

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

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Epstein–Barr virus (EBV) has been suggested to play a role in hepatocellular carcinoma (HCC). However, reports on detailed EBV transcript analyses in HCCs are limited. It was shown recently that expression of the transforming *BARF1* (*Bam*HI A rightward open reading frame 1) gene of EBV is restricted to latently EBV-infected epithelial malignancies, i.e. nasopharyngeal carcinoma and gastric carcinoma. The aim of this study was to test the presence of EBV in Dutch HCCs. A semiquantitative DNA PCR-enzyme immunoassay (PCR-EIA) for the *Bam*HI W fragment of EBV was used to assess the presence of EBV in frozen and paraffin-embedded tissues of 16 HCCs. In addition, several RNA detection techniques, i.e. nucleic acid sequence-based amplification (NASBA), RT-PCR, RNA *in situ* hybridization (RISH) and immunohistochemistry (IHC), were applied. Five of 16 HCCs and two of four hepatitis C virus hepatitis samples were weakly positive for EBV DNA by PCR-EIA. Using sensitive RNA transcription techniques, no transcripts were found for *BARF1*, EBNA-1 and BARTs (*Bam*HI A rightward transcripts) in any of the liver tissues tested. In addition, RISH for EBER1/2 and BARTs and IHC for EBNA-1, LMP-1 and ZEBRA, performed on the paraffin-embedded tissue of the PCR-EIA-positive cases and on adjacent non-neoplastic liver tissues, were negative. The absence of epithelial-specific *BARF1* transcripts and other EBV transcripts and proteins in the EBV DNA PCR-positive cases argues strongly against a role for EBV in HCC.

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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 *Bam*HI 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

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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 EBV-positive lymphocytes by EBV 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 paraffin-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. Paraffin-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 EBV1/2 RISH (van Beek *et al.*, 2002).

Corresponding paraffin-embedded material of EBV DNA PCR-EIA-positive cases were tested by EBV1/2 RISH and IHC for the presence of EBV.

All cases were subjected to EBV RNA transcript analysis. Prior to DNA and 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 BGPCO₃–BGPCO₅ 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 (1×10^4 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' bio-gtgaagtcacaacaagcc-3'; EBV14, 5'-ctctgtagtgattggacc-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 $2 \times \text{SSC}$ 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-aactgacacttagagctctggaggactt-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 10^4 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).

EBV1/2, BARTs and β -actin RISH. Paraffin-embedded tissues of the EBV DNA PCR-positive HCCs were subjected to a non-radioactive EBV1/2 RISH using the Dig-labelled antisense EBV1/2 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-labelled antisense run-off transcripts from the A3A4–BARF0 construct (J. van Beek and others, unpublished data) was used as probe. The respective sense probe served as control. The A3A4–BARF0 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 *BARF0* (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, *Bam*HI 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.

Case no.	Clinical data Age Sex		HCC					Corresponding non-neoplastic liver tissue					HCV	
			Frozen material			PET		Frozen material			PET			
			EBV DNA	<i>BARF0</i> / EBNA-1	<i>BARF1</i> NASBA	EBER1/2 and BART RISH	EBNA-1, LMP-1 and ZEBRA IHC	EBV DNA	<i>BARF0</i> / EBNA-1	<i>BARF1</i> NASBA	EBER1/2 and BART RISH	EBNA-1, LMP-1 and ZEBRA IHC		
1	16	F	-	-	-	-	-	-	-	-	-	-	-	+†
2	23	F	-	-	-	-	-	-	-	-	-	-	-	+†
3	73	M	±	-	-	-	NA	NA	NA	NA	-	NA	NA	+†
4	U	U	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
5	80	F	±	-	-	-	-	NA	NA	NA	-	-	-	-†
6	78	F	-	-	-	-	-	NA	NA	NA	-	-	-	-†
7	11	M	±	-	-	-	-	NA	NA	NA	-	-	-	-†
8	69	M	-	-	-	-	-	NA	NA	NA	-	-	-	+†
9	35	F	-	-	-	-	-	NA	NA	NA	-	-	-	+†
10	68	F	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
11	78	M	±	-	-	-	-	NA	NA	NA	-	-	-	+†
12	59	M	-	-	-	-	-	NA	NA	NA	-	-	-	-†
13	61	M	-	-	-	-	-	NA	NA	NA	-	-	-	-†
14	22	M	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
15	27	F	-	-	-	-	-	NA	NA	NA	-	-	-	-†
16	54	M	±	-	-	-	-	NA	NA	NA	-	-	-	-†
Chronic HCV-positive non-neoplastic liver tissue														
17	49	F	±	-	-	-	-	-	-	-	-	-	-	+‡
18	42	F	±	-	-	-	-	-	-	-	-	-	-	+‡
19	29	M	-	-	-	-	-	-	-	-	-	-	-	+‡
20	45	M	-	-	-	-	-	-	-	-	-	-	-	+‡
Controls														
21 normal	28	F	-	-	-	-	-	-	-	-	-	-	-	-†
22 normal	2	F	-	-	-	-	-	-	-	-	-	-	-	-†
EBV-positive GC			+	+	+	+	+	+	+	+	+	+	+	+*
JY cell line			+	+	+	+	+	+	+	+	+	+	+	+
Distilled water			-	-	-	-	-	-	-	-	-	-	-	-

