The Study of Epstein-Barr Virus Encoded microRNAs in Nasopharyngeal Carcinoma Cells

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ABSTRACT

The study of Novel Epstein-Barr Virus Encoded Micro-RNAs in Nasopharyngeal Carcinoma Cells

Submitted by: LUNG, Raymond Wai Ming for the degree of Doctor of Philosophy in Anatomical and Cellular Pathology at The Chinese University of Hong Kong

Infection with the Epstein-Barr virus (EBV) is a strong predisposing factor in the development of nasopharyngeal carcinoma (NPC). Many viral gene products including EBNA1, LMP1 and LMP2 have been implicated in NPC tumorigenesis, although the *de novo* control of these viral oncoproteins remain largely unclear.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs with a size around 18-24 nucleotides with significant roles in regulating gene expression by either transcriptional silencing or translational suppression. As gene regulators, recent miRNA studies have emphasized the contribution of aberrant miRNA expression in cancer development. The recent discovery of EBV encoded viral miRNAs (ebv-miRNAs) in lymphoid malignancies has prompted us to examine the NPC-associated EBV miRNAs. In this study, we have systematically examined the NPC associated EBV genome for viral-encoded miRNA expression. By constructing small cDNA libraries from a native EBV positive NPC cell line (C666-1) and a xenograft (X2117), we screened about 3000 clones and detected several small EBV fragments, within which two novel ebv-miRNAs in the BARTs region were identified. These two newly identified miRNAs, now named miR-BART21 and miR-BART22, were proven to be abundantly expressed in most NPC samples by both Northern blot and QRT-PCR analysis.

Based on matching analysis between different EBV strains, we found two nucleotide variations in miR-BART21 and four nucleotide changes in miR-BART22. Interestingly, two nucleotide variations upstream of mature

miR-BART22 likely favor its biogenesis by Drosha/DGCR8 processing and we experimentally confirmed this augmentation by *in-vitro* Drosha digestion, and thus may underline the high and consistent expression of miR-BART22 in NPC tumors.

We attempted to predict the potential viral and cellular targets of miR-BART21 and miR-BART22 by public available computer programs, miRanda and RNAhybrid. A number of potential cellular mRNA targets were suggested, although many failed to be validated by luciferase reporter assay. However, we found a putative miR-BART22 binding site in the LMP2A-3'UTR. Although the LMP-2A transcript is consistently detected in NPC, only 6 out of 26 (23%) primary NPC tumors show weak LMP-2A expression by immunohistochemistry (IHC). The expression levels of miR-BART22 and LMP-2A mRNA have also been determined in eleven of these tumors. Interestingly, the LMP-2A mRNA expression level did not directly correlate with protein expression, and relatively low expression levels of miR-BART22 miRNA were observed in all 3 LMP-2A positive-primary tumors. The suppressive effect of miR-BART22 on LMP-2A was also experimentally validated by a series of dual luciferase reporter assays using reporter constructs containing the putative or mutated recognition site at the LMP-2A 3'UTR. By co-transfection of different amounts of miR-BART22 with the LMP-2A-3'UTR expression vector in reporter assay, we confirmed that miR-BART22 suppressed the LMP-2A protein level in a dose-dependent manner. Furthermore, transfection of miR-BART22 into HEK293 cells that had been stably transfected with pcDNA3.1-LMP-2A, which contains a complete LMP-2A *ORF* and 3'UTR, readily suppressed levels of the LMP-2A protein.

Taken together, this thesis shows that two newly identified EBV-encoded miRNAs are highly expressed in latent EBV infection in NPC. Frequent expression of miR-BART22 can be explained partially by a specific EBV strain that is associated with NPC in our locality. Our findings emphasize the role of miR-BART22 in modulating LMP-2A expression. Because LMP-2A is a potent immunogenic viral antigen that is recognized by the cytotoxic T cells (CTLs), down-modulation of LMP-2A expression by mir-BART22 may permit escape of EBV-infected cells from host immune surveillance.

摘要

艾巴氏病毒(Epstein-Barr virus, EBV)感染與鼻咽癌(Nasopharyngeal carcinoma, NPC)病變是互相關聯的。 雖然目前仍不清楚病毒潛伏感染怎樣影響上皮細胞的變異，但是許多病毒蛋白已證實對腫瘤的發展有極大關連，其中包括 EB 核抗原(EBNA1)與病毒潛伏膜蛋白(LMP-1 and LMP-2)。

MicroRNAs(miRNAs, 譯爲微 RNA)是一類長度大約 18-24 個核苷酸的細小、而非編碼的核糖核酸(RNA)分子，它主要透過分解靶基因(mRNA)或抑制其翻譯，從而調節轉錄後基因的蛋白表達。 研究表明，miRNAs 的異常表達往往與癌症發展是密切相關。而最近科學家在淋巴系統惡性腫瘤中發現 EBV 亦會製造 miRNAs(ebv-miRNAs)。 這個發現促使我們對審查與鼻咽癌相關的 ebv-miRNAs 表達的興趣。 通過 EBV 陽性的鼻咽癌細胞株(C666-1)和異種皮移植(X2117)抽取出來的互補去氧核糖核酸(cDNA)，我們製造了兩個小 cDNA 文庫 (cDNA libraries)，在大約 3000 個克隆的分析中，我們發現了幾個 EBV 的小片段，其中包括兩個現已命名爲 miR-BART21 和 miR-BART22 的新 ebv-miRNAs。 運用印跡雜交(Northern Blot)和定量實時 PCR 技術(QRT-PCR)分析, 證明了這兩個新的 miRNAs 會在鼻咽癌樣本中最充分表達。

基於不同病毒株的匹配分析，我們分別在 BART21 和 BART22 附近的序列中發現序列了兩個和四個核苷酸變異。 經過仔細的計算機程序分析後，我們懷疑當中兩個在 BART22 序列附近的核苷酸變異，極有可能會影響其成熟進程，我們亦運用了體外消化實驗去證實了這一點。 這亦可能是解釋爲何 miR-BART22 在鼻咽癌腫瘤一貫高表達的其中一個原因。

我們試圖運用流行的計算機程序(miRanda 和 RNAhybrid)來預測 miR-BART21 和 miR-BART22 的潛在目標。 結果得到一些潛在的細胞靶基因目標建議，可惜許多建議目標都不能夠通過熒光素酶報告分析的驗證。 然而，我們發現在 LMP-2A 靶基因的 3'端未轉譯區 (3'UTR)有一個 miR-BART22 的假定結合位置。 許多文獻報告都認爲 LMP-2A 靶基因在差不多每個鼻咽癌樣本中都能表達出來，但根據我們對二十六個鼻咽癌樣本作出的免疫組織化學分析結果,當中只有六個 (23%)腫瘤樣本能查看出微弱的 LMP-2A 蛋白表達。 在其中的十一個腫瘤樣本的詳細分析中，我們發覺 LMP-2A 靶基因和蛋白的表達並沒有直接關聯，而當中只有三個 miR-BART22 表達相對較低的樣本中才能偵查出 LMP-2A 蛋白的表達。 運用雙熒光素酶報告分析實驗，我們成功地驗證 miR-BART22 可以透過在 LMP-2A 靶基因 3' 端未轉譯區的假定結合位置來抑制其目標蛋白的翻譯。 我們亦證實 miR-BART22 是劑量依賴性地抑制 LMP-2A 蛋白表達。 另一方面， 在已穩定轉染完整的 LMP-2A 開放閱讀框(Open reading frame)及 3'端未轉譯區的 HEK293 細胞株中， 短暫性轉染 miR-BART22 可以有效率地壓抑 LMP-2A 蛋白翻譯。

概括而論，我們在這論文報告新發現了兩個在鼻咽癌樣本中高表達的 ebv-miRNAs。 而本土化獨特的 EB 病毒株可能是導致 miR-BART22 高表達的其中一個原因。 我們的研究結果強調 miR-BART22 對調節 LMP-2A 表達的作用。 由於 LMP-2A 是一種容易被毒殺性 T 淋巴球(CTLs)識別的強力免疫病毒抗原，EB 病毒感染的細胞可能會利用 miR-BART22 去低調製 LMP-2A 的蛋白表達,從而逃被細胞的免疫監視。

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LIST OF PUBLICATIONS

Publications arising from thesis

1. **Lung RW,** Tong JH, SungYM, Leung PS, Ng DC, Chau SL, Chan AW, Ng EK, Lo KW, To KF. Modulation of LMP2A expression by a Newly Identified EBV-encoded microRNA miR-BART22. ***Neoplasia,*** **2009** Nov; 11(11): 1174-84.
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1. Choy EY, Siu KL, Kok KH, **Lung RW**, Tsang CM, To KF, Kwong DL, Tsao SW, Jin DY. An Epstein-Barr Virus-encoded microRNA targets PUMA to promote host cell survival, ***J. Exp. Med.*** **2008** Oct27; 205(11):2551-60.
2. Wong QW, **Lung RW**, Law PT, Lai PB, Chan KY, To KF, Wong N. MicroRNA-223 is Commonly Repressed in Hepatocellular Carcinoma and Potentiates Expression of Stathmin1. ***Gastroenterology.*** **2008** Jul;135(1):257-69.

Selected Abstracts in scientific meetings

(1) **Lung RW**, Tong JH, Sung YM, Lo KW, To KF. Identification of Epstein-Barr Virus microRNA in Nasopharyngeal Carcinoma. **100th AACR Annual Meeting 2009.**

(2) Tong JH, **Lung RW**, Sung YM, Leung PS, Chan AW, Lo KW, To KF. Differential expression of EBV microRNAs in Nasopharyngeal Carcinoma cells. **MicroRNA and Cancer Keystone Symposia 2007.**

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ABBREVIATIONS

Ago	Argonaute
BART	*Bam*H1-A region rightward transcript
BIC	*B-cell Integration Cluste*
BL	Burkitt's lymphoma
bp	Basepair
C.elegans	*Caenorhabditis elegans*
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
Ci	Curie
CLL	Chronic Lymphocytic Leukemia
DAPI	4'6-diamidino-2-phenylindole
DGCR8	DiGeorge syndrome critical region gene-8
DGCR8	DiGeorge syndrome Critical Region gene-8
DMSO	Dimethyl Sulfoxide
DNA	Deoxy-ribonucleic acid
dNTP	Deoxribonucleoside triphosphate
E.coli	Escherichia coli
EBV	Epstein-Barr Virus
ebv-miRNA	EBV encoded microRNAs
eIF	eukaryotic Initiation Factor
FBS	Fetal bovine serum
GEM	Gemcitabine
HD	Hodgkin's Disease
HLA	Human leukocyte antigen
Hr	Hour
IHC	Immunohistochemical staining
IM	Infectious mononucleosis
IP	Immunoprecipitation
ISH	in situ hybridization
Kb	Kilobase
KDa	Kilo dalton
LCLs	Lymphoblastoid cell lines
LMP-2A	Latent membrane protein 2A
m^7G	7-methyl-guanosine
MCS	Multi cloning site
mg	Milligram
MICB	*MHC class I polypeptide-related sequence B*
min	minute
miR, miRNA	microRNA
miRISC	miRNA-containing RNA-induced silencing complex

miRNAs	MicroRNAs
ml	Milliliter
mM	Millimolar
MTT	3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
NaCl	Sodium Chloride
NPC	Nasopharyngeal carcinoma
nt	Nucleotide
℃	Degree Celsius
ORFs	Open reading frames
PAGE	Polyacrylamide Gel Electrophoresis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PK	Proteinase K
QRT-PCR	Quantatitive reverse transcription polymerase chain reaction
RISC	RNA induced silencing complex
RNA	Ribosonucleic acid
rpm	Rotation per minute
rRNA	ribosomal RNA
RT	Reverse Transcription
SDS	Sodium Dodecylsulfate
SDS	Sodium dodecyl sulfate
sec	Second
siRNA	Small interfering RNA
SV40	Simian virus 40
TAg	T Antigen
tRNA	Transfer RNA
TSA	Trichostatin A
U	Unit activity
UTR	Untranslated Region
wt	Wild type
ng	Nanogram
μg	Microgram
μl	Microliter

CHAPTER 1: INTRODUCTION

1.1 Epstein-Barr Virus (EBV)

1.1.1 Discovery of EBV

In the 1950s, Dr. Denis Burkitt first described a new lymphoma affected children in equatorial Africa. This lymphoma was named as Burkitt's lymphoma (BL). Due to the high incidence rate of yellow fever in the same region, Burkitt originally postulated that BL might also be induced by infection of insect vectored virus similar to that of yellow fever (Burkitt, 1958, 1962; Haddow, 1964). Through further investigation on the unusual epidemiology of BL, Tony Epstein and his colleagues finally identified human herpes-like viral particles in the endemic BL-derived cell lines under the electron microscope examination. They named this virus Epstein-Barr Virus (EBV) (Epstein *et al.*, 1964).

Subsequent work on EBV found out that healthy adults were also positive in serological tests against EBV (Henle & Henle, 1966), however the antibody titers to EBV antigen in BL patients were much higher than the control groups (Henle & Henle, 1966; Klein *et al.*, 1968). Further studies of EBV recovered from BL patients had proven that the virus had the ability to transform peripheral leukocytes *in vitro* into EBV latently infected B-lymphoblastoid cell lines (LCLs) (Henle *et al.*, 1967). Furthermore, the detection of a small amount of EBV DNA in a non-virus producing line "virus-free" from BL (Raji) indicated that EBV is the first virus identified to take an important role in the induction of human malignancies (Zur Hausen & Schulte-Holthausen, 1970).

1.1.2 Molecular Biology of EBV

EBV, also known as human herpesvirus 4 (HHV4), shares a similar structure with other herpesviruses, in which the viral genome is wrapped with a toroid-shaped protein core. The EBV genome is linear and double stranded with a size about 184kb (Pritchett *et al.*, 1975; Raab-Traub *et al.*, 1980). The genome contains around 80 *ORFs* but only 70 *ORF*s are expressed during virus replication. There are 0.5kb internal tandem repeats (TR) of the same sequence at both termini and 6 to 12 internal repeats (IR1) which divide the genome into short and long unique sequence domains (U_S and U_L) (Given & Kieff, 1979; Given *et al.*, 1979; Cheung & Kieff, 1981, 1982; Hayward *et al.*, 1982). EBV was the first herpesviruses to be completely cloned and sequenced in 1984. Since the sequence was obtained from a *Bam*HI fragment-cloned library, the promoters and open reading frames (ORFs) are all referred to the specific *Bam*HI fragments, from A to Z, in descending order (Baer *et al.*, 1984; Polack *et al.*, 1984).

1.1.3 Lytic and latent infection of EBV

EBV preferentially infects B-cells by binding its envelop glycoproteins, gp350 and gp42, to the CD21 receptor and human leukocyte antigen (HLA) class II molecules respectively (Nemerow *et al.*, 1987; Borza & Hutt-Fletcher, 2002). After penetration, the viral genome is transported to the nucleus, in which it immediately circularizes and initiates viral gene transcription. The infected B-cells, which prohibit virus replication would subsequently become a latently infected proliferating lymphoblasts, and eventually develop into transformed LCL.

Although it has been difficult to demonstrate direct EBV lytic infection in *in vitro* systems, virus-infected B-cell do elicit a potent T-cell response against viral antigens. In young adult, primary EBV infection often elicits infectious mononucleosis (IM) in which the serum from the patient became strongly positive to EBV antibodies during the emergence of IM symptoms (Henle *et al.*, 1968; Niederman *et al.*, 1968). Interestingly, the virus will not be completely eliminated and will ultimately establish persistence within memory B cells permanently (Niederman *et al.*, 1970).

The lytic EBV infection process can be studied in latently EBV infected cell lines using several chemical inducers (Luka *et al.*, 1979; Hudewentz *et al.*, 1980; Ben-Sasson & Klein, 1981). EBV replication is initiated in the nucleus with the expression of two immediate early lytic genes, *BZLF1* and *BRLF1*. BZLF1 and BRLF1 proteins are transcription factors that alter host cellular responses and facilitate the expression of other early lytic genes for replication such as BHRF1, BSMLF1 and BMRF1 for replication (Chevallier-Greco *et al.*, 1986; Cox *et al.*, 1990; Holley-Guthrie *et al.*, 1990; Quinlivan *et al.*, 1990). The activation of these early genes is followed by viral genome replication and late protein expression for virus packaging. The free virions will finally be released from the cells.

1.1.4 EBV Latent Genes Expressions

Latent EBV infection, which is common among the majority of adults, is thought to be a contributor to EBV-related malignancies. There are only 12 viral genes selectively expressed during latent infection. Based on their expression pattern, four different types of EBV latency infection program have

been classified, denoted as latency 0, I, II, III. In latency III, all 12 viral genes are expressed, including six EBV nuclear antigens (*EBNA-1,2,3A,3B,3C* and *LP*), three latent membrane proteins (*LMP-1*, *LMP-2A* and *LMP-2B*), transcripts from *Bam*HIA region (*BARTs*) and two small EBV-encoded RNAs (*EBER1* and *EBER2*) (Gregory *et al.*, 1990; Rowe *et al.*, 1992). Due to the high expression of all latent genes, latency III cells will stimulate the host immunosuppressive effect, leading to the onset of several illnesses such as post-transplant lymphoproliferative disease (PTLD) and infectious mononucleosis etc (Timms *et al.*, 2003). EBV gene expression in latency II is limited to *EBNA-1*, *LMPs*, *EBERs* and *BARTs*. This type of latency can be found in tissue from NPC, gastric carcinoma, T-cell lymphoma and Hodgkin's disease (Herbst *et al.*, 1991; Brooks *et al.*, 1992; Imai *et al.*, 1994). EBNA-1 is the only viral protein expressed in latency I, which has the phenotype found in BL (Rowe *et al.*, 1987). In a healthy carrier, EBV remains silent in memory B-cells which circulate in the peripheral blood (latency 0), EBNA-1 is the only viral protein expressed during cell division (Hochberg *et al.*, 2004), albeit the *LMP-2A* transcript is also detected at very low level (Babcock *et al.*, 1998) EBERs and BARTs are expressed in all latency types (Brooks *et al.*, 1993; Chen *et al.*, 1999b). The EBV latency pattern and their associated malignancies are summarized in Table 1.1

Table 1.1 Pattern of viral gene expression and their associated malignancies in different forms of EBV latency

Latency	*EBNA-1*	*EBNA2,3,LP*	*LMP-1*	*LMP2A/B*	*EBERs*	*BARTs*		Diseases
0	+/-[1]	-	-	-[2]	+	+	➢	**Healthy carriers**
I	+	-	-/+[3]	-/+[3]	+	+	➢	**Burkitt's lymphoma (BL);**
							➢	**Gastric carcinoma (GC)[4]**
II	+	-	+	+	+	+	➢	**Hodgkin's disease (HD);**
							➢	**Nasopharyngeal carcinoma (NPC);**
							➢	**T/NK lymphoma; T-cell lymphoma;**
							➢	**Primary effusion lymphoma (PEL)**
III	+	+	+	+	+	+	➢	**Infectious mononucleosis (IM);**
							➢	**AIDS-associated lymphoma;**
							➢	**Post-transplant lymphoproliferative disease (PTLD)**

[1] *EBNA-1* is only be expressed during cell replication

[2] *LMP2A* is expressed in some reports (Cohen, 2005)

[3] *LMP1* and *LMP2* are expessed in some reports (Cohen, 2005)

[4] *LMP2A* is expressed in a subset of EBV-positive gastric carcinoma (Takada, 2000; Luo *et al.*, 2005)

Abbreviations: EBNA, Epstein-Barr virus nuclear antigen; EBNA-LP, Epstein-Barr virus nuclear antigen leader protein; LMP, Latent membrane protein; EBERs, Epstein-Barr virus encoded RNAs; BARTs, *Bam*H1-A region rightward transcripts

1.1.4.1 EBV Nuclear Antigens (EBNAs)

EBNA-1 is the only viral protein which is expressed in all EBV infected cells. It can be transcribed from different promoters according to the EBV stages (Deacon *et al.*, 1993). The Qp promoter, located in the *Bam*HI Q fragment, is used in infected cells with latency I and II. In latency III, EBNA-1 is transcribed from the Cp and Wp promoters. The Fq promoter is activated to transcribe EBNA-1 during lytic infection. EBNA-1 protein can bind to the sequence-specific repeat elements on the plasmid origin of viral replication, oriP in order to maintain the circular episomal genome of EBV (Yates *et al.*, 1984). In addition, EBNA-1 can transactivate other *EBNAs* and *LMP-1* expression from episomal DNA, probably through viral Cp and Wp promoters (Nonkwelo *et al.*, 1996; Kang *et al.*, 2001).

The presence of an internal glycine-alanine repeat domain in EBNA-1 protein resists ubiquitin-proteosomal degradation, thus preventing it from being cleaved into small viral piptides for presentation through MHC class I pathway, which is a class I restricted cytotoxic T-Cells (CTLs) response (Levitskaya *et al.*, 1997). The functions of other EBNA proteins are showed in Table 1.2.

Table 1.2 Functions of EBNAs proteins

Protein	Required for transformation	Functions
EBNA-1	**Yes**	➢ **Episomal maintenance;** ➢ **Up-regulates viral genes**
EBNA-2	**Yes**	➢ **Up-regulates viral and cellular genes**
EBNA-3	**-A, -C: Yes** **-B: No**	➢ **Inhibits EBNA-2 activity;** ➢ **Up-regulates cellular genes**
EBNA-LP	**Probably No**	➢ **Augments EBNA-2 activity**

Abbreviations: EBNA, Epstein-Barr virus nuclear antigen;
EBNA-LP, Epstein-Barr virus nuclear antigen leader protein.

Table adapted from Cohen JI, 2006 (Cohen, 2006)

1.1.4.2 Latent Membrane Proteins (LMPs)

1.1.4.2.1 LMP-1

LMP-1 is the known EBV oncoprotein. This is also the first EBV protein demonstrated to have transforming ability in rodent fibroblasts (Wang *et al.*, 1985). Furthermore, expression of LMP-1 is essential for B-cell transformation in both cell lines studies and in transgenic mice experiments (Kaye *et al.*, 1993; Kulwichit *et al.*, 1998).

LMP-1 is a functional homologue of the constitutively active form of CD40, a member of tumor necrosis factor receptor (TNFR) family (Uchida *et al.*, 1999). There are 2 functional domains on the C-terminal of LMP-1, namely the C-terminal activation regions 1 and 2 (CTAR1 and 2). TNF-associated factors (TRAFs) can interact either directly CTAR1 or indirectly with CTAR2 via the TNF receptor-associated death domain (TRADD) for activation (Huen *et al.*, 1995; Eliopoulos & Young, 2001). They can promote cell growth and differentiation responses by initiating the activities of several signaling molecules including phosphatidylinositol 3-kinase (PI3K), Janus-activated kinase 3 (JAK-3), mitogen-activated protein kinase kinase kinases (MAPKKKs) and nuclear factor kappa B (NF-κB) (Devergne *et al.*, 1996; Eliopoulos & Young, 2001).

LMP-1 is also involved in other signaling pathways for cancer development. For example, it can promote apoptotic resistance by enhancing A20 and Bcl2 expression (Fries *et al.*, 1996; Wang *et al.*, 1996). Furthermore, it can contribute to both growth stimulation and immune system suppression by activating cellular gene expression, such as of intracellular adhesion molecule (ICAM-I), lymphocyte function associated antigen-1 (LFA-1), HLA class I, II

and TAP (Zhang *et al.*, 1994; Rowe *et al.*, 1995; Devergne *et al.*, 1998).

Recent findings revealed that expression of LMP-1 in B-cells can stimulate the expression of two oncogenic host miRNAs, miR-146a and miR-155 (Mrazek *et al.*, 2007). Due to the presence of two NF-κB response elements in their promoter regions, LMP-1 may mediate up-regulation of these two miRNAs via NF-κB signaling pathways (Motsch *et al.*, 2007; Cameron *et al.*, 2008; Gatto *et al.*, 2008). Interestingly, expression of these two miRNAs can contribute to the EBV latency maintenance. For examples, miR-155 can reduce both EBV copy number and EBNA1 expression in latently-infected cells (Lu *et al.*, 2008a), and miR-146a can inhibit the expression of some EBV lytic genes such as *BZLF1* (Godshalk *et al.*, 2008). Consistent with the findings in papillary gland carcinoma (He *et al.*, 2005a) and cervical cancer (Wang *et al.*, 2008b), our QRT-PCR results in the biopsy study also demonstrate the up-regulation of miR-146a in nasopharyngeal carcinoma (NPC), one of the EBV-associated diseases (unpublished observations). Another research finding in diffuse large B-cell lymphoma and BL cells also reported that LMP1 can down-regulate a major oncogene, TCL1 expression by up-regulating miR-29b expression (Anastasiadou *et al.*, 2009). Together with our previous finding that ebv-miRNAs, such as miR-BART1, 16 and 17, can also downmodulate LMP-1 protein expression (Lo *et al.*, 2007). Thus, LMP-1, the main viral oncoprotein, is a key molecule taken an integral part in the complicated miRNA signaling network for cancer development.

1.1.4.2.2 LMP-2s

The viral *LMP-2* gene encodes two integral membrane proteins, *LMP-2A*

and *LMP-2B*. The LMP-2A promoter is located upstream of the *LMP-2* gene while *LMP-2B* is expressed from the same bi-directional promoter as *LMP-1* (Laux *et al.*, 1989). Being transcribed from a different promoter, LMP-2B represents an N-terminal truncated form of LMP-2A, in which only 119 amino acids in N-terminal cytoplasmic domains of LMP-2A is missing (Sample *et al.*, 1989). Due to the lack of a functional amino terminal domain found in LMP-2A, LMP-2B can associate with LMP-2A and negatively modulate LMP-2A activity in B-cells (Rovedo & Longnecker, 2007).

Although *LMP-2s* are not essential for B-cell transformation *in vitro* (Longnecker, 2000), experiments in transgenic mice show that expression of LMP-2A in B-cell receptor (BCR) deficient B-cells can rescue them from apoptosis (Caldwell *et al.*, 1998). LMP-2A replenishes BCR function with the BCR-like amino terminal domain, immunoreceptor tyrosine-based activation motif (ITAM), which mimics receptor function by constitutively activating the Syk tyrosine kinases (Fruehling & Longnecker, 1997). However, the Src family kinase, LYN protein, also binds to the Y112 motif on the LMP-2A active terminus. This interaction sequesters LYN kinase from BCR and eventually inhibits BCR signaling pathways (Miller *et al.*, 1994; Dykstra *et al.*, 2001). The amino-terminal of LMP-2A also contains two PYmotifs (PPXY motifs) which are associated with Nedd4 ubiquitin proteins to down-regulate both LMP-2A and LMP-2A-induced phosphorylated tyrosine kinases (PTKs) activities (Ikeda *et al.*, 2001). Consequently, the normal BCR signaling pathway is blocked and the AKT induced transcription of the viral early lytic gene, *BRLF1*, is inhibited, resulting in maintenance of viral latency (Miller *et al.*, 1995; Mori & Sairenji, 2006). LMP-2A also contributes to EBV-associated epithelial malignancies.

It can affect cellular growth properties, transform epithelial cell lines and inhibit differentiation (Scholle *et al.*, 2000; Morrison *et al.*, 2003). LMP-2A constitutively activates PI3K/Akt pathways in both epithelial cells and B cells by employing different mechanisms (Fruehling *et al.*, 1998; Lu *et al.*, 2006). Recent studies in gastric carcinoma cell lines suggested that LMP-2A expression favors cancer development by down-regulating *PTEN* and up-regulating *survivin* expression (Hino *et al.*, 2008; Hino *et al.*, 2009). The signaling pathways engaged by *LMP-2A* are summarized in Figure 1.1.

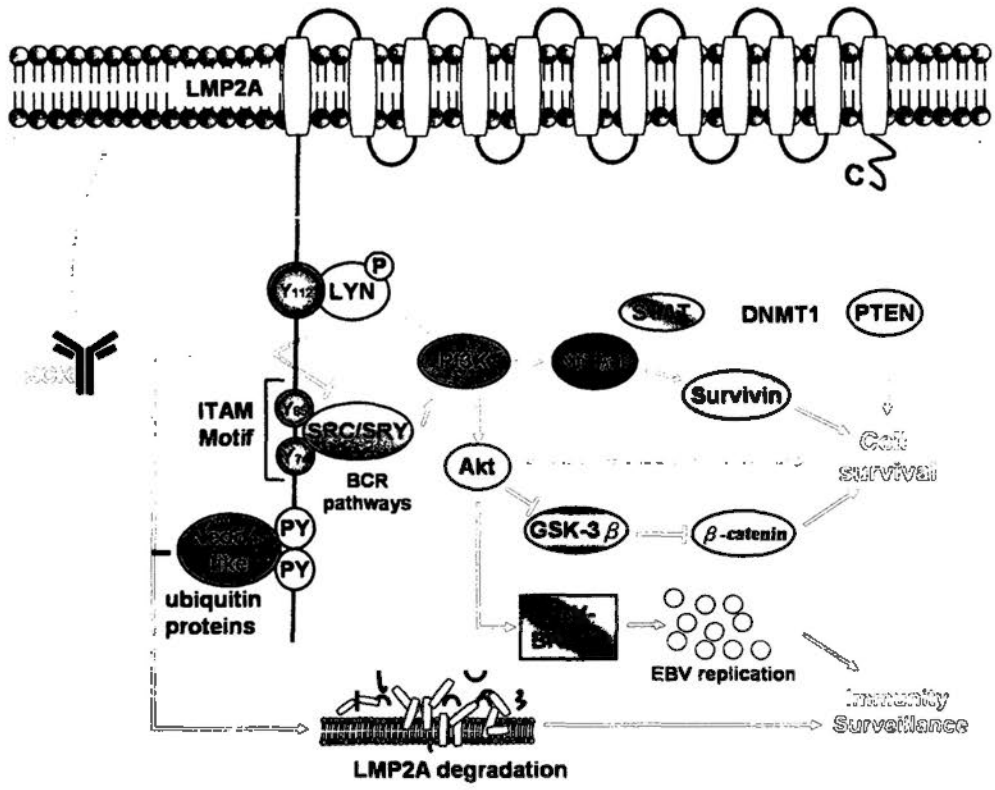


Figure 1.1 Structure and function of LMP-2A

Epstein-Barr virus latent membrane protein LMP-2A contains 12 transmembrane domains, 27 amino acids C-terminal and 119 amino acid functional N-terminal that contain 12 tyrosine residues. Three of the main tyrosine residues are indicated as circle Y, in which Tyr74 and Tyr85 form on ITAM motif with similar function to active BCR. However, the interaction between LMP-2A and LYN sequesters the latter protein from BCR, resulting in the inhibition of B-cell signaling pathways. The two phosptyrosine (PY) motifs, indicated as circle PY, recruit NEDD4-like ubiquitin proteins to promote degradation of both LYN and LMP-2A itself through a ubiquitin-dependent mechanism.

1.1.4.3 EBV Encoded Small RNAs (EBERs)

In addition to viral latent proteins, EBV also transcribes non-coding RNAs in all forms of latency programs. This virus actually expresses EBERs in around 5×10^6 copies per infected cell (Howe & Shu, 1989). Nowadays, detection of EBER transcripts has been extensively used for EBV detection in human biopsies. EBERs, first identified in 1981 by Prof. Joan A. Steitz's research team contain two species, EBER1 and EBER2, with the size of approximately 170 nucleotides (Lerner *et al.*, 1981). They are confined to the nucleus, where they form complexes with autoantigen La protein, ribosomal protein L22 (formerly EBER-associated protein EAP) and interferon-inducible, double-stranded RNA activated protein kinase R (PKR) (Lerner *et al.*, 1981; Howe & Steitz, 1986; Clarke *et al.*, 1991; Toczyski & Steitz, 1991; Fok *et al.*, 2006a; Fok *et al.*, 2006b). Although EBERs are nonessential for EBV-induced transformation in B-cells, expression of EBERs in B-cells has been demonstrated to have oncogenic roles *in vitro* and *in vivo* (Komano *et al.*, 1999; Laing *et al.*, 2002; Niller *et al.*, 2003; Yajima *et al.*, 2005). They have been shown to facilitate cell survival by inducing interleukin-10 (IL-10) expression and resisting interferon-inducible apoptosis in Burkitt's lymphoma cell lines (Kitagawa *et al.*, 2000; Nanbo *et al.*, 2002). In epithelial malignancies, EBERs neither induce cellular transformation nor increase tumorigenicity, but confer an apoptotic phenotype in epithelial cells (Wong *et al.*, 2005). On the other hand, EBERs were proven to support cell growth by stimulating insulin-like growth factor 1 (IGF-1) secretion in both EBV-positive gastric carcinoma and NPC cell lines (Iwakiri *et al.*, 2003; Iwakiri *et al.*, 2005)

1.1.4.4 *Bam*HI-A Rightward Transcripts (BARTs)

BARTs are multi-spliced transcripts that are originally discovered by cDNA library analysis in NPC xenograft and later reported in other EBV-infected individuals (Hitt *et al.*, 1989; Gilligan *et al.*, 1990; Karran *et al.*, 1992; Chen *et al.*, 1999a). Since expression of BARTs is selectively high in EBV-positive NPC and gastric cancer cells, it has long been speculated that BARTs have important functional roles in these EBV-associated epithelial malignancies (Cai *et al.*, 2006). In the early study of BART transcripts, some *ORFs* in the spliced cDNA transcripts were investigated. For example, RPMS1 and A73 *ORFs* were identified to be translated into protein *in vitro* (Smith *et al.*, 2000). The important roles of these two "proteins" for cancer development were experimentally confirmed. RPMS1 and A73 have been shown to be negative regulators of Notch signaling and of PKC and Src tyrosine kinase, respectively (Smith *et al.*, 2000; Zhang *et al.*, 2001; de Jesus *et al.*, 2003; Li *et al.*, 2005). However, expression of these two viral proteins has not been confirmed in natural EBV-infection samples (Al-Mozaini *et al.*, 2009).

Recent studies on viral miRNA have identified 23 EBV miRNAs (ebv-miRNAs) from latently infected B cells (Pfeffer *et al.*, 2004; Cai *et al.*, 2006; Grundhoff *et al.*, 2006; Landgraf *et al.*, 2007). Among them, 20 miRNAs (miR-BART1 to miR-BART20) are derived from the BART transcripts. The details of miR-BARTs will be discussed later in this chapter.

1.2 Nasopharyngeal Carcinoma (NPC)

1.2.1 Histopathology and Epidemiology

Nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck cancer arising from the nasopharynx (Sham *et al.*, 1990). The World Health Organization (WHO) has classified NPC into 3 categories according to histology (Shanmugaratnam & Sobin, 1991). Type 1 is keratinizing squamous cell carcinoma (SCC), which is the common type of NPC found in Western world (Marks *et al.*, 1998). Type 2 is non-keratinizing carcinoma and type 3 is undifferentiated epithelial carcinoma. Because of usually prominent lymphocytic infiltration, type 3 is also described as *lymphoepithelioma* of nasopharynx. More than 97% of NPC in Southern China belongs to this type (Marks *et al.*, 1998)

NPC exhibits distinctive ethnic and geographic distribution. This is a rare tumor in most parts of the world (below 1 per 100,000 persons per year). However, the incidence rate is remarkably high among southern Chinese, especially those of Cantonese origin (McDermott *et al.*, 2001). In Hong Kong, males are reported to have a higher NPC incident rate than females with a sex ratio of about 2.8:1. This is the fifth most common cancer in males in the region. The incidence rate and mortality rate are about 20.7 and 7.8 per 100,000 persons (Hong Kong Cancer Registry 2007; http://www3.ha.org.hk/cancereg/e_stat.asp).

1.2.2 Etiology

Although the cause of NPC is still poorly understood, a number of etiological factors have been extensively investigated. These include environmental factors such as the consumption of salted fish; other factors

involve genetic predisposition and EBV infection.

1.2.2.1 Environmental Factors

Consumption of Cantonese-style salted fish was first proposed to be an etiological factor for NPC in 1971 (JHC, 1971). A large number of studies conducted in Cantonese migrants in different parts of Asia had been carried out to investigate the hypothesis (Yu *et al.*, 1986; Ning *et al.*, 1990; Sriamporn *et al.*, 1992; Lee *et al.*, 1994; Armstrong *et al.*, 1998). Case-control studies suggested that consumption of salted fish during childhood was the primary cause of NPC among Cantonese (Yu *et al.*, 1985; Yu *et al.*, 1989). These studies had provided additional evidence for the earlier finding from our group member that feeding rats with salted fish could promote NPC development (Huang *et al.*, 1978). Since the extract from salted fish could elevate the expression of EBV early antigen (EA) in B-cells (Shao *et al.*, 1988), consumption of salted fish might indeed promote NPC development through EBV activation.

In addition to salted fish consumption, traditional preserved food such as pickled vegetables, fermented beans, salted and fermented eggs, might also increase the risk of NPC development (Yu *et al.*, 1988; Ning *et al.*, 1990; Lee *et al.*, 1994; Yuan *et al.*, 2000). Potential carcinogens, including volatile nitrosamines, have been consistently detected in these preserved foods (Huang *et al.*, 1981; Shao *et al.*, 1988; Zou *et al.*, 1994). Besides, consumption of tobacco, alcohol and Chinese herbal medicine was also suggested to be associated with NPC carcinogenesis by case-controlled studies (Yu, 1990; Hildesheim *et al.*, 1992). Other high risk events such as occupational exposures to certain toxic pollutants have been reported to increase NPC incidence. Such pollutants

included formaldehyde, smoke and chlorophenols and heavy metal (Blair *et al.*, 1986; Mirabelli *et al.*, 2000; Yu & Yuan, 2002).

1.2.2.2 Genetic Factors

The distinct geographical distribution of NPC incidence suggests a genetic involvement to this cancer. It has been proven by the observation that high NPC incidence among the Chinese immigrants from southern China to non-endemic areas are retained even by the second and third generations (Buell, 1974; Zeng & Jia, 2002). Moreover, around 10% of NPC cases have a family history of the disease (Ng *et al.*, 2009).

Human leukocyte antigens (HLA) genes are highly polymorphic and encode HLA molecules that are essential for the presentation of foreign antigens to the host immune system. An association between HLA locus and NPC was first reported in 1974, in which the HLA-A2 phenotype was related to an increased risk of NPC among Cantonese (Simons *et al.*, 1974; Simons *et al.*, 1975). Later on, studies on HLA loci demonstrated that an increased risk of NPC might be associated with HLA-BW46, BW59, B98 and DR9, and negatively associated with A11 and B3 (Chan *et al.*, 1983; Chan, 1990; Hildesheim *et al.*, 2002). Furthermore, high-resolution *HLA* genotype screening among Chinese has shown that people with HLA-A*0207, but not HLA-A*0201, have an increased NPC risk. In fact, HLA-A*0207 is the common genotype among Chinese (Hildesheim *et al.*, 2002). In addition to the HLA locus, recent genome-wide linkage scanning in familial nasopharyngeal carcinoma recognized three susceptibility loci at 4p15.1-q12 (Feng *et al.*, 2002); 3p21.31-21.2 (Xiong *et al.*, 2004); 5p13-15 (Hu *et al.*, 2008). However, the NPC-related genes in these

regions need to be further investigated.

Other genetic polymorphisms are also involved in NPC susceptibility. For example, a case-control experiment demonstrated that subjects carrying the c2/c2 genotype of the *cytochrome P450 2E1 (CYP2E1)* gene experience an increase risk of NPC by 2.6 fold. The variant form of *CYP2E1* (c2 allele) can possess higher enzymatic activity to activate nitrosamines, a carcinogen found in preserved food (Hildesheim *et al.*, 1997). Genetic polymorphism of *glutathione S-transferase M1* (*GSTM1*) may also relate to NPC. GSTM1 is a critical enzyme involved in the detoxification of several tobacco-related carcinogens, maintaining genome integrity and cancer susceptibility. A study of GSTM1 indicated that null genotype of this gene is correlated with a two-fold increase in risk for NPC (Nazar-Stewart *et al.*, 1999; He *et al.*, 2009).

1.2.2.3 EBV infection

The complex multi-step process of NPC carcinogenesis is not only influenced by host genetics and environmental factors, but also related to EBV infection. It is observed that clonal EBV genome and viral latent gene expression are detected in almost all primary NPCs (Figure 1.2) (Raab-Traub & Flynn, 1986; Lo & Huang, 2002; Raab-Traub, 2002; Lo *et al.*, 2004).

EBV is known to reside in NPC as latency II infection, where only few viral latent genes are expressed (Table 1.1). As discussed previously in section 1.1.4, EBV acquires tumorigenic potential through the expression of a set of viral latent genes. However, tumor cells are capable of processing and presenting those viral proteins on the cell surface in conjunction with the major histocompatibility complex (MHC) (Lee, 2002; Gottschalk *et al.*, 2005).

In NPC, viral protein expression is limited to EBNA1, LMP-1 and 2. Among them, LMP-2A has a particularly stronger immunogenicity than the two other NPC-expressed viral proteins (Brooks *et al.*, 1992; Khanna *et al.*, 1998; Lee *et al.*, 2000; Leen *et al.*, 2001). In this regard, limiting LMP-2A protein expression would potentially be advantagous for NPC cells to escape host immune surveillance.

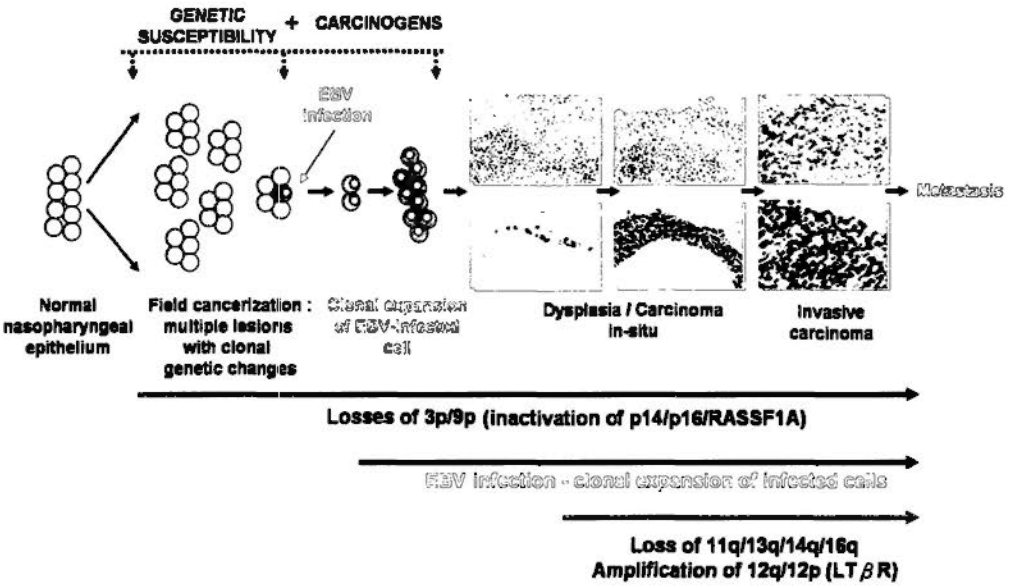


Figure modified from (Lo *et al.*, 2004)

Figure 1.2 Multiple steps of Nasopharyngeal Carcinoma tumorigenesis

NPC carcinogenesis is a complex process, in which EBV infection is consistently detected in invasive carcinoma and high-grade dysplastic lesion prior to tumor expansion. Expression of EBV latent genes can enhance tumor transformation, so clonally expansion of the EBV genome in the early stages of NPC may activate the progression to invasive carcinoma. Recent findings in our team have shown that 12p12.1-13.3 amplification, with the functional *LTβR* oncogene overexpressed, is highly associated with NPCs (Or *et al.*, 2010). This has added even more complexity to NPC tumorigenesis.

1.2.3 Molecular Pathogenesis

1.2.3.1 Global Genetic Changes

NPC is a multistep process, in which multiple genetic variations are present. In early genetic studies, several chromosomal abnormalities had been identified by karyotyping analysis on NPC cell lines and biopsies (Lo & Huang, 2002). Most of these genetic alterations were later confirmed by multi-color spectral karyotyping (SKY), with additional breakpoints identified at 3p21, 3q26, 5q31, 6p21-25, 7p14-p22, and 8q22 (Wong *et al.*, 2003).

Comparative genomic hybridization (CGH), a more accurate method for genetic lesion detection, was also employed to study the DNA copy gains and losses in NPC by several groups. These studies suggested numerous genetic variations of multiple chromosomal regions in NPCs (Hui *et al.*, 1999; Wong *et al.*, 2003) (Chien *et al.*, 2001; Fang *et al.*, 2001). Through this method, gain and loss of chromosome 12 and 3p were suggested to be the most frequently observed chromosomal abnormalities associated with NPC.

