

Cyclin D1 expression is induced by viral BARF1 and is overexpressed in EBV-associated gastric cancer

Thorsten Wiech · Elisabeth Nikolopoulos ·
Silke Lassman · Timo Heidt · Anja Schöpflin ·
Mario Sarbia · Martin Werner · Yuko Shimizu ·
Emna Sakka · Tadamasu Ooka · Axel zur Hausen

Received: 3 October 2007 / Revised: 7 January 2008 / Accepted: 6 February 2008 / Published online: 25 April 2008
© Springer-Verlag 2008

Abstract Approximately 10% of gastric carcinomas (GC) worldwide are associated with Epstein–Barr virus (EBV). GC is one of the most frequent human malignancies associated with EBV. The latent expression of the EBV-oncogene BARF1 is restricted to epithelial malignancies. To investigate the underlying BARF1-related mechanisms of oncogenic epithelial transformation, we analyzed gene expression profiles of a BARF1-transfected epithelial (HaCaT+) and the corresponding BARF1-negative (HaCaT-) cell line by cDNA microarray analysis. Real-time PCR was performed to confirm the cDNA microarray results. In addition, immunohistochemistry and fluorescence in situ hybridization were performed on a tissue microarray of 181 GC including 11 EBV-associated GC. Among other genes cyclin D1 expression was significantly

upregulated in HaCaT+ on the transcriptional and protein level. Cyclin D1 protein expression in GC revealed a significant overexpression of cyclin D1 in EBV-associated GC ($p < 0.012$) but not in EBV-negative GC. Cyclin D1 FISH showed that cyclin D1 overexpression was not due to gene amplification in EBV-associated GC. Cyclin D1 is induced in HaCaT+ by BARF1 and is overexpressed in EBV-associated GC indicating an interaction of viral BARF1 and cyclin D1.

Keywords Epstein–Barr virus · Gastric carcinogenesis · BARF1 · Viral oncogene · Cyclin D1

Introduction

Although the incidence of gastric cancer is declining, it still remains the fourth most common type of cancer and the second leading cause of cancer-related death worldwide [6]. The association of EBV with approximately 10% of gastric carcinomas (GC) and endemic nasopharyngeal carcinoma (NPC) is well established [18, 28]. With an incidence of approximately 88,000 new cases each year worldwide, GC is one of the most frequent EBV-associated human malignancies. However, the exact role of EBV in the carcinogenesis of GC and NPC remains to be elucidated, though very recent findings indicate an important and independent role of EBV in gastric carcinogenesis [1]. Recently, the expression of the EBV-encoded oncogene BARF1 (i.e., *Bam*HI A rightward open reading frame 1) has been reported in EBV-associated GC [15, 27, 36]. Latent BARF1 expression is restricted to epithelial EBV-associated malignancies [8, 11, 15, 24, 27, 36]. BARF1 induces malignant transformation of rodent fibroblasts and transfection of the BARF1 gene enhances the tumorigenic-

T. Wiech · E. Nikolopoulos · S. Lassman · T. Heidt ·
A. Schöpflin · M. Werner · Y. Shimizu · A. zur Hausen (✉)
Institute of Pathology, University Hospital Freiburg,
Breisacherstr, 115A,
79106 Freiburg, Germany
e-mail: axel.zurhausen@uniklinik-freiburg.de

M. Sarbia
Institute of Pathology, Technical University,
Munich, Germany

Y. Shimizu · E. Sakka · T. Ooka
Laboratoire de Virologie Moléculaire, UMR5537, CNRS,
Faculté de Médecine R.T.H. Laennec,
Université Claude Bernard Lyon-1,
Lyon, France

Present address:
M. Sarbia
Institute of Pathology,
Nymphenburg,
Munich, Germany

ity of BL-derived Louckes and Akata cells [26, 31, 32]. In addition, BARF1 is able to immortalize primary monkey epithelial cells [33]. Transfection of the BARF1 gene has been reported to result in growth activation of monkey epithelial cells, in which the viral sequence was specifically localized in double minute chromosomes [9]. Thus it appeared that BARF1 was implicated not only in cell immortalization but also in malignant transformation. It has been demonstrated recently that the first 54 amino acids of BARF1 are sufficient to both, induce malignant cell transformation, and activate Bcl2 expression [25]. Bcl2 activation was also observed in EBV-negative human Akata B cell transfected by BARF1 [26]. However, the oncogenic mechanism induced by BARF1 is not yet known.

