Unique Transcription Pattern of Epstein-Barr Virus (EBV) in EBV-carrying Gastric Adenocarcinomas: Expression of the Transforming BARF1 Gene

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ABSTRACT

Approximately 10% of gastric adenocarcinomas worldwide are associated with human EBV. These carcinomas generally do not express the latent membrane protein 1 (LMP1), the major known EBV oncoprotein.

Recently, another EBV gene [i.e., BARF1 (BamHI A rightward open reading frame)] was shown to have transforming and immortalizing capacities. Therefore, in this study, we investigated the expression of BARF1 in EBV-carrying gastric adenocarcinomas in relation to the expression of other latent EBV transcripts.

In the present study, 10 of 132 gastric adenocarcinomas tested positive for EBV using EBER1/2-RNA in situ hybridization. We demonstrate BARF1 gene transcription in nine EBV-carrying gastric adenocarcinomas (with sufficient RNA quality) using the BARF1-specific nucleic acid sequence-based amplification assay. In addition, we also detected other latent EBV transcripts (i.e., BARF0-, LMP2A-, and Q/K-driven EBNA1 transcripts in these carcinomas using reverse transcription-PCR analysis).

No expression of LMP1, EBNA2, and ZEBRA (either at transcription or protein level) was found. In addition, two cases were positive for BHRF1 transcripts, the viral bcl-2 homologue. Thus, together with BARF1 transcription, a unique and distinct EBV latency type has been found in EBV-associated gastric adenocarcinomas.

Because BARF1 exerts immortalizing effects on human epithelial cells in vitro and EBV-carrying gastric adenocarcinomas lack the expression of LMP1, the BARF1 gene might act as the viral oncopogene in EBV-carrying gastric carcinomas. The BARF1 gene offers an alternative way for EBV-mediated oncogenesis other than LMP1.

INTRODUCTION

Gastric cancer is the second leading cause of cancer-related mortality worldwide, and clinical prognosis is very poor. Apart from the accepted role of Helicobacter pylori in the pathogenesis of gastric carcinomas, the human γ-herpesvirus EBV is present in ~10% (range, 2–16%) of human gastric adenocarcinomas worldwide (1–3). Furthermore, EBV is associated with 80%–100% of the rare lymphoepithelioma-like gastric carcinomas (4) and is also present in ~35% of the gastric stump carcinomas (5). The pathogenic role of EBV in gastric adenocarcinomas remains still undefined. The latency type of EBV in gastric adenocarcinomas is distinct from the known EBV latency types (e.g., in Burkitt’s lymphomas and nasopharyngeal carcinomas; Ref. 6). This is mainly due to the expression of LMP2A and the absence of LMP1 expression in gastric adenocarcinomas; Ref. 6). This is mainly due to the expression of LMP1 oncoprotein (7), although LMP1 expression has occasionally been reported (8, 9).

Apart from LMP1, another EBV gene (i.e., BARF1) has recently been determined as a transforming and immortalizing EBV gene (10, 11). The BARF1 open reading frame is located within a 40-kb fragment of the EBV genome and encodes a M, 33,000 protein. This 40-kb fragment encompasses the BamHI D to BamHI A regions of the EBV genome and is able to immortalize primary monkey and human epithelial cells in vitro (12, 13). Wei et al. (11) recently demonstrated that BARF1 is involved in the immortalization of primary monkey epithelial kidney cells. Furthermore, it has been demonstrated that transfection of BARF1 into the rodent fibroblasts cell line BALB/c 3T3 or in the EBV-negative B cell line Louckes resulted in tumorigenic transformation (10, 14). Injection of the transfected murine fibroblasts into newborn rats led to development of aggressive BARF1-expressing tumors, whereas injection of the transfected Louckes cell line induced only small tumors that disappeared 3 weeks after injection.

Recently, Stockbicine et al. (15) reported that BARF1 is a functional homologue of the human CSF receptor. The CSF receptor is the gene product of the human proto-oncogene c-fms. This homology between BARF1 and c-fms is especially interesting in the context that c-fms and CSF1 have been suggested to modulate neoplastic mammary epithelial cell proliferation (16).

