Original article

The oncogenic potential of hepatitis B virus rtA181T/surface truncation mutant

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Abstract

Background: Previously, a less prevalent lamivudine-resistant mutant (rtA181T) was discovered in Taiwanese patients, in which a stop codon in the surface gene concomitantly occurred, leading to impaired secretion of hepatitis B virus (HBV) surface antigen. The rtA181T mutant also conferred drug resistance to adefovir. We discovered a 39-year-old patient with advanced hepatocellular carcinoma, who was seropositive for HBV e antigen but seronegative for HBV surface antigen. Nucleotide sequence analysis revealed the presence of polymerase rtA181T/surface truncation mutant in both the serum and hepatoma samples. Surprisingly, this patient has never received lamivudine or adefovir antiviral therapy. Here, we aimed to evaluate the oncogenic potential of HBV rtA181T/surface truncation mutant.

Methods: Site-directed mutagenesis experiments followed by transactivation assays were performed in HepG2 cells to evaluate the transactivation activities of the corresponding pre-S/S truncation mutant for c-Myc, c-Fos and Simian virus 40 promoters. NIH3T3 cells stably expressing the mutant were used to assess the tumourigenicity in nude mice.

Results: Transactivation experiments revealed that the corresponding pre-S/S truncation mutant was capable of transactivating the Simian virus 40 and human c-Myc promoters but not the c-Fos promoter. NIH3T3 cells stably expressing this mutant were tumourigenic in four of the five nude mice tested.

Conclusion: Our data indicate that an HBV polymerase rtA181T/surface truncation mutant could emerge spontaneously without previous antiviral treatment. The presence of this mutant in a patient with advanced hepatocellular carcinoma as well as its oncogenic potential warrants careful re-evaluation of the current strategy of prolonged antiviral therapy.

Introduction

Lamivudine is a nucleoside analogue with a potent inhibitory effect on the RNA-dependent DNA polymerase of hepatitis B virus (HBV) [1]. Owing to its effective antiviral effect, this drug is now widely used as a major regimen against chronic HBV infection [2]. If lamivudine is administered for a short period of time, clearance of HBV e antigen (HBeAg) is observed in only a minority of patients. Rapid relapses occur upon the withdrawal of lamivudine and is largely attributed to the persistence of HBV covalently closed circular DNA in hepatocytes. Prolonged usage of lamivudine is therefore recommended, leading to significant improvement of HBeAg seroconversion. However, emergence of lamivudine-resistant mutants could occur in a significant proportion of patients [3]. Recently, adefovir dipivoxil has been approved for the treatment of chronic hepatitis B [2]. Only a small percentage of treatment-naive patients treated by adefovir develop resistant mutants and this drug is also effective in patients developing lamivudine resistance. Although this is a great improvement in treating chronic hepatitis B, the emergence of viral mutants that are cross-resistant to both drugs remains a worrisome issue [3].

Lamivudine-resistant viruses usually possess characteristic amino acid substitutions over domains B or C of the RNA-dependent DNA polymerase, such as rtM204V, accompanied by upstream rtL180M mutations and the rtM204I mutation alone [3]. Previously, we have discovered an additional resistant mutant, rtA181T, in Taiwanese patients receiving prolonged lamivudine therapy [4]. The rtA181T mutant occurred in 4 of 23 (17.4%) patients with lamivudine resistance. Strikingly, the rtA181T mutant was also
recognized as a resistant mutant to adefovir [5,6]. Thus, this mutant appears to confer cross resistance to both drugs.

Here, we report a treatment-naive patient with advanced hepatocellular carcinoma who was negative for HBV surface antigen (HBsAg), but positive for HBeAg. Molecular analysis revealed that the rtA181T/surface truncation mutants constituted major viral population in this patient, wherein a stop codon was generated concomitantly in the surface reading frame (sW172stop).

**Methods**

**Patient**

A 39-year-old Taiwanese male had been diagnosed as an HBV carrier with HBsAg-positive serum since 1984 in our liver clinic. He was followed at various local hospitals, but at irregular intervals. Abdominal fullness and fatigue developed 2 weeks before visiting our liver clinic on 10 March 1998. Serologically he was HBsAg-negative in 1998. His mother was also an HBV carrier and died of hepatocellular carcinoma. Physical examination revealed hepatomegaly with a palpable firm liver margin. The patient denied any alcoholic history. Because this patient was a known HBV carrier, HBeAg and HBV DNA were tested subsequently.

