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Hepatitis delta virus induces specific DNA methylation processes in Huh-7 liver cancer cells

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ABSTRACT

Hepatitis delta virus (HDV) is a small, defective RNA virus that can infect only individuals carrying hepatitis B virus. HBV/HDV co-infection results in more severe liver disease than HBV single infection and more rapid progression to cirrhosis and hepatocellular carcinoma (HCC). The epigenetic events involved in hepatocyte transformation towards malignancy in this context are poorly known. Here we report that, in Huh-7 cells, HDV induces DNMT3b expression and is associated to E2F1 transcription factor hypermethylation. Moreover our cell cycle analysis showed that HDV induces G2/M arrest. These findings suggest that HDV could play a role in HCC development at least in part by altering DNA methylation events. A better understanding of the molecular mechanisms involved in HDV-related carcinogenesis could help to identify new therapeutic targets.

1. Introduction

Hepatitis delta virus (HDV) consists of a circular single-stranded RNA genome of 1679 nucleotides which assembles two viral proteins (HDAg-L and HDAg-S) but does not encode its own polymerase, instead, employing a cellular enzyme for its replication [1]. HDV transmission requires the hepatitis B virus (HBV) surface antigen (HBsAg), [2,3] which is the reason why this defective virus infects only individuals in the presence of hepatitis B virus [4]. Chronic HDV replication enhances liver damage relative to HBV alone leading to cirrhosis and hepatocellular carcinoma (HCC) at annual rates of 4% and 2.8%, respectively [5]. The mechanism(s) behind this enhancement is currently unknown. Tumor suppressor gene silencing through aberrant addition of methyl groups to specific regions of the DNA such as CpG islands is a frequent epigenetic event in HCC [6]. The transfer of methyl groups to the DNA is catalyzed by DNA methyltransferases (DNMTs). Among those DNMT1 and DNMT3b are also transiently recruited to DNA break sites [7]. HCC development is a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes (TSGs) [8]. Proteomic analyses of Huh-7 hepatocellular carcinoma cells transfected with HDV RNA, HDAg-S, or HDAg-L have shown that at least 32 proteins display differential expression in the presence of HDV components [9]. These dysregulated proteins are known to be involved in different cellular processes such as nucleic acid metabolism, protein metabolism, cellular transport, signal transduction, apoptosis, and cell growth. However, how HDAgs regulate host cell mechanisms of epigenetic control of gene expression remains poorly understood. Up to date, the ability of some viruses in inducing the methylation of host DNA is well documented [10–12]. In the present study we evaluated the expression levels of the DNA methyltransferases DNMT1 and 3b in an attempt to elucidate these molecular mechanisms using an in vitro model the human hepatoma cell line (Huh-7) expressing the HDV antigens. Moreover we evaluated the methylation levels of 24 genes involved in HCC development in order to find out a possible role for the observed DNMT3b overexpression. A better understanding of the interaction between HDV and the epigenetic host cell machinery can provide new insights for antiviral strategies or new therapeutic targets to prevent HDV-associated HCC.

2. Materials and methods

2.1. Plasmids, antibodies, and chemicals

Plasmid pSVL(D3) (kindly donated by John M. Taylor, Fox Chase Cancer Center, Philadelphia, PA) contains three copies of the

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complementary DNA (cDNA) of the HDV genome in the expression vector pSVL (Pharmacia, Uppsala, Sweden). The antibody that recognizes both small and large HDV antigen (HDAg-S and HDAg-L), also provided by John M. Taylor, Fox Chase Cancer Center, Philadelphia, PA, was raised in rabbits by inoculation of HDAg expressed in *Escherichia coli*. Rabbit polyclonal antibodies directed against STAT3 and its phosphorylated form, DNMT1, DNMT3b, were purchased from Cell Signaling Technology, Inc. (Euroclone, Milan). Mouse monoclonal antibody β-actin (clone Ac74) was from Sigma–Aldrich (Steinheim, Germany). STAT3 inhibitor (NSC74859) was obtained from Merck Chemicals Inc. (Milan, Italy).

