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Absence of Epstein–Barr virus DNA in the tumor cells of European hepatocellular carcinoma

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Abstract

The Epstein–Barr virus (EBV) has recently been associated with hepatocellular carcinoma (HCC) arising in Japanese patients. We analyzed 82 cases of HCC from Germany and the U.K. for the presence of EBV DNA and viral gene products within tumor cells. Initial screening of whole sections using quantitative (Q)-PCR detected EBV DNA in 9/58 U.K. cases and in 9/24 German cases; in positive cases viral load was very low, ranging between 1.4 and 49.1 copies of the EBV genome/1000 cell equivalents, compared to much higher values for EBV-positive Hodgkin's disease and nasopharyngeal carcinoma controls (range, 714–3259/1000 cells). EBV DNA was not detected in the tumor cells of any of the Q-PCR-positive cases either by Q-PCR of pure tumor cell populations isolated by laser capture microdissection or by isotopic in situ hybridization. Furthermore, none of the German or U.K. HCC tumors tested positive for EBER or EBNAI expression in tumor cells. Our results provide strong evidence that HCCs from the U.K. or Germany are not associated with EBV.
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Keywords: Epstein–Barr virus; Hepatocellular carcinoma; In situ hybridization; Quantitative PCR; Laser capture microdissection

Introduction

The association between the ubiquitous Epstein–Barr virus (EBV) and several epithelial cancers including undifferentiated nasopharyngeal carcinoma (UNPC) (Klein, 1979), a subset of gastric adenocarcinomas (Shibata and Weiss, 1992), and lymphoepitheliomas from a variety of sites (Iezzoni et al., 1995), is now well established. Two recent studies have suggested the possibility that hepatocellular carcinoma (HCC) might be a new addition to the list of EBV-associated diseases. In the first of these (Sugawara et al., 1999), monoclonal EBV DNA was detected by Southern blot hybridization in 13/35 (37%) of HCC tumors of Japanese patients, 10 of which contained EBV only in tumor tissue and not in nontumor tissues. Staining of three tumor specimens using human sera specific for the EBV-encoded nuclear antigen (EBNA) complex revealed nuclear labeling in a fraction (7–13%) of the tumor cell population. Western blot analysis and RT-PCR detected Qp-driven EBNA1 expression and the *Bam*H1A transcripts, but not expression of any other latent genes, including the EBERs. This observation suggested the possibility of a novel EBER-negative form of EBV latent infection and raised concerns regarding the use of EBER in situ hybridization as a means of determining the EBV status of tumors. In the second study (Sugawara et al., 2000), EBV was detected by PCR in HCC from both hepatitis C virus (HCV)- and hepatitis B virus (HBV)-positive patients, although EBV was present more frequently in the HCV group.

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Definitive designation of a tumor as "EBV-associated" requires unequivocal demonstration of the EBV genome or virus gene products in the tumor cell population. In this regard, PCR and Southern blotting are generally considered unsatisfactory since, when used alone, they cannot localize the virus to specific cell types. On the other hand, immunohistochemistry for detection of essential latent proteins, such as EBNA1, may lack specificity and sensitivity.

To overcome these difficulties, we have applied two methods to detect the EBV genome in HCC samples from the U.K. and Germany. In the first method we employed real-time quantitative (Q)-PCR combined with laser capture microdissection (LCM) to quantify EBV copy number in tumor cells isolated from HCC samples. In the second method we used a highly sensitive isotopic in situ hybridization method to localize EBV DNA to specific cell types. In neither of these approaches could we detect EBV DNA in the tumor cell population. We conclude that EBV is not associated with HCC originating in patients from the United Kingdom or Germany.

