No role for Epstein–Barr virus in Dutch hepatocellular carcinoma: a study at the DNA, RNA and protein levels

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INTRODUCTION

Recent reports show a significant increase in hepatocellular carcinoma (HCC) in the United States (Parkin et al., 1999). Worldwide, primary liver cancer is the fifth most common malignancy, of which HCC is the most prevalent form (El-Serag & Mason, 1999).

Apart from alcohol, the pathogenesis of HCC is closely linked to chronic hepatitis B (HBV) and C (HCV) virus infection (IARC, 1994, 1998). In contrast to HBV, there is no evidence to date to support a direct oncogenic role for HCV in the development of HCC. It is thought that HCC develops via HCV-induced chronic liver injury progressing to fibrosis and cirrhosis.

The human pathogenic gammaherpesvirus Epstein–Barr virus (EBV) is associated with diverse human lymphoid and epithelial malignancies. The association of EBV with endemic nasopharyngeal carcinoma (NPC) and approximately 10% of gastric carcinoma (GC) is well established. With 876,000 new cases of GC worldwide (Parkin et al., 1999), EBV-positive GC is the most frequent EBV-associated malignancy. EBV is found in all tumour cells and is transcriptionally active in both GCs and NPCs. Recently, EBV has been linked to the pathogenesis of HCC, i.e. another epithelial malignancy (Sugawara et al., 1999a, 2000).

Sugawara and co-workers reported high loads of EBV DNA in HCCs (Sugawara et al., 1999a). In addition, the presence of EBV was closely correlated with the HCV-positive status of the patient. These authors reported a unique EBV latency type, characterized mainly by the absence of EBER1/2 transcripts and the presence of EBV-encoded nuclear antigen-1 (EBNA-1) and BamHI A rightward transcripts (BARTs), as determined by RT-PCR analysis. In addition, EBNA-1 protein expression was demonstrated also by immunofluorescence in a subset of tumour cells using human sera known to contain EBNA-1 antibodies. These authors also demonstrated in vitro that EBNA-1 enhanced
the replication of HCV (Sugawara et al., 1999b). In contrast, a recent report by Chu and co-workers did not find any EBV DNA in 41 American HCCs by PCR but did find EBER-positive lymphocytes by EBER RNA in situ hybridization (RISH) (Chu et al., 2001a). Using immunohistochemistry (IHC), this group found two cases of HCC positive for EBNA-1 and one case positive for ZEBRA.

We and others have shown recently the expression of the transforming and immortalizing BARF1 (BamHI A rightward open reading frame 1) gene of EBV in NPC and gastric adenocarcinoma (Sbih-Lammali et al., 1996; Brink et al., 1998; zur Hausen et al., 2000). In addition, we demonstrated that BARF1 transcription is restricted to EBV-associated epithelial malignancies and is not found in either EBV-associated lymphoid malignancies or benign lesions of epithelial origin (Hayes et al., 1999). Transcript analysis has been shown to be important to confirm the role of EBV in the pathogenesis of EBV-related malignancies; although EBV DNA was present in breast and oral carcinomas, no relevant EBV transcripts were found in these malignancies, suggesting strongly that EBV is not playing a role in these cancers (Bonnet et al., 1999; Brink et al., 2000; McCall et al., 2001; Chu et al., 2001b; Cruz et al., 2000).

To elucidate whether transcriptionally active EBV is present in neoplastic and non-neoplastic liver tissue, we tested 16 primary liver tumours, corresponding normal liver tissue (if available) and four cases of chronic HCV hepatitis for the presence of EBV by means of DNA PCR, RT-PCR, NASBA (nucleic acid sequence-based amplification), RISH and IHC. In addition, HCV status was evaluated in corresponding non-neoplastic, paraffin-embedded liver tissue (n = 15) by IHC using a monoclonal antibody directed to the NS3 region of HCV.

## METHODS

### Clinical materials.
Snap-frozen and corresponding paraaffin-embedded liver tissues were collected retrospectively at the Department of Pathology of the Vrije Universiteit Medical Center, Amsterdam, The Netherlands. Tissue specimens consisted of frozen material of 16 HCCs with two adjacent non-neoplastic liver tissues, four tissues of non-neoplastic chronic HCV hepatitis and two normal liver tissues. Paraaffin-embedded material tested comprised five of the above-mentioned 16 HCCs, 13 of 16 non-neoplastic liver tissues adjacent to the 16 HCCs and two of four HCV-positive cases; all specimens are listed and summarized in Table 1.

