Expression of viral microRNAs during primary infection of Epstein-Barr virus in B cells

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Expression of viral microRNAs during primary infection of Epstein-Barr virus in B cells

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This certifies that the masters Thesis
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Yonsei University

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Jung Hwa Lee

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Expression of viral microRNAs during primary infection of Epstein-Barr virus in B cells

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(Directed by Professor Jeon Han Park)

MicroRNAs (miRNAs) are single-stranded RNAs (ssRNAs) of 19–25 nucleotides and regulate gene expression at the posttranscriptional level by base pairing with target mRNAs, which leads to mRNA cleavage or translational repression.

Recently, many virus-encoded miRNAs have been identified, but the functions of most virus-derived miRNAs are unknown. The identification of virus-encoded miRNAs implicates that viruses would employ RNA silencing mechanism in the regulation of host and viral gene expression and host immune defense mechanism. Epstein-Barr virus (EBV) is the first virus demonstrated to encode miRNAs and encodes five miRNAs located in *BHRF1* mRNA and intronic regions of the *BART*s.

Northern blot analysis of reported five EBV miRNAs in cell lines of different latency types was performed to analyze expression pattern of EBV miRNAs. Mir-BHRF1s were detected in all different latent cell types tested; at significant levels in B95.8 cells but at only low levels in other cells, such as Rael, Mutu III, IM9wt and IM9mt. Mir-BARTs were detected in B95.8 and Rael cells. There was no significant difference between latent infected cells and lytic cycle-induced B95.8 cells.

In this study, to investigate the expression pattern of EBV miRNAs in early infection of B cells, Akata, a EBV-negative Burkitt's lymphoma (BL) cell line, was infected with EBV and time course expression of EBV miRNAs was analyzed following infection. Mir-BHRF1s were expressed during an initial infection step. Mir-BHRF1s were coordinately expressed with EBNA2 that was also expressed in an early infection step.

To determine the function of mir-BHRF1-3 on predicted target $p21^{WAF1}$, $p21^{WAF1}$ expression was investigated in EBV-positive cells which express mir-BHRF1-3, and the effect of mir-BHRF1-3 on cell cycle progression and apoptosis was analyzed in EBV-infected B cells. 2'-O-methyl oligonucleotides, specifically inhibiting individual miRNAs, were transfected into EBV negative Akata cells and then these transfectants were infected with EBV. 2'-OMe-mir-BHRF1-3 resulted in an increase of apoptosis, implicating that mir-BHRF1-3 functions in cell survival maintenance. A DNA damaging agent, cisplatin, failed to induce $p21^{WAF1}$ protein expression in LCL-1 cells representing high level of mir-BHRF1-3 expression, whereas induced increased

expression of $p21^{WAF1}$ in IB4 cells with low level of mir-BHRF1-3 expression. However, expression of $p21^{WAF1}$ mRNA induced by cispatin was shown to be increased in both cells. Thus, these data suggest that mir-BHRF1-3 functions in blocking apoptosis and maintaining cell survival through the regulation of 21^{WAF1} expression at the posttranscriptional level. Through inhibition of 21^{WAF1} expression, mir-BHRF1-3 can induce EBV-infected cells to bypass G1/S checkpoint and therefore contribute to maintenance of EBV latent infection and EBV-induced tumorigenesis.

Mir-BHRF1s were expressed in initial infection phase and coordinately expressed with EBNA2. Mir-BHRF1-3 induced cell survival maintenance, regarded as participating in latent infection maintenance and EBV-associated tumorigenesis by regulating cell cycle checkpoint.

Key words : microRNA, Epstein-Barr virus, mir-BHRF1-3, $p21^{WAF1}$

Expression of viral microRNAs during primary infection of Epstein-Barr virus in B cells

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Department of Medical Science

The Graduate School, Yonsei University

< Directed by Professor Jeon Han Park >

I. Introduction

MicroRNAs (miRNAs) are single-stranded RNAs (ssRNAs) of 19–25 nucleotides found in all metazoan eukaryotes, and with >200 members per species in higher eukaryotes. MiRNAs are one of the largest gene families, accounting for ~1% of the whole genome[1-3]. MiRNAs function as guide molecules in post-transcriptional gene silencing by base pairing with target mRNAs. Recent studies have revealed that miRNAs play key roles in diverse regulatory pathways, including control of developmental timing, hematopoietic cell differentiation, apoptosis, cell proliferation, and organ development[4,5].

MiRNA genes are transcribed by RNA polymerase II (pol II) to generate the primary transcripts (pri-miRNAs)[6]. The initiation step in miRNA processing is mediated by the nuclear RNase III enzyme Drosha–DGCR8 complex known as the microprocessor complex, which cleaves pri-miRNA to release the ~70-nt pre-miRNA hairpin intermediate[7-10]. Pre-miRNA constitutes a transport complex together with Exportin-5 and its cofactor Ran (the GTP-bound form)[11]. Following export, the cytoplasmic RNase III Dicer participates in the second processing step to produce miRNA duplexes[12]. The duplex is separated and usually one strand is selected as the mature miRNA, whereas the other strand is degraded. Incorporated into the RNA-induced silencing complex (RISC), miRNA acts to guide RISC to mRNAs bearing complementary sequences. RISC binding to mRNA can inhibit gene expression by one of two mechanisms. If the mRNA bears an essentially perfectly complementary sequence, binding induces cleavage by the RISC component Ago2, leading to mRNA degradation. However, if the mRNA contains an imperfectly complementary target, RISC binding may instead induce translational inhibition. Unlike mRNA cleavage, which can be mediated by a single RISC, translation inhibition is highly cooperative and may require binding by several RISCs, potentially bearing different miRNAs, to be effective[13,14].

Recently, many virus-encoded miRNAs have been identified, but as with their cellular counterparts, the functions of most virus-derived miRNAs are unknown; however, viral miRNAs are expected to function as regulators of viral and cellular

gene expression for their survival in infected cells. Recently, host-encoded miRNAs have been shown that can modulate viral replication via interaction with target sites in viral transcripts[15,16]. To date, virus-encoded miRNAs have been identified in the following classes of viruses: herpesviruses; Epstein-Barr virus (EBV), Kaposi sarcoma-associated virus (KSHV), mouse gammaherpesvirus 68 (MHV68), and human cytomegalovirus (HCMV), polyomavirus; simian virus 40 (SV40) and retroviruses[-17-24].

