression of hTERT protein also occurred at 48 h after transfection. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

AZA (Fig. 5A). The results of Western blot showed that DNMT1 was down-regulated by 53.12 ± 3.1% and the expression of hTERT was down-regulated by 43.35 ± 4.3% in protein level (Fig. 5B).

DETECTION OF THE METHYLATION STATUS OF *hTERT* PROMOTER BY MS-PCR

As shown in Figure 6, HCVc transfection affected the methylation status of hTERT. Overexpression of HCVc could increase DNA methylation level, while inhibiting the JAK/STAT3 pathway decreased it. After we inhibited DNMT1 with AZA, the methylation status of hTERT was also down regulated and the expression of hTERT went down. This phenomenon indicated that HCVc expression was possibly associated with the methylation level of hTERT promoter through JAK/STAT3 pathway.

DISCUSSION AND CONCLUSION

Hepatitis C Virus (HCV) is one of the leading causes of chronic liver disease worldwide. It was estimated that approximately 3% of the world population was infected [Ripoli et al., 2011] and might ultimately cause hepatocellular carcinoma. Therefore, the underlying mechanism of the carcinogenicity of HCV infection is a hot spot of research. A major difficulty to do HCV research is that HCV keens to mutation. Therefore, to find the commonness in all types of hepatitis C virus is of critical importance to elucidate the pathogenesis of hepatitis C. The core protein of HCV (HCVc) is a structural protein encoded by the most conservative region of HCV genome and plays an important role in tumor genesis. Thus, HCVc is considered to be an important research objective.

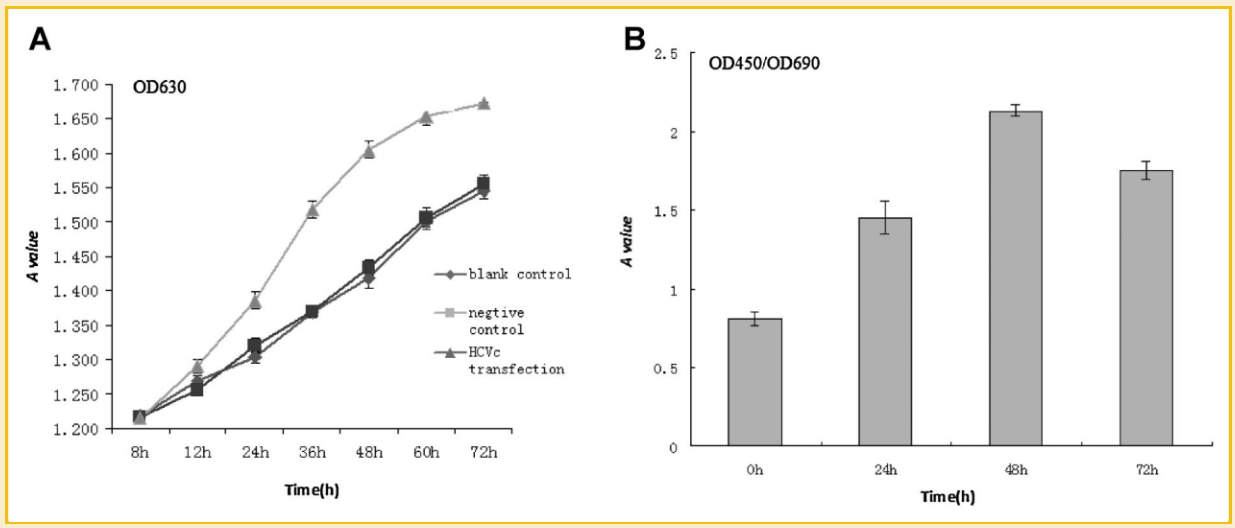

Fig. 2. The proliferation ability and activity of hTERT of Huh7 after HCVc transfection. A: The HCVc transfected Huh7 cell lines grew faster than the nontransfected cell lines, but there was no significant difference between empty plasmid transfected group and untransfected group. B: ELISA result showed that the activity of hTERT was improved after HCVc transfection, and the highest point was at 48 h after transfection.