Snap-frozen tissues were screened by EBV DNA PCR-enzyme immunoassay (PCR-EIA). Applying PCR-EIA to screen for EBV in GC, we have shown recently that PCR-EIA-negative cases were never found to be positive by EBER1/2 RISH (van Beek et al., 2002).

Corresponding paraaffin-embedded material of EBV DNA PCR-EIA-positive cases were tested by EBER1/2 RISH and IHC for the presence of EBV.

All cases were subjected to EBV RNA transcript analysis. Prior to RNA isolation, the sandwich sections, i.e. the first and last section (of this material), were stained with haematoxylin–eosin and controlled microscopically for the presence of tumour cells. The tissue sections in between were used for DNA and RNA analysis.

### RNA and DNA isolation.
RNA was isolated from 12 cryosections, each 5 μm thick, using 1 ml of guanidium/phenol-based RNazol reagent (Cinna Biotex). The purity and concentration of the isolated RNA were determined by spectrophotometry.

The remaining solution of the RNazol procedure was used for subsequent DNA isolation using the High Pure PCR Template Purification (HPPTP) kit (Roche), according to the instructions of the manufacturers. A 10 μl sample of the 200 μl elute was used for PCR.

### β-globin PCR.
To test for DNA quality, PCR for the human β-globin gene was performed using the BGPC0–BGPCO3 primer set, as described previously (de Roda Husman et al., 1995). The 209 bp PCR products were visualized by gel electrophoresis on a 1:5 % agarose gel and evaluated as compared to the positive control (1 x 107 JY cells per reaction).

### EBV PCR-EIA (BamHI W PCR).
To detect EBV, isolated DNA was subjected to EBV14/20 BamHI W repeat PCR using a biotinylated EBV20 primer (bioEBV20, 5’–gtgaagaacctaaacagc–3’); EBV14, 5’–ctctggtagtggctggacc–3’), as described recently (van Beek et al., 2002). Briefly, 5 μl biotinylated BamHI W PCR product was coated onto a 96-well streptavidin-coated microtitre plate (Roche) in 50 μl washing buffer containing 2 x SSC and 0.5 % Tween-20. After 1 h, unbound sample was washed away. PCR products were denatured for 15 min at room temperature using 0.2 M NaOH. Samples were hybridized with the digoxigenin (Dig)-labelled EBV probe (5’ Dig-aactgacacatagctggagcct–3’) (Eurogentec), at a concentration of 10 pmol ml–1 in washing buffer, for 60 min at 37 °C. After washing the plates, samples were incubated for 1 h with the alkaline-phosphatase-labelled anti-Dig Fab fragments (Roche). Samples were washed five times and visualized using the Sigma Fast pNPP substrate (Sigma). Absorbance (405/630 nm) values were measured after 30 min and overnight substrate incubation. About 104 cells of the JY cell line, an EBV-positive lymphoblastoid B cell line, were used as positive control and distilled water was included as negative control. The cut-off value was defined as three times the average negative control. The JY cell line was obtained previously and has been described extensively by Brink et al. (1997, 1998).

### EBER1/2, BARTs and β-actin RISH.
Paraaffin-embedded tissues of the EBV DNA PCR-positive HCCs were subjected to a non-radioactive EBER1/2 RISH using the Dig-labeled antisense EBER1/2 run-off transcripts as probe (Jiwa et al., 1993; van Beek et al., 2002). As control, the respective sense probe was used as described previously. In addition, a BARTS RISH using the Dig-labeled antisense run-off transcripts from the A3A4–BARTF0 construct (J. van Beek and others, unpublished data) was used as probe. The respective sense probe served as control. The A3A4–BARTF0 fragment has been shown to be present in all BARTs described to date (J. van Beek and others, unpublished data) and not in potentially coding BARTF0 (Smith et al., 2000).

RNA quality was assessed by β-actin RISH as described previously (van Beek et al., 2002).