Cyclin D1 is a positive regulator of the cell cycle and a proto-oncogene. Binding of cyclin D1 to cyclin-dependent kinase Cdk4 initiates phosphorylation of Rb which results in the release of E2F and transcriptional activation of S-phase genes [5]. Aberrant overexpression of cyclin D1 leads to a disturbed cell cycle regulation. Cyclin D1 gene amplification or high expression of its protein has been shown to be frequent events in diverse human malignant tumors [14, 16, 22]. Besides gene amplification, the transcription of cyclin D1 mRNA and the expression of its protein may be modulated by genetic polymorphisms [2, 12, 23].

In this study, we used gene expression profiling and identified cyclin D1 expression induced by BARF1 in a BARF1-transfected epithelial HaCaT cell line and analyzed cyclin D1 expression and gene amplifications in EBV-associated and EBV-negative gastric carcinomas. Our data underline recent findings that EBV-associated gastric carcinomas comprise a distinct clinico-pathological entity of gastric carcinoma [29, 35].

Materials and methods

Cell lines HaCaT cells were transfected by pBABE vector containing BARF1 sequence and selected by 500 µg/ml of neomycine. BARF1 sequence was at first amplified by PCR using two primers, then inserted in the *Bam*H1- and *Eco*R1-linearized pBABE vector. After amplification of BARF1 recombinant pBABE vector, HaCAT cells were transfected. At 24-h post-transfection, 500 µg/ml of neomycine was added into the culture medium, then neo-resistant cells were isolated after 3 weeks culture (E. Sakka et al., submitted).

RNA isolation, sample processing, and array hybridization Total RNA was extracted with Tri-reagent (Sigma, France) and treated twice with DNase 1 (Stratagene). The quality of total RNA and biotin-labeled cRNA of all samples was checked by calculating the ratio of absorbance at 260 and 280 nm (between 1.9 to 2.1) and agarose gel electrophoresis.

Further sample processing, including labeling, hybridization, and image scanning was performed using standard Affymetrix protocol. In brief, 5 µg of total RNA from each cell line, T7-oligo(dT) primers, and Superscript II RT (GibcoBRL®) were used for first strand cDNA synthesis. After second strand synthesis, in vitro transcription was performed using IVT Transcript Labeling Kit (Ambion, Inc.) to generate biotinylated cRNA targets. cRNA targets were fragmented at 94°C for 35 min and 15 µg of biotinylated cRNA fragments was hybridized to HG-U133A chips (Affymetrix, Santa Clara, CA, USA) at 45°C for 16 h. The arrays were washed and stained with 10 µg/ml streptavidin–phycoerythrin. After signal amplification with biotinylated anti-streptavidin antibodies, the HG-U133A chips, containing 22,283 probe sets representing 14,564 human genes, were scanned using the GeneChip® Scanner 3000.

Quality control and data analysis CEL files, containing the signal intensity value and the standard deviation for each probe, were generated using the GeneChip operating software (Affymetrix, Santa Clara, CA, USA). As a quality control criterion, the ratios of 3' signal to 5' signal of the housekeeping gene GAPDH were between 0.9 and 3.8. The signals of the hybridization controls BioB, BioC, BioD, and Cre were in a linear relationship of intensity. Furthermore, the scale factors between the arrays did not vary by fourfold and the background intensities did not exceed 60. For every probe, the difference expression between each pair of samples was weighted by the absolute of the mean difference. We denoted this measure by the vector $m = m_{\text{probe}_1, \dots, \text{probe}_{22.283}}$. Note that m_{probe_i} takes positive values for genes that were overexpressed in the modified sample or otherwise negative values. The probes were sorted with respect to m separately in both trials. Furthermore, we determined the pool of probes where $m > 0$ in both trials and sorted the resulting lists with respect to m . cDNA synthesis and TaqMan analysis Reverse transcription of mRNA was performed using oligo(dT) primers and Superscript™ II RT 200 U/µl (GibcoBRL®).

For validation of the expression array results, a semi-quantitative real-time PCR (TaqMan, Applied Biosystems, Foster City, USA) for selected genes (cyclin D1, defensin beta 1, protease inhibitor 3 SKALP, DiGeorge syndrome critical region gene 8, DEAD box polypeptide 58) was performed. For cyclin D1 the primers, forward primer CCC TGA CGG CCG AGA AG, the reverse primer AGG TTC CAC TTG AGC TTG TTC AC, and the probe 6-FAM-AGG AGC TGC TGC AAA TGG AGC TG-TAMRA were used. For relative quantification the housekeeping gene TATA box binding protein (TBP) was amplified. For the other genes, the TaqMan® Gene Expression Assays (Applied Biosystems) were used according to the manufacturer's instructions. The ratio of the amount of the gene

to the amount of the TBP at the beginning of the reaction was determined with assumption of maximum efficiency using the formula: $S_{\text{gene}}/STBP=2^{-Ct} (TBP)/2^{-Ct} (\text{gene})$ with S being the starting amount and Ct the cycle number of the amount exceeding the detection threshold.

