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Expression Profile of MicroRNAs in Epstein-Barr Virus-Infected AGS Gastric Carcinoma Cells

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Latent infection with Epstein-Barr virus (EBV) is responsible for multiple types of malignancies, including 10% of all gastric carcinomas. The microRNA (miRNA) expression in several EBV-infected AGS gastric carcinoma cell lines was determined. Infected cells expressed the viral BamHI A rightward transcript (BART) miRNAs at high levels and had consistently decreased expression of a small fraction of cellular miRNAs with specific downregulation of tumor suppressor miRNAs. These changes likely reflect expression of the viral noncoding RNAs and not latent protein expression.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus in which latent infection is associated with a number of malignancies, including multiple types of B-cell lymphomas, nasopharyngeal carcinoma (NPC), and gastric carcinoma (GC) (1, 2). EBV expresses a diverse subset of latent proteins with oncogenic potential in lymphomas in immunocompromised patients but a much more restricted set of viral proteins in malignancies in patients with intact immune systems. In addition, EBV expresses multiple noncoding RNAs during all types of latency, including two clusters of microRNAs (miRNAs). The largest set of miRNAs, the BamHI A rightward transcript (BART) miRNAs, are 44 miRNAs processed from the introns of the BARTs that are expressed at high levels in EBV epithelial malignancies (3–5). Infection of the gastric carcinoma cell line, AGS, with EBV has recently been shown to induce a more transformed phenotype, with dramatic changes in the cellular expression profile despite the expression of only one latent viral protein, EBNA1, to any significant level (6). A significant fraction of the changes in cellular expression likely reflect expression of the BART RNAs (6).

To profile the expression pattern of the BART miRNAs in infected AGS cells, five additional clonally infected lines were established by incubation of induced Akata-BX1 cells with uninfected AGS cells and selection for green fluorescent protein (GFP)-positive, G418-resistant clones. The viral protein expression pattern was determined by Western blotting and was nearly identical to that of the original cell line, with expression of only EBNA1 at a significant level (Fig. 1A). A small and somewhat variable amount of LMP1, which has previously been shown to not affect cellular gene expression, was expressed in these cell lines (6). Analysis of EBER expression by using quantitative reverse transcription (RT)-PCR that amplifies a conserved region revealed that all six EBV-infected AGS cell lines (AGS-EBV) expresses both EBER1 and EBER2, with consistently higher levels of EBER2 than are expressed by the Jijoye lymphoid cell line (Fig. 1B). Importantly, each clone had improved colony formation in soft agar, indicating that the transformed phenotype of latent EBV infection in this cell line was highly reproducible (Fig. 1C and D).

To profile the expression level of the viral miRNAs in these cell lines, small-RNA libraries were created using an Illumina TrueSeq small-RNA sample preparation kit and sequenced on a HiSeq 2000 sequencing system. The libraries are specific for miRNAs, as the RNAs are selected for size and the 3′ adapter is specific for RNA containing a 3′ hydroxyl group characteristic of miRNAs. In total, eight libraries were sequenced, two libraries from the parental AGS cell line, two libraries from the original AGS-EBV cell line, and one library each from new clones 1 and 2, as well as a library from the NPC cell line C666 and one from the NPC xenograft C15 for comparison. After processing was performed to remove adapter sequences, reads were aligned to the human (hg19) and viral (NC_007605) genomes using the Bowtie short read aligner. Confirming the size selection, the vast majority of reads were 20 to 24 bases. Reads of 16 to 25 nucleotides in length that overlapped by at least 16 nucleotides with mature miRNA coordinates obtained from miRBASE were counted as miRNA reads in the analysis in order to not exclude miRNAs with differential 5′ and 3′ processing, which is common in EBV miRNAs (Table 1) (7). A small fraction of the reads from the parental cells aligned to the EBV genome, although most were extremely short reads and likely nonspecific. However, very low levels of authentic EBV miRNAs were detected in these samples. It is not clear if this is due to some minor level of contamination during sample preparation or a low level of background EBV infection in the parental cell line. The numbers of total reads and miRNAs sequenced are shown in Table 1.