EBV, a member of the human herpesvirus subfamily, is a ubiquitous infectious agent, infecting more than 90% of the world's population[25]. EBV is associated with a variety of human tumours including B-cell malignancies such as Hodgkin's disease (HD), lymphoproliferative disease arising in immunosuppressed patients, some T-cell lymphomas, and epithelial tumours such as nasopharyngeal carcinoma (NPC) and gastric cancer. Infection by EBV *in vitro* easily transforms resting B cells from human peripheral blood into actively proliferating lymphoblastoid cell lines (LCLs)[26-29].

Latency I, II, and III are based on patterns of expression of the EBV latent genes. During latency I, associated with Burkitt's lymphoma, EBNA-1 and EBERs are expressed. Latency II has been associated with Hodgkin's disease, T-cell non-Hodgkin's lymphoma, and NPC. EBV gene expression is usually limited to EBNA-1, EBERs, LMP-1, LMP-2A, and LMP-2B. The final pattern of gene expression, latency III, occurs mainly in immunocompromised individuals suffering from posttransplant lymphoproliferative disorders, AIDS-related proliferative disorders, and in

lymphoblastoid cell lines. Latency III usually involves the unrestricted expression of all EBNAs, EBERs, and LMPs[30]. Viral proteins and RNAs expressed during latent infection have an important role in EBV-associated tumorigenesis[31].

EBV was the first virus demonstrated to encode miRNAs. Cloning from a B cell line latently infected with EBV identified five miRNAs. mir-BHRF1 (mir-BHRF1-1 to mir-BHRF1-3) is located within the mRNA of the *BHRF1* (Bam HI fragment H rightward open reading frame 1) gene encoding a distant Bcl-2 homolog and mir-BART1 and mir-BART2 are located in intronic regions of the *BART* (Bam HI-A region rightward transcript) gene. The function of the EBV miRNAs has not been established, but computational predictions suggest that some of these miRNAs may target chemokines, cytokines, and apoptosis and cell growth control genes such as p53[17].

To establish successful infection, viruses employ many components of host gene expression machinery while simultaneously evolving mechainsms for evading innate host cell immune responses against virus, such as the IFN production to inhibit viral replcation or apoptosis induction resulting lysis of taget cells infected with viruses. MiRNAs give an alternative way for viruses to turn off host cell defense factors. The identification of virus-encoded miRNAs implicates that viruses would employ RNA silencing mechanism in the regulation of host and viral gene expression and host immune defense mechanism. Thus, the roles of EBV miRNAs involved in viral replication, latent infection, B cell transformation and tumorigenesis are predicted.

In this study, to investigate the expression of EBV miRNAs in early infection of B

cells, expression pattern of miRNAs was analyzed at different time points following infection. Through miRNA target prediction, mir-BHRF1-3 is predicted to have a complementary binding site to 3'UTR of $p21^{WAF1}$. To determine the function of mir-BHRF1-3 on predicted target $p21^{WAF1}$, $p21^{WAF1}$ expression was investigated in EBV-positive cells which express mir-BHRF1-3 and the effect of mir-BHRF1-3 on cell cycle progression and apoptosis was analyzed in EBV-infected B cells.

II. Materials and Methods

1. Cell culture

Akata, a Burkitt's lymphoma-derived cell line carrying EBV, EBV-negative Akata, Ramos, a EBV-negative BL cell line, MutuI and Rael, EBV-positive latency type I BL cell lines, MutuIII, a latency type III BLCL, B95-8, a EBV-positive B cell line, and IB4 and LCL-1, lymphoblastoid cell lines, were maintained in a Roswell Park Memorial Institute (RPMI 1640) medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/㎖ penicillin, and 100 μg/㎖ streptomycin at 37°C in 5% humidified CO_2 atmosphere. IM9, a lymphoblastoid cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) containing 10% FBS, 100 U/㎖ penicillin, and 100 μg/㎖streptomycin.

2. Northern blot analysis

Total RNA (50 μg) from each cell line was resolved on 12.5% urea-polyacrylamide gels and transferred electronically to Hybond-N^+ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). Oligonucleotides complementary to miRNAs labeled at the 5′ end with ^{32}P-γ-ATP were used as probes. Prehybridization and hybridization were carried out using ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA, USA) following the manufacturer's recommendation. The sequences of the oligonucleotide probes were as follow; 5'-

GCAGCTCACCTTCCACTAAGA–3' (miR-BART1), 5'-GCAAGGGCGAAT GCAGAAAAT-3' (miR-BART2), 5'-AACTAAGGGGCTGATCAGGTTA-3 (mir-BHRF1-1), 5'-TTCAATTTCTGCCGCTTTTGATA-3' (mir-BHRF1-2), 5'-GTGTGCT TACAC ACTTCCCGTTA-3' (mir-BHRF1-3). For the control, mir-16 was used and its sequence is 5'-GCCAATATTTACGTGCTGCTA-3' (mir-16). The membranes were washed at low stringency in 2× SSC, 0.05% SDS at room temperature for 30 min and at high stringency in 0.1 SSC, 0.1% SDS at room temperature for 40 min. The blots were then exposed to X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan) at -80℃.

3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using TriZol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (5 μg) was used for the first-strand cDNA synthesis with Superscript^{TM}Ⅱ reverse transcriptase (Invitrogen) and random hexamers (Invitrogen). Sequence-specific primers for p21^{WAF1} and GAPDH were used for PCR amplification. Primer sequences are as follows: p21^{WAF1}, forward 5'-CCGAAGT CAGTTCCTTGTGG-3' and reverse 5'- GGATTAGGGCTTCCTCTTGG-3'; GAPDH, forward 5'-CGGGAAGCTTGTGATCAATGG-3' and reverse 5'-GGCAG TGATGGCA TGGACTG-3'.

4. Purification of B95-8 EBV virus

EBV was purified from B95.8 cells (3x10^6 cells/㎖) which had been treated with 30

ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St Louis, MO, USA) for 5 days. The supernatant was collected and concentrated by ultrafiltration using Centricon Plus-80 units (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. The supernatant was filtered through a 0.45-μm-pore-size membrane (Millipore), divided into 1 ㎖ aliquots, and stored at −80°C until used.

5. Virus infection

EBV negative Akata cells were incubated with EBV (purified by ultrafiltration from the marmoset B95.8 cell line) at a density of 10^7/㎖ for 3 h at 37°C in a 5% CO_2 incubator. Cells were then resuspended in culture medium and cultivated. After indicated times of cultivation, cells were harvested and analyzed for Northern blot and RT-PCR analysis.