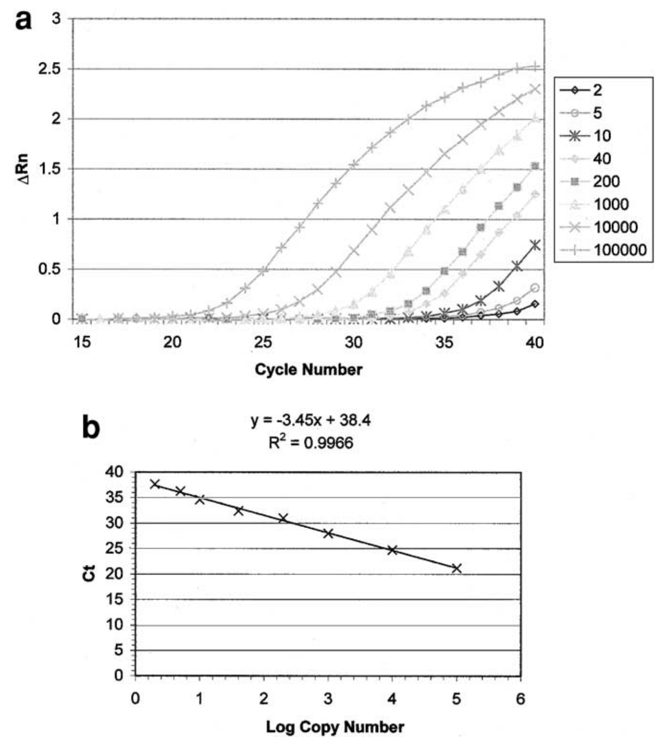

Fig. 1. Detection of EBV genomes by Q-PCR. Serial dilutions of Namalwa DNA containing 10^5, 10^4, 10^3, 200, 40, 10, 5 and 2 EBV genomes were amplified using primer/probe combination specific for EBV Pol and cellular B2m sequences in a multiplex PCR and the fluorescent signals detected using an ABI Prism 7700. (a) Change in FAM fluorescent intensity (ΔRn) plotted against cycle number; replicates are omitted for clarity. (b) Calibration curve generated by plotting the Ct value for each sample, defined as the fractional cycle number at which the amplification plot crosses the threshold (solid horizontal line), against the initial number of EBV copies. The *y*- intercept corresponds to the number of cycles required to detect a single episome and was consistently less than 40 cycles.

Results

Q-PCR assay to quantify EBV genome load and cellular input

We used a Taqman multiplex PCR assay to simultaneously detect EBV Pol and cellular B2m sequences. Amplification of the endogenous B2m reference gene served as a control for the input of cellular DNA. A typical Pol amplification plot showing changes in fluorescence intensity against cycle number from a series of Namalwa DNA dilutions (10^5, 10^4, 10^3, 200, 40, 10, 5, and 2 EBV genomes) is shown in Fig. 1A. A calibration curve (Fig. 1B) generated from the same data shows a strong linear relationship between the Ct values and the $\log_{10}$ value of the initial EBV copy number ($r = 0.99$) and the dynamic range extends over at least five orders of magnitude. We could reproducibly detect as little as two EBV genomes using this assay, although, as expected by Poisson distribution, this standard was amplified inconsistently (data not shown). Amplification plots for B2m obtained using the same Namalwa DNA dilutions showed a similar sensitivity and dynamic range (data not shown). No significant difference was seen in the sensitivity or efficiency of the Pol and B2m amplifications generated from Namalwa standards diluted in water or in a background of 600 ng EBV-negative DNA (data not shown).

Q-PCR analysis of whole sections of tumor

All cases were initially screened by Q-PCR. The EBV genome was detected in 9/58 U.K. cases (15.5%), but in all these cases the genome copy number was very low (range of 1.5–49.1 copies/1000 cell equivalents). Likewise, 9/24 (37.5%) of the German tumors contained low copy numbers of the EBV genome (range 1.4–38.5 copies/1000 cell equivalents). Q-PCR analysis of positive controls revealed much higher viral loads compared with HCC specimens (Table 1). EBV was not detectable in negative controls.

Q-PCR analysis of tumor cells isolated by LCM

The nine U.K. cases in which the EBV genome was detected by Q-PCR of whole sections were subject to LCM (Fig. 2) for isolation of tumor cells followed by Q-PCR. Despite the collection of multiple tumor clusters from different locations, the EBV genome was not detected in any of these samples. B2m detection revealed that adequate numbers of tumor cells had been sampled in all cases (Table 2). EBV was regularly detectable in microdissected tumor cells from EBV-positive NPC controls (Table 2).