2.2. Cell culture and transfection

Human hepatoma cells (Huh-7) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 ng/ml streptomycin (all products were from Invitrogen, Milan, Italy). Subconfluent cultures of Huh-7 cells were transfected using lipofectamine 2000 (Invitrogen, Milan, Italy) and incubated at 37 °C with Opti-MEM medium for 6 h in a humidified, 5% CO_2 incubator. The medium was then replaced with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum without antibiotics, and transfected cells were incubated for another 9 days, with medium changes every second day.

2.3. Indirect immunofluorescence (IFL)

We fixed Huh-7 cells, plated and transfected on coverslips, for 10 min in 4% paraformaldehyde at room temperature and permeabilized them with 0.2% Triton X-100 for 2 min. We washed the coverslips three times in phosphate-buffered saline solution (PBS) and incubated them with anti-HDAg (1:100 in PBS) overnight at 4 °C. After three washes in PBS, cells were incubated for 1 h at room temperature with rhodamine labeled anti-rabbit antibodies (Jackson Lab) diluted at 1:100. After rinsing three times in PBS, coverslips were mounted on microscope slides using Vectashield H1-200 (DBA Milan, Italy). For fluorescence microscopy, slides mounted for immunofluorescence were observed using a Nikon Eclipse E600 microscope. Percentages of HDAg-positive or HDAg-negative cells over total cells were calculated by counting >200 cells in randomly selected fields.

2.4. RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Milan, Italy) and subsequently treated with deoxyribonuclease I. Quantitative real time PCR was performed starting from 50 ng of purified RNA using the one step quantifast SYBR Green RT PCR KIT (Qiagen). For real-time polymerase chain reaction (PCR), the following Human SYBR Green QuantiTect Primer Assays (all purchased from Qiagen) were used: human DNMT1 (QT00034335), human DNMT3b (QT00032067) and human E2F1 (QT00016163). Reactions were set up in 96-well plates using a 7700HT Real-Time PCR System (Applied Biosystems, Foster City, CA), and all samples were assayed in triplicate. Optical data obtained were analyzed using the default and variable parameters available in the SDS software package (version 1.9.1; Applied Biosystems, Foster City, CA). Expression levels of target gene were normalized using the housekeeping control genes: TATA binding protein (TBP, QT00000721).

2.5. Immunoblot analysis

For total protein extracts, cells were washed once with ice-cold phosphate-buffered saline (PBS) and scraped from culture dishes in the presence of lysis buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol) supplemented with protease inhibitor cocktail (COMPLETE; Roche Diagnostics, Mannheim, Germany), 1 mM phenylmethylsulphonyl fluoride and 1 mM sodium orthovanadate. Equal amounts of protein extracts were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and electroblotted onto nitrocellulose (Whatman, Dassel, Germany). Membranes were blocked for 1 h at room temperature with PBS containing 0.1% (vol/vol) Tween 20 and 5% (wt/vol) non-fat milk powder before the overnight incubation at 4 °C with the above primary antibodies in blocking solution. After three washes with blocking buffer, the membranes were incubated for 90 min at room temperature with horseradish peroxidase–conjugated goat anti-mouse or goat antirabbit secondary antibodies (BioRad, Hercules, CA) diluted 1:4000. Membranes were washed extensively with washing solution and antigen–antibody complexes were detected by enhanced chemiluminescence (ECL; Amersham Biosciences) and the signal was detected on an X-ray film (Amersham Biosciences) according to the manufacturer's instructions. For quantitative measurement, films were scanned by densitometry and the spots corresponding to proteins were analyzed by using the Image J software.

2.6. EpiTect Methyl Signature qPCR Array

DNA extracted from Huh-7 control cells and from those transfected with pSVL(D3) were used to perform the EpiTect Methyl Signature qPCR Array (cat. no. MEAH-031A-12 Sabiosciences, Milan Italy) following the manufacture instructions. Briefly 1 μg of DNA was subjected to a digestion with methylation sensitive or methylation-dependent restriction enzyme. These enzymes will digest the unmethylated and methylated DNA, respectively. Following digestion, the remaining DNA is quantified by real-time PCR in each individual enzyme reaction using primers that flank the promoter (gene) region of interest. The relative fractions of hypermethylated, intermediately methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest.