1.2.3.2 Oncogenes

Conventional CGH analysis is an important tool to study putative NPC-associated oncogenes. However, due to the relatively low resolution (~10Mb), the tumor related genes have been difficult to localize. Instead, a higher-resolution array-based CGH (aCGH) method has been used in NPC study. This commercial CGH microarray, AmpliOnc I microarray (Vysis), contains 58 known common amplicons in cancers. Using this array, amplifications of several putative oncogenes in NPC were identified by our group. They are including *MYCL* (1p34.2), *TERC* (3q26.3), *ESR* (6q25.1) and *PIK3CA* (3q26.3)

(Hui *et al.*, 2002). Furthermore, an active mutation (A3140G) in the *PIK3CA* gene was also observed in NPC cells (Or *et al.*, 2006). With the help of another home-made array which contained 1803 BAC clones (obtained from Prof. J.W. Gray, UCSF), our group further identified cyclin D1 (*CCND1*, 11q13) as a putative oncogene in NPCs (Hui *et al.*, 2005). Furthermore, lymphotoxin beta receptor (*LTβR*, 13p13.31) had also been identified as a putative oncogene by using the Agilent Human Genome CGH Microarray 44B (Agilent Technologies Inc) (Or *et al.*, 2010). The oncogenic potential of these two genes in NPC cells had been demonstrated by *in vitro* functional studies. Studies from other groups suggested that several oncogenes such as c-myc, ras, c-met, bcl-2 were over-expressed in primary NPC (Lu *et al.*, 1993; Porter *et al.*, 1994; Qian *et al.*, 2002). Although the regulatory mechanism of these oncogenes in NPC is largely unknown, their high expression has suggested an involvement in NPC development.

1.2.3.3 **Tumor Suppressor Genes (TSGs)**

Alterations of well-known TSGs, such as *Rb* and *p53*, are not common in NPC (Effert *et al.*, 1992; Sun *et al.*, 1993). However, inactivation of TSGs has been identified in minimal deleted regions. Loss of heterozygosity (LOH) in the *p14ARF*, *p15 and p16* TSGs on 9p21 was identified in up to 85% of NPC cases. In the microdissected primary NPC samples, inactivation of *p14ARF* (54%), *p15* (40%), *and p16* (77%) was confirmed to be the result of both homozygous deletion and promoter hypermethylation (Huang *et al.*, 1994; Lo *et al.*, 1996; Kwong *et al.*, 2002). Promoter hypermethylation is a common mechanism for NPC's TSGs inactivation. Through the extensive LOH and epigenetic studies in

NPC, an increasing number of NPC-related TSGs had been reported. They included *RASSF1A* (3P21.3) (Lo *et al.*, 2001), *SLC1* (11q23.2) (Hui *et al.*, 2003), *DLEC1* (3p22.3) (Kwong *et al.*, 2007), *DLC1* (8p22.3) (Seng *et al.*, 2007), and *WIF* (12q14.3) (Chan *et al.*, 2007), *POLydactylt 1* (*MIPOL1*) (14q13.1-13.3) (Cheung *et al.*, 2009) and protein tyrosine phosphatase receptor type G (*PTPRG*) (3p14-21) (Cheung *et al.*, 2008)

1.3 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a group of abundant small non-coding RNAs with the size of around 18-24 nucleotides. They are produced by endogenous enzymatic (Drosha/DGCR8 and Dicer) digestion of RNA transcripts containing hairpin structures. Mature miRNAs function as negative gene regulators through complementary sequence pairing to the 3' untranslated region (3'UTR) of the target transcripts by inducing either mRNA degradation or translational repression (Bartel, 2004). Given that miRNAs can regulate gene expression by imperfect complementary binding to the 3'UTR of the target mRNA, one miRNA is expected to regulate over 100 predicted cellular targets. Therefore, it is estimated that about 30% of cellular protein-coding genes are, in fact, regulated by miRNAs (Krek *et al.*, 2005; Lewis *et al.*, 2005; Lim *et al.*, 2005). As gene regulators, mammalian miRNAs play key roles not only in various biological processes including development, differentiation, but also in cancer development.

The first miRNA, lin-4, was discovered in *Caenorhabditis elegans* (*C.elegans*) by two research teams (Lee *et al.*, 1993; Wightman *et al.*, 1993). They observed that *lin-4* contained sequences complementary to the 3' UTR of

lin-14 mRNA and functioned as a repressor of lin-14 protein expression, a novel protein that regulated *C.elegans* development. Since lin-14 was not conserved in other species, not much attention was paid to this miRNA as it was regarded as a molecule for nematode-specific developmental regulation (Ambros, 2008).

Only in 2000, the second small RNA from *C.elegans*, *let-7*, was discovered and identified as a temporally regulated RNA generated from a longer double strand hairpin structure precursor (Reinhart *et al.*, 2000). *Let-7* regulates *lin-41* expression through the imprecise antisense base-pairing to the 3'UTR of *lin-41* mRNA (Slack *et al.*, 2000). Subsequently, *let-7* was found to be highly conserved and expressed in a wide range of animal species (Pasquinelli *et al.*, 2000). This accelerated the extensive identification of small RNA in different species like *C.elegans*, *Drosophila melanogaster*, and in the human genome (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee & Ambros, 2001). This new class of 18-24-nt noncoding regulatory RNAs were named miRNAs. In 2004, the first group of animal virus-encoded miRNA was identified in EBV (Pfeffer *et al.*, 2004). Currently, 23 EBV-encoded miRNAs have been identified in the EBV genome (Pfeffer *et al.*, 2004; Cai *et al.*, 2006; Grundhoff *et al.*, 2006).

1.3.1 MiRNAs Biogenesis

Similar to protein coding mRNAs, miRNA biogenesis starts with the transcription of primary transcript, named pri-miRNA (Figure 1.3). The transcription is mediated by either RNA polymerase II (Pol II) as polycistronic primary transcripts (Lee *et al.*, 2004) or RNA polymerase III (Pol III) (Borchert *et al.*, 2006). Pri-miRNAs possess a 5' methyl guanosine cap structure and a polyA tail at the 3' end. In humans, over 70% of the pri-miRNAs are located

within introns (intronic miRNAs) of either coding or non-coding transcripts (Kim & Kim, 2007). Due to sequence complementarity within the pri-miRNA transcript, distinct hairpin structures were formed.

The stem-loop pri-miRNA is sequentially processed by two ribonuclease III-like enzymes (RNase III), Drosha and Dicer. In the nucleus, the stem-loop structures of the pri-miRNAs are recognized by the dsRNA binding protein, DiGeorge syndrome critical region gene-8 (DGCR8) or Pasha in *Drosophila* and *C.elegans*. DGCR8 then forms a complex with Drosha and other proteins called the "microprocessor" that crops the pri-miRNA transcript into small precursor miRNAs (pre-miRNAs) with a size around 70-nt (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Landthaler *et al.*, 2004; Han *et al.*, 2006). The cleavage site on the stem-loop is approximately 11-bp from the ds/ssRNA junction (Figure 1.4). Some other proteins, like DEAD box RNA helicase subunits and SMAD protein, may also be critical for a subset of Drosha-mediated miRNA processing (Fukuda *et al.*, 2007; Davis *et al.*, 2008). Drosha produces pre-miRNAs by cleaving pri-miRNAs or mRNAs that contain long hairpin structures. If the cleavage sites are located within an exonic region, spliced out pre-miRNAs can destabilize the protein coding transcript and down-regulate it protein synthesis (Han *et al.*, 2009). On the other hand, intronic miRNAs can be processed cotranscriptionally without affecting the mature mRNA levels (Kim & Kim, 2007). In addition, other studies have suggested an alternative miRNA biogenesis mechanism which is not dependent on Drosha. The spliced intronic pre-miRNAs, named mirtrons, are processed by an alternative pathway other than Drosha cleavage in the nucleus (Figure 1.3) (Berezikov *et al.*, 2007; Kim & Kim, 2007; Okamura *et al.*, 2007; Ruby *et al.*, 2007).

All the pre-miRNAs and mirtrons are actively transported to the cytoplasm by the nuclear transporter, exportin-5 (Yi *et al.*, 2003; Okamura *et al.*, 2007). In the cytoplasm, pre-miRNAs are further processed by the second RNase III-like enzyme, Dicer, into around 22-nt double-strand duplex containing the mature miRNA (Figure 1.3 and Figure 1.4). In human, Dicer interacts with TRBP (TAR RNA-biding protein), PACT (PRK activator), Argonaute (AGO) 1-4 to mediate the pre-miRNA processing and the assembly of the miRNA-containing RNA-induced silencing complex (miRISC) (Chendrimada *et al.*, 2005; Haase *et al.*, 2005; Lee *et al.*, 2006; Kok *et al.*, 2007). Based on the stability of the 5' end duplex, AGO selects only one strand of mature miRNAs to be incorporated into the RISC and guides the complex to search for the 3'UTR of the target mRNA. The opposite strand, known as *miRNA** is usually destroyed quickly by the miRISC complex. However in some case, *miRNA** can be survived and function as mature miRNA (Schwarz *et al.*, 2003; Gregory *et al.*, 2005). Although all AGO family members, AGO-1-4, are associated with miRNAs in human, only AGO-2 is proven to guide target RNA cleavage (Meister *et al.*, 2004). However, a recent study in *Drosophila* indicated that AGO-1 is indeed the main AGO protein contributing to miRNA regulatory mechanism in that organism (Easow *et al.*, 2007; Hong *et al.*, 2009; Kawamata *et al.*, 2009).

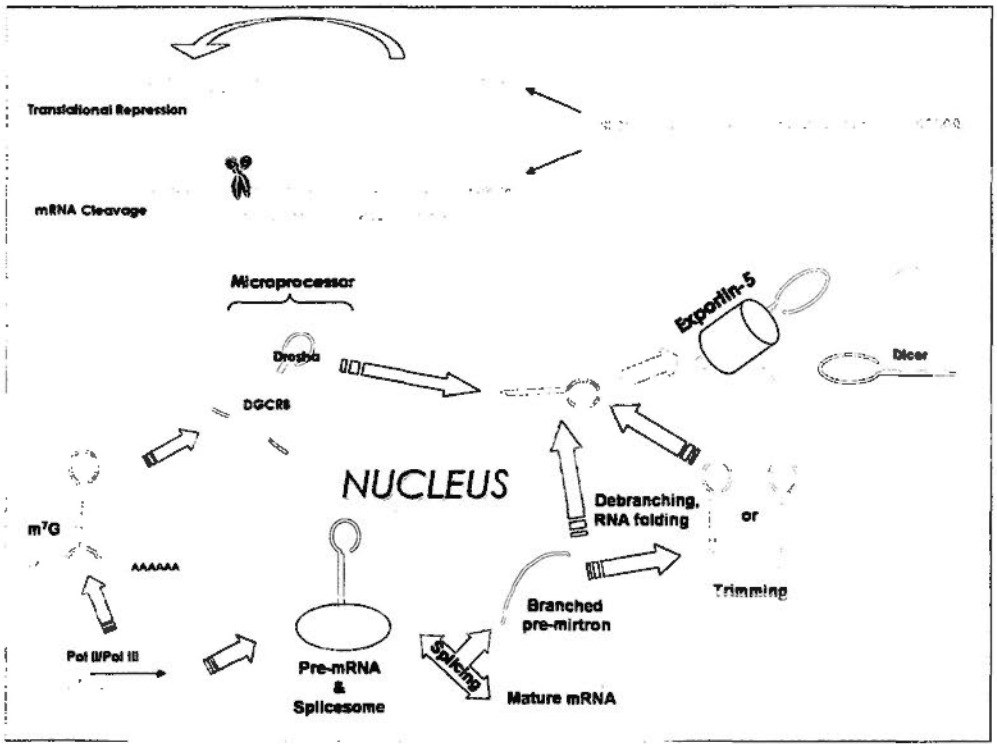


Figure 1.3 MiRNA biogenesis pathways

Mature miRNAs can be produced from either the canonical miRNA pathway (green arrows in the nucleus) or mirtron pathway (yellow arrows in the nucleus). Details are described in the text.

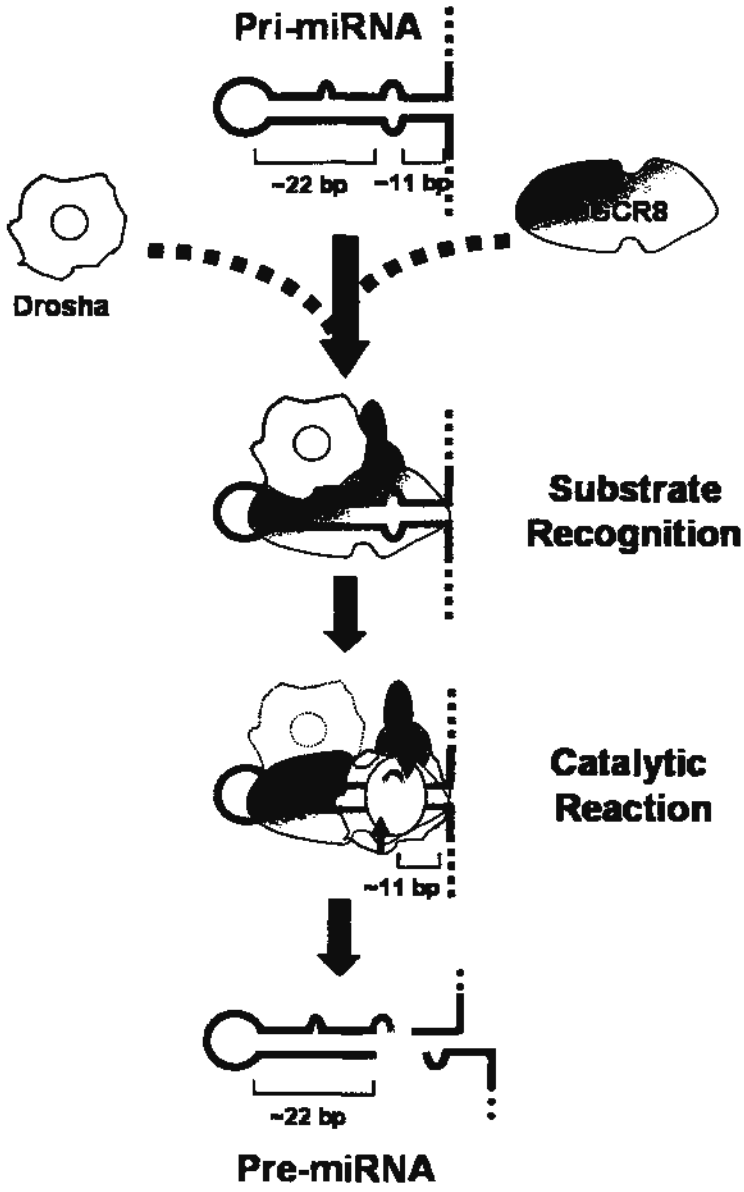


Figure modified from (Han *et al.*, 2006)

Figure 1.4 Junction anchoring model for pri-miRNA processing

The cleavage process involves in 2 steps. First, DGCR8 interacts with the stem of a pri-miRNA, which is ~33 bp from the terminal loop (substrate recognition step). After the initial recognition step, Drosha will transiently interact for the cleavage and release of the pre-miRNA (catalytic reaction step). The cleavage centre is placed ~11 bp from the ss/ds RNA junction.

1.3.2 Post-transcriptional Gene Silencing by MiRNAs

The function of miRISC is to recognize target mRNAs and subsequently modulate their expression. The miRNA binding site is mainly located in the 3'UTR, and in some cases, 5'UTR of the target transcript (Lytle *et al.*, 2007). The nearly perfect complementarity between miRNAs and their binding sites favors mRNA cleavage (Zeng *et al.*, 2003). On the other hand, imperfect complementarity between the miRNA and its target is mainly for inducing translational repression or RNA degradation. It was found that the 5' end of the miRNA is particularly important to miRNA-target association, especially the 2^{nd}- to 8^{th}-nt (seed region) (Lai, 2002; Doench & Sharp, 2004; Brennecke *et al.*, 2005). Nevertheless in some cases, poor a seed match can be compensated by strong 3' base pairing sequence (Lewis *et al.*, 2005).

While the mechanism of how miRISC mediates gene silencing still remain elusive, multiple possibilities have been suggested (Figure 1.5). For example, the miRNA can block translation of 7-methyl-guanosine (m^7G) capped mRNA at the initiation step by interfering with the cap-binding protein that directs the ribosome to the cap structure (Humphreys *et al.*, 2005). This hypothesis is further proven by the identification of a specific motif on the hAGO2 that is highly similar to the cap-binding domain on eukaryotic initiation factor 4E (eIF4E, a well-known cap-binding protein). The presence of this motif on AGO is likely to preclude the recruitment of eIF4E to the m^7G of the mRNA target (Kiriakidou *et al.*, 2007). MiRISC has also been reported to interfere with mRNA translation by recruiting other mRNA regulatory proteins, such as RNA helicases (RCK/p54 and MOV10), for RNA degradation (Meister *et al.*, 2005; Chu & Rana, 2006), and eIF6, for preventing the productive assembly of the

ribosome on to the cap structure (Chendrimada *et al.*, 2007). The ribosome-free mRNA will finally be repressed and aggregate into cytoplasmic processing bodies (P-bodies) for either degradation or storage (Figure 1.5) (Liu *et al.*, 2005).

Other studies also support that miRNAs regulate translation in post-initiation steps (Nottrott *et al.*, 2006; Petersen *et al.*, 2006; Lytle *et al.*, 2007). This is mainly carried out by deadenylation leading to decay of the target mRNA, in which GW182 proteins play critical roles by directly interacting with AGO proteins (Eulalio *et al.*, 2008). GW182 proteins recruit the deadenylase complex and poly(A)-binding protein (PABP) towards its C-terminal to degrade target transcripts (Behm-Ansmant *et al.*, 2006; Fabian *et al.*, 2009; Zipprich *et al.*, 2009). While the different miRISC regulatory mechanisms may not be mutually exclusive, further investigations are necessary to obtain a clear miRISC regulatory network (Figure 1.5).

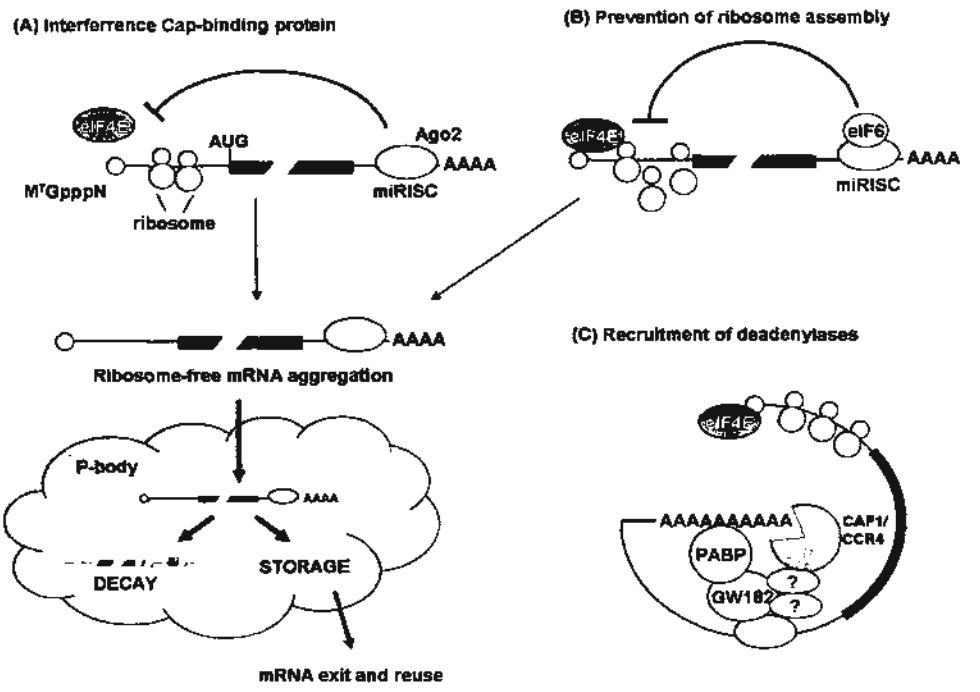


Figure modified from (Pillai *et al.*, 2007) and (Fabian *et al.*, 2009).

Figure 1.5 Possible mechanisms of miRNA-mediated repression

Three proposed miRISC-mediated gene silencing mechanisms are shown. Details are described in the text.

1.3.3 MiRNAs and Cancer

The first two miRNAs, *lin-4* and *let-7*, were originally identified to regulate developmental timing in *C.elegans*. Since then, miRNAs were recognized as a major group of regulatory molecules for gene expression, in which aberrant expression of miRNAs was predicted to be involved in cancer development.

The first study of involvement of miRNAs in cancer was reported in 2002. Calin et al. identified two clusters of miRNAs, *miR-15a* and *16-1*, located in the well-known 30-kb deleted region on chromosome 13 in chronic lymphocytic leukemia (CLL) patients. They subsequently confirmed in blood samples that these two miRNAs were down-regulated in the majority (~68%) of CLL patients (Calin *et al.*, 2002). This observation suggested that *miR-15a/16-1* might be involved in the pathogenesis of CLL with the 30-kb deletion. These two miRNAs were later confirmed to have tumor suppressive activity by controlling cell cycle and apoptosis in a variety of cancer cells such as lymphoma, prostate cancer and non-small cell lung carcinoma (Cimmino *et al.*, 2005; Bonci *et al.*, 2008; Chen *et al.*, 2008; Bandi *et al.*, 2009).

1.3.3.1 MiRNAs with Tumor Suppressive Activities

Although originally identified in *C.elegans*, *let-7* was the first miRNA experimentally proved to function as a tumor suppressor gene by inhibiting the expression of the proto-oncogene, *RAS* in lung cancer (Johnson *et al.*, 2005). Overexpression of the *RAS* oncogene is common in lung cancer. Johnson et al further showed that overexpression of *RAS* was strongly correlated with reduction of *let-7* expression in lung cancer tissue. Another independent group previously reported that expression of *let-7* is reduced in lung cancer, and this

correlated with poor cancer prognosis (Takamizawa *et al.*, 2004). Recently, the let-7 complementary binding site polymorphism on the KRAS 3'UTR was shown to be associated with an increased risk of oral and non-small cell lung carcinoma. Hence, *let-7* may be the key tumor suppressor gene in cancer involving KRAS expression (Chin *et al.*, 2008; Christensen *et al.*, 2009). Shortly after Johnson's report, two well-known oncogenes, *MYC* and *HMGA2*, were validated as targets of let-7 as well (Lee & Dutta, 2007; Sampson *et al.*, 2007).

Another well studied tumor suppressor miRNA is *miR-34*. This miRNA is the downstream target of p53 tumor suppressor gene upon DNA damage. In addition, expression of miR-34 alone in both primary and tumor-derived cell lines could induce cell cycle arrest (Chang *et al.*, 2007; He *et al.*, 2007). One independent group even demonstrated that miR-34 inhibited the silent information regulator 1 (SIRT1) expression, resulting in stabilization of p53 and its downstream targets, PUMA and p21 by increasing their acetylation. Furthermore, p53 might activate itself by suppressing the expression of SIRT1 through miR-34, which is a transcriptional target of p53 (Yamakuchi *et al.*, 2008). Recently, some tumor suppressor miRNAs such as miR-29c, miR-200a and miR-100, have been identified in NPC (Sengupta *et al.*, 2008; Shi *et al.*, 2009; Xia *et al.*, 2009). Other well-characterized examples of tumor suppressor miRNAs are listed in Table 1.3.

1.3.3.2 MiRNAs with Oncogenic Activities

The miR-17-92 cluster, which consists of six miRNAs, is known to be over-expressed in B-cell lymphoma and lung cancer (Ota *et al.*, 2004; Hayashita

et al., 2005; He *et al.*, 2005b). This cluster of miRNAs is located within a region on chromosome 3 that is frequently amplified in human B-cell lymphoma and lung cancer (Hayashita *et al.*, 2005; Johnson *et al.*, 2005). He et al. directly showed that miRNAs in this cluster are overexpressed in B-cell lymphoma cell lines with chromosomal amplification. Furthermore, they demonstrated in a mouse model that increased expression of miR-17-92 cluster can accelerate the tumorigenesis of c-Myc-induced B cell lymphoma. Later, another research group demonstrated that c-myc can increase the expression of miR-17-92, which in turn suppressed the function of angiogenesis protein Thrombospondin 1 (TSP1) (Dews *et al.*, 2006). Interestingly, *miR-17-92* does not simply function as an oncogene. Another independent study suggested that myc protein can induce expression of both E2F1 and miR-17-92, whilst miR-17-92 in turn inhibited E2F1 expression (O'Donnell *et al.*, 2005). Based on this observation, a new regulatory mechanism in which c-myc fine-tunes its oncogenic properties by miRNA expression has been suggested.

MiR-155, is processed from a non-coding RNA transcribed from the *B-cell Integration Cluster* (BIC) (Lagos-Quintana *et al.*, 2002). Overexpression of miR-155 has been detected in several solid tumors such as breast cancer (Volinia *et al.*, 2006), colon cancer (Wang *et al.*, 2008b) and lung cancer (Yanaihara *et al.*, 2006). Moreover, up-regulation of miR-155 has been detected in human leukemia and lymphoma (Eis *et al.*, 2005; Kluiver *et al.*, 2005). Several biological activities of miR-155 in tumorigenesis have been suggested. For example, it suppresses apoptosis by blocking the caspase-3 activity and induces cell cycle arrest by targeting the tumor protein p53 inducible nuclear protein 1 (TP53INP1) (Gironella *et al.*, 2007; Ovcharenko *et al.*, 2007).

Interestingly, Kaposi's sarcoma herpes virus (KSHV) and Marek's disease virus (MDV) have recently been shown to encode miRNAs with high homology to miR-155, namely miR-K12-11 (KSHV) (Skalsky *et al.*, 2007) and MDV-miR-M4 (Zhao *et al.*, 2009). In addition, EBV has been reported to up-regulate miR-155 expression through the NF-κB pathways (Gatto *et al.*, 2008; Lu *et al.*, 2008a). As a result, these viruses may contribute to cancer development by increasing miR-155 homologous miRNA expression. As important gene regulators, some miRNAs were reported to have oncogenic effects in NPC. They include miR-141, miR-17-92 and miR-155 (Chen *et al.*, 2009; Zhang *et al.*, 2010). Other well-characterized oncogenic miRNAs are listed in Table 1.4.

Table 1.3 Examples of miRNAs with tumor suppressive effects

miRNA	Related Cancers	Validated Cellular Targets	References
Let-7	Burkitt's lymphoma; colon cancer; lung cancer;	RAS and HMGA2	(Takamizawa *et al.*, 2004; Johnson *et al.*, 2005; Lee & Dutta, 2007; Sampson *et al.*, 2007; Peng *et al.*, 2008)
miR-15a-16-1	CLL; pituitary adenoma	BCL-2;	(Bottoni *et al.*, 2005; Cimmino *et al.*, 2005)
miR-24	Lung adenocarcinoma; papillary carcinoma; pancreatic carcinoma	P16(INK4a) and ALK4	(Bottoni *et al.*, 2005; Yanaihara *et al.*, 2006; Lee *et al.*, 2007; Porkka *et al.*, 2007; Lal *et al.*, 2008; Nam *et al.*, 2008; Nikiforova *et al.*, 2008; Wang *et al.*, 2008a)
miR-26a	Ovarian carcinoma; papillary carcinoma; prostate cancer	Enhancer of Zeste homolog 2 (E2H2) and SMAD1	(He *et al.*, 2005a; Luzi *et al.*, 2008; Wong & Tellam, 2008)
miR-29	Lung adenocarcinoma; AML; CLL.	MCL1, Laminin, TCL1	(Pekarsky *et al.*, 2006; Mott *et al.*, 2007; Sengupta *et al.*, 2008)
miR-124a	Colon cancer; lung cancer; medulloblastoma; ALL	CDK6	(Lujambio & Esteller, 2007; Pierson *et al.*, 2008; Agirre *et al.*, 2009; Garzon *et al.*, 2009)

Abbreviation: ALL: Acute Lymphoblastic leukemia;
AML: Acute Myeloid leukemia;
CLL: Chronic Lymphocytic leukemia;

Table 1.4 Examples of miRNAs with oncogenic effects

miRNA	Related Cancers	Validated Cellular Targets	References
miR-17-92	B-cell lymphoma; lung cancer, NPC	E2F1; thromobospondin-1; PTEN; p21	(Ota *et al.*, 2004; He *et al.*, 2005b; Johnson *et al.*, 2005; O'Donnell *et al.*, 2005; Dews *et al.*, 2006; Fontana *et al.*, 2008; Xiao *et al.*, 2008; Chen *et al.*, 2009)
miR-21	Breast cancer; CLL; cervical cancer; Liver cancer	PTEN; tropomyosin 1; programmed cell death 4 (PDCD4); Maspin	(Iorio *et al.*, 2005; Meng *et al.*, 2007; Zhu *et al.*, 2007; Lu *et al.*, 2008b; Zhu *et al.*, 2008)
miR-155	B-cell lymphoma; Burkitt's lymphoma; breast cancer; colon cancer; lung cancer; CLL; NPC	tumor protein p53 inducible nuclear protein 1 (TP53INP1); Btb and CNC homolog 1 (BACH-1)	(Eis *et al.*, 2005; Kluiver *et al.*, 2005; Volinia *et al.*, 2006; Yanaihara *et al.*, 2006; Gironella *et al.*, 2007; Ovcharenko *et al.*, 2007; Skalsky *et al.*, 2007; Wang *et al.*, 2008b; Chen *et al.*, 2009)
miR-200a	Ovarian cancer, NPC	ZEB1; SIP1;	(Gregory *et al.*, 2008; Hu *et al.*, 2009; Saydam *et al.*, 2009; Xia *et al.*, 2009)
miR-210	Breast cancer; pancreatic tumor	Ephrin-A3; E2F3; MNT (MYC antagonis)	(Camps *et al.*, 2008; Fasanaro *et al.*, 2008; Giannakakis *et al.*, 2008; Greither *et al.*, 2009; Zhang *et al.*, 2009)
miR-221, 222	Breast cancer; NSCLC; prostate cancer; thyroid papillary carcinoma	P27Kip1; CDKN1C/P57; CDKN1B/P27	(Galardi *et al.*, 2007; Visone *et al.*, 2007; Fornari *et al.*, 2008; Garofalo *et al.*, 2008; Miller *et al.*, 2008; Sun *et al.*, 2009)

Abbreviation: CLL: Chronic Lymphocytic leukemia;
NSCLC: Non small cell lung carcinoma
NPC: Nasopharyngeal carcinoma

1.3.4 Viral-Encoded MiRNAs

The first group of viral-encoded miRNAs was reported by Thomas Tuschl's research team in 2004. They used molecular cloning method to identify five miRNAs in human B-cells infected with the prototype EBV strain B95.8, which carries a 12-kb deletion in the BART region of the EBV genome (Pfeffer *et al.*, 2004). Subsequent work on other latently infected B cells further identified a total of 23 EBV encoded miRNAs (Cai *et al.*, 2006; Grundhoff *et al.*, 2006). Among them, three miRNAs arise from in the untranslated region of the viral early lytic gene, *BHRF* (miR-BHRF1-1, miR-BHRF1-2 and miR-BHRF1-3). Expression of those miRNAs is predicted in EBV latency III program. The rest of the 20 miRNAs (miR-BART1 to miR-BART20) are located into two clusters within the non-coding BART region, which is generally highly expressed in most EBV infected epithelial cells (Figure 1.6).

Currently, over 60 virus encoded miRNAs have been reported from different human DNA viruses, with most of them derived from herpesviruses (Table 1.5). Although one functional miRNA was reported to be produced by Dicer in human immunodeficiency virus 1 (HIV-1) (Ouellet *et al.*, 2008), other RNA viruses are unlikely to produce miRNA (Pfeffer *et al.*, 2005). A possible reason is that processing of miRNA precursor in RNA virus by Drosha might lead the cleavage of the viral genome and result in degradation.

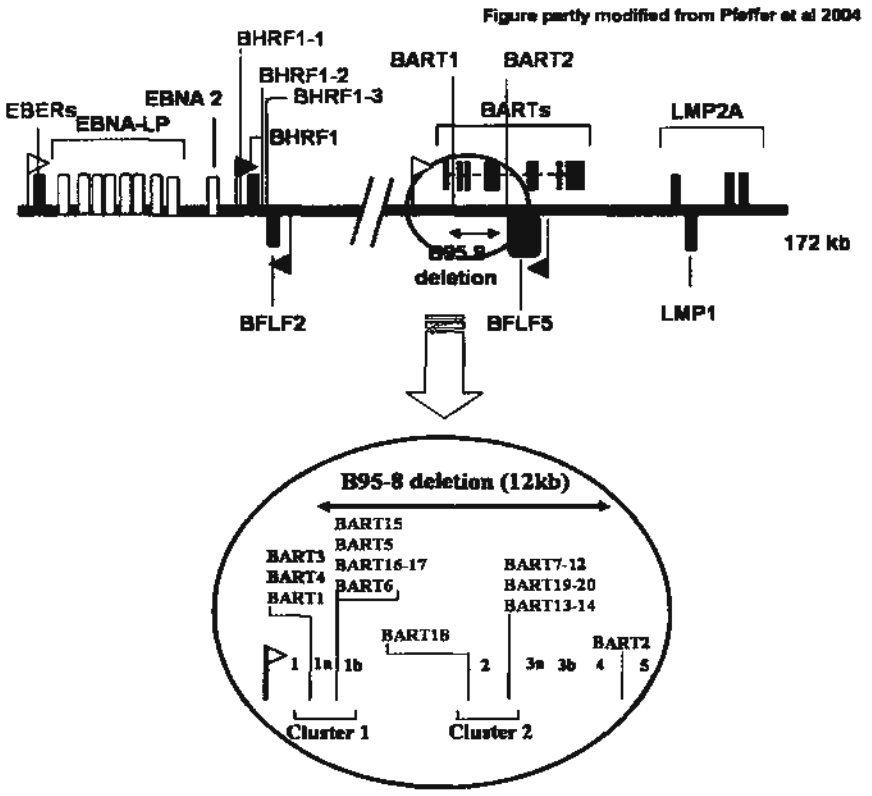


Figure 1.6 Location of miRNAs in EBV genomes

The locations of the miRNAs in the EBV genome were listed. The first five identified EBV-miRNAs and non-coding RNAs (EBERs and BARTs) are named in red and blue colors, respectively (*upper panel*). MiRNAs located in the B95.8 deleted BART region are further illustrated inside the red circle in the lower panel.

Table 1.5 Known viral encoded miRNAs in human

Family	Genus (Name)	Number of pre-miRNAs	References
Herpesvirus	Simplexvirus (HSV-1; HSV-2)	HSV-1: 8 HSV-2: 3	(Cui *et al.*, 2006; Tang *et al.*, 2008; Umbach *et al.*, 2008; Tang *et al.*, 2009; Umbach *et al.*, 2009)
	Cytomegalovirus (CMV)	11	(Grey *et al.*, 2005; Pfeffer *et al.*, 2005)
	Rhadinovirus (KSHV)	12	(Cai *et al.*, 2005; Pfeffer *et al.*, 2005; Samols *et al.*, 2005; Grundhoff *et al.*, 2006)
	Lymphocryptovirus (EBV)	23*	(Pfeffer *et al.*, 2004; Cai *et al.*, 2006; Grundhoff *et al.*, 2006)
Polyomavirus	(BKV)	1	(Seo *et al.*, 2008)
	(JCV)	1	(Seo *et al.*, 2008)
	(MCV)	1	(Seo *et al.*, 2009)
Adenovirus	(AV)	1	(Sano *et al.*, 2006)
Retrovirus	Lentivirus (HIV)	1	(Ouellet *et al.*, 2008)

*Two more EBV pre-miRNAs were identified from this thesis

Abbreviation: KSHV: Kaposi's sarcoma-associated herpesvirus
EBV: Epstein-Barr Virus
HIV: Human immunodeficiency virus
MCV: Merkel Cell Polyomavirus

1.3.5 Function of viral miRNA

Gene regulation by miRNAs is particularly useful and convenient for the virus machinery. Unlike other regulatory proteins, miRNAs are processed from small pri-miRNAs, with a minimal size of around 200 nucleotides. The small pri-miRNAs can be tightly packed into a relatively small viral genome. Furthermore, miRNAs are non-coding RNAs which are presumably non-immunogenic molecules, yet they can regulate several target genes to enhance infected cell survival.

1.3.5.1 Viral mRNA targets

Identification and validation of miRNA targets is the most difficult part of an miRNA study. In spite of this, some viral targets of viral miRNA have been easily identified because they are transcribed antisense to the pre-miRNA sequence. Interestingly, the target genes of viral miRNAs mainly correspond to the control of host immune system and virus replication. The first viral miRNA target was identified in simian virus (SV40). The SV40 encoded miRNA, miR-S1, is expressed in the late stage of infection and it down-regulates the expression of viral T antigen (TAg) by perfect complementarity to its target mRNA. The infected cell then becomes less sensitive to cytotoxic T-cells and is protected from the host immune system (Sullivan *et al.*, 2005). MiR-S1 is evolutionary conserved with miRNAs from other human polyomaviruses including JCV and MCV. They are all transcribed antisense oto their early TAg, and negatively regulate the expression of TAg in infected cells, like SV40 (Seo *et al.*, 2008; Seo *et al.*, 2009).

A similar example is observed in EBV. EBV miR-BART2 was found to lie

opposite to the viral DNA polymerase gene, *BALF5* (Figure 1.6, *upper panel*) (Pfeffer *et al.*, 2004). Another research group confirmed later that miR-BART2 can down-regulate BALF5 via siRNA cleavage of the binding site (Barth *et al.*, 2008). Being an important protein for DNA replication, down regulation of BALF5 may facilitate virus entry to latency. Apart from the siRNA cleavage mechanism, EBV-miRNAs can also modulate the expression of other viral genes by binding to the 3'UTRs of target transcripts. Our laboratory has previously reported that LMP-1 is down-regulated by three cluster 1 miR-BARTs, miR-BART1-5p, miR-BART16 and miR-BART17-5p. Although LMP-1 exhibits oncogenic properties in some cell types, overexpression of LMP-1 results in increased sensitivity to apoptosis and reduced NF-κB activity. For this reason, EBV-miRNAs may contribute to cancer maintenance by tightly regulating LMP-1 expression in infected cells (Lo *et al.*, 2007).

HSV-1 expresses a total of seven miRNAs in latent infection (Umbach *et al.*, 2009). One of these miRNAs, miR-H2, is transcribed antisense to the viral *immediate early protein (ICP0)* mRNA, yet it can only down-regulate *ICP0* expression at the translational level, despite sequences that are fully complementary. Umbach et al further demonstrated that another HSV-1 miRNA, miR-H6, can target another immediate early protein mRNA, ICP4, through imperfect binding. Suppression of ICP activities inhibits the transcription of other viral genes and results in maintenance of viral latency. HSV-1-miR-H3 and H4 are also proposed to inhibit expression of ICP34.5 as they are transcribed from the antisense strand of this gene (Umbach *et al.*, 2008). The silencing effect of miR-H3 and H4 on ICP34.5 was finally demonstrated by HSV-2 miRNAs, miR-I and miR-II, which share a similar genomic location and

sequence to HSV-1-miR-H3 and H4, respectively (Figure 1.7) (Tang *et al.*, 2009). As a known neurovirulent factor, down-regulation of ICP34.5 may protect the latent infected cells from neurovirulent effects during virus reactivation.

Although eleven miRNAs are produced from hCMV, the function of only one miRNA, miR-UL112-1, has been identified. MiR-UL112-1 is able to target viral immediate-early protein IE1, also known as IE72 and U123, through two partially complementary elements located in the 3'UTR (Grey *et al.*, 2007; Murphy *et al.*, 2008). The expression level of IE1 is initially high but gradually declines after hCMV infection. Grey et al. also demonstrated that premature expression of miR-UL112 in the early stage of hCMV infection resulted in a decrease in viral replication rate. This example again shows that miRNAs contribute to the establishment and maintenance of viral latency. On the whole, modulation of viral protein expression by viral miRNAs might be a common approach for viruses to maintain/enter viral latency and protect their infected cells from the host antiviral immune response. The known viral targets of other viral miRNAs are summarized in Table 1.6.

1.3.5.2 Cellular mRNA targets

Herpesviruses are the leading cause of human disease after influenza virus. Once a human being is infected with herpesviruses, the viruses may stay in the host for life-long latency with subsequent reactivation. For this reason, viruses need to protect themselves by regulating cellular signaling pathways in order to escape the host defense system. Viral encoded miRNAs, a new group of gene regulators, seem to participate in "autoregulation", and inhibit translation of

cellular mRNAs bearing imperfect complementary sites in their 3'UTRs, having similar function to cellular miRNAs.

The miRNAs from three of the four miRNA-producing herpesviruses are able to regulate cellular genes for cancer development (Table 1.7), with KSHV miRNAs highly preferring to target cellular genes. An interesting example is miR-K12-11, which shares the same seed sequence as human miR-155; thus, they are predicted to regulate a similar set of targets. For example, they regulate the expression of the transcriptional suppressors, *BTB and CNC homolog 1* (*BACH1*), which contain several putative binding sites for these two miRNAs in their 3'UTRs (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Although the function of *BACH1* in KSHV replication is not clear, up-regulation of miR-155 was observed in several tumors (see section 1.2.3.2). For this reason, miR-K12-11, which is supposed to down-regulate the same set of miR-155 target genes, might also be involved in tumor development.

A cluster of KSHV miRNAs, including mir-K1, K3-3p, K6-3p and K11, are able to down-regulate the expression of an important tumor suppressor gene, *thrombospondin1 (THBS1)* (Samols *et al.*, 2007). THBS1 is a potent anti-angiogenic regulator involved in several biological processes including cell to cell adhesion and anti-proliferation (Lawler, 2002). Down-regulation of *THBS1* by KSHV miRNAs may assist the development of Kaposi's sarcoma *in vivo*. Recently, the pro-apoptotic protein, *Bcl-2-associated factor 1* (*BCLAF1* or *bcf*) and the natural killer (NK) cell ligand, *MHC class I polypeptide-related sequence B* (*MICB*), were identified as cellular targets of KSHV miRNAs (Nachmani *et al.*, 2009; Ziegelbauer *et al.*, 2009). Intriguingly, unrelated

miRNAs deriving from hCMV and EBV were also reported to inhibit *MICB* expression through independent binding sites within the 3'UTR of the target transcript (Table 1.7) (Stern-Ginossar *et al.*, 2007; Nachmani *et al.*, 2009). As MICB is the stress-inducible cell surface ligand recognized by NK cells for immune responses, down-regulation of *MICB* by viral miRNAs might be an important process for herpesviruses-infected cells (stress induction) to escape the host immune defense system.

EBV miRNAs can also modulate cellular gene expression. For example, miR-BART5, which is strongly expressed in epithelial cancers, is able to inhibit the expression of the pro-apoptotic protein, p53-upregulated modulator of apoptosis (PUMA). Suppression of endogenous miR-BART5 in NPC cell line (C666-1) has been demonstrated to stimulate PUMA-dependent apoptosis (Choy *et al.*, 2008), suggesting that miR-BART5 may protect EBV-infected cells from p53 induced apoptosis. Another ebv-miRNA, miR-BHRF1-3 has been proposed to down-regulate the expression of a chemokine named *CXC-chemokine ligand 11* (*CXCL11*), a potent interferon-inducible T-cell chemoattractant (Xia *et al.*, 2008). Specifically high expression of miR-BHRF1-3 in viral latency III and during virus replication may be important for active EBV infected cells to be shielded from cytotoxic T cell elimination *in vivo*.

In summary, the identified cellular and viral targets of virus-encoded miRNAs are similar in function. They enable the virus to prolong their culture in infected cells by either evading host immune response or escaping viral-induced apoptosis.

Table 1.6 Viral targets of human viral miRNAs

Virus	miRNA	Target	Function	References
HSV-1	miR-H2-3p	ICP0	Immediate-early protein	(Umbach *et al.*, 2008)
	miR-H3	ICP34.5	Neuro-virulence factor	
	miR-H4	ICP34.5	Neuro-virulence factor	
	miR-H6	ICP4	Immediate-early protein	
HSV-2	miR-I	ICP4	Immediate-early protein	(Tang *et al.*, 2008; Tang *et al.*, 2009)
	miR-II	ICP34.5	Neuro-virulence factor	
	miR-III	ICP34.5	Neuro-virulence factor	
HCMV	miR-UL112-1	IE1*	Immediate-early protein	(Grey *et al.*, 2007; Murphy *et al.*, 2008)
EBV	BART2	BALF5	Virus DNA polymerase	(Barth *et al.*, 2008)
	BART1-5p	LMP-1	Viral oncogene	(Lo *et al.*, 2007)
	BART16			
	BART17-5p			

* IE1 also known as IE72 or UL123

miRNA	Sequence (5' to 3')	Homology
JCV miR-J1-5p	UUCUGAGGCCUGGGAAAAGCAU-	57%
SV-40 miR-S1-5p	-UGAGGGGCCUGAAAUGAGCCUU	
JCV miR-J1-3p	UGCUUGAUCCAUGUCCAGAGUC	75%
SV-40 miR-S1-3p	-GCCUGUUUCAUGCCCUGAGU-	
HSV-1 miR-H2	CUGAGCCAGGGACGAGUGCGACU	73%
HSV-2 miR-III	UCUGAGCCUGGGUCAUGCGCGA--	
HSV-1 miR-H3	-UGGGGAUGUGCGGUUGGGA--	85%
HSV-2 miR-I	UUUGGGACUCUGCGGUUGGGAGC	
HSV-1 miR-H4	CUUGCCUGUCUAACUCGCUAGU	57%
HSV-2 miR-II	CGUGCUUGCCUAGCGAACUCA-	
miR-K12-11	UUAAUGCUUAGCCUGUGUCCGA-	57%
miR-155	UUAAUGCUAAUCGUGAUAGGGGU	

Figure 1.7 Conservation between different miRNAs

The miRNA sequences were extracted from miRBASE (http://www.mirbase.org/) and the seed region (2nd to 8th nucleotides were highlight with green color).

