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Original Article

Diverse Expression Patterns of EBV Oncogenes (*LMP2A, EBV-Encoded microRNA, and EBV-encoded dUTPase*) in EBV Associated Gastric Carcinoma and their Association with Viral Loads

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Abstract

The chromogenic in situ hybridization (CISH) test is the gold standard for detecting Epstein-Barr virus (EBV)-associated gastric carcinoma (GC). Real-time (RT) PCR method is also a sensitive test that can detect the viral load in samples. As such, three EBV oncogenes were investigated in this study. RNA extraction and cDNA synthesis were performed on GC tissues of nine patients, who were previously confirmed to have EBVGC subtype. In addition, 44 patients that had positive RT-PCR but negative CISH results were also included as the control group. TaqMan RT-PCR analysis was performed to determine the expression of EBV-encoded microRNAs, and the expression of EBV-encoded *dUTPase*, as well as *LMP2A*, was analyzed by SYBR Green RT-PCR. EBV-encoded microRNAs and *LMP2A* were identified in 2 out of 9 (22%) EBVGC subtypes. In addition, EBV-encoded *dUTPase* was detected in 4 out of 9 (44.5%) EBVGC subtypes. EBV-encoded *dUTPase* was also expressed in a sample of the control group. The expression of *LMP2A*, EBV-encoded microRNAs, and EBV-encoded *dUTPase* viral oncogenes in patients with high EBV viral loads indicates that these expressions correlate with viral loads. Our findings indicate that the EBV-encoded *dUTPase* gene may have a role in EBVGC patients' non-response to treatment and might be considered a Biomarker-targeted therapy.

Keywords: *DUTPase*, Epstein-Barr virus, Gastric carcinoma, *LMP2A*, MicroRNA

1. Introduction

Epstein-Barr virus (EBV), with a significant role of 1.5%-2%, has been documented in all types of cancer worldwide, and EBV viral genes are expressed at different periods in cancerous growths (1, 2). EBV-associated gastric carcinoma (EBVGC) is considered one of the four subtypes of GC based on molecular features (3). EBVGC often shows a latency pattern that has been observed with Epstein-Barr nuclear antigen 1 (*EBNA1*), Epstein-Barr virus-encoded small RNAs (*EBER*), and BamHI fragment A rightward transcript (*BART*) microRNA expressions. Various studies consider the latent form of EBV in GC varies between latency types l and 2 (4, 5). Although the critical role of EBV in different types of cancer is well documented, the exact mechanism of carcinogenicity of this virus in the EBVGC subtype is not yet clear.

The *Bllf3* open reading frame of EBV encodes the EBV-encoded *dUTPase* enzyme (6). Although a large number of patients with primary infection or activated

EBV infection have EBV-encoded *dUTPase* antibodies, no antibodies against EBV-encoded *dUTPase* have been detected in healthy individuals carrying EBV (7). It has been shown that EBV-infected cells at the lytic stage express high amounts of EBV-encoded *dUTPase* protein (7). Several studies have demonstrated the importance of the tumor's microenvironment in the growth and progression of the disease. Indeed, EBV-encoded *dUTPase* affects the tumor's microenvironment by inducing IL-6 and IL-10 expression and increasing the expression of BIC/microRNA-155 (*pri-miR-155*) and *CCL20* (8).

It has been shown that EBV-encoded *LMP2A* plays an important role and exerts its biological actions through different mechanisms, including activating the signaling phosphatidylinositol kinase *PI3CK* and serine-threonine kinase AKT, activating the Notch pathway in epithelial cells in breast and GCs (9), enhancing survival (10), and maintaining viral latency through inhibiting the normal activation of the B cell receptor (11).

Recent studies have shown that EBV can encode viral microRNAs, which, like cellular microRNAs, play an essential role in various physiological processes, such as differentiation, immune signaling, apoptosis, proliferation, and tumorigenesis. EBV encodes at least 40 microRNAs from two regions of the viral genome. While the *BART* microRNA clusters are located between the intronic regions of the *BARTs*, the *BHRF1* microRNA cluster is located immediately upstream and downstream of the *BHRF1* open reading frame (12). Although many studies have shown that EBV-encoded microRNAs are involved in EBV malignancy, limited data are available regarding the role of EBV-encoded microRNAs in GC (12).