In situ hybridization for BamH1 W repeats

All nine of the U.K. tumors and five of the German cases that tested positive for the EBV genome by Q- PCR analysis of whole sections were assayed by a highly sensitive in situ hybridization assay to detect the EBV genome. All of these

Table 1
Details of samples in which the EBV genome was detected by Q-PCR of whole sections

Case/control number	Number of copies of B2m	EBV genome copy number (assuming two copies of B2m/diploid cell)	No. of EBV copies per 1000 diploid cell equivalents
UK1	912	11	24.1
UK2	6026	11	3.7
UK3	13,571	18	2.7
UK4	11,242	276	49.1
UK5	3465	3	1.7
UK6	2921	2	1.4
UK7	2977	6	4.0
UK8	11,758	9	1.5
UK9	37,450	76	4.1
GER1	1447	2	2.8
GER2	3163	11	7.0
GER3	1593	7	8.8
GER4	1931	2	2.1
GER5	250	2	16.0
GER6	5707	4	1.4
GER7	156	3	38.5
GER8	3934	11	5.6
GER9	251	3	24.0
NPC1	1852	1219	1316
NPC2	2261	3684	3259
NPC3	1799	2224	2472
HD1	6036	2555	847
HD2	479	171	714

Note. Included as controls are whole sections of EBV-positive NPC and HD tumors.

cases were negative despite detection of the EBV genome in controls (Fig. 3).

EBER in situ hybridization/EBNA1 immunohistochemistry

EBER RNAs were not detected in tumor cells of any of the nine U.K. HCC samples shown to be positive by whole-section PCR, or in any of the 24 German tumors. By contrast strong reactivity was seen in positive controls (data not shown). In a single case from the U.K., isolated EBER-positive cells in the tumor stroma were noticed (case number UK1). Immunoreactivity with the monoclonal antibody 2B4-1 and 1H4-1 was also not detectable in these tumors, whereas control EBV-positive NPC tumors showed typical granular nuclear staining (data not shown).

Discussion

Two studies have suggested the possibility that hepatocellular carcinomas may represent yet another addition to the growing list of EBV-associated tumors (Sugawara et al., 1999, 2000). Interestingly, in the first of these, EBNA1

Table 2
Absence of the EBV genome in nine cases of HCC analysed by LCM-Q-PCR

Case/control number	Number of copies of B2m	EBV genome copy number (assuming two copies of B2m/diploid cell)	Copies of EBV per diploid cell equivalent
UK1	335	0	0
UK2	767	0	0
UK3	813	0	0
UK4	711	0	0
UK5	693	0	0
UK6	364	0	0
UK7	642	0	0
UK8	1757	0	0
UK9	1659	0	0
NPC1	23	73	6.3
NPC2	43	117	5.4

Note. EBV could be regularly detected in EBV-positive NPC controls, even when only small numbers of cells were collected.