Table 1.7 Cellular targets of human viral miRNAs

Virus	miRNA	Target	Function	References
KSHV	miR-K12-5, -9, -10	BCLAF1	Pro-apoptotic factor	(Ziegelbauer *et al.*, 2009)
	miR-cluster (K12-1, -3-5p, -6-3p, -11 etc)	THBS1	Angiogenesis inhibitor	(Samols *et al.*, 2007)
	miR-K12-7	MICB	Natural-killer cell ligand	(Nachmani *et al.*, 2009)
	miR-K12-11	BACH1	Transcriptional suppressor	(Gottwein *et al.*, 2007; Skalsky *et al.*, 2007)
HCMV	miR-UL112	MICB	Natural-killer cell ligand	(Stern-Ginossar *et al.*, 2007)
EBV	BHRF1-3	CXCL11	Chemokine, T-cell attractant	(Xia *et al.*, 2008)
	BART5	PUMA	Pro-apoptotic factor	(Choy *et al.*, 2008)
	BART2-5p	MICB	Natural-killer cell ligand	(Nachmani *et al.*, 2009)

Abbreviation: BCLAF1: Bcl-2-associated factor (also called bcf);

THBS1: thrombospondin1;

MICB: MHC class I polypeptide-related sequence B;

BACH1: BTB and CNC homology1;

CXCL11: CXC-chemokine ligand 11;

PUMA: p53-up-regulated modulator of apoptosis.

1.4 AIM OF THESIS

With their limiting coding capacity, tiny miRNAs represent a particularly efficient and accessible tool for viruses to regulate specific gene expression. The recent discovery of EBV-encoded miRNAs in lymphoma cells and the identification of several cancer-related targets of miR-BARTs suggests the importance of EBV miRNAs in tumorigenesis including NPC (see previous section for information). Whereas miR-BARTs are important in complex virus-host interactions and contribute to human tumorigenesis, currently identified EBV miRNA are either cloned from EBV positive lymphoma cell lines or confirmed in lymphoma cells by computer prediction from the EBV sequence. Thus, EBV-encoded miRNA involved in NPC may have been missed in previous discovery processes. These "NPC specific viral miRNAs" may be important for NPC carcinogenesis.

In order to understand the contribution of EBV-encoded miRNAs in NPC development, we have three objectives in this thesis. The **first objective** is to systematically examine the local NPC-associated EBV genome for viral-encoded miRNAs. Such information would enhance our understanding of the potential role of EBV miRNA in NPC development. The distinct expression pattern of ebv-miRNAs may also serve as a potential diagnostic marker for NPC.

Based on the conventional small RNA library cloning methods developed by the Bartel lab (Lau *et al.*, 2001), we constructed complementary DNA (cDNA) libraries from a native EBV-positive NPC cell line (C666-1) and xenograft (X2117). In the libraries, we identified within the BART region two novel ebv-miRNAs, named miR-BART21 and miR-BART22. We also confirmed the high expression of these two miRNAs in NPC cell lines, xenografts and primary

tumor biopsies.

For the above findings, the **second objective** of my thesis is to explain the high expression of miR-BART21 and miR-BART22 in endemic NPC samples. By comparing sequences between different EBV strains, we identified several nucleotide variations in the flanking regions of mature miR-BART21 and miR-BART22. Among them, two nucleotide variations upstream of the mature miR-BART22 in the local EBV strain are likely to enhance its biogenesis by Drosha/DGCR8 processing.

By computational methods for miRNA targeting prediction, we found several interesting cellular targets for miR-BART22. This observation has led to the **third objective**, which is to identify and validate targets of the newly identified miRNAs using bioinformatic analysis and functional screening. The bioinformatics software we used for miRNA target prediction were miRanda (Enright *et al.*, 2003; John *et al.*, 2004) and RNA hybrid (Rehmsmeier *et al.*, 2004), as we had previous experience using these programs to identify EBV miRNA targets (Lo *et al.*, 2007; Choy *et al.*, 2008). We finally established that *LMP-2A*, an important viral latent gene, is one of the downstream targets of miR-BART22.

In summary, this thesis sought to characterize two novel ebv-miRNAs, miR-BARTR21 and miR-BART22, which are highly expressed in NPC. Specific sequence variations within the prevalent EBV strain in our locality might contribute to the high miR-BART22 expression level in our NPC samples. Further investigation of the functional role of miR-BART22 in modulating the expression of the viral *LMP-2A* gene may facilitate a deeper understanding of NPC development.

In summary, the aims of the study are follows:

1. To identify the ebv-miRNAs in NPC by constructing small RNA libraries from EBV-positive NPC cel line and xenograft.
2. To elucidate the possible contribution of sequence polymorphism in the biogenesis of ebv-miR-BART22.
3. To identify target(s) of ebv-miR-BART22.

CHAPTER 2: MATERIALS AND METHODS

2.1 General Materials

2.1.1 Reagents

Reagents	Company
3M Sodium Acetate	Ambion Inc
40% Acrylamide/bis 19:1 Gel Solution	BioRad Laboratories
40% Acrylamide/bis 29:1 Gel Solution	BioRad Laboratories
Bromophenol Blue	Sigma (Fluka) Company
Chloroform	BDH
Diethyl pyrocarbonate (DEPC)	Sigma Chemical Company
Dimethyl sulfoxide (DMSO)	Sigma Chemical Company
Ethanol	BDH
Ethidium bromide (EtBr)	Sigma Chemical Company
Formamide	Sigma Chemical Company
Gel loading buffer II (Denaturing PAGE)	Ambion Inc
GeneJuice transfection reagent	Novagen Inc
Glacial Acetic Acid	BDH
Glycerol	Sigma Chemical Company
Hi-Di Formamide	Applied Biosystems
Isopropanol	BDH
Lipofectamine™2000	Gibco Invitrogen Corporation
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma Chemical Company
Non-radioactive cell Assay	Promega Corporation
Nuclease free Water	Ambion Inc
Phenol/Chloroform/isoamyl alcohol 25:24:1	Gibco Invitrogen Corporation
Precision Plus Protein™ Standards (Dual Color)	BioRad Laboratories
Protein loading buffer pack (Western)	Fermentas Life Sciences
Sephadex™ G-50 Fine	Amersham Biosciences
Super optimal culture (S.O.C) medium	Gibco Invitrogen Corporation
SYBR Gold	Gibco Invitrogen Corporation
Trizol® reagent	Gibco Invitrogen Corporation
Trypan Blue	Gibco Invitrogen Corporation
Tween20	Sigma Diagnostic
Xylene Cyanol FF	Sigma (Fluka) Company

2.1.2 Chemicals

Chemicals	Company
4-Morpholinepropanesulfonic acid (MOPS)	Sigma Chemical Company
Agarose, regular	Biowest
Ammonium persulfate (APS)	Sigma Chemical Company
Boric Acid	BioRad Laboratories
Complete Protease Inhibitor Cocktail Tablets	Roche Applied Bioscience
Ethylenediaminetetraacetic acid (EDTA)	BDH
Glycine	Sigma Chemical Company
LB. Broth	USB Corporation
LB. Agar	USB Corporation
Phosphate buffer saline (PBS) pellet	Sigma Chemical Company
Sodium Chloride (NaCl)	BDH
Sodium Citrate	BDH
Sodium deoxycholate	Sigma Chemical Company
Sodium dodecyl sulfate (SDS)	BioRad Laboratories
Sodium floride (NaF)	Sigma Chemical Company
Sodium orthovanadate (Na_3VO_4)	EMD Biosciences
Trizma® base (Tris)	Sigma Chemical Company

1.1.3 Buffers

Prepared Buffers	Reagents
Blotting buffer, 10× (Western blot)	0.25M Trizma Base, 1.92M Glycine (10% Methanol were added for 1X blotting buffer)
Dissolving buffer, 8× (Western blot)	3M Trizma Base (pH8.8) (add SDS to 0.4% after autoclave)
DNA loading Dye, 6×	10mM Tris, 0.03% Bromophenol Blue, 0.03% Xylene Gyanol FF, 60mM EDTA (pH 8.0, 60% Glycerol
Oligo annealing buffer, 10×	100mM Tris (pH 8.0), 10mM EDTA, 1M NaCl
RIPA (Whole cell lysis buffer)	1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 10mM Sodium orthovanadate (Na_3VO_4), 50mM Sodium floride (NaF), Dissolving in PBS
Running buffer	1X Blotting buffer, 0.1% SDS
SSC solution, 20×	1 L of Stock solution containing: 175.3g NaCl; 88.2g Sodium Citrate (pH 7.0)
Stacking buffer, 4× (Western blot)	0.5M Trizma Base (pH6.8) (add SDS to 0.4% after autoclave)
Stripping buffer (Northern blot)	0.1%SDS and 0.1% SSC
Tris buffer saline (TBS)	150mM NaCl, 50Mm Tris. (pH7.5)
Tris buffer saline tween (TBST)	TBS, 0.01% Tween
ULTRAhyb-Oligo hybridization Buffer	Ambion

1.1.4 Cell Culture

Reagents and Culture Plastic	Company
Defined Keratinocyte-SFM (KSFM)	Gibco Invitrogen Corporation
Fetal Bovine Serum (FBS)	Gibco Invitrogen Corporation
Geneticin (G418)	Roche Applied Science
GlutaMAX™-I	Gibco Invitrogen Corporation
Karatinocyte-SFM(KSFM)	Gibco Invitrogen Corporation
Minimum Essential Medium (MEM)	Sigma Chemical Company
Opti-MEM® I Reduced-Serum Medium	Gibco Invitrogen Corporation
Penicillin-Streptomycin, 100x	Gibco Invitrogen Corporation
RPMI powder	Sigma Chemical Company
RPMI Solution	Gibco Invitrogen Corporation
Tissue culture flask (25, 80 cm^2)	BD Labware Company
Tissue culture plate (96-, 24- and 6-well)	Iwaki Company
Trypsin, 0.05% with EDTA	Gibco Invitrogen Corporation

1.1.5 Nucleic Acids

Nucleic acids	Company
Custom designed oligonucleotides	Gibco Invitrogen Corporation, Tech Dragon Ltd
Deoxyuncleotide Trisphosphate dNTP set	GE Healthcare
GeneRuler™ 1 kb DNA Ladders	Fermentas Life Sciences
miRNA mimics	Qiagen
GeneRuler™ Ultra Low Range DNA Ladders	Fermentas Life Sciences
SiRNA duplex	Qiagen
phi X-174 RF DNA-Hae III Digest	GE Healthcare
Taqman Probe for QRT-PCR	Applied Biosystems

1.1.6 Enzymes

Enzymes	Company
*EcoR*I	New England Biolabs
*Hind*III	New England Biolabs
*Spe*I	New England Biolabs
T4 DNA ligase	Gibco Invitrogen Corporation
T4 Polynucleotide Kinase (PNK)	New England Biolabs
Taq DNA polymerase	Fermentas Life Sciences
*Xba*I	New England Biolabs
*Xho*I	New England Biolabs

1.1.7 Equipments

Equipments	Company
7500 *Real-Time* PCR System	Applied Biosystems
7900HT Fast *Real-Time* PCR System	Applied Biosystems
9800 Fast Thermal Cycler	Applied Biosystems
ABI PRISM® 3130XL Genetic Analyzer	Applied Biosystems
NanoDrop-1000 UV-VIS Spectrophotomer	NanoDrop Technologies
Victor^{3TM} Multilabel Counter	Perkin Elmer

1.1.8 Kits

Kit	Company
BigDye Terminator Cycle Sequencing Kit	Applied Biosystems
DAB (,3'-diaminobenzidine) Detection Kit	Sigma Chemical Company
Dual Luciferase Reporter® Kit	Promega
EBV probe ISH kit	Novocastra, UK
Flag® Tagged Protein Immunoprecipitation Kit	Sigma Chemical Company
High Capacity cDNA Archive Kit	Applied Biosystems
MAXIscript kit	Ambion
microRNA Isolation Kit, Human Ago2	Wako Pure Chemical Industries, Ltd
miRCat^{TM} cloning Kit	Integrated DNA Technologies
MirVana^{TM} miRNA Isolation Kit	Ambion Inc
miScript Reverse Transcription Kit	Qiagen
miScript SYBR Green PCR Kit	Qiagen
PureLink^{TM} Hi Pure mini, midi prep Kits	Gibco Invitrogen Corporation
QIAquick Gel Extraction Kit	Qiagen
QIA PCR purification Kit	Qiagen
QIAamp DNA FFPE Tissue Kit	Qiagen
TaqMan Universal PCR Master mix	Applied Biosystems
TaqMan Universal PCR Master mix, No UNG	Applied Biosystems
TOPO TA Cloning® Kit	Gibco Invitrogen Corporation
Western Chemiluminescent HRP Substrate	Millipore

1.1.9 Software and Web Resources

Software and web resources	Company/URL
ABI PRISM® 3130 Genetic Analyzer Data Collection Software v1.1	Applied Biosystems
GenBank database	http://www.ncbi.nlm.nih.gov/BLAST
Image J software	http://rsb.info.nih.gov/ij/
MFOLD	http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi
MiRanda	http://www.microrna.org/
MiRBASE	http://microrna.sanger.ac.uk/
National Center for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/
Primer 3	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/

2.2 NPC Biopsies

Twenty-five nasopharyngeal carcinoma (NPC), three normal nasal pharyngeal (NP), nine Hodgkin's disease (HD) and one infectious mononucleosis (IM) endoscopic biopsies were used in both Northern blot analysis and immunohistochemical staining (IHC). The EBV DNA for the miR-BART22 sequence variation (Chapter 3) study was collected from the oral cavities of local NPC patients. All specimens were obtained from the patients prior to treatment with informed consent from the Prince of Wales Hospital, The Chinese University of Hong Kong. All biopsies were paraffin-embedded, sectioned at around 5μm and subjected to histological diagnosis by Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong. The presence of EBV in the tissue was detected by EBV-encoded small RNA (EBER) in situ hybridization (ISH).

2.3 NPC CELL LINES AND XENOGRAFTS

2.3.1Cell lines

A panel of NPC cell lines was commonly used in this study (Table 2.1). They included five NPC cell lines, namely C666-1, HK1, HK1-EBV, HONE1, HONE1-EBV and two immortalized normal nasal pharynx (NP) epithelial cell lines, NP69 and NP460. The C666-1 cell line, which consistently carried EBV in long-term culture, was established in our laboratory from an NPC xenograft (X666) derived from an undifferentiated NPC tumor biopsy (Cheung *et al.*, 1999). HK1 is a well-differentiated NPC cell line established from a recurrent squamous carcinoma of the nasopharynx of a Chinese male (Huang *et al.*, 1980). HONE1 was derived from the biopsy taken form poorly-differentiated squamous

cell carcinoma of the nasal pharynx (Glaser *et al.*, 1989). HK1-EBV and HONE1-EBV were established by co-culturing the corresponding parental cell lines with Akata cells which carry a recombinant EBV tagged green fluorescent protein (GFP) and a neomycin resistance gene (Neo^r). The cells were selected by G418, and the presence of EBV in the cells was confirmed with GFP expression (Lo *et al.*, 2006). NP69 and NP460 were two normal immortalized NP epithelial cell lines established by SV40T oncogenes and telomerase reverse transcriptase (hTert) integration, respectively (Tsao *et al.*, 2002; Li *et al.*, 2006). They were commonly used as the EBV-negative normal epithelial cell control.

For comparison purposes, EBV-positive marmoset leukocyte, B95.8, and three native EBV-containing human lymphoid cells, Akata, Namalwa and Raji, were included. They were available from the American Type Culture Collection (ATCC). For luciferase reporter assay analysis, human embryonic kidney cell lines HEK293 and 293FT, which is a HEK293 derivative expressing the simian virus 40 (SV40) T antigen, were purchased from ATCC and Gibco Invitrogen Corporation respectively.

Cell lines C666-1, HK1, HONE1, HK1-EBV, HONE1-EBV, B95.8, Akata, Namalwa, Raji were cultured in RPMI plus 10% FBS. To maintain the EBV genome inside the cells, G418 selection was applied for HK1-EBV (100μg/ml), HONE-EBV (300μg/ml) and Akata (300μg/ml). NP69 cells were grown in Keratinocyte serum free medium (KSFM) supplemented with prequalified human recombinant Epidermal Growth Factor 1-53 and Bovine Pituitary Extract (BPE). NP460 cells were cultured in medium containing KSFM and Defined KSFM in 1:1 ratio. Cell lines HEK293 and 293FT cells were grown in MEM containing 10% FBS. All cell lines were kept in humidified incubator with 5%

CO_2 at 37 °C. Cells at around 80% confluence were routinely split or harvested for RNA and protein extraction.

2.3.2 Xenografts

Four NPC xenografts, X666, X2117, C15 and C17, were used in the study. X666 and X2117 were established in our laboratory (Huang *et al.*, 1989). On the other hand, C15 and C17 were kindly provided by Prof. Pierre Busson at the Institut Gustave Roussy, France (Busson *et al.*, 1988). All the xenografts were continuously maintained by inoculating subcutaneously into nude mice. Xenografts removed from the mice were snap-frozen and stored at -80°C until protein or RNA extraction. The information of all NPC cell lines and xenografts are summarized in Table 2.1

Table 2.1 Summary of NPC cell lines and xenografts in the study

	Characteristic	EBV	Reference
Cell lines			
C666-1	Undifferentiated Carcinoma	positive	(Cheung *et al.*, 1999)
HK1	Well-differentiated squamous cell carcinoma	Negative	(Huang *et al.*, 1980)
HK1-EBV	Well-differentiated squamous cell carcinoma	Positive	(Lo *et al.*, 2006)
HONE1	Poorly-differentiated squamous cell carcinoma	Negative	(Glaser *et al.*, 1989)
HONE1-EBV	Poorly-differentiated squamous cell carcinoma	Positive	(Lo *et al.*, 2006)
NP69	Normal nasopharyngeal epithelial cells	Negative	(Tsao *et al.*, 2002)
NP460	Normal nasopharyngeal epithelial cells	Negative	(Li *et al.*, 2006)
Xenografts			
X666	Undifferentiated carcinoma	Positive	(Huang *et al.*, 1989)
X2117	Undifferentiated carcinoma	positive	(Huang *et al.*, 1989)
C15	Undifferentiated carcinoma	positive	(Busson *et al.*, 1988)
C17	Undifferentiated carcinoma	positive	(Busson *et al.*, 1988)

2.4 Induction of EBV lytic cycle in NPC cell lines

To compare the viral lytic effect with different agents, C666-1 and HK-EBV cells were treated with 100ng/ml of Gemcitabine (GEM, Eli Lilly and Company) alone, or together with either 300nmol/L of Valporic acid (VPA, Sigma Chemical Co.) or 100ng/ml Trichostatin A (TSA, Sigma Chemical Co.) for 48 days. Western Blots were performed as described in section 2.11 with anti-EA-D (BMRF1) antibody. The result of the lytic effect is shown in Figure 2.1.

2.5 Plasmid DNA preparation

2.5.1Plasmids

Construction of specific expression vectors will be discussed later in this chapter. The information of basal plasmids used in this study is listed in Table 2.2.

Table 2.2 Basal plasmids used in this study

Name:	Selectable marker	Vector Map	Source:
pcDNA3.1-(+)	Ampicillin	Figure 2.2	Gibco Invitrogen Corporation
pEGFP-C1	Kanamycin	Figure 2.3	Clotech company
pFLAG/HA-DGCR8	Ampillicin	Figure 2.4	Addgene Inc (Landthaler *et al.*, 2004)
pcDNA4/TO/cmycDrosha	Ampillicin	Figure 2.4	Addgene Inc (Landthaler *et al.*, 2004)
pRL-CMV	Ampillicin	Figure 2.5	Promega Corporation
pMIR-REPORT™	Ampillicin	Figure 2.5	Ambion Inc

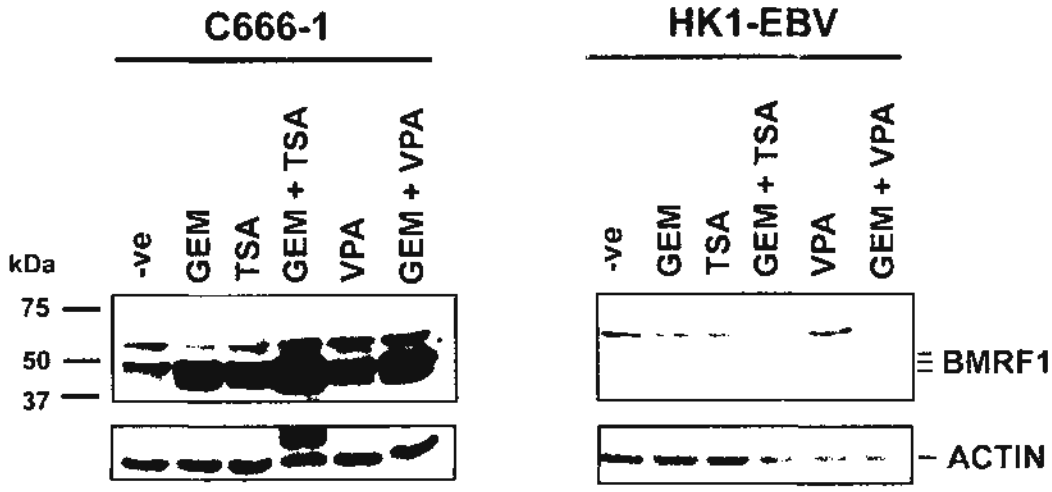
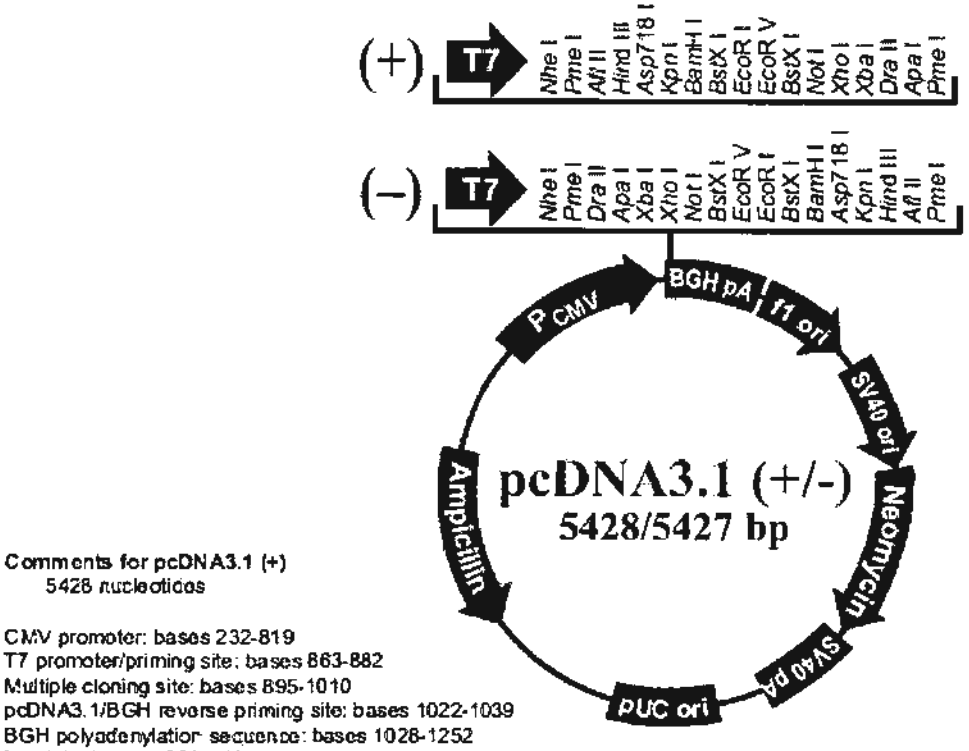


Figure 2.1 Lytic inductive effect of different agents in NPC cell lines

Expression of early viral lytic protein BMRF1 was detected by Western blot analysis. Cells with no treatment (-ve) were included for comparison. Expression of actin was included as a protein loading control. The three main detected forms of BMRF1 are indicated (48kDa unphosphorylated form and 50 and 52kDa phosphorylated forms) (Tsurumi, 1993).

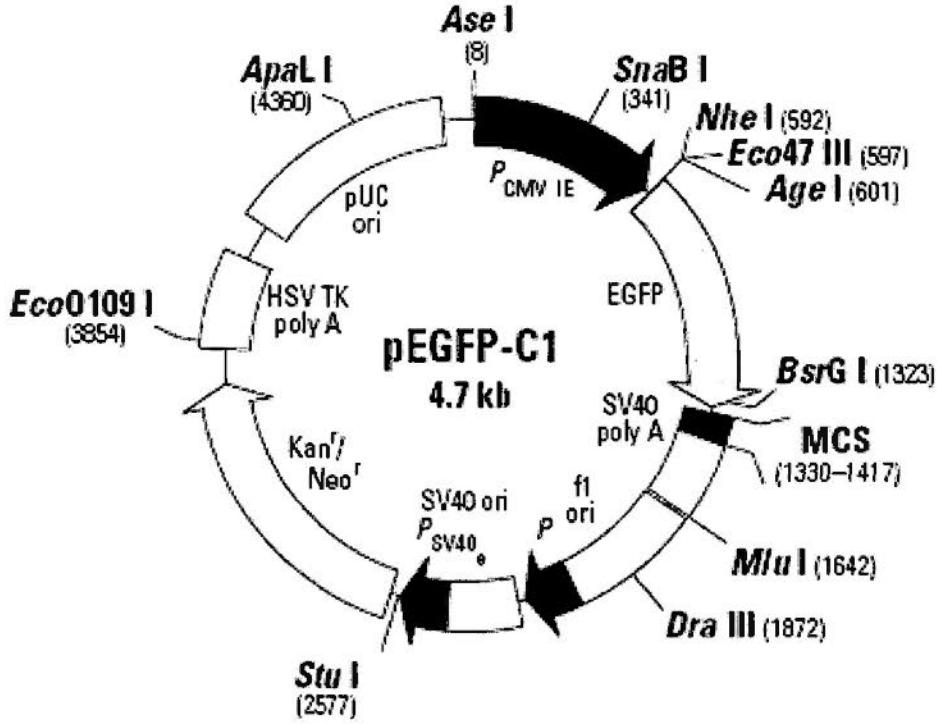


Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

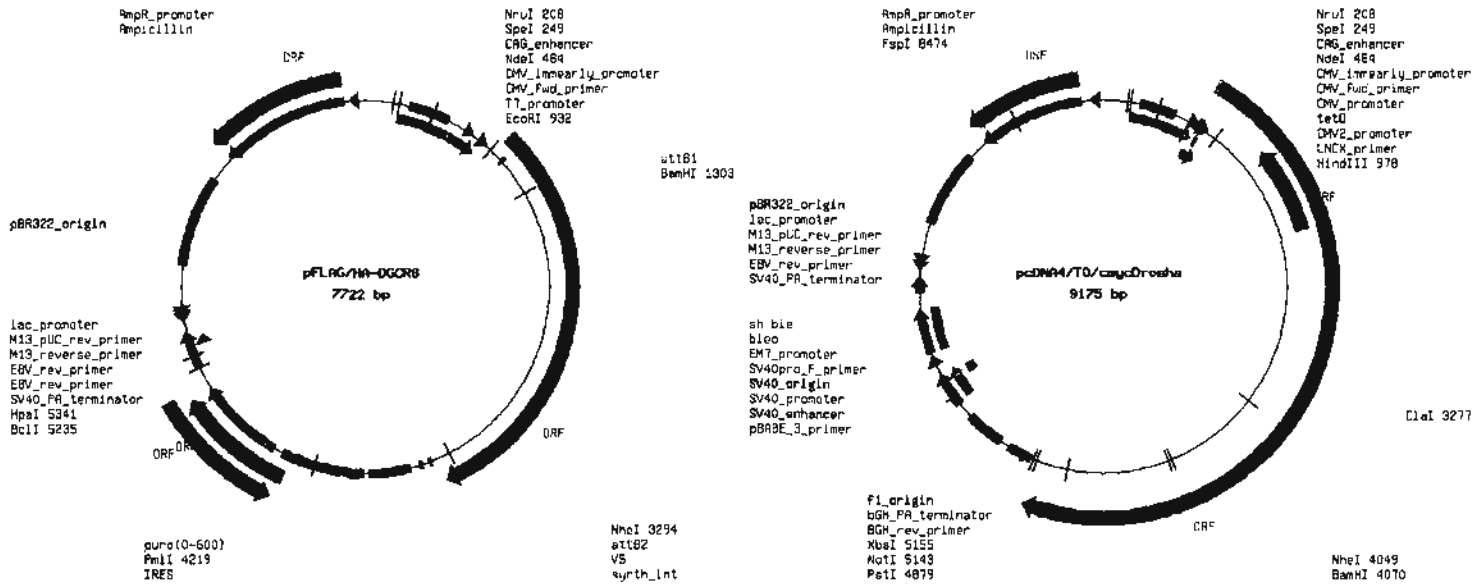
(Source: http://tools.invitrogen.com/content/sfs/manuals/pcdna3.1_man.pdf)

Figure 2.2 Restriction map of pcDNA3.1 (+/-)



(Source: http://www.clontech.com/images/pt/dis_vectors/PT3028-5.pdf)

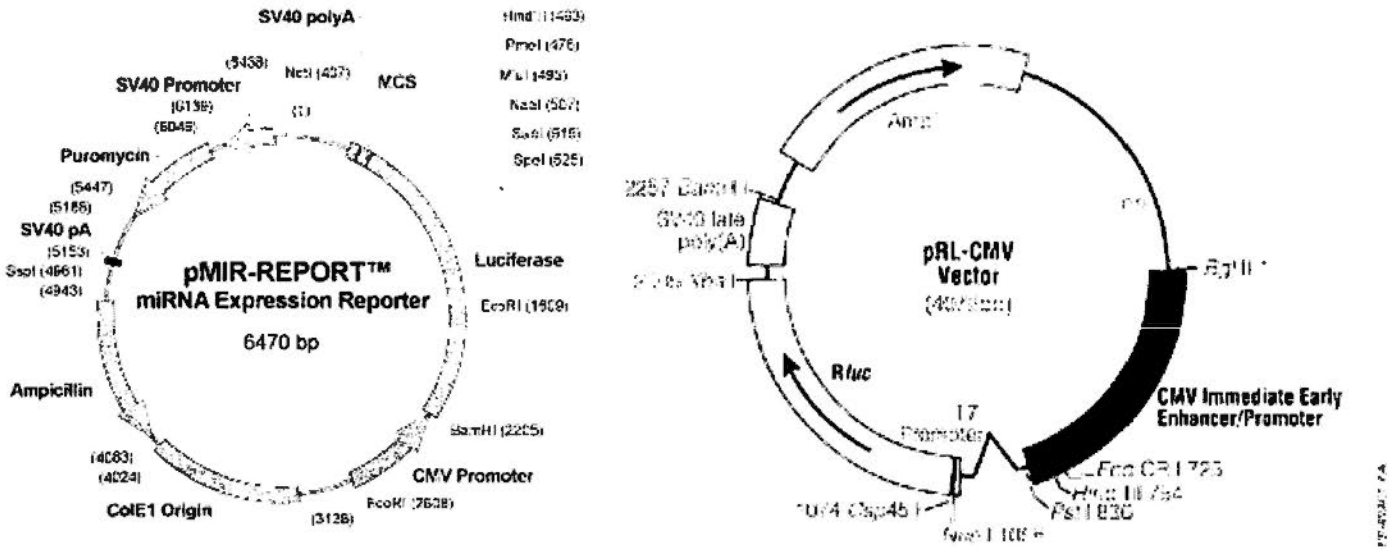
Figure 2.3 Vector map of pEGFP-C1



(Source: http://www.addgene.org/pgvec1?f=c&identifier=PUBMED.15589161&cmd=findpub)

(Landthaler *et al.*, 2004)

Figure 2.4 Vector maps of pFLAG/HA-DGCR8 and pcDNA4/to/cmycDrosha



(Source: pMIR-REPORT™: http://www.ambion.com/catalog/Image.php?639)

(Source: pRL-CMV: http://www.promega.com/catalog/catalogproducts.aspx?categoryname=productleaf_265)

Figure 2.5 Vector maps of pMIR-Reporter™ and pRL-CMV

2.5.2Preparation of Competent E.coli

A fresh bacteria colony (TOP-10 and DH5α) was incubated in 2 ml of L.Broth and grown for 3-4 hr. The bacteria were diluted into 100ml LB Broth and grown for about 2 hr until the OD_{550} reached 0.2. The bacterial culture was chilled on ice for 15 min and the pellet was harvested by centrifugation at 2500 rpm at 4°C for 15 min. The pellet was resuspended in 50ml of ice cold 0.1M $CaCl_2$ (pH 6.0) and kept on ice for 15 min to 2 hr. The bacteria were centrifuged again at 2500 rpm for 5 min, and resuspended in 5 ml fresh 14% glycerol and 0.1M $CaCl_2$. Aliquots of the suspension in Eppdendorf tubes were snap-frozen in liquid nitrogen and stored at -80°C until use.

2.5.3Bacterial Transformation

For transformation of the plasmid DNA, E.coli DH5α and TOP10 were used. The frozen competent bacteria (see 2.5.2) were thawed slowly on ice. Around 500 ng of plasmid DNA was added into 50μl of bacterial suspension and kept on ice for 10 min. The competent cell-DNA mixture was then heated at 42°C for 2 min or 37°C for 4 min, diluted to 250μl of L.Broth or S.O.C. medium followed by shaking at 37°C for 45 min at 200 rpm. One hundred micoliters of mixture was plated on the L.Broth plate in the presence of suitable antibiotic (100μg/ml of ampicillin or 50μg/ml of kanamycin). The transformed bacteria were incubated overnight at 37°C for colony selection.

2.5.4Plasmid DNA Extraction

For small scale plasmid DNA preparation, a single bacterial colony was inoculated into 3ml of LB Broth medium with a suitable concentration of

antibiotic for at least 8 hr at 37°C with shaking. The bacterial culture was pelleted by high speed centrifugation and resuspended in 0.1ml of solution 1 (50mM glucose, 25mM TrisCl pH8.0 and 10mM EDTA pH8.0) for incubation at room temperature for 5 min. Afterwards, 0.2ml of solution 2 (0.2M NaOH and 1% SDS) was added to the suspension with gentle mixing, and the mixture was kept on ice for 10 min. The mixture was mixed gently with 0.15ml of ice-cold solution 3 (3M potassium acetate and 0.1M glacial acetic acid) and incubated on ice for 5 min for neutralization. The debris was centrifuged down and the supernatant was precipitated with 0.7 volume of isopropanol. The DNA-isopropanol mixture was incubated at room temperature for 2 min, and the DNA was pelleted by centrifugation at full speed for 30 min at 4°C. The DNA pellet was air dried and dissolved in 40µl of water. Large scale plasmid DNA preparation was done by PureLink™ Hi Pure midi preparation Kits (Invitrogen) following the manufacturer's instructions.

2.6 PAGE Northern Blot Analysis

2.6.1Polyacrylamide Gel Electrophoresis for miRNA

To prevent RNase contamination, the gel plate and apparatus were soaked with 0.5M NaOH for 15 min and then rinsed thoroughly with RNase-free water. The 12% polyacrylamide/8M Urea denaturing gel was prepared from a 15 ml mixture of 7.2g urea, 1× TBE, 4.8ml of 40% acrylamide (acryl:bis acryl = 19:1), 75µl of 10% APS and 15µl TEMED.

Ten micrograms of total RNA was denatured with an equal volume of 2× Gel Loading buffer II (Ambion Inc.) at 95°C for 5 minutes and chilled on ice immediately. The sample was then resolved on a 12% polyacrylamide/8M Urea

gel using the Bio-Rad Protein II minigel system. The electrophoresis was performed at constant voltage (140V) until the faster dye reached the bottom of the gel. The gel was removed from the glass plate, stained with SYBR GOLD for 3-10 minutes and the RNA stained image was recorded. The gel was then rinsed with 0.5× TBE. RNA in the gel was electrophoretically transferred onto a Nytran Supercharge membrane (Schleicher & Schuell, Dassel, Germany) using a semi-dry electroblotter (BioRad Laboratories) at a constant voltage of 20V for 1 hr. The transferred RNA on the membrane was fixed by UV cross-linking twice at 1200× 10μJ for 30 sec using a UV crosslinker (Stratagene).

2.6.2Probe Labeling

Oligonucleotides complementary to target RNA sequences were end-labeled with γ-32P ATP as probes for Northern blots. Fifty microliters of the labeling mixture containing 1.5μl of 10μM oligonucleotides, 15U of T4 PNK and 5μl of 10× PNK buffer were incubated at 37°C for 1 hour followed by 70°C for 15 min to stop the reaction. The mixture was directly used for hybridization. Sequences of the oligonucleotide probes for the Northern blot are listed in Table 2.3.

Table 2.3 Sequences of oligonucleotide probes for Northern blot

Target miRNAs	Sequence (5'→ 3')	Size
hsa-miR-16	CGCCAATATTTACGTGCTGCTA	22
ebv-miR-BART21	GTTAGTTGCCTTCACTAGTG	20
ebv-miR-BART22	ACTACTAGACCATGACTTTGTAA	23
ebv-miR-BART1-5p	CAGCACGTCACTTCCACTAAGA	22
ebv-miR-BART2-5p	GCAAGGGCGAATGCAGAAAATA	22
ebv-miR-BART5	GATGGGCAGCTATATTCACCTT	22
ebv-miR-BART10	TGTACAGAACCAAAGAGGTGGC	22
ebv-miR-BHRF1-1	AACTCCGGGGCTGATCAGGTTA	22
ebv-miR-BHRF1-2	TCAATTTCTGCCGCAAAAGATA	22
ebv-miR-BHRF1-2*	GCTATCTGCTGCAACAGAATTT	22
ebv-miR-BHRF1-3	TGTGCTTACACACTTCCCGTTA	22
U6 RNA	GCAGGGGCCATGCTAATCTTCTCTGTATCG	30

2.6.3Hybridization and Signal Detection

` Membrane were pre-hybridized with 5ml of ULTRAhyb®-oligo hybridization buffer at 42°C for 1 hr. Labeled probe (section 2.6.2) were then added directly to the membrane in hybridization buffer with for overnight hybridization at 30°C.

After hybridization, the membrane was rinsed twice with primary wash buffer (6X SSC/0.2% SDS), followed by washing 3 times with the same buffer at room temperature for 5 min each, then washed once with 2× SSC/0.5% SDS at 42°C for 15 min. After the final wash, the blot was wrapped in plastic wrap and exposed to the X-ray film (Fujifilm Corporation) at -80oC overnight. The exposure time was dependent on the expression level of the target miRNA.

2.7 Total DNA and RNA Extraction

2.7.1DNA Extraction

For sequencing analysis, DNA was purified from different samples including FFPE tissue, flesh biopsies, xenografts and cell lines. QIAamp DNA FFPE Tissue Kit (Qiagen) was used to extract DNA from FFPE tissue following

the manufacturer's protocol.

Homogenized biopsies, xenografts and cell lines were washed twice with 1× PBS and then resuspend in 10ml nucleic lysis buffer (10mM Tris-Cl, 0.4M NaCl and 5mM EDTA). Following centrifugation at 1000 rpm for 15 min at 4°C, the pellet was collected and resuspended in 3ml nucleic lysis buffer together with 120µl proteinase K (10mg/ml) and 400µl 10%SDS. Samples were digested at 55°C overnight. Following proteinase digestion, the supernatant was transferred into a new 15ml tube and DNA was precipitated by adding 350µl 3M sodium acetate (pH 5.2) and 10ml ice cold 100% isopropanol with gentle shaking. The DNA precipitate was transferred into a new 15ml tube containing 10ml of 75% ethanol. The DNA was rocked for an hour to remove salt and then transferred into a new 1.5ml tube. The DNA pellet was dried by incubating at 65°C for 15 min. The DNA was resuspended in 100µl of TE buffer and its concentration was determined using a NanoDrop-1000 UV-VIS Spectrophotomer.

2.7.2RNA Extraction

For quantitative analysis, total RNA was extracted from cell lines, xenografts and biopsies using TRIzol® reagent. Homogenized xenografts, biopsies and cell lines at 80% confluence in culture dishes were washed twice with 1× PBS and then mixed with 1 ml TRIzol reagent. Samples were incubated at room temperature for 5 min to permit complete dissociation of nucleoprotein. Chloroform was added at a ratio of TRIzol: Chloroform, 10:2. The mixture was vigorously vortexed for 15 sec and allowed to stand at room temperature for 3-5 min. Following centrifugation at 14000 rpm for 15 min at

4°C, the aqueous phase containing RNA was carefully collected and precipitated by isopropanol in a ratio TRIzol: Isopropanol, 2:1. The mixture was kept at room temperature for 10 min and then centrifuged at 14000 rpm for 30 min at 4°C. The RNA pellets were dried at 65°C for 15 min and resuspended in nuclease free water. The concentration of RNA was determined using a NanoDrop-1000 UV-VIS Spectrophotomer. All RNA was kept at -80°C. The extraction of a small RNA-enriched fraction will be discussed in section 2.18.1.

2.8 Quantitative RT-PCR (QRT-PCR)

For traditional QRT-PCR, one microgram RNA was reverse transcribed with High Capacity cDNA Archive Kit following the manufacturer's protocol. In brief, the 15μl reaction mixture contained RNA, 0.15μl of 100mM dNTP, 1.5μl of 10× random primers (Invitrogen), 0.19μl of 20U/μl RNase inhibitor, and 1μl of 50U/μl MultiScribe™ Reverse Transcriptase. The reaction was performed at 16°C for 30 min, 42°C for another 30 min and 85°C for 5 min. All cDNA were stored at -80°C until use.

For SYBRGreen QRT-PCR, 10μl of PCR reaction containing 1μl of cDNA, 5μl of 2× SYBRGreen Reaction mix, 0.2μl of each of the 10μM forward and reverse primers was amplified. If a Taqman probe was used for the reaction, one micro-liter of cDNA was added to the 10μl PCR reaction containing 5μl of 2× Taqman® Universal PCR Master Mix, 0.05μl of 10μM Taqman probe, 0.2μl of each of the 10μM with as the follow thermal cycling condition: 95 °C for 10 min, 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. The threshold cycle number (Ct) at which the emission intensity of fluorescent signal was detected. The specificity of the SYBRGreen PCR product was confirmed by adding

dissociation steps at the end of PCR reaction, in which the PCR products were subjected to an increase in temperature from 55°C to 95°C and the fluorescence measurements were taken at each temperature increment. The fluorescent signal was plotted versus temperature with a single peak on the plot around 80°C indicating the specificity of the PCR product.