Immunohistochemistry Immunohistochemistry (IHC) for cyclin D1 was carried out as described previously [21]. A tissue microarray (TMA), which contained among others 92 carcinomas of the gastric cardia and 89 distal GC [20], was evaluated for cyclin D1 expression. The expression of cyclin D1 as tested by IHC was evaluated by light microscopy. The percentage of cyclin D1-positive tumor cells was determined according to the following category: 0 (0%), 1 (1–4%), 2 (5–24%), 3 (25–49%), 4 (50–74%), and 5 (75–100%) [21]. The cyclin D1 expression was correlated to the EBV status which has previously been described [20]. Statistical analyses were performed using Fisher's exact test. *p* values <0.05 were considered as significant.

Western immunoblotting To analyze cyclin D1 protein expression in BАРF1-transfected and mock control HaCaT cells we performed Western immunoblotting as previously described using a 10% polyacrylamide gel [13]. The monoclonal cyclin D1 antibody (clone SP4, DCS, Germany; 1:5,000 dilution) detected the 36-kDa cyclin D1 protein. Equal amounts of protein were loaded on the gel as tested by Bradford assay, Ponceau staining, and β -actin Western immunoblotting (clone AC-15, Sigma, Germany; 1:1,000; Fig. 2b).

Fluorescence in situ hybridization For fluorescence in situ hybridization (FISH) analysis, a PathVysion cyclin D1 DNA probe kit (Vysis; Downers Grove, UK) was used according to the manufacturer's recommendations. The kit consists of directly labeled fluorescent DNA probes specific for the PRAD1/cyclin D1 gene locus (11q13.3) and a DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1–q11.1). Tissue pretreatment was performed as previously described [34]. Counterstaining of nuclei was performed using diaminobenzidine. Nuclei from normal squamous epithelium or lymphocytes deposited separately on the same slide were used as controls of hybridization efficiency and specificity.

Results

Stable BАРF1 transfection of epithelial HaCaT cells

Transcriptional expression of BАРF1 was analyzed by RT-PCR prior to cDNA microarray analyses. RNA of the EBV-positive BАРF1-expressing P3HR1 and Louckes cell line

served as positive controls. Specific full-length transcripts of BАРF1 were restricted to P3HR1 cells and BАРF1-transfected HaCaT cells (Fig. 1) while no BАРF1 transcripts were detected in vector control cells. In addition, BАРF1 protein expression was tested in both transfectants and as expected the largest part of BАРF1 protein was secreted into the culture medium [7, 19].

cDNA array analysis of BАРF1-transfected epithelial HaCaT cells

The gene expression profile of BАРF1-transfected epithelial HaCaT cells was compared to its pBABE vector control HaCaT cells. Detailed analysis revealed 1,010 genes to be differentially expressed. Most of them showed a decreased expression (94.3%) whereas only a small percentage was upregulated (5.7%). A summary of the 20 most significantly downregulated genes in BАРF1-transfected HaCaT cells is given in Table 1.

Functional gene groups up- or downregulated in BАРF1-transfected HaCaT cells

Analyzing the results with respect to functional groups, it is interesting that 22.9% of genes induced by BАРF1 expression in the epithelial background of the HaCaT cells could be grouped to transcription (Table 2). In addition, rather unexpected no significant number of genes was found to be induced in the group of cell proliferation in HaCaT cells. The results are summarized in Table 2.

Validation of microarray results by quantitative RT-PCR and Western blotting

In order to evaluate the data obtained by microarray analysis, Taqman analysis was performed for the following genes, i.e., *mad2*, *bcl-xl*, protease inhibitor-3, DiGeorge syndrome 8, human defensin beta 1, *DEAD*, and cyclin D1. The results are shown in Fig. 2a with the given fold change as tested by Taqman analysis. Cyclin D1 expression was

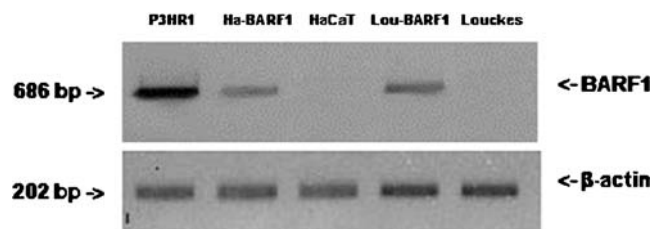


Fig. 1 Upper panel: specific transcription of full-length BАРF1 (686 bp) in the EBV-positive epithelial cell line P3HR1 cells, BАРF1-transfected HaCaT cells (*Ha-BАРF1*) and BАРF1-transfected Louckes cells (*Lou-BАРF1*) tested by RT-PCR. No BАРF1 transcripts are detected in the pBABE HaCaT and pZip Louckes cells. Lower panel: RT-PCR reveals β -actin transcripts in all tested cell lines