**Serology**

HBeAg and antibodies against hepatitis C virus were assayed using an enzyme immunoassay kit (qualitative HBeAg and HCV-II; Abbott Laboratories, North Chicago, IL, USA). HBsAg and antibodies against hepatitis D virus were assayed using radioimmunoassay kits (Austria-II and anti-delta; Abbot Laboratories). Serum HBV DNA levels were determined with a Hybrid Capture assay (Digene Hybrid Capture II HBV DNA Test; Digene Corp., Gaithersburg, MD, USA). The detection limit was 1.4×10^5 copies/ml.

**Immunohistochemistry**

Immunohistochemistry was performed according to the protocol described previously [7]. For detection of HBV core and surface antigens, 1:500 dilution of rabbit polyclonal antibody against the HBV core antigen (Dako Corporation, Santa Barbara, CA, USA) and 1:1,000 dilution of mouse monoclonal antibody against the 'a' epitope of HBsAg (Chemicon, Tenecula, CA, USA) were used as the primary antibodies, respectively.

**DNA extraction and PCR**

The methods for DNA extraction and PCR were as described previously [4]. PCR was performed with primers M1 5'-CTTGGGAAACAGGCCTACAGC-3' (nucleotides [nt] 2836–2856, sense) and M2 5'-CCAATTATGTAGCCCCATGAAG-3' (nt 896–876, antisense). The reaction was carried out in a DNA thermal cycler (Perkin–Elmer Cetus, Norwalk, CT, USA). The PCR product was sequenced using the sense primer in an automatic DNA sequencer (CEQ 2000; Beckman Instruments, Inc., Fullerton, CA, USA). Alternatively, the PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and 20 clones with inserts were selected for sequence analysis.

**Plasmid construction and site-directed mutagenesis experiment**

A plasmid, pCMVHBV, was generated by inserting one copy of a greater-than-unit-length HBV genome (3.37 kb, nt 1820–2990, adw subtype and GenBank accession number X02763) into a vector, pRC/CMV (Invitrogen, San Diego, CA, USA) [4]. To express wild-type and mutant pre-S/S proteins, two plasmids, pIRES-pre-S/S and pIRES-pre-S/St, were generated. To construct pIRES-pre-S/St, PCR was performed using pCMVHBV as the template. The primers used were P1, 5'-GAACAAGAGCTACAGCATGG-3' (nt 2841–2860, sense) and P2, 5'-GTTTTAAATGTAATCCCTGAGAC-3' (nt 840–819, antisense). The PCR product was blunt-ended and inserted into pIRESbleo (Clontech, Palo Alto, CA, USA). Site-directed mutagenesis was performed using a PCR-based strategy. The details of this method have been described previously [4]. The resulting PCR product was blunt-ended and inserted into pIRESbleo to generate pIRES-pre-S/St, which carried the rtA181T/surface truncation mutation. Because both pIRES-pre-S/S and pIRES-pre-S/St contained the promoter of pre-S2/S gene, they could express large, middle and small surface proteins.

To construct pGL3-cFos-P and pGL3-cMyc-P, the promoter areas of c-Fos and c-Myc were amplified from chromosomal DNA. The primers were PF1 5'-GCTCGAGCCCCGAGGCTGAGG-3' (nt 21–41, sense) and PF2 5'-TGGAGTATTCCGGTGTAGCAGGCGG-3' (nt 740–721, anti-sense) for c-Fos promoter and PM1 5'-GGCCTCTCAAGTACGTGGCC-3' (nt 29–50, sense) and PM2 5'-CAGCGAGTCTAAGCCGAGGCG-3' (nt 818–799, antisense) for c-Myc promoter. The resulting DNA fragments were blunt-ended and inserted into pGL3-Basic (Promega, Madison, WI, USA) upstream of the luciferase gene. The plasmid, pGL3-SV40-P, was the same as pGL3-Promoter (Promega), which contained the Simian virus 40 (SV40) promoter sequence upstream of the luciferase gene.

**Cell culture and transactivation assay**

NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.
HepG2 cells were maintained in minimal essential medium containing 10% fetal bovine serum. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen). The efficiency of transfection was monitored by cotransfecting a β-galactosidase expressing plasmid, pCMVβ (Clontech).