For miRNA QRT-PCR, the miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit were used according to the manufacturer's instructions. The principle of miScript is illustrated on Figure 2.6. In brief, 10μl of reverse transcription reaction mixture containing 1μg of RNA, 0.5μl of miScript Reverse Transcriptase Mix and 2μl of 5× miScript RT buffer were incubated at 37°C for 60 min, then 5 min at 95 °C to inactivate any enzyme activities. Thirty microliters of nuclease free water were added to dilute the cDNA for real-time PCR. The PCR reaction (10μl) contained 5μl of 2× QuantiTect SYBR Green PCR Master Mix, 1μl of 10× miScript Universal primer, 0.5μl of 10μM miRNA specific primer and 1μl of cDNA sample was performed with the following thermal cycling condition: 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 sec, 55°C for 30 sec and 60°C for 30 sec. The results of the relative fold difference in the target gene expression relative to the house-keeping gene (β-actin or RNU6B snRNA) were calculated using $2^{-\Delta\Delta Ct}$. Analysis of each sample was performed in triplicate. The primers used for QRT-PCR are listed in Table 2.4

Table 2.4 Primers for QRT-PCR

Primer name	Sequence (5'→ 3')	Size	Melting temp.
ACTIN-69F	CTGGCACCCAGCACAATG	18bp	57°C
ACTIN-69R	GCCGATCCACACGGAGTACT	20bp	59°C
BRLF1-F	AATTTACAGCCGGGAGTGTG	20bp	56°C
BRLF1-R	AGCCCGTCTTCTTACCCTGT	20bp	58°C
BZLF-F	GCACATCTGCTTCAACAG	18bp	52°C
BZLF-R	CGTGAGGTCAGTATATAC	18bp	47°C
EBNA1-1162F	TCATCATCATCCGGGTCTCC	20bp	56°C
EBNA1-1229R	CCTACAGGGTGGAAAAATGGC	21bp	56°C
EGFP 82F:	CGACAACCACTACCTGAGCA	20bp	57°C
EGFP 82R:	GAACTCCAGCAGGACCATGT	20bp	57°C
Notch 1-F:	CCGCAGTTGTGCTCCTGAA	19bp	58°C
Notch 1-R	ACCTTGGCGGTGTCGTAGCT	20bp	61°C
LMP2A-F	CGGGATCACTCATCTGAACACATA	24bp	56°C
LMP2A-R	GGCGGTCACAACGGTACTAACT	22bp	59°C
Taqman Probe[1]	(FAM)-CAGTATGCCTGCCTGTAATTGTTGCG-(TAMRA)	26bp	--
Spec- RNU6B	ACGCAAATTCGTGAAGCGTT	20bp	56°C
Spec-BART10	TACATAACCATGGAGTTGGCTGT	23bp	56°C
Spec- BART21	CACTAGTGAAGGCAACTAAC	20bp	51°C
Spec-BART22	TTACAAAGTCATGGTCTAGTAGT	23bp	51°C

[1] The primers and probe designed for LMP-2A QRT-PCR were refer to (Bell *et al.*, 2006)

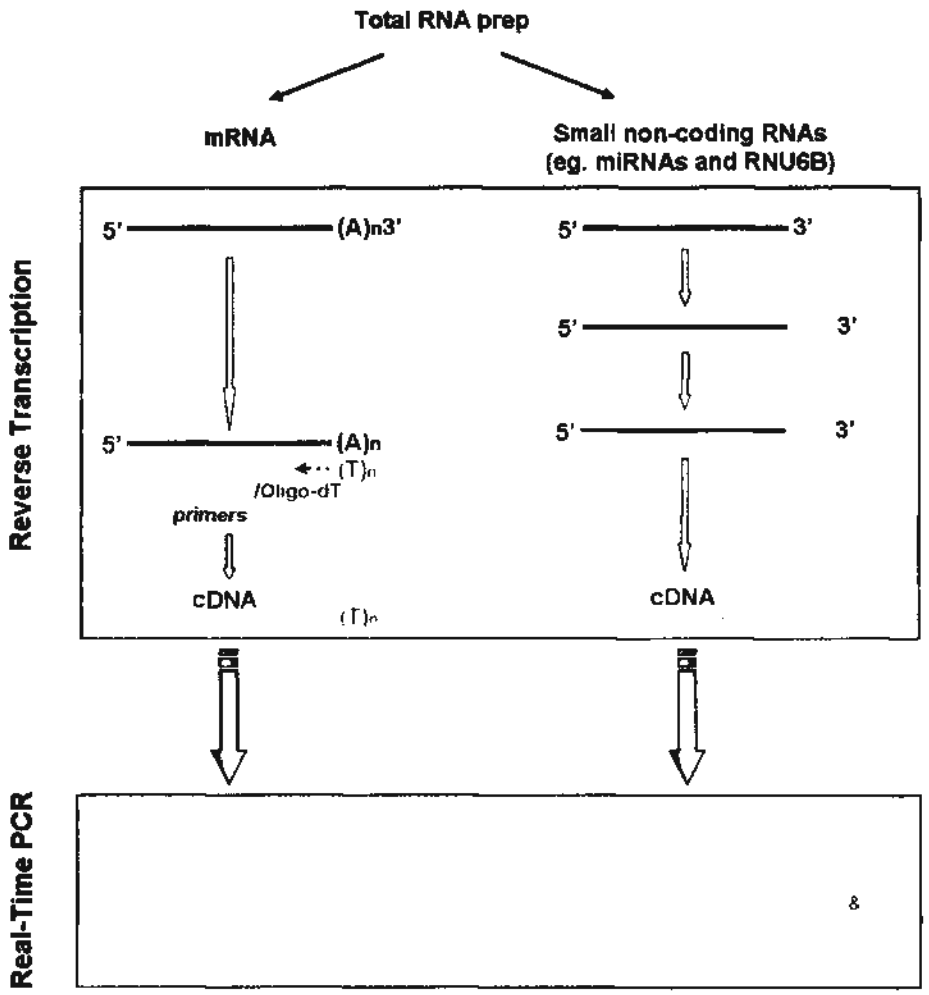


Figure modified from (miScript System Handbook, Qiagen)

Figure 2.6 miScript principle

RNAs are polyadenylated by poly(A) polymerase and subsequently converted into cDNA by reverse transcriptase with oligo dT priming in miScript Reverse Transcriptase Mix (Step 1). The cDNA can be used for real-time PCR quantification of both miRNA (using miRNA specific primer and miScript Universal Primer) (*right panel*) and specific mRNA (using gene-specific primers) (*left panel*).

2.9 hAGO-2 Immunoprecipitation

Immunoprecipitation (IP) was performed in C666-1 cells using the anti-hAGO-2 antibody microRNA Isolation Kit following the manufacturer's protocol (Wako Company). This antibody has been confirmed to work efficiently for IP (Azuma-Mukai *et al.*, 2008). Anti-Flag M2 antibody (Sigma Chemical Company) was used in a parallel experiment as the negative control. In brief, the cell pellet collected from 2 plates of 50% confluency was resuspended in 2.2 ml cell lysis buffer (Wako) and kept on ice for 5 min. Afterwards, the mixture was centrifuged at 14000 rpm at 4°C for 20 min. The supernatant was split into three aliquots, 1 ml for hAGO-2 bead IP, 1 ml for Anti-Flag M2 bead IP, and 0.2 ml was kept as input total RNA for comparison. The antibody beads for the reaction were pre-washed as described in the manufacturer's protocols before adding to the cell lysate. The antigen-antibody reaction was mixed by rotator overnight at 4 °C followed by purification of the miRNA fraction. Total RNA was extracted with the TRIzol method and dissolved in 20μl of nuclease free water as described in section 2.7. Four micro-liters of RNA were loaded for Northern blot analysis.

2.10 Protein Extraction

Cells were washed twice with PBS, harvested and centrifuged in a microfuge. The cell pellet was resuspended in 100μl RIPA buffer (section 2.1.3), supplemented with 1× protease inhibitor cocktail (Roche). The mixture was freeze-thawed twice using liquid nitrogen and 37°C and then left on ice for 30 min. The mixture was subsequently centrifuged at 14000 rpm at 4 °C for 15 min to remove all cell debris. The protein supernatant was transferred to a new

1.5ml tube. The protein concentration was determined by BCA Protein Assay Reagent (Thermo Scientific) with reference to serial dilutions (0.1-2mg/ml) of bovine serum albumin (BSA). The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein (Smith *et al.*, 1985).

2.11 Western Blot Analysis

Proteins were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a mini protein gel apparatus (BioRad). Twenty micrograms of protein samples were mixed with 5× protein loading buffer, denatured at 95°C for 5 min and chilled on ice for 5 min before loading. Samples were run in 1× running buffer at a constant voltage of 120V. Precision Plus Protein™ Standards (BioRad) were used as size markers.

Following electrophoresis, the proteins in the gel were transfered to a PVDF membrane (GE Healthcare Life Science) using a Mini Trans-Blot® Cell at approximately 120V for 1 hr in 1× blotting buffer. The membrane with transfered proteins was then incubated with 5% dried milk in 1× TBST for 30 min to block non-specific protein binding, followed by incubating overnight at 4 °C or 1 hr at room temperature with the primary antibody in 5% dried milk (or BSA) in 5 ml of TBST. The concentrations of antibodies used in Western blots are listed in Table 2.5. After washing the membrane with three changes of 20 ml TBST at room temperature, the membrane was incubated in secondary antibody diluted in 5% dried milk in 5ml of TBST. Excess antibody was removed by washing the membrane as before. Antibody conjugated signals were visualized by exposure to X-ray film using Western Chemiluminescent

HRP Substrate (Millipore). All information about the buffer preparations and materials used in Western blot is described in Section 2.1.1.

Table 2.5 Concentrations of antibodies used in Western blot

1° Antibodies	Company	Dilution
Mouse monoclonal Anti-EBV EA-D (BMRF1) (6D1)	(Chemicon) Millipore.	1000
Mouse monoclonal anti-GAPDH (G8795)[1]	Sigma Chemical Co.	200000
Mouse monoclonal anti-β-Actin (AC15)	Sigma Chemical Co.	200000
Mouse monoclonal anti-E2F3 (PG30)	Sigma Chemical Co.	1000
Mouse anti-EBV Transcription Factor R (BRLF1)	ARGENE	1000
Mouse anti-EBV Z Replication Activator (ZEBRA)	ARGENE	1000
Mouse monoclonal Anti-AGO2 antibody (9E8.2)	(Upstate) Millipore	5000
Mouse monoclonal GFP	Clontech Company	100000
Rabbit monoclonal Anti-AKT (#9272)	Cell Signaling	2000
Rabbit monoclonal anti-phospho-AKT(Ser473) (#9271)	Cell Signaling	2000
Rat monoclonal LMP2A (15F9)	AbD SeroTec.	2000
Biotinylated 2° Antibodies		
Polyclonal goat anti-rabbit	DAKO Company	250000
Polyclonal rabbit anti-mouse[1]	DAKO Company	250000
Polyclonal rabbit anti-rat	DAKO Company	250000

[1] To detect GAPDH, membrane incubated with primary/secondary antibody for 15 min at room temperature is enough.

2.12 Immunohistochemistry (IHC)

The protocol for LMP-2A Immunohistochemical staining is described in Heussinger et al. with some modification (Heussinger *et al.*, 2004). In brief, the 5μm paraffin sections were dewaxed in xylene and rehydrated in a series of graded ethanol series and finally washed in TBS. Endogenous peroxidase activity was blocked with 3% H_2O_2 at room temperature for 20 min. Antigen retrieval was performed by using a pressure cooker with 10nM citrate buffer (pH 6.0) for 4 min. The tissue sections were then washed with TBS and blocked with serum in TBS with 3% BSA for 10 min. Primary antibody against LMP-2A (15F9, AbD SeroTec; 1:50 dilution) was applied and incubated for 2 hr. Subsequently, the section was incubated with biotinylated secondary antibody (DAKO; 1:100 dilution) for 1 hr. The signal was visualized by using the 3, 3'-diamino-benzidine DAB detection kit (Sigma) and hematoxylin counter staining was performed. The presence of EBV in the paraffin sections was confirmed by EBER *in situ* hybridization which was carried out with an EBV probe ISH kit (Novocastra, Newcastle, U.K.).

2.13 Cell Poliferation Assay

The cell proliferation rate was measured by non-radioactive cell Assay kit (Promega Corporation). Briefly, 2×10^3 transfected cells were distributed in a 96-well plate and incubated at 37°C with 5% CO_2. Cell viability was estimated by adding 15μl of dye solution to each well and incubation at 37°C for 4 hours to allow the cells to convert the tetrazolium of the dye solution into a formazan product. Afterwards, 100μl of solubilization solution was added to solubilize the formazan product. The absorbance at OD_{570} was recorded using a Victor^{3TM}

Multilabel Counter.

2.14 Flow Cytometry

All cells after transfection were grown in normal culture medium. The cells should grow to about 60% confluency at the time of harvesting for analysis. The cells were washed in 5 ml ice-cold PBS and finally resuspended in 100 µl cold PBS, followed by gently adding 5 ml of ice-cold 70% EtOH. Cell suspensions were then fixed by incubation at 4 °C overnight or at least 30 min. The cell pellet was collected by centrifugation at 1000 rpm for 5 min; it was then stained in 800 µl of PBS containing 50µg/ml propidium iodide (Sigma) and 10µg/ml of RNaseA. Flow cytometric analysis was performed using a flow cytometer FACS Calibur (BD Biosciences, CA, USA) after incubating the samples at 37 °C for 30 min. Data were analyzed by Modfit LT™ with standard settings.

2.15 Transfection

Cells for transfection were normally growth to 50-80% confluency. Plasmid transfections on 293FT and HEK293 cells were performed using GeneJuice transfection reagent (Novagen) following the manufacturer's protocol. The ratio of GeneJuice: DNA was 3µl: 1µg unless otherwise specified. For each well in a 24-well plate format, 0.75µl GeneJuice was mixed with 20µl Opti-MEM® medium and incubated at room temperature for 5 min. Following incubation, 0.25µg of plasmid DNA was added and the GeneJuice-DNA mixture was further incubated for another 15 min. The entire mixture was added dropwise to the cells in normal culture medium; the plate was then gently rocked

to ensure even distribution. To transfect cells in a 6-well plate and 100mm dish format, the volume of the mixture prepared for transfection was increased by 4- and 24-fold, respectively. To normalize the transfection efficiency for some comparative experiments, 10% pEGFP-C1 was included in the transfection mixture.

All siRNA duplex and miRNA mimics were purchased from Qiagen. The target sequence of siLMP-2A with dTdT overhang modification was AACUCCCAAUAUCCAUCUGCU (Bell *et al.*, 2006). The sequences of the miRNA mimics used are listed in Table 2.6. Allstars negative control (Qiagen) was used for both siRNA and miRNA transfection experiments. In the experiments, 20nM siRNA or miRNA mimic were used to transfect cells in a 6-well plate format. Protein and RNA were extracted 24 hours post-transfection. All transfections were carried out by lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. In a 24-well plate format, 0.6μl of 20μM RNA to 1μl of lipofectamine™ 2000 was mixed in 100μl of Opti-MEM® medium, and incubated at room temperature for 15 min; the mixture was then evenly added to the culture cells.

Table 2.6 Sequences of miRNA mimics

Name of miRNA mimic	Sequence (5'→3')	Length (nt)
miR-BART12	UCCUGUGGUGUUUGGUGUGGUU	21
miR-BART22	UUACAAAGUCAUGGUCUAGUAGU	23
miR-M2-BART22	UAAGAUACUCAUGGUCUAGUAGU	23
miR-M3-BART22	AUUCUAUGUCAUGGUCUAGUAGU	23

2.16 DNA SEQUENCING ANALYSIS

All DNA sequencing reactions were performed with the Big Dye Terminator v1.1 Cycle sequencing kit. In a 10μl sequencing reaction, 1μl of Big Dye, 0.32μl of 10μM sequencing primer, and 1.5μl 5× sequencing buffer were mixed together with 100ng plasmid DNA or 0.5μl PCR products amplified from genomic DNA. The sequences of some common sequencing primers are listed in Table 2.7. The sequencing reaction was carried out in 9800 Fast Thermal Cycler (Applied Biosystems) with thermal cycling condition as follows: 1 min at 96°C, followed by 30 cycles of 10sec at 96°C, 5sec at 50°C and 1min at 60°C. To remove unincorporated nucleotides, sequencing products were purified with Sephadex™ G-50 (GE Healthcare Life Science). The eluted product was mixed with 10μl of Hi-Di formamide, denatured at 95°C for 5 min and chilled on ice for another 5 min prior to the sequencing analysis. The sequences of the samples were analyzed using an ABI PRISM® 3130XL Genetic Analyzer.

Table 2.7 Sequences of sequencing primers

Primer Name	Sequence (5'→3')	Size (nt)	Tm (°C)
pMIR-REPORTERTM vector			
pMIR-F1	AGGCGATTAGTTGGGTA	17	58
pMIR-R2	CGGAGGAGTTGTGTTTGT	18	57
pcDNA3.1-(+)			
CMV-F	TCTAAAAGCTGCGGAATTGT	20	53
T7-F	TAATACGACTCACTATAGGG	20	50
BGH10-R	TAGAAGGCACAGTCGAGG	18	54

2.17 Small cDNA Library

2.17.1 Extraction of the small RNA fraction (<200 nt)

The enriched small-RNA fraction for cDNA cloning was collected with the miVana miRNA Isolation Kit according to the manufacturer's instructions (Ambion). Two plates of 50% confluence C666-1 and 10mg of X2117 xenograft were used as the starting materials for extraction. The RNA samples were finally eluted in 50 µl of nuclease free water and the concentrations were determined by a NanoDrop-1000 UV-VIS Spectrophotomer. Twenty micrograms of enriched RNAs were used for library construction.

2.17.2 Library Construction

The small RNA libraries were constructed by using miRCat^{TM} cloning kit (Integrated DNA Technologies, IA), which was modified from the most common small RNA cloning method (Lau *et al.*, 2001). The outline of the procedure is shown in Figure 2.7. In brief, both a 3'-acetylated linker and a 5' linker were ligated to small RNAs for cDNA synthesis followed by PCR amplification and cloning. An internal RNA control (miSPIKE^{TM}) of 21-nts was included as a size marker during RNA purification and 3' linker ligation reaction. Due to the absence of a 5' phosphate group on the miSPIKE^{TM}, this internal control would be eliminated in the subsequent cloning steps. In order to increase the sequencing efficiency, the small RNAs (miRNA + linker) were serially ligated (concatemerized) before cloning. All cloning used the TOPO-TA cloning® kit and sequencing was performed using an ABI PRISM® 3130xl DNA Sequencer.

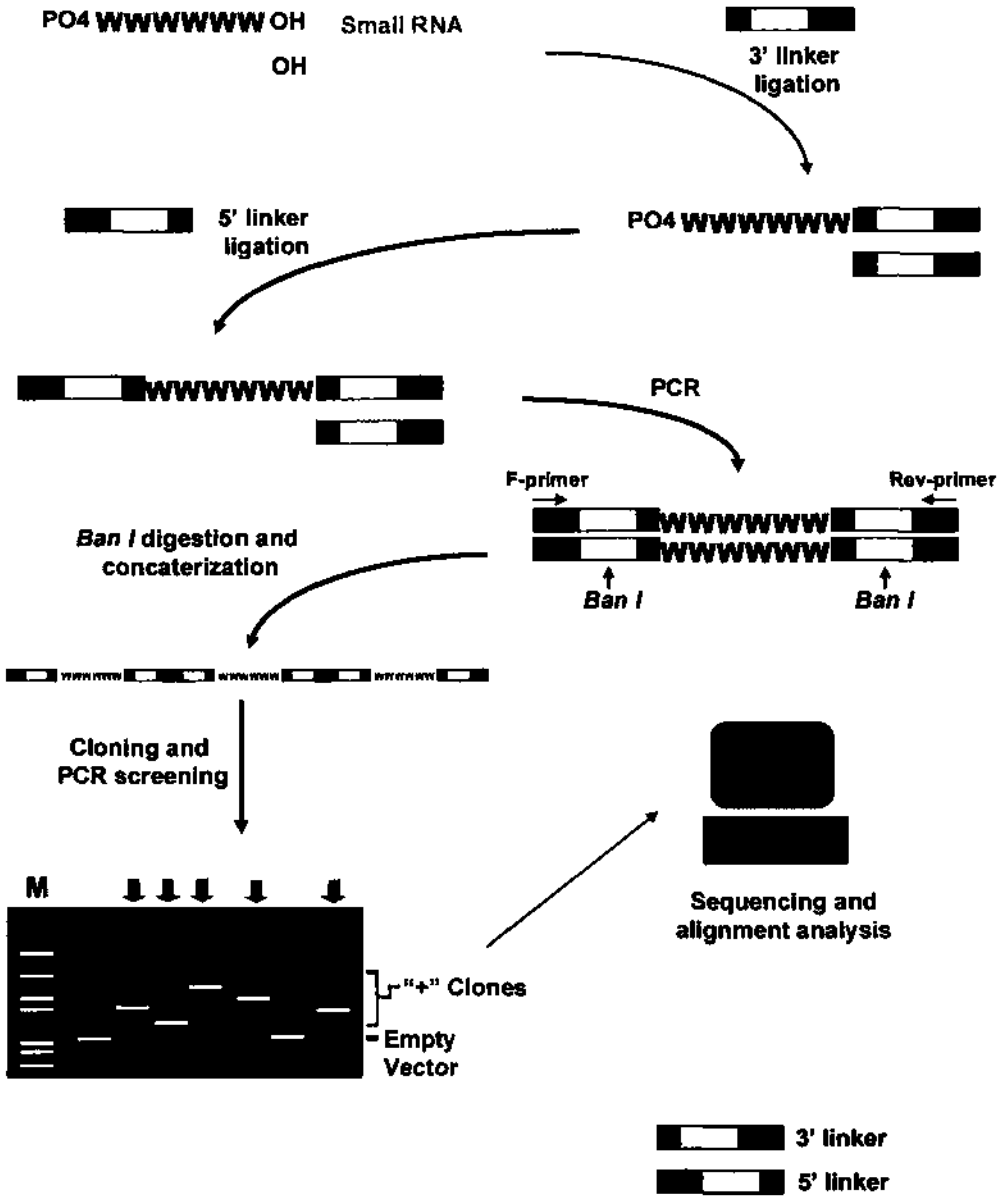


Figure 2.7 Outline of the library construction

The construction of a small RNA library is illustrated in this figure. The red arrows in the cartoon gel photo indicate the positive clones for sequence analysis.

2.17.3 Identification and Structure Prediction of miRNAs

Isolated RNA fragments longer than 18nt were annotated to the genome. Known miRNAs were identified by blasting the sequences to "miRBase" (Grundhoff *et al.*, 2006). The remaining sequences were individually blasted to NCBI databases. Putative precursor sequences of the cloned EBV fragments were extracted from the EBV genome (AJ507799) and further examined for their pre-miRNA structures by using the MFOLD program (Zuker, 2003). The most stable predicted structure was trimmed to a size of less than 80 nucleotides.

2.18 *In Vitro* Drosha Digestion

2.18.1 In vitro primary miRNA transcripts

The digestion substrate was prepared by *in vitro* transcription from one microgram of T7 promoter-containing miR-BART22 PCR product (321nt) using MAXIscript kit (Ambion) following the manufacturer's protocol. The PCR templates were amplified from genomic DNA of either C666-1 or Namalwa cells. The primer sequences for PCR reaction are listed in Table 2.8.

2.18.2 Digestion Preparation and Signal Detection

For *in vitro* digestion, Drosha and Flag-DGCR8 expression vectors (Figure 2.3) (Landthaler *et al.*, 2004) were co-transfected into 293FT cells at a 2:1 ratio. The Drosha/DGCR8 enzymatic complex was purified using the Flag® Tagged Protein immunoprecipitation kit (Sigma, Saint Louis) 48 hours post-transfection. Digestion was performed by mixing 100ng RNA with the precipitated complex at 37 °C for 1.5 hours. Digested products were visualized after 8% PAGE by SYBR Gold staining (Invitrogen) and Northern blot analysis. The outline of the

procedure of *in vitro* pri-miRNA processing assay is shown in Figure 2.8.

Table 2.8 Primer sequences for T7-added PCR

Primer name	Sequence (5'→ 3')	Size (nt)	Tm (°C)
T7-C666-BART22-F	TAATACGACTCACTATAGGGCTA ATATCA	29	55
T7-Nam-BART22-F	TAATACGACTCACTATAGGGCTA ATAACC	29	55
T7-BART22-R	CCCAAGGCAGGTAAACATTG	20	54

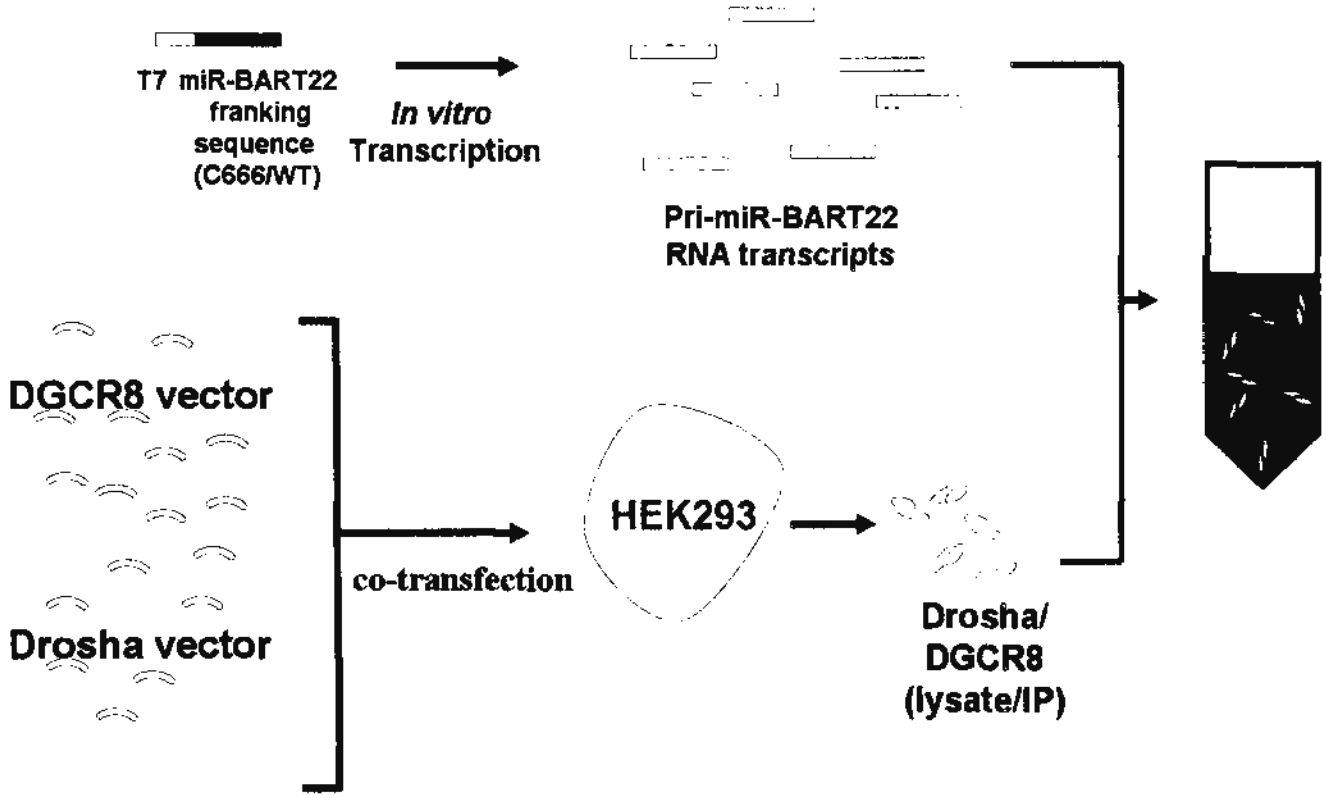


Figure 2.8 Outline of the *in vitro* pri-miRNA processing assay

2.19 MicroRNA Functional Studies

2.19.1 Potential Target Prediction of miRNAs

Both miRanda (Enright *et al.*, 2003; John *et al.*, 2004; Kiriakidou *et al.*, 2004) and RNA hybrid programs (Rehmsmeier *et al.*, 2004) were used to predict the potential targets of miR-BART21 and BART22. Human 3' UTR sequences were first extracted from BioMart (http://www.ensembl.org/Multi/martview) and subsequently analyzed by the miRanda program. In order to obtain more putative targets, we loosened the stringency in the miRanda prediction by adjusting the energy threshold to -15kcal/mol and the cut-off score to 90. Before experimental validation, interesting potential targets were cross-analyzed using the RNAhybrid program with the default settings. The LMP-2A reference sequence for target prediction was from NCBI (AB290724).

2.19.2 Cloning

2.19.2.1 MicroRNA Expression Vectors

The miRNA expression plasmids were made by inserting the PCR products, which contains miRNA flanking sequences of about 300nt, into the pcDNA3.1 expression vector via *Hind* III and either *Xba*I or *Xho*I sites. All the PCR products were generated using C666-1 DNA as template and PCR primer sequences listed in Table 2.9.

Table 2.9 Primer sequences for miRNA expression vector constructions

Primer	Sequences (5'→ 3')	Size (nt)	Tm (°C)
BART1-F	GGT*AAGCTT*ATGCTGCTGGTGT	22	58
BART1-R	GGC*TCTAGA*TGGTCATGTTTCCCT	24	58
BART14-F	GGT*AAGCTT*GGACGGCTGAC	20	58
BART14-R	GGC*TCTAGA*AAAGGCCTGCTGT	22	59
BART16-F	GGT*AAGCTT*CTGATGCTCTGTGG	23	58
BART16-R	GGC*TCTAGA*TGGATTGGACCAAC	23	57
BART21-F	AGCC*AAGCTT*GCTGGGCAGAGAA TGTTTGT	30	65
BART21-R	ACCG*CTCGAG*TAAGGGGAGGGG AAAGCTAAA	31	66
BART22-F	AGCC*AAGCTT*ACTTCATGGGTCC CGTAGTG	30	64
BART22-R	ACCG*CTCGAG*CCACACTGCTAAG GCAGTCA	30	68

Restriction Enzyme sites are underlined: AAGCTT (*EcoRI*); TCTAGA (*XbaI*); CTCGAG (*Xho I*).

2.19.2.2 Luciferase Reporter Vectors

Construction of the firefly luciferase reporter plasmids containing CMV-driven Flyfire pMIR-REPORT (Figure 2.4) was described previously (Wong *et al.*, 2008). The predicted or mutated binding site containing inserts were prepared by annealing synthetic oligonucleotides and cloning into the pMIR-REPORT. Oligonucleotides (50μM each) were annealed in DNA annealing buffer containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 100mM NaCl. One hundred microliters of the reaction were denatured at 95°C for 10 min and then annealed at room temperature for 30 min. Annealed oligonucleotides were diluted by 50,000-fold for cloning.

Two micrograms pMIR-REPORT was digested with *Spe*I and *Hind*III at 37°C for 2 hours. Following enzyme digestion, digested vector was subjected to agarose gel electrophoresis and eluted into 20μl water after purification by using QIAquick Gel Extraction Kit (Qiagen). One microliter of insert was ligated to 3μl of purified vector in the 5μl reaction mix using High Density T4 ligase (Invitrogen) at room temperature for 1 hour. Ligated product was transformed into bacteria as described in section 2.5.3. Eight colonies were randomly picked for PCR to check for the presence of an insert. Usually, the size of the PCR product from a positive clone was slightly different from the PCR product using pMIR-REPORT vector as a template. The correct sequence of the insert was double confirmed by sequence analysis as previous described in section 2.17. Only plasmids bearing the correct insert sequence were used for the luciferase reporter assay. The sequences of the primers used for both PCR and sequence analysis are listed in Table 2.10.

Table 2.10 Sequences of oligonucleotides used for firefly luciferase reporter plasmids construction

Primer	Sequences (5'→ 3')
T1-S	CTAGTGATCGCCTGCCACTTCCACAGCAA
T1-AS	AGCTTTGCTGTGGAAGTGGCAGGCGATCA
T2-S	CTAGTAACCCCACGGAGCAGGGCAACATTGCAGGGA
T2-AS	AGCTTCCCTGCAATGTTGCCCTGCTCCGTGGGGTTA
T3-S	CTAGTAGACTATGCATACACTGAATTTAGA
T3-AS	AGCTTCTAAATTCAGTGTATGCATAGTCTA
T4-S	CTAGTAGACCTGTGTGCTGTATTTAA
T4-AS	AGCTTTAAATACAGCACACAGGTCTA
BART12-S	CTAGTAACCACACCAAACACCACAGGAA
BART12-AS	AGCTTTCCTGTGGTGTTTGGTGTGGTTA
BART14-S	CTAGTATCCCTACTACTGCAGCATTTAA
BART14-AS	AGCTTTAAATGCTGCAGTAGTAGGGATA
BART16-S	CTAGTAGAGCACACACCCACTCTATCTAAA
BART16-AS	AGCTTTTAGATAGAGTGGGTGTGTGCTCTA
BART21-S	CTAGTGTTAGTTGCCTTCACTAGTGA
BART21-AS	AGCTTCACTAGTGAAGGCAACTAACA
BART22-S	CTAGTACTACTAGACCATGACTTTGTAAA
BART22-AS	AGCTTTTACAAAGTCATGGTCTAGTAGTA
BART1-5p-S	CTAGTCACAGCACGTCACTTCCACTAAGAA
BART1-5p-AS	AGCTTTCTTAGTGGAAGTGACGTGCTGTGA
LMP2A-WT-S	CTAGTCCTGTGTGACCCCTCACTTTGTACA
LMP2A-WT-AS	AGCTTGTACAAAGTGAGGGGTCACACAGGA
LMP2A-M1-S	CTAGTCCTGTGTGACCCCTCAA
LMP2A-M1-AS	AGCTTTGAGGGGTCACACAGGA
LMP2A-M2-S	CTAGTCCTGTGTGACCCCTCAGTATCTTCA
LMP2A-M2-AS	AGCTTGAAGATACTGAGGGGTCACACAGGA
LMP2A-M3-S	CTAGTCCTGTGTGACCCCTCACATAGAAGA
LMP2A-M3-AS	AGCTTCTTCTATGTGAGGGGTCACACAGG

2.19.3 Construction of a stable LMP-2A transfectant

The pcDNA3.1-LMP-2A expression plasmid was constructed by inserting 2kb of RT-PCR product, which included a complete LMP-2A *ORF* and 3'UTR, into the pcDNA3.1 expression vector via the *Eco*RI and *Xba*I sites. The RT-PCR product was amplified from RNA in B95.8 cells by using the LMP-2A-F primer: 5'-ACG GAA TTC *TGC TGC AGC TAT G*-3' (EBV coordination: 166092-166105) and LMP2A-R primer: 5'-CGT TCT AGA *GCA CAT TGG GTT TAT TGG*-3' (EBV coordination: 5840-5856). The presence of the LMP-2A coding sequence and the complete 3'UTR was confirmed by sequencing.

After introducing pcDNA3.1-LMP2A plasmid into HEK293 cells, stable LMP-2A transfectants were selected by growth in medium containing 600μg/ml G418 (Geneticin, invitrogen) for four weeks. A single colony was isolated and allowed to grow in medium containing 300μg/ml of G418. Expression of LMP-2A in the stable clones was confirmed by both Western blot analysis and IHC using the LMP-2A specific antibody (clone 15F9). The results of LMP-2A specific IHC are shown in Figure 2.9.

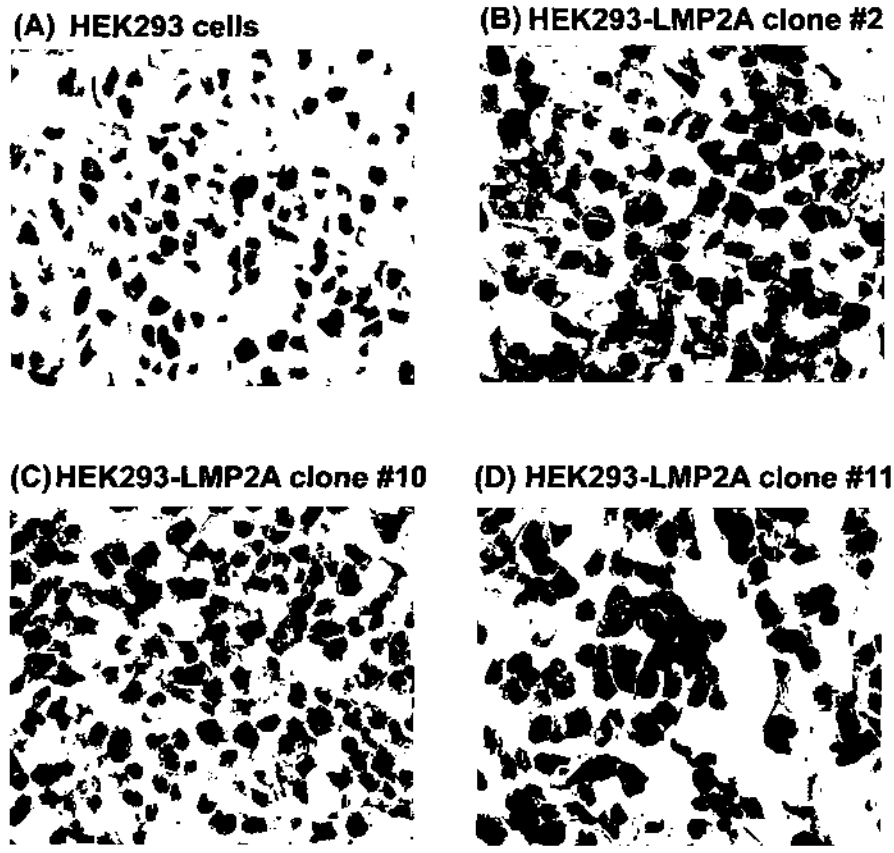


Figure 2.9 LMP-2A specific IHC staining on three individual LMP-2A expressing stable clones

2.19.4 Luciferase Reporter Assay

Cells (1×10^5) grown in 24-well plates were co-transfected with miRNA and reporter plasmid for analysis. All miRNA expression plasmid transfection experiments were carried out using GeneJuice transfection reagent (Novagen) in a 24-well plate format. Transfection complexed containing 50ng of reporter plasmid, 2ng of pRL-CMV control reporter plasmid and 200ng of the indicated miRNA expression plasmid were prepared for each transfection. For miRNA mimic transfections, a mixture containing 50nM of miRNA mimic, 800ng reporter vector and 80ng control reporter vector was prepared with lipofectamine 2000 (Invitrogen, CA). Cell lysates were collected for analysis 48 hours post-transfection. Cells were first rinsed with PBS, followed by addition of 100μl of 1× passive lysis buffer (Promega), and the cells were allowed to lyse at room temperature for 20 min with mild rocking. Later, 20μl of lysate were collected to assay enzyme activities using the Dual Luciferase Reporter Kit (Promega). One-hundred microliters of a 2-fold diluted LARII substrate was added to the lysate, and the luminescent signal of firefly luciferase activity was measured immediately. This was followed by the addition of 100μl of the 2-fold diluted Stop and Glo buffer that contains the substrate for renilla luciferase to simultaneously quench the firefly luciferase reaction and initiate the renilla luciferase reaction. The transient renilla signal was immediately recorded. Three individual experiments were performed and *student t-test* was used for statistical analysis. A P-value <0.05 was considered as statistically significant.

CHAPTER 3: IDENTIFICATION OF NOVEL EBV-ENCODED MICRORNAS IN NPC

3.1 Introduction

Shortly after Pfeffer's group reported the identification of five EBV encoded miRNAs in an EBV infected B-cell line (Pfeffer *et al.*, 2004), over 80 viral-derived miRNAs have been discovered. Interestingly, almost all miRNAs producing viruses are members of the polyomaaviridae and herpesviridae families, which carry DNA genomes and can establish lifelong latent infection in their host. They include Epstein-Barr Virus (EBV) (Pfeffer *et al.*, 2004; Cai *et al.*, 2006; Grundhoff *et al.*, 2006), Kaposi's sarcoma associated herpesvirus (KSHV) (Samols *et al.*, 2005; Grundhoff *et al.*, 2006), Herpes simplex virus 1 (Cui *et al.*, 2006; Gupta *et al.*, 2006; Umbach *et al.*, 2008), Human Cytomegalovirus (hCMV) (Dunn *et al.*, 2005; Grey *et al.*, 2005; Pfeffer *et al.*, 2005) and Simian virus 40 (SV40) (Sullivan *et al.*, 2005).

Until 2006, 23 ebv-miRNAs were identified into two distinct regions (BHRF1 flanking region and BART region) on the EBV genome. Among them, 20 miR-BARTs (miR-BART1 to miR-BART20) could be further clustered into two groups according to their genomic location (Figure 1.6). Similar to the *BART* transcripts, miR-BARTs are generally highly expressed in most EBV infected epithelial cells (Cai *et al.*, 2006; Kim do *et al.*, 2007). Nasopharyngeal carcinoma (NPC) is well-known to be associated with Epstein-Barr virus (EBV), and the detection of clonal EBV genomes can be found in almost all invasive carcinoma in our locality. However, only a few viral latent proteins are expressed in this undifferentiated lymphoepithelial cancer. Thus, it is plausible

that EBV augments cancer development through miR-BARTs. While in most instances the function of the EBV-miRNAs remains unclear, recent discoveries suggest great importance for miR-BARTs in modulating both viral and cellular gene expression (Cullen, 2009; Ghosh *et al.*, 2009).

Although EBV may exploit RNA silencing as a convenient mechanism for gene regulation, the currently identified EBV miRNA are either primarily cloned from EBV positive lymphoma cell lines or confirmed in lymphoma cells following computer prediction. Data on EBV miRNA expression in epithelial malignancy are very scanty.

This chapter aims to investigate the EBV miRNA expression profile in our local NPC cells by constructing miRNA libraries from the EBV positive NPC cell line (C666-1) and xenograft (X2117). Such information would enhance our understanding of NPC tumorigenesis, thus providing new diagnostic and therapeutic prospects for future developments. The reason to use local NPC samples is that the EBV strains in Hong Kong are different from other localities in many respects, e.g. The LMP-1 variants in the majority of EBV isolates from NPC are China 1 strain with a 30-bp deletion in comparison to B95.8 strain (Cheung *et al.*, 1998). Most significantly, C666-1 cells carry a relatively wild-type EBV strain. The BART RNAs spanning region deleted in B95.8 strain is retained in C666-1 EBV (de Jesus *et al.*, 2003).

3.2 Materials and Methods

3.2.1 Cell Lines

For comparison purposes, five cell lines not listed in Table 2.1 were subjected to analysis in this chapter. Among them, four EBV positive cell lines are of lymphoid origin. They are the Burkitt's lymphoma cell lines Raji (Pulvertaft, 1965), Namalwa (Klein *et al.*, 1972) and recombinant EBV-containing Akata (Kanda *et al.*, 2004), as well as an in house EBV re-infected lymphoblastoid cell line, CB14022. A marmoset cell line carrying the prototype EBV strain B95.8 was used as a positive control. It has been previously shown that all the earliest identified EBV-miRNAs a strongly expressed in B95.8 cells (Pfeffer *et al.*, 2004). All cell lines were cultured in RPMI plus 10% FBS unless otherwise specified. Akata cells were cultured in selective medium with 500μg/ml of G418.

3.2.2 Tumor Samples

For Northern blot and QRT-PCR analysis, three nasopharyngeal (NP) and 14 NPC biopsies were obtained from the Prince of Wales Hospital, The Chinese University of Hong Kong. The presence of EBV in the NPC samples was confirmed by EBER ISH. Information on NP and NPC biopsies is shown in Table 3.1. The methods of RNA preparation and Northern blot analysis are described in detail in section 2.6.

3.2.3 Detection of BHRF-1 mRNA expression

Expression of BHRF1 mRNA was analyzed by an RT-PCR-based assay as previous described (Oudejans *et al.*, 1995). RNA extraction and RT-PCR were

performed as described in sections 2.7.2 and 2.8. In brief, RNA was extracted by Trizol reagent and 1μg of total RNA was reversed transcribed by the High Capacity cDNA Archive Kit (Applied Biosystems) in a 20μl reaction containing random primers. One microliter of total cDNA was used to perform PCR reaction for 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min in a 25μl reaction containing 0.25mM dNTPs (Amersham), 0.2μl of each 10μM primer, 1× PCR reaction buffer, 1.6μl of 25mM $MgCl_2$ and 0.2μl of Taq polymerase (Fermentas). PCR products were analyzed in a 2% agarose gel. To transfer DNA to the membrane for Southern blot analysis, DNA in the agarose gel was first depurinated in 0.25M HCl for 5 min, then denatured in washing buffer (0.5M NaOH, 1.5M NaCl) for 20 min, followed by a wash with neutralizing buffer (0.5M Tris Ph7.0, 3.0M NaCl) for another 20 min. DNA was then transferred to the positively-charged Nylon membrane (Amershan) with 20× SCC overnight. Synthetic oligonucleotides complementary to the lytic form of BHRF1 mRNA were end-labeled with γ-^{32}P ATP using T4 polynucleotide kinase as described in section 2.6.2. Hybridization and washing were carried out as described for Northern blot analysis described in section 2.6.3. The sequences of primers and probes are listed below:

Actin 207-F: TAAGGAGAAGCTGTGCTACGTC

Actin 2097-R: GGAGTTGAAGGTAGTTTCGTGG

BHRF1 5'-H2: GTCAAGGTTTCGTCTGTGTG (Lear *et al.*, 1992)

BHRF1 3'-H3: TTCTCTTGCTGCTAGCTCCA (Lear *et al.*, 1992)

BHRF1 probe: ATGCACACGACTGTCCCGTATACAC (Oudejans *et al.*, 1995)

Table 3.1 Information of NP and NPC biopsies

Code	Sex	Age	Tumor Staging for Nasopharynx[1]		EBV
NPC			**STAGE (UICC)**	**TNM Group**	
NPC01	M	43	Local NP Recurrent		+
NPC02	M	48	IV B	T3N1M0	+
NPC03	M	50	II B	T2Bn1M0	+
NPC04	M	71	IV A	T4N2M0	+
NPC05	F	48	IV B	T3N3aM0	+
NPC06	M	66	II B	T2Bn0M0	+
NPC07	M	42	IV C	T3N2M0	+
NPC08	M	47	I	T1N0M0	+
NPC09	M	48	IV A	T1N3bM0	+
NPC10	M	49	III	T3N0M0	+
NPC11	F	55	II B	T2bN0M0	+
NPC12	F	55	I	T1N0M0	+
NPC13	F	63	IV B	T4N2/3M0	+
NPC14	M	43	II B	T2bN1M0	+
Normal NP control			**Presenting symptoms**		
NP01	M	25	allergic rhinitis		-
NP02	M	46	nasal discharge		-
NP03	F	57	blood stained nasal discharge		-

[1] See Appendix 1 for detail of tumor staging in Nasopharynx (AJCC 6th Edition)

3.2.4Sequencing analysis

Sequencing analysis was preformed as described in section 2.16. We used a forward PCR primer for sequencing reactions. The primer sequences for PCR and sequencing were as follow:

SEQ-BHRF1-1F: CCTTTAGGAAGCACCACGTC (41377:41396)

SEQ-BHRF1-1R: CCCAACCTTTTAATGGCAAA (41634:41615)

SEQ-BHRF1-2,3F: GACACAGTGCCCATGCATTA (42747:42766)

SEQ-BHRF1-2,3R: TGGGCTGCAGTATAGGCTCT (43129:43110)

The numbers in the parentheses indicate the location of the primers in the viral genome (AJ507799). Multiple sequence alignments were performed by using the CLC Sequence viewer (www.clcbio.com/index.php?id=28).