6. miRNA inhibitors

2'-*O*-methyl oligonucleotides were synthesized and purified using high-performance liquid chromatography (HPLC) at Integrated DNA Technologies (Integrated DNA Technologies, Inc., Coralville, IA, USA). The sequences of the 2'-*O*-methyl oligonucleotides were 5'-Cy3ACUCCGGGGCUGAUCAGGUUA-3' for 2'-OMe miR-BHRF1-1, 5'-6-FAMUUCAAUUUCUGCCGCAAAAGAUA-3' for 2'-OMe miR-BHRF1-2 and 5'-Cy3GUGUGCUUACACACUUCCCGUUA-3' for 2'-OMe miR-BHRF1-3. All the nucleotides in the inhibitors contain 2'-OMe modifications at every

base and a 5' Cy3 or FAM containing amino linker.

7. Transfections

To transfect miRNA inhibitors, 4 $\mu\ell$ siPORT NeoFx (Ambion, Inc., Austin, TX, USA) was diluted into Opti-MEM I (Invitrogen, Grand Island, NY, USA) to 50 $\mu\ell$ for each sample and incubated for 10 min at room temperature. MiRNA inhibitors were diluted into Opti-MEM I to 50 $\mu\ell$ resulting a final concentration of 30 nM. Diluted siPORT NeoFx and diluted miRNA inhibitors were mixed and incubated for another 10 min at room temperature to allow transfection complexes to form and then dispensed into the empty wells of 12-well culture plates. Added to the complex was 900 $\mu\ell$ of diluted cell suspension containing $1x10^5$ cells. The cells were cultivated for 24 h, and then assayed for FACS analysis.

8. Western blot analysis

Cells were washed once in PBS, and solubilized by sonication in sample buffer (130 mM Tris-HCl, pH6.8, 2% SDS, and 10 % (v/v) glycerol). Cells were solubilized at a concentration of $5x10^6$ cells /200$\mu\ell$ sample buffer, boiled for 5min, and then sonicated briefly to reduce the viscosity. For protein quantitation, the bicinchoninic acid (BCA) assay was performed using the BCA kit (Pierce, Rockford, IL, USA) by following the manufacturer's protocol. Equivalent amounts of extract protein were resolved in 12% SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane (Bio-Rad

Laboratories, Richmond, CA, USA). The blot was then incubated with primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized with enhanced chemiluminescence western blotting detection reagents (Amersham, Little Chalfont, UK)

9. DNA-damaging agents

Cisplatin [*cis*-platinum(II)-diammine dichloride] and doxorubicin (adriamycin) were purchased from Sigma Chemical Co. Cisplatin and doxorubicin were used at concentrations of 10 ㎍/㎖ and 0.4 μM respectively and each agent was titrated for ability to induce apoptosis by dose-response experiments in the EBV negative Akata cell line. Cisplatin was dissolved at 50 ㎎ in 3 ㎖ of DMSO and stored at -20℃. Doxorubicin was dissolved at 50 ㎎/㎖ in H_2O and stored at -20℃.

10. Apoptosis assessment by annexin V staining

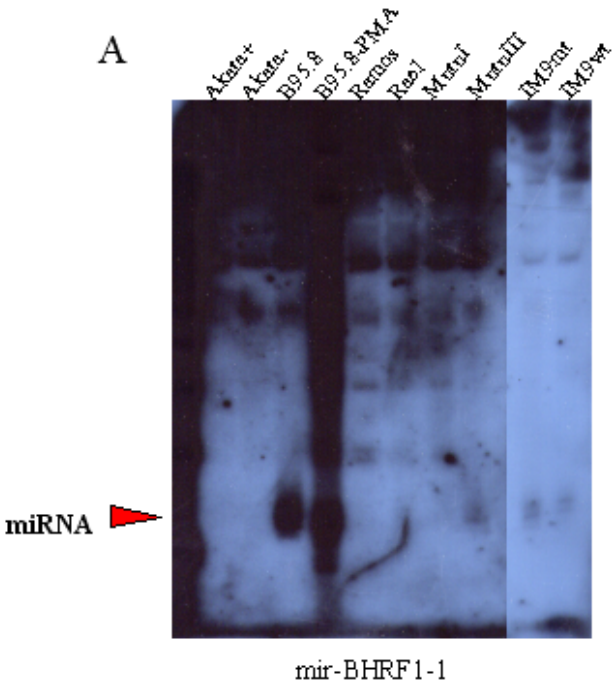
Cells were stained with the Annexin V fluorescein isothiocyanate (FITC) kit according to the manufacturer's protocol (Biosource, Camarillo, CA, USA). To quantitate the apoptotic cells, prepared cells were washed with cold PBS and then resuspended in binding buffer at a concentration of $1x10^6$cells/㎖. Then, 5 ㎕ of annexin V-FITC and 10 ㎕ of propidium iodine (PI) (Sigma Chemical Co.) were added to cells. After incubation for 15 min at room temperature 300 ㎕ of binding buffer was added and then analyzed with FACStar flow cytometer (Becton Dickinson, San Jose, CA, USA).

III. Results

1. Analysis of EBV miRNA expression pattern in different types of EBV latency

To analyze expression pattern of EBV miRNAs in EBV negative or EBV positive, latently infected cell lines represented in three different latency stages that are characterized by the expression of different subsets of the latent genes, northern blot analysis was performed. The expression of five EBV miRNAs was analyzed. Mir-BHRF1-1 was detected in B95.8, Rael, Mutu III, IM9wt, and IM9mt (Fig. 1A). Mir-BHRF1-2 and mir-BHRF1-3 were detected in B95.8, Rael, and Mutu III. In B95.8 cells, mir-BHRF1 expression was represented at significant levels, but in other cells, Rael, Mutu III, IM9wt and IM9mt, expressed at only low levels. Mir-BART1 expression was detected in B95.8 and Rael cells and mir-BART2 expression was detected in B95.8 cells. All of the five reported EBV miRNAs were detected in B95.8 cells (Fig. 1B). In the cases of mir-BHRF1-2 and mir-BHRF1-3, the ~60-nt mir-BHRF1 pre-miRNA as well as the 22-nt mature mir-BHRF1 were readily detectable by Northern blot analysis as reported previously[15]. To examine whether EBV miRNAs might be differentially expressed upon induction of the virus lytic cycle or EBV lytic replication activated any EBV miRNA expression, EBV miRNA expression was analyzed in PMA-treated B95.8 cells. EBV miRNA expression was detected in both latently infected and PMA-treated B95.8 cells, indicating that gene regulation is

mediated by viral miRNAs during both latent and lytic cycle of EBV. For five EBV-encoded miRNAs, there was no enhanced expression after PMA treatment, which suggests that PMA treatment, resulting in induction of lytic replication, has little or no effect on EBV miRNA expression (Fig. 1B; lane 3, 4).


