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Expression of Viral MicroRNAs in Epstein-Barr Virus-Associated Gastric Carcinoma▽

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Epstein-Barr virus (EBV) is associated with about 6 to 16% of gastric carcinoma cases worldwide. Expression of the EBV microRNAs (miRNAs) was observed in B cells and nasopharyngeal carcinoma cells infected with EBV. However, it is not clear if the EBV miRNAs are expressed in EBV-associated gastric carcinomas (EBVaGCs). We found that BART miRNAs but not BHRF1 miRNAs were expressed in EBV-infected gastric carcinoma cell lines and the tumor tissues from patients as well as the animal model. The expression of viral miRNAs in EBVaGCs suggests that these EBV miRNAs may play important roles in the tumorigenesis of EBVaGCs.

About 6 to 16% of gastric carcinoma cases worldwide are associated with Epstein-Barr virus (EBV) (20). EBV is proposed to play a causative role in EBV-associated gastric carcinoma (EBVaGC) as the virus is found in almost all tumor cells and the viral DNA shows monoclonality in the cancer (11). However, how EBV causes EBVaGC is unclear because the well-known EBV oncogenes, such as those coding for LMP1, EBNA2, and EBNA3s, are not expressed in EBVaGC (14).

MicroRNAs (miRNAs) are 19- to 25-nucleotide-long single-stranded RNAs processed from transcripts having stem-loop structures (2, 4, 8). miRNAs have diverse functions, including the regulation of cellular differentiation, proliferation, and apoptosis (1, 3, 15, 21). miRNAs might also function as tumor suppressors (7, 10) or oncogenes (6, 10, 13).

Pfeffer et al. (19) cloned five EBV miRNAs (miR-BHRF1-1, miR-BHRF1-2, miR-BHRF1-3, miR-BART1, and miR-BART2) from Burkitt's lymphoma cells latently infected with the B95-8 EBV strain. By Northern blotting, expression of these EBV miRNAs was confirmed in various B-cell lines. Recently, two research groups identified additional EBV miRNAs from the introns of the BART gene (5, 12). The EBV miRNAs may play important roles in the tumorigenesis of EBVaGC. As a first step to test this possibility, we have investigated whether the EBV miRNAs are expressed in EBV-positive gastric carcinoma cell lines and EBVaGC cases.

Expression of EBV miRNAs in EBV-infected gastric carcinoma cell lines. The expression profile of EBV miRNAs was analyzed by Northern blotting in EBV-infected gastric carcinoma cell lines. SNU-719 is a naturally derived EBV-infected gastric carcinoma cell line (17, 18), and the green fluorescent protein (GFP)-expressing cell line AGS-EBV-GFP is an AGS cell line artificially infected with a recombinant EBV (16). Previously, we reported that the EBV gene expression pattern of SNU-719 is uniquely similar to that of EBVaGC (17). Total RNA was resolved in a 15% polyacrylamide–urea gel before being transferred to a Zeta-Probe blotting membrane (Bio-Rad Laboratories, Hercules, CA). Oligonucleotide complementary to each mature EBV miRNA (12, 19) was end labeled with [γ-^{32}P]ATP and T4 kinase. The initially identified five EBV miRNAs (19) were all expressed in B95-8 as expected (Fig. 1A). In contrast, SNU-719 and AGS-EBV-GFP expressed only miR-BART1 and miR-BART2 between them. Similar to other reports (19), the expression level of miR-BART2 was very low compared with that of miR-BART1. In addition to the mature miRNAs, the EBV pre-miRNAs were also detected sometimes, but not always (data not shown).

The majority of the newly identified EBV miRNAs (5, 12) are located within the region deleted in the B95-8 EBV strain (Fig. 1B). Among the newly identified EBV miRNAs, miR-BART3, miR-BART5, miR-BART7, miR-BART10, and miR-BART12 were chosen to examine their expression. SNU-719 and AGS-EBV-GFP expressed all of the newly identified BART miRNAs, while B95-8 did not express any of them except miR-BART3 (Fig. 1A).

To clarify that the failed detection of BHRF1 miRNAs in the gastric carcinoma cell lines was not due to variances in the EBV strains, sequences around these miRNAs were analyzed. Even though there were 10 nucleotide changes in SNU-719 compared with B95-8, the sequences of the putative pre-BHRF1 miRNAs of SNU-719 were the same as those of B95-8 (data not shown). Thus, EBV miRNAs seem to be differentially regulated in different EBV-infected cell lines.