2. Materials and Methods

2.1. Specimen's Characteristics and Ethic Statements

Fifty-three fresh frozen tissues matching formalin-fixed paraffin-embedded (FFPE) specimens *were collected from surgical resections in the tumor bank at the Cancer Institute of Imam Khomeini Hospital, Tehran, Iran.* Nine EBVGC subtype patients confirmed by RT-PCR and CISH test results were selected as the case group. In addition, 44 patients with positive RT-PCR but negative CISH test results were considered the control group. The patient and control groups were selected by the standard CISH test. The mean age of the control group was 53 years (range: 33 to 80), and the female-male ratio was 1/4. On the other hand, the nine EBVGC phenotype patients had a median age of 61 years. The mean age was 58.6 (range: 40 to 74), and the female-male ratio was 1/9.

This study was approved by the clinical research Ethics Committee of the Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1397.324), and all specimens were named numerically before being included in the study.

2.2. EBV DNA Detection by Quantitative Real-Time PCR and *EBER1* Detection by CISH

Briefly, DNA was extracted from fresh frozen tissues using DNeasy Blood and Tissue Kits (QIAGEN, Germany), according to the protocol. The extracted DNA was analyzed to identify and quantify the EBV genome (the DNA sequence of the single-copy gene encoding *EBNA1*) by Gene Proof Epstein-Barr Virus PCR Kit (Gene Proof, Czech), as instructed by the manufacturer.

Epstein-Barr virus-encoded small RNA1 (*EBER1*) CISH Detection Kit (Master Diagnostica, Spain) was also used to identify specific EBV *EBER1* RNA sequences in FFPE of surgical resections to confirm EBVGC phenotype.

2.3. RNA Extraction and cDNA Synthesis

Briefly, the viral RNA was extracted from 30-50 mg of tissue pieces, and the master digestion reagents were used from the extraction kit of Dneasy Blood & Tissue Kits (QIAGEN, Germany). Next, 500 µl of RNX solution (TRIzol) was added to ensure tissue homogenization. After the formation of the pellet, 30 µl of elution buffer was added. The extracted RNA concentration was immediately measured by the NanoDrop 2000 spectrophotometer (Thermo Fisher

Scientific, USA). A total of 1000 ng RNA of the samples was treated with DNase I to remove the residual DNA, and then cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). The cDNA master mix contents included 1000 ng RNA in 4 µl of 5× RT reaction buffer, 1 µl of dNTP mix (40 µM), 1 µl of random hexamer (300 µM), 0.5 µl of RNase inhibitor (40 U/µl), 0.5 µl of reverse transcriptase (20 U/µl), and nuclease-free water to adjust 20 µl of the final volume. The cDNA synthesis was performed for 10 min at 25°C, followed by 30 min at 55°C, with a final enzyme denaturation for 5 min at 85°C.

2.4. Primer Design

To design primers for *LMP2A*, EBV-encoded *dUTPase*, and beta-actin (*ACTB* as a reference gene) genes, the reference gene was first taken from the NCBI-Gene website and then imputed as a template in the online Primer-BLAST software. Default parameters were considered for the primer design, and only the product size was changed between 100 and 150. The designed primers are listed in table 1.

Table 1. Primer design for SYBR Green Real time PCR

Oligo Name	Sequence (5'- 3')
ACTB-F	CCACCATGTACCCTGGCATT
ACTB-R	CGGACTCGTCATACTCCTGC
dUTPase-F	GTCCGGTCACGTCTCATGTT
dUTPase-R	GTACTGGGGGTGGTTGATGG
LMP-2A-F	TCCTTCTGGCACGACTGTTC
LMP-2A-R	CCCCATTCGGTCAGGATAGC

2.5. EBV-encoded microRNA (*BART1, BART2, BHRF1*) Detection by TaqMan Real-Time PCR

To detect the EBV-encoded microRNA (*BHRF1* and *BART* clusters), TaqMan RT-PCR was used, as described by Amoroso et al. (doi:10.1128/JVI.01528-10). Quantitative PCRs were performed using the Rotor-Gene™ 6000 (QIAGEN, Germany). Briefly, the 20 µl reaction consisted of 5 µl of cDNA and 15 µl of the master mix, which was prepared using 1 µl of the 10 µM of the forward and reverse primers, 5 µM of prob, 10 µl of TaqMan master mix (Roche, Switzerland), and 4 µl of nuclease-free water. The cycling conditions consisted of 5 min at 95°C to activate the hot-start polymerase, followed by 40 cycles of 10 sec at 95°C, 20 sec at 60°C, and detection for 20 sec at 72°C. At each stage, positive and negative control were used. The results of *BART1*, *BART2*, and *BHRF1* expressions (CT value) were determined automatically using the device software.