It has been reported that hTERT played an important role in hepatic carcinoma. Tahara examined the expression of hTERT in liver tissue specimens of 105 cases (including hepatic carcinoma, chronic hepatic disease tissue, and normal liver tissue). The result showed that hTERT was positive in 85% hepatic carcinoma tissues and 55% chronic hepatic disease tissues, but was negative in all normal liver tissues [Tahara et al., 1995]. Further investigation demonstrated that the activity of hTERT was closely related with the

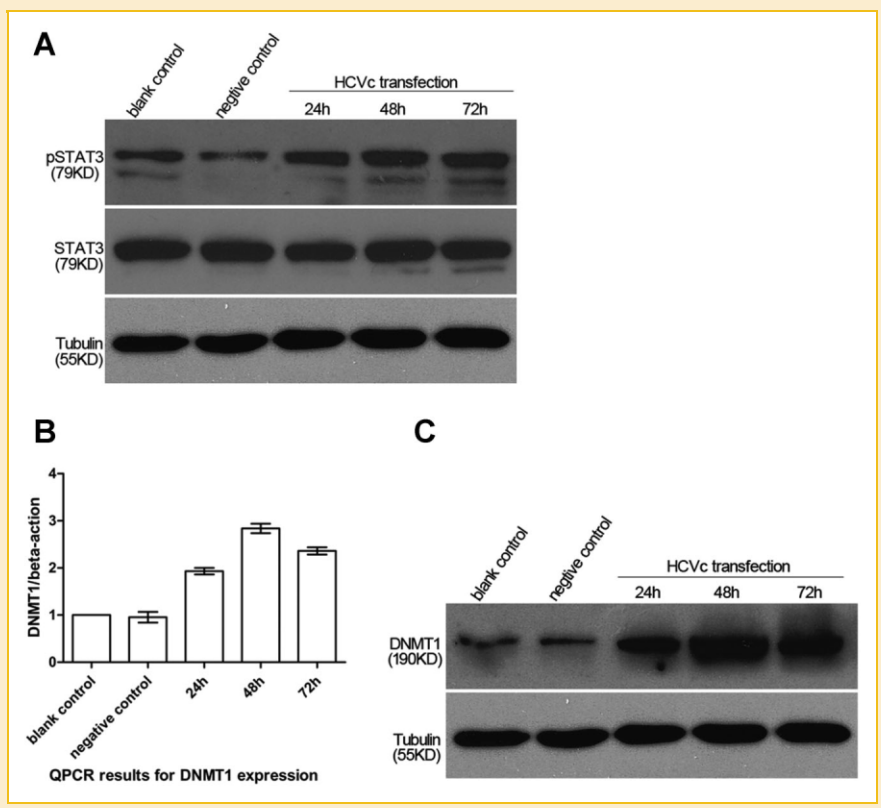

Fig. 3. The expression of STAT3, pSTAT3, and DNMT1 after HCVc transfection. A: The result of Western blot showed pSTAT3 was up-regulated after the transfection of HCVc. However, the expression of STAT3 showed no significant changes after HCVc transfection. B: The result of Q-PCR showed DNMT1 expression was up regulated by 135.3 ± 8.4%, 172.6 ± 6.9%, and 135.4 ± 15.6% respectively (at 24, 48, and 72 h) after transfection of HCVc. C: The Western blot of DNMT1 showed the expression of DNMT1 protein was up regulated after transfection of HCVc and the highest expression level appeared also at 48 h.