A
Akata+
Akata-
B95.8
B95.8-PMA
Ramos
Raz1
Mutu1
MutuIII
IM9mt
IM9wt
miRNA
mir-BHRF1-1

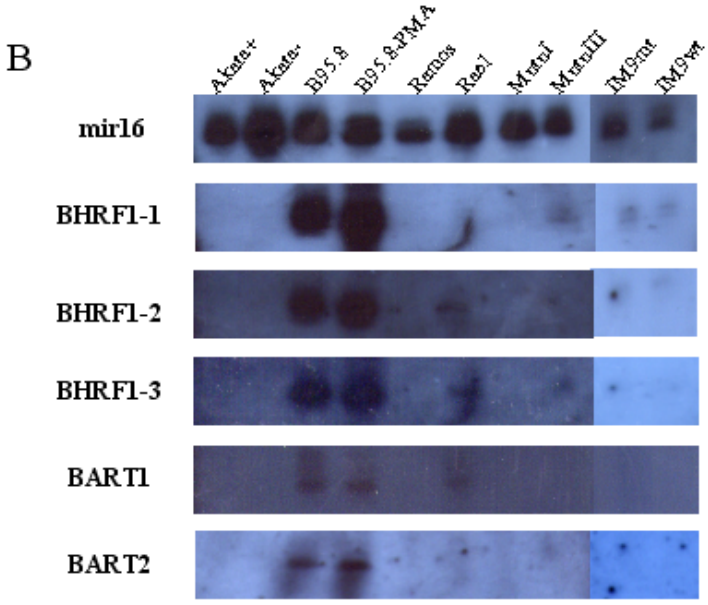
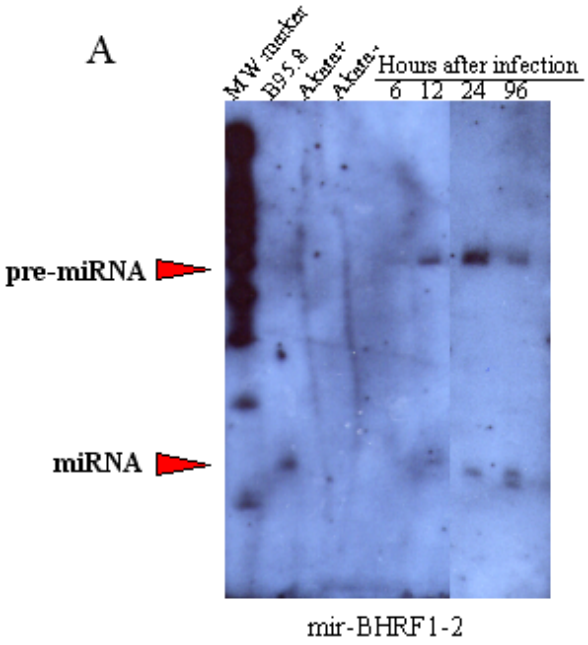

B
Akata+
Akata-
B95.8
B95.8-PMA
Ramos
Raz1
Mutu1
MutuIII
IM9mt
IM9wt
mir16
BHRF1-1
BHRF1-2
BHRF1-3
BART1
BART2

Figure 1. Expression pattern of EBV miRNAs in cell lines of different latency types

A. Northern blot analysis for mir-BHRF1-1 in cell lines of different latency types. Total RNAs isolated from EBV negative cell lines (EBV-negative Akata and Ramos) and from latently infected (EBV-positive Akata, B95.8, Rael, MutuI, MutuIII, IM9wt and IM9mt) or PMA-induced (PMA-treated B95.8) EBV positive cell lines were used for Northern blot analysis. B. Northern blot analysis for EBV miRNAs, mir-BHRF1-1, mir-BHRF1-2, mir-BHRF1-3, mir-BART1, and mir-BART2. The expression of human mir-16 is shown as a positive control.

2. EBV miRNA expression pattern during EBV infection in B cells

To investigate the expression pattern of EBV miRNAs during an initial step of EBV infection, EBV negative Akata cells were infected with EBV from lytic cycle induced B95.8 cells by PMA treatment and then viral miRNA expression was analyzed at indicated time points. At 6 h, 12 h, 24 h, and 96 h after infection, total RNAs were extracted from infected Akata cells for Northern blot analysis to determine the expression pattern of viral miRNAs. As shown in Figure 2, mir-BHRF1-1, mir-BHRF1-2, and mir-BHRF1-3 were detected at 6 h after infection in both mature forms and their fold-back precursor forms, while mir-BART1 and mir-BART2 expression were not detected until 96 h after infection in precursor forms as well as mature forms. These results indicate that EBV mir-BHRF1s are expressed at an early infection stage.


A
MW marker
B95.8
Akata+
Akata-
Hours after infection
6 12 24 96
pre-miRNA
miRNA
mir-BHRF1-2

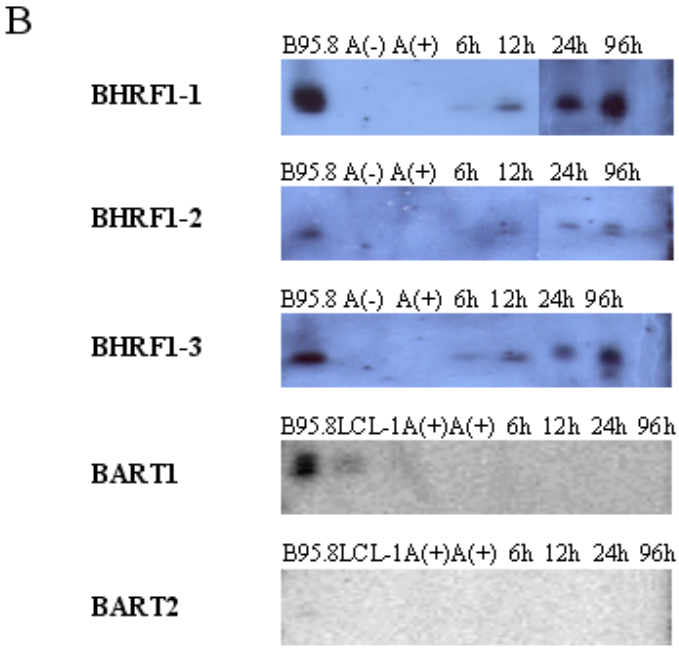

B
B95.8 A(-) A(+) 6h 12h 24h 96h
BHRF1-1
B95.8 A(-) A(+) 6h 12h 24h 96h
BHRF1-2
B95.8 A(-) A(+) 6h 12h 24h 96h
BHRF1-3
B95.8LCL-1A(+)A(+) 6h 12h 24h 96h
BART1
B95.8LCL-1A(+)A(+) 6h 12h 24h 96h
BART2

Figure 2. Time-course expression of EBV miRNAs in EBV-infected Akata cells.A. Northern blot analysis for mir-BHRF1-2. At 6 h, 12 h, 24 h, and 96 h after infection, total RNAs were ectracted from EBV-infected Akata cells, separated on denaturing gels, transferred to membranes and hybridized with a ^{32}P-labelled oligonucleotide specific for mir-BHRF1-2. The position of migration of the mature miRNAs (miRNA) and its fold-back precursors (pre-miRNA) is indicated as arrows. B95.8 was used as a positive control. B. Northern blot analysis for EBV miRNAs at indicated time points after infection.