Real-time PCR
Total RNA was isolated from transformed NIH3T3 cells using TRI reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA). To eliminate contaminated DNA, the extracted RNA was dissolved in diethylpyrocarbonate-treated water containing 10 mmol/l of MgCl₂ and incubated with 100 µg/ml of RNase-free DNase I for 30 min at 37°C. EDTA was added to a final concentration of 30 mmol/l and the mixture was heated at 95°C for 5 min to stop the reaction. A fragment of HBV DNA flanked by S1, 5′-TCTCCGGAGACTGGGAC-3′ (nt 126–145, sense) and S2, 5′-ATAGCAGCAGGATGAAGGG-3′ (nt 425–406, antisense) was amplified by PCR. As an internal control, a fragment of β-actin cDNA flanked by B1, 5′-CACCAACTGGGACGACATGG-3′ (nt 301–320, sense) and B2, 5′-AGGATCTTCATGAGGTAGTC-3′ (nt 651–532, antisense), was amplified by reverse transcriptase-PCR. Both PCR products were cloned into pRC2.1-TOPO (Invitrogen) for in vitro transcription.

To perform the real-time PCR assay, HBV surface and β-actin RNA fragments were generated by in vitro transcription using the MEGAscript T7 kit (Ambion Inc., Austin, TX, USA). The RNA product was diluted serially (10-fold) to generate a set of standards containing 10⁶ to 10⁵ copies. Real-time PCR was subsequently performed in LightCycler (Roche Diagnostics Corporation, Indianapolis, IN, USA). The primers used for real-time PCR were S1/ S2 for HBV pre-S/S mRNA and B1/B2 (for β-actin RNA). The copy numbers of these two RNA species were derived from NIH3T3 cells that had to fit the standard curves, otherwise both were diluted 10- and 100-fold in parallel and the assays were repeated.

Tumourigenicity assay
Male athymic BALB/c nude mice were obtained from the National Animal Experimental Centre (Taipei, Taiwan). The mice were maintained in specific pathogen-free conditions and used when 4 weeks old. NIH3T3 cells stably transfected with pIRESbleo (mock control), pIRES-pre-S/S or pIRES-pre-S/St were harvested by trypsinization. A total of 10⁶ cells with a viability >95% were injected subcutaneously into the backs of nude mice. Five sets of experiments were performed for each construct. The formation of tumours was monitored until week 15. Afterwards, the mice were sacrificed and tumours were resected for study.

Results
The patient was HBsAg-positive in 1984, but became HBsAg-negative in two consecutive tests (taken 1 week apart) in 1998. However, he was HBeAg-positive and the serum HBV DNA level was >10⁵ copies/ml. He was also negative for anti-hepatitis C virus antibodies, anti-hepatitis D virus antibodies and anti-HIV antibodies. Because this patient was a known HBV carrier, immunohistochemistry was performed to verify the carrier status. Serum α-fetoprotein level was 79 ng/ml. Biochemical studies revealed that serum aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase and alkaline phosphatase were 129 U/l, 79 U/l, 81 U/l and 133 U/l, respectively. Serum bilirubin level and prothrombin time were normal. Echo-guided liver biopsy was performed. Tumour and non-tumour parts of liver tissue were obtained. Computer tomography revealed multiple confluent liver tumours (Figures 1A & 1B). Histological examination revealed hepatocellular carcinoma (Figure 1C). Computer tomography showed tumour metastasis to lung (Figure 1D). The patient received palliative hepatic artery chemoembolization for one session. Thereafter, only conservative treatment was given. The tumours progressed rapidly and the patient expired 2 months later.

In the non-cancerous part of the liver tissue obtained, some hepatocytes were positive for HBsAg and hepatitis B core antigen (Figure 2A), indicating that this patient was indeed an HBV carrier and the
seronegativity of HBsAg was caused by secretion failure and not production failure. Subsequently, the pre-S/S gene was PCR amplified and sequenced from the serum sample, cancerous liver tissue and non-cancerous liver tissue. A G-to-A point mutation was found at codon 181 of the reverse transcriptase (GCT to ACT). This mutation simultaneously generated a stop codon in the S reading frame (TGG to TGA, codon 172). The rtA181T/sW172stop was detected in all samples by direct sequencing. However, the mutation was found to coexist with the wild-type sequence. The PCR products derived from serum and cancerous liver tissue were subsequently cloned into a vector and 20 clones were sequenced, respectively. Of the 20 clones, 12 (60%, serum) and 18 (90%, liver) clones harboured the rtA181T/sW172stop mutation. The nt sequence of the wild type pre-S/S gene derived from this patient was submitted to GenBank (accession number EU835940).