*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 [32 P]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 RNA 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 μ l 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 blotting in 10 \times SSC and hybridized to specific [32 P]ATP end-labelled oligonucleotide probes. The absolute sensitivity of the *BARF1* NASBA assay was determined to detect 10–100 RNA templates (Brink *et al.*, 1998).

IHC. To detect EBV-specific proteins in paraffin-embedded tissue, monoclonal antibodies against LMP-1 (CS1-4, Dako), ZEBRA (Dako) and EBNA-1 (2B4-1, provided by F. Grässer, Hamburg, Germany) were used. Antibodies were visualized with a streptavidin–biotin–horseradish peroxidase complex and the DAB/H₂O₂ staining method, as described previously (Jiwa *et al.*, 1995).

In brief, tissues were boiled for 15 min in a citrate buffer (0.1 M l⁻¹, pH 6.0) before incubation with the anti-EBNA-1 antibody (2B4-1) (Grässer *et al.*, 1994). The antibody was then incubated overnight at room temperature with a 1:50 diluted antibody (final concentration 44 μ g ml⁻¹). Detection of the antibody was performed with a streptavidin–biotin–horseradish peroxidase complex. The peroxidase was visualized by incubation for 3 min in 0.2 mg ml⁻¹ DAB, 0.003% H₂O₂ and 0.12% nickel ammonium sulphate in 50 mmol l⁻¹ Tris/HCl, pH 7.6, followed by silver enhancement of the DAB–nickel precipitate.

To detect HCV by IHC in paraffin-embedded tissue, a monoclonal antibody (mouse IgG₁, clone HCV88; BioGenex) directed against the NS3 region of HCV was used. The IHC staining procedure was carried out according to the instructions of the manufacturer. HCV-positive, paraffin-embedded liver tissue was used as positive control.

RESULTS

β -globin PCR and *Bam*HI 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 *Bam*HI 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, BARTs and β -actin RISH

Since we have demonstrated recently that a negative *Bam*HI 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 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.