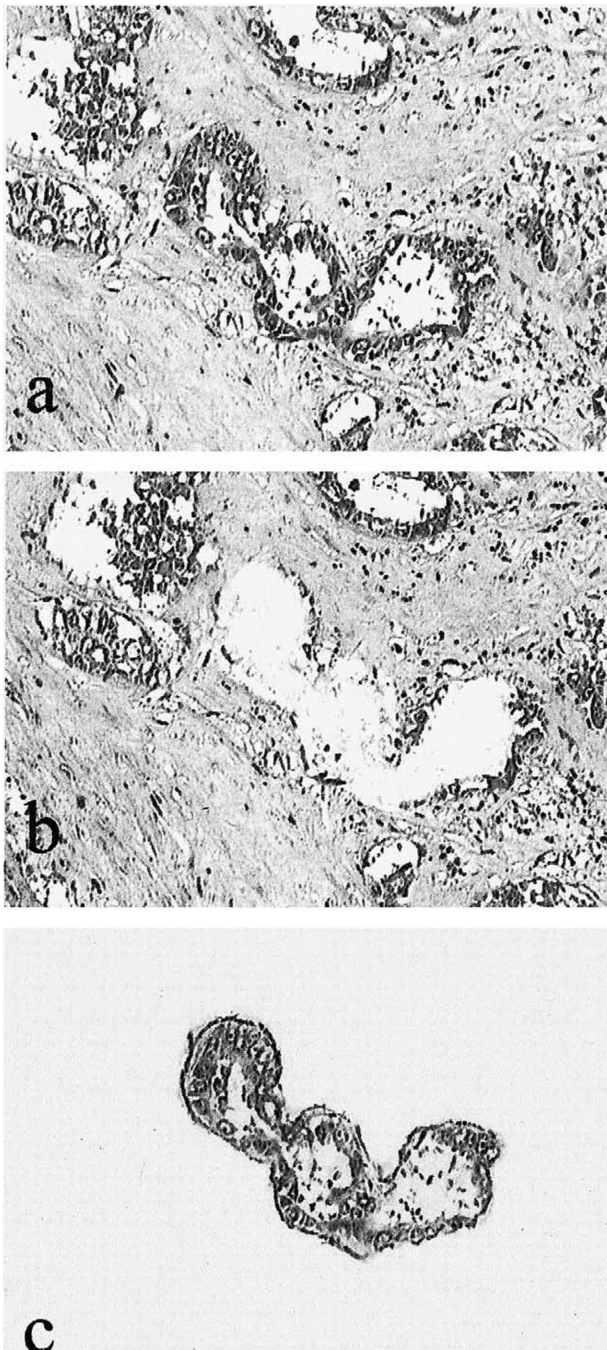

Fig. 2. Laser capture microdissection of a single cluster of tumor cells from an HCC lesion. (a) and (b) Sections before and after capture, respectively. (c) The microdissected cells on the Eppendorf cap after capture. In the analysis of EBV copy number multiple tumor clusters were pooled and analyzed by Q-PCR.

expression and the presence of the *Bam*H1A transcripts were detected in the absence of EBER expression, suggesting that HCC might be characterized by a unique form of viral latency. Moreover, EBV genomes detected in this study were shown to be monoclonal by analysis of the terminal repeat region. This finding points to the presence of EBV in a monoclonal cell population and implicates the tumor cells as the likely source of the viral DNA. However, more recently, Chu and colleagues (Chu et al., 2001) used a variety of approaches to detect EBV in a series of American HCCs. Although none of their 41 samples were EBV-positive by PCR for either LMP1 or EBNA4 DNA, they were able to detect immunoreactivity for EBNA1 in the tumor cells of two cases using the 2B4-1 monoclonal reagent and two further cases contained BZLF1-positive or EBER-positive nontumor cells.

EBV detection in tumors has generally employed either methods such as PCR (and RT-PCR) or Southern/Western blotting, or in situ approaches such as immunohistochemistry or in situ hybridization for the detection of virus gene products. Unfortunately, each of these approaches has significant drawbacks; PCR and blotting methods do not rule out the detection of EBV in nontumor cells, and in situ methods to detect gene expression may be uninformative where there is restricted virus gene expression. Furthermore, the reagents currently available to detect the only essential EBV gene product, EBNA1, are not entirely specific and show low sensitivity (Grasser et al., 1994; Murray et al., 1996; Brink et al., 2000).

Because of these methodological difficulties, we decided to target the EBV genome for detection using two distinct but complementary approaches. First we combined Q-PCR with LCM to quantify EBV DNA in isolated tumor cells, and second, we employed a highly sensitive in situ hybridization technique to detect the *Bam*H1W repeats of the EBV genome.

Initial screening of whole tumor sections by Q-PCR detected EBV in a proportion of cases. Importantly, however, the copy number was very low in these cases. A number of EBV-positive cases was then subject to microdissection followed by Q-PCR. Although, B2m copy number was high in all these microdissected samples, EBV DNA was not detectable in isolated tumor cells from any case. Application of an isotopic in situ hybridization for detection of viral DNA confirmed these results. In one of the previous studies EBV was only detected (by EBNA staining) in a fraction of the tumor cell population (Sugawara et al., 1999). We believe that sampling error during the LCM process is unlikely to have explained our negative findings, since multiple tumor clusters from different locations within each tumor were analyzed.

Our study provides compelling evidence that the small quantity of EBV that can be regularly detected during the gross analysis of European HCC tumor tissue does not originate from the tumor cell population. The most likely source of this viral DNA is from EBV-infected bystander lymphocytes; such EBV-infected lymphocytes have previously been detected in liver specimens (Niedobitek et al., 1997). Many cases of HCC occur in a background of cirrhosis and chronic liver disease may itself be immunosuppressive. In combination with the possible application of immunosuppressive therapy for liver disease and cancer chemotherapy in some patients this could be responsible for

an increase in the EBV-infected B cell pool which could be more readily detected by whole-section PCR.

We conclude that HCC, at least from the United Kingdom or Germany, is not an EBV-associated disease. Further studies employing robust methodologies, such as those described here, are required to determine if EBV is associated with HCC from other geographical locations.

Materials and methods

Tissues

Paraffin wax embedded tissue blocks from a total of 82 patients with histologically confirmed HCC were used in this study. Fifty-eight patients were diagnosed at the Queen Elizabeth Hospital, Birmingham, U.K. and a further 24 cases were retrieved from the files of the Pathological Institute in Erlangen, Germany.

