syphilis, leishmaniasis, Q fever, toxoplasmosis, cryptococcosis, infectious mononucleosis, herpes zoster, legionnaires' disease, and typhoid fever, were not suspected clinically.

In conclusion we suggest that the time correlation between interferon alpha-2b treatment and the appearance of granulomas in the bone marrow, and their disappearance when the treatment had been discontinued, suggest that the interferon caused the granuloma formation. The histology of the granulomas seems to be most compatible with drug hypersensitivity, as mycobacteriosis was ruled out.

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J Clin Pathol 1995;48:880-882

Hepatitis C virus replication in hepatocellular carcinoma

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Abstract

Hepatitis C virus (HCV) replication is reported in both tumour and non-tumour tissue in a case of hepatocellular carcinoma. Viral replication was established by showing the presence of minus strand HCV RNA by PCR amplification, after excluding residual reverse transcriptase activity of *Taq* polymerase. No minus strand was found in serum derived virion RNA. PCR amplified products from both tumour and non-tumour parenchyma were sequenced in the 5' non-coding region and shown to be identical. The genotype of this Indonesian patient was found to be 1b (or II), the most prevalent type in the Far East.

(*J Clin Pathol* 1995;48:880-882)

Keywords: hepatitis C virus; hepatocellular carcinoma; replication; polymerase chain reaction.

A high incidence of markers of hepatitis C virus (HCV) infection has been reported in association with non-hepatitis-B virus hepatocellular carcinoma.¹ Since the virus is a positive polarity single stranded RNA virus its replication is not thought to involve a DNA intermediate and the possibility of integration of viral sequences into the host genomic DNA, or the overexpression of a viral transactivating function leading to a dysregulatory effect on cell growth.

The questions which we have addressed in this study of a case of HCV associated hepatocellular carcinoma were first, whether the

virus is actively replicating in a liver bearing a tumour; second, whether it is replicating in the tumour tissue itself; and finally, whether the type of virus found in the tumour was genetically identical to that found in the surrounding non-tumorous parenchyma and in serum collected at the same time.

Case report

The patient was an Indonesian male, aged 56 years, with chronic active HCV hepatitis and cirrhosis possibly related to a blood transfusion for a bleeding peptic ulcer in 1976. Serum markers of previous hepatitis A and B infections were present (HAV IgG+, HBsAg-, anti-HBsAg+, anti-HBcAg+, HBeAg-, HBV DNA-). Anti-HCV antibodies were detected both by second generation enzyme linked immunosorbent assay (ELISA) and by recombinant immunoblot assay (RIBA) (anti-c22P + + + +, anti-c33c + + + +, anti-100-3P + + + +, anti-NS5 + + + +), and serum HCV RNA was detected by polymerase chain reaction (PCR) amplification.² A hepatocellular carcinoma was diagnosed after computerised axial tomography was done to investigate the development of hepatosplenomegaly and ascites. The carcinoma was found to occupy mostly segment 8 of the right lobe. Laparotomy was carried out to resect the affected liver lobe but the procedure was abandoned because of dissemination of the tumour to the left lobe. From the specimens removed for histology at the time of surgery, tissue was made available for the present study.

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Accepted for publication
21 February 1995

Figure 1 Liver cell carcinoma with a conspicuous lymphoid infiltrate. Haematoxylin and eosin stain, $\times 300$.

HISTOPATHOLOGY

Histopathological examination confirmed the presence of a clear cell hepatocellular carcinoma on a background of micronodular cirrhosis, with cells arranged in trabeculae, rosettes, and pseudoacinar patterns. There was

marked nuclear pleomorphism and hyperchromatism, with scattered binucleate and multinucleated cells and numerous mitoses. A delicate fibroblastic stroma was seen with a conspicuous lymphoid infiltrate (fig 1).

Methods

Tumour and non-tumour tissue in that order were separately dissected and homogenised in ice cold guanidinium isothiocyanate using a motor driven teflon homogeniser, and total RNA was extracted following the procedure of Chomczynski and Sacchi.³ cDNA was synthesised from the final RNA pellet dissolved in sterile water, using both a minus polarity primer for amplifying genomic RNA and a plus polarity primer for amplification of minus strand RNA (replicative intermediate). The primers used for cDNA synthesis and PCR amplification of the 5' non-coding region of HCV were 5'-ATGAATCACTCCCCTGTG 3' (sense) and 5'-CAAACGTAACACCAACCG 3' (antisense) for first PCR and 5'-CTTCACGCA-GAAAGCGTC 3' (sense) and 5'-GATG-CACGGTCTACGAGACC 3' (antisense) for the nested PCR. In addition serum collected a week before surgery was also processed for RNA extraction and cDNA synthesis followed