### RT-PCR.
EBV-specific primers for EBNA-1 and BARF0, and primers specific for the U1 small nuclear ribonucleoprotein-specific A protein (snRNP U1A), have been described previously (Oudejans et al., 1995; Bijl et al., 1996). Prior to the RT reaction, RNA was centrifuged for 15 min, washed with 70 % ethanol and air-dried. Reverse transcription and subsequent PCR were performed as described previously (Brink et al., 1997). PCR products were analysed on 1 : 5 % agarose gels, transferred to nylon filters by alkaline Southern blotting and hybridized to specific oligonucleotide probes.
**Table 1. EBV detection at the DNA, RNA and protein levels in HCC tissue**

HCC, hepatocellular carcinoma; HCV, chronic hepatitis C virus infection; NASBA, nucleic acid sequence-based amplification; EBV PCR, BamHI W DNA PCR-EIA (+, positive; ±, weak positive; –, negative); RISH, RNA *in situ* hybridization; IHC, immunohistochemistry; JY, EBV-positive lymphoblastoid cell line used as positive control; PET, paraffin-embedded tissue; NA, no material available for testing; U, unknown.

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**Chronic HCV-positive non-neoplastic liver tissue**

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<tr>
<td>20</td>
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**Controls**

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*EBV-positive GCs express EBNA-1 but not LMP-1 and ZEBRA (zur Hausen et al., 2000).
†HCV tested by IHC on paraffin-embedded tissue.
‡HCV tested by serology.
labelled with [32P]ATP to determine their specificity. After standard (i.e. overnight) exposure, X-ray films were developed. The sensitivity of the EBNA-1 and BARF0 RT-PCR was determined to detect the transcripts in one EBV-positive cell (Brink et al., 1997).

**BARF1 NASBA.** NASBA is an isothermal in vitro amplification method with simultaneous activity of RT, T7 DNA polymerase and RNase H, which enables a reliable and sensitive detection of target RNA in the presence of DNA, independently of splice sites (Kievits et al., 1995). The BARF1 NASBA reaction was carried out as described previously (zur Hausen et al., 2000). Briefly, 100 ng total RNA per reaction was amplified at 41˚C in 20 μl reaction volumes containing 10 pmol of either primer, 15% DMSO, 40 mM Tris/HCl, pH 8.5, 12 mM MgCl2, 70 mM KCl, 4 mM DTT, 1 mM each dNTP, 2 mM rATP, rUTP, rCTP, 1-5 mM rGTP and 0.5 mM ITP (BioMerieux). Reaction products were evaluated by gel electrophoresis using 1-5% agarose in TBE, transferred from the gels to the nylon filters (Genescreen plus; Lifescience Products) via capillary phoresis using 1866 (BioMerieux). Reaction products were evaluated by gel electrophoresis using 1-5% agarose in TBE, transferred from the gels to the nylon filters (Genescreen plus; Lifescience Products) via capillary phoresis using 1

**RESULTS**

### β-globin PCR and BamHI W PCR-EIA

To assess the quality of isolated DNA, a β-globin DNA PCR was performed. All samples showed sufficient DNA quality for further DNA amplification procedures.

Compared to the positive control, 5 of 16 HCCs (31.25 %) were weakly EBV positive by PCR-EIA (data are summarized in Table 1) and 11 HCCs were completely negative for EBV DNA, as determined with the sensitive BamHI W repeat PCR.

In addition, the non-neoplastic liver tissues adjacent to the EBV DNA-negative HCCs (n=2, Table 1) did not contain EBV DNA. Of the four non-neoplastic chronic HCV cases, two were EBV positive, although this positivity was weak as compared to the positive control; the other two cases were completely EBV negative.