Expression profile of EBV miRNAs in an EBVaGC animal model. Four-week-old female athymic nude mice (BALB/c *nu*/*nu*) were purchased from Japan SLC, Inc. (Hamamatsu,

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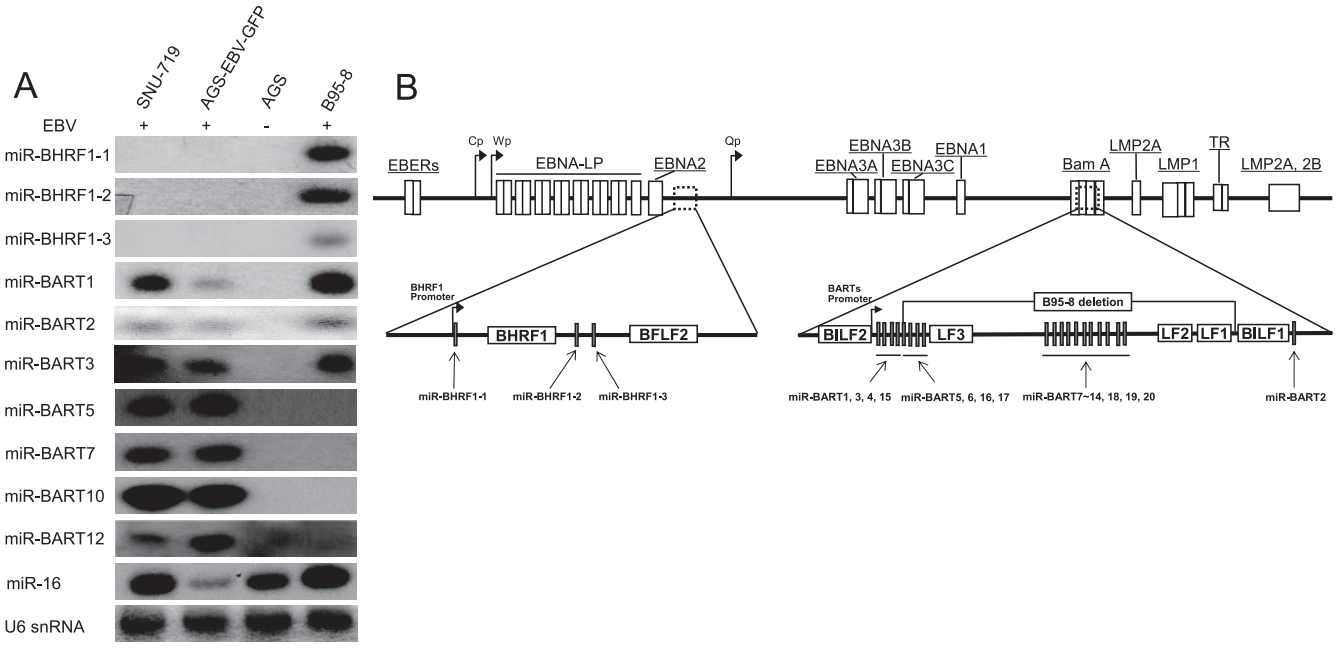

FIG. 1. Expression profile of EBV miRNAs in EBV-infected gastric carcinoma cell lines. (A) Northern blot for EBV miRNAs using total RNA isolated from gastric carcinoma cell lines. An EBV-positive B-cell line, B95-8, was used as a positive control. An EBV-negative gastric carcinoma cell line, AGS, was included as a negative control. The quality and quantity of the loaded RNA were assessed by reprobing the blot for U6 snRNA. The expression of human miR-16 was assessed as a reference. Blots were stripped and reprobed several times. Before reprobing, complete stripping of the blot was confirmed by exposing the membrane to film for a week. (B) Locations of the EBV miRNAs. The top panel shows the position of the latent genes and promoters (arrows). The lower panels show the position of the EBV miRNAs, which are marked as gray boxes.

Japan), and were maintained under specific-pathogen-free conditions. SNU-719 cells were injected subcutaneously into a flank of the mouse. All of the animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals of the Catholic University of Korea. Tumor was induced 40 to 45 days after the inoculation and showed similar characteristics of EBVaGCs (Sang Taek Oh et al., unpublished data). Four tumor-bearing nude mice were sacrificed to analyze the expression pattern of EBV miRNAs. The tumors expressed all of the tested BART miRNAs but not any of the BHRF1 miRNAs (Fig. 2). miR-BART2 was detectable at a low level only after X-ray film had been exposed for an extended period (data not shown). A relatively higher level of expression of BART miRNAs was noticable for tumors compared with the injected SNU-719 cells. Elevated BART miRNA expression was also observed in a nasopharyngeal carcinoma (NPC) tumor passaged in nude mice (C15) compared with that in an NPC cell line (5).