Because LMP1 is generally not expressed in EBV-carrying gastric adenocarcinomas, we studied here the expression of BARF1 as an alternative way for EBV-mediated oncogenesis in relation to the expression of other latent EBV genes.

MATERIALS AND METHODS

Cell Lines. The EBV-positive lymphoblastoid B cell line JY was used as a positive control for the expression of the EBV transcripts.

The EBV-negative Louckes cell line, Louckes1–5, transfected with a BARF1 expression construct (14), was kindly provided by Dr. T. Ooka (Laboratoire de Virologie Moléculaire, Centre National de la Recherche Scientifique, Lyon, France). The EBV-positive C15 tumor cell line derived from a nasopharyngeal carcinoma was kindly provided by Dr. B. Griffin (Imperial College School of Medicine, London, United Kingdom; Ref. 17).

Clinical Material. Paraffin-embedded gastric adenocarcinomas (n = 132), of which also frozen material was available, collected at the Department of Pathology of the University Hospital Vrije Universiteit (Amsterdam, the Netherlands), were tested by EBER1/2-RISH for the presence of EBV. Corresponding snap-frozen material of these EBV-positive gastric carcinomas and 10 gastric control tissues, including 5 EBV-negative gastric carcinomas and 5 specimens of normal gastric epithelium, were used for the RNA EBV-transcript analysis. Before RNA isolation, the sandwich frozen sections (of this material) were H&E stained and microscopically checked for the presence of tumor cells.

EBER1/2-RISH. Paraffin-embedded tissue from 132 gastric carcinomas was subject to a nonradioactive EBER1/2-RISH using the Digoxigenin-labeled antisense and sense EBER1/2 probe, as described previously (18).

Oligonucleotide Primers and Probes. All EBV-specific primers (i.e., EBNA1, EBNA2, BARF0, LMP1, LMP2A, BHRF1, and ZEBRA; Ref. 19) and primers specific for the U1 small nuclear ribonucleoprotein-specific A protein (20) have been described previously.
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RESULTS

EBER1/2-RISH. Ten (7.6%) of 132 gastric adenocarcinomas tested positive for EBV by EBER1/2-RISH. Using the EBER1/2 antisense probe, nuclear EBER1/2 expression was detected in the majority of, if not all, neoplastic cells of these gastric adenocarcinomas (Fig. 1). Nuclear staining was also found in the positive controls used (i.e., JY cell line and one EBV-positive Hodgkin’s lymphoma). No expression was seen using the EBER1/2 sense probe.

Assessment of RNA Quality. Of the corresponding snap-frozen tissues of the 10 EBV-positive gastric adenocarcinomas, 9 revealed a sufficient RNA quality for further transcript analysis (summarized in Table 1), as was shown by clearly visible 18S and 28S rRNA bands and U1 small nuclear ribonucleoprotein-specific A protein mRNA RT-PCR.

BARF1 Transcription by NASBA. The nine remaining EBV-positive gastric adenocarcinomas were tested for BARF1 transcription using the NASBA (i.e., an alternative RNA amplification method that enables a reliable and sensitive detection of target RNA in the presence of DNA independent of splice sites). Indeed, all nine EBV-positive gastric adenocarcinomas did show expression of BARF1-RNA using the sensitive BARF1-NASBA assay, revealing a 203-bp fragment (Fig. 2a). In contrast, EBV-negative gastric adenocarcinomas (Fig. 2b) and normal gastric control tissue (data not shown) did not show transcription of BARF1. As shown in Fig. 2, a and b, the positive controls (i.e., Loukes1–5 cell line and C15 tumor cell line) showed BARF1 transcripts, whereas the negative control (distilled water) was negative.