Each of the EBV-infected cell lines had a large fraction of their total miRNA pool derived from the virus, with 57% of all miRNAs in the C15 NPC tumor encoded by EBV (Fig. 2). The AGS-EBV cell lines have an expression pattern consistent with the pattern of the NPC cells and other epithelial EBV malignancies in which the BART miRNAs are very highly expressed and the BHRF1 miRNAs are rarely expressed (3). Expression of the BHRF1 miRNAs was essentially negligible in the C15 and C666 NPC samples, while a few hundred reads were detected in the AGS-EBV cells (see Data Set S1 in the supplemental material). As these miRNAs have been

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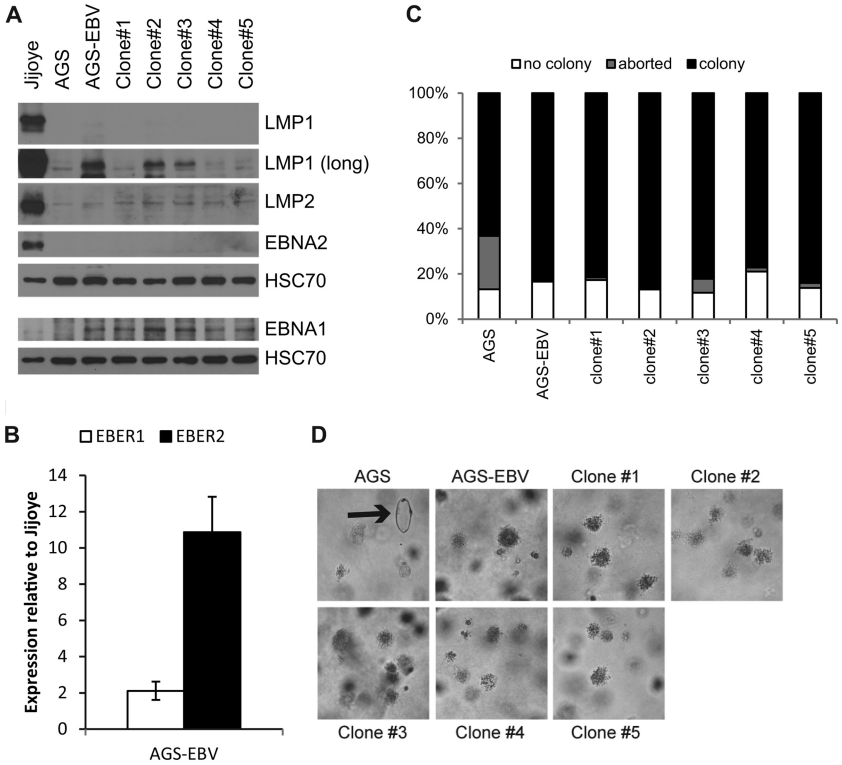

FIG 1 Multiple clonal infections of AGS cells are anchorage independent with restricted viral expression. (A) Western blot for latent viral protein expression in AGS-EBV clonal cell lines. The five new clones are compared to the original AGS-EBV cell line, to parental cells as a negative control (AGS), and to a type III Burkitt's lymphoma as a positive control (Jijoye). Western blots were probed with anti-LMP1 (S12 hybridoma supernatant), anti-LMP2 (clone 14B7; abcam), anti-EBNA1 (OT1x monoclonal antibody), anti-EBNA2 (PE2 monoclonal antibody), or anti-HSC70 (Santa Cruz; sc-7298) as a loading control. An overexposed lane of LMP1 is shown to demonstrate the small amount of LMP1 present in some of the cell lines. (B) Quantitative RT-PCR for EBER expression in AGS-EBV cells. The bars represent the average EBER expression levels across two RNA preparations from each of the six AGS-EBV cell lines relative to the average from three independent RNA preparations from Jijoye cells. PCR was performed with the Qiagen Quantifast SYBR green RT-PCR kit and the following primers: EBER1, 5′-ACCTACGCTGCCCTAGAGGT and 3′-TGGGAAGACAACCACAGACA; EBER2, 5′-GGAGGAGAAGAGAGGCTTCC and 3′-ATAGCGGACAAG CCGAATAC. (C and D) Soft agar assay of AGS-EBV cells. Cells were seeded in F12 medium with 0.5% Bacto agar and allowed to grow for 10 days. Colonies were then stained with 0.005% crystal violet and scored for growth (C) or photographed at ×100 magnification (D). AGS cells infected with EBV grow larger colonies in soft agar, with a lack of the hollow, aborted colonies seen in the parental cells. An example of an aborted colony is indicated by an arrow in panel D. All five new clones of infected cells show similar phenotypes of increased anchorage-independent growth.

