Case Report

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Hepatitis B virus DNA integration in tumour tissue of a non-cirrhotic HFE-haemochromatosis patient with hepatocellular carcinoma

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Abstract

Co-existence of multiple causes of liver injury increases the risk of hepatocellular carcinoma (HCC) development. HCC usually develops in patients with cirrhosis although it may also occur in individuals with no or mild liver disease, in particular in cases with hepatitis B virus (HBV) infection.

Here we report the case of a 43 year-old man with HFE-haemochromatosis, seronegative for hepatitis B and C infections, who developed HCC in the absence of severe liver damage. Both tumoural and non-tumoural liver DNA extracts were tested by nested-PCR and primers specific for four different HBV genomic regions in order to evaluate the presence of occult HBV infection. Only X gene sequences were detected in tumour (but not in non-tumour) DNA extracts. HBV-Alu PCR showed a HBV integration involving a 5'-deleted X gene with an intact enhancer-II/basal-core promoter region. The viral-host junction sequencing revealed that this integrant was located upstream of the partitioning-defective-6-homolog-gamma gene (PARD6G) and real time-PCR quantification demonstrated that PARD6G was overexpressed in tumour compared to non-tumour liver tissues.

In conclusion, the combination of HFE-haemochromatosis and occult HBV infection in this patient might have led to a sequel of cellular events that determined the development of HCC even in the absence of cirrhosis.

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Introduction

Hepatocellular carcinoma (HCC) is largely the most common malignancy of the liver worldwide [1–3]. It may occur as a consequence of every pathologic condition able to provoke chronic liver damage, and cirrhosis is recognized to be a nodal step in hepatocarcinogenesis. The risk of HCC development is strongly increased by the co-existence of two or more causes of liver injury, particularly when hepatitis B virus (HBV) is involved, such as the case of HBV carriers chronically co-infected with hepatitis C virus (HCV) or exposed to aflatoxin B1 [1–3]. Apart from the long-lasting hepatitis produced by chronic infection, HBV may exert a direct oncogenic role through several different mechanisms, including viral DNA integration into the host genome as well as production of proteins with pro-oncogenic properties [1–3] and it may promote liver cell transformation and cancer even independently of cirrhosis development [1]. Moreover, much evidence indicates that HBV may maintain its pro-oncogenic role also in the case of occult infection, defined as the persistence of viral DNA at intrahepatic level despite the absence of circulating HBV surface antigen (HBsAg) [1,2,4]. Here we report and discuss the results of molecular analyses performed on liver tissue specimens of a non-cirrhotic HBsAg negative patient with HFE-haemochromatosis who developed HCC.

Case report

In 1990, a 43-year-old man was diagnosed with hereditary haemochromatosis, based on biochemical, clinical and histological criteria. Serum ferritin (SF) was 3990 ng/ml. He underwent first weekly phlebotomy, and, after one year, a twice year maintenance treatment. The diagnosis was confirmed in 1997 by HFE gene sequencing that documented homozygosity for the c.845G>A (p.C282Y) change of the HFE gene.

In February 2008, he was diagnosed with HCC by imaging (two nodules of 2.5 cm and 3.6 cm each) and histology. SF was 240 ng/ml and all liver tests were normal. He was negative for all serum markers of HBV and HCV infection, although he showed results of blood analyses performed in 1990 where he tested positive for antibodies to HBV core antigen (anti-HBc). The HCC nodules were treated by chemoembolization.

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Fig. 1. Schematic representation of HBV DNA integration site. Shaded box, HBV sequence. The region corresponding to the basic core promoter (BCP) and enhancer II is indicated within the viral sequences by the filled black box. Numbering of nucleotides is according to HBV ayw subtype. Open box, *PARD6G* coding sequence. Bold arrowheads, orientation of the ORF. The distance between the HBV integration site and the gene ATG is indicated (3.7 kb).

In November 2009, the patient underwent orthotopic liver transplantation (OLT) due to HCC recurrence. At present, the patient is undergoing the planned post-OLT follow-up with no evidence of HCC recurrence.
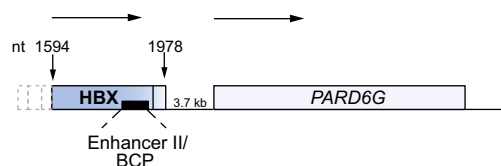
At the time of OLT, small portions of tumoural and non-tumoural tissue specimens were collected and immediately stored at −80 °C for subsequent molecular investigations. The non-tumoural liver tissue was histologically examined, showing a preserved liver architecture, absence of significant fibrosis and/or inflammatory infiltrates, minimal iron deposits.