Figure 2 (A) 2% agarose-ethidium bromide gel electrophoresis of HCV cDNA amplified by RT-PCR in the 5' non-coding region. Lanes 1 and 14: size markers from EcoRI + HindIII digested lambda DNA. Lane 10: size markers from Hae digested ϕ X174 DNA. Lane 2: tumour (T) RNA, + strand. Lane 3: T RNA, - strand. Lane 4: T RNA, - strand, RNase-treated cDNA. Lane 5: non-tumour (NT) RNA, + strand. Lane 6: NT RNA, - strand. Lane 7: NT RNA, - strand, RNase treated cDNA. Lane 8: serum RNA, + strand. Lane 9: serum RNA, - strand, RNase treated cDNA. Lane 11: negative control, RNA stage. Lane 12: negative control, cDNA stage. Lane 13: negative control, PCR stage. (B) HCV sequences from the 5'NC region obtained from tumour and non-tumour liver tissues and from serum. NT⁺ = non-tumour PCR product from plus strand; NT⁻ = non-tumour PCR product from minus strand; T⁺ = tumour PCR product from plus strand; S⁺ = serum PCR product from plus strand; 1a and 1b = prototype sequences for types 1a and 1b.⁵

by PCR amplification in the HCV 5' non-coding region, as previously described.⁴ The PCR product was precipitated and directly sequenced using a commercial sequencing kit (Pharmacia) based on the dideoxy chain termination reaction and the genotype was established from the 5' non-coding sequence according to Simmonds.⁵

Results

PCR amplification of RNA derived from serum as well as tumorous and non-tumorous liver tissue clearly showed the presence of minus strand HCV RNA in both tumour and non-tumour tissue (fig 2A, lanes 3 and 6), whereas no minus strand was found in serum (fig 2A, lane 9). Plus strand RNA, on the other hand, was found in all three samples (fig 2A, lanes 2, 5, and 8). Possible reverse transcriptase activity of *Taq* polymerase was excluded by first boiling the cDNA preparations for 5 min, then cooling them quickly on ice and digesting them with RNase (0.1 µg/ml) for 20 min at 37°C,⁶ before PCR amplification using our standard conditions (fig 2A, lanes 4 and 7).

Sequencing of the PCR product amplified from plus strand revealed the presence of the same majority species in the three specimens (fig 2B). The same sequence was also obtained from the PCR product generated from the minus strand of non-tumour tissue. Because there was an insufficient amount of PCR product, sequencing of PCR-amplified cDNA from the minus strand from tumour tissue was not performed. By comparing the 5' non-coding sequences obtained with the corresponding consensus sequences of various HCV genotypes⁵ we were able to establish the genotype of this patient as type 1b (or II) as shown in fig 2B. HCV type 1a shows two specific changes at positions -237 and -107 which discriminate 1a and 1b (fig 2B).⁷

Our study has shown conclusively that HCV replication occurred in the tumour tissue of a case of hepatocellular carcinoma associated with chronic HCV infection. This was established from the presence of minus strand in the tumour tissue after excluding possible reverse transcriptase activity associated with *Taq* polymerase. The finding of a conspicuous inflammatory infiltrate of the tumour tissue may reflect the presence of the virus. Sequence analysis of the viral genomic RNA from serum and from tumour and non-tumour liver tissues revealed the presence of the same isolate. To ensure that the tissue-derived sequence was not caused by contamination from blood the

sequence was also obtained from the PCR product generated from the minus strand. Finally the genotype was established as being type 1b (or II), the commonest type to be found in the Far East.

Discussion

This study illustrates the reported association between HCV and hepatocellular carcinoma and shows the ability of the virus to replicate in the tumour tissue, in agreement with one previous report,⁸ but not with another⁹ where no minus strand was detected in the tumour tissue. The possibility that the *Taq* polymerase preparation might have contained reverse transcriptase activity was however not excluded in the first study.⁸ HCV RNA detected by polymerase chain reaction has also been reported both in cancerous and non-cancerous liver tissue by another group¹⁰ but the presence of the replicative intermediate of minus polarity was not investigated and blood contamination cannot be excluded.

Finally we may speculate that the presence of HCV in the tumour tissue which has been documented in this study may offer the possibility of devising new strategies for the eradication of tumour cells, both primary and metastatic.

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