2.7. Cell cycle analysis

Cells were harvested the day before the experiment and fixed with 70% cold ethanol over night at −20 °C. After centrifugation at 2000 rpm for 5 min cells were washed twice with PBS and RNase I was added at concentration of (100 μg/ml). Finally cells were stained with propidium iodide (PI) (50 μg/ml in PBS) before loading on TALI® Image Cytometer (LifeTechnologies, Milan, Italy). Results are expressed as mean and S.E.

2.8. Statistics

Results were expressed as means ± S.E.M. For statistical comparison, significance was evaluated using the Student *t* test. Values of $P < 0.05$(*) and $P < 0.005$(**) or $P < 0.001$ (***) were considered statistically significant.

3. Results

3.1. DNMT1, DNMT3b expression levels in HDV expressing cells

To investigate whether HDV modulates DNA methyltransferases (DNMTs) expression, we reproduced an in vitro system that was reported to allow HDV replication [13]. Huh-7 cells were transfected with pSVL(D3) plasmid coding for HDAg-S and HDAg-L and, at regular intervals of 3-6-9 days, proteins were extracted and examined by immunoblot analysis for the presence of the two HDAg isoforms (Fig. 1A). The efficiency of transfection was

comparable among different experiments and typically amounted to approximately 45% as determined by counting HDAg-positive cells under the fluorescence microscope in six randomly selected fields (Fig 1B). Only 9 days post transfection both antigens were expressed and for this reason all the subsequent experiments were performed at this time point. We then evaluated DNMT1 and 3b levels in HDV expressing cells in order to better understand a possible role of HDV in HCC-associated epigenetic alteration. DNMT3b mRNA and protein expression resulted upregulated upon HDV transfection by quantitative real time PCR (and immunoblot detection (HDV vs mock $P = 0.01$) (Figs. 1 and 2). No changes were observed for DNMT1 mRNA and protein expression levels.

3.2. STAT3 inhibition decreases DNMT3b expression

As it was reported that large delta antigen activates the signal transducer and activator of transcription 3 (STAT3) [28] and that STAT3 is involved in the regulation of DNMT1 expression [14] we evaluated STAT3 phosphorylation in pSVL(D3) transfected cells.

Noteworthy, as shown in Fig. 2, phosphorylation at Tyrosine 705 residue of STAT3 protein and its total form resulted increased in HDV overexpressing cells. In order to test whether STAT3 is responsible for DNMT3b overexpression we measured its mRNA and protein levels upon treatment with the STAT3 inhibitor NSC74859 in Huh-7 cells expressing the HDV antigens. As it is shown in Fig. 3A and B, DNMT3b was significantly decreased after treatment with NSC either in mock or HDV transfected cells at the mRNA and protein levels, suggesting that HDV induces DNMT3b upregulation through STAT3 in Huh-7 cells. On the other hand no effect of NSC treatment on DNMT1 expression was observed (data not shown) and the reason of this discrepancy could rely on the different type of cell cultures used in these different studies.

3.3. E2F1 hypermethylation and cell cycle analysis in Huh-7 cells expressing HDV

As altered promoter methylation of tumor suppressor genes is frequently found in HCC, we then sought to perform a methylation profile of 24 genes involved in HCC using the EpiTect Methyl Signature qPCR Array in Huh-7 cells expressing HDVAg as compared to