3. Latent gene expression during EBV infection in B cells.

To investigate the expression pattern of EBV latent genes at an early infection step and analyze the relationship with EBV miRNA expression pattern, EBV latent gene expression was analyzed following EBV infection using RT-PCR analysis. To determine mRNA level of EBV latent genes, EBNA2, LMP1, and LMP2A, RT-PCR analysis was performed in EBV-infected Akata cells at 6 h, 12 h, 24 h, and 96 h after infection. As shown in Figure 3, EBNA2 expression was detected from 6 h after infection and LMP1 expression from 24 h after infection. LMP2A expression was not detected to 96 h after infection (data not shown). EBNA2, essential for B-cell immortalization, was expressed earlier than any other latent genes during EBV infection, and LMP1 expression, activated by EBNA2, was represented after then. Thus, mir-BHRF1 is expressed within 6 h after infection, early time points of EBV infection, similar to EBNA2 expression.

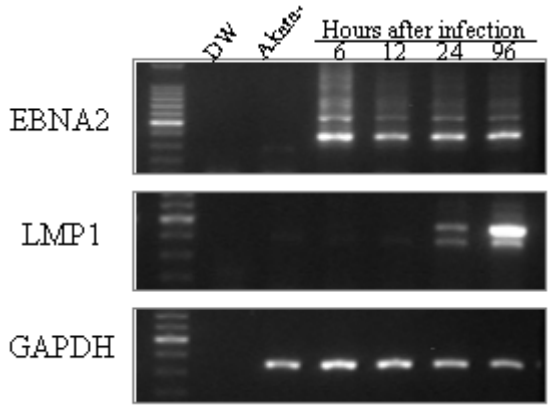


Figure 3. Expression pattern of EBV latent genes in EBV-infected Akata cells.RT-PCR analysis of viral gene expression in EBV-infected Akata cells at indicated time points after infection was done. GAPDH is shown as an internal control.

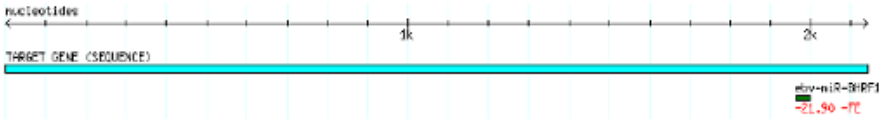
4. Target prediction: mir-BHRF1-3 has a binding site complementary to 3'UTR of $p21^{WAF1}$.

Towards $p21^{WAF1}$ expression, acting as a critical regulator of the cell cycle and DNA replication, there has been previous studies that EBNA2, a essential protein for B cell immortalization, induces $p21^{WAF1}$ expression[31]. However, it was also reported that without other EBV genes, EBNA2 alone results in cell growth retardation[31]. Previous studies showed increased $p21^{WAF1}$ mRNA level[32] but no one showed increased protein level. This discrepancy remains unsolved until now, but recent discovery that miRNAs play a role involved in the regulation of the cell cycle checkpoint proposeed a possibility that EBV-encoded miRNAs function as a posttranscriptional regulator of $p21^{WAF1}$ expression and we used a computational method to search whether $p21^{WAF1}$ has potential binding sites for EBV miRNAs. Through Microinspector, a web-based tool for searching miRNA binding sites in a given target mRNA sequence[33], we found out mir-BHRF1-3 has a binding site complementary to 3'UTR of $p21^{WAF1}$ (Fig. 4).

A

POSITION	SEQUENCE OF TARGET	NAME OF MIRNA	SEQUENCE OF MIRNA	FREE ENERGY	LINK (SEC.STRUCTURE .ps)
1964	tctcggctccccatgtgtcctggttcccgttt	ebv-miR-BHRF1-3	uaacgggaaguguguaagcacac	-21.9	Link

Results in .CSV format (Right click and 'Save as')



B

Figure 4. Results of a Microinspector analysis searching for EBV miRNA binding sites in the 3'UTR sequences of $p21^{WAF1}$.

A. The interaction of mir-BHRF1-3 with 3'UTR of $p21^{WAF1}$ is identified at position 1964. B. Representation of a secondary structure graphic displaying the interaction between mir-BHRF1-3 and target $p21^{WAF1}$.

5. The function of mir-BHRF1-3 in apoptosis induction

To investigate the function of mir-BHRF1-3 to predicted target $p21^{WAF1}$, the effect of mir-BHRF1-3 on cell proliferation and apoptosis was examined using a miRNA inhibitor, 2'-O-methyl oligonucleotides specifically inhibiting mir-BHRF1-3. To perform this experiment, 2'-OMe-mir-BHRF1-3 was transfected into EBV negative Akata cells and then these transfected cells were infected with EBV purified form B95.8 cells. Flow cytometric analysis using annexin-V/PI staining showed that 1 day after infection, in EBV-infected Akata cells 2'-OMe-mir-BHRF1-3 had no effect in apoptotic induction compared with untransfected EBV-infected Akata cells (Fig. 5A). Whereas 4 days after infection, 2'-OMe-mir-BHRF1-3 increased the level of apoptosis. Figure 5B shows that 4 days after EBV infection of Akata negative cells transfected with the miRNA inhibitor, 2'-OMe-mir-BHRF1-3, resulted in enhanced apoptotic induction compared with transfection of 2'-OMe-mir-BHRF1-2 and untransfected Akata cells which did not affect the apoptosis. These data shows that inhibition of mir-BHRF1-3 induces apoptosis in EBV-infected cells and which suggests that the function of mir-BHRF1-3 is related to cell survival maintenance in EBV-infected cells.