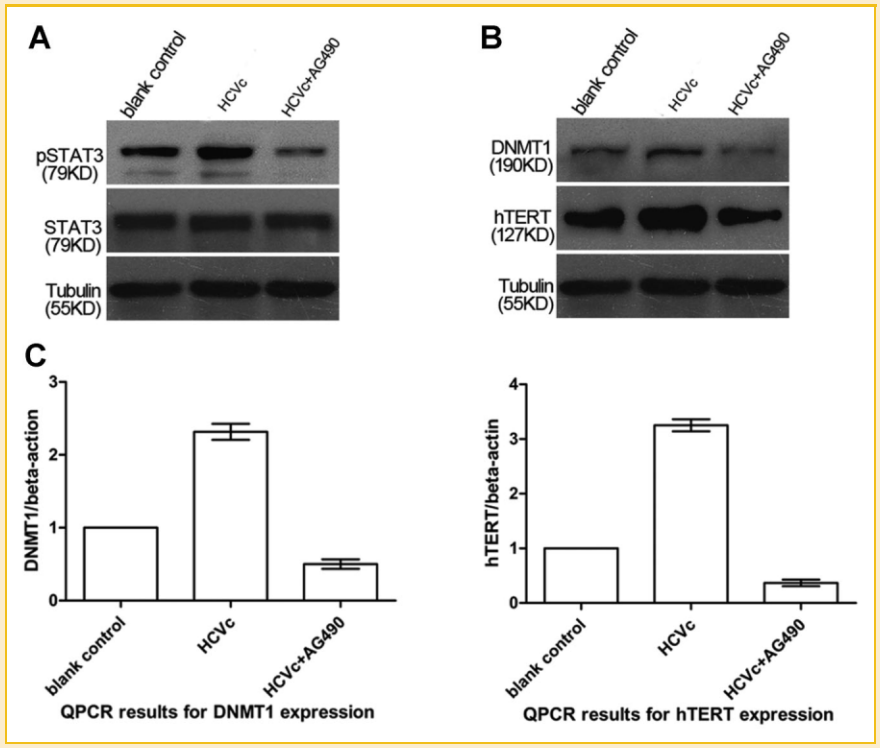

Fig. 4. Expression of hTERT and DNMT1 after AG490 treatment. A: Western blot result showed pSTAT3 was downregulated by 48.7 ± 3.6% in comparison with the control group after AG490 treatment. The effect of upregulation of pSTAT3 by HCVc transfection could be reversed by AG490 treatment. B: The expression of hTERT and DNMT1 protein were also down regulated by 30.2 ± 4.5% and 37.3 ± 5.6% after AG490 treatment. C: The results of Q-PCR showed the same trend as did Western blot. DNMT1 and hTERT were downregulated by 50.1 ± 6.5% and 63.3 ± 0.6% in RNA level in comparison with the blank control group.

occurrence of hepatoma, and was increased following the progress of disease. Inhibition of the activity of hTERT in hepatocellular carcinoma cells can induce apoptosis of hepatoma cells, cell cycle arrest and cell growth delay [Zhang et al., 2002a; Liu et al., 2004]. We found in our present study that HCVc transfection could lead to up-regulation of hTERT expression in hepatoma cell lines. As we know, autonomous repair of telomeres by the enzyme telomerase is considered to be an important process whereby malignant cells avoid replicative senescence, and the induction of the enzyme is a key event in the multi-step process of hepatocarcinogenesis [Hytiroglou and Theise, 2006]. However, the mechanism of hTERT upregulation by HCVc remains unclear.

Epigenetic regulation means heritable changes in gene function that occur without a change in the DNA sequence, so that cells have different patterns of gene expression and biological function with the same sequence of DNA. Epigenetic regulation includes DNA methylation, histone acetylation, histone methylation, and non-coding RNA regulation, etc. Epigenetic regulation plays an important role not only in organ and individual development but also in the development and progression of many diseases, especially in tumorgenesis [Schneider et al., 2002; Yang et al., 2005]. DNA methylation is the most common form of epigenetic regulation. In most cases, affinity of transcription factors is weakened after DNA methylation, transcription factors cannot effectively combine with regulatory sequences and gene transcription is inhibited. However, epigenetic regulation of hTERT is a bit special. Large-scale analysis on tissue samples of bladder, brain, heart, breast, colon and kidney for hTERT promoter sequences showed that in telomerase-positive cells hTERT core promoter region had a high degree of methylation [Guilleret and Benhattar, 2004; Choi et al., 2007]. Guilleret et al. [2002] analyzed the hTERT promoter core sequence of 56 tumor cell lines and tumor tissues from different organs, indicating that the activity of hTERT promoter and the activity of telomerase were positively correlated with the degree of hTERT promoter methylation. They selected telomere enzyme-positive cell line Lan-1, Hela and Col15 cells to do a further study and found that hTERT was deceased after AZA treatment, which indicated that demethylation could reduce the expression of hTERT and epigenetic regulation could be one important potential pathway in the process of hTERT upregulation by HCVc. In this study, we found the same phenomenon in hepatoma cell line Huh7. According to the results shown in Figure 6, we found that in Huh7, DNMT1 mainly affected the core promoter region around −625 to −525 bp but not the CpG region near the initiation site (−25 to 75 bp).

As we know, DNA methyltransferases showed high expression in a variety of tumor cells and were closely related with methylation of promoters of tumor suppressor genes. However, the effect of overexpression of DNMT in hepatoma cells on TERT was still in dispute, and DNMT1 seems to play a major role in this process [Choi et al., 2010; Meeran et al., 2010]. In this study, DNMT1 was upregulated after HCVc transfection (Fig. 3) and expression of hTERT was also up regulated correspondingly, which indicated HCVc affected hTERT through DNMT1 by an unknown mechanism.