3.2.5Transfection study in NP69 cells

In order to increase the transfection efficiency of NP69 cells, FuGENE6 Transfection Reagent (Roche) was employed. In a 6-well plate format, NP69 cells were seeded to 70% confluency the day before transfection. Transfection complexes were prepared according to the manufacturer's standard protocol (FuGENE: DNA ratio = 3:2). The transfection efficiency of NP69 was evaluated by employing the pEGFP-C1 (Figure 2.3). The GFP signal from the transfected cells was examined under a fluorescence microscrope at 24 hours post-transfection. Flow cytometry analysis was performed 24 hours after transfection. The procedure for flow cytometry analysis was described in section 2.13. The cells for MTT analysis were trypsinized and seeded again in 96-well plate (2000 cells/well) 5 hr after transfection. The procedure for MTT analysis is described in section 2.14.

3.2.6Statistical analysis

Results of the MTT assay and QRT-PCR expression analysis were compared using the *student's t-test*. Analyses of each sample were performed in triplicate with the mean +SD shown. A *p*-value <0.001 is denoted as (**) and *p*-value <0.05 is denoted as (*)

All other methods, such as Northern blot, Western blot, Ago2 co-IP and the MTT assay, are described in Chapter 2. The sequences of synthetic oligonucleotides used as probes in Figure 3.11 and Figure 3.14 are listed below:

EBER1-5p: TAGGGCAGCGTAGGTCCT;

EBER1-3p: AAACATGCGGACCACCAGCTGG;

EBER2-5p: GTGTGTCCGAAACCACTA;

EBER2-3p: ACAAGCCGAATACCCTTCTC

3.3 Results

3.3.1 Aberrant expression of ebv-miRNAs in NPC

Expression of five ebv-miRNAs (miR-BHRF1s and miR-BART1 and 2) was first characterized in B-cell lines (Pfeffer *et al.*, 2004). To study the significance of miRNAs in NPC, the expression pattern of the early identified ebv-miRNAs in a variety of EBV-positive NPC samples was examined by Northern blot analysis. A panel of NPC samples for the analysis included a native EBV-positive NPC cell line C666-1, two EBV-reinfected cell lines (HK1-EBV, HONE1-3EBV), two xenografts (X666 and X2117) and two biopsies.

Northern blot analysis of NPC samples suggested that miR-BART1 and miR-BART2 were consistently detected in almost all EBV positive samples with expression at different levels (Figure 3.1). In addition, abundant expression of miR-BART5 was observed in most NPC samples (Figure 3.2). The expression of miR-BART5 was not detected in B95.8 because the region that gives rise to miR-BART5 is deleted (Figure 1.6). It is interesting to note that although EBV infection of Namalwa, Raji, CB14022 and B95.8 cells is in latency III, the expression of miR-BART1 and miR-BART2 in these cells highly varied. For example, miR-BART1, which was highly expressed in Namalwa and B95.8 at comparable levels, was not detected in lymphoblastoid (LCL) cell, CB14022. On the other hand, miR-BART2 expression in Namalwa cells was only barely detectable by blotting analysis. Similar results were also observed for the EBV latency II infected cells. Expression of miR-BART2 in C666-1 is apparently higher than that in HK1-EBV and HONE1-EBV reinfected cells, while the expression of miR-BART1 was similar among these three cell lines of epithelial

origin (Figure 3.1*B*). These observations suggested that the distinct miR-BART expression pattern in various EBV positive samples reflect their unique function for specific cell environments.

Unlike the ubiquitous expression of miR-BARTs, expression of miR-BHRF1s demonstrated vast variation in different types of EBV samples. We did not detect expression of EBV-miR-BHRF1s in any of the tested NPC samples including cell lines, xenografts and biopsies. As a control, expression of miR-BHRF1-1, miR-BHRF1-2 and miR-BHRF1-3 was detected in latency III infected cells (Namalwa, CB14022, Raji and B95.8). Nevertheless, although miR-BHRF1 genes are located within the intronic region and 5'UTR of BHRF1 mRNA, it is interesting to observe the expression of BHRF1 mRNA in NPC samples including C666-1, X666 and X2117. Weak expression of BHRF1 mRNA was further confirmed in two of the four NPC biopsies by Southern blot analysis of the membrane transferred RT-PCR products from agarose gel (Figure 3.4).

By analyzing the viral DNA sequences from a variety of EBV positive cell lines, two distinct single nucleotide variations were observed in the pre-miR-BHRF1 sequence (Figure 3.5). However, the nucleotide variations should not affect the production of either miR-BHRF1-2 or 3. The EBV sequences in CB14022 cells in which miR-BHRF1-2 and 3 are expressed, are the same as all miR-BHRF1-2 and 3 negative EBV samples (C666-1, X666 and X2117). Thus, the absence of expression of miR-BHRF1s in NPC can not due to differenced in the EBV strains infecting.

In contrast to previous reports that induction of lytic EBV replication in Akata, MUTU1 and Daudi infected cells (latency I) can facilitate miR-BHRF1s

expression (Cai *et al.*, 2006; Xing & Kieff, 2007), our data show that induction of lytic EBV replication does not activate pre- and mature miR-BHRF1 expression in C666-1 cells, which exhibit EBV latency type II infection (Figure 3.6).

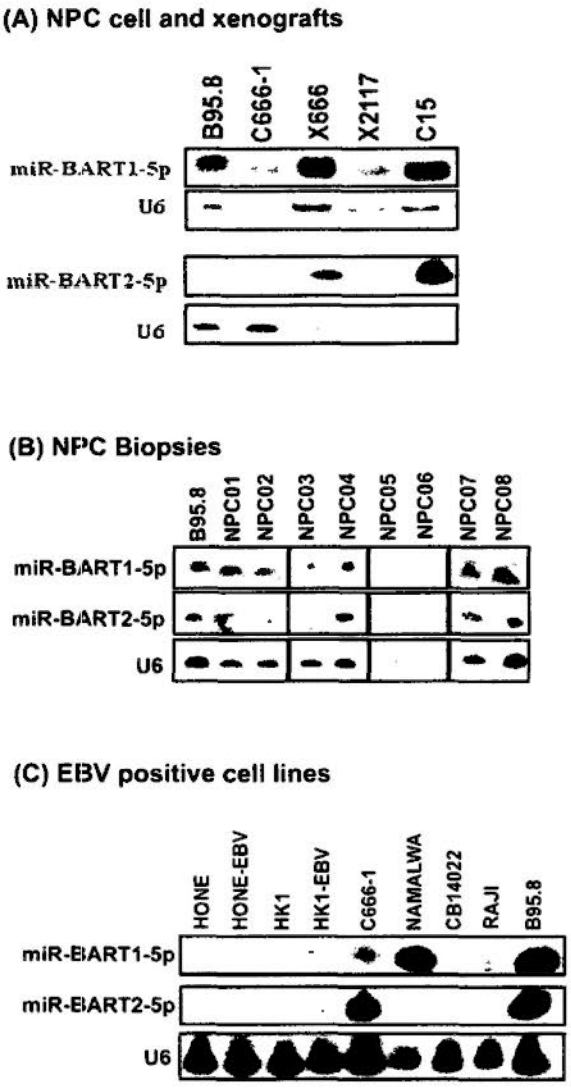


Figure 3.1 Expression of ebv-miR-BART1 and BART2 in NPC samples

Northern blot analysis indicates the expression of miR-BART1 and BART2 in several NPC samples including cell lines and xenografts (*Panel A*); NPC biopsies (*Panel B*) and other EBV infected cell lines (*Panel C*). In panel C, EBV positive cell lines (Namalwa, CB14022 and Raji) were included for comparison. Expression of miRNAs in B95.8 cells provided a positive control. Detection of U6 RNA expression serves as an RNA loading control.

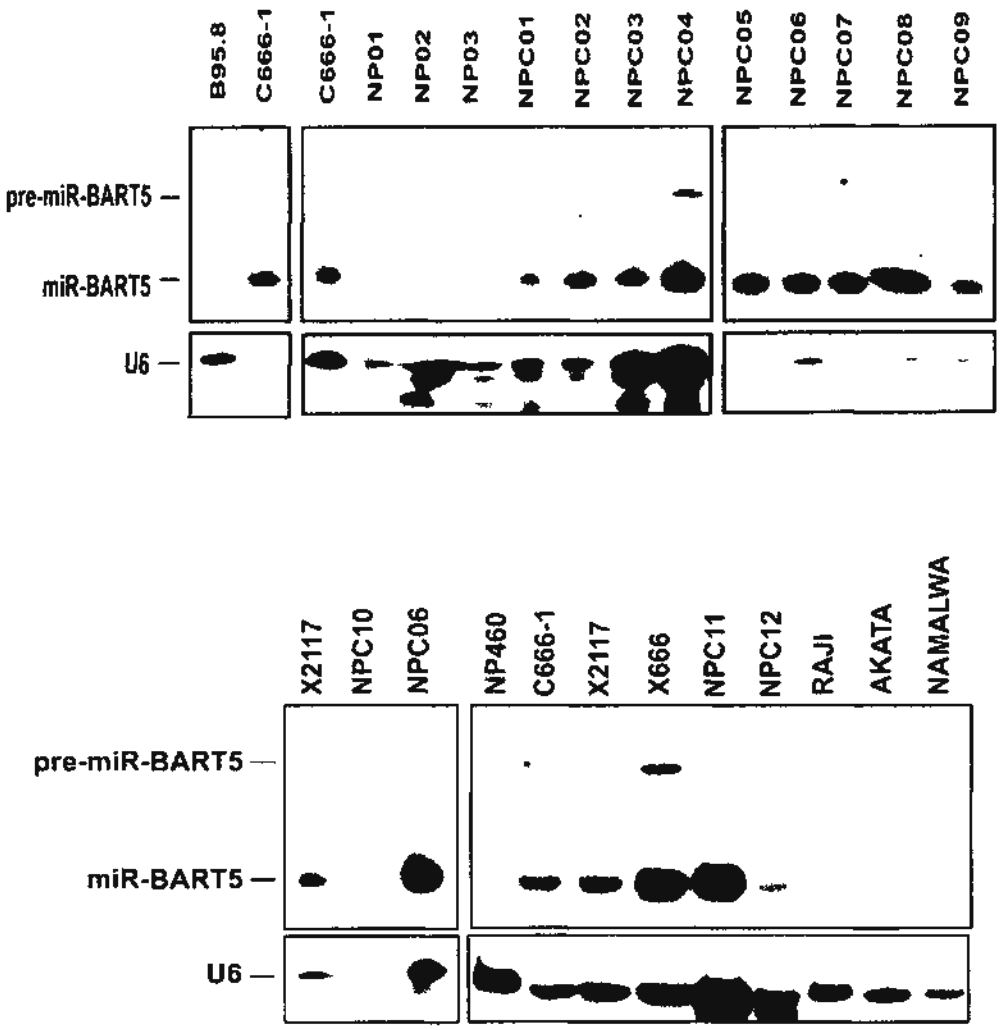


Figure 3.2 Expression of miR-BART5 in NPC

Northern blot analysis to show expression of miR-BART5 in NPC biopsies (*Upper panel*) and different EBV infected cell lines including lymphoid cell lines (*Lower panel*). Normal epithelial biopsies (NP01-03) (*Upper panel*) and cell line NP460 (*Lower panel*) were blotted as negative controls. Detection of U6 RNA expression serves as an RNA loading control.

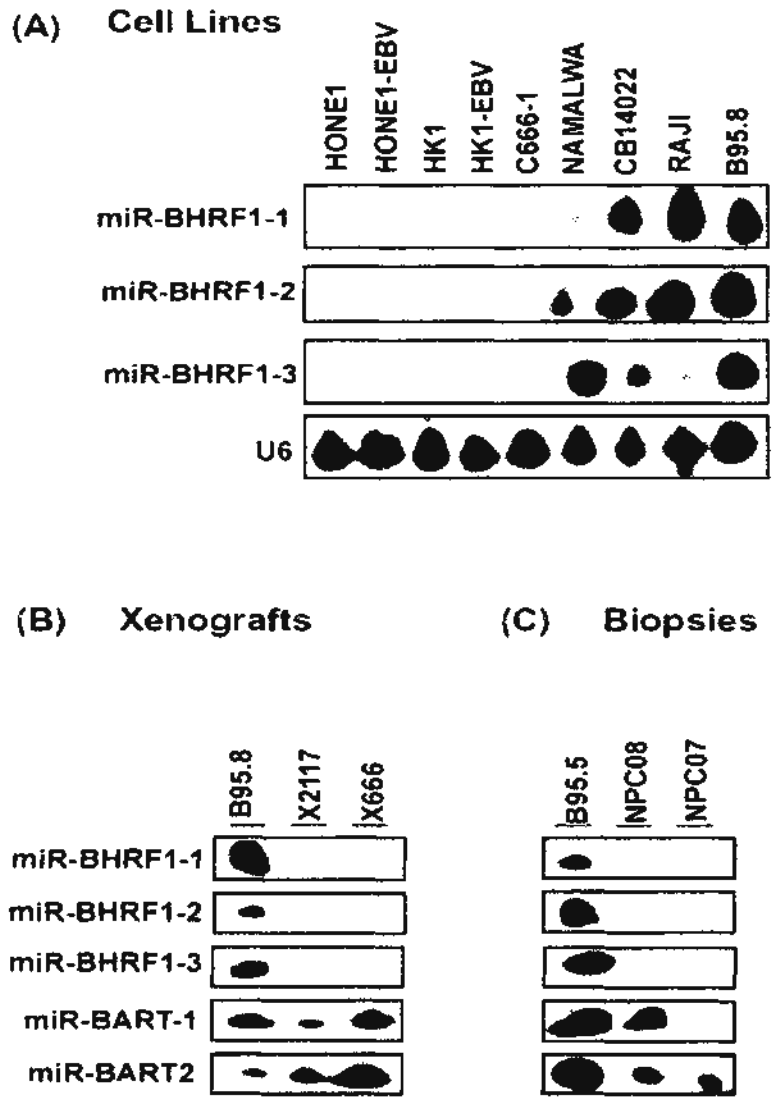


Figure 3.3 Expression of ebv-miR-BHRF1s in NPC samples

Northern blot analysis indicates expression of miR-BHRF1s in several NPC samples including cell lines (*Panel A*), NPC xenografts (*Panel B*) and two NPC biopsies (*Panel C*). EBV positive cell lines (Namalwa, CB14022 and Raji) were included for comparison. Expression of miRNAs in B95.8 cells provided a positive control. Detection of U6 RNA expression served as an RNA loading control.

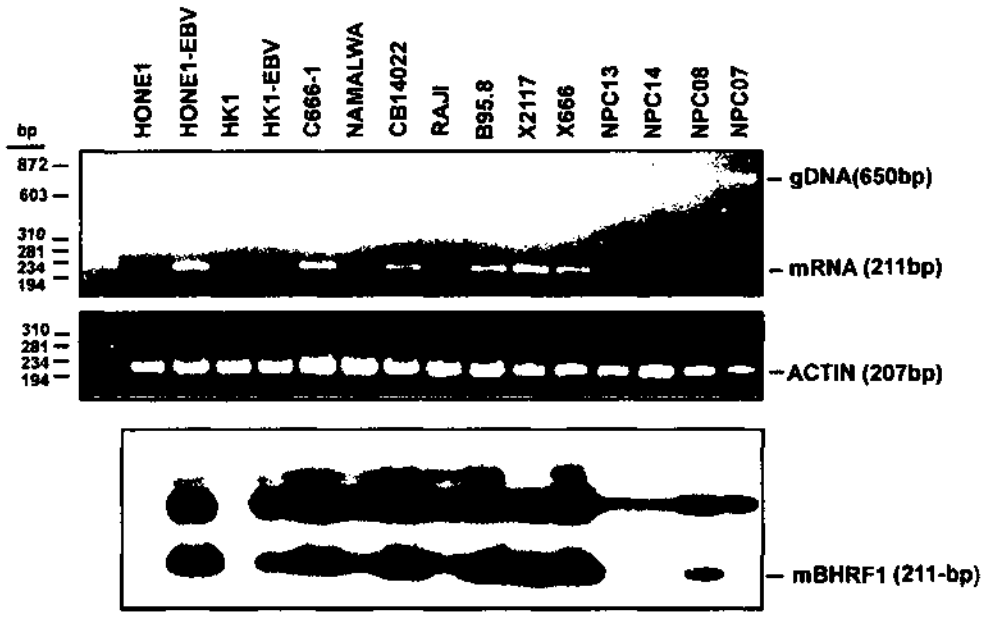


Figure 3.4 RT-PCR detection of the lytic spliced BHRF1 transcript

RT-PCR and blotting signal on the membrane-transferred RT-PCR agarose gel shows the expression of the lytic BHRF1 transcript (211-bp). The 650-bp band detected in some cases indicates amplification of viral genomic DNA. The presence of RNA for RT-PCR was confirmed by the detection of actin mRNA expression (207-bp).

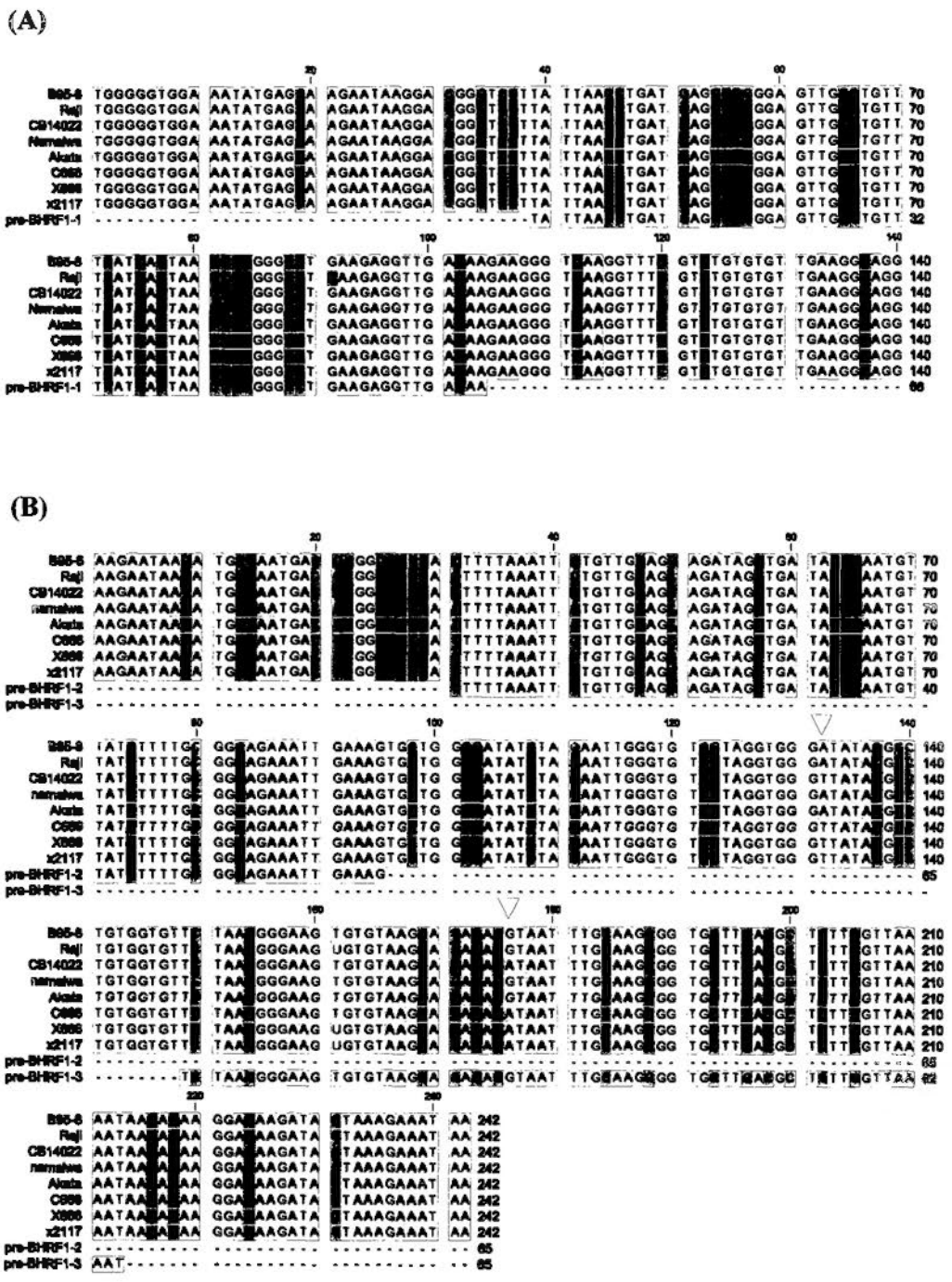


Figure 3.5 Alignment of pre-miR-BHRF1s in different EBV strains

Alignment of pre-miR-BHRF1-1 (AJ507799, 41433:41568) (*Panel A*) and pre-miR-BHRF1-2, 3 (AJ507799, 42818:43059) (*Panel B*). The two positions of nucleotide variation are indicated by the red arrowheads.

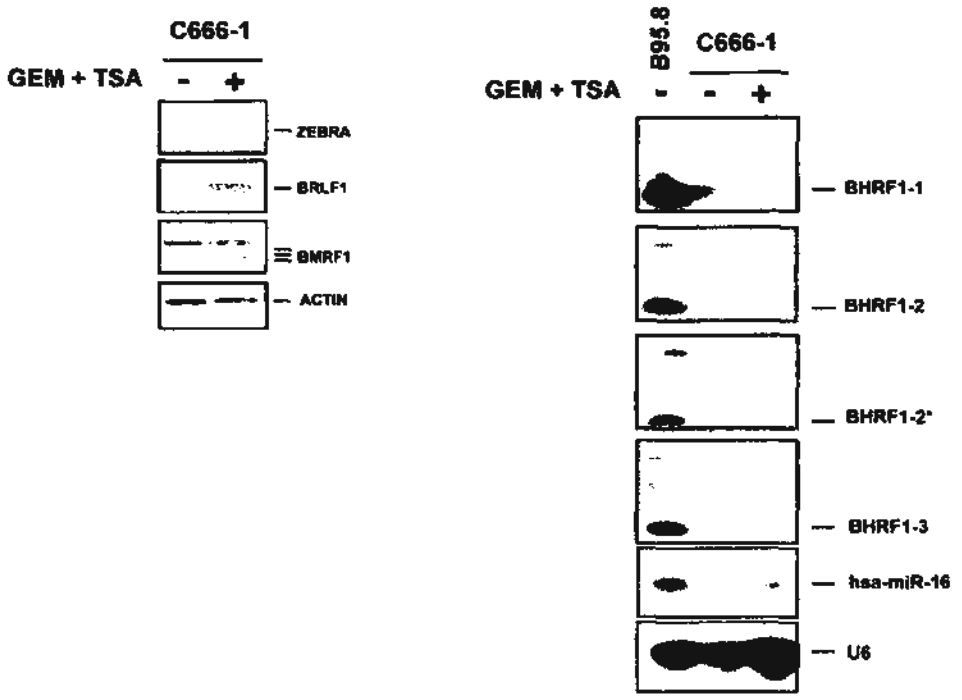


Figure 3.6 Induction of EBV lytic replication in C666-1 cells unable to activate miR-BHRF1s expression.

C666-1 cells were cultured in either normal medium (indicated as "-") or medium containing 100ng/ml of Gemcitabine (GEM) and Trichostatin A (TSA) (indicated as "+") for 48 days. Cells entering into viral lytic replication are indicated by the expression of a number of viral lytic proteins, including ZEBRA, BRFL1 and BMRF1, analyzed by Western blot (*Left Panel*). Expression of ebv-miR-BHRF1s is demonstrated by Northern blot (*Right Panel*). B95.8 sample was included as positive control. Detection of hsa-miR-16 was included to confirm the presence of miRNAs in the tested samples. RNA loading was normalized by U6 snRNA detection.

3.3.2 Identification of Novel ebv-miRNAs from NPC small cDNA libraries

3.3.2.1 Small cDNA libraries

Two independent cDNA libraries were constructed from C666-1 and X2117 cells as described in section 2.17 and Figure 2.7. The construction from C666-1 small RNAs is illustrated in Figure 3.7 as an example. A total of 2928 clones with sequence $\geqq$ 18 nucleotides were obtained, including 1813 clones from C666-1 and 1115 clones from X2117. The known miRNAs and human sequences were annotated by blasting individual extracted sequences against miRBASE (Griffiths-Jones, 2004) and GenBank databases. Sequences that match the EBV genome were identified by BLAST search against the full length EBV genome (accession number: AJ507799). The results indicate that several kinds of cellular and EBV fragments are present in the libraries, while many of them are known microRNAs (Table 3.2 and Table 3.3).

In the C666-1 library, 615 and 277 clones matched to known EBV and human miRNAs, respectively, based on the miRBase (Version 11.0) search. In addition to the known miRNAs, 811 clones were human sequences that mostly correspond to rRNA and tRNA fragments. Interestingly, 32 sequences that aligned to the EBV genome showed no match to any known miRNAs previously reported in lymphoid cells. According to their sequence similarity, these novel EBV transcripts could be classified into 3 groups. Group 1 and group 2 represent EBV fragments from positions 145515-145534 and 147203-147215, respectively. Group 3 contains multiple short EBV

fragments with homology to EBERs. Examples of the sequencing results from each group are displayed in Figure 3.8. Their sequences and total number of clones are listed in Table 3.4.

The distribution of small RNAs in the X2117 library is similar to that in C666-1, except that a higher percentage of human cellular miRNAs are found in X2117. On the whole, the most abundant EBV miRNA clones are miR-BART9 (n=467) and miR-BART10 (n=251).

To establish whether some of the isolated RNA fragments could represent potential novel ebv-miRNAs, putative precursor sequences with sizes of around 70 nucleotides contained the cloned fragments from the EBV genome were subjected to structure predictions using the MFOLD program (Zuker, 2003). A precursor miRNAs (pre-miRNAs) hairpin structure was produced from each of the putative precursor sequences from Group 1 and Group 2 EBV fragments, which are now named miR-BART21 and miR-BART22, respectively (Figure 3.9). Group 3 contained multiple short fragments from EBER 1 and 2, whose secondary structures contain a number of stable stem loops (Glickman *et al.*, 1988). It is interesting to find that some of the cloned fragments are located in regions of stem structure (Figure 3.10).

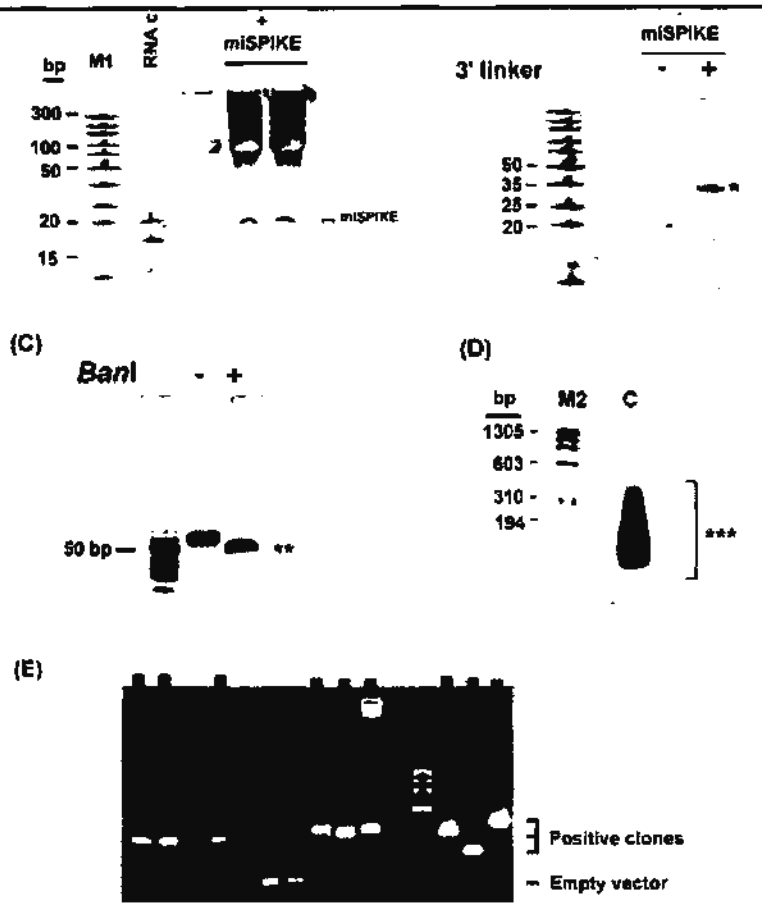


Figure 3.7 Construction of a small RNA library from C666-1 cells

(*A*) Small RNA (≤ 200-nt) were mixed with an internal RNA marker "miSPIKE" and separated by 15% PAGE. Synthetic RNA oligonucleotides of 18 and 24-nt were loaded as size markers. (*B*) The isolated RNA ligated to the3' linker (indicated as *) was visualized and isolated from the PAGE analysis. (*C & D*) After ligation of both 3' and 5' linkers, the cDNA was amplified by PCR, followed by digestion with *Ban*I (**) restriction enzyme for concatamerization. The concatamer migrate as a fuzzy ladder (***) on the 2% agarose gel. The DNA migrating above 100bp was excised, purified and cloned as described in section 2.17. (*E*) PCR screening was used to identify positive clones (red arrows) for further sequencing and annotation analysis.

Table 3.2: Distribution of small RNAs in cloned libraries

	C666-1	X2117
Human Sequences	**812**	**167**
Total known hsa-miRNAs	**277**	**433**
Total known ebv-miRNAs[a,b]	**615**	**504**
BART1-5p (-3p)	25 (9)	6 (2)
BART2-5p	1	0
BART3 (3*)	30 (11)	6 (1)
BART4	11	2
BART5	22	5
BART6-5p (-3p)	18 (17)	0 (0)
BART7	45	64
BART8 (8*)	15 (7)	16 (10)
BART9	179	288
BART10	169	82
BART11-5p (-3p)	7 (4)	0 (0)
BART12	6	1
BART13 (13*)	1 (0)	3 (1)
BART14 (14*)	5 (2)	0 (2)
BART16	7	0
BART17-3p (-5p)	14 (9)	6 (0)
BART18-5p	1	0
BART19-3p (-5p)	0 (0)	8 (1)
EBV fragments (Group 1)[c]	1	0
EBV fragments (Group 2)[d]	14	1
EBV fragments (Group 3)[e]	17	2
Total number of EBV fragments	**32**	**3**
Unknown sequences	77	8
Total clones for analysis	**1813**	**1115**

[a] All miR-BHRF1s, BART15 and BART20 were not cloned;
[b] Ebv-miRNA nomenclature is recommended by miRBase registry (http://www.mirbase.org/)
[c] MiR-BART21 clones;
P[d] MiR-BART22 clones
[e] EBV sequences from EBV encode RNA s (EBERs).

Table 3.3 Distribution of known hsa-miRNAs in cloned libraries

Hsa-miR[a]	C666-1	X2117	Hsa-miR	C666-1	X2117
let-7a	17	0	miR-101	1	0
let-7b	11	0	miR-103	6	0
let-7c	0	2	miR-103*	1	0
let-7d	10	0	miR-106a	2	0
let-7f	16	0	miR106b	10	1
let-7i	6	3	miR-125b	0	8
miR-15a	0	1	miR-148b	0	3
miR-15b	6	10	miR-184	0	1
miR-16	2	0	miR-184*	1	0
miR-17-3p*	1	0	miR-186	0	1
miR-17-5p	25	0	miR-191*	1	0
miR-18b	6	0	miR-193a	12	0
miR-19b	3	0	miR-200a	2	29
miR20a	6	0	miR-200b	20	72
miR-20b	0	1	miR-200c	21	47
miR-21	22	6	miR-205	0	4
miR-23a	4	65	miR-221	0	1
miR-23b	0	64	miR-300d	0	1
miR-23b*	0	1	miR-320a	17	1
miR-24	0	2	miR-331	5	0
miR-25	1	12	miR-365	5	0
miR-26a	1	4	miR-374b	0	1
miR-27a	0	1	miR-423-5p	7	0
miR-29b	0	1	miR-429	0	2
miR-30e	0	1	miR-449	14	0
miR-32a	0	1	miR-484	0	1
miR-34a	10	3	miR-505	0	2
miR-92a	0	64	miR-532-5p	0	1
miR-92b	0	3	miR-551b	0	1
miR-93	5	0	miR-574-5p	0	1
miR-99a	0	10	**Total clones**	**277**	**433**

[a] MiRNA nomenclature is recommended by miRBase registry (http://www.mirbase.org/)

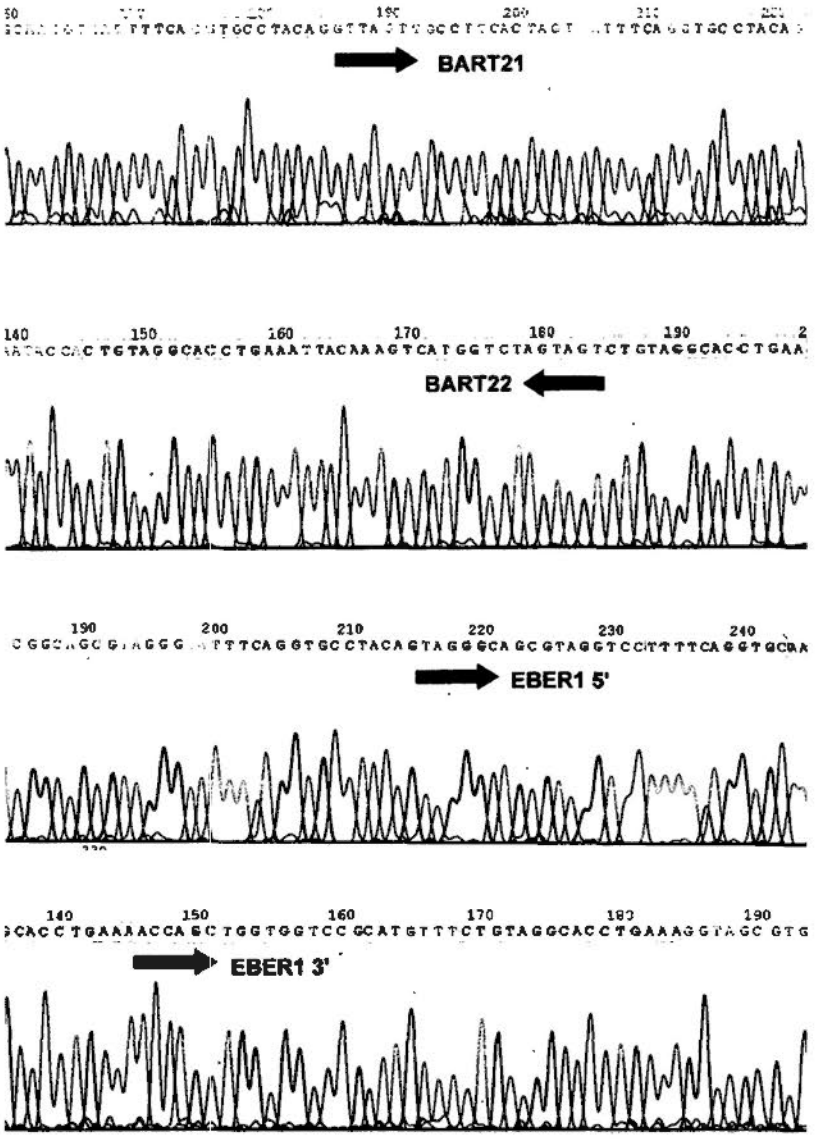


Figure 3.8 Identification of EBV fragments in the cloned libraries

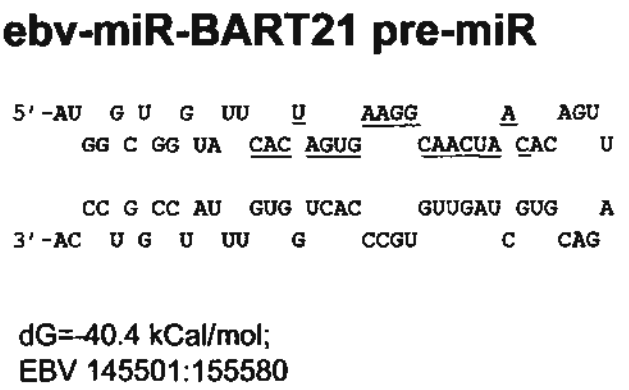
The cloned small EBV fragments were shown. Orientation of the cloned sequence is indicated with the black arrow. Small EBV fragments and adaptor sequences in the figure are highlighted with blue-green and purple colors respectively.

Table 3.4 Sequence and Genomic Location (AJ507799) of EBV encoded RNAs that do not match known miRNAs

	RNA fragment	Sequence (5' – 3')	Length	C666-1	X2117	Position
Group 1	miR-BART21	CACUAGUGAAGGCAACUAAC	20	1	0	145515:145534
Group 2	miR-BART22	UUACAAAGUCAUGGUCUAGUA(GU)[a]	21-23	14	1	147203:147215
Group 3	EBER1-5p	(A)AGGACCUACGCUGCCCU(A)	17-19	8	0	6629:6646
	EBER1 (1)	GACUCUGCUUUCUGCCGU	18	1	0	6742:6759
	EBER1 3p	(UACC)AGCUGGUGGUCCGCAUGUUU	20-24	5	1	6772:6794
	EBER2 (1)	UAGUGGUUUCGGACACAC	18	1	0	6972:6989
	EBER2-3p	GAGAAGGGUAUUCGGCUUGU	20	2	0	7098:7117
	EBER2-5p	AGGACAGCCGUUGCCCUAGUGGUUU	25	0	1	6956:6980

Sequence variations of the cloned fragments were indicated by parentheses

[a] Two out of 15 sequences had two nucleotides shorter



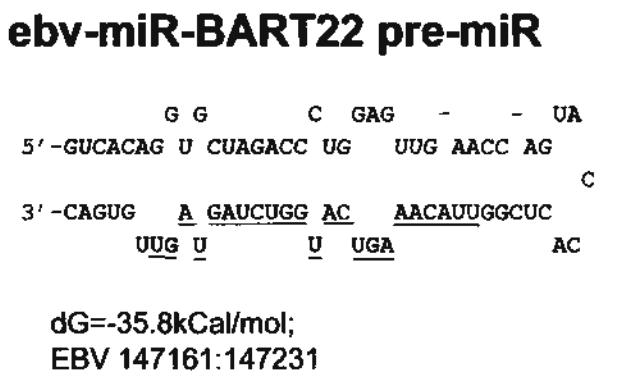
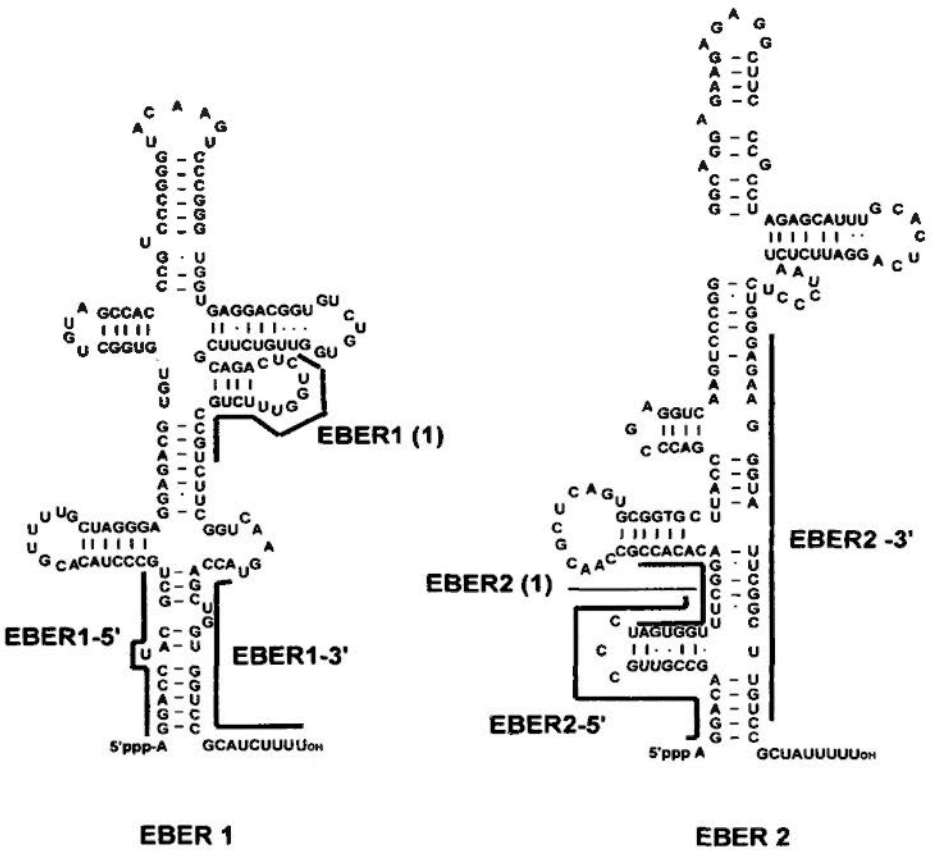


Figure 3.9 Fold back precursor sequences of ebv-miR-BART21 and ebv-miR-BART22

Stem-loop structures of miR-BART21 (*upper panel*) and miR-BART22 (*lower panel*) are illustrated. The cloned mature miRNA sequences are indicated by underlined letters. The folding energy (dG) and EBV genomic sequences (AJ507799) are listed below.



Modified from Glickman JN *et al.*, J Virol (1988)

Figure 3.10 Positions of the cloned EBER fragments on EBER1/2

Predicted EBER 1 and EBER 2 secondary structures are shown. The positions of the cloned small RNAs are indicated with red lines.

3.3.2.2 Detection of cloned EBV fragments

The expression of cloned EBV fragments in EBV-infected NPC tumors were tested by Northern blot analysis. Expression of 5' and 3' end small EBER1 fragments could be readily detected in EBV positive NPC samples including two NPC biopsies. However, we could only detect the expression of two EBER1 end terminal but not any of the small EBER2 fragments in EBV positive samples by the same detection method (Figure 3.11).

The expression of miR-BART21 and miR-BART22 in EBV-infected NPC tumors were validated by Northern blot analysis. The cell line C666-1, three xenografts (X666, X2117, C15) and two primary tumor biopsies were examined. The EBV-negative immortalized normal nasopharyngeal epithelial cell line, NP460, was included as a negative control. As a comparison for expression in another EBV-associated tumor type, three lymphoid cell lines, Akata, Namalwa and Raji, were examined at the same time. Expression of miR-BART21 was detected in C666-1, X666, X2117 and one of the two primary NPC biopsies (Figure 3.12, *upper panel*). In contrast, miR-BART22 was expressed in all EBV positive cells but particularly highly in NPC samples (Figure 3.12, *lower panel*). These results suggested that the two novel EBV miRNAs are preferentially expressed in NPC cells. Because only a small amount of RNA could be isolated from the primary NPC biopsies, we designed a sensitive QRT-PCR assay for the detection of these novel EBV miRNAs in tissue specimens. The relative expression levels of miR-BART21 and miR-BART22 in cell lines and xenografts as measured by QRT-PCR were similar to those determined by Northern blot analysis (Figure 3.12). By employing QRT-PCR, we confirmed high and consistent expression of these two EBV miRNAs in primary NPC tumors (Table 3.5).

3.3.2.3 Characterization of the Novel Ebv-miRNAs

The miR-BART21 and BART22 expression plasmids (pcDNA3.1-BART21 and pcDNA3.1-BART22) were constructed by inserting the PCR product, which contained miR-BART with flanking sequence (~300nt), into the pcDNA3.1 expression vector via *Hind* III and *Xho*I sites, where the expression of miRNA was driven by the CMV promoter. All PCR products were generated using C666-1 DNA as template with primer sequences listed in Table 2.9.

To investigate whether the expression vectors could be used for study, the expression and silencing properties of these vectors were studied in transient transfection assays. Northern blot analysis was chosen to confirm the expression of miR-BART21 and miR-BART22 in 293FT cells at 24 hours post-transfection by expression vector. As shown in Figure 3.13 (*upper panel*), both expression vectors produced mature miRNAs with a size similar to the corresponding endogenous miRNA in C666-1. The relative expression of miRNAs in the transfectants is also be quantified by QRT-PCR. The silencing activities of these miRNAs were confirmed by the dual luciferase reporter assay. A miRNA expression vector was co-transfected into 293FT cells together with the control renilla reporter vector (Rluc) and firefly reporter vector (Fluc) bearing a single perfectly complementary sequence to the corresponding transfected miR-BART22 vector. The result in Figure 3.13 (*lower panel*) shows that Fluc activity was significantly reduced by 70% in the cells transfected with pcDNA3.1-BART22. We further confirmed that miR-BART22 was associated with Ago2 in C666-1 cells by co-immunoprecipitation (Figure 3.14). On the other hand, Fluc activity was only slightly inhibited by co-transfection with pcDNA3.1-BART21 although the difference was statistically significant.

To elucidate the regulatory role of these two novel ebv-miRNAs on cell proliferation, MTT assay was performed on NP69 cells transfected either with pcDNA3.1, pcDNA3.1-BART21, or pcDNA3.1-BART22. The result in Figure 3.15 showed that expression of BART21 and BART22 on NP69 did not affect the proliferation rate. Further analysis by flow cytometry using PI staining showed that expression of these two microRNAs on NP69 cells did not affect cell viability and DNA content in cell cycle (Figure 3.16).