The frozen liver specimens were divided into two parts and total DNA and RNA were extracted from each of them. DNA extracts were processed for investigating a possible occult HBV infection through well established procedures [4]. The non-tumoural liver specimen proved negative for all four different viral genomic sequences tested (S, Core, Pol, X genes), while in the neoplastic tissue only the sequence corresponding to the X gene proved positive, suggesting the possible presence of viral DNA integration into the host genome. In order to test this hypothesis, DNA extracts from both tumoural and non-tumoural tissues were examined through HBV-*Alu* PCR approach. Briefly, DNA was extracted from the frozen liver specimens by standard procedures. After the first 10 cycles of tag introducing amplification, the *Alu* primers were destroyed by UDG treatment, taking advantage of incorporated dUTPs in the primers. HBV primers specific for core, X and S regions [5] were used.

Ten pM of each primer was added for the next amplification, for which a "touchdown" PCR technique was applied. Five μl of the amplified products was subjected to nested PCR with internal specific HBV primers. PCR was performed using the Expand high-fidelity PCR system (Roche Diagnostics, Mannheim, Germany). PCR products were separated by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining. Southern blot experiments were performed to show the specificity of the amplification products.

The HBV-*Alu* PCR approach made it possible to reveal integrated HBV DNA in the host genome of the tumoural tissue, while no viral integrants were detected in the non-neoplastic specimen. The PCR products testing positive for HBV integration were those obtained using HBV primers designed for the X region.

Nucleotide sequences of the PCR product containing the viral-host junction were determined using nested PCR primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applera, Foster City, CA) according to the manufacturer's instructions. The sequencing products were resolved in an automatic DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applera). Nucleotide sequences of HBV DNA were characterized through alignment using the CLUSTAL W program. The cellular flanking sequences were assessed using the BLAST search system. This approach allowed a complete characterization of the integration. The HBV integrant was 384 nucleotides long (from nucleotide position [nt] 1594 to nt 1978, numbering from the unique *Eco*RI site of ayw subtype) and included a 5′-terminal truncated X gene, the precore region and 78 nt of the core gene. Therefore, the HBV integrant comprised the basic core promoter (BCP)/enhancer-II regulatory regions, the direct repeats DR-1 and DR-2, the unique viral polyadenylation signal, as well as the HBV genomic sequences corresponding to the second subelement of the post-transcriptional regulatory element and the pgRNA encapsidation signal ε [2,6]. The viral integrant was located 3743 nucleotides

upstream of the coding sequence for *PARD6G*, on chromosome 18. Orientation of the HBV genome open reading frame (ORF) is the same as the cellular gene orientation (Fig. 1). Considering that the BCP/enhancerII HBV sequence in the integration site could be disruptive to normal cellular gene transcription, *PARD6G* gene expression was assessed in the HCC specimen, in the corresponding non-tumoural liver tissue and in normal liver tissue samples from three liver-disease-free individuals (who undergone liver resection or needle biopsy during abdominal surgery) used as control, by a specific real time RT-PCR approach. Briefly, tissue specimens were homogenized by the use of TissueRupter instrument (Qiagen, Milano, Italy) in 500 μl of TRIzol reagent (Invitrogen, Paisley, Scotland) as recommended by the manufacturer. Samples containing equal amounts (5 μg) of total cellular RNA were treated with RQ1 RNase-free DNase (Promega, Madison, WI) for 1 h at 37 °C and then used as template for first-strand cDNA synthesis with Super Script reverse transcriptase (Invitrogen) and oligo(dT) primers. The cDNA samples were then amplified in Master Mix buffer (Roche Diagnostics). The sequences and nucleotide positions of the primers and probes for *PARD6* real-time PCR assay were as follows: *PARD6G*-F, 5′-ACCAGCGCAACAACGT-3′ (nt 918 to 933) *PARD6G*-R, 5′-GTCGTTGTCCTCATCGCTC-3′ (nt 1066 to 1048); *PARD6G*/FL (FRET hybridization probe), 5′-CGCGGCGGCCGCGCGTTGGGCA-FL-3′ (nt 938 to 959); and *PARD6G*/LC (FRET hybridization probe), 5′-LC-ReD640-CGGGACCGCCCTCGGACGGCACC-PH-3′ (nt 963 to 985). Real-time PCR by use of the "utility channel" of a Cobas TaqMan 48 instrument was performed under the following conditions: 95 °C for 10 min and then 60 cycles of 95 °C for 30 s, 57 °C for 20 s, and 72 °C for 20 s. The plasmid pCRII-PAR6, containing one copy of the *PARD6G* gene sequence (nt 163 to 1297), was used as a standard for *PARD6G* cDNA quantification. The plasmid was digested with *Eco*RI (New England Biolabs GmbH, Frankfurt, Germany), and the *PARD6G* sequence was gel purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI). The concentration of purified *PARD6G* DNA was determined with an ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE), and the corresponding copy number was calculated. A series of 10-fold dilutions of the plasmid pCRII-PAR6 was used as a standard for *PARD6G* cDNA quantification. The *h-G6PDH* housekeeping gene Light Cycler set (Roche Diagnostics) was used to normalize the RNA samples. All experiments were reproducible and were carried out in triplicate or quadruplicate.