Expression of EBV miRNAs in human gastric carcinoma tissues. Paraffin-embedded human gastric carcinoma tissues were procured in accordance with the Institutional Review Board Policies of the Catholic University of Korea. To select EBVaGC cases, in situ hybridization for EBER was carried out (Fig. 3A) (17). Four EBER-positive (G7 to G10) and six EBER-negative (G1 to G6) gastric carcinoma cases were chosen for analysis. RNA was purified from 200-μm-thick slices of the EBVaGC paraffin block. The obtained total RNAs (12 to 57 μg) were used at once without normalizing the amounts. On the gel, distinct 5S rRNA bands were visible from the control cell lines, but the RNA samples from the tumor tissues appeared as smears

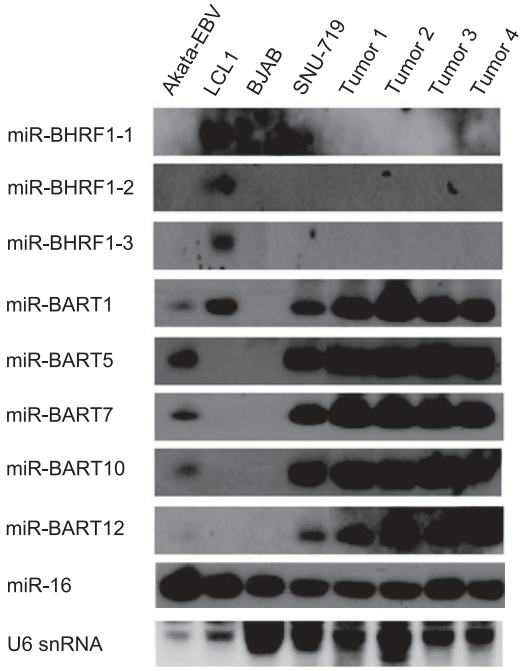

FIG. 2. Expression profile of EBV miRNAs in an EBVaGC animal model. Total RNA was resolved in a gel and transferred to a membrane. Each mature EBV miRNA was hybridized with a specific probe labeled with [γ-^{32}P] ATP. Akata-EBV was used as a positive control for BART miRNAs, and LCL1 was used as a positive control for BHRF1 miRNAs. BJAB was used as a negative control. The loading amount for each RNA sample was monitored by Northern blotting of U6 snRNA. Blots were stripped and reprobed several times. Before reprobing, complete stripping of the blot was confirmed by exposing the membrane to film for a week.