EBV Transcript Analyses by RT-PCR. Using RT-PCR all nine carcinomas expressed BARF0 transcripts. Furthermore, we found Q-promoter-driven EBNA1 transcription in eight of the nine cases. In contrast, no Cp/Wp-promoter-driven EBNA1 transcripts could be demonstrated. In accordance with previously published results, no LMP1 and EBNA2 transcripts were found, whereas seven of nine cases displayed LMP2A expression at the RNA level (shown in Fig. 3). Two cases actually displayed BHRF1 transcripts driven by the H2/HF-promoter (data not shown). In contrast, no Cp/Wp-promoter-driven BHRF1 transcripts were found and no expression of ZEBRA transcripts. All RT-PCR and NASBA results are summarized in Table 1.

**Table 1 Summary of results in nine EBV-positive gastric carcinomas**

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Fig. 1. EBER1/2-RISH in a gastric adenocarcinoma. EBER1/2 signals (antisense Dig-labeled riboprobes) are located in the nuclei of the carcinoma cells.
In this study, we found EBV in 7.6% of the gastric adenocarcinomas. Because gastric adenocarcinomas generally do not express LMP1—until now the major EBV oncogene—BARF1 expression in EBV-carrying gastric carcinomas may be an alternative way for EBV-mediated gastric carcinogenesis. BARF1 has recently been determined as a transforming gene in rodent fibroblasts and as an immortalizing gene in primary monkey epithelial cells (10, 11). In this context, it is interesting that stockbine et al. (15) recently demonstrated that the \textit{BARF1} gene encodes a novel \textit{CSF-1} receptor. BARF1 shares a subtle, highly localized region of homology with several members of the tyrosine kinase receptor family, including the cellular proto-oncogene \textit{c-fms}, which encodes the \textit{CSF-1} receptor. \textit{CSF-1 and c-fms} expression have been suggested to be involved in the modulation of neoplastic mammary epithelial cell proliferation (16). According to Storga et al. (29), \textit{c-fms} is expressed in gastric adenocarcinomas, but the role of \textit{c-fms} in gastric carcinogenesis has not been further elucidated. Theoretically, BARF1 might act as a homologue of \textit{c-fms} proto-oncogene in immortalizing gastric epithelium, but additional studies concerning the role of \textit{c-fms} and \textit{BARF1} in gastric carcinomas need to support this hypothesis. Only recently, Cohen and Lekstrom (30) demonstrated that \textit{BARF1} is dispensable for B-cell transformation and interacts with the cellular IFN production. However, the recombinant EBV mutant used by Cohen and Lekstrom (29) still contained the transforming domain of \textit{BARF1} (AA 1–54), which might have influenced their results. As shown by the \textit{in vitro} immortalizing and transforming capacities in epithelial cells (Ref. 11; and supported by our data \textit{in vivo}), we suggest that \textit{BARF1} exerts different functions in lymphoid and epithelial cells: in the latter \textit{BARF1} might be involved in the lytic cycle, acting as an early protein, whereas in epithelial cells \textit{BARF1} has immortalizing/transforming capacities.

**In this study, we found EBV in 7.6% of the gastric adenocarcinomas.**

**DISCUSSION**

In the present study, we demonstrate a novel EBV latency pattern in EBV-carrying gastric adenocarcinomas (Table 2), predominantly based on the presence of \textit{BARF1} transcripts and the absence of LMP1 expression. This is the first time that transcription of the transforming \textit{BARF1} gene has been demonstrated in EBV-carrying gastric adenocarcinomas. The novel latency pattern is characterized by transcription of the transforming \textit{BARF1} gene, \textit{EBER1/2}, Q-promoter-driven \textit{EBNA1}, \textit{BARF0}, \textit{LMP2A}, and the absence of \textit{LMP1} and \textit{EBNA2} transcription. Although \textit{BARF1} is designated as an early gene in lytic infection in B-lymphocytes, the transforming \textit{BARF1} is exclusively transcribed as a latent gene in EBV-associated epithelial malignancies (i.e., NPC; Refs. 21 and 27) and gastric carcinomas (this study). Shih-Lammali \textit{et al.} (27) previously demonstrated weak \textit{BARF1} transcription in two of five North African NPCs using a reverse Northern blotting technique. In addition, Brink \textit{et al.} (21) and Hayes \textit{et al.} (28) used a more sensitive technique (i.e., NASBA) and found \textit{BARF1} expressed in almost all NPCs and not in lymphoid malignancies or productive EBV infection (oral hairy leukoplakia).