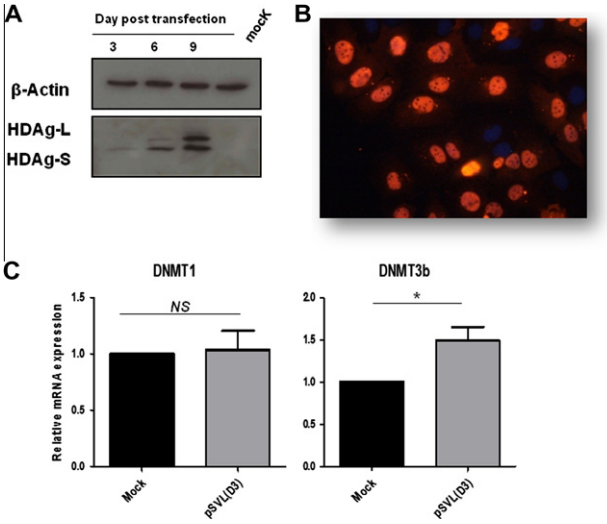

Fig. 1. Huh-7 cells were transfected with pSVL(D3) and the expression of the two isoforms of the HDV antigen was analyzed at 3, 6, and 9 days post-transfection by immunoblot analysis (A). Immunofluorescence detection of HDV antigens in Huh-7 cells at 9 days post transfection (B). Quantitative real time PCR analysis for mRNA expression level of DNMT1 and DNMT3b in mock and pSVL(D3) expressing cells (C).

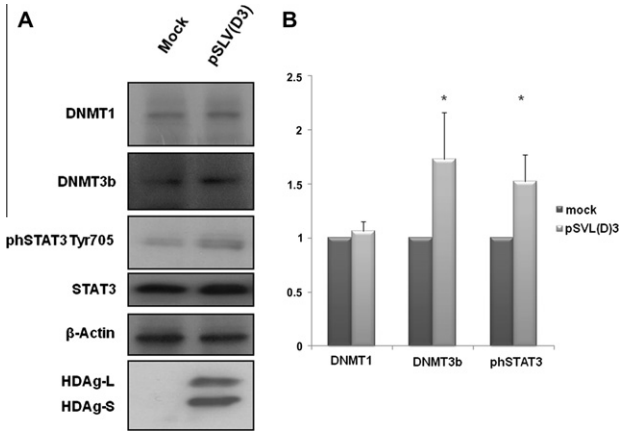

Fig. 2. Immunoblot detection of DNMT1, DNMT3b, and STAT3 proteins and its phosphorylation at Tyr705 residue in mock and pSVL(D3) expressing cells (A). After 9 days from transfection, cells were lysed and an equal amount of proteins were loaded on 10% polyacrylamide gel and separated by electrophoresis and immunoblotted with the respective antibodies normalized to β-actin expression as loading control. (B) Quantification plot of DNMT1, 3b, phSTAT3 and STAT3 normalized to β-actin.

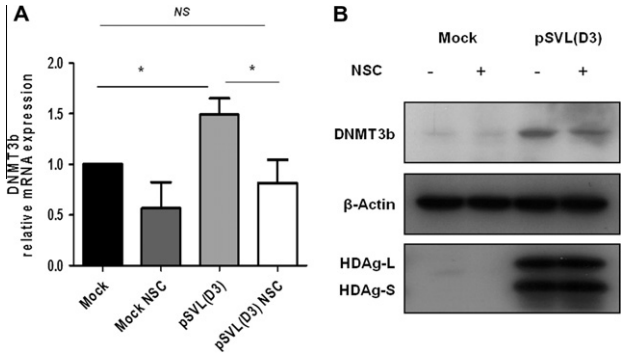

Fig. 3. DNMT3b mRNA expression levels by qRealTime PCR in mock and pSVL(D3) expressing cells before and after treatment with STAT3 inhibitor (NSC74859) at concentration of 100 μM for the last 48 h of transfection (A). Immunoblot detection and DNMT3b proteins in mock and pSVLD3 expressing cells before and after treatment with STAT3 inhibitor (NSC74859) at concentration of 100 μM for the last 48 h of transfection. β-Actin levels served as a loading control (panel B).

control cells. Among the different genes investigated only E2F1 resulted hypermethylated in the presence of HDV (Fig. 4A).

Since E2F1 family plays a crucial role in the control of cell cycle [15], we then sought to perform a cell cycle analysis in Huh-7 cells before and after HDV transfection. Fig. 4B shows that HDV increases of almost 2-fold the percentage of the cells in G2/M phase (26% ± 2 HDV vs 14% ± 1 mock, $P = 0.01$) and slightly reduces the percentage of the cells in S phase as compared to mock cells (65.5% ± 0.5 mock vs 54.5% ± 2 HDV $P = 0.02$) suggesting that HDV induces G2/M cell cycle arrest.