### EBER1/2, BARFs and β-actin RISH

Since we have demonstrated recently that a negative BamHI W PCR-EIA never shows positivity in the EBER1/2 RISH, an EBER1/2 and BARTs RISH was performed on the paraffin-embedded tissue of the weak PCR-EIA-positive HCCs (n=5) and chronic HCV hepatitis cases (n=2). In these cases, no EBER1/2 transcripts or BARTs were found either in hepatocytes (Fig. 1b, d) or in lymphocytes. In contrast, an EBV-carrying GC used as positive control did show nuclear EBER1/2 transcripts (Fig. 1a) and cytoplasmic BARTs (Fig. 1c) in tumour cells. The β-actin RISH used to control for RNA quality revealed cytoplasmic signals with the antisense probe and no signals with the sense probe in all cases. The absence of detectable EBV transcripts in HCCs indicates that EBV DNA is not present in tumour cells. In addition, EBER1/2 and BARTs RISH was also negative in the adjacent non-neoplastic liver tissues, of which 13 of 16 samples were not available for testing.

### EBNA-1 and BARF0 RT-PCR and BARF1 NASBA

We performed RT-PCR analysis for EBNA-1 and BARF0 on all 24 liver tissues. Prior to transcript analysis, an U1A RT-PCR was performed, showing sufficient RNA quality for subsequent RT-PCR and NASBA analysis.

None of the 24 cases tested positive for EBNA-1, BARF0 and BARF1 after gene-specific amplification and subsequent hybridization with specific radioactive-labelled oligonucleotide probes under standard conditions (overnight exposure). The control RNA of the JY cell line (Fig. 2) and an EBV-carrying GC were clearly positive after overnight film exposure. After extreme prolonged exposure (more than 72 h), very faint hybridization signals were seen in two cases for BARF0 and in one case for EBNA-1, which is clinically not relevant (see Discussion).

### EBV IHC

Standard IHC for EBNA-1, LMP-1 and ZEBRA did not reveal any protein expression in the EBV DNA PCR-EIA-positive HCCs tested (n=4) and the two EBV-positive chronic HCV cases. In addition, 13 of 16 available adjacent non-neoplastic paraffin-embedded liver tissues were also negative for EBV protein expression.

### HCV IHC

Of the 13 available normal liver tissues, six revealed a specific staining comparable with the positive control (Table 1, case 17, liver biopsy of HCV-positive patient). In three of six cases, histology was compatible with chronic HCV infection. None of the neoplastic liver tissues showed specific staining for HCV.
DISCUSSION

Besides its established association with endemic NPC and approximately 10% of GCs worldwide, recent reports have linked EBV to other epithelial malignancies, i.e. HCC (Sugawara et al., 1999a, 2000).

Fig. 1. (a) EBER1/2 RISH in a GC. EBER1/2 transcripts (antisense Dig-labelled riboprobes) are located in the nuclei (red) of all carcinoma cells. (b) EBER1/2 RISH in an EBV DNA (PCR)-positive HCC. No EBER1/2 transcripts (antisense Dig-labelled riboprobes) are detected in any nuclei of the carcinoma cells. (c) BARTs RISH in a GC. BARTs signals (antisense Dig-labelled riboprobes) are located in the cytoplasm (dark brown) of all carcinoma cells. (d) BARTs RISH in an EBV DNA (PCR)-positive HCC. No BARTs signals are seen in the cytoplasm (antisense Dig-labelled riboprobes) of any carcinoma cell.

In the present study, we applied a recently developed DNA PCR-EIA for the detection of the BamHI W repeat fragment of EBV in HCCs (van Beek et al., 2002). We detected weak signals for EBV DNA in 5 of 16 HCCs (31-25%) and in two of four HCV-related hepatitis cases. No EBV DNA was detected in non-neoplastic and normal liver tissue.

Using EBER1/2 and BARTs RISH, none of the EBV DNA-positive cases revealed transcripts for EBER1/2 or BARTs. In addition, we did not detect transcripts of latent EBNA-1, BARF0 and BARFI in any of the liver tissues after radioactive hybridization of the amplification products and subsequent overnight film exposure, as determined by RT-PCR or NASBA, respectively. We and others have shown recently that transcription of the transforming BARFI gene of EBV is restricted to latently EBV-infected epithelial malignancies (Sbih-Lammali et al., 1996; Brink et al., 1998; zur Hausen et al., 2000). No BARFI transcription has been detected in EBV-associated lymphoid malignancies or benign
EBV-related epithelial diseases (Hayes et al., 1999). Using the highly sensitive BARFI NASBA, which detects up to 10–100 copies of BARFI RNA targets, no specific BARFI transcripts in the EBV DNA PCR-positive HCCs and HCV-related hepatitis cases could be detected. This suggests strongly that EBV is not present in the carcinoma cells and, therefore, it is unlikely that EBV plays a role in hepato cellular carcinogenesis.