**Additional IHC studies using \textit{BARF1}-specific monoclonal antibodies are needed to show expression at the protein level in EBV-carrying gastric adenocarcinomas.**

**EBV-carrying gastric adenocarcinomas were tested immunohistochemically for \textit{EBNA1}, \textit{LMP1}, and \textit{ZEBRA}. Using the anti-\textit{EBNA1}-specific rat monoclonal antibody 2B4 –1, 5 of 10 carcinomas revealed staining for \textit{LMP1} or \textit{ZEBRA}. Data are protein expression using the 2B4 –1 anti-\textit{EBNA1} antibody. None of the 10 carcinomas revealed staining for \textit{EBNA1}, \textit{LMP1}, and \textit{ZEBRA}. Using the \textit{anti-EBV-associated gastric carcinomas}.**

**IHC.** EBV-carrying gastric adenocarcinomas were tested immunohistochemically for \textit{EBNA1}, \textit{LMP1}, and \textit{ZEBRA}. Using the anti-EBNA1-specific rat monoclonal antibody 2B4 –1, 5 of 10 carcinomas showed protein expression of \textit{EBNA1}. Interestingly, one gastric carcinoma that was tested negative by RT-PCR for \textit{EBNA1} did show protein expression using the 2B4–1 anti-EBNA1 antibody. None of the 10 carcinomas revealed staining for LMP1 or ZEBRA. Data are summarized in Table 1. EBV-positive control (i.e., \textit{JY} cell line) tested positive for \textit{EBNA1}, LMP1, and ZEBRA.

**DISCUSSION**

In the present study, we demonstrate a novel EBV latency pattern in EBV-carrying gastric adenocarcinomas (Table 2), predominantly based on the presence of \textit{BARF1} transcripts and the absence of LMP1 expression. This is the first time that transcription of the transforming \textit{BARF1} gene has been demonstrated in EBV-carrying gastric adenocarcinomas. The novel latency pattern is characterized by transcription...
BARF1 TRANSCRIPTION IN EBV-CARRYING GASTRIC CARCINOMAS

Griffin, B. E., and Karran, L. Immortalization of monkey epithelial cells by specific EBNA1 protein using the 2B4–1 anti-EBNA1 antibody. One gastric carcinoma that did show EBNA1 protein expression tested negative in RT-PCR for EBNA1. This result might reflect nonspecific binding of this antibody, as has been described and discussed extensively recently by Cruz et al. (32). The EBNA1 protein expression in EBV-carrying gastric adenocarcinomas has also been demonstrated previously by other groups using IHC (33, 34). The absence of LMP1 (either at the transcription and protein level) and the presence of BARF0 is in line with recently published data of Suguita et al. (6). The absence of LMP1 in these carcinomas distinguishes this novel EBV latency type from the EBV latency type seen in NPCs, which is another EBV-associated epithelial malignancy. Interestingly, we also found BHRF1, the viral bcl-2 homologue, in two cases. The meaning of this remains to be determined.

In conclusion, in this study we showed that a novel EBV latency pattern in EBV-carrying gastric adenocarcinomas is present, especially characterized by BARF1 transcript expression and the absence of LMP1 (either at the RNA and protein level). BARF1 might act as the viral oncogene in the development of EBV-carrying gastric adenocarcinomas. Additional studies are needed, including the development of BARF1-specific antibodies and the application of morphological techniques like RISH and IHC. Functional assays with BARF1 are necessary to determine its role in (gastric) carcinogenesis. BARF1 might be a novel therapeutic target for EBV-carrying gastric adenocarcinomas.

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REFERENCES


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