shown to increase upon induction of viral replication, the BHRF1 miRNA reads may be due to expression in a small subset of cells with permissive infection. Overall, the pattern of abundance of the individual BART miRNAs was quite consistent across the AGS-EBV clones and the NPC samples, suggesting similar processing in these different cell types. However, this pattern of BART abundance is distinct from that reported in other profiling studies done in NPC, and there is little consistency for one pattern of BART miRNA abundance in the literature (5, 7–9). This may reflect differences in techniques and informatics between studies.

In order to determine whether there were any changes in the abundance of cell-derived miRNAs in response to EBV infection of AGS cells, the aligned human sequences were imported into the Partek genomic suite software, which was used to calculate significant changes in human miRNA levels. miRNA changes with a P value of <0.05 were considered significant, while those that rep-

TABLE 1 Summary of small-RNA sequencing results

Library	No. of reads			No. of miRNAs		% viral miRNAs
	Total	Mapped to human	Mapped to EBV	Human	EBV	
Parental AGS 1	35,111,900	33,305,259	445,435	22,884,865	7,705	0.03
Parental AGS 2	19,200,981	18,250,058	1,343,213	14,988,219	5,134	0.03
AGS-EBV 1	56,873,929	51,935,642	4,317,658	41,696,846	3,913,670	8.58
AGS-EBV 2	24,244,558	21,212,801	4,529,355	16,841,905	2,484,335	12.85
Clone 1	30,010,016	24,889,072	5,441,869	18,042,838	4,060,515	18.37
Clone 2	23,432,180	19,467,911	5,176,603	12,263,106	3,163,597	20.51
C666	24,241,258	18,475,830	7,185,207	8,748,402	5,693,242	39.42
C15	37,510,160	29.354.352	11,383,451	3,076,020	4,137,077	57.36

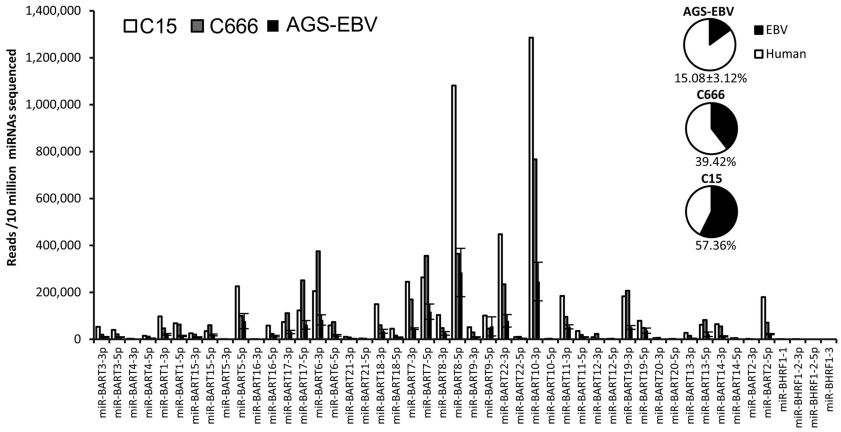

FIG 2 EBV miRNA counts from small-RNA libraries. The number of sequencing reads mapped to the mature miRNAs of EBV is indicated per 10 million total miRNAs to normalize for sequencing depth in each library. The value displayed for AGS-EBV is an average of results for four sequencing libraries, two from the original AGS-EBV cell line and one each from the first two newly made clones. The inset displays the relative contribution of EBV to the total pool of miRNAs from each of the cell lines. Again, the value for the AGS-EBV cells is an average of results for four samples, with the standard error of the mean indicated by error bars.

resented less than 0.05% of the total pool were excluded. A small subset of the human miRNAs was downregulated upon EBV infection, with no miRNAs increased by infection. Interestingly, the downregulated miRNAs included several miRNAs that act as tumor suppressors (see Data Set S1 in the supplemental material). The miRNA expression profile of the two NPC line sequences was substantially different from that of the AGS cells. It is likely that the considerable differences in the cellular miRNA profiles between AGS-EBV and the C15 NPC may reflect the contribution of viral proteins expressed in the C15 NPC tumor, including abundant LMP1.