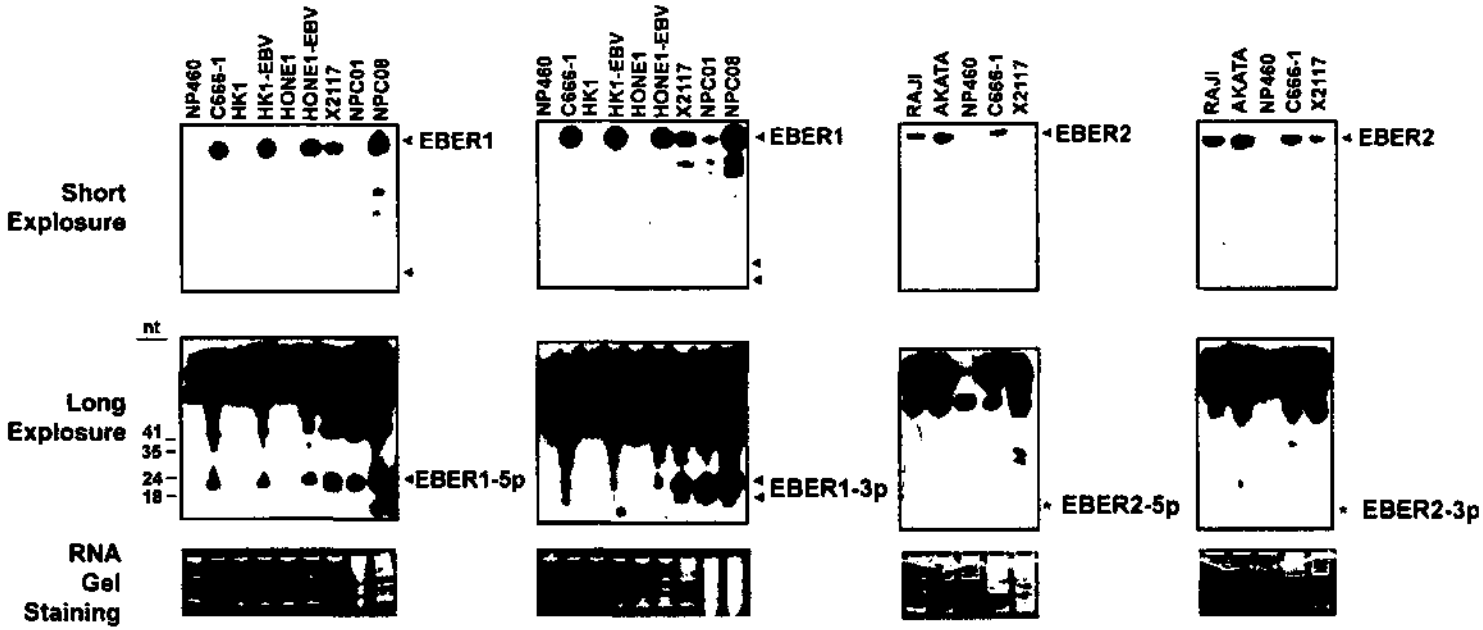


Figure 3.11 Detection of small EBER end terminal fragment in NPCs

Expressions of small EBER fragments in NPCs are validated by Northern blot analysis. Locations of EBER1, EBER2 and EBER1 small fragments are indicated with arrows. NP460 cell were included as negative control. The predicted location of EBER2 small fragment is indicated with (*).

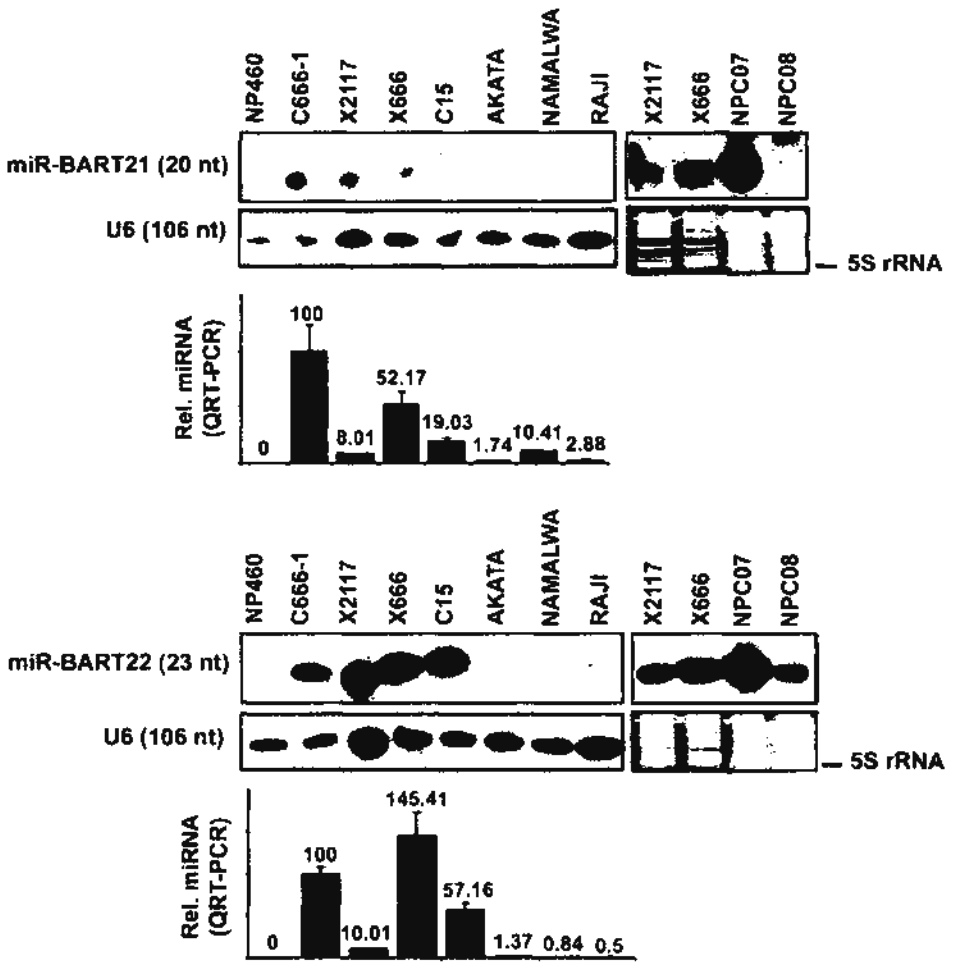


Figure 3.12 Expression of novel ebv-miRNAs in EBV positive samples

Representative Northern blot results for the expression of miR-BART21 and 22 in cell lines, NPC xenografts and NPC biopsies are displayed. The normal epithelial cell line NP460 was included as negative control. U6 RNA or SYBR Gold staining PAGE was used to control for RNA loading. The miRNA expression in the sample was assayed by QRT-PCR and compared with that in C666-1 (set at 100). The analysis of each sample was performed in triplicate with the mean +SD shown.

Table 3.5 Expression of novel ebv-miRNAs in NPC samples

Sample	BART21	BART22
C666-1	100	100
NPC1	9.17	36.39
NPC2	52.57	123.18
NPC3	31.85	41.07
NPC4	26.97	18.85
NPC5	47.44	76.54
NPC6	3.15	2.06
NPC7	29.36	39.17
NPC8	25.81	28.05
NPC9	8.71	30.68
NPC10	61.72	64.04
NPC11	12	55.2
NP1	0	0
NP2	0	0
NP3	0	0

Expression on C666-1 was set at 100 for comparison.

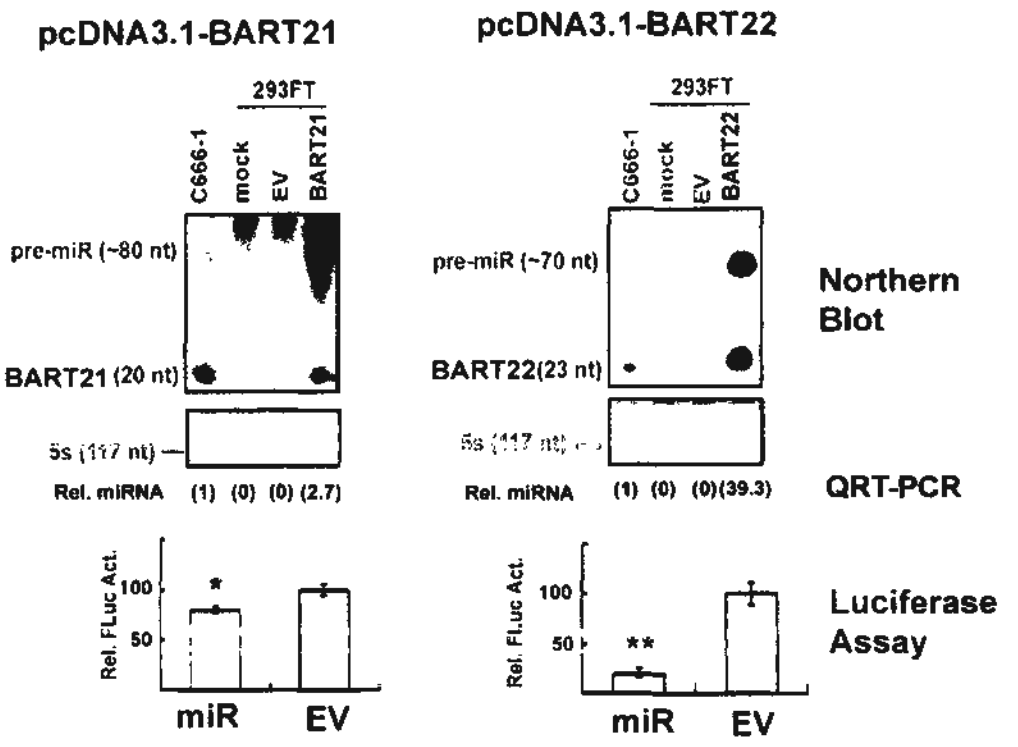


Figure 3.13 Expression of novel ebv-miRNAs in transient assays

Expression of ebv-miRNAs by DNA3.1-BART21 and pcDNA3.1-BART22 (*upper panel*) was tested by transfecting 293FT cells with either empty vector (EV) or a specific ebv-miRNA expression vector, as indicated. Detection of specific miRNAs in C666-1 and 293FT (mock) were included as positive and negative controls respectively. The silencing effect of miRNA expression vectors was demonstrated by the luciferase reporter assay (lower panel). 293FT cells transfected with the reporter plasmid, containing a perfectly complementarity sequence to the corresponding miRNA in its 3'UTR, plus an empty vector or its miRNA expression vector (miR). The relative FLuc activity was normalized with RLuc. The analysis of each sample was performed in triplicate with the mean +SD shown. Statistical analysis by *Student-t test* was used with a *p*-value of <0.001 denoted as (**) and a *p*-value of <0.05 denoted as (*)

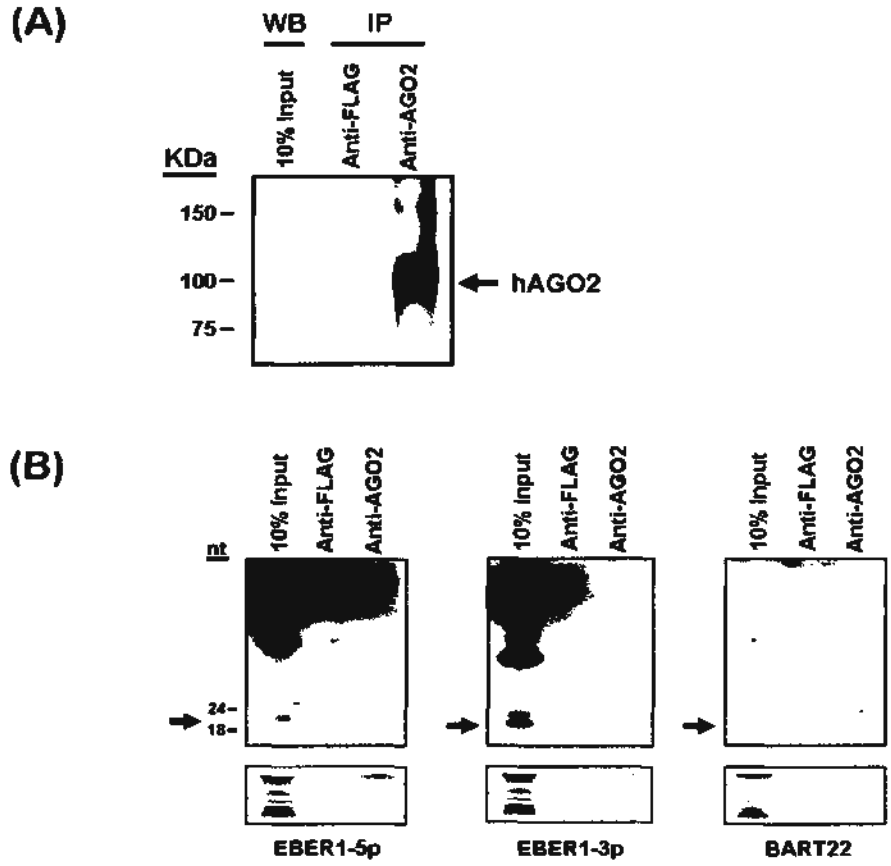


Figure 3.14 Biochemical detection of AGO2 associated miR-BART22

(A) Western blot (WB) analysis of hAGO-2 protein from C666-1 whole cell lysate before (Input) and after immunoprecipitation (IP) with anti-Ago2 antibody. (B) Monoclonal antibody against FLAG tag (lane 2) and against hAgo2 (lane 3) were used for IP from C666-1 cell lysate. AGO-2 associated miRNAs were analysed by Northern blot with synthetic oligonucleotide probe for the specific small EBV fragments listed below the figure. Locations of the small RNA fragments are indicated with arrows. RNA loading was visualized by SYBR Gold stained PAGE. RNA without immunoprecipitation (lane 1) was included as positive a control.

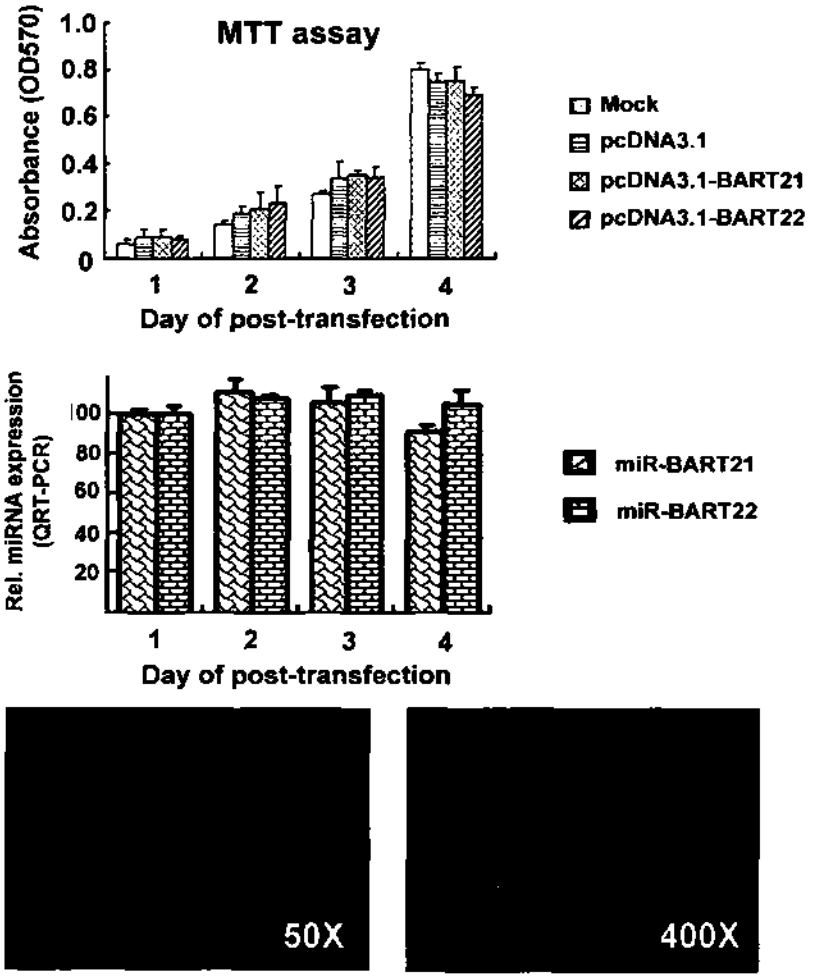
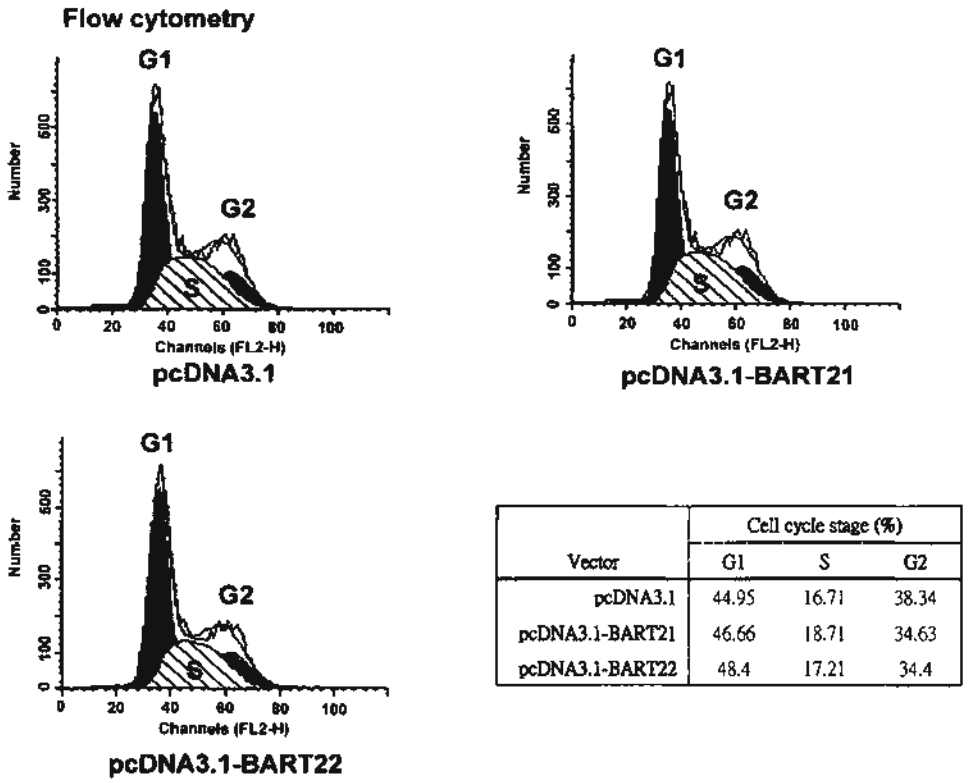


Figure 3.15 MTT assays on miRNAs transfected NP69 cells

Representative results from three individual MTT assay are shown. Expression vectors (0.2μg/well in a 96-well plate) for transfection are indicated on the right. The miRNA expression of the transfected cells in each of post-transfection day was assayed by QRT-PCR and compared with that on the first day (set at 100). The analysis of each sample was performed in five replicates with the mean +SD shown. The transfection efficiency of NP69 cells was evaluated by pEGFP-C1 plasmid transfection, and the representative images were shown in the lower panel.



Vector	Cell cycle stage (%)		
	G1	S	G2
pcDNA3.1	44.95	16.71	38.34
pcDNA3.1-BART21	46.66	18.71	34.63
pcDNA3.1-BART22	48.4	17.21	34.4

Figure 3.16 Flow cytometry analysis of novel ebv-miRNAs on NP69

NP69 cells were transfected with expression vectors listed below each figure and harvested for PI staining 24 hours after transfection. The distribution of three main phases (G1, S and G2) of cell cycle is summarized in the table shown in the bottom right corner.

3.3.3 Nucleotide polymorphisms affect Drosha processing of miR-BART22

Since pre-miR-BART10 (AJ507799; 147304-147380) and miR-BART22 (147161-147231) are in close proximity in the EBV genome, miR-BART10 and miR-BART22 are thought to share the same RNA transcript in miRNA biogenesis. However, the expression ratio of miR-BART22 to miR-BART10 was significantly higher in C666-1 than in Namalwa cells ($p<0.05$) as shown by both Northern blotting and QRT-PCR analysis (Figure 3.17). This observation suggested that the biogenesis of miR-BART10 and miR-BART22 was different between these two cell types. To evaluate if nucleotide variation(s) play a role in the biogenesis of these novel miRNAs, the flanking sequences of miR-BART21 and miR-BART22 from C666-1 (EU828629) and Raji (AJ507799) were compared. We employed the Raji-EBV sequence for analysis because Raji is commonly accepted as a reference EBV sequence. Based on matching analysis, we found two single nucleotide variations in miR-BART21 and four nucleotide changes in miR-BART22 (Figure 3.18*A*).

By MFOLD analysis, the predicted secondary structures of the miR-BART21 primary transcript (~300 nucleotides) from Raji-EBV and C666-EBV strains were highly similar (Figure 3.19). In contrast, the secondary structure predicted for miR-BART22 was dissimilar between the Raji-EBV and C666-EBV. Folding predictions for pri-miR-BART22 (147137:147456) from Raji-EBV suggested the presence of a small side-branched stem-loop adjacent to the mature miR-BART22 sequence (Figure 3.20, *left panel*). However, this stem-loop was not predicted for the C666-EBV derived miR-BART22 sequence (Figure 3.20, *middle panel*). It is possible that the nucleotide variations found

in the C666-EBV associated miR-BART22 underlie the predicted structural difference between the EBV strains. Although all four nucleotide variations in miR-BART22 are positioned distal from the hairpin structure, changes of two nucleotides (147144 A>T and 147146 C>A) could readily affect the stem-loop formation (Figure 3.20, *right panel*). Remarkably, these two specific nucleotide variations were identified in all 17 primary tumors tested from Hong Kong NPC patients (Figure 3.21). In fact, the formation of the small side stem-loop might impair miRNA maturation by concealing the DGCR8 recognition site during pri-miRNA processing (Han *et al.*, 2006). To elucidate whether this nucleotide polymorphism of pri-miR-BART22 affects miRNA maturation, we examined the digestion efficiency of Drosha/DGCR8 enzymatic complex by incubating immunoprecipitated flag-tagged Drosha/DGCR8 with *in-vitro* transcribed EBV RNA substrates (147137-147456) corresponding to the C666-1 and Namalwa EBV genome (section 2.18), which has the same sequence as Raji (Figure 3.21). PAGE analysis showed digested products of ~70-bp, which could correspond to either miR-BART10 or miR-BART22 pre-miRNA (Figure 3.20 and Figure 3.22 *left panel*). To further confirm that the digested product contained pre-miR-BART22, Northern blot analysis with a complementary miR-BART22 oligonucleotide was carried out on the PAGE transferred to membrane. We found that *in vitro* digestion of pre-miR-BART22 from C666-1 was more efficient in Drosha/DGCR8 processing compared to Namalwa (Figure 3.22 *right panel*). Our findings lead us to hypothesize that nucleotide polymorphisms within the pri-miR-BART22 transcript can augment its maturation in NPC, and explain at least in part the varying transcript levels in different EBV strains.

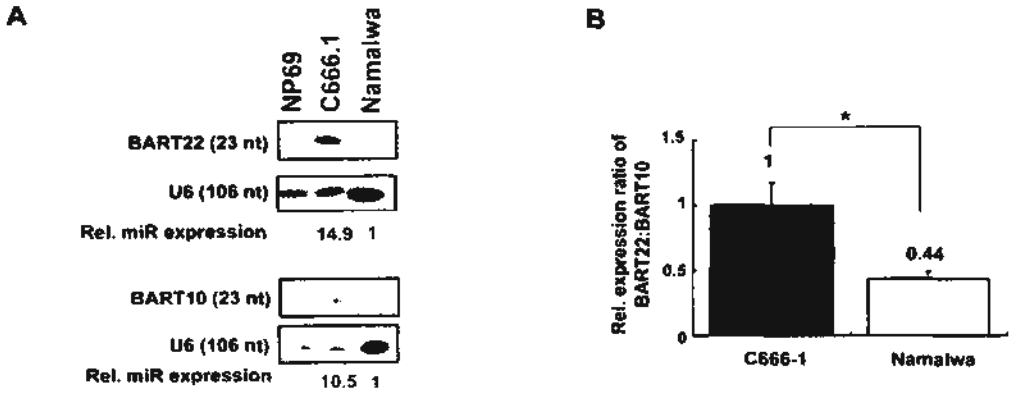


Figure 3.17 MiR-BART10 and miR-BART22 expression in C666-1 and Namalwa cells

(A) Northern blot analysis demonstrates that the relative expression of miR-BART22 to BART10 in C666-1 is significantly higher than that in Namalwa ($p<0.05$). Representative results from three independent experiments are shown. The relative expression level for each miRNA was calculated with reference to Namalwa (set as 1) after normalization to the U6 level. (B) The expression ratio of miR-BART22 to miR-BART10 is significantly higher in C666-1 than Namalwa cells as measured by QRT-PCR ($p<0.05$). The expression of miR-BART22 is compared to miR-BART10 after normalization to EBNA1. The results shown are the mean+SD from three independent experiments.

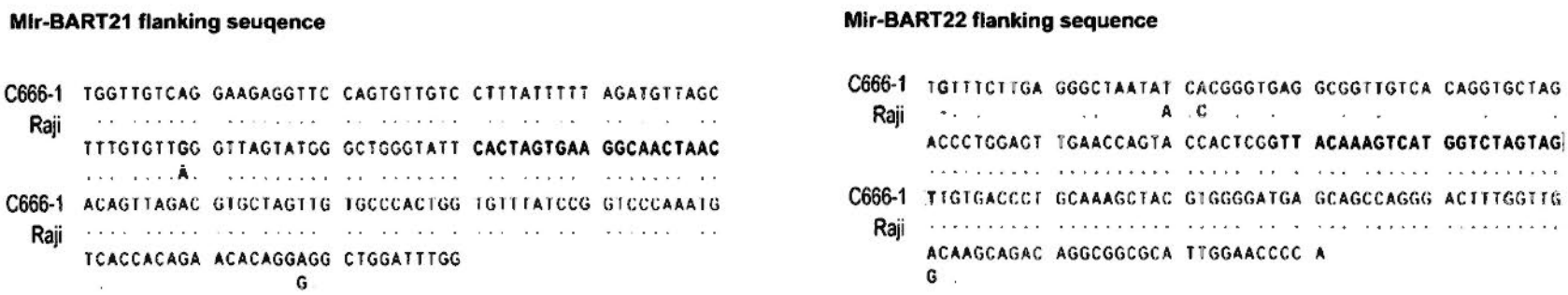


Figure 3.18 Nucleotide polymorphisms in pri-miR-BART-21 and 22 from C666-1-EBV

The nucleotide variations in pri-miR-BART21 (AJ507799, 145435:145614) *(left)* and pri-miR-BART22 (147126:147305) *(right)* relative to Raji-EBV are shown in color. The mature miRNA sequences are shown in gray boxes.

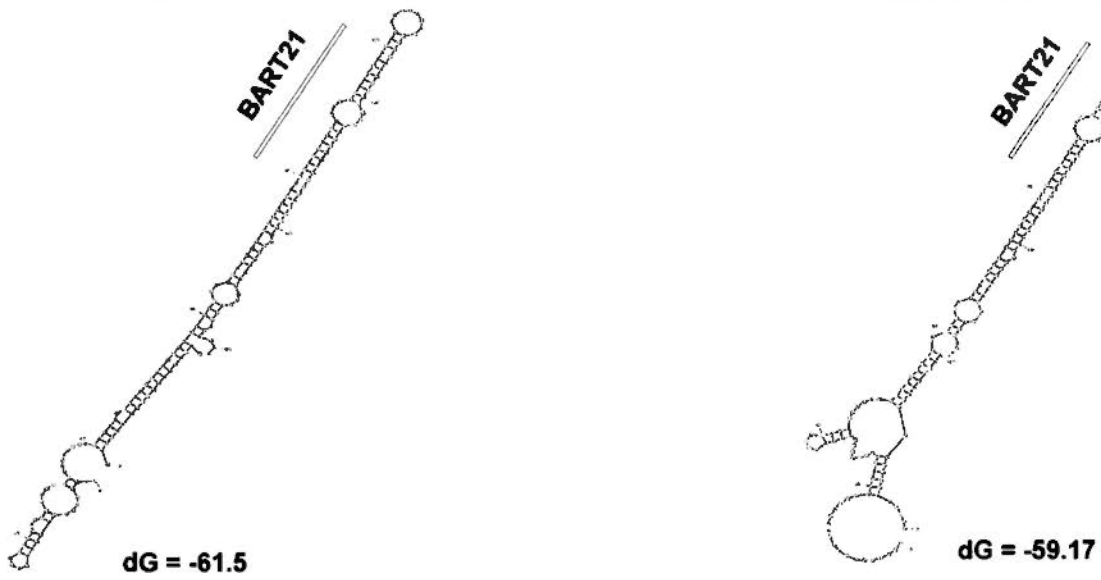


Figure 3.19 Predicted secondary structures of pre-miR-BART21

The secondary structure of pri-miR-BART21 from C666-EBV and Raji-EBV sequences (refer to AJ507799, 145435:145614) were predicted by the MFOLD program with standard settings. The fold energy (dG, kcal/mol) and the location of miR-BART21 are indicated.

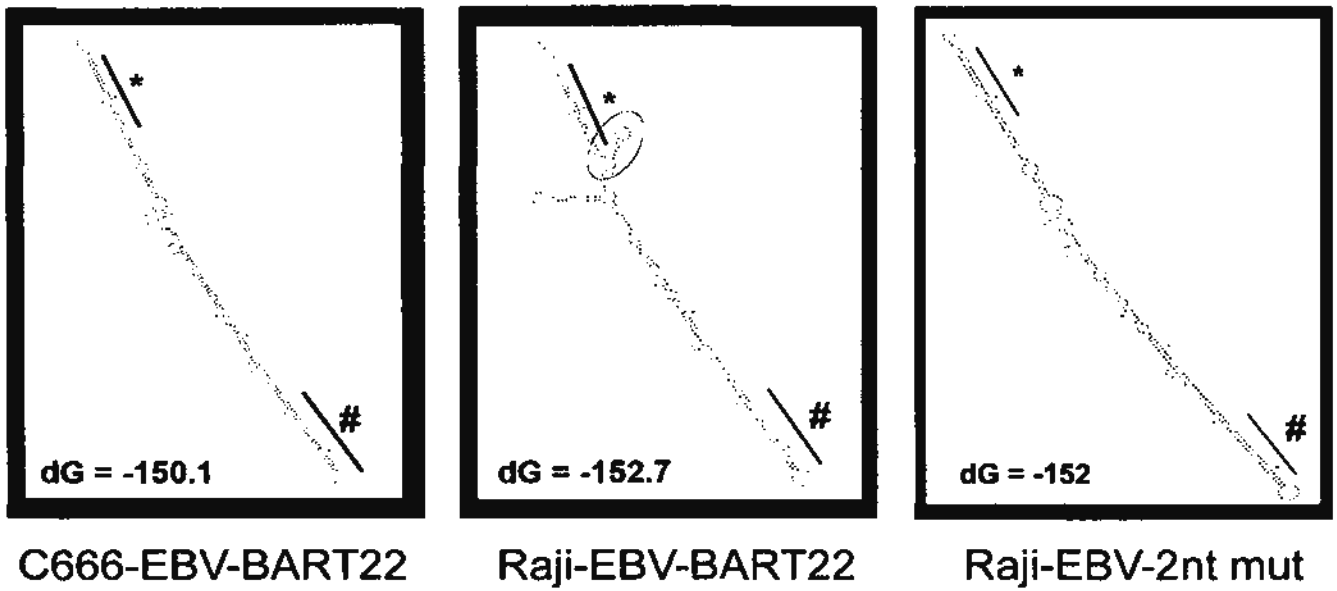


Figure 3.20 Predicted secondary structures of pre-miR-BART22

The predicted secondary structures of C666-1-EBV miR-BART22 (EU828629, 8087:8267) *(left)*, Raji-EBV miR-BART22 (AJ507799, 147137:147456) *(middle)*, and the two nucleotide variations of Raji-EBV, Raji-EBV-2nt mut (147144 A>T and 147146 C>A) *(right)* are shown. The folding energies (dG) in kcal/mol are indicated.

Raji	TAACCCGGGT	GAGGCGGTTG	TCACAGGTGC	TAGACCCTGG	AGTTGAACCA	GTACCACTCG	GTTACAAAGT	CATGGTCTAG	TAGTTG
C666.1	..T.A.....	....	....	....	....	....	....	....	......
GD1	..T.A.....	....	....	....	....	....	....	....	......
BC-1	....	....	....	....	....	....	....	....	......
IM9	....	....	....	....	....	....	....	....	..C...
Jiyoye	....	....	....	....	....	....	....	....	......
Mutu 1	....	....	....	....	....	....	....	....	......
Namalwa	....	....	....	....	....	....	....	....	......
NPC-T1	..T.A.....	....	....	....	....	....	....	....	......
NPC-T2	..T.A.....	....	....	....	....	....	....	....	......
NPC-T3	..T.A.....	....	....	....	....	....	....	....	......
NPC-T4	..T.A.....	....	....	....	....	....	....	....	......
NPC-T5	..T.A.....	....	....	....	....	....	....	....	......
NPC-T6	..T.A.....	....	....	....	....	....	....	....	......
NPC-T7	..T.A.....	....	....	....	....	....	....	....	......
NPC-T8	..T.A.....	....	....	....	....	....	....	....	......
NPC-T9	..T.A.....	....	....	....	....	....	....	....	......
NPC-T10	..T.A.....	....	....	....	....	....	....	....	......
NPC-T11	..T.A.....	....	....	....	....	....	....	....	......
NPC-T12	..T.A.....	....	....	....	....	....	....	....	......
NPC-T13	..T.A.....	....	....	....	....	....	....	....	......
NPC-T14	..T.A.....	....	....	....	....	....	....	....	......
NPC-T15	..T.A.....	....	....	....	....	....	....	....	......
NPC-T16	..T.A.....	....	....	....	....	....	....	....	......
NPC-T17	..T.A.....	....	....	....	....	....	....	....	......
C15	....	....	....	....	....	....	....	....	......
C17	....	....	....	....	....	....	....	....	......
C18	....	....	....	....	....	....	....	....	......
X666	..T.A.....	....	....	....	....	....	....	....	......
X1915	..T.A.....	....	....	....	....	....	....	....	......
X2117	..T.A.....	....	....	....	....	....	....	....	......
X99186	..T.A.....	....	....	....	....	....	....	....	......

Figure 3.21 Alignment of pri-miR-BART22 sequences from different EBV strains

Alignment of pri-miR-BART22 (AJ507799, 147142:147227) sequences from seventeen NPC samples (NPC-T1 to NPC-T17), EBV infected cell lines and NPC xenografts. The sequences of Raji (M35547), IM9 (EU828628), Mutu 1 (EU828632), and NPC samples GD1 (AY961628) and C18 (EU828627) were extracted from GenBank for analysis. The sequences of other samples were directly obtained by sequencing in our laboratory. The two nucleotide variations critical for the small side stem-loop formation are shown inside the pink boxes.

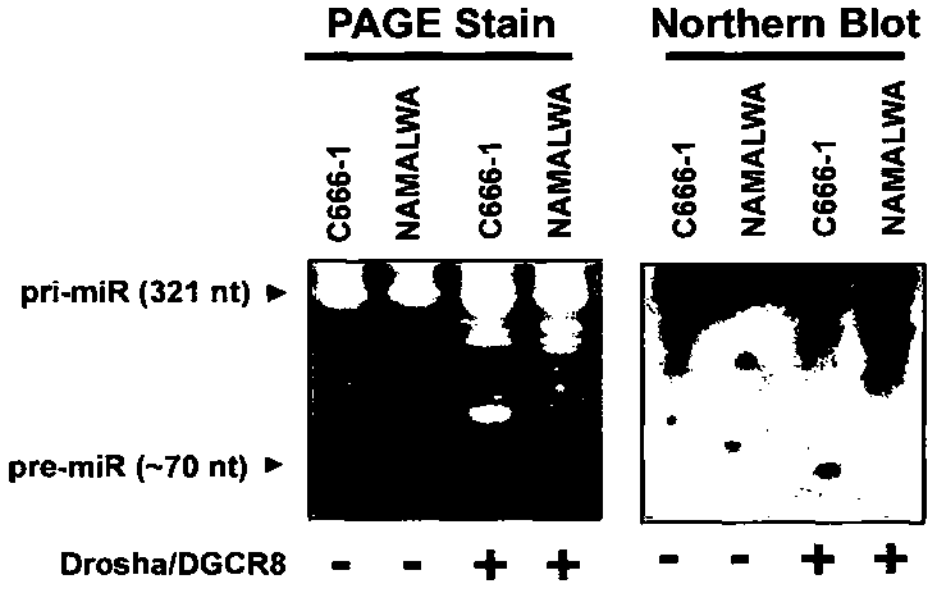


Figure 3.22: *In vitro* Drosha/DGCR8 processing of pri-miR-BART22 transcript.

C666-1-EBV and Namalwa-EBV pri-miR-BART22 transcripts (pri-RNA) (AJ507799, 147137:147456) were *in vitro* transcribed and incubated with bead-bound Flag-tagged Drosha/DGCR8 complex (+) or beads containing empty vector transfected cell extract (-) (section 2.18.2). The digested products were separated on 8% PAGE for analysis *(left)*. The presence of pre-miR-BART22 with a size around 70nt was confirmed by Northern blot with a miR-BART22 complementary oligonucleotide probe *(right)*. A representative result from at least three individual blots is shown.

3.4 Discussion

Besides the production of viral latent proteins, it is believed that EBV contributes to NPC development through expressing abundant non-coding viral RNAs, including EBERs and miRNAs. In this chapter, we examined miR-BART1, BART2 and BART5 as examples to confirm that ebv-miR-BARTs are abundantly expressed in NPC samples (Figure 3.1 and figure 3.2). Similar observations had also been reported in another epithelial cancer, gastric carcinoma (Kim do *et al.*, 2007). Thus, we postulated that ebv-BARTs should function in epithelial cancer development. Although expression of BHRF1 mRNA in NPC cell samples (including C666-1 cells) was observed, miR-BHRF1s were not detected in the same sample by Northern blot analysis (Figure 3.3 and figure 3.4). Furthermore, different from previous reports that EBV replication can stimulate the expression of miR-BHRF1s in cell lines (Cai *et al.*, 2006; Xing & Kieff, 2007), we found that induction of the EBV lytic cycle in C666-1 cells did not activate expression of miR-BHRF1s. These observations suggested that biogenesis of miR-BHRF1s might be controlled by more complex mechanisms in NPC.

In this chapter, we report the identification of two new ebv-miRNAs, miR-BART21 and miR-BART22, from screening 2928 clones by a traditional small RNA library cloning method in the NPC cell line (C666-1) and xenograft (X2117). They are both located in the BART cluster 2 region (Figure 3.23). These two newly identified miRNAs are consistently expressed in NPC cells and primary tissues. In line with our finding, Meister and co-workers have recently reported the identification of several novel EBV miRNAs by massive sequencing of 47000 clones that were derived from two NPC biopsies. We note that two

EBV miRNAs identified in their study, ebv-miR-BART21-3p and ebv-miR-BART22, are identical in sequence to our newly discovered miR-BART21 and miR-BART22, respectively (Zhu *et al.*, 2009). Our study has clearly established the relevance of miR-BART21 and miR-BART22 in NPC, and demonstrates the feasibility of direct cloning of small cDNA libraries as an efficient approach for the discovery of novel viral miRNAs.

In the library screening, miR-BART15 and miR-BART20 were not detected. In fact, a previous study in our group suggested that miR-BART15 is undetectable by Northern blot in all native infected, re-infected epithelial cell lines, as well as in Hela cells overexpressing EBV RNA transcripts from the C666-1 strain (Lo *et al.*, 2007). Nevertheless, Cosmopoulos and coworkers have recently reported the detection of BART15 and BART20 expression in C666-1 cell using real time RT-PCR (Cosmopoulos *et al.*, 2009).

We also identified some small RNA fragments from another abundant non-coding EBV RNA (EBER). Among them, we could only validate the presence of the fragments from EBER1 by Northern blot analysis (Figure 3.11). Although some reports have suggested that another non-coding virus-associated (VA) RNA from adenovirus can be processed to a functional mature microRNA (sva-RNA) (Aparicio *et al.*, 2006; Sano *et al.*, 2006), EBER1 is unlikely to undergo microRNA biogenesis because of the following reasons. Firstly, the predicted secondary structure of EBER1 is unlikely to be a pre-miRNA (Figure 3.10). Secondly, EBER1 is confined to the nucleus and does not associate with exportin 5, a protein which facilitates nucleus export of premature miRNAs (Fok *et al.*, 2006a). Thirdly, it was not cleaved by Dicer in an *in vitro* experiment (Sano *et al.*, 2006). And in this chapter, we further confirmed that small

EBER1 fragments (EBER1-3p and EBER1-5p) do not bind to Ago2, an integral part in RISC complex (Figure 3.14).

It was shown in previous reports that microRNA biogenesis can be altered by sequence polymorphisms located in either the mature microRNA sequence or microRNA flanking sequence (Gottwein *et al.*, 2006; Duan *et al.*, 2007; Jazdzewski *et al.*, 2008). By comparing sequences between different EBV strains, we identified the presence of a strain specific EBV polymorphism, especially at nt147144 A>T and nt147146 C>A, which predisposes to enhanced biogenesis of miR-BART22 and thus increases its expression level in our local NPC samples.

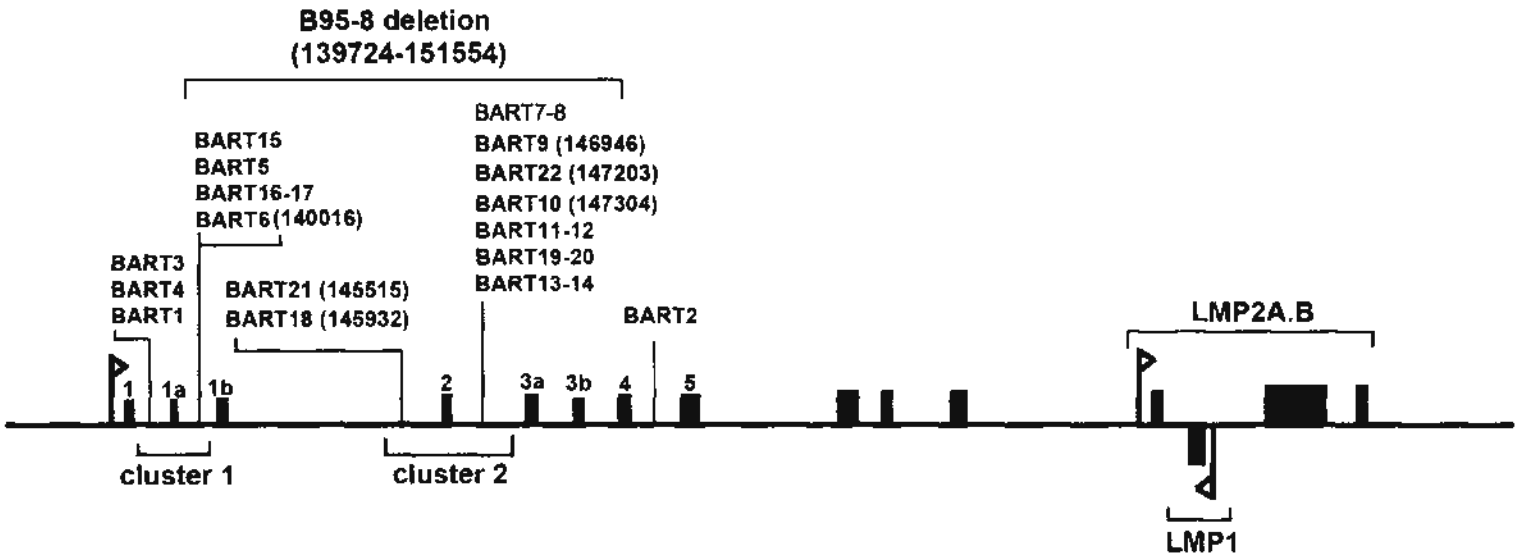


Figure 3.23 MiR-BART21 and miR-BART22 are located in the miR-BART cluster 2 region.

CHAPTER 4: FUNCTIONAL ANALYSIS OF MIR-BART22

4.1 Introduction

EBV resides in NPC in latency II and the expression of latent proteins is restricted to EBV nuclear antigen 1 (EBNA1), latent membrane protein 2 (LMP-2A and LMP-2B), and in around 20-50% of NPC, oncogenic latent membrane protein 1 (LMP-1) (Fahraeus *et al.*, 1988; Young *et al.*, 1988; Niedobitek *et al.*, 1992; Raab-Traub, 2002). In addition, two types of non-coding RNAs, EBERs and BARTs, are expressed in all NPC. Interestingly, given the selectively high expression of BARTs in EBV positive epithelial cancers including NPC, the function of these transcripts is not clear. The recent discovery of ebv-miRNAs within the BARTs region may help to reveal the function of these long non-coding transcripts. There is strong evidence suggesting that miRNAs are an important class of regulatory non-coding RNA species, which negatively regulate gene expression through complementary sequence pairing to the 3' untranslated regions (3' UTR) of target genes by inducing either mRNA degradation or translational repression (Bartel, 2004). While in most instances the function of ebv-miR-BARTs remains unknown, current findings suggest similar regulatory roles of miR-BARTs in gene expression (Cullen, 2009; Ghosh *et al.*, 2009).