Site-directed mutagenesis experiments were performed to obtain pIRES-pre-S/S and pIRES-pre-S/St for expression of the wild-type and mutant pre-S/S proteins, respectively. Their abilities of transactivating c-Fos, c-Myc and SV40 promoters were tested in HepG2 cells (Figure 2B). The results indicated that the pre-S/St protein was capable of transactivating c-Myc and SV40 promoters for 5.1 ± 1.2 and 4.5 ± 1.1-fold, respectively. The pre-S/St protein could not transactivate c-Fos promoter.

Tumourigenicity experiments were performed using NIH3T3 cells stably transfected with pIRESbleo, pIRES-pre-S/S and pIRES-pre-S/St. The amounts of intracellular pre-S/S mRNA were evaluated by real-time PCR. The results indicated that 0, 9 ± 1.3 and 11 ± 1.6 copies of pre-S/S mRNA (per 100 copies of β-actin RNA) were found in NIH3T3 cells stably transfected with pIRESbleo (mock), pIRES-pre-S/S and pIRES-pre-S/St, respectively. These cells were injected into nude mice subcutaneously. It was found that four of five mice injected with NIH3T3 cells carrying pIRES-pre-S/St generated tumours, whereas none of the cells carrying pIRESneo or pIRES-pre-S/S generated tumours (Figure 2C).

Discussion

Prolonged usage of lamivudine has led to development of the rtA181T/surface truncation mutant in some Taiwanese patients [4]. Interestingly, a mutation corresponding to the same position of the nt sequence has also occurred in chronic hepatitis B-infected woodchuck receiving lamivudine therapy [8,9]. Because the rtA181T/V mutant also

Figure 2. Immunohistochemistry analysis of HBV core and surface antigens in the non-cancerous liver tissue

(A) Immunohistochemistry analysis of hepatitis B virus (HBV) core (upper) and surface (lower) antigens in the non-cancerous liver tissue. An arrow indicates core antigen in the nucleus. (B) Transactivation activities of pIRESbleo, pIRES-pre-S/S, pIRES-pre-S/St on c-Fos, c-Myc and Simian virus 40 promoters. *Mutant versus either wild type or mock (P<0.001). (C) Tumourigenicity of NIH3T3 cells stably transfected with pIRES-pre-S/St in nude mice.
confers resistance to adefovir, this mutant could become a prevalent cross-resistant mutant in the future [5,6]. In 1998, neither lamivudine nor adefovir was available in Taiwan, indicating that the rtA181T mutation could emerge in lieu of drug selection. The mechanism whereby this mutant emerged in this patient is unclear. An alarming feature was that this patient developed advanced hepatocellular carcinoma and his mother also died of hepatocellular carcinoma.

Previous studies reported transactivation activities of a truncated pre-S mutant isolated from the integrated HBV gene in hepatocellular carcinoma, which was capable of transactivating promoters of oncopgenes [10]. Transactivation activity occurred only when the truncation points were located within a defined region of surface gene, named the ‘transactivity-ON-region’ [11]. The truncated pre-S mutant was thus considered to play a role in hepatocarcinogenesis. Despite an appealing hypothesis, the integrated HBV gene was usually silent with no detectable protein generated. The situation for rtA181T mutant was different, wherein the virus replicated actively and generated significant amounts of viral proteins. The truncation point of surface gene in the rtA181T mutant is, however, located at the border of the transactivity-ON-region and the transactivating activity of this mutant has not been investigated until now. In this study, moderate but definite transactivation activity was found in the rtA181T mutant. To our knowledge, none of the patients harbouring the rtA181T mutant after lamivudine or adefovir therapy were reported to develop hepatocellular carcinoma. Other cellular or viral factors must be involved.

In our tumourigenecity experiments, the pre-S/S proteins alone were expressed in the absence of HBV replication. In humans, however, full-length replicating HBV genomes are present and all HBV viral proteins including X protein are expressed. With the help of X protein, the oncogenic potential of rtA181T/SW172stop mutant could be further enhanced. This possibility should be carefully examined in the future. The wild-type large surface protein has been shown to induce endoplasmic reticulum stress and is considered toxic to cells. Therefore, an alternative possibility is that the wild-type large surface protein is more toxic to cells than the mutant. Consequently, only the mutant is capable of transforming cells. This possibility cannot be excluded at this time.

In summary, we have identified the rtA181T/surface truncation mutant in a treatment-naive hepatoma patient, who was HBsAg-negative but HBeAg positive. This discovery warranted careful re-evaluation of the current strategy of prolonged antiviral therapy.

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Disclosure statement

The authors declare no competing interests.

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