To confirm these findings and expand the number of samples tested, Exiqon miRNA PCR arrays were performed. A total of 10 arrays were performed using three unique preparations of parental AGS RNA, two from the original AGS-EBV cell line, and one each from the five individual clones of AGS-EBV cells (see Data Set S2 in the supplemental material). The data from the PCR arrays largely confirmed the sequencing data, with many of the same miRNAs downregulated in response to EBV infection (Table 2). The fold changes tended to be larger with the PCR method than with the sequencing method, which may reflect normalization to housekeeping genes for the PCR data whereas the sequencing data are normalized to the total human miRNA pool. This result would also be consistent with EBV infection's decreasing the overall abundance of human miRNAs, perhaps due to competition for processing with the viral miRNAs or reported effects on Dicer function (10). Two miRNA families, the let-7 family and the miR-200 family, both of which are known tumor suppressor families, were heavily represented in the downregulated miRNAs (11, 12). These findings confirm previous findings indicating decreased expression of the miR-200 family of miRNAs in EBV-infected AGS cells and the observation that the levels of miR-200 family members are lower in EBV-positive GCs than in EBV-negative GCs (13, 14). Both methods of profiling indicated that miR-143, which has also been shown to act as a tumor suppressor in a number of different types of cancer, was significantly decreased (15–17).

To evaluate the potential effects of viral protein expression on host tumor suppressor miRNAs, EBNA1, the one viral protein significantly expressed in AGS cells, was overexpressed in uninfected AGS cells and the effects on a subset of the cellular tumor suppressor miRNAs was assessed using quantitative RT-PCR (Fig. 3A). Expression of EBNA1 at substantially higher levels than that of EBV-infected cells reduced miR-143 levels but did not affect the five other miRNAs tested (Fig. 3B). To ensure that the trace levels of LMP1 expressed in these cells was not responsible for the decreased expression of the tumor suppressor miRNAs, a dominant negative LMP1 was overexpressed to inhibit any residual LMP1

TABLE 2 Host miRNAs downregulated by EBV infection of AGS cells[a]

	Downregulation determined by:			
	Sequencing		RT-PCR	
miRNA	*P* value	Fold change	*P* value	Fold change
let-7 family				
hsa-let-7a-5p	0.0397	−2.226	0.0442	−1.608
hsa-let-7b-5p	0.0533	−1.912	0.0135	−2.555
hsa-let-7f-5p	0.0434	−1.696	0.1935	−1.681
hsa-mir-98-5p	0.0471	−2.219	0.0617	−3.566
miR-200 family				
hsa-miR-200a-3p	0.1446	−1.624	0.0133	−2.315
hsa-miR-200b-3p	0.0225	−1.757	0.0352	−2.498
hsa-miR-200c-3p	0.0480	−1.684	0.0328	−1.857
hsa-miR-429	0.0717	−1.838	0.0195	−2.591
Others				
hsa-miR-143-3p	0.1416	−2.974	0.0014	−6.090
hsa-miR-146b-5p	0.1337	−1.954	0.0234	−4.106
hsa-miR-148a-3p	0.0760	−1.997	0.0087	−3.135
hsa-miR-181b-5p	0.1252	−2.004	0.0400	−2.589
hsa-miR-23a-3p	0.0080	−1.840	0.0261	−2.073
hsa-miR-24-3p	0.1091	−1.945	0.0336	−1.807
hsa-miR-27a-3p	0.0149	−1.345	0.0350	−1.980
hsa-miR-29a-3p	0.0345	−1.343	0.0226	−2.318
hsa-miR-92b-3p	0.1022	−1.843	0.0482	−2.305

[a] miRNA changes were considered highly confident with a *P* value of <0.05 by one method and showed a confirmatory change with a *P* value of < 0.20 by the other method. miRNAs that represent <0.05% of the total pool (based on sequencing data) were excluded.