Following the findings in chapter 3 that miR-BART21 and miR-BART22 are highly expressed in NPC samples, we proceeded to investigate how these two miRNAs contribute to NPC development. In this chapter, we aim to identify the targets of these newly identified miRNAs using bioinformatics and functional approaches. At the time of this study, two bioinformatics software programs were in wide use for miRNA target prediction, namely miRanda (Enright *et al.*,

2003; John *et al.*, 2004) and RNA hybrid (Rehmsmeier *et al.*, 2004). Although these two programs were initially designed to predict miRNA target genes in *D. Melanogaster*, we previously explored them to successfully identify some viral miRNA targets (Lo *et al.*, 2007; Choy *et al.*, 2008).

4.2 Materials and Methods

4.2.1 Tumor samples

For LMP-2A IHC, all nasopharyngeal (NP) and the first 11 NPC biopsies were the same as the ones used in Chapter 3. The rest of the biopsies, isolated from other EBV-positive NPC, infectious mononucleosis (IM) or Hodgkin Disease (HD), were obtained from the Prince of Wales Hospital, The Chinese University of Hong Kong. Information on the patients is listed in Table 4.4. Human experiments were approved by the local ethics committee.

4.2.2 Western Blot

Western blots were performed as described in section 2.11. For LMP-2A protein expression analysis in cell lines, fifty micrograms of protein were used for the western blot. However, five micrograms of protein was used to analyse the transgene expression in stable clones or transient transfectants. The density of the Western signals was measured by Image J software as described previously.

4.2.3 DNA sequencing

DNA extraction and sequencing analysis were performed as described in section 2.7.1 and section 2.16, respectively. In brief, the DNA fragment of interest from the EBV genome was amplified by PCR using 10ng of genomic DNA. The amplification product was checked by 2% agarose gel electrophoresis and visualized by EtBr staining. Roughly one microliter of PCR product was directly used for a 10μl sequencing reaction using the forward PCR primer. The sequences of the primers for PCR are listed below:

LMP2A-3'UTR-F: TGGCGCTTTGCTATGAATTA (5457:5476)

LMP2A-3' UTR-R: AGGTTCACGTCCAGCTTCTC (5804:5823)

The numbers in the parentheses indicate the location of the primers in the viral genome (AJ507799). Multiple sequence alignment was performed by using CLC Sequence viewer (www.clcbio.com/index.php?id=28).

4.2.4 Construction of luciferase reporter vectors

Construction of firefly luciferase reporter vectors was described previously (Wong *et al.*, 2008). In brief, two micrograms of sense (S) and antisense (AS) oligonucleotides corresponding to encoded a single miRNA test target were annealed in 30 mmol/L HEPES buffer (pH7.4) containing 100 nmol/L potassium acetate and 2 mmol/L magnesium acetate, and the annealed oligos were cloned downstream of the cytomegalovirus (CMV) promoter-driven firefly luciferase cassette in a pMIR-REPORT vector (Ambion, Inc) via *SpeI* and *Hind III* sites. The dual luciferase reporter assay was performed as described in section 2.19. The sequences of oligonucleotides used for cloning are listed below:

T1-S: 5'-CTAGTGATCGCCTGCCACTTCCACAGCAA-3'

T1-AS: 5'-AGCTTTGCTGTGGAAGTGGCAGGCGATCA-3'

T2-S: 5'-CTAGTAACCCCACGGAGCAGGGCAACATTGCAGGGA-3'

T2-AS: 5'-AGCTTCCCTGCAATGTTGCCCTGCTCCGTGGGGTTA-3'

T3-S: 5'-CTAGTAGACTATGCATACACTGAATTTAGA-3'

T3-AS: 5'-AGCTTCTAAATTCAGTGTATGCATAGTCTA-3'

T4-S: 5'-CTAGTAGACCTGTGTGCTGTATTTAA-3'

T4-AS: 5'-AGCTTTAAATACAGCACACAGGTCTA-3'

BART12-S: 5'-CTAGTAACCACACCAAACACCACAGGAA-3'

BART12-AS: 5'-AGCTTTCCTGTGGTGTTTGGTGTGGTTA-3'

BART14-S: 5'-CTAGTATCCCTACTACTGCAGCATTTAA-3'

BART14-AS: 5'-AGCTTTAAATGCTGCAGTAGTAGGGATA-3'

BART16-S: 5'-CTAGTAGAGCACACACCCACTCTATCTAAA-3'

BART16-AS: 5'-AGCTTTTAGATAGAGTGGGTGTGTGCTCTA-3'

BART21-S: 5'-CTAGTGTTAGTTGCCTTCACTAGTGA-3'

BART21-AS: 5'-AGCTTCACTAGTGAAGGCAACTAACA-3'

BART22-S: 5'-CTAGTACTACTAGACCATGACTTTGTAAA-3'

BART22-AS: 5'-AGCTTTTACAAAGTCATGGTCTAGTAGTA-3'

BART1-5p-S: 5'-CTAGTCACAGCACGTCACTTCCACTAAGAA-3'

BART1-5p-AS: 5'-AGCTTTCTTAGTGGAAGTGACGTGCTGTGA-3'

LMP2A-WT-S: 5'-CTAGTCCTGTGTGACCCCTCACTTTGTACA-3'

LMP2A-WT-AS: 5'-AGCTTGTACAAAGTGAGGGGTCACACAGGA-3'

LMP2A-M1-S: 5'-CTAGTCCTGTGTGACCCCTCAA-3'

LMP2A-M1-AS: 5'-AGCTTTGAGGGGTCACACAGGA-3'

LMP2A-M2-S: 5'-CTAGTCCTGTGTGACCCCTCAGTATCTTCA-3'

LMP2A-M2-AS: 5'-AGCTTGAAGATACTGAGGGGTCACACAGGA-3'

LMP2A-M3-S: 5'-CTAGTCCTGTGTGACCCCTCACATAGAAGA-3'

LMP2A-M3-AS: 5'-AGCTTCTTCTATGTGAGGGGTCACACAGGA-3'

4.2.5 RT-PCR analysis of LMP-2A

Expression of LMP-2A mRNA was confirmed by traditional RT-PCR. In brief, one microgram of total RNA was reverse transcribed by High Capacity cDNA Archive Kit (Applied Biosystems) in a 20μl reaction containing random primers. One microliter of total cDNA was used to perform the PCR reaction

for 22 (ACTIN) or 40 (LMP-2A) cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min in 25μl reaction containing 0.25mM dNTPs (Amersham), 0.2μl of each 10μM primer, 1XPCR reaction buffer, 1.6μl of 25mM $MgCl_2$ and 0.2μl of Taq polymerase (Fermentas). Due to the low LMP-2A mRNA expression in C666-1, another 10 additional cycles of PCR reaction were performed in order to achieve a clear-cut result. The PCR products were analyzed in a 2% agarose gel. The sequences of the primers are listed below:

LMP2A-Exon-1-F: 5'-CGGGATCACTCATCTGAACACATA-3'

LMP2A-Exon-3-R: 5'-CATGTTAGGCAAATTGCAAA-3'

Actin-209F: 5'-TAAGGAAGAAGCTGTGCTACGTC-3'

Actin-209R: 5'-GGAGTTGAAGGTAGTTTCGTGG-3'

4.2.6 IHC

Immunohistochemical staining for LMP-2A was performed as described in section 2.12.

4.2.7 LMP-2A stable transfectants

Construction of stable LMP-2A transfectants is described in section 1.19.3 and the expression of LMP-2A in the stable clones was confirmed by IHC (Figure 2.9).

4.2.8 Preparation of small RNA markers

Chemically synthesized 18- and 24-mer RNA oligonucleotides were purchased from IDT Inc. The sequences of the synthetic oligonucleotides are listed below:

18-mer: rArGrCrGrUrGrUrArGrGrGrArUrCrCrArArA;

24-mer: rGrGrCrCrArArCrGrUrUrCrUrCrArArCrArArUrArGrUrGrA

Two hundred micro moles of RNA oligonucleotides were separated and purified on 15% PAGE. RNA was eluted from the gel strip by incubating the gel with 200µl of elution buffer (0.5M NH_4OAc, 1mM EDTA and 0.1% SDS) at 55°C for 1 hour, followed by incubation at 4°C overnight with shaking. After elution, the RNA-containing buffer was collected into a new tube for RNA precipitation. For RNA precipitation, 1µl of glycogen (10mg/ml) and 600µl of 100% EtOH were added to the RNA-containing buffer and incubated on dry ice for 10 min. The RNA pellet was collected by centrifugation at 4°C for 15 min at maximum speed, washed once with 75% EtOH and re-dissolved in 20µl of RNase free water. One to two microliters of RNA was used as a marker.

Thirty-five-mer and 41-mer size markers were prepared by ligating the modified DNA oligo 17.91X onto 18- and 24-mer RNA following the miRNA cloning protocol available from the Bartel Lab: (http://web.wi.mit.edu/bartel/pub/protocols.html) (England *et al.*, 1977). The sequence of 17.91X is AppCTGTAGGCACCATCAddA (available from IDT Inc.).

4.2.9 Statistical analysis

Results from QRT-PCR, luciferase reporter assays and protein expression analyses were compared using the Student's t-test. The results were analyzed from at least three independent experiments. A $p \leq 0.05$ was considered significant.

All other methods are described in detail in Chapter 2.

4.3 Results

4.3.1 Prediction of potential cellular target(s) of miR-BART21 and miR-BART22

To attempt the prediction of any potential cellular targets of miR-BART21 and miR-BART22 by miRanda and RNAhybrid programs, we first retrieved all human 3'UTR using MartView (www.ensembl.org/Multi/martview). The 3'UTRs were fed separately into miRanda and the RNAhybrid program to predict potential targets using standard parameters. The total score of predicted binding site(s) $\geqq 90$ in miRanda and the minimum free energy (r_mef) $\geqq$ -18kCal/Mol in RNAHybrid were considered. Potential binding sites were further checked for cross species conservation using UCSC Human BLAT. The top 100 potential targets of each miRNA ranked by miRanda are listed in appendix II and III.

At the first glance, several potential cellular targets appeared to be involved in the regulation of immunity, cell cycle and apoptosis (Table 4.1 and 4.2). However, in transient transfection assay, pcDNA3.1-BART21 vector could only produce a low level of mature miRNA which was not enough to exert sufficient suppressive effect for miRNA-target validation (Figure 3.13). Thus, we could only focus on the targets of miR-BART22. The E2F3 transcription factor has drawn high attention because we have identified a total of three miR-BART22 putative binding sites on its 3'UTR by using another computer prediction program, miTarget (Table 4.3) (Kim *et al.*, 2006). Furthermore, expression of E2F3 in two EBV negative epithelial cells, NP69 and HONE1 is higher than that in C666-1 (Figure 4.1 *left panel*). Western blot analysis was directly performed to further investigate the expression of E2F3 after ectopic expression of

miR-BART22 in two EBV negative NPC cells, HONE1 and NP69. However, western blot showed the negative result that E2F3 protein level did not change between mock, miR-BART21 and miR-BART22 transfectants in these two cell lines (Figure 4.1, *middle and right panel*).

E2F3 was found to be a false positive target of miR-BART22. Simultaneously, we also failed to validate all potential targets listed in Table 4.2 by luciferase reporter assays using pcDNA3.1-BART22. The high false positive rate generated from our prediction may be due to the fact that the algorithm design of these two programs is not sensitive enough for the viral miRNA target searching. This, in turn, was probably caused by insufficient experimental data concerning the interaction between viral miRNAs and their cellular mRNA targets. However, by using miRanda and RNAhybrid programs, previous reports were able to identify a specific viral mRNA target that interacts with viral encoded miRNAs (Grey *et al.*, 2007; Lo *et al.*, 2007). Subsequent analysis therefore focused on viral mRNAs potentially targeted by miR-BART22.

Table 4.1: Examples of predicted cellular mRNAs of miR-BART21

Gene ID*	Gene Function	Alignment (miRanda score and energy)
	Immunity	
MICB (ENST00000252229)	MHC class I polypeptide-related sequence B	BART21: 3' CAATCAACG-GAAGTGATCAC 5' MICB: 5' CCAGGCTGCAGTTCACTGGTG 3' (99, -19.58kCal/Mol)
	Apoptosis, cell proliferation	
TP53 (ENSG00000141510)	Cellular tumor antigen p53	BART21: 3' CAATCAA-CGGAAGTGATCAC 5' TP53: 5' ACAAGTTGGCCTGCACTGGTG 3' (110, -24.16kCal/Mol)
BCL2L13 (ENSG00000099968)	Bcl-2-like 13 protein (Protein Mil1)	BART21: 3' CAATCAACGGAAGTGATCAC 5' BCL2L13: 5' TTCAGCCCTTTTCGCTAGTG 3' (103, -18.99kCal/Mol) BART21: 3' CAATCA-ACGGAA--GTGATCAC 5' BCL2L13: 5' GTTTCTCAGCCTTGGCACTAGTG 3 (103, -20.51kCal/Mol)

*Gene symbol is indicated as recommended by HUGO

Table 4.2 Examples of predicted cellular mRNAs of miR-BART22

Gene ID*	Gene Function	Alignment (miRanda score and energy)
	Cell cycle	
E2F3 (ENSG00000112242)	Transcription factor	BART22: 3' TGATGATCTGGT---ACTGAAACATT 5' E2F3 5' ACACCTA-ATCAGTTTAGCTTTGTGT 3' (131, -24.45kCal/Mol)
ATM (ENSG00000149311)	Serine-protein kinase ATM (Ataxia telangiectasia mutanted)	BART22: 3' TGATGATCTG-GTACTGAAACATT 5' ATM: 5' ACTACT-GACTCGTGTATTAGTGA 3' (100, -21.00kCal/Mol)
	Apoptosis, cell proliferation	
MAKP3K3 (ENSG00000135341)	Mitogen-activated protein kinase kinase kinase 5 (Apoptosis signal-regulating kinase 1)	BART22 : 3'TGATG-ATCTGGT--ACTGAA-ACATT 5' MAKP3K3: 5'TCTGCTTAGACCACCTGGTTTATGTGA 3' (101, -21.92kCal/Mol)
FAS (ENSG000000261033)	Tumor necrosis factor receptor superfamily member 6 precursor	BART22: 3' TGAT--GA---TCTGGTACTGAAACATT 5' FAS: 5' ACTGCTCTCAGAGAAAGTAGCTTTGTGA 3' (98, -17.54kCal/Mol)
CASP3 (ENSG00000164305)	Caspase-3-precursor	BART22: 3' TGATGATCTGG-TACTG-AAACATT 5' GASP3 : 5' CTTACTAGACCTGTAACTTTTGTAA 3' (119, -23.62kCal/Mol)

*Gene symbol is indicated as recommended by HUGO

Table 4.3 Predicted miR-BART22 binding sites on E2F3 (Accession number: NM_001949.3)

Alignment	Position	Free Energy (5')	Free Energy (3')	Free Energy	SVM Score
3'-UGAUGAUCUGGUACUGAAACAUU-5' || || ||| :||||||: 5'-UCUGCUU--CCA--GCUUUGUGU-3'	3605	-4.70	-6.40	-15.20	0.866
3'-UGAUGA-UCUGGUACUGAAACAUU-5' |||| | | ||| ||||||| 5'-CCUACGGGGUACGUGAUUUUGUAA-3'	4491	-3.40	-9.00	-19.30	0.650
3'-UGAUGAUCUGGUAC---UGAAACAUU-5' |||||| | ||: ||||||: 5'-ACUACUA-AUCAGUUUAGCUUUGUGU-3'	4552	-4.70	-6.96	-16.00	0.901

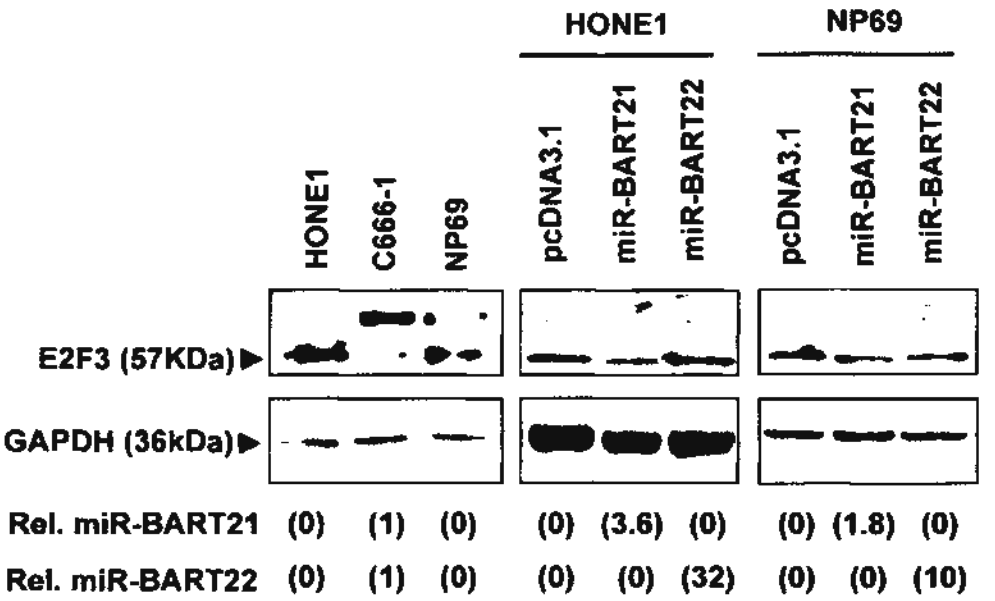


Figure 4.1 Effect of miR-BART22 on protein expression of E2F3

Western blot analysis for E2F3 protein expression in EBV negative cells, HONE1 and NP69, and EBV-positive NPC cells, C666-1, indicate that EBV infection may regulate the expression of E2F3 (*left panel*). However, transient transfection of miR-BART22 into both HONE1 and NP69 did not alter E2F3 protein expression levels suggesting that E2F3 is not the direct target of miR-BART22. The miRNA expression in the samples was assayed by QRT-PCR and compared with that in C666-1 (set at 1). Representative data were selected from two independent experiments.

4.3.2 Interaction of BART22 with the 3'UTR of LMP-2A mRNA

Using the default setting of the miRanda program, we found a putative miR-BART22 binding site in the LMP2A-3'UTR (AJ507799; 5546-5568). The predicted putative target site is highly conserved between different EBV strains (Figure 4.2). A total of 16 nucleotides including the seed sequence (2-8nt) of miR-BART22 are perfectly complementary to the target site (Figure 4.3, upper panel). To study the predicted suppressive effect of miR-BART22 on LMP-2A, a series of dual luciferase reporter assays were performed in 293FT cells. The reporter constructs contained the predicted or mutated binding sequences that were cloned using synthetic oligonucleotides into the 3'UTR of the pMIR-REPORT plasmid. Using a miRNA expression vector in reporter assays, we found that miR-BART22 exerts a strong inhibitory effect on the LMP-2A 3'UTR (42%, $P < 0.001$). On the other hand, repression was eliminated when the complementarity of the seed region was either deleted or mutated, for example, LMP2A-M1-M3 in Figure 4.3. This observation suggested that the repressive property of BART22 on the LMP2A 3'UTR is both functional and specific.

To further confirm the importance of seed sequence complementarity in the BART22-LMP-2A interaction, we performed additional luciferase assays of LMP2A-M1-M3 reporter plasmids with miR-BART22 and two miRNA mimics, miR-M2-BART22 and miR-M3-BART22, in which the mutated seed regions on the LMP2A-M2 and M3 reporters should be compensated (Figure 4.4). The miRNA mimics exerted different levels of suppression on the 3'UTR BART22, indicating that the miRNA mimics were functionally active. However in co-transfection with LMP2A-WT 3'UTR, only miR-BART22, but not

miR-M2-BART22 and miR-M3-BART22, was able to successfully reduce translation. Specifically, mutant miR-M3-BART22 with restored complementarity to LMP2A-M3 failed to exert an inhibitory effect on its corresponding mutated reporter. While miR-M2-BART22 could significantly inhibit translation on the LMP2A-M2 mutated reporter, it was only slightly below the control level and clearly did not exhibit profound repression ability (Figure 4.4). Together, our data would hence suggest that the seed interaction between miR-BART22 and LMP2A-3'UTR is unique and the replacement of seed pairing by other complementary sequences provides negligible or only a partial suppressive effect.

We further investigated whether LMP2A-3'UTR might contain putative binding sites for other miR-BARTs. By loosening the MiRanda program parameters for prediction, target sites for miR-BART1, 12, 14 and 16 were suggested but none could be successfully validated by luciferase reporter assay (Figure 4.5). This further implied the uniqueness of the interaction between miR-BART22 and LMP2A-3'UTR, thus the expression regulation of LMP2A.

```
B95-8       CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
Akata       CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
BC1         CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
C17         CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
C15         CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
X1915       CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
X2117       CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
C666-1      CGTCATCTGG CTCTCCTGTG TGACCCCTCA CTTTGTACAG ACTTTTGGCA
LMP2A-UTR   ---------- ----CCTGTG TGACCCCTCA CTTTGTAC-- ----------
```

Figure 4.2 Conservation of the 3'UTR of the LMP-2A gene in different EBV strains

Direct sequencing results of the putative miR-BART22 binding site on the 3'UTR of the LMP-2A gene from different samples are illustrated. The B95.8 sequence is extracted from GenBank (accession number: X01995) and is shown as a reference sequence.

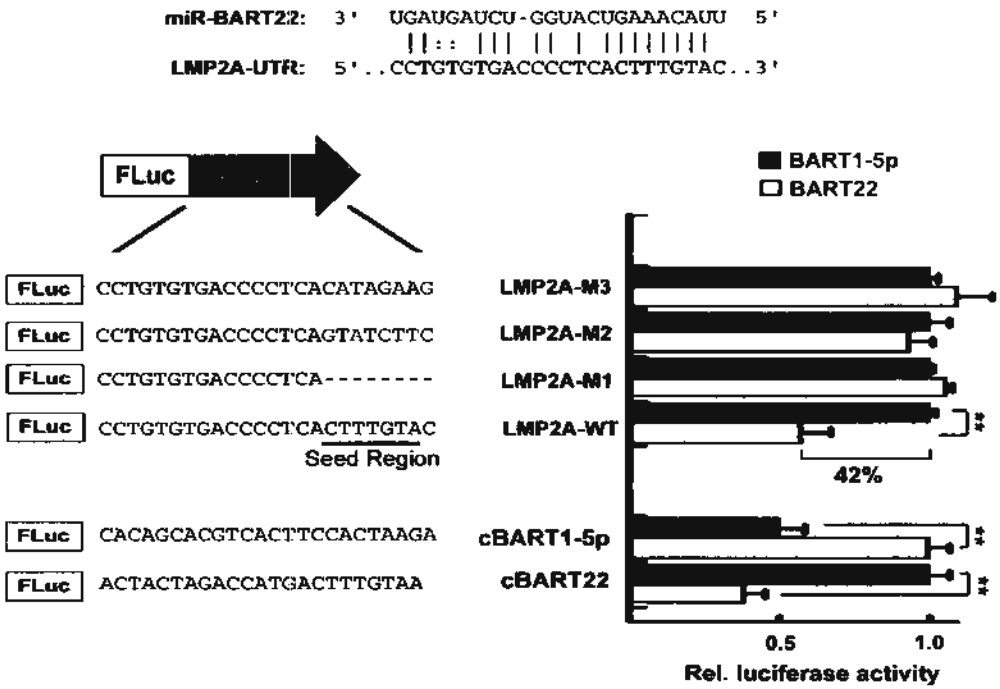


Figure 4.3 Viral LMP-2A is a potential target of miR-BART22

The base complementarity suggests that the putative miR-BART22 target site is at the 3'UTR of LMP-2A (AJ507799.2, 5545:5568) *(upper panel)*. The luciferase reporter vector carrying wild-type (LMP2A-WT) and mutated (LMP2A-M1 to M3) LMP2A sequence in the 3'UTR are shown. The seed binding region is underlined and the base substitutions used are marked in red. As a control, reporter vectors carrying miRNA complementary sequence in the 3' UTR are constructed (cBART1-5p and cBART22). The relative luciferase activity was normalized to renilla luciferase control and results were taken from at least three independent experiments. Data shown are the mean + SD. Statisctical analysis by *Student-t test* was used, and a p-value < 0.001 is denoted as (**).

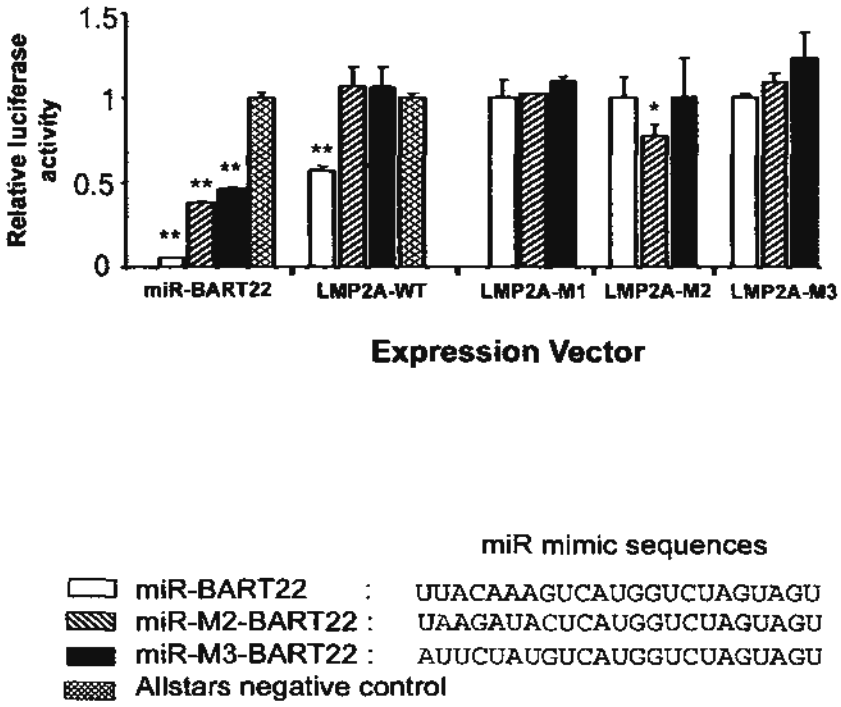


Figure 4.4 Uniqueness of the interaction between miR-BART22 and the LMP-2A-3'UTR

The sequences of miRNA mimics are displayed. The base substitutions that restore seed-mutated complementarity are labeled in red (miR-M2-BART22 and miR-M3-BART22). The relative firefly luciferase activity was normalized to the renilla luciferase control and results were taken from at least three independent experiments. Data shown are the mean + SD. Statistical analysis by *Student-t test* was used, and a p-value < 0.001 is denoted as (**).

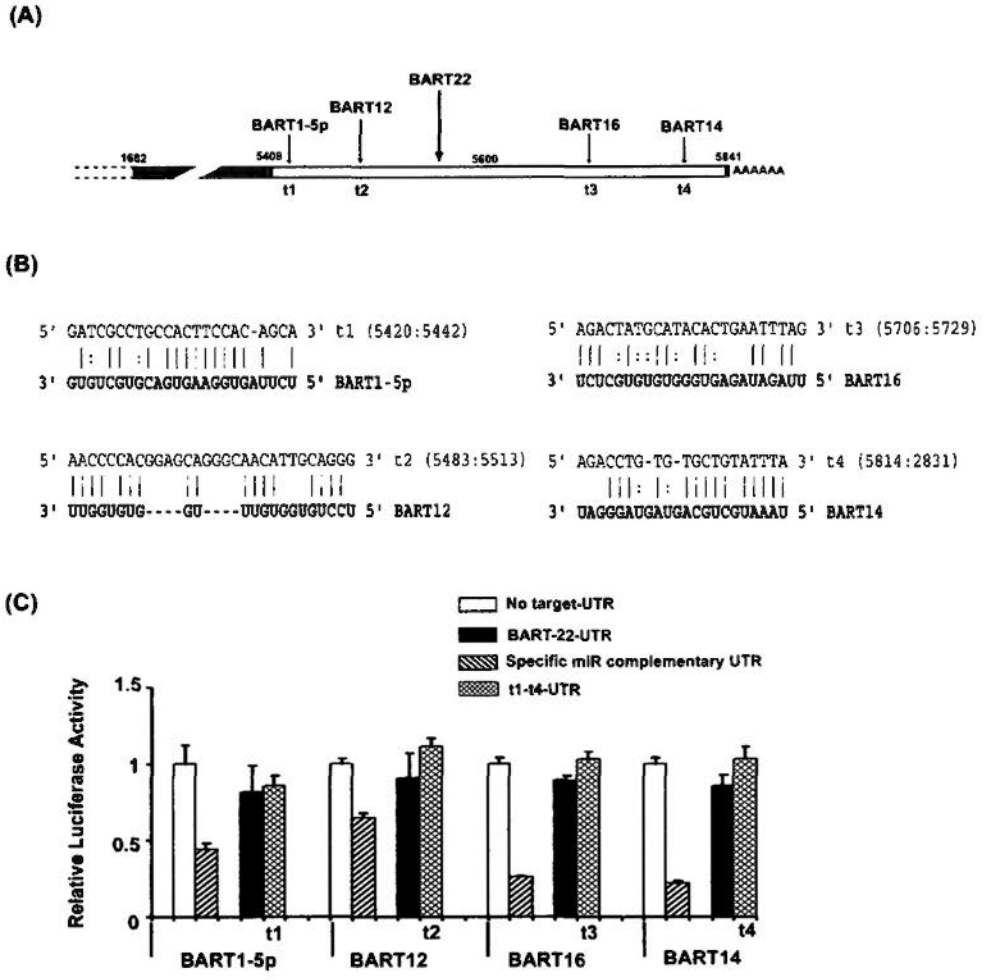


Figure 4.5 Predicted LMP-2A-3'UTR binding sites for other miR-BARTs

(A) Schematic diagram showing the location of predicted target sites (t1-4) on the 3'UTR of the LMP-2A gene (open bar) according to GenBank accession no. AJ507799. (B) The alignment of the target sites to the corresponding miR-BARTs is shown. The target sites were cloned into luciferase reporters for analysis. (C) Luciferase reporter assays of t1-4 containing constructs in the presence of indicated miR-BARTs were performed in 293FT cells. Reporter activity was normalized to the renilla luciferase control. Luciferase activity from the construct containing no miRNA target in its 3'UTR (white bar) was set at 1. Data shown are the mean + SD from three independent experiments.

4.3.3 Differential expression of LMP-2A in NPC

In order to investigate whether LMP-2A is commonly expressed in NPC, we performed both RT-PCR and Western Blot on a panel of NPC samples from Southern China. Although the LMP-2A RNA transcript could be detected in C666-1 cells and NPC xenografts X666 and X2117, none of them showed detectable LMP-2A protein levels in western blot analysis (Figure 4.6). We also failed to detect the expression of LMP-2A protein in a series of EBV positive cell lines (Figure 4.7). Nevertheless, we were able to detect weak focal expression of LMP-2A in X2117 by immunohistochemical staining (IHC). As a control, strong LMP-2A staining was demonstrated in an EBV-positive infectious mononucleosis (IM) and six of the eight Hodgkin's disease/lymphoma (HD) biopsies (Table 4.4). This finding suggested that IHC analysis has a higher sensitivity than Western blotting for the detection of LMP-2A expression. By IHC, we were able to detect weak LMP-2A expression in 6 out of 26 (23%) primary NPC tumors (Figure 4.8). The expression levels of miR-BART22 and LMP-2A mRNA have also been determined in eleven of these tumors (Table 4.5). Interestingly, the LMP-2A mRNA expression level was not directly correlated with protein expression. Furthermore, a relatively low expression level of miR-BART22 miRNA was observed in all three LMP-2A positive-primary tumors, as well as in X2117 (NPC6, 8 and 11 in Table 4.5 and Figure 4.8). These findings supported the possible regulatory role of miR-BART22 in LMP-2A expression.

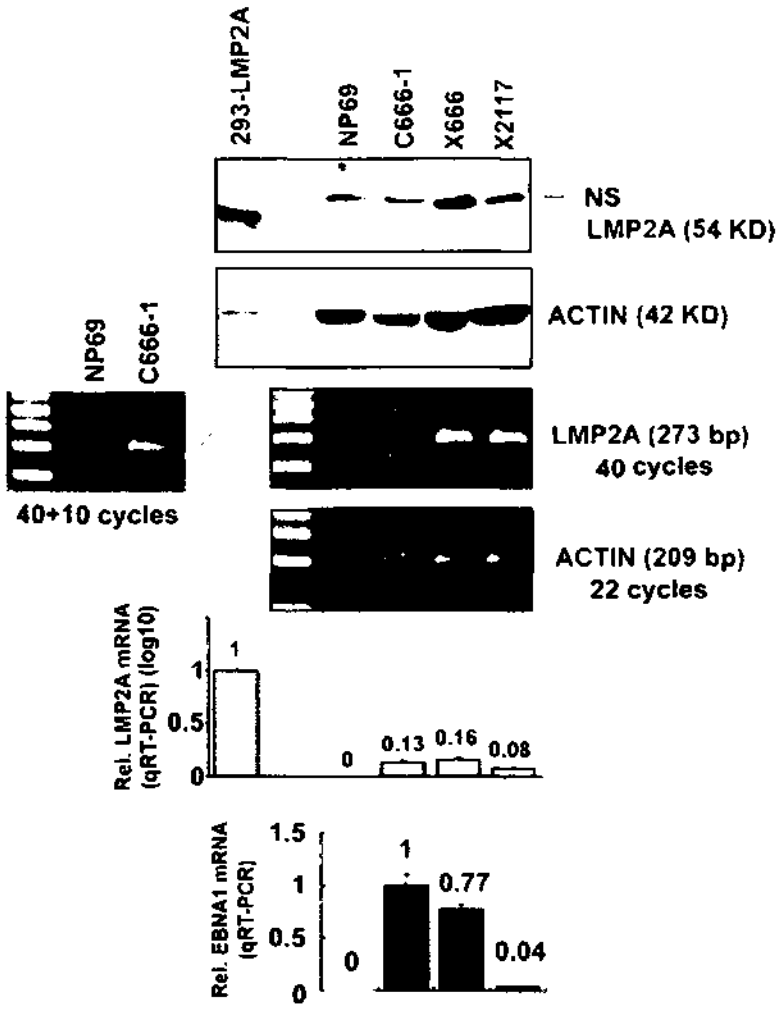


Figure 4.6 Western blot analysis of LMP-2A expression in NPC samples

One NPC cell line (C666-1) and two xenografts (X666 and X2117) were subjected to analysis. Protein samples from LMP-2A transfected 293FT cells (293-LMP-2A) and NP69 were included as the positive and negative controls, respectively. The non-specific bands (NS) are labeled. The LMP-2A and EBNA1 mRNA expression levels in the same sample were confirmed by RT-PCR and QRT-PCR, respectively. The QRT-PCR results were normalized to GAPDH and are shown as the mean ± SD from three independent experiments. The expression levels of LMP-2A in 293-LMP-2A, and of EBNA1 in C666-1 were set at 1.

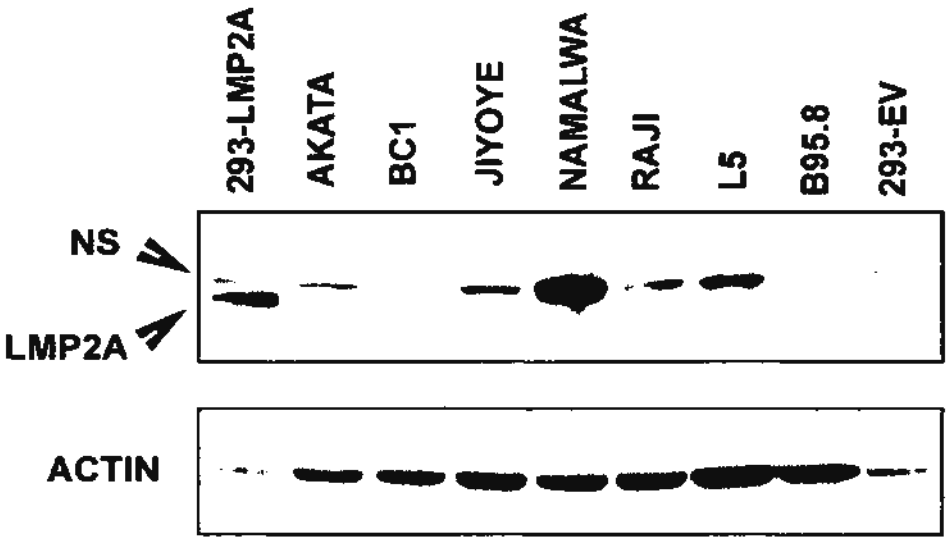


FIGURE 4.7 Detection of LMP-2A protein in EBV positive cell lines

LMP-2A expression was analyzed by western blot. Protein samples from LMP-2A transfected 293FT cells (293-LMP-2A) and pcDNA3.1 empty vector transfected cells (293-EV) were included as the positive and negative controls, respectively. Non-specific bands (NS) are labeled.

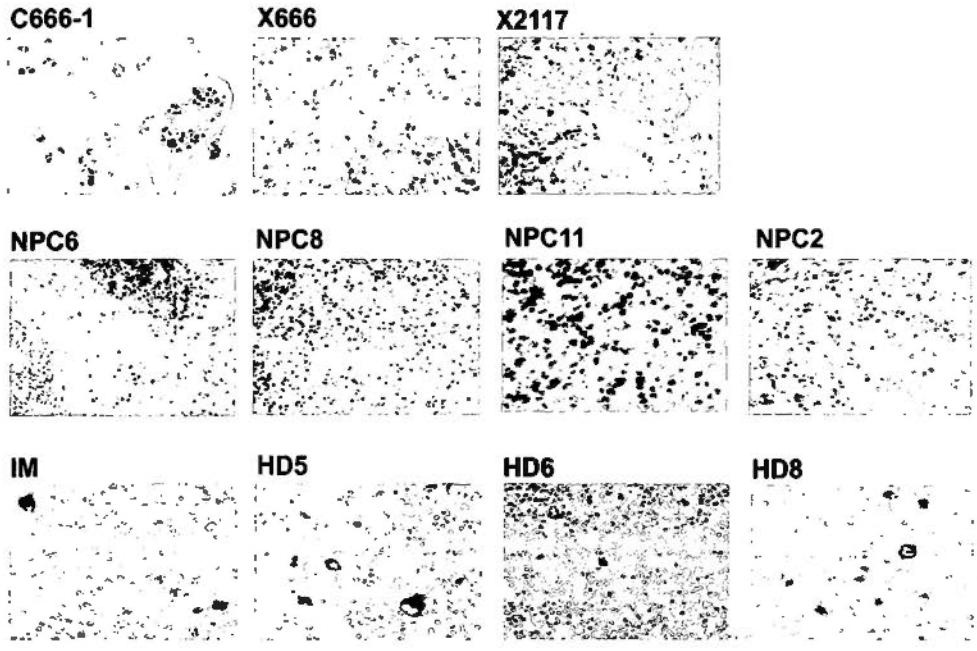


Figure 4.8 LMP-2A protein expression in NPC, IM and HD specimens

LMP-2A expression was not detected in the NPC cell line C666-1 and xenograft X666 and only focally weak LMP-2A expression was detected in xenograft X2117(*Upper panel*). Weak expression of LMP-2A was noted in three NPC samples (NPC6, 8, 11) and a negative example (NPC2) was included *(Middle panel)*. Strong expression of LMP-2A was detected in Infectious Mononucleosis (IM) and three tissues which positive for Hodgkin Disease (HD) were included as controls (*lower panel*).

Table 4.4 Expression of LMP-2A in HD and IM samples

	Code	Sex	Age	LMP-2A (IHC)
Hodgkin's disease (HD)	HD1	M	34	Positive
	HD2	M	22	Positive
	HD3	M	56	Positive
	HD4	M	29	Negative
	HD5	F	78	Positive
	HD6	G	58	Positive
	HD7	G	31	Negative
	HD8	M	68	Positive
Infectious mononucleosis (IM)	IM	F	17	Positive
Nasopharyngeal Carcinoma (NPC)	NPC12	M	43	Negative
	NPC13	F	65	Negative
	NPC14	F	54	Negative
	NPC15	F	35	Positive
	NPC16	M	50	Negative
	NPC17	M	51	Negative
	NPC18	M	78	Negative
	NPC19	M	39	Negative
	NPC20	M	57	Negative
	NPC21	M	50	Positive
	NPC22	M	41	Negative
	NPC23	M	54	Negative
	NPC24	M	45	Negative
	NPC25	M	70	Negative
	NPC26	M	52	Positive

Table 4.5 Expression of miR-BART22 and LMP-2A in NPC samples

Sample	BART22	LMP2A mRNA*	LMP2A IHC
C666-1	100	100	Negative
NPC1	36.39	<1	Negative
NPC2	123.18	226	Negative
NPC3	41.07	242	Negative
NPC4	18.85	258	Negative
NPC5	76.54	257	Negative
NPC6	2.06	<1	Positive
NPC7	39.17	233	Negative
NPC8	28.05	335	Positive
NPC9	30.68	264	Negative
NPC10	64.04	NA	Negative
NPC11	55.2	183	Positive
NP1	0	0	Negative
NP2	0	0	Negative
NP3	0	0	Negative

Expression level of C666-1 was set at 100; NA, Not analyzed;
*Expression level was normalized to EBNA1.

4.3.4 Suppression of LMP-2A protein expression by miR-BART22

To establish whether an interaction between miR-BART22 and the LMP2A-3'UTR occurs, two experiments were designed. Firstly, the dose effect of miR-BART22 on LMP-2A expression was studied by co-transfection of different amounts of miR-BART22 with the LMP-2A expression vector that included the full length of the LMP-2A 3'UTR. MiR-BART22 suppressed the LMP-2A protein level in a dose-dependent manner without an apparent effect on LMP-2A mRNA levels (Figure 4.9). In addition, miR-BART22 expression had no obvious effect on the EGFP control protein. Secondly, transfection of miR-BART22 into HEK293 cells that had been stably transfected with pcDNA3.1-LMP-2A (Figure 4.10) readily suppressed the LMP-2A protein level. The transfection again had no significant effect on LMP-2A mRNA expression. These results strongly suggest that LMP-2A is a direct target of miR-BART22, which specifically represses LMP-2A expression at the post-transcriptional level.

Previous reports suggested that expression of LMP-2A can induce AKT activity in C666-1 cells (Shair *et al.*, 2008) and activate the Notch signaling pathway in epithelial cells (Anderson & Longnecker, 2009). We asked whether transient transfection of miR-BART22 in LMP-2A expressing HEK293 stable clones could suppress Notch-1 mRNA expression and mildly suppress AKT activation (Figure 4.11 and Figure 4.12). Although suppression of both Notch and AKT pathways may not favor cancer cell growth, forced expression of miR-BART22 in normal epithelial cells did not induce any change in cell proliferation (Figure 3.15). These observations were consistent with the recent finding that overexpression of LMP-2A in C666-1 cells does not affect cell growth and migration although it can activate the PI3K/Akt pathway (Shair *et al.*,

2008). Thus, we believe that downregulation of LMP-2A by miR-BART22 may contribute to cancer development by other means, such as permitting the infected cells to escape from the host immune surveillance.

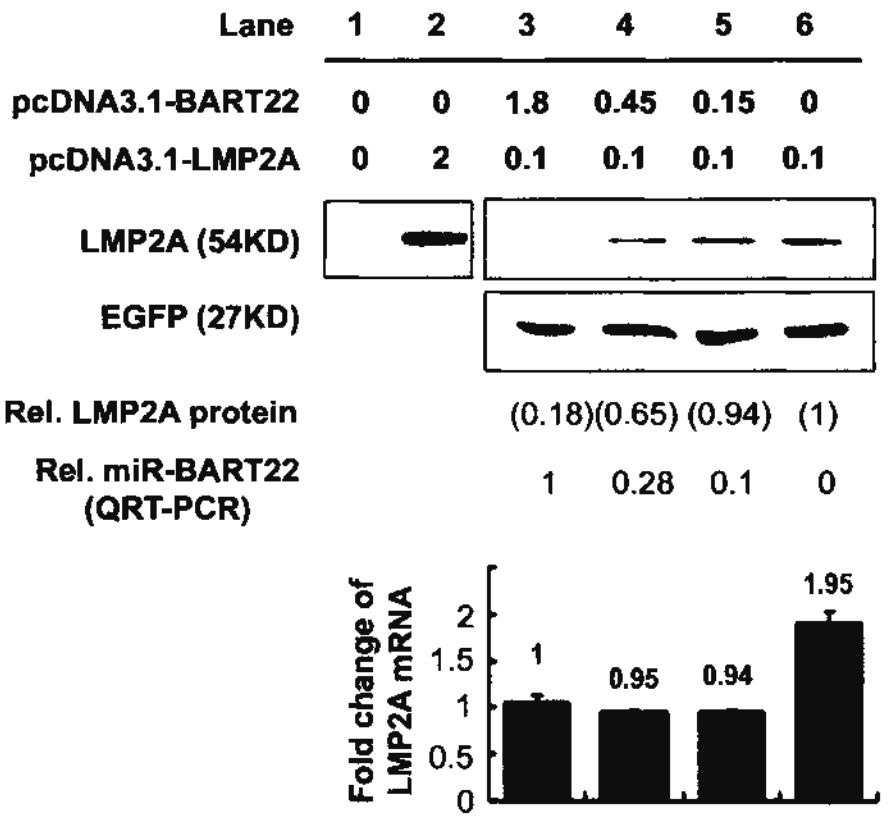


Figure 4.9 Suppression of LMP-2A protein expression by miR-BART22 in a dose-dependent manner

Western blot of LMP-2A and EGFP in 293FT cells transiently co-transfected with 0.1μg/well pEGFP-C1 plasmid in a 6-well plate format. The amount of the expression plasmids are indicated (lane 3-6), with 2μg of total DNA made up with pcDNA3.1 as carrier. LMP-2A expression level was normalized to EGFP. MiR-BART22 (normalized to U6) and LMP-2A (normalized to actin) RNA expression levels in the same sample were analyzed by QRT-PCR and are shown relative to lane 3.