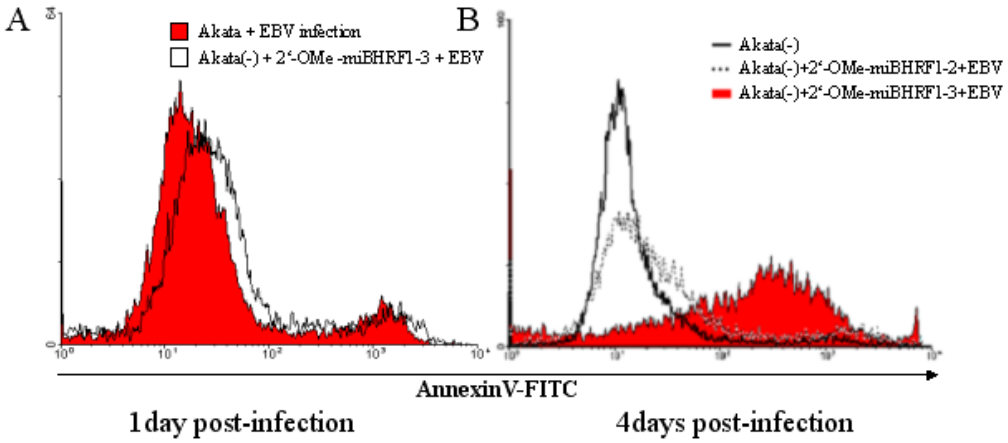


Figure 5. Inhibition of mir-BHRF1-3 induces apoptosis in EBV-infected cells.

To inhibit mir-BHRF1-2 and mir-BHRF1-3, antisense 2'-O-methyl-oligonucleotides corresponding to each miRNA were transfected into EBV-negative Akata cells and then the transfected cells were infected with B95.8 EBV. Then, 1 day and 4 days after infection, apoptotic assay was performed using annexin-V/PI staining.

6. Analysis of $p21^{WAF1}$ expression after cisplatin-induced DNA damage

To analyze the expression level of $p21^{WAF1}$ induced by the DNA damaging agent in EBV-positive cells, RT-PCR and Western blotting were performed in LCL-1 and IB4 cells treated with cisplatin. After treatment of LCL-1 and IB4 cells with 10 ㎍/㎖cisplatin protein extracts and total RNAs were prepared at the indicated times. Western blot analysis showed that in LCL-1 cells, showing significant level of mir-BHRF1-3 of which expression was detected in Northern blot analysis, $p21^{WAF1}$did not accumulate in response to cisplatin whereas $p21^{WAF1}$ protein accumulated after exposure to cisplatin in IB4 cells, representing a low level of BHRF1-3 expression. The slight decrease in $p21^{WAF1}$ was observed 20 h after treatment, and this result is consistent with previously reported data that 16 h after treatment the decrease of $p21^{WAF1}$ expression sometimes occurs in the EBV-infected cells due to apoptotic cells and proteolytic cleavage of $p21^{WAF1}$ by activated caspases[33]. RT-PCR analysis was performed to determine the expression level of $p21^{WAF1}$ mRNA. $p21^{WAF1}$ mRNA level was increased following cisplatin treatment in both cell lines and these data implicate that the failure of $p21^{WAF1}$ protein accumulation in EBV-infected cells does not result from reduced transcription (Fig. 6). Thus, mir-BHRF1-3 inhibits $p21^{WAF1}$ expression at the post-transcriptional level and this result provides a possibility that through cell cycle regulation, EBV miRNA functions in contributing towards B cell immortalization.

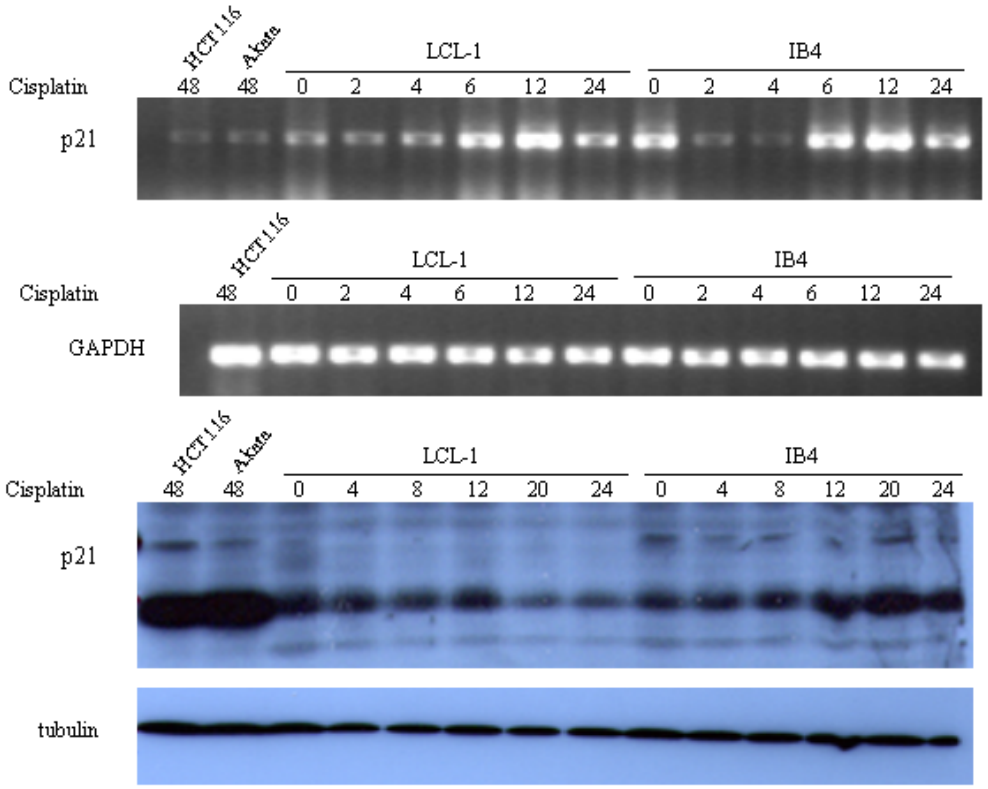


Figure 6. p21^{WAF1} expression after cisplatin-induced DNA damage.

After treatment of LCL-1 and IB4 cells with 10 ㎍/㎖ cisplatin, RT-PCR and Western blot analysis were performed using total RNAs and protein extracts harvested at the indicated times. HCT116 cells treated with 1 μM doxorubicin and EBV-negative Akata cells treated with 0.4 μM doxorubicin were used as positive controls.

IV. Discussion

MiRNAs have been identified in several mammalian viruses, including herpesviruses, such as EBV, KSHV, and HCMV, SV 40 and the human immuno-deficiency virus (HIV) [17-24]. The functions of most viral miRNAs remains unknown, but potential functions have been proposed for several viral miRNAs and they are expected to regulate the expression of viral and host genes for their survival in infected cells. Recently, a cellular miRNA was revealed that can mediate antiviral defense in human cells[34].