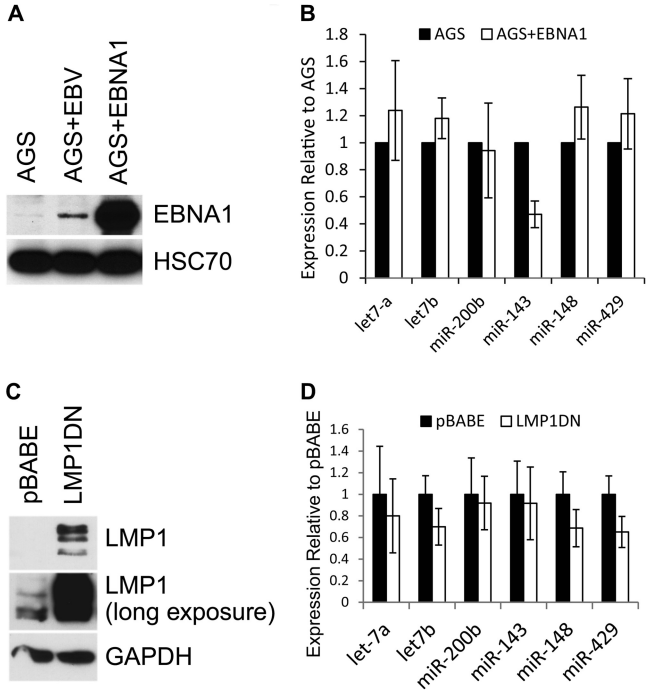

FIG 3 Lack of regulation of tumor suppressor miRNAs by EBV latent proteins. (A) Western blot demonstrating the overexpression of EBNA1. Parental AGS cells were stably transfected with the pc3OriPE vector that contains the EBV *oriP* sequence as well as the EBNA1 open reading frame. Western blots were probed with anti-EBNA1 (OT1x monoclonal antibody) or anti-HSC70 (Santa Cruz; sc-7298) antibody as a loading control. (B) Quantitative RT-PCR for six indicated tumor suppressor miRNAs that are downregulated by EBV infection. PCR was performed using miScript primer assays and the miScript RT-PCR system (Qiagen) from RNA prepared using TRIzol. The data were normalized to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are displayed as the average of the results for three independent RNA preparations. The error bars display the standard errors of the means. (C) Western blot illustrating the overexpression of the LMP1 dominant negative construct. Stable cell lines were made with the pBABE-LMP1DN construct (LMP1DN) or vector control (pBABE) in AGS-EBV cells. Western blots were probed with anti-LMP1 (S12 hybridoma) or anti-GAPDH (Santa Cruz; sc-25778) antibody. An overexposed lane is shown to demonstrate the level of expression of the dominant negative construct over the small amount of wild-type LMP1 in infected cells. (D) Quantitative RT-PCR for tumor suppressor miRNAs in the LMP1DN cell line. PCR was performed as described for panel B.

function in the AGS-EBV cells (Fig. 3C) (18). Inhibition of LMP1 did not increase expression of any of the miRNAs tested (Fig. 3D).

These results suggest that latent EBV infection modulates the expression pattern of a subset of host miRNAs largely through a mechanism that is independent of latent protein expression. Although it is likely that a variable number of cells in each of the infected cell lines has some expression of replicative genes, the consistent effects on cellular miRNA expression suggest that the changes occur in the majority of cells which are latently infected. Given the dramatic level of EBV BART miRNA expression, it is possible that these host miRNA levels are changed as a secondary effect of EBV miRNAs on expression of transcriptional regulators. Additionally, other EBV noncoding RNAs such as the EBERs may contribute to these effects. Taken together, the data presented demonstrate a significant shift in the total miRNA pool in epithelial cells infected with EBV with very restricted viral protein expression. These changes include both a decrease in host tumor suppressor miRNAs and elevated expression of viral miRNAs with demonstrated oncogenic potential.

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