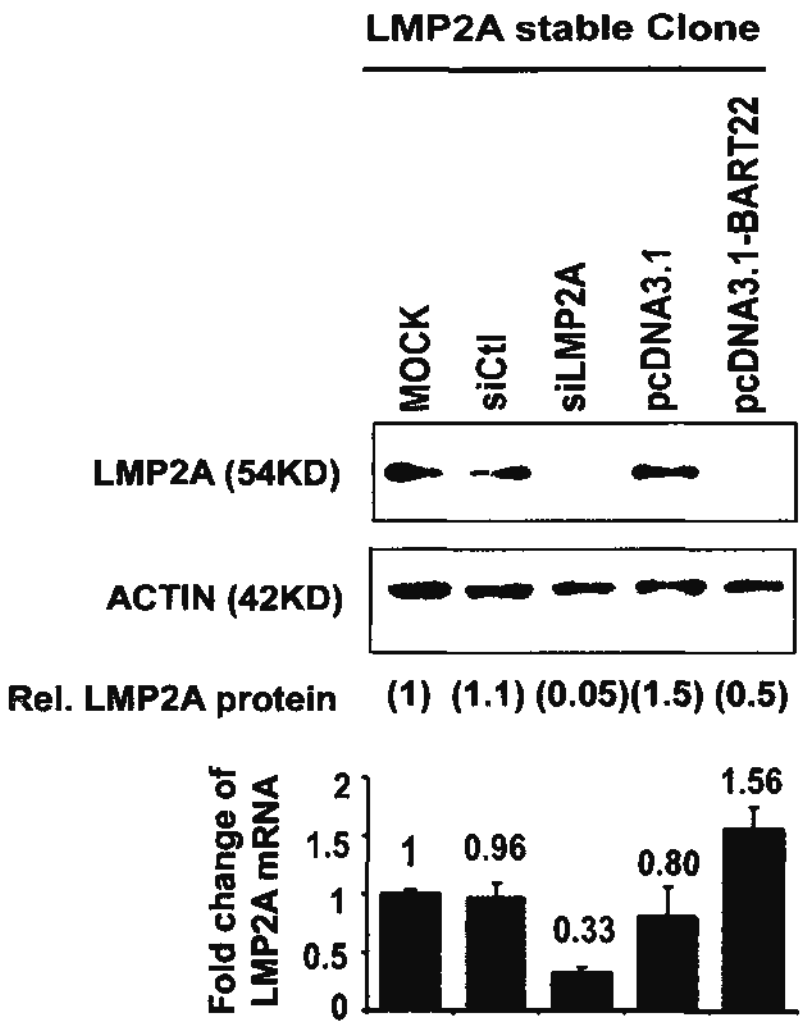


Figure 4.10 Suppression of LMP-2A protein expression by miR-BART22 a in stable clone

Representative western blot results indicate that miR-BART22 represses the expression of LMP-2A protein. An siRNA control (siCtl) and an LMP-2A specific siRNA (siLMP-2A) were included as controls. The LMP-2A expression level was normalized to actin and the level relative to mock transfection (set at 1) was calculated. LMP-2A mRNA expression in the same sample was assayed by QRT-PCR.

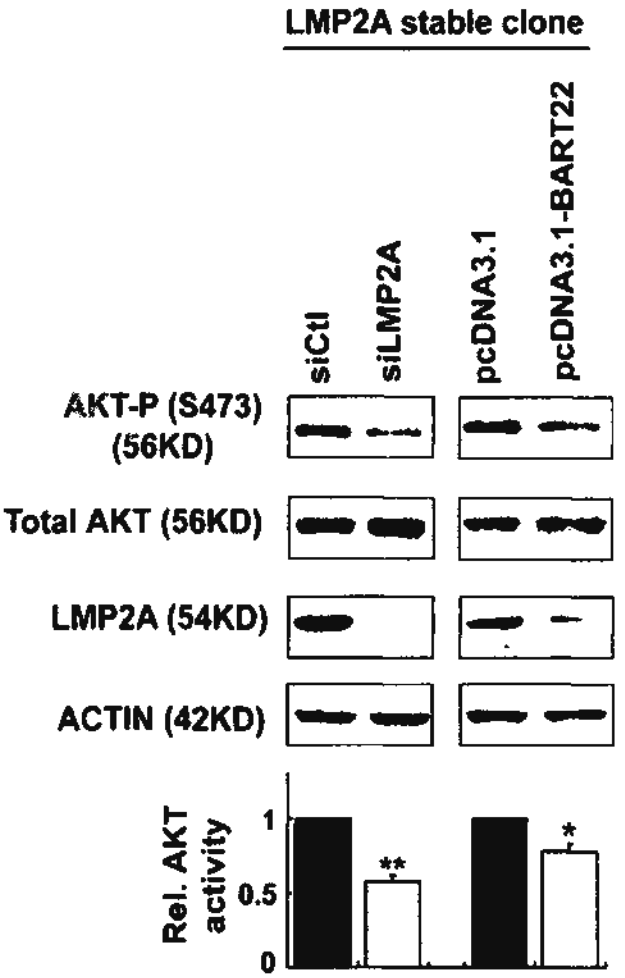


Figure 4.11 Inhibition of AKT activity by miR-BART22

Inhibition of LMP2A-mediated AKT activity by miR-BART22 was examined using western blot. HEK293-LMP2A stable cells transfected with an siRNA control (siCtl) and LMP-2A specific siRNA (siLMP-2A) were included as controls. A representative blot from three independent experiments is shown in panel A. The AKT activity was calculated by the expression level of phosphorylated AKT relative to total AKT, with the activity relative to a control transfection (set at 1) shown. Statistical analysis by *Student-t test* was used and compared with the control transfection, p-value <0.05 is denoted as (*) and <0.001 is denoted as (**).

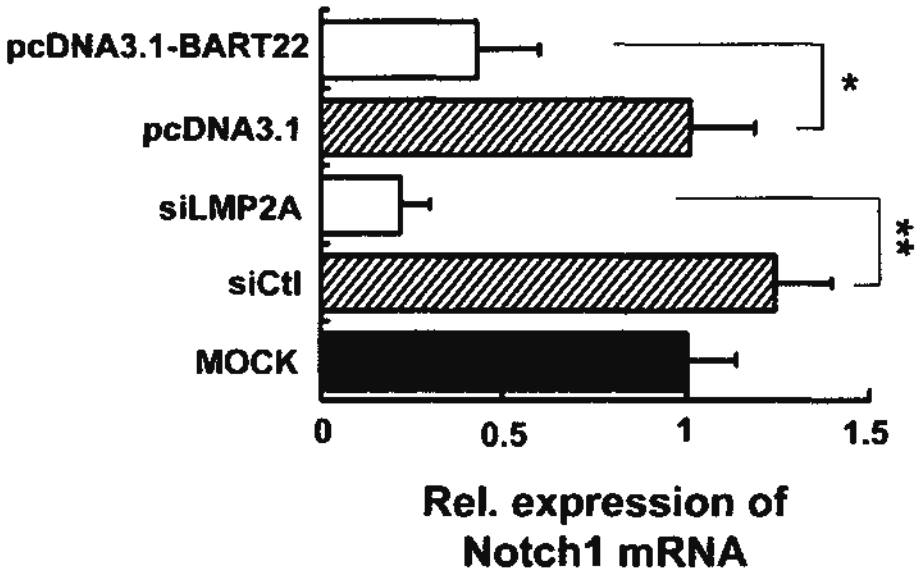


Figure 4.12 Downregulation of Notch1 expression by miR-BART22

The relative expression of Notch-1 of transfected cells was analyzed by QRT-PCR. Notch-1 expression levels were normalized to actin and compared with the mock transfection (set at 1). All data shown in the figure are the mean ± SD from three independent transfection experiments. Statistical analysis by *Student-t test* was used to compare the relative expression to the control transfection, p-value <0.05 is denoted as (*) and <0.001 is denoted as (**).

4.4 Discussion

Although the LMP-2A transcript was previously reported to be detected in most NPC samples (Brooks *et al.*, 1992; Busson *et al.*, 1992; Heussinger *et al.*, 2004), our IHC data indicate that only 23% of primary NPC tumors from our locality have detectable LMP-2A protein expression. Transcriptional regulation of LMP-2A in EBV-infected cells by epigenetic and viral latent protein mechanisms has been reported previously (Gerle *et al.*, 2007; Anderson & Longnecker, 2008; Fernandez *et al.*, 2009). In this chapter, we showed that LMP-2A can also be modulated at the translational level by miR-BART22. Moreover, while seed complementarity of the LMP-2A-3'UTR with other known ebv-miRNAs has been suggested, we have not been able to confirm these associations in a reporter assay (Figure 4.4), implying a unique role for miR-BART22 in the translational regulation of LMP-2A. We were able to demonstrate LMP-2A protein expression in NPC biopsies, which tend to have relatively low expression of miR-BART22 (Table 4.5). However, not all biopsies with low miR-BART22 expression have detectable LMP-2A, indicating that LMP-2A expression might also be regulated by other pathways. Such multiple regulatory mechanisms have also been implicated in LMP-1 modulation (Lo *et al.*, 2007).

There are several potential benefits of suppressing LMP-2A expression during NPC development. Previous reports showed that NPC cells are capable of processing and presenting endogenously synthesized proteins to CTLs. LMP-2A in particular is more strongly immunogenic than two other NPC expressed viral proteins, namely EBNA1 and LMP-1 (Brooks *et al.*, 1992; Khanna *et al.*, 1998; Lee *et al.*, 2000; Leen *et al.*, 2001; Lo *et al.*, 2007). In this

regard, limiting LMP-2A protein expression would have potential advantages for NPC cells to escape host immune surveillance, and thus LMP-2A expression in NPC is predictably low. As discussed in Chapter 1, immunomodulatory effects of other ebv-miRNAs have recently been demonstrated. For example, down-regulation of LMP-1 expression by miR-BARTs may favor immune escape by decreasing the antigen processing function of NPC cells (Lo *et al.*, 2007). In primary effusion lymphoma, miR-BHRF1-3 can target CXCL-11/I-TAC, an IFN-inducible T-cell attracting chemokine (Xia *et al.*, 2008). Furthermore, miR-BART2-5p can repress expression of the cellular stress-induced immune molecule, MICB (Nachmani *et al.*, 2009). Apart from immunogenicity aspects, LMP-2A, as opposed to LMP-1, could suppress NF-κB levels resulting in an anti-proliferative effect (Stewart *et al.*, 2004). Moreover, LMP-2A expressing epithelial cells could also inhibit telomerase reverse transcriptase activity, an enzyme important for cell immortalization and transformation (Chen *et al.*, 2005). In addition, our preliminary data showed that forced expression of LMP-2A in C666-1 cells could stimulate the expression of one of the viral early lytic genes (*BRLF1*). This is probably through the activation of the PI3K/Akt signaling pathway by LMP-2A, as previous described in Burkitt's lymphoma cells (Figure 4.13) (Mori & Sairenji, 2006; Shair *et al.*, 2008). Since LMP-2A has diverse functional roles in epithelial cells, its expression level is therefore required to be tightly regulated during the development of NPC.

Our group has previously reported that miR-BARTs regulate LMP-1 expression (Lo *et al.*, 2007) and miR-BART5 affects the expression of the cellular target gene PUMA (Choy *et al.*, 2008). In this study, we further identified miR-BART22 as a modulator of an important oncogenic and

immunogenic viral gene, LMP-2A (Figure 4.14). In concordance with our previous reports, we have thus provided evidence for the vital roles of EBV-encoded miRNAs in regulating oncogenic and immunogenic viral latent protein expression, which holds importance for the progression and survival of EBV-infected NPC.

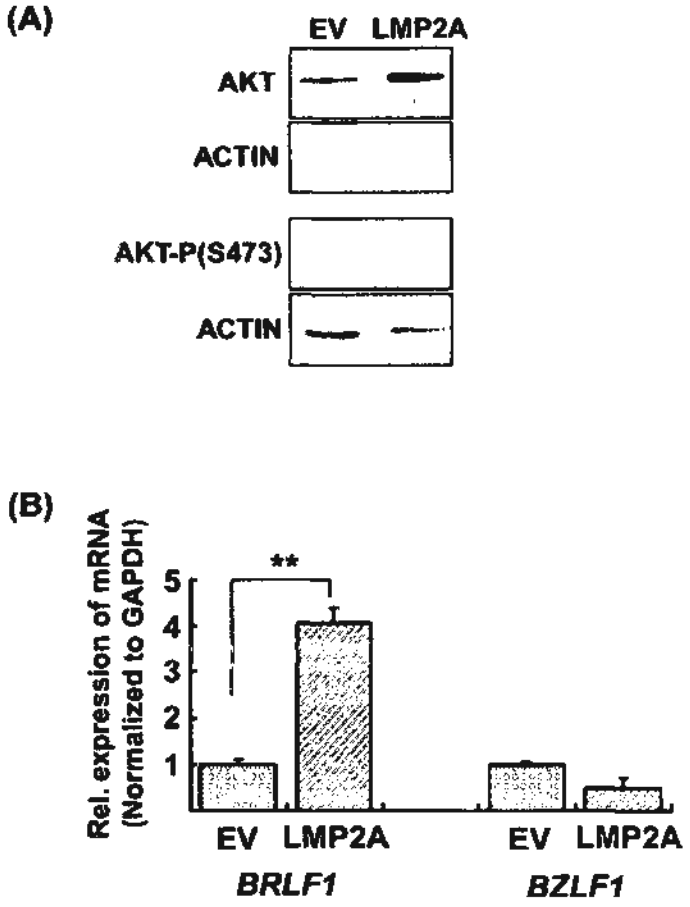


Figure 4.13 Activation of BRLF1 mRNA expression by LMP-2A

C666-1 cells were transfected with either an LMP-2A expression vector (LMP-2A) or empty vector (EV) for 24 hours. Protein and RNA were extracted from the same sample for parallel analysis. The representative western blot in Panel A shows the activation of Akt phosphorylation by LMP-2A. Expression of viral early lytic genes, BRLF1 and BZLF1, was measured by QRT-PCR and normalized to GAPDH (Hs00266705; Applied Biosystems). Triplicate individual experiments were done for both western blots and QRT-PCR, and *student-t test* was used for statistical analysis with a p-value <0.001 denoted by (**). The sequences of the primers and antibodies used are list in Table 2.4 and 2.5 respectively.

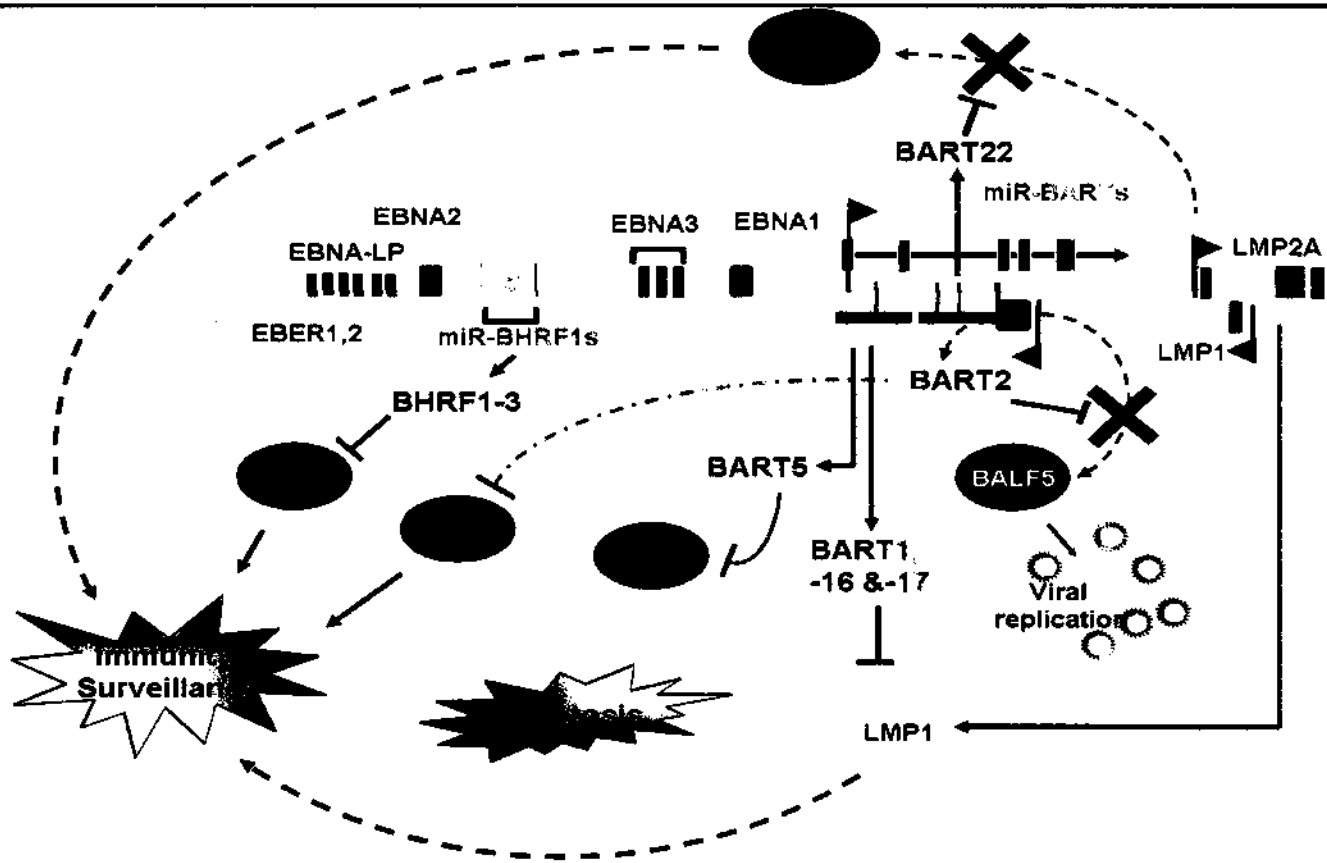


Figure 4.14 Function of ebv-miRNAs in host cell survival

CHAPTER 5: DISCUSSION AND PROPOSED FUTURE STUDIES

5.1 General findings

In spite of their high expression in NPC, the function of the two EBV non-coding RNA transcripts, EBERs and BARTs, remains obscure. Unlike EBER RNAs, which are highly expressed in infected cells in all states of latency, BARTs are preferentially expressed in cells of latency II status such as in NPC and gastric carcinoma. The recent discovery of a novel viral-encoded miRNAs in the *BART* region indicates that EBV, particularly in latency II, may explore this efficient regulatory system to facilitate cancer development and/or avoid host immune and antiviral responses.

In this thesis, we identified two novel ebv-miRNAs which are located in the *BART* region and are highly expressed in local NPC samples. Frequent expression of miR-BART22 can be explained partially by a specific EBV strain that is associated with NPC in our locality. By bioinformatics and functional analysis, *LMP-2A* has been successfully identified as the direct downstream target of miR-BART22. Because LMP-2A is a potent immunogenic viral antigen that is recognized by cytotoxic T cells (CTLs), down-modulation of LMP-2A expression by miR-BART22 may permit escape of EBV-infected cells from host immune surveillance.

5.2 LMP-2A protein expression regulated by human miRNAs

We observed in chapter 4 that the correlation between LMP-2A (IHC) and miR-BART22 expression is not absolute (Table 4.5). Furthermore, while LMP-2A mRNA is consistently detected in NPC and latent EBV infected B

lymphocytes (Qu & Rowe, 1992; Tierney *et al.*, 1994), we have failed to detect protein expression of LMP-2A in a variety of tested EBV positive cell lines (Figure 4.6 and Figure 4.7). These observations indicate that the expression of LMP-2A may also be regulated by other mechanism(s) at the posttranscriptional level.

Understanding LMP-2A regulation is important in EBV related cancer biology, especially in immunotherapy development. It is worthwhile to continue to investigate whether cellular molecules can regulate LMP-2A expression. Using the computer program "Probability of Interaction by Target Accessibility" (PITA) (Kertesz *et al.*, 2007), together with the previously described miRanda and RNAhybrid, several putative homo sapiens miRNA (hsa-miRNA) binding sites on LMP-2A-3'UTR are suggested (Table 5.1). The good complementarities between some predicted miRNAs and their potential binding sites in the LMP-2A-3'UTR (Table 5.2) suggests that these miRNAs may modulate LMP-2A expression. However, further experimental analysis should be conducted to determine if those miRNAs contribute to LMP-2A regulation.

Table 5.1 Predicted hsa-miRNA binding sites on LMP-2A-3'UTR (AJ507799; 5408-5856)

		MiRanda		RNAhybrid		PITA
miRNA	**Position***	**Score**	**MFE**	***P* Value**	**MFE**	**Score**
hsa-miR-15a	5809	102	-20.46	0.05106	-27.5	-16.02
hsa-miR-134	5681	122	-30.56	0.004978	-33.9	-14.93
hsa-miR-296-3p	5526	105	-22.42	0.003262	-35.3	-16.29
hsa-miR-423-5p	5540	98	-25.61	0.007576	-33.4	-10.46
hsa-miR-497	5811	98	-22.61	0.025405	-29.2	-17.82
hsa-miR-510	5528	108	-24.35	0.050451	-28.4	-10.01
hsa-miR-550	5652	103	-24.65	0.043588	-30	-11.45
hsa-miR-615-5p	5604	98	-32.67	0.001269	-38.2	-13.44
hsa-miR-638	5529	94	-36.31	0.006972	-37	-21.84
hsa-miR-648	5620	102	-21.64	0.091312	-26.3	-13.51
hsa-miR-658	5580	116	-33.29	0.013909	-33.9	-14.01
hsa-miR-663	5609	97	-27.79	0.014686	-33.8	-14.04
hsa-miR-744	5733	108	-30.21	0.012162	-32.2	-20.46
hsa-miR-760	5741	93	-28.36	0.010931	-31.9	-17.38
hsa-miR-920	5640	93	-20.64	0.071232	-27.3	-13.9
hsa-miR-1275	5794	94	-22.97	0.066624	-26.4	-11.66

* Position is refer to the AJ507799

Table 5.2 Alignment of LMP-2A-3'UTR with predicted hsa-miRNAs

Human miRNA	Alignment[a]
hsa-miR-15a	Target 5′ CCAGACC--UGUGUGCUGCA 3′ miR 3′GUGUUUGGUAAUACACGACGAU 5′
hsa-miR-134	Target 5′ GCCCCUCCGAGU-GACCAGUCACC 3′ miR 3′ GGGGAGAC-CAGUUGGUCAGUGU 5′
hsa-miR-296-3p	Target 5′ UAGCC-CCGGGCCCAGCCCUCC 3′ miR 3′ CCUCUCGGAGG--UGGGUUGGGAG 5′
hsa-miR-423-5p	Target 5′ AUCUGGCUCUCCUGUGUGACCCCUCAC 3′ miR 3′ UUUCAGAGCGAGAG-----AC-GGGGAGU 5′
hsa-miR-497	Target 5′ CCAGACCU--GUGUGCUGUA 3′ miR 3′ UGUUUGGUGUCACACGACGAC 5′
hsa-miR-550	Target 5′ AGCUCACCUAUGGUCACUCAGGCACG 3′ miR 3′CCCGAGA----AUGAGGGAGUCCGUGA 5′
hsa-miR-1275	Target 5′ CGUGAACCUCCCCCCCAG 3′ miR 3′ CUGUCGGAGAGGGGGUG 5′

[a]Alignment was suggested by RNAhybrid program

5.3 Identification of ebv-miRNA downstream targets

A single miRNA is expected to silence hundreds of target transcripts at the post-transcriptional level. Hence further identification of more ebv-miRNAs downstream targets is critical to understand how EBV is involved in cancer development. In the previous studies, several computer prediction programs were successfully employed to identify targets of ebv-miRNAs. In this thesis, the viral *LMP-2A* gene was identified and confirmed as a target of miR-BART22. The same strategy was explored to investigate the target of ebv-miR-BARTs in our laboratory but no new downstream target was finally validated. On the other hand, some reports suggested that genuine miRNA targets might escape *in silico* predictions if they lack seed match associations with the 3'UTR of the target transcripts. For example, miR-24 can directly down-modulate E2F2 and MYC expression eventhough it contains a poorly conserved 7-mer exact seed match with their 3'UTRs (Lal *et al.*, 2009). Despite the successful use of *in silico* methods for viral miRNA target identification, the number of false positives/negatives remains high. These observations suggest the need to obtain miRNA targets by other suitable and reliable approaches.

MiRNAs also affect the expression levels of target mRNAs (Lim *et al.*, 2005). In view of this, our department has successfully employed gene expression profiling together with *in silico* predictions to identify some miRNA targets in cancer cells: for example, downregulation of *Stathmin 1* by miR-223 in HCC (Wong *et al.*, 2008) and *SLC16A1* by miR-124 in medulloblastoma (Li *et al.*, 2009). However, identification of miRNA targets by proteomic studies suggested that miRNA mainly suppress protein expression without any significant change in mRNA level (Baek *et al.*, 2008; Selbach *et al.*, 2008). By

using 2D-PAGE and mass spectrometry analysis, tumor suppressor tropomyosin 1 (*TPM1*) was confirmed as a downstream target of miR-21 in MCF7 breast cancer cell lines (Zhu *et al.*, 2007). This approach highlights the feasibility of using proteomic approaches for miRNA target prediction although the secondary effects of miRNAs may score as false positives.

Direct biochemical methods may provide an easy and reliable alternative means for miRNA target identification (Beitzinger *et al.*, 2007; Easow *et al.*, 2007; Karginov *et al.*, 2007). This method combines RISC co-immunoprecipitation with gene expression analysis (Figure 5.1). Transfection of either control or miR-BART expression vectors into Hela cells can be subjected to pull down by monoclonal Ago1 and Ago2 antibodies. Since Ago protein is a core component of RISC, the pull down complex from miR-BARTs transfectants should contain miR-BARTs-targeted transcripts. The bound cellular transcripts can be extracted and analyzed by microarray profiling or high throughput sequencing. Potential targets can further be analyzed by miRNA recognition sites in their 3'UTR regions. The key advantage of this method is that it can enrich the direct targets of miR-BARTs without any consideration of the down-regulatory pathways involved.

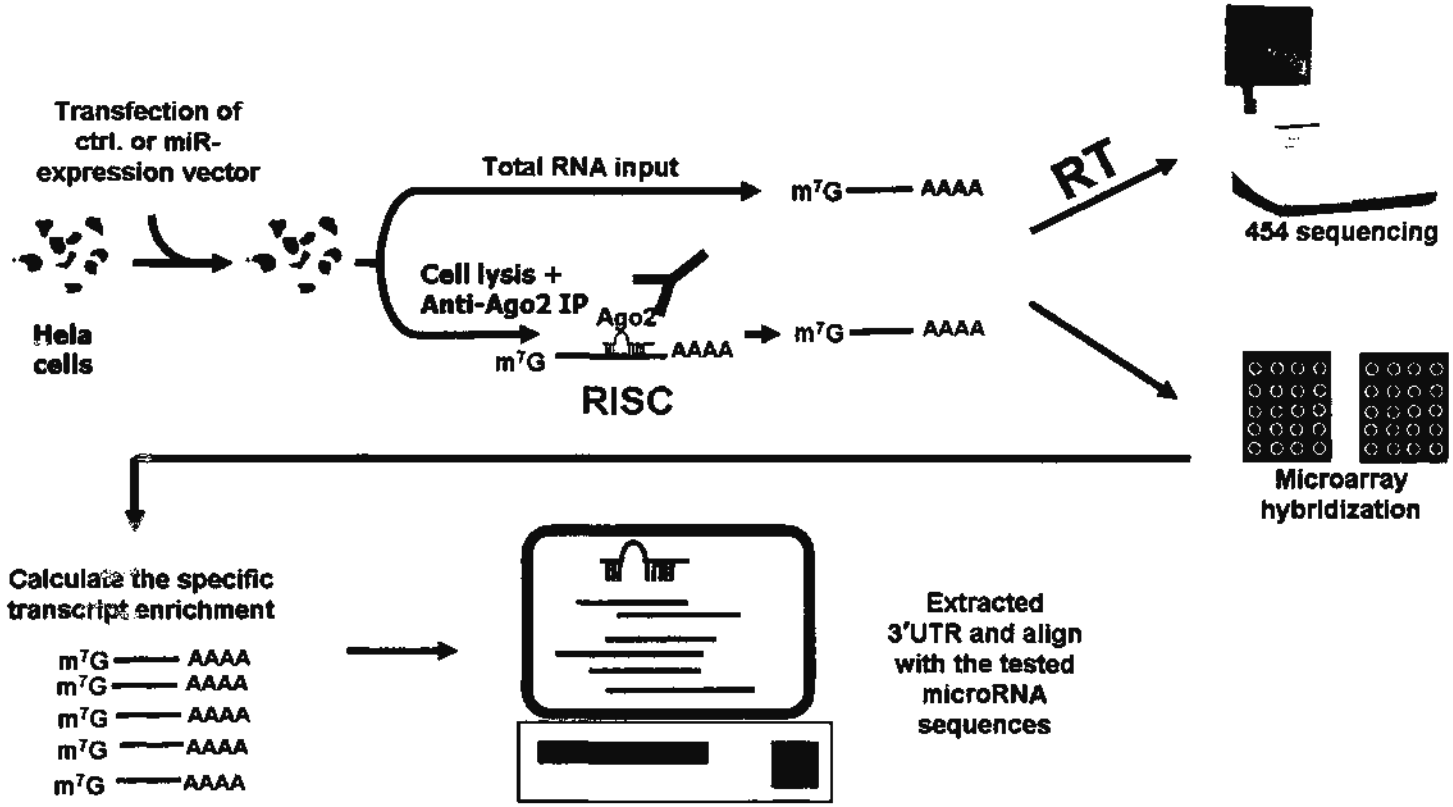


Figure 5.1 Schematic diagram for identification of miR-BARTs downstream targets by Ago immunoprecipitation

APPENDIX I
TUMOR STAGING (AJCC 6TH EDITION) FOR NASOPHARYNX

Primary tumor (T)

TX	Cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor confined to nasopharynx
T2	Tumor extends to soft tissue
T2a	Tumor extends to the oropharynx and/or nasal cavity without parapharyngeal extension.
T2b	Any tumor with parapharyngeal extension
T3	Tumor invades bony structures and/or paranasal sinuses
T4	Tumor invades intracranial extension and/or involvement of cranial nerves, infratemporal fossa, hypopharynx, orbit, or masticator space

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Unilateral metastasis in lymph node(s), 6 cm or less in greatest dimension, above supraclavicular fossa
N2	Bilateral metastasis in lymph node(s), 6 cm or less in greatest dimension, above supraclavicular fossa
N3a	Metastasis in lymph node(s) more than 6 cm in dimension
N3b	Metastasis in lymph node(s) residing wholly or in part in the supraclavicular fossa

Distant Metastasis (M)

MX	Cannot be assessed
M1	Distant metastasis

STAGE	GROUP		
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II A	T2a	N0	M0
Stage II B	T1	N1	M0
	T2a	N1	M0
	T2b	N0, N1	M0
Stage III	T1	N2	M0
	T2	N2	M0
	T3	N0, N1, N2	M0
Stage IV A	T4	N0, N1, N2	M0
Stage IV B	Any T	N3	M0
Stage IV C	Any T	Any N	M0

APPENDIX II

The top 100 potential targets of miR-BART21

Rank	Score	Gene ID	Description	MFE (*kca/mol*)
1	123	ENSG00000134760	Desmoglein-1 precursor	-26.39
2	123	ENSG00000159082	Synaptojanin-1	-24.81
3	123	ENSG00000139154	AE binding protein 2	-21.9
4	122	ENSG00000147592	lactamase beta 2	-23.28
5	118	ENSG00000134668	SPOC domain containing 1	-22.16
6	118	ENSG00000176095	Inositol hexaphosphate kinase 1	-22.12
7	118	ENSG00000127483	HP1-BP74	-21.74
8	117	ENSG00000136842	Tropomodulin-1	-21.63
9	117	ENSG00000138336	CXXC finger 6	-21.12
10	116	ENSG00000123416	Tubulin alpha-ubiquitous chain	-23.84
11	116	ENSG00000152270	cGMP-inhibited 3'5'-cyclic phosphodiesterase B	-22.26
12	116	ENSG00000118058	Zinc finger protein HRX	-21.83
13	116	ENSG00000152078	transmembrane protein 56	-21.77
14	116	ENSG00000184005	Alpha-N-acetylgalactosaminide alpha-26-sialyltransferase 3	-21.11
15	116	ENSG00000028203	Vezatin	-20.99
16	116	ENSG00000127688	Gigaxonin	-20.84
17	115	ENSG00000180694	transmembrane protein 64	-21.55
18	115	ENSG00000138386	NGFI-A-binding protein 1	-21.49
19	115	ENSG00000182489	XK-related protein 2	-20.48
20	114	ENSG00000110436	Excitatory amino acid transporter 2	-24.33
21	114	ENSG00000081692	jumonji domain containing 4	-23.91

22	114	ENSG00000119547	One cut domain family member 2	-23.17
23	114	ENSG00000189357	CDNA FLJ43859 fis clone TESTI4007382	-22.47
24	114	ENSG00000196712	Neurofibromin	-22.41
25	114	ENSG00000133059	Receptor-interacting serine/threonine-protein kinase 5	-20.13
26	113	ENSG00000112773	family with sequence similarity 46 member A	-23.71
27	113	ENSG00000134108	ADP-ribosylation factor-like protein 8B	-22.4
28	113	ENSG00000111669	Triosephosphate isomerase	-22.12
29	113	ENSG00000103160	hydroxysteroid dehydrogenase like 1	-22.06
30	113	ENSG00000187957	delta-notch-like EGF repeat-containing transmembrane	-21.78
31	113	ENSG00000066032	Alpha-2 catenin	-21.51
32	113	ENSG00000112182	Transcription regulator protein BACH2	-21.5
33	113	ENSG00000186310	Nucleosome assembly protein 1-like 3	-21.5
34	113	ENSG00000107077	JmjC domain-containing histone demethylation protein 3C	-20.82
35	112	ENSG00000112715	Vascular endothelial growth factor A precursor	-22.99
36	112	ENSG00000103061	solute carrier family 7 member 6 opposite strand	-22.92
37	112	ENSG00000122566	Heterogeneous nuclear ribonucleoproteins A2/B1	-22.11
38	112	ENSG00000074706	phosphoinositide-binding protein PIP3-E	-21.77
39	112	ENSG00000131781	Dimethylaniline monooxygenase [N-oxide-forming] 5	-20.55
40	111	ENSG00000156750	aquaporin 7 pseudogene 2	-20.7
41	111	ENSG00000139496	Nucleoporin p58/p45	-20.23
42	110	ENSG00000129270	Matrix metalloproteinase-28 precursor	-24.17
43	110	ENSG00000141510	Cellular tumor antigen p53	-24.16
44	110	ENSG00000173786	2'3'-cyclic-nucleotide 3'-phosphodiesterase	-22.01
45	110	ENSG00000167528	zinc finger protein 641	-21.8
46	110	ENSG00000134253	Tripartite motif protein 45	-21.48

47	110	ENSG00000135108	F-box only protein 21	-20.53
48	109	ENSG00000189377	DMC	-21.74
49	109	ENSG00000186714	sarcoma antigen NY-SAR-79	-20.75
50	109	ENSG00000198668	Calmodulin	-20.3
51	109	ENSG00000047230	cytidine triphosphate synthase II	-20.29
52	109	ENSG00000069869	E3 ubiquitin-protein ligase NEDD4	-20.27
53	109	ENSG00000101193	Protein C20orf11	-20.22
54	108	ENSG00000187773	PREDICTED: hypothetical protein XP_950106 isoform 3	-21.33
55	108	ENSG00000166435	X-ray radiation resistance associated 1 protein	-21.1
56	108	ENSG00000197930	ERO1-like protein alpha precursor	-21.09
57	108	ENSG00000143507	Dual specificity protein phosphatase 10	-20.73
58	108	ENSG00000170915	Membrane progestin receptor beta	-20.48
59	108	ENSG00000062598	Engulfment and cell motility protein 2	-20.1
60	107	ENSG00000162946	Disrupted in schizophrenia 1 protein	-23.64
61	107	ENSG00000198203	Sulfotransferase 1C1	-22.8
62	107	ENSG00000075891	Paired box protein Pax-2	-22.48
63	107	ENSG00000084733	Ras-related protein Rab-10	-20.36
64	107	ENSG00000007237	Growth-arrest-specific protein 7	-20.31
65	107	ENSG00000161813	La-related protein 4	-20.25
66	107	ENSG00000189043	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	-20.04
67	106	ENSG00000090857	pyruvate dehydrogenase phosphatase regulatory subunit	-22.24
68	106	ENSG00000155729	potassium channel tetramerisation domain containing 18	-22.07
69	106	ENSG00000107937	Nucleolar GTP-binding protein 1	-21.5
70	106	ENSG00000154930	Acetyl-coenzyme A synthetase 2-like mitochondrial precursor	-20.84
71	105	ENSG00000136848	DAB2 interacting protein isoform 2	-23.66

72	105	ENSG00000136444	radical S-adenosyl methionine domain containing 1	-22.27
73	105	ENSG00000197915	Homerin.	-21.59
74	105	ENSG00000169083	Androgen receptor	-20.63
75	105	ENSG00000116783	Serine/threonine-protein kinase TNNI3K	-20.32
76	105	ENSG00000167526	60S ribosomal protein L13	-20.17
77	104	ENSG00000121680	Peroxisomal membrane protein PEX16	-23.11
78	104	ENSG00000196505	ganglioside induced differentiation associated protein 2	-21.86
79	104	ENSG00000086205	Glutamate carboxypeptidase 2	-21.55
80	104	ENSG00000181072	Muscarinic acetylcholine receptor M2	-21.25
81	104	ENSG00000170802	Human T-cell leukemia virus enhancer factor	-20.75
82	104	ENSG00000166224	Sphingosine-1-phosphate lyase 1	-20.34
83	104	ENSG00000173542	Mps one binder kinase activator-like 1A	-20.28
84	104	ENSG00000206270	Sialidase-1 precursor	-20.25
85	103	ENSG00000065534	Myosin light chain kinase smooth muscle	-23.51
86	103	ENSG00000196233	Ligand-dependent corepressor	-22.06
87	103	ENSG00000135862	Laminin gamma-1 chain precursor	-21.81
88	103	ENSG00000125089	SH3 domain and tetratricopeptide repeats-containing protein 1	-21.75
89	103	ENSG00000144959	arylacetamide deacetylase-like 1	-21.62
90	103	ENSG00000143479	Dual specificity tyrosine-phosphorylation-regulated kinase 3	-21.59
91	103	ENSG00000072756	tRNA-nucleotidyltransferase 1 mitochondrial precursor	-21.17
92	103	ENSG00000153914	Splicing factor arginine/serine-rich 12	-21.15
93	103	ENSG00000100829	Homeobox and leucine zipper protein Homez	-20.93
94	103	ENSG00000144802	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta isoform a	-20.77
95	103	ENSG00000126067	Proteasome subunit beta type 2	-20.72
96	103	ENSG00000175787	Zinc finger protein 169	-20.57

97	103	ENSG00000099968	Bcl-2-like 13 protein	-20.51
98	103	ENSG00000182533	Caveolin-3	-20.31
99	102	ENSG00000114302	cAMP-dependent protein kinase type II-alpha regulatory subunit	-23.14
100	102	ENSG00000185591	Transcription factor Sp1	-21.75

Rank, score and miminum free energy (MFE) were determined by miRanda.

APPENDIX III

The top 100 potential targets of miR-BART22

Rank	Score	Gene	Description	MFE (*kcal/mol*)
1	138	ENSG00000130150	Motile sperm domain-containing protein 2	-25.2
2	136	ENSG00000171316	Chromodomain-helicase-DNA-binding protein 7	-24.68
3	135	ENSG00000146039	solute carrier family 17	-25.76
4	133	ENSG00000197893	nebulin-related anchoring protein isoform C	-27.17
5	133	ENSG00000119414	Serine/threonine-protein phosphatase 6	-23.81
6	132	ENSG00000154736	ADAMTS-5 precursor	-24.06
7	131	ENSG00000119318	UV excision repair protein RAD23 homolog B	-24.75
8	131	ENSG00000112242	Transcription factor E2F3	-24.45
9	130	ENSG00000129151	Gamma-butyrobetaine dioxygenase	-26.5
10	129	ENSG00000104635	solute carrier family 39	-22.17
11	129	ENSG00000133026	Myosin-10	-20.61
12	129	ENSG00000134308	14-3-3 protein theta	-20.43
13	128	ENSG00000168216	liver regeneration p-53 related protein	-22.45
14	127	ENSG00000196209	OTTHUMP00000029985.	-26.12
15	127	ENSG00000164307	Adipocyte-derived leucine aminopeptidase precursor	-24.39
16	127	ENSG00000197702	Alpha-parvin	-22.81
17	127	ENSG00000181744	Protein C3orf58 precursor	-22.69
18	126	ENSG00000182957	spermatogenesis associated 13	-23.67
19	126	ENSG00000138698	Rap1 GTPase-GDP dissociation stimulator 1	-23.39
20	126	ENSG00000204899	OTTHUMP00000018497.	-20.99
21	126	ENSG00000092529	Calpain-3	-20.89

22	126	ENSG00000108733	Peroxisome assembly protein 12	-20.7
23	125	ENSG00000197381	Double-stranded RNA-specific editase 1	-25.05
24	125	ENSG00000066185	Zinc finger MYND domain-containing protein 12	-21.98
25	125	ENSG00000125944	Heterogeneous nuclear ribonucleoprotein R	-21.46
26	125	ENSG00000186184	DNA-directed RNA polymerase I subunit D	-21.33
27	125	ENSG00000157890	MEGF11 protein	-20.65
28	124	ENSG00000111196	Protein mago nashi homolog 2	-25.15
29	124	ENSG00000110851	PR domain zinc finger protein 4	-24.89
30	124	ENSG00000186056	CDNA FLJ46197 fis clone TEST14007565	-24.54
31	124	ENSG00000143614	Transcriptional repressor p66 beta	-24.2
32	124	ENSG00000134962	Beta klotho	-22.69
33	124	ENSG00000156531	PHD finger protein 6	-22.06
34	124	ENSG00000123728	Ras-related protein Rap-2c precursor	-21.56
35	124	ENSG00000172115	Cytochrome c	-21.26
36	123	ENSG00000054523	Kinesin-like protein KIF1B	-25.85
37	123	ENSG00000130985	Ubiquitin-activating enzyme E1	-23.76
38	123	ENSG00000135318	5'-nucleotidase precursor	-23.46
39	123	ENSG00000112246	Single-minded homolog 1	-22.64
40	123	ENSG00000197959	Dynamin-3	-22.49
41	123	ENSG00000044524	Ephrin type-A receptor 3 precursor	-21.82
42	123	ENSG00000196865	NHL repeat containing 2	-21.69
43	122	ENSG00000137070	Interleukin-11 receptor alpha chain precursor	-26.58
44	122	ENSG00000186106	Ankyrin repeat domain-containing protein 46	-23
45	122	ENSG00000011258	mbt domain containing 1	-22.86
46	122	ENSG00000158125	Xanthine dehydrogenase/oxidase	-22.65

47	122	ENSG00000180370	Serine/threonine-protein kinase PAK 2	-21.32
48	122	ENSG00000100307	Chromobox protein homolog 7	-20.83
49	122	ENSG00000064225	Type 2 lactosamine alpha-23-sialyltransferase	-20.64
50	122	ENSG00000183878	Ubiquitously transcribed Y chromosome tetratricopeptide repeat protein	-20.29
51	122	ENSG00000135387	GPI-anchored protein p137	-20.13
52	121	ENSG00000064961	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related	-25.15
53	121	ENSG00000119689	Dihydrolipoyllysine-residue succinyltransferase component of 2- oxoglutarate dehydrogenase complex mitochondrial precursor	-25.05
54	121	ENSG00000101974	Probable phospholipid-transporting ATPase IG	-24.84
55	121	ENSG00000128714	Homeobox protein Hox-D13	-23.82
56	121	ENSG00000148672	Glutamate dehydrogenase 1 mitochondrial precursor	-23.35
57	121	ENSG00000187186	Small inducible cytokine A27 precursor	-22.98
58	121	ENSG00000157654	A-kinase anchor protein 2	-22.87