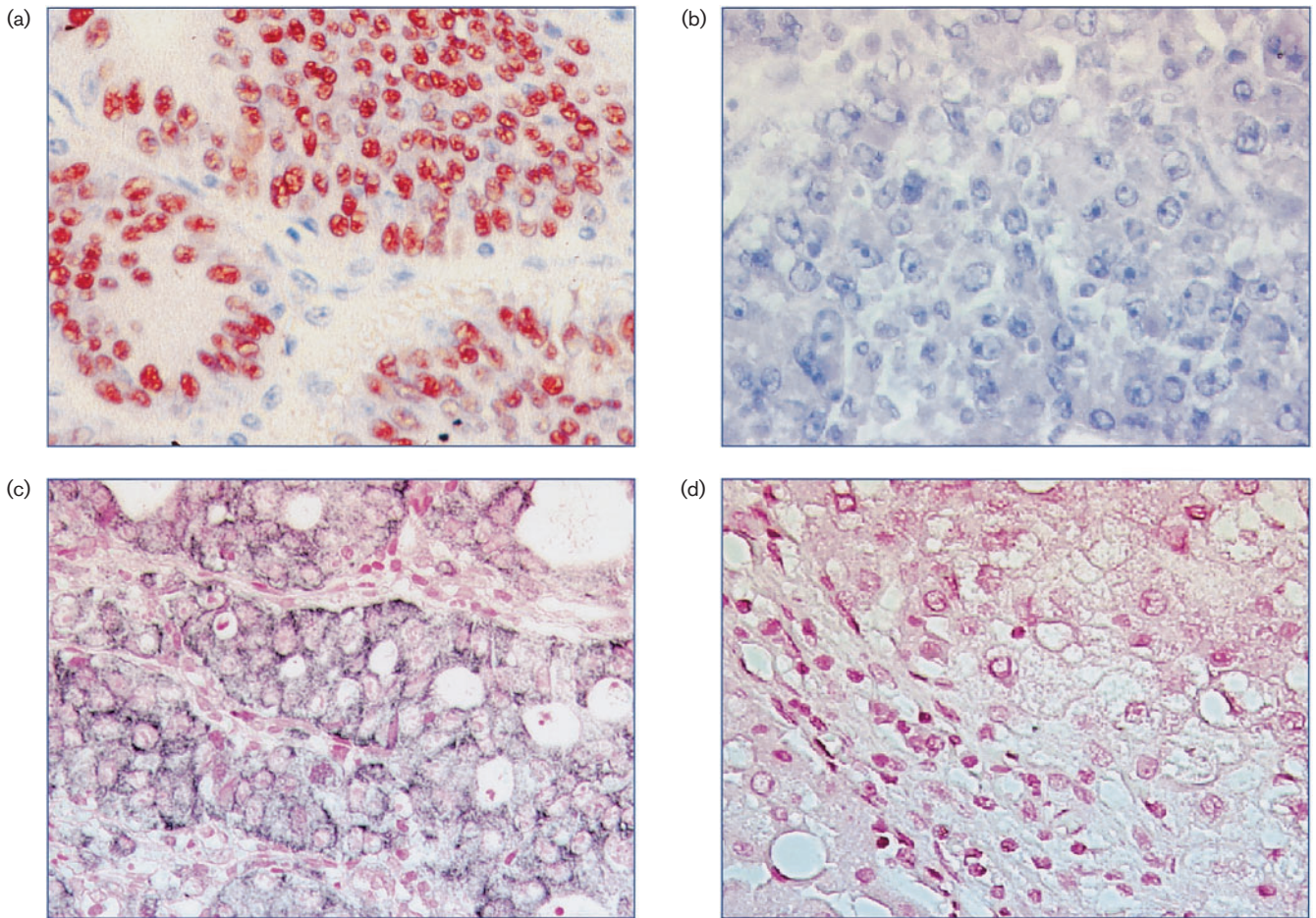


Fig. 1. (a) EBER1/2 RISH in a GC. EBER1/2 transcripts (antisense Dig-labelled riboprobes) are located in the nuclei (red) of all carcinoma cells. (b) EBER1/2 RISH in an EBV DNA (PCR)-positive HCC. No EBER1/2 transcripts (antisense Dig-labelled riboprobes) are detected in any nuclei of the carcinoma cells.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

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).

In the present study, we applied a recently developed DNA PCR-EIA for the detection of the *Bam*HI *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.

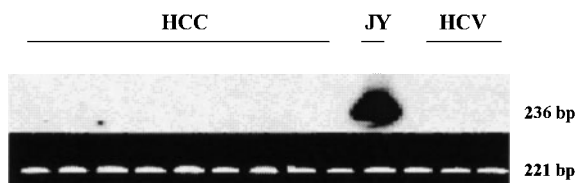


Fig. 2. Southern blot analysis for Q promoter-driven EBNA-1 RT-PCR products in a panel of nine HCCs and three chronic HCV hepatitis samples (top panel). The EBV-positive JY cell line served as positive control. Ethidium bromide-stained gel of U1A RT-PCR products to demonstrate sufficient quality of RNA (bottom panel).

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 *BARF1* 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 *BARF1* gene of EBV is restricted to latently EBV-infected epithelial malignancies (Sbihi-Lammali *et al.*, 1996; Brink *et al.*, 1998; zur Hausen *et al.*, 2000). No *BARF1* transcription has been detected in EBV-associated lymphoid malignancies or benign

EBV-related epithelial diseases (Hayes *et al.*, 1999). Using the highly sensitive *BARF1* NASBA, which detects up to 10–100 copies of *BARF1* RNA targets, no specific *BARF1* 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 hepatocellular 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 unspecific 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 *BARF1* in our series of HCCs argues strongly against an important role for EBV in the carcinogenesis of HCC in the Dutch population tested.

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