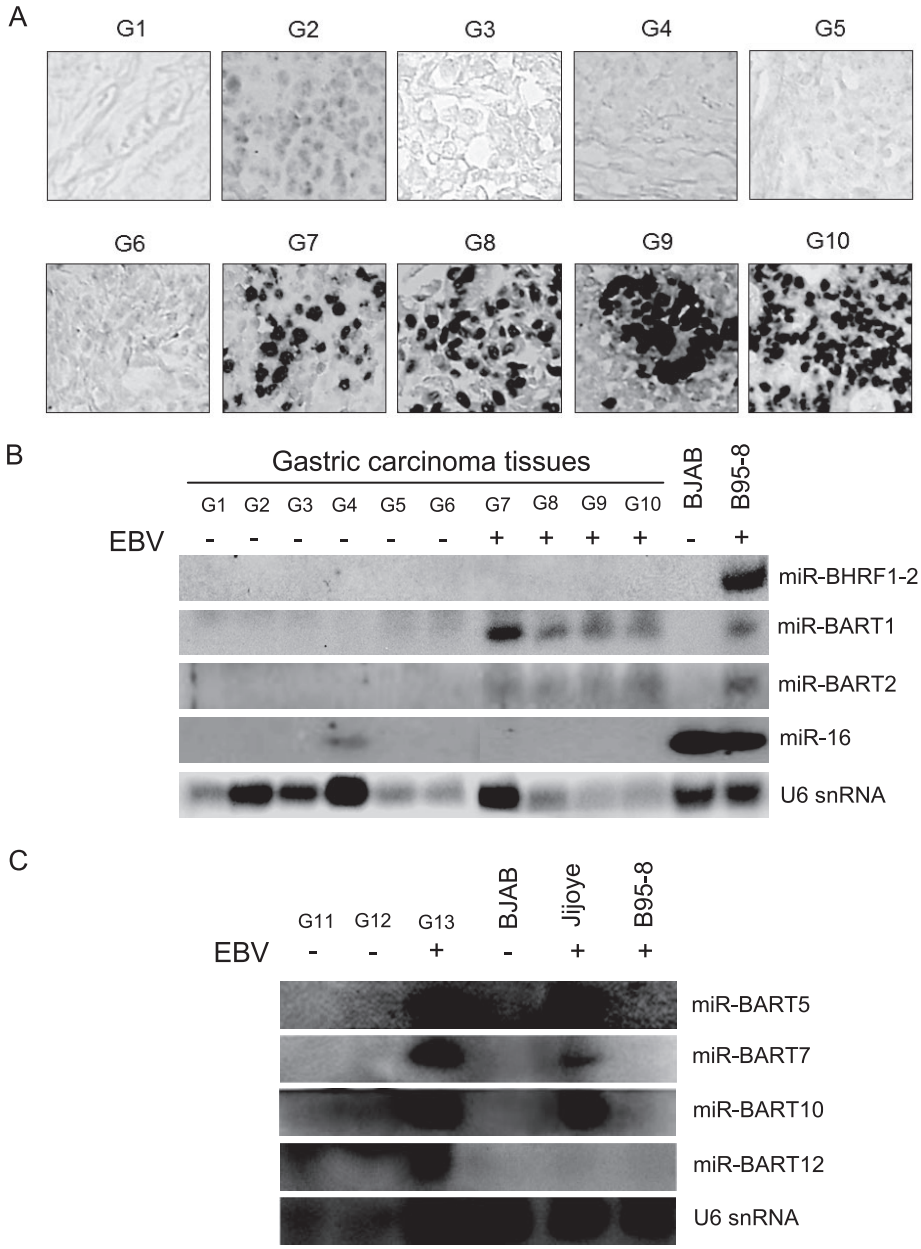

FIG. 3. Expression of EBV miRNAs in the gastric carcinoma tissues. (A) The gastric carcinoma tissues were fixed and hybridized with an EBER-specific DNA probe. Under a light microscope, positive staining was observed as black granules at the site of hybridization (original magnification, ×200). (B) Northern blotting for EBV miR-BART1, miR-BART2, and miR-BHRF1-2 was performed. The expression of human miR-16 was assessed for comparison. The loading amount for each RNA sample was monitored by Northern blotting of U6 snRNA. All of the data were obtained using one blot. Before reprobing, complete stripping of the blot was confirmed by exposing the membrane to film for a week. G1 to G10, gastric carcinoma tissues. (C) Northern blotting for EBV miR-BART5, miR-BART7, miR-BART10, and miR-BART12 was performed as described above.

except for the G4 tumor, in which a faint 5S rRNA band was detectable (data not shown). Extended storage of the paraffin blocks at room temperature as well as the fixing process of the tumor tissue might have resulted in degradation of RNA.

In spite of the poor RNA quality, we could detect miR-BART1 and miR-BART2 expression in all of the EBVaGC cases (Fig. 3B). BHRF1 miRNAs were undetectable in any of the tumor tissues, similar to what was observed for the EBV-positive gastric carcinoma cell lines (Fig. 3B) (data not shown). To analyze the expression pattern of the newly identified BART miRNAs, additional gastric carcinoma cases were screened (data not shown) and one EBV-positive and two EBV-negative cases were selected. The quality of the purified RNA from the tumor tissues was not good, as judged from the U6 snRNA bands. However, miR-BART5, miR-BART7, and miR-BART10 were detected in the EBVaGC case as well as in the Jijoye cell line (Fig. 3C). After prolonged exposure, miR-BART 12 was detected in the EBV-positive tumor tissue but

not in Jijoye cells (Fig. 3C). This may be due to its limited expression in B cells, as shown in Fig. 1 and Fig. 2 as well as in other reports (5, 12).

Unlike in the control cell lines, miR-16 was undetectable in all the tumor samples except tumor G4 (Fig. 3B). This can be explained by the poor RNA quality or by frequent deletion and down-regulation of miR-16 in some tumors (7).

As many EBV miRNAs including some BART miRNAs are evolutionally conserved, they are expected to play important roles in the viral life cycle (5, 9). It is puzzling that B95-8 shows full transforming activity and viral production in vitro, even though the majority of BART miRNAs are deleted in B95-8. BART miRNAs may play important roles in EBV-induced epithelial cell transformation but not in B-cell transformation. Or BART miRNAs could be essential for cell survival in vivo but dispensable in vitro. The highly expressed BART miRNAs may play important roles in immune evasion of the EBV-associated epithelial tumors in vivo. These EBV miRNAs may also support cell proliferation or suppress apoptosis under low oxygen tension found inside the tumor tissue. To clarify the role of EBV miRNAs in EBV-associated tumors, further research is warranted.

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