To establish successful infection, viruses downregulate innate antiviral defense mechanisms of host cells coincident with employing many components of host gene expression machinery. Viruses encode proteins that function in preventing the host cell defense mechanism as a mechanism of evading host cell antiviral response. The identification of virus-encoded miRNAs represents that viruses can utilize miRNA machinery to inactivate host antiviral factors through gene silencing of target mRNA[24]. In addition, viruses that establish the latent infection enable for miRNAs to modulate host gene expression by providing enough time to modify the host cell machinery contrary to viruses in lytic cycle[21].

In this study, the expression pattern of EBV miRNAs during an initial step of EBV infection was investigated. Mir-BHRF1-1, mir-BHRF1-2, and mir-BHRF1-3 were detected from 6 h after infection in both mature forms, and their fold-back precursor

forms, while mir-BART1 and mir-BART2 expression were not detected until 96 h after infection. These data indicates that mir- BHRF1s are expressed in early infection step, within 6 h after infection, as shown in Northern blot analysis, and with EBNA2 and EBNA-LP, which were also detected within 6 h after infection, coordinately expressed. During early infection, mir-BHRF1s were predicted to regulate the expression of viral and cellular genes.

Mir-BHRF-1s expression is observed in different latency types, B95.8, Rael, Mutu III, IM9wt and IM9mt, and mir-BARTs expression is represented in only several cell limes, such as B95.8 and Rael. During latent infection and lytic cycle induction, EBV miRNAs were expressed at latent infection and EBV lytic cycle induction was shown to have no effect on miRNA expression in B95.8 cells.

Recently Hatfield et al. reported that stem cell division is regulated by the miRNA pathway in the fruit fly *Drosophila melanogaster*[35]. In this study, they revealed that miRNA pathway modulates cell cycle of the germline stem cell (GSC)s by affecting the G1/S transition through repressing the cyclin-dependent kinase (CDK) inhibitor Dacapo (Dap; a homologue of the p21/p27 family of CDK inhibitors) using GSCs carrying a mutation in *dicer-1*, a gene essential for miRNA biogenesis. GSCs mutant for dicer-1 was delayed in the G1 to S transition, which is dependent on Dap. These data show that miRNAs are required for stem cells to bypass the normal G1/S checkpoint. The discovery that the mechanism which makes stem cells insensitive to environmental signals that normally stop the cell cycle was medaited by miRNAs

suggests the possibility that this mechanism is also applied to cancer cells.

$p21^{WAF1}$ has been reported to bind and inhibit proliferating cell nuclear antigen, resulting in blocking DNA replication in addition to inhibiting cyclin-CDK complexes[36,37]. Towards $p21^{WAF1}$ expression, there has been a previous data that showed EBNA2 induces $p21^{WAF1}$ expression[31]. EBNA2 has been known to be required for B cell immortalization by EBV infection. The transactivation function of EBNA2 is essential for promotion of cell-cycle progression in EBV-immortalized B-lymphocytes since EBNA2 activates transcription of other EBV genes such as LMP1, EBNA3A, EBNA3C and EBNA-LP, which are also essential to the B-cell transformation[38]. However, it had been reported that EBNA2 alone, in the absence of other EBV genes, can retard cell growth. Through the investigation of the mechnism underlying cell growth retardation induced by EBNA2, it was revealed that EBNA2 can induce $p21^{WAF1}$ expression, which was mediated through p53[31]. In this study, stable or transient transfection of EBNA2 results in enhanced $p21^{WAF1}$ expression at both mRNA and protein level, implicating that EBNA2 induces cell growth retardation through $p21^{WAF1}$ induction but there was no data showing increased $p21^{WAF1}$ protein expression in EBV-infected cells. Other studies also revealed that the DNA damaging agent only increases $p21^{WAF1}$ mRNA level while $p21^{WAF1}$ protein level was shown to fail to accumulate in EBV-infected B cells and all LCLs analyzed[32].

This discrepancy has not resolved until now, but we found out the role of miRNAs involved in regulation of the cell cycle checkpoint. The possibility that EBV-encoded

miRNAs regulate the expresssion of $p21^{WAF1}$ at the posttranscriptional level was considered and we had searched whether $p21^{WAF1}$ has potential binding sites for EBV miRNAs using Microinspector, a web tool for analysis of a user-defined RNA sequence for the occurrence of binding sites for known and registered miRNAs, and finally found that mir-BHRF1-3 has a complementary binding site to 3'UTR of $p21^{WAF1}$.

To determine the function of mir-BHRF1-3 on predicted target $p21^{WAF1}$, $p21^{WAF1}$ expression in EBV-positive cells which express mir-BHRF1-3 was investigated and the effect of mir-BHRF1-3 on cell cycle progression and apoptosis was analyzed in EBV-infected B cells. In EBV-infected cells, inhibition of mir-BHRF1-3 resulted in enhanced apoptotsis induction, suggesting that the function of mir-BHRF1-3 was related to cell survival maintenance in EBV-infected cells. Analysis of $p21^{WAF1}$ expression level induced by the DNA damaging agent showed that in LCL-1 cells, with a significant level of mir-BHRF1-3 expression, $p21^{WAF1}$ did not accumulate in response to cisplatin but $p21^{WAF1}$ accumulated after exposure to cisplatin in IB4 cells, representing a low level of BHRF1-3 expression whereas $p21^{WAF1}$ mRNA level was increased by cisplatin treatment in both cells and these results implicate that mir-BHRF1-3 inhibits $p21^{WAF1}$ expression at the post-transcriptional level. The function of mir-BHRF1-3 in downregulating 21^{WAF1} expression can enable for EBV-infected cells to overcome G1/S checkpoint and resulting abnormal cell cycle control contributes to maintenance of EBV latent

infection and EBV-induced tumorigenesis, supporting that miRNA pathway involved in tumorigenesis by inducing uncontrolled, abnormal growth, a characteristic of cancer cells, through cell cycle regulation.

Mir-BHRF1s were expressed in different latency types while mir-BARTs were expressed in only several cell lines. Mir-BHRF1s were expressed in initial infection phase and suggested to coordinately expressed with EBNA2, regulating viral and cellular genes and especially, mir-BHRF1-3 induced cell survival maintenance. Mir-BHRF1-3 is regarded as contributing on latent infection maintenance and EBV-associated tumorigenesis.

V. Conclusion

In this study, to investigate the expression pattern of EBV miRNAs in early infection of B cells, Akata, a EBV-negative BL cell line, was infected with B95.8-derived EBV and the expression of EBV miRNAs was analyzed at different time points after EBV infection. Through computational target prediction, we found out mir-BHRF1-3 has a complementary binding site to 3'UTR of $p21^{WAF1}$. To determine whether mir-BHRF1-3 inhibits the expression of $p21^{WAF1}$ at the post-transcriptional level, we investigated that inhibition of mir-BHRF1-3 has any effect on cell cycle progression and apoptosis in EBV-infected cells and analyzed the expression of $p21^{WAF1}$ induced by a DNA damaging agent in EBV-positive cell lines representing different expression level of mir-BHRF1-3.