2.6. EBV-encoded *dUTPase* and *LMP2A* mRNA Detection by SYBR Green Real-Time PCR

To detect the expression of EBV-encoded *dUTPase* and *LMP2A*, SYBR Green PCR was performed on the first-strand cDNA using Rotor-Gene™ 6000-6 Plex Instrument (QIAGEN, Germany) with Fast Start Universal SYBR Green Master (Roche, Switzerland). Briefly, 2.5 µl of 1:10 diluted cDNA were added to 22.5 µl of a master mix consisting of 12.5 SYBR Green Master and 1 µl (10 pmol) of forward and reverse primers. The cycling conditions consisted of 10 min denaturation at 95°C to activate the hot-start polymerase, followed by 40 cycles of 15 sec at 95°C, 20 sec at 59°C, and 20 sec at 72°C (data collection was conducted in this step). The melting curve analysis was performed from 65-98°C in 0.5°C/s increments to assess the specificity of RT-PCR products.

2.7. Primer Efficiency and Validation by SYBR Green Real-Time PCR

To construct relative calibration curves, three duplicates of cDNA as 1:10 serial dilutions were prepared for SYBR Green RT-PCR. Primers with more than 85% efficiency in calibration curves had acceptable performance.

2.8. Statistical Analysis

The SPSS Software (version 24) was employed to process the data with Spearman's rank correlation coefficient and the Kruskal-wills test for clinical data analysis.

3. Results

3.1. Patients' Data

Fifty-three fresh frozen GC tissues were included in this study, which had positive TaqMan RT-PCR test results with identified viral loads. Nine out of 53, who had been confirmed to have EBVGC subtype by positive CISH *EBER1* results on formalin-fixed paraffin-embedded, were considered the case group. In addition, 44 patients with positive RT-PCR and negative CISH test results were also included as the control group.

The mean age of the control group was 53 years (range: 33 to 80), and the female-male ratio was 1/4. The nine EBVGC phenotype patients had a median age of 61 years. The mean age was 58.6 years (range: 40 to 74), and the female-male ratio was 1/9.

3.2. Expression of EBV-Encoded microRNAs (*BART1, BART2, BHRF1*) and *LMP2A* in Gastric Carcinoma Tissues

The expression of *LMP2A* and EBV-encoded microRNAs (*BART1*, *BART2*, and *BHRF1*) was investigated in 53 GC tissues, including EBVGC subtypes that previously had been confirmed by the CISH *EBER1* and RT-PCR results, as well as the control group. EBV-encoded microRNAs and *LMP2A* were identified in 2 out of 9 EBVGC subtypes (22%) simultaneously, and both of them showed a high viral load (513,656 and 8,818,367, respectively). Viral loads of other samples were much less than these two. None of the patients in the control group had *LMP2A* and EBV-encoded microRNAs (*BART1*, *BART2*, and *BHRF1*) expression.

3.3. Expression of EBV-Encoded *dUTPase* Gene in Gastric Carcinoma Tissues

EBV-encoded *dUTPase* expression assay was developed in 53 GC tissues, including nine EBVGC subtypes as the case group that had been confirmed by RT-PCR and the CISH positive results and also 44 GC patients with RT-PCR results. EBV-encoded *dUTPase* was detected in 4 out of 9 (44.5%) patients, and also 1 gastric carcinoma sample of the control group expressed EBV-encoded *dUTPase*. Overall, 5 out of 53 (9.4%) GC patients with positive RT-PCR results expressed EBV-encoded *dUTPase*, most of which had a high EBV viral load.

4. Discussion

The treatment of tumors can improve through concentration on the understanding of their biology (13). The identification of the genomic features of GC subgroups creates an appropriate roadmap for classifying patients, performing clinical trials, and providing therapeutic goals (3). The recognition of virus virulence markers also makes it possible to detect high-risk groups in cancer progression (14).

This study highlights the role of EBV-encoded *dUTPase* in EBVGC patients. A previous study suggested that some lytic EBV genes can be expressed in EBVGC tumor tissues (4), but no study assayed the *bllf3* (EBV-encoded *dUTPase*) gene. As such, EBV-encoded *dUTPase* was investigated and detected in 4 out of 9 (44.5%) EBVGC phenotypes. One sample of the control group from GC that had positive RT-PCR but negative CISH test results also expressed EBV-encoded *dUTPase*. This may be due to the fact that although the CISH assay is the gold standard to detect EBV *EBER* with high specificity, it has a lower sensitivity than RT-PCR. Therefore, it is possible that this sample of the control group actually belongs to the case group, which was not detected in the CISH test.