3.4. Effect of azacytidine treatment on E2F1 expression in Huh-7 expressing the HDV antigens

In order to test whether E2F1 hypermethylation resulted in a downregulation of the mRNA expression we analyzed the E2F1 expression by qRT-PCR. As expected the hypermethylated gene resulted downregulated at the mRNA level in HDV expressing cells (mock vs HDV $P = 0.01$) (Fig. 5B). We then tested whether by modulating DNMT3b, using a specific inhibitor (azacytidine), we would be able to reverse the decreased mRNA expression of the selected genes. First, a time-dose-dependent experiment to assess the effect

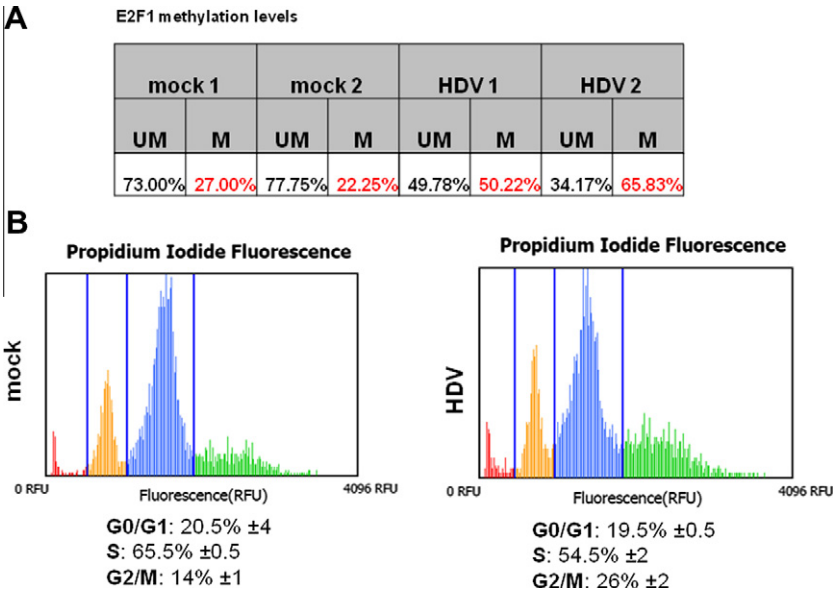

Fig. 4. Percentage of unmethylated (UM) and methylated (M) DNA of E2F1 promoter in Huh-7 cells expressing HDV antigens as compared to control cells quantified by Sabioscience EpiTect Methyl Signature qPCR Array (A). Cell cycle analysis in HDV expressing cells as compared to control cells (B).

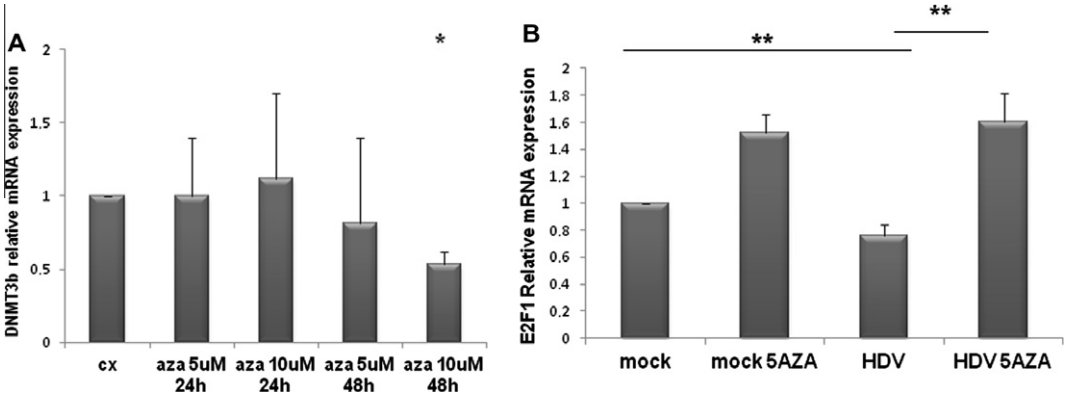

Fig. 5. Time-dose-dependent effect of azacytidine treatment on DNMT3b expression. The plot represents quantitative real-time PCR of cells treated at different time points and at different concentrations (A). E2F1 mRNA expression in Huh-7 cells expressing HDV antigens before and after treatment with azacytidine at concentration of 10 μM for 48 h (B).