DNA extraction from whole tumor sections

For all tissues DNA was extracted from 16-μm paraffin sections using the DNeasy Tissue Kit (Qiagen Ltd., Cat. No. 69504), according to the manufacturer's instructions. Controls, also cut at the same time as test samples, included paraffin wax embedded pellets of EBV-negative D674 cells, the EBV-positive line, Namalwa, which is diploid and carries two copies of the EBV genome (Whitaker, 1985; Lawrence et al., 1988), as well as EBV-positive NPC and Hodgkin's disease (HD) specimens. Initial screening for EBV DNA was performed using Q-PCR.

Real time Q-PCR assays

Amplification of target DNA sequences was followed by real-time monitoring of changes in fluorescence intensity using dual-labeled fluorogenic Taqman probes (Heid et al., 1996). EBV DNA was detected using primers to specifically amplify a fragment of the EBV DNA polymerase (Pol) gene in combination with a FAM-labeled probe (Gallagher et al., 1999). Primers for the amplification of the human beta 2 microglobulin (B2m) gene in combination with a VIC-labeled probe were used to determine the input cellular DNA in each sample and normalize for variations in the number of cells taken for analysis or DNA recovery (Summers et al., 2001). All primer and probe sequences (Table 3) were obtained from PE Biosystems. EBV Pol and control B2m sequences were detected simultaneously using a multiplex PCR, in which the B2m amplification reaction was primer limited so as to avoid depletion of PCR reagents. Amplification reactions were performed in 50 μl volumes containing 25 μl Taqman Universal master mix (PE Biosystems), 0.5 μl forward and reverse Pol primers (20 μM), 1 μl 5 μM FAM-labeled Pol probe, 1 μl 3 μM B2m forward primer, 1 μl 4 μM B2m reverse primer, 0.5 μl 5 μM VIC-labeled B2m probe, 10.5 μl water, and 10 μl test DNA.

Table 3
Primer and probe sequences

EBV polymerase gene
Forward primer: 5′ AGT-CCT-TCT-TGG-CTA-GTC-TGT-TGA-C 3′
Reverse primer: 5′ CTT-TGG-CGC-GGA-TCC-TC 3′
Dual-labeled fluorogenic probe: 5′ (FAM) CAT-CAA-GAA-GCT-GCT-GGC-GGC-CT (TAMRA) 3′
Beta-2 microglobulin gene
Forward Primer: 5′ GGA-ATT-GAT-TTG-GGA-GAG-CAT-C 3′
Reverse Primer: 5′ CAG-GTC-CTG-GCT-CTA-CAA-TTT-ACT-AA 3′
Dual-labeled fluorogenic probe: 5′ (VIC)-AGT-GTG-ACT-GGG-CAG-ATC-ATC-CAG-CTT-C (TAMRA) 3′

Following activation of the uracil-*N*-glycosylase (2 min at 50°C) and the Amplitaq Gold (10 min at 95°C), the reaction mixtures were amplified for 40 cycles (15 s at 95°C, 60 s at 60°C) and the fluorescent signals generated by each sample detected using an ABI Prism 7700 Sequence Detection System (PE Biosystems).

Analysis of Q-PCR data

Changes in FAM and VIC fluorescence intensity (corresponding to amplification of Pol and B2m, respectively) were plotted against cycle number using the Sequence Detection Software v1.63 and the resultant amplification curves used to determine the corresponding Ct value for each sample, defined as the fractional cycle number at which the amplification curve crosses a threshold level (set as 10 times the standard deviation of the baseline). Since this Ct value is proportional to the initial amount of starting material, the number of Pol and B2m copies in the test samples could then be deduced from suitable standard curves. The Pol calibration curve was obtained from the amplification of serial dilutions (in water) of Namalwa DNA containing 10^5, 10^4, 10^3, 200, 40, 10, 5, and 2 Pol copies (assuming diploid Namalwa cells carry two EBV genomes and that each cell contains 6.6 pg DNA); a linear calibration curve was then generated by plotting Ct values (*y*-axis) against $\log_{10}$ Pol copy number (*x*-axis) from which the initial number of EBV genomes in the unknown samples could be determined. Similarly a B2m calibration curve was generated from the same Namalwa DNA dilutions and used to determine the cell input in each sample (assuming two B2m genes per diploid cell).