The results showed an overexpression of *PARD6G* in the neoplastic tissue, compared to non-neoplastic and normal liver samples where *PARD6G* expression was similar (Table 1).

Case Report

Discussion

The case reported here presents several peculiar and conceptually intriguing aspects. First of all, HCC developed in the absence of cirrhosis, an event generally infrequent, also in haemochromatosis patients. Secondly, the patient had been positive for anti-HBV antibodies in the past, but at the time of HCC diagnosis he tested negative for all HBV serum markers. In addition, the non-tumoral liver tissue was negative for all four different HBV genomic sequences tested, thus apparently excluding a typical occult HBV infection, which is defined as the intrahepatic long-lasting persistence of episomal viral DNA and recognized through detection of at least two different viral genomic regions [7]. Furthermore, no viral integrants were found in the non-neoplastic specimen. On the contrary, the analysis of the tumour tissue revealed the presence of HBV X gene sequences that were shown to be integrated into the host genome once examined by *Alu*-PCR technique. Altogether, these data suggest that HBV infection had occurred but the virus was cleared from the liver, persisting solely as DNA integrated into the genome of some hepatocytes which subsequently expanded clonally as tumour cells.

The ability of HBV DNA to integrate in the host genome is considered to be a key event in HBV-related hepatocarcinogenesis. HBV DNA integration occurs randomly in the context of the human genome and may involve multiple sites of different chromosomes [2,8,9]. Nevertheless, viral DNA very frequently integrates near or within fragile sites or other repetitive regions of the human genome as Alu sequences and microsatellites that are prone to instability in tumour development and progression [10].

There is evidence demonstrating that some conditions modifying cellular homeostasis may increase the frequency of insertional events. In particular, it has been shown that exposure to oxidative stress may favour HBV DNA integration [8]; in the case reported here it can be speculated that iron accumulation created the conditions for HBV DNA integration through the continuous oxidative stress determined in the hepatocytes.

Integrated HBV DNA might contribute to hepatocellular malignant transformation either through the production of mutated viral proteins such as truncated X- or preS/S-proteins, which often possess transcriptional *trans*-activator properties and are able to activate signalling pathways implicated in tumorigenesis [11–13], or through the insertion of viral DNA into cellular genomic regulatory regions or coding regions with consequent modification of gene expression or structural and functional alteration of the produced cellular proteins [1,2,14]. This evidence has very recently been strongly strengthened by a study in which a genomewide survey of HBV integration in liver cancer genomes has shown that most HBV breakpoints in HCC occur inside or near coding genes. Moreover, regardless of whether HBV integration was at promoter, exon or intron, all genes recurrently affected in the tumour samples show much higher expression levels compared to the non-tumour samples [9].

In the case reported here, an activation of PARD6G expression is demonstrated by the real-time PCR findings, showing a strong overexpression of *PARD6G* transcripts in the neoplastic tissue compared to the non-neoplastic specimen as well as to normal control liver tissues. HBV integrant could have had a key role in the upregulation of PARD6G expression since the viral sequence integrated in proximity of the *PARD6G* open reading frame contains the enhancer II/BCP HBV regulatory region. The deregulation of PARD6G expression is of particular relevance since it encodes for a protein that is part of the Par6 complex, which is involved in the establishment of cell polarization and in the polarized migration of cells [15]. The Par6 complex binds to atypical protein kinase C (aPKC) and to a Rho family GTPase, RacI, that is a critical downstream effector of oncogenic PKCι in multiple cell types [16]. Furthermore, Par6 is a target of the transforming growth factor-beta (TGFβ) and mediates TGFβ-induced epithelial-to-mesenchymal transition (EMT), a process very likely responsible for tumour invasion and metastasis, as recently demonstrated in breast cancer [17].

In conclusion, in the case reported here a sequence of virological and cellular events probably led to the development of HCC even in the absence of cirrhosis. It is likely that an occult HBV infection had occurred in this patient, and that the continuous cellular oxidative stress determined by iron accumulation in the hepatocytes had favoured the integration of viral genomic sequences into cellular DNA, which persisted in the liver after the clearance of the virus. The peculiar site of cellular integration and the presence of *cis*-regulatory elements in the integrated viral sequences might have promoted mechanisms leading to a deregulation of PARD6G expression and to the alteration of oncogenic pathways.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Case Report

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Case Report