The weak positivity of EBV DNA in some liver tissues might be explained by amplification of EBV DNA in the lymphoid infiltrate or blood. Since the presence of EBV DNA in blood is well characterized, it might reflect a high EBV DNA load in these patients. Unfortunately, no data are available concerning the presence of EBV in the peripheral blood of the patients used in this retrospective study. Although not definitive, this seems to be the most likely explanation because more than 90% of the healthy Dutch population is latently infected with EBV.

In two recent studies from Japan, EBV DNA was found in 37 and 40% of HCCs by PCR (Sugawara et al., 1999a, 2000). The incidence of EBV DNA in HCC was correlated closely to a positive HCV status. It is interesting that we find in our population with a low prevalence of HCC (<3/6/100 000) the same prevalence of EBV DNA – although weak – as Sugawara and co-workers did in a population with a high prevalence of HCC (<48–9/100 000).

It has been published that the expression of latent EBNA-1 and BARF0 transcripts could be detected in three HCCs (Sugawara et al., 1999a). In our study, we did not find BARF0 or EBNA-1 transcription after standard overnight film exposure. Only after extreme prolonged exposure were very weak hybridization signals seen in 2 of 16 cases for BARF0 and in 1 of 16 cases for EBNA-1, corresponding to less than one transcript in the RNA analysed. However, as compared to previous data of our group (Brink et al., 1998), this intensity should correspond to less than one copy in the analysed RNA, definitely indicating clinically non-relevant transcription. It has been shown previously by Chen and co-workers that circulating EBV-positive peripheral B cells express BARF0 transcripts (Chen et al., 1999). This would also explain the negative BARTs RISH results in our study. It has been estimated by Deamant and co-workers that 1 in 1000 to 1 in 10 000 lymphocytes are EBV positive in EBV-seropositive individuals (Deamant et al., 1993).

Although we did not find EBER1/2 or BARTs RISH-positive lymphocytes in routine histology sections, we cannot exclude the presence of EBV-positive lymphocytes in the larger amount of frozen tissue used for PCR testing.

The second Japanese study from the same group confirmed the presence of EBV DNA in HCV-positive HCCs (Sugawara et al., 2000). Unfortunately, no EBV gene expression was studied. The relevance of EBV DNA in tumours without evidence of EBER1/2 transcripts is a controversial issue. EBER1/2 RISH is used routinely to detect EBV transcripts in EBV-associated diseases and is well accepted as a standard marker of latent EBV infection. Recent data of EBV in HCC and breast carcinomas suggested an EBER-negative EBV-latency type (Sugawara et al., 1999a; Bonnet et al., 1999). In the latter case, recent literature shows that EBV is not associated convincingly with breast carcinomas (Brink et al., 2000; McCall et al., 2001; Chu et al., 2001b) because this association was based purely on the presence of EBV DNA by PCR and the use of an EBNA-1 antibody that has been shown to cross-react with cellular antigens (Cruz et al., 2000). Also, no EBNA-1 transcripts could be demonstrated in these breast carcinomas (Brink et al., 2000).

A recent study from the United States investigated 41 American HCCs for the presence of EBV (Chu et al., 2001a). Using DNA PCR for LMP-1 and EBNA-4, they did not find any case positive for EBV DNA. However, they did find in one case one EBER RISH-positive lymphocyte and in two other cases EBNA-1-positive tumour cells, as tested by IHC. They also found in one case ZEBRA expression in tumour cells, as demonstrated by IHC. No transcription analysis was performed on these cases. Because of the EBV DNA negativity and the known un specific background staining using IHC with the antibodies, the authors concluded that EBV does not play a role in American HCCs.

In conclusion, the absence of latent EBNA-1, BARF0 transcripts and the absence of transcripts of the transforming, epithelial-specific EBV-gene BARFI in our series of HCCs argues strongly against an important role for EBV in the carcinogenesis of HCC in the Dutch population tested.

ACKNOWLEDGEMENTS

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REFERENCES