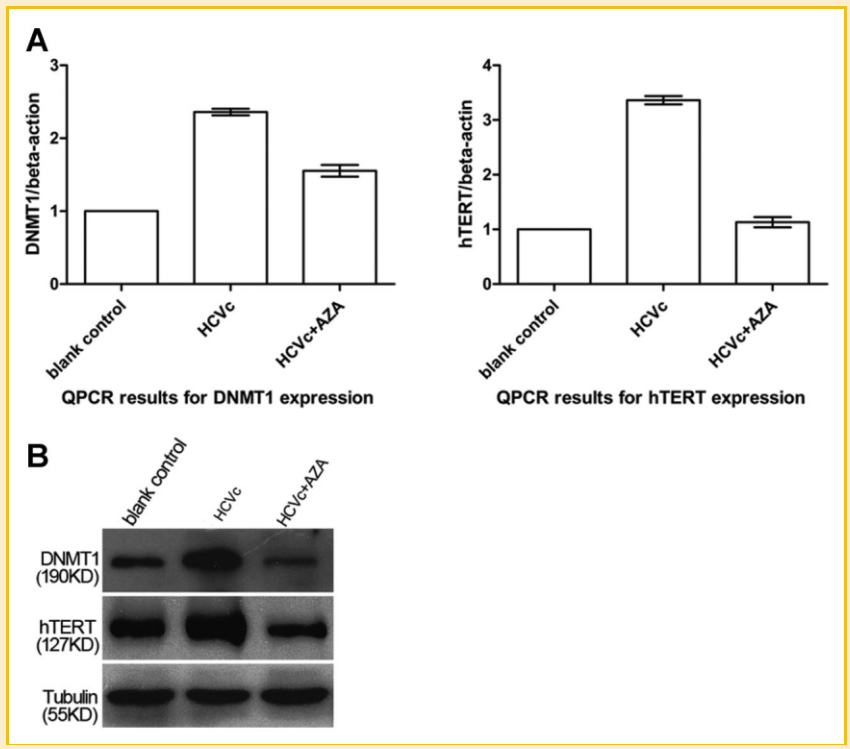

Fig. 5. The expression of hTERT after inhibition of DNMTs by AZA. A: The results of Q-PCR showed the treatment with AZA led to the similar result as with AG490 treatment. The upregulation effect of DNMT1 and hTERT mRNA by HCVc transfection was abolished after the treatment of AZA. B: Western blot showed DNMT1 was down regulated by 53.12 ± 3.1% after AZA treatment. Correspondingly, the expression of hTERT was also down regulated by 43.35 ± 4.3% in protein level.

According to the results of recent studies, we speculated that JAK/STAT3 pathway may be the bridge connecting the two.

Signal transducers and activators of transcription factors belong to the STATs family which includes seven family members. STATs could be activated by janus kinase (JAK) and turned into the active phosphorylation form. STAT3 is an important member of STATs family. Persistent activation of STATs, particularly STAT3, has been implicated in the pathogenesis of a whole spectrum of malignancies [Zhang et al., 2002b, 2005b]. It has been reported that in malignant T lymphocytes, STAT3 induced transcription of DNMT1 by binding to STAT3 SIE/GAS-binding sites identified in promoter of the DNMT1 gene. This finding indicated that in hepatoma cell line Huh7, the same mechanism might exist and JAK/STAT3 pathway might connect HCVc to DNMT1. In our study, we found that the transfection of HCVc could up-regulate the expression of pSTAT3 (STAT3 level remained the same) and DNMT1 (Fig. 3). Treatment with AG490 (JAK/STAT3 pathway inhibitor) could inhibit the up-regulation of DNMT1 caused by HCVc transfection (Fig. 4) and reverse the methylation status of hTERT (Fig. 6). These results indicated that showed HCVc might upregulate DNMT1 through JAK/STAT3 pathway.

In conclusion, HCVc infection may increase the risks of hepatoma by promoting the methylation of the promoter of *hTERT* through upregulation of DNMT1 by JAK/STAT3 pathway. Epigenetic regulation plays an important role in the HCVc-JAK/STAT3-DNMT1-hTERT process in hepatoma cell line, and epigenetic regulation may affect DNA methylation of hTERT. However, our findings are still restricted in Huh7 cell line and further investigations are needed to unveil the specific mechanism.

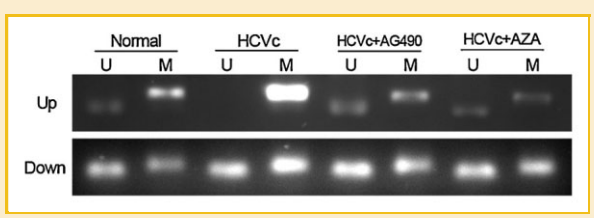

Fig. 6. Detection of the methylation status of hTERT promoter by MS-PCR. The transfection of HCVc could affect the methyaltion status of the promoter in hTERT's upstream region (−625 bp from transcription start site) and increase the degree of methyaltion. This effect disappeared after treatment with AG490 (STAT3 pathway inhibitor) or AZA (DNMT1 inhibitor). We could deduce that HCVc affected the expression of hTERT through epigenetic pathway by STAT and DNMT1. There's no significant change of the methyaltion status of downstream region of hTERT promoter (encompassing the transcription and translation start sites).

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