The *dUTPase* family is the new target of antimicrobial and anti-cancer therapies, which arises in human tumor cells. It has been shown that in human cells, *dUTPase* silencing siRNA can increase sensitivity to the anti-cancer drug 5-fluorodeoxyuridine (15). Studies on human *dUTPase* have shown that the *dUTPase* enzyme could be a prognostic marker in prognosis and metastasis in colorectal carcinoma (16, 17). Many factors are associated with the development of chemoresistance to 5-fluorouracil (5-FU) drugs. Some studies have reported that *dUTPase* expression is an important mediator of resistance to chemotherapeutic agents in the class of thymidylate synthase inhibitors, such as 5-FU *in vivo* and *in vitro* (these agents are used to treat gastrointestinal, breast, and neck cancers) (18). In this study, clinical data showed that 4 out of 5 EBV-encoded *dUTPase* patients did not respond to the treatment, and there was no

record of the fifth patient. Our study showed that the expression of *dUTPase* may be correlated with EBVGC patients' non-response to treatment. Since the sample size of the present study was small, we recommend that future studies investigate the role of EBV-encoded *dUTPase* in larger sample sizes. In addition, we recommend they assay the level of EBV-encoded *dUTPase* protein (the product of the *bllf3* gene) in the EBVGC subtype.

The present study showed that 2 out of 9 (22%) patients with EBVGC subtypes express *LMP2A* and EBV-encoded microRNAs. However, none of the patients in the control group had *LMP2A* expression. The *LMP2A* protein has been detected in EBV-related malignant tumor biopsies, which induces transformation, anchorage independence, increased motility, and differentiation inhibition, all of which are functions that can cause malignant cell growth (9). A previous study reported that *LMP2A* was expressed in 40% of patients with EBVGC subtypes (19). This data suggest that *LMP2A*-targeted treatment is suitable for some patients. In this study, 2 out of 9 fresh frozen GC specimens from the EBVGC subtype (22%) expressed *LMP2A*, both of which had the highest viral load. We recommend that more studies should be performed on the correlation of high viral loads with the expression of *LMP2A* in fresh frozen samples.

EBV encodes numerous microRNAs that have been recognized to stimulate EBV-associated diseases by targeting the host genes and self-viral mRNA (20). Two cluster regions of the EBV genome, including *BHRF1* encoding three microRNA precursors (*BHRF1* to *3*) that generate four mature microRNAs and the *BART* region containing 22 microRNA precursors (*BART1* to *22*) that produce 44 mature microRNAs (21).

Some studies have also shown the increased level of some EBV-encoded microRNAs in tumor biopsy specimens of 10 to 100-fold (22). A study reported that microRNA *BARTs* expressed more than 10% of the total pool of microRNAs in EBVGC tumor cells (23). A study on surgically resected tissues from 10 EBVGC subtypes reported that from 44 known viral microRNAs, 40 were expressed at different levels; however, no *BHRF* cluster microRNA was detected *(24).*

Some studies showed that microRNA *BART*s were expressed highly in EBV-associated Hodgkin lymphoma and GC, but microRNA *BART*s were barely detectable (21). A study suggested that 18 out of 52 EBVGC cases identified microRNA *BART* (34%), but none of them expressed the *BHRF1* cluster (25).

In this study, 2 out of 9 (22%) patients that had been confirmed as EBVGC patients were reported to have EBV-encoded microRNAs (*BART1*, *BART2*, and *BHRF1*). None of the patients in the control group expressed EBV-encoded microRNA. Given that the detection of *BHRF1* is rare, in this study, two samples expressed *BHRF1* with the highest viral loads; therefore, high EBV viral load may be associated with *BHRF1* expression. In this study, the details of the microRNA members were not studied.

Our results showed the expression of oncogenes, such as EBV-encoded micro RNAs, *LMP2A*, and EBV-encoded *dUTPase*, in EBVGC in the case (EBVGC phenotype confirmed by the gold standard test) and the control group (the presence of EBV reported only by RT-PCR test results), as well as their correlation with response to treatment and viral load. The samples with the highest viral load expressed EBV-encoded micro RNAs and *LMP2A*. EBV-encoded *dUTPase* in EBVGC was expressed in patients with progressive disease who also did not respond to treatment. Although the sample size was small, the results of this study pave the way for further research in this area.

Authors' Contribution

Study concept and design: M. S, A. N., T. M. A. and F. N.

Analysis and interpretation of data: M. S. and A. N.

Drafting of the manuscript and wrote final manuscript: M. S., A. N. and S. M. M.

Statistical analysis: M. S. and M. Y.

Ethics

This study was approved by the clinical research ethics committee of the Tehran University of Medical Science. (IR.TUMS.SPH.REC.1397.324) and all specimens were named by numeral before study inclusion.

Conflict of Interest

The authors declare that they have no conflict of interest.

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