of azacytidine treatment on DNMT3b expression was performed. Fig. 5A shows that 10 μM of azacytidine for 48 h significantly downregulated the DNMT3b expression in Huh-7 cells. Moreover, azacytidine treatment significantly recovered the E2F1 expression (HDV vs HDV + aza $p = 0.00$). (Fig. 5B).

4. Discussion

Viruses are among the most important human carcinogens [16] and their ability in inducing DNMTs expression for some of them is already documented [17–21]. The mechanisms behind the activation of DNMTs are challenging due to lack of relevant reports on the regulation of their expression [20]. To our knowledge this is the first report showing that HDV increases DNMT3b overexpression through STAT3 activation. Several tumours are characterized by activated STAT3 protein [22,23] and STAT3 inhibitors are under development for the treatment of certain cancers [24]. Constitutive activation of STAT3 in disease indicates an imbalance of stimulatory and downregulatory pathways [25]. DNMTs are critical in epigenetic events through the addition of methyl groups to DNA. Promoter hypermethylation mediated by DNMTs triggers the inactivation of tumor suppressor genes (TSGs). Maintenance of methylation pattern is achieved by DNMT1 function during DNA replication while new or de novo methylation is primarily catalyzed by DNMT3a and DNMT3b [26]. The latter may play an oncogenic role during tumorigenesis, and its genetic variants have been consistently associated with risk of several cancers including HCC [27]. In our study we demonstrated that HDV antigens induced DNMT3b expression and STAT3 increased phosphorylation. The latter is in agreement with a previous study [28] where the authors demonstrated that HDV large Antigen is responsible for STAT3 and NF-kB activation via oxidative stress. We further demonstrated that inhibition of STAT3 through NSC74859 treatment blocked DNMT3b upregulation. In a previous study Mota et al., [9] performed a proteome analysis using a different in vitro model of Huh-7 stably expressing the HDV antigens. Although they did not found overexpressed DNMT3 levels, when a computational analysis of protein interactions was performed, they found a subset of genes (NASP, TPI, and PABP2), previously reported to interact through the euchromatic histone methyltransferase SETDB1 which also interacts with de novo DNA methyltransferases DNMT3A and DNMT3B promoting gene silencing in human cancers. Moreover we found that E2F1 promoter was hypermethylated in Huh-7 cells expressing HDV antigens. The E2F family plays a crucial role in the

control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA [29,30] or RNA tumor viruses [31]. This protein binds preferentially to retinoblastoma protein pRB in a cell-cycle dependent manner. It can mediate both cell proliferation and p53-dependent/independent apoptosis [32]. Our cell cycle analysis showed that HDV induces G2/M cell cycle arrest which is a common feature of a number of viral infections [33]. In order to link cell cycle arrest to tumorigenesis, we speculate that it is possible that the infected hepatocyte may acquire one or more mutations that allow it to escape G2 arrest and apoptosis and proceed through the cell cycle, providing an apoptosis-resistant clone of cells with altered cell cycle regulation as suggested by Kannan [34].

Recently, it has been reported that HDV exerts an epigenetic control over hepatitis B virus transcription/replication activity [35]. Whether the DNMTs plays a crucial role in the interaction between the HBV and HDV needs to be assessed. In conclusion, knockdown of DNMTs could represent an alternative anticancer strategy for the treatment of HCC as already suggested [36]. Advances in our understanding of the molecular basis of HDV-induced malignancies, will help to identify new targets for future therapies, contributing to reduce the burden of these diseases.

Acknowledgments

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