All standards and test samples were analyzed in duplicate (10, 5, and 2 copy number standards were run in triplicate). Each experiment included DNA samples prepared from EBV-positive and EBV- negative controls, as well as water-only (no template) controls. Samples were considered negative if the Ct values exceeded 40 cycles.

Laser capture microdissection

All U.K. cases identified to contain the EBV genome by Q-PCR analysis of whole sections were subject to LCM

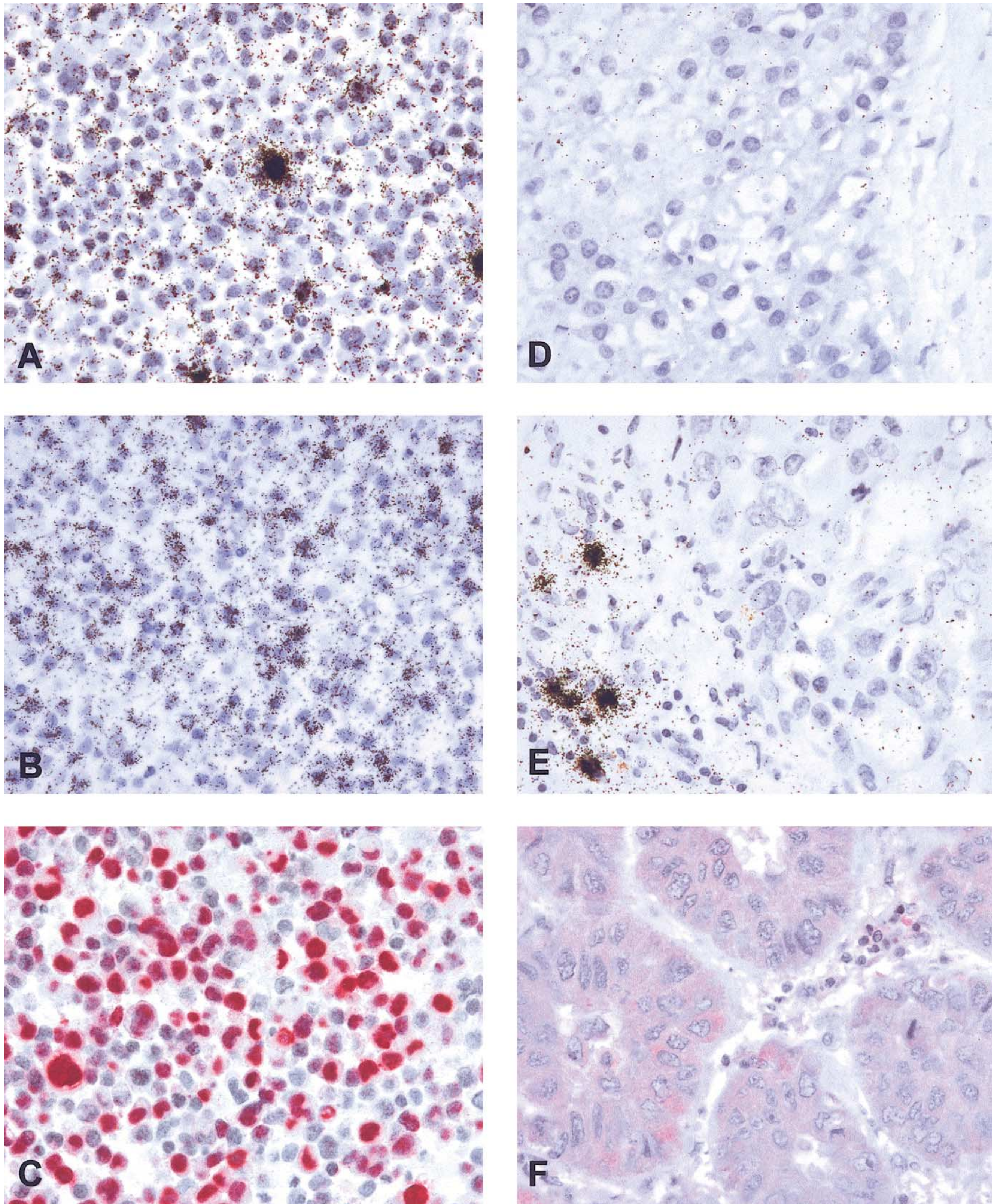

Fig. 3. In situ hybridization for the detection of EBV DNA using a ^{35}S-labeled probe reveals the presence of the virus in most cells of the B95.8 lymphoblastoid cell line (A). Using in situ hybridization with ^{35}S-labeled RNA probes, expression of the EBERs is detected in the vast majority of B95.8 cells (B). Similarly, expression of EBNA1 is detected by immunohistochemistry in most B95.8 cells (C, red labeling). Application of these methods to hepatocellular carcinomas reveals absence of viral DNA from the tumour cells (D) and lack of expression of the EBERs (E) or EBNA1 (F) in the epithelial tumor cells. Note a few EBER-expressing lymphoid cells in the tumor stroma of a hepatocellular carcinoma in E.