Table 1 Summary of the 20 most significantly down regulated genes in BARF1-transfected HaCaT cells

Symbol	Gene name	Accession	fc change
SKALP	<i>Homo sapiens</i> protease inhibitor 3, skin-derived (SKALP)	NM_002638.1	-19.91
ENO1L1	Human alpha enolase like 1	U88968.1	-10.48
	Human elafin gene	L10343	-9.32
	Translocase of inner mitochondrial membrane 17	BC004439.1	-6.45
	14-3-3 protein mRNA	U28964.1	-6.08
SKP1A	S-phase kinase-associated protein 1A (p19A)	NM_006930.1	-5.83
	Capping protein (actin filament)	BC005338.1	-5.7
	HSPC274 protein	NM_014145.1	-5.64
	Similar to heat shock protein, 110 kDa	BC002526.1	-5.52
SPRR1B	Small proline-rich protein 1B (cornifin)	NM_003125.1	-5.48
MTAP44	Interferon-induced, hepatitis C-associated microtubular aggregate protein (44 kDa)	NM_006417.1	-5.09
RAB1	RAB1, member RAS oncogene family	NM_004161.1	-5.07
JNK2B1	JNK2 beta1 protein kinase	U35002.1	-5.02
IL1A	Human interleukin 1-alpha	M15329.1	-5.01
SPTLC1	Serine palmitoyltransferase, long chain base subunit 1	NM_006415.1	-4.57
ANX14	Annexin 14	AF196478.1	-4.54
SCCA2b	<i>Homo sapiens</i> mRNA for SCCA2b	AB046400.1	-4.33
	Human mitochondrial NAD(P) ⁺ -dependent malic enzyme	M55905.1	4.19
RHAMM	Hyaluronan receptor	U29343.1	-4.14
IL8	Interleukin 8 C-terminal variant	AF043337.1	-4.11

found 1.53-fold upregulated in BARF1-transfected HaCaT cells by microarray analysis. A triplicate testing revealed a 2.5-fold overexpression of cyclin D1 in BARF1-transfected HaCaT cells as tested by Taqman analysis.

In addition, protein expression of cyclin D1 was assessed by Western blotting analysis. Cyclin D1 protein expression was upregulated in BARF1-transfected HaCaT cells whereas this upregulation was not found in the BARF1-negative mock control HaCaT cells (Fig. 2b).

Cyclin D1 expression in EBV-positive and EBV-negative GC

Since cyclin D1 protein expression has been implicated in gastric carcinogenesis recently [3, 4, 10, 17, 30], we

Table 2 Comparison of functional gene groups up- or downregulated in BARF1-transfected HaCaT cells

	HaCaT BARF1+	
	Gene expression induced by BARF1	Gene expression downregulated by BARF1
Genes	58	952
Apoptosis	5.7%	4.9%
Cell cycle	5.7%	6.4%
Cell proliferation	–	5.4%
Cell adhesion	2.9%	1.4%
Transcription	22.9%	10.2%
Metabolism	5.7%	9.4%
Mitosis	2.9%	1.8%

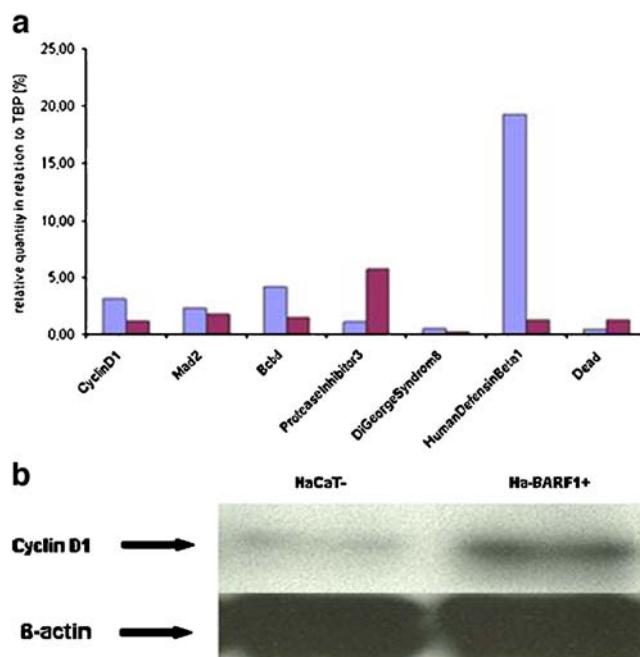


Fig. 2 **a** Results of Taqman RT-PCR analyses of selected genes up- or downregulated by BARF1 in HaCaT cells: bars in blue represent HaCaT BARF1-transfected cells and bars in red BARF1-negative vector control HaCaT cells. Y-axis indicates the relative quantity in relation to TBP. Bars represent the average of 3 (cyclin D1, DiGeorge syndrome 8, human defensin beta 1 and bcl-x1) and 2 (Mad2, DEAD, protease inhibitor 3) independent analyses. **b** Upper panel: equal amounts of protein (25 µg/lane) were loaded on a 10% SDS page gel. Western immunoblotting reveals strong protein expression of the 36 kDa cyclin D1 protein in BARF1-transfected HaCaT cells in contrast to the BARF1-negative mock control-transfected HaCaT cells. Lower panel: Equal loading was confirmed by β-actin protein expression

decided to test its expression in a large cohort of GC in order to assess the biological importance of cyclin D1 expression in EBV-associated epithelial malignancies. One hundred eighty-one formalin-fixed and paraffin-embedded GC specimens spotted on a tissue microarray were tested for cyclin D1 expression by immunohistochemistry. Of 181 GC, 157 were available for cyclin D1 IHC. Of 11 previously described EBV-positive GC, nine revealed a strong overexpression of cyclin D1 (Fig. 3a–b). In contrast, only 50 EBV-negative GC revealed a strong expression of cyclin D1 within the tumor cells. This difference was statistically significant with a p value <0.0012 .

Cyclin D1 overexpression in EBV-associated GC is not due to gene amplification

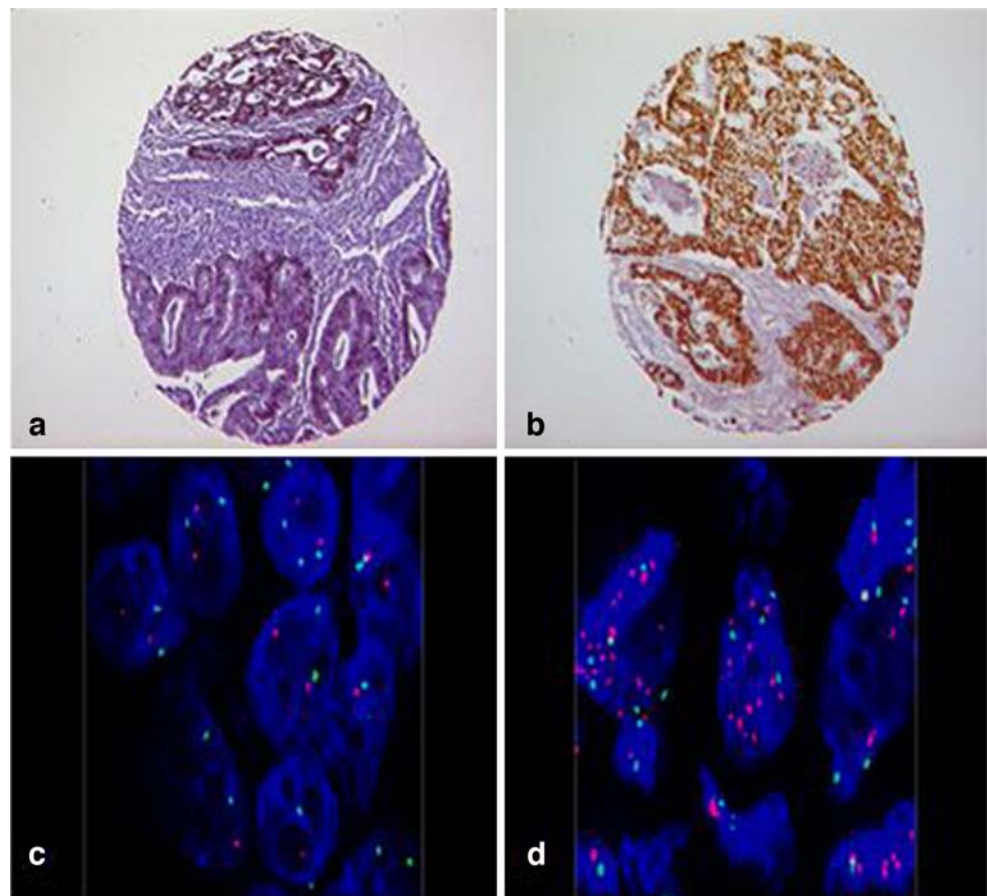
To test whether cyclin D1 overexpression in EBV-associated GC was due to amplification, we performed cyclin D1 FISH. Five of nine EBV-associated and 25 of 50 EBV-negative GC which were shown to overexpress cyclin D1 were left to cyclin D1 FISH analysis because of multiple usage of the TMA and due to the FISH pretreatment protocol. None of the five EBV-associated GC revealed an

amplification of the cyclin D1 gene (Fig. 3c). In contrast, 23 of the EBV-negative GC overexpressing cyclin D1 revealed gene amplification of the cyclin D1 gene (Fig. 3d).

Discussion

Latent expression of the viral oncogene BARP1 is restricted to epithelial EBV-associated malignancies [8, 11, 15, 24, 27, 36]. The BARP1 gene is the only viral oncogene of EBV that is expressed in EBV-associated GC. In order to investigate BARP1-mediated effects on gene transcription in epithelial cells, we analyzed the gene expression profile of BARP1-transfected HaCaT cells using Affymetrix HG-U133A expression microarray chips. The number of differentially expressed genes in HaCaT+ was 1,010 genes, and it is of interest that the majority of differentially expressed genes in HaCaT+ (94.3%) were downregulated and only a small proportion of genes were upregulated (5.7%). This might reflect that BARP1 mainly contributes to carcinogenesis by possibly downregulating genes which are involved in the maintenance of the cellular integrity and indeed the analysis of the genes downregulated (Table 2) mainly

Fig. 3 **a** Specific nuclear EBER transcripts (*dark brown*) in the tumor cells of a gastric carcinoma as tested by RNA in situ hybridization. **b** Immunohistochemistry reveals strong nuclear (*brown*) cyclin D1 expression in an EBV-associated gastric carcinoma. **c** No cyclin D1 gene amplification was detected in an EBV-associated gastric carcinoma by cyclin D1 FISH (*red*: cyclin D1; *green*: centromer). **d** Cyclin D1 FISH in an EBV-negative gastric carcinoma overexpressing cyclin D1 reveals cyclin D1 gene amplification (*red*: cyclin D1; *green*: centromer)



revealed major differences in differentially expressed genes involved in transcription, cell proliferation, and metabolism.

The analysis of up- and downregulated genes in BARF1-transformed HaCaT cells reveals that BARF1 expression induces a number of significant changes in gene expression in an epithelial cell line. Some of these changes have been described previously and others have not been associated with gastric carcinogenesis before [4, 25]. Especially genes such as the human defensin beta, skin-derived protease inhibitor-3 and others with significant fold changes of the expression level are of interest for further studies. In the present study, we selected cyclin D1 to further test its importance in EBV-related gastric carcinogenesis. The finding that cyclin D1 expression is induced upon BARF1 transfection in epithelial cells on the transcriptional and protein level is of particular interest. Cyclin D1 expression has previously been associated with gastric carcinogenesis [3, 4, 10, 17, 30]. In our study, 37.6% of GC revealed overexpression of cyclin D1 as tested by IHC. Other studies show a range between 23.7% and 47% of cyclin D1 expressing GC [3, 10, 17]. In a recent study, Chang et al. report a more frequent expression of cyclin D1 in EBV-associated GC [4]. However, data on cyclin D1 gene amplification in GC are very sparse and only few reports range between 10% and 16% coinciding with a fair agreement of overexpression and amplification of the CCND1 gene [3, 10]. Prior to this study, no data about cyclin D1 gene amplification in EBV-associated GC were available. The fact that in our study none of the EBV-associated GC which overexpressed cyclin D1 revealed amplification of the cyclin D1 gene and most of the EBV-negative GC showed amplification indicates that BARF1 of EBV is able to induce cyclin D1 overexpression in these GC. As to what extent if there is a direct or indirect interaction between BARF1 and cyclin D1 is currently under investigation. Among others, BARF1 siRNA experiments will be helpful to determine the modality of BARF1 and cyclin D1 interactions.

In summary, the present study identifies new molecular targets of EBV-related carcinogenesis transcriptionally regulated by the viral oncogene BARF1. Our data strongly indicate a cyclin-D1-mediated role of BARF1 in gastric carcinogenesis. The results of the present gene expression profiling will further help to define the molecular background of BARF1-related carcinogenesis and its influence of its expression in GC.

Acknowledgments We like to acknowledge the excellent technical assistance of Mrs. Bokla and Deepa Pantulu, both from the Institute of Pathology, University Hospital Freiburg, Germany. We thank Axel Walch for helpful discussions with cyclin D1 FISH analyses.

Conflict of interest statement None declared.

References

1. Au WY, Pang A, Chan EC, Chu KM, Shek TWH, Kwong YL (2005) Epstein–Barr virus-related gastric adenocarcinoma: an early secondary cancer post hemopoietic stem cell transplantation. *Gastroenterology* 129:2058–2063
2. Betticher DC, Thatcher N, Altermatt HJ, Hoban P, Ryder WD, Heighway J (1995) Alternate splicing produces a novel cyclin D1 transcript. *Oncogene* 11:1005–1011
3. Bizari L, Borim AA, Leite KR, Gonsalves Fde T, Cury PM, Tajara EH, Silva AE (2006) Alterations of the CCND1 and HER-2/neu (ERBB2) proteins in esophageal and gastric cancers. *Cancer Genet Cytogenet* 165:41–50
4. Chang MS, Lee HS, Jung EJ, Kim CW, Lee BL, Kim WH (2005) Cell-cycle regulators, bcl-2 and NF-kappaB in Epstein–Barr virus-positive gastric carcinomas. *Int J Oncol* 27:1265–1272
5. Coqueret O (2002) Linking cyclins to transcriptional control. *Gene* 299:35–55
6. Correa P, Piazuelo MB, Camargo MC (2004) The future of gastric cancer prevention. *Gastric Cancer* 7:9–16
7. De Turenne-Tessier M, Jolicoeur P, Middeldorp JM, Ooka T (2005) Expression and analysis of the Epstein–Barr virus BARF1-coded protein from a tetracycline-regulatable adenovirus system. *Virus Res* 109:9–18
8. Decaussin G, Sbih-Lammali F, De Turenne-Tessier M, Bouguermouh A, Ooka T (2000) Expression of BARF1 gene encoded by Epstein–Barr virus in nasopharyngeal carcinoma biopsies. *Cancer Res* 60:5584–5588
9. Gao Y, Lu YJ, Xue SA, Chen H, Wedderburn N, Griffin BE (2002) Hypothesis: a novel route for immortalization of epithelial cells by Epstein–Barr virus. *Oncogene* 21:825–835
10. Gao P, Zhou GY, Liu Y, Li JS, Zhen JH, Yuan YP (2004) Alterations of cyclin D1 in gastric carcinoma and its clinicopathological significance. *World J Gastroenterol* 10:2936–2939
11. Hayes DP, Brink AA, Vervoort MB, Middeldorp JM, Meijer CJ, van den Brule AJ (1999) Expression of Epstein–Barr virus (EBV) transcripts encoding homologues to important human proteins in diverse EBV associated diseases. *Mol Pathol* 52:97–103
12. Hosokawa Y, Gadd M, Smith AP, Koerner FC, Schmidt EV, Arnold A (1997) Cyclin D1 (PRAD1) alternative transcript b: full-length cDNA cloning and expression in breast cancers. *Cancer Lett* 113:123–130
13. Kurz AK, Graf D, Schmitt M, Vom Dahl S, Haussinger D (2001) Tauroursodeoxycholate-induced choleresis involves p38 (MAPK) activation and translocation of the bile salt export pump in rats. *Gastroenterology* 121:407–419
14. Lung JC, Chu JS, Yu JC, Yue CT, Lo YL, Shen CY, Wu CW (2002) Aberrant expression of cell-cycle regulator cyclin D1 in breast cancer is related to chromosomal genomic instability. *Genes Chromosomes Cancer* 34:276–284
15. Luo B, Wang Y, Wang XF, Liang H, Yan LP, Huang BH, Zhao P (2005) Expression of Epstein–Barr virus genes in EBV-associated gastric carcinomas. *World J Gastroenterol* 11:629–633
16. Moonen L, Ong F, Gallee M, Verheij M, Horenblas S, Hart AA, Bartelink H (2001) Apoptosis, proliferation and p53, cyclin D1, and retinoblastoma gene expression in relation to radiation response in transitional cell carcinoma of the bladder. *Int J Radiat Oncol Biol Phys* 49:1305–1310
17. Muller W, Noguchi T, Wirtz HC, Hommel G, gabbert HE (1999) Expression of cell-cycle regulatory proteins cyclin D1, cyclin E, and their inhibitor p21 WAF1/CIP1 in gastric cancer. *J Pathol* 189:186–193
18. Rickinson AB, Kieff E (2002) Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM (eds) *Fields virology*. Lippincott-Raven, Philadelphia, pp 2575–2627

19. Sall A, Caserta S, Jolicoeur P, Franqueville L, de Turenne-Tessier M, Ooka T (2004) Mitogenic activity of Epstein–Barr virus-encoded BARP1 protein. *Oncogene* 23:4938–4944
20. Sarbia M, Hausen AZ, Feith M, Geddert H, von Rahden BH, Langer R, von Weyhern C, Siewert JR, Höfler H, Stein HJ (2005) Esophageal (Barrett's) adenocarcinoma is not associated with Epstein–Barr virus infection: an analysis of 162 cases. *Int J Cancer* 117:698–700
21. Sarbia M, Stahl M, Fink U, Heep H, Dutkowski P, Willers R, Seeber S, Gabbert HE (1999) Prognostic significance of cyclin D1 in esophageal squamous cell carcinoma patients treated with surgery alone or combined therapy modalities. *Int J Cancer* 84:86–91
22. Sauter ER, Yeo UC, von Stemm A, Zhu W, Litwin S, Tichansky DS, Pistrutto G, Nesbit M, Pinkel D, Herlin M, Bastian BC (2002) Cyclin D1 is a candidate oncogene in cutaneous melanoma. *Cancer Res* 1:3200–3206
23. Sawa H, Ohshima TA, Ukita H, Murakami H, Chiba Y, Kamada H, Hara M, Saito I (1998) Alternatively spliced forms of cyclin D1 modulate entry into the cell cycle in an inverse manner. *Oncogene* 16:1701–1712
24. Sbih-Lammali F, Djennaoui D, Belaoui H, Bouguermouh A, Decaussin G, Ooka T (1996) Transcriptional expression of Epstein–Barr virus genes and proto-oncogenes in north African nasopharyngeal carcinoma. *J Med Virol* 49:7–14
25. Sheng W, Decaussin G, Sumner S, Ooka T (2001) N-terminal domain of BARP1 gene encoded by Epstein–Barr virus is essential for malignant transformation of rodent fibroblasts and activation of BCL-2. *Oncogene* 20:1176–1185
26. Sheng W, Decaussin G, Ligout A, Takada K, Ooka T (2003) Malignant transformation of Epstein–Barr virus-negative Akata cells by introduction of the BARP1 gene carried by Epstein–Barr virus. *J Virol* 77:3859–3865
27. Seto E, Yang L, Middeldorp JM, Sheen TS, Chen JY, Fukayama M, Eizuru Y, Ooka T, Takada K (2005) Epstein–Barr virus (EBV)-encoded BARP1 gene is expressed in nasopharyngeal carcinoma and EBV-associated gastric carcinoma tissues in the absence of lytic gene expression. *J Med Virol* 76:82–88
28. Takada K (2000) Epstein–Barr virus and gastric carcinoma. *J Clin Pathol: Mol Pathol* 53:255–261
29. van Beek J, zur Hausen A, Klein Kranenbarg E, van de Velde CJH, Middeldorp JM, van den Brule AJC, Meijer CJLM, Bloemena E (2004) EBV-positive gastric adenocarcinomas: a distinct clinicopathologic entity with a low frequency of lymph node involvement. *J Clin Oncol* 15:664–670
30. Wang Q, Tsao SW, Ooka T, Nicholls JM, Cheung HW, Fu S, Wong YC, Wang X (2006) Anti-apoptotic role of BARP1 in gastric cancer cells. *Cancer Lett* 238:90–103
31. Wei MX, Ooka T (1989) A transforming function of the BARP1 gene encoded by Epstein–Barr virus. *EMBO J* 8:2897–2903
32. Wei MX, Moulin JC, Decaussin G, Berger F, Ooka T (1994) Expression and tumorigenicity of the Epstein–Barr virus BARP1 gene in human Louckes B-lymphocyte cell line. *Cancer Res* 54:1843–1848
33. Wei MX, de Turenne-Tessier M, Decaussin G, Benet G, Ooka T (1997) Establishment of a monkey kidney epithelial cell line with the BARP1 open reading frame from Epstein–Barr virus. *Oncogene* 14:3073–3082
34. Werner M, Mattis A, Aubele M, Cummings M, Zitzelsberger H, Hutzler P, Höfler H (1999) 20q13.2 amplification in intraductal hyperplasia adjacent to in situ and invasive ductal carcinoma of the breast. *Virchows Arch* 435:469–472
35. Young LS, Rickinson A (2004) Epstein–Barr virus: 40 years on. *Nat Rev Cancer* 4(10):757–768
36. zur Hausen A, Brink AA, Craanen ME, Middeldorp JM, Meijer CJ, van den Brule AJ (2000) Unique transcription pattern of Epstein–Barr Virus (EBV) in EBV-carrying gastric adenocarcinomas: expression of the transforming *BARP1* Gene. *Cancer Res* 60:2745–2748