1. Mir-BHRF1s were detected in different latency types, B95.8, Rael, Mutu III, IM9wt and IM9mt, and mir-BARTs were detected in B95.8 and Rael cells. EBV miRNA expression showed no difference between latent infected cells and lytic cycle-induced B95.8 cells.

2. Mir-BHRF1-1, mir-BHRF1-2, and mir-BHRF1-3 were detected from 6 h after infection and the expression of mir-BART1 and mir-BART2 was not detected until 96 h after infection.

3. Using Microinspector, $p21^{WAF1}$ was predicted as a target of mir-BHRF1-3.

4. Inhibition of mir-BHRF1-3 induced apoptosis of EBV-infected Akata cells.

5. $p21^{WAF1}$ protein induced by cisplatin did not accumulate in LCL-1 cells with high level of BHRF1-3 expression, whereas accumulated in IB4 cells, representing low level of BHRF1-3 expression. In both cells, $p21^{WAF1}$ mRNA expression was increased in response to cisplatin.

These data indicates that mir-BHRF-1 was expressed in an initial infection phase and thus mir-BHRF-1 is proposed to coordinately express with EBNA2 and EBNA-LP, which are expressed during early infection, and regulate the expression of viral and/or cellular genes. In EBV-infected cells, inhibition of $p21^{WAF1}$, predicted as a target of mir-BHRF1-3 through computational prediction, resulted in enhanced apoptosis. DNA damaging agent only induces increased $p21^{WAF1}$ mRNA expression, not protein expression. These results implicates that mir-BHRF1-3 functions in inhibition of $p21^{WAF1}$ expression at the post-transcriptional level and therefore blocking apoptosis and inducing cell survival maintenance.

This study indicates that EBV-encoded mir-BHRF1-3 can play a role in EBV-induced B cell immortalization through inhibition of $p21^{WAF1}$ as a possibility that virus-encoded miRNAs can regulate cell cycle progression through inhibition of gene

expression playing a critical role in the cell cycle checkpoint to bypass cell cycle arrest signal and contribute to continuous cell cycle progression and abnormal growth inducing successful viral infection and virus-associated tumorigenesis.

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국 문 요 약

Epstein-Barr virus 에 의한 B 세포 초기감염 과정에서 viral microRNA 의 발현

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이 정 화

MicroRNA (miRNA)는 약 22 뉴클레오타이드로 이루어진 작은 비번역 RNA 로 전사후 단계에서 진핵세포의 유전자 발현을 조절한다. MiRNA 는 표적 mRNA 의 3'-UTR 에 염기서열 특이적으로 결합하여 표적 mRNA 의 분해나 번역 억제를 유도하며, 세포의 증식이나 분화, 세포사멸, 발달과정에서 중요한 역할을 하는 유전자의 발현을 조절한다고 알려져 있다.

최근에 바이러스가 miRNA 를 발현하고 있음이 밝혀졌다. MiRNA 를 발현하는 바이러스로 최초로 보고된 Epstein-Barr virus (EBV)는 BHRF1 mRNA 와 BART 인트론에 존재하는 5 개의 miRNA 를 발현하며 세포 증식과 사멸의 조절자, B 세포 특이적인 케모카인과 사이토카인,

전사 조절자, 신호 전달 경로에서 역할을 하는 물질이 바이러스 miRNA의 표적으로 예측되었다.

바이러스 miRNA의 발견은 바이러스가 숙주와 바이러스의 유전자 조절과 바이러스에 대한 숙주의 방어 기작 조절에서 RNA 침묵 기작을 이용함을 나타낸다. EBV miRNA는 바이러스 복제, 잠복감염, B 세포 형질전환과 종양 형성에 관여하여 중요한 역할을 할 것으로 예상된다.

본 연구에서는 EBV의 B 세포 초기 감염 과정에서 EBV miRNA의 발현을 조사하기 위하여 EBV-negative Burkitt's lymphoma (BL) 세포주인 Akata 세포주에 EBV를 감염시키고 시간별로 EBV miRNA의 발현을 조사하였다. Microinspector를 통해 mir-BHRF1-3의 표적으로 예측된 $p21^{waf1}$ 의 발현을 mir-BHRF1-3을 발현하는 EBV-positive 세포에서 조사하였고, EBV 감염 세포에서 mir-BHRF1-3가 세포주기 진행과 세포사멸에 미치는 영향을 알아보기 위해 mir-BHRF1-3을 발현을 2'-OMe-mir-BHRF1-3로 저해 후 유도되는 세포사멸의 변화를 살펴보았다.

mir-BHRF1의 발현이 감염 6시간 후부터 관찰되는 것을 Northern blot을 통해 확인하였다. 즉, mir-BHRF1은 EBV의 B 세포 감염 초기 과정에서 발현되어 EBNA2와 EBNA-LP와 같이 초기 감염 단계에서 바이러스와 숙주 세포의 유전자 발현을 조절하는 기능을 할 것으로 예측되었다.

mir-BHRF1-3의 발현 저해 결과 EBV 감염 B 세포에서 세포사멸의

증가가 유도었으며 mir-BHRF1-3을 발현하는 세포에서 DNA 손상 물질인 cisplatin에 의해 유도되는 $p21^{WAF1}$의 발현을 조사한 결과 높은 수준으로 mir-BHRF1-3을 발현하는 LCL-1 세포에서는 $p21^{WAF1}$ 단백질의 발현이 cisplatin에 의해 유도되지 않았으나, mir-BHRF1-3의 발현량이 낮은 IB4 세포의 경우에는 $p21^{WAF1}$ 단백질의 발현이 증가하는 것이 관찰되었다. 반면, $p21^{WAF1}$ mRNA의 발현은 LCL-1과 IB4 세포에서 모두 cisplatin에 의해 증가된 것으로 나타났다.

이상의 결과는 EBV 감염초기에 발현되는 mir-BHRF1-3가 전사후 단계에서 $p21^{WAF1}$ 발현의 조절을 통해 EBV 감염 세포의 세포사멸 유도를 막고 세포주기의 계속적인 진행을 유도함으로써 바이러스가 효과적인 감염을 이룩하는데 작용하고 있다는 것을 시사한다.

핵심 되는 말: microRNA, Epstein-Barr virus, mir-BHRF1-3, $p21^{waf1}$