followed by Q-PCR: Sections measuring 4 μm were cut onto uncoated slides, taken to water, and stained in hematoxylin. This was followed by thorough dehydration and two washes in xylene. LCM was performed on dry sections using the PixCell II system (Arcturus Engineering Inc. Mountain View, CA). Multiple individual clusters of tumor cells from different regions of each tumor were microdissected (Fig. 2) and pooled onto the same cap. Clusters of tumor cells containing prominent lymphocytes or other nonmalignant cells were not collected. DNA was extracted from LCM-procured tumor cells using the LCM/DNA extraction kit (Arcturus Ltd.) or the DNeasy kit (Qiagen Ltd.).

Isotopic in situ hybridization

All U.K. and German cases identified as PCR-positive by whole-section PCR were investigated using an isotopic in situ hybridization method to detect EBV DNA provided sufficient tissue was left in the block. The plasmid pBS-W harboring the *Bam*HI W fragment of the EBV genome was labeled with [^{35}S]dCTP (1200 Ci/mmol, NEN Life Science, Cologne, Germany) by nick translation using a commercially available kit (Amersham, Braunschweig, Germany). In situ hybridization was carried out as described previously (Niedobitek et al., 1991). Controls were pelleted paraffin embedded B95.8 cells and these served as surrogates for clinical samples containing latent EBV episomes. In brief, paraffin sections were dewaxed, rehydrated, treated with 0.2 N hydrochloric acid and pronase (0.5 mg/ml, Boehringer, Mannheim, Germany), acetylated in 0.1 M triethanolamine pH 8.0, 0.25% (v/v) acetic anhydride, dehydrated through graded ethanols, and air dried. An amount of 25 μl of hybridization mixture (50% deionized formamide/2× SSC/ 10% dextran sulphate/100 μg/ml carrier DNA/10 mM DTT/1 ng labeled probe) was added per slide. Tissue and probe DNA were simultaneously denatured at 95°C for 2 min, followed by hybridization at 37°C overnight. Subsequently, slides were washed in 50% formamide/2× SSC/10 mM DTT at 37°C for 4 h, dehydrated through graded ethanols, air dried, and dipped in Ilford G5 photographic emulsion. Slides were stored at 4°C for 3 to 12 days, developed, fixed, and counterstained. This method was previously shown to detect a single copy of the EBV genome in AW Ramos cells (Niedobitek et al., 1991).

Immunohistochemistry for EBNA1

In parallel, immunohistochemistry was performed for detection of EBNA1 protein on all German cases and on all U.K. cases in which Q-PCR of whole sections detected EBV. Briefly, sections were taken to water and endogenous peroxidase activity blocked in 3% hydrogen peroxide/methanol, prior to microwave antigen retrieval in citric acid buffer pH 5.8 for 50 min at full power (750 W). Following an endogenous biotin-blocking step (Dako Ltd., Cat No. X0590), sections were incubated in monoclonal antibody reactive against EBNA1 (either 2B4-1 or 1H4-1, gifts from E. Kremmer) for 1 h at RT. After a brief wash in TBS pH 7.6, mouse anti-rat antibody (Dako Ltd., Cat No. E0468) was then applied for 30 min prior to detection using the StreptABComplex/HRP duet mouse/rabbit kit (Dako Ltd., Cat No. K0492.). Visualization was by the standard diaminobenzidine reaction.

EBER in situ hybridization

In situ hybridization for EBER-1 and EBER-2 was also carried out on those U.K. cases identified as EBV DNA positive by whole-section Q-PCR and on all German cases. Digoxigenin- or ^{35}S-labeled probes were prepared by in vitro transcription of recombinant plasmids (pBSJJJ1 for EBER-1 and pBSJJJ2 for EBER-2) in the presence of digoxigenin- or ^{35}S-labeled nucleotides (Niedobitek et al., 1991). Sense probes and known EBV-positive and -negative controls were included in all runs.

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