The role of the Epstein-Barr virus-encoded BARF1 gene expressed in human gastric epithelial cells

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Cite this article as: Li S, Zhang F, Li J, et al. The role of the Epstein-Barr virus-encoded BARF1 gene expressed in human gastric epithelial cells. Turk J Gastroenterol 2020; 31(11): 775-81.

ABSTRACT

Background/Aims: The study aimed to explore the effects of Epstein-Barr virus–-encoded BARF1 in human gastric epithelial cells (GES-1).

Materials and Methods: A eukaryotic expression vector carrying BARF1 gene (pcDNA3.1-BARF1) was constructed. The pcDNA3.1-BARF1 was transfected into GES-1 cells, and they were selected by G418. The GES-1 cells lines that expressed BARF1 (GES-1-BARF1) were obtained. The cycle of GES-1-pcDNA3.1 cells (GES-1 cells transfected with empty vector), GES-1-BARF1 cells (GES-1 cells transfected with BARF1), and TPA-GES-1-BARF1(GES-1-BARF1 cells stimulated by 12-O-tetradecanoylphorbol-13-acetate (TPA) were analyzed by flow cytometry. Colony formation in soft agar and tumorigenicity of the transfected cells in mice with severe combined immunodeficiency (SCID) were also observed.

Results: The morphology of GES-1-BARF1 cells were changed from the original shuttle to round, the adhesion between the cells and bottle wall was weakened, and the cells showed overlapping growth. The proliferation rate of GES-1-BARF1 and TPA-GES-1-BARF1 cells were faster than GES-1 and GES-1-pcDNA3.1 cells; the S phase was significantly prolonged for GES-1-BARF1 and TPA-GES-1-BARF1. GES-1-BARF1 and TPA-GES-1-BARF1 cells formed colonies in soft agar, with a cloning rate of 24.2% (58/240) and 40.0% (96/240), respectively; GES-1 and GES-1-pcDNA3.1 cells did not form colonies in soft agar. Tumors were formed in mice with SCID after injecting TPA-GES-1-BARF1 cell groups. Tumor formation did not occur in mice with SCID after injecting GES-1 and GES-1-pcDNA3.1 cell groups, but nodules were formed in the mice with SCID after injecting GES-1-BARF1 cell groups.

Conclusion: GES-1-BARF1 cells malignant transformation was induced by transfected BARF1 gene and TPA stimulation. This result indicated that tumor formation not only require oncogenes, but also the stimulation of cancer-promoting substance.

Keywords: Gastric cancer, transfection, oncogene

INTRODUCTION

Gastric cancer is the second most common malignant neoplasm causing death worldwide (1). Its occurrence and development involved a variety of factors such as *Helicobacter pylori* infection (2), smoking (3–5), alcohol drinking (3, 6), and dietary factors (5–7). Despite extensive investigations for related factors, the etiology of gastric cancer (GC) has not yet been established.

In 1990, Burke et al. (8) first reported a case of Epstein-Barr virus (EBV)-positive gastric carcinoma. EBV is a common human tumor virus and was shown to be associated with many human diseases such as nasopharyngeal carcinoma (NPC), lymphomas, Burkitt lymphoma, and Hodgkin lymphoma (9, 10). Approximately 10% of GCs worldwide are caused by EBV infection (11-13).

BARF1 gene has been recognized in recent years as a new oncogene encoded by EBV. BARF1 protein expression can be detected in approximately 90% of EBV-related GC tissues (14, 15). It can promote epithelial cell immortalization and transformation functions (16-19), and it is expressed in EBV-positive NPC and GC tissues during latency and in parallel with the more widely studied protein LMP1 (20, 21). Particularly in EBV-positive GC, BARF1 is expressed in the absence of LMP1, and it possibly plays an important role as an EBV oncogene in the occurrence and development of EBV-related GC (15, 22, 23). Therefore, in this study, the role of *BARF1* gene in malignant transformation of human gastric epithelial cells was investigated by establishing GES-1/*BARF1* cells.

MATERIALS AND METHODS

Cell Lines

The marmoset B-lymphoblastoid cell line (B95-8) that is infected with EBV, it is as a source of *BARF1*; Gastric epithelial cells (GES-1). The GES-1 cell lines were cultivated

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Received: **November 24, 2018** Accepted: **December 7, 2019**

DOI: **10.5152/tjg.2020.18827**

Table 1. The primers used in this study.

Primer	Sequences (5′–3′)	Position	Amplicon size (bp)
BARF1#1	CCGGAATTCGCCACCATGGCCAGGTTCATCGCTC	165504-165522	666
BARF1#2	TGCTCTAGATTATTGCGACAAGTATCCAG	166150-166169	
BARF1#3	CCAGGTTCATCGCTCAGCTC	165508-165527	524
BARF1#4	CATAACACCGCCATTTGCCG	166012-166031	

Note: Primers 1 and 2 for the purpose of gene sequences, and primers 3 and 4 for the detection.
bp: base pair.

in Dulbecco's modified Eagle's medium, and the B95-8 cells were cultivated using RPMI 1640 (Solarbio Corporation, Beijing, China), supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin, the concentration of each antibiotic was 100 U/mL) under standard conditions. Cells were maintained as a subconfluent monolayer at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2)/95% air. Exponentially proliferating cells were harvested with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid, resuspended in fresh medium, and seeded in new flasks. The cells (2×10^6) were collected and washed with phosphate-buffered saline (PBS). The cells were then separated into two cryotubes and were immediately frozen in liquid nitrogen or stored at –70°C until further use.

Mice with SCID

SCID were used for the study (4-6 weeks of age). They were fed under specific pathogen-free conditions.

Primer Design and Synthesis

BARF1 primers were designed according to GenBank-provided *BARF1* gene sequences, and restriction sites were added at both ends of the primers (Table 1).

The Eukaryotic Vector Construction and Transfection of GES-1 Cells and Cloning

RNA was extracted from B95-8 cells, and *BARF1* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR). The polymerase chain reaction (PCR) products of BARF1 were cloned into the pUM-T vector and transformed into *E. coli* DH5α. The positive clones were screened. The plasmids were extracted from the selected clones, which were digested with EcoRI and XbaI. The pcDNA3.1 eukaryotic vectors were digested with EcoRI and XbaI. BARF1 and pcDNA3.1 were cut from 1.0% agarose gel, and isolated, purified using a Qiaquick Gel Extraction kit according to the manufacturer's protocol, respectively. Then, pcDNA3.1 and BARF1 were ligated, which were transfected into *E. coli* DH5α; the positive clones were identified; and the plasmids were extracted.

The eukaryotic vector pcDNA3.1-BARF1 was transfected into the human gastric epithelial cell line GES-1 by Lipofectamine2000 (according to the kit instructions). The cells were digested 3 days after transfection and inoculated into a 48-well plate with 200 cells per well. Then, resistant clones were screened with a culture medium containing 300 μg/mL G418.

BARF1 Gene Expression in Transfected Cells

The cell RNA was extracted from GES-1-BARF1 resistant clones, and *BARF1* was amplified by reverse transcriptase PCR (using primer 3 and 4 sets, products 524 base pair [bp], as shown in Table 1).

Cell Counting Kit-8 Assay

During the logarithmic growth phase, 200 μL of cells (cell count of approximately 3×10^3 cells/mL) were added into every well of 96-well plates (four wells for each cell group). The cell growth was detected using the method of the cell counting kit-8 (CCK-8, according to the kit instructions), using spectrophotometry to measure the cell concentration after culturing for 24 hours, 48 hours, and 72 hours at 450 nm. The CCK-8 assay was repeated three times. The results were recorded.

The Cell Proliferation Cycle Assay

The logarithmic growth phase cells (cell count of approximately 1×10^6 cells/mL) were collected after 0.25% trypsin digestion and were washed in cold PBS. Then, 70% precooled ethanol was added, and the cells were fixed at 4°C overnight. The supernatant was removed after 2,000 g centrifugation, and the cells were then washed in 2 mL cold PBS. Then, 300 μL PBS, 2 μL (1:1,000) RNA enzyme water, and 70 μL propidium iodide (PI) dye mixed in the dark for 15 minutes were added. A 300-mesh nylon filter

was used to filter the cells. The cell cycle was analyzed using flow cytometry.

Growth in Soft Agar

Anchorage-independent cell growth was assayed in the upper layer with 0.6 mL complete cell growth medium (containing approximately 4×10^2 cells/mL and 0.33% low-melting-temperature soft-agar) and plated onto prepared in advance 0.6 mL complete cell growth medium containing 0.66% soft agar using a 24-well plate. Each group of cells was inoculated into four wells and labeled. The plate was maintained for 2 weeks at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cell clone formations in soft agar were observed, counted, and photographed with a camera every day. The cells that divided three times, that is, proliferated into eight cells, were defined as a clone, and the clone formation rate was calculated according to the following formula: clone formation rate (%)=number of clones/cells inoculated×100%.

Tumorigenicity Experiments

Mice with SCID (4-6 weeks of age) were inoculated with 1 mL cell suspension (cell concentration 2×10^7 living cells/mL) of GES-1, GES-1/pcDNA3.1, GES-1-BARF1, and TPA-GES-1-BARF1 cell groups. Each experimental group consisted of three mice. Tumorigenicity was observed for 1 month after injection into mice with SCID.

RNA was extracted from tumor tissues, and the expression of BARF1 in tumor tissues was then detected by RT-PCR.

Tumor tissues were detected by the hematoxylin and eosin (H&E) staining and immune staining.

Statistical Analysis

The data were expressed as the mean±standard deviation, and a two-tailed Student's *t*-test was used for statistical analysis by the Statistical Package for the Social Sciences (IBM Corp.; Armonk, NY, USA) software. Statistical significance was set at $p\leq0.05$.

RESULTS

Amplification of the Target BARF1 Gene

BARF1 gene was amplified using cDNA of B95-8 RNA as a template, and about 666 bp products for *BARF1* were observed and consistent with the expected result.

BARF1 Gene Expression in Transfected Cells

Resistant clones were obtained after 2 weeks of G418 screening, whereas non-transfected cells died. Three resistant clones from each transfected cell were selected and amplified. Total RNA was extracted from these positive clone cells (including *BARF1* gene-transfected cells and empty vector-transfected cells), and 524 bp amplification products were obtained by reverse transcriptase PCR (Figure 1).

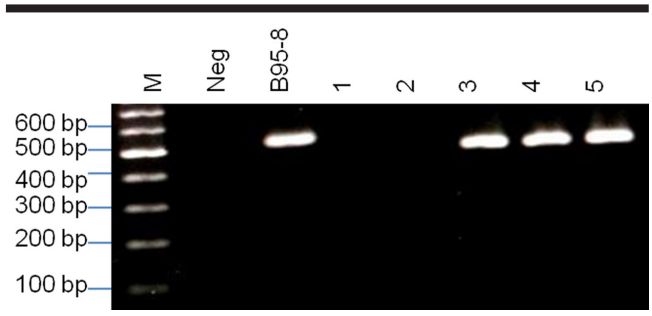

Figure 1. The expression of *BARF1* gene in transfected cells were identified by RT-PCR.
M1:100 bp ladder. Neg was negative control using sterile double distilled water as a template. B95-8 was positive control of the B95-8 cell cDNA as template. 1 was amplification using GES-1 cell cDNA as template; 2 was amplification using transfected empty vector cell cDNA as template; 3, 4, 5 was amplification using three transfected *BARF1* gene cell cDNA as template, respectively. RT-PCR: reverse transcription polymerase chain reaction; bp: base pair.

Alteration of BARF1 Affects GES-1 Cell Growth

Each cell group GES-1-BARF1, TPA-GES-1-BARF1, GES-1, TPA-GES-1 and GES-1-pcDNA3.1 was synchronized, and their cell growth activity was evaluated by CCK-8 at 24 hours, 48 hours, and 72 hours (Figure 2). The cell growth activities of GES-1-BARF1 and TPA-GES-1-BARF1 was higher than that of GES-1, TPA-GES-1, and GES-1/pcDNA3.1 cell groups, and these differences became increasingly higher with increasing incubation time.

Alteration of BARF1 Affects the Cell Proliferation Cycle

The cells were fixed and stained using PI fluorescence agent. The cell cycle distribution of GES-1-BARF1 cells, TPA-GES-1-BARF1 cells, GES-1 cells, and GES-1-pcDNA3.1 cells were then detected by flow cytometry (Figure 3). The results of flow cytometry showed that the G1 phase of GES-1-BARF1 cells and TPA-GES-1-BARF1 cells was shortened ($p<0.05$) and the S phase of these cells was significantly prolonged ($p<0.05$) compared with that of GES-1-pcDNA3.1 cells and GES-1 cells.

Colony Formation Assay

To determine the activity of these cell groups, the cell growth capacity was examined in 0.33% agar. Approximately 240 living cells from each of the cell groups were

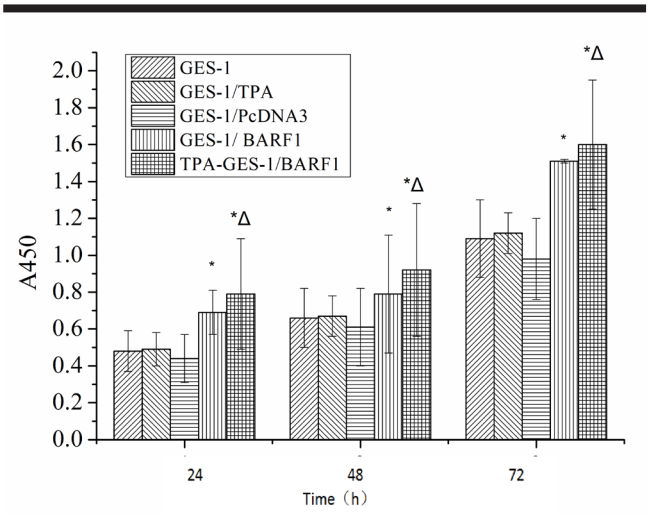

Figure 2. The cell growth activity was valuated at 24 hours, 48 hours, and 72 hours by CCK-8
The CCK-8 assay was done in triplicate and showed standard deviations. The proliferation of null GES-1 cells treated with TPA was shown, and the differences in growth between BARF-1 expressing clones were shown.
Note: Compared GES-1-BARF1 cell group with the control group normal GES-1 (or TPA-GES-1, transfected empty vector GES-1 group), *p<0.05; compared TPA-GES-1-BARF1 with GES-1-BARF1 (or transfected empty vector GES-1, TPA-GES-1, GES-1) group, △p<0.05. CCK-8: cell counting kit-8; TPA: 12-O-tetradecanoylphorbol-13-acetate.

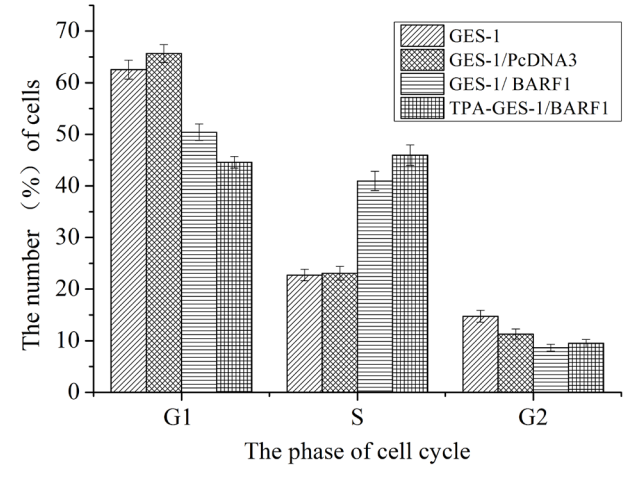

Figure 3. (a) The cell cycle distribution of the normal GES-1 cells groups: G1: 62.53%, G2: 14.75%, S: 22.72%. (b) The cell cycle distribution of transfected empty vector cell groups: G1: 65.66%, G2: 11.28%, S: 23.06%. (c) The cell cycle distribution of transfected BARF1 gene cell groups: G1: 50.40%, G2: 8.65%, S: 40.95%. (d) The cell cycle distribution of TPA stimulation of transfected BARF1 gene cell groups: G1: 44.55 %, G2: 9.49 %, S: 45.96 %. TPA: 12-O-tetradecanoylphorbol-13-acetate.

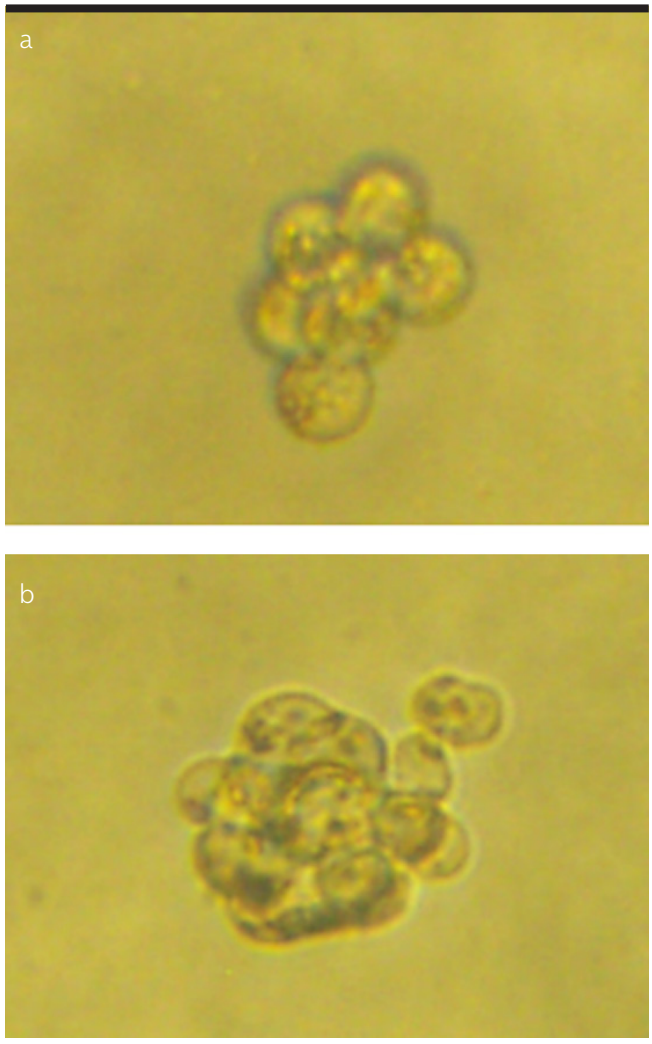

Figure 4. a, b. (a) The typical clone of transfected BARF1 gene cell groups. (b) The typical clone of TPA stimulation of transfected BARF1 gene cell groups. TPA: 12-O-tetradecanoylphorbol-13-acetate.

seeded in each well of a 24-well plate with 0.33% agar. Colony formation was checked daily until 14 days. On day 5, colony formation was observed under an inverted microscope for transfected with the BARF1 cell groups and their TPA stimulation cell groups, and the number of colony formation for every cell group was counted every 2 days as shown in Table 2. Colony formation of the transfected with BARF1 cell groups and their TPA stimulation cell groups in soft agar under an inverted microscope were presented after culturing for 14 days (Figure 4). The clone formation rate of the transfected with the BARF1 cell groups and their TPA stimulation cell groups was approximately 24.2% (58/240) and 40.0% (96/240) (average of value obtained from four subclones), respectively,

Table 2. The number of clones formed by every cell group.

	Clones/day			
Groups	5	8	11	14
GES-1- BARF1-1	6	15	37	60
GES-1- BARF1-2	2	11	32	57
GES-1- BARF1-3	3	13	35	59
GES-1- BARF1-4	2	9	30	56
Average for GES-1/ BARF1	4.5	12.0	33.5	58.0
TPA-GES-1-BARF1-1	7	19	48	99
TPA-GES-1-BARF1-2	6	17	43	97
TPA-GES-1-BARF1-3	5	13	39	93
TPA-GES-1-BARF1-4	6	15	42	95
Average for TPA-GES-1/BARF1	6.0	16.0	43.0	96.0

TPA: 12-O-tetradecanoylphorbol-13-acetate

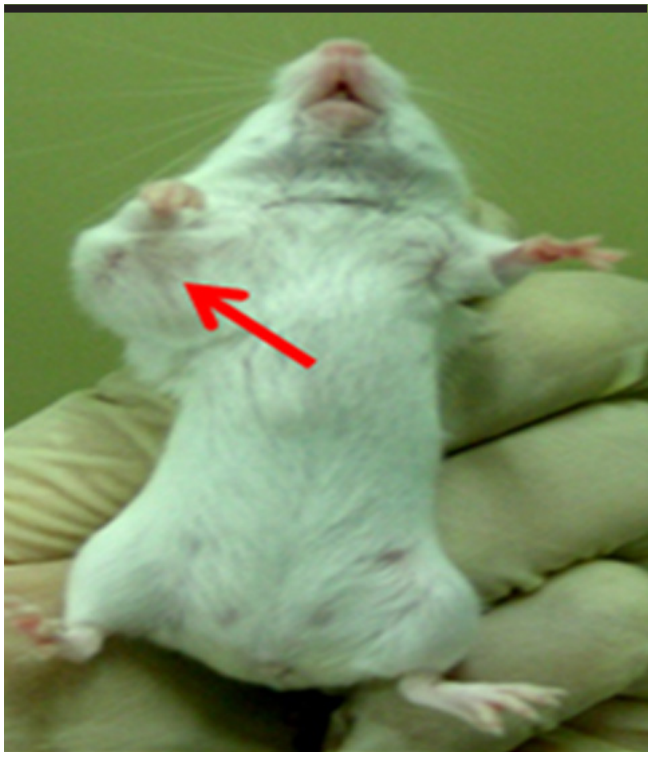

Figure 5. Tumor formation in mice with SCID. The red arrow was the location of SCID mouse tumorigenic situation induced by TPA stimulation of cell groups expressing *BARF1* gene. SCID: severe combined immunodeficiency; TPA: 12-O-tetradecanoylphorbol-13-acetate.

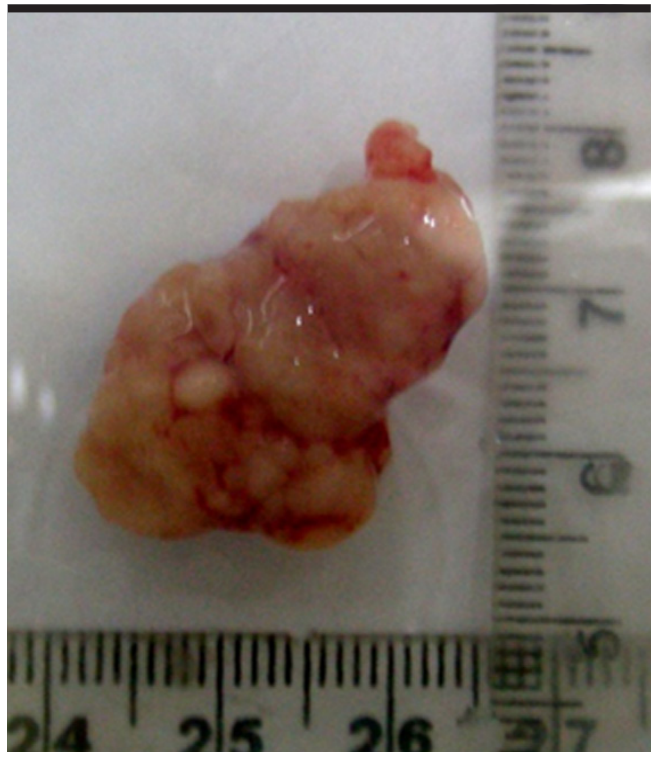

Figure 6. Tumor morphology.

whereas the normal control GES-1 and transfected with vector alone cell groups did not give any positive colonies, and the cells died.

Tumorigenicity of BARF1-Expressing Human Gastric Epithelial Cells in Mice with SCID

To determine whether BARF1 expression contributes tumorigenicity to EBV-negative human gastric epithelial cells, all four cell groups, including GES-1-BARF1, TPA-GES-1-BARF1, GES-1, and GES-1-pcDNA3.1 (2×10^7 cells/mouse), were injected into mice with SCID (three mice with SCID for each cell group). An apparent mass formation could be palpated in mice with SCID mice with GES-1-BARF1 and TPA-GES-1-BARF1 cell groups after 5 days. However, the mice inoculated with GES-1-BARF1 cell group did not form tumors, and only a nodule was formed; the three mice inoculated with TPA-GES-1-BARF1 cell groups formed tumors eventually (Figure 5). The tumor size (cm) was approximately 1.8×2.0 (Figure 6), whereas those inoculated with GES-1 cells and GES-1-pcDNA3.1 cells were unable to induce any tumors in mice with SCID.

RNA was extracted from tumor tissues, and the expression of BARF1 in tumor tissues was then detected by RT-

PCR. The results showed that *BARF1* gene was positive, indicating that *BARF-1* gene expression was present in tumor tissues. The tumor tissues were stained with H&E and confirmed as adenocarcinoma (data not show); the tumor tissues were immunohistochemically stained (using serum of BARF1-positive patient with nasopharyngeal carcinoma as antibodies) in the cell membrane or cytoplasm, and the results showed that BARF1 expression was positive (data not show).

DISCUSSION

EBV is a DNA oncogenic virus. Recently, great attention has been paid to the role that the EBV-encoded *BARF1* gene plays in gastric carcinogenesis. EBV-encoded *BARF1* is expressed in EBV-positive malignancies such as nasopharyngeal carcinoma, EBVaGCs, B-cell lymphoma, and nasal natural killer/T-cell lymphoma, and has been shown to have an important role in oncogenesis (24–27). In this study, a role for the EBV-encoded *BARF1* gene in GC occurrence and development was detected through the construction of human gastric epithelial cell lines carrying the *BARF1* gene.

The oncogenic properties of BARF1 have been previously documented in other cell types (such as EBV-negative nasopharyngeal carcinoma cells, primate epithelial cells, rodent fibroblasts, HEK-293) forced to ectopically express BARF1 (28–31).From our results, GES-1-BARF1 cells became rounder, bigger, and more fusiform than GES-1 cells. The GES-1-BARF1 cells were digested easily using trypsin. The adhesion of GES-1-BARF1 cells was reduced, and they could grow in suspension medium. Both CCK-8 and colony formation assays inferred that BARF1 might be a growth-promoting factor in the malignant transformation of gastric epithelial cells. However, mice with SCID could not form tumors with GES-1-BARF1 cells. It is worth noting that the mice inoculated with TPA-GES-1-BARF1 cell groups formed tumors eventually. These results show that tumor formation is a process of multi-stage and multi-factor interactions. Although the *BARF1* gene is an oncogene, it alone is insufficient to cause tumorigenesis. The tumorigenicity experiments were carried out using the oncogene *BARF1* as an initiator and using TPA as a cancer-promoting factor. Mice with SCID formed tumors through the interaction of oncogenes and tumor promoters.

Zur et al. (15) previously demonstrated that 9 of 10 cases were *BARF1* gene positive from 132 cases of EBV-positive gastric adenocarcinomas, which indicates that BARF1 plays an important role in the malignant transformation of gastric epithelial cells and in the maintenance of the malignant phenotype.

The migration and invasion of assays can be performed to assess the effects of BARF1 on cell migration and invasion by transwell insert chambers: the expression of BARF1 could be effectively enhance the ability of migration and invasion compared to the control, respectively, which indicat improve effects of BARF1 on the migration and invasion of GC.

In conclusion, in this study, the cell cycle S phase was extended for GES-1-BARF1 cells and TPA-GES-1-BRAF1 cells. Malignant transformation was induced by *BARF1* gene transfection, which induced the characteristics of tumor cell growth in the *BARF1* gene-transfected cells. All of these findings suggest that the *BARF1* gene as an oncogene of EBV may play an important role in the malignant transformation of gastric epithelial cells.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of North China University of Science and Technology.

Informed Consent: *N/A.*

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – S.L., K.Z., Jintao L.; Design - S.L., K.Z., Jintao L.; Supervision - S.L., K.Z.; Resource - S.L.; Materials - K.Z., Jintao L.; Data Collection and/or Processing - F.Z., J.L., X.H.,W.Z.; Analysis and/or Interpretation - S.L., K.Z., Jintao L.; Literature Search - F.Z., J.L., X.H.,W.Z.; Writing - S.L., K.Z., Jintao L.; Critical Reviews - S.L., K.Z., Jintao L.

Acknowledgements: The authors would like to thank School of Basic Medical Sciences, North China University of Science and Technology, Tangshan, China for their support and assistance in this study.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: This study was supported by the Project of Science and Technology for overseas scholars in Hebei Province (No. CY201620), and the Project of North China University Science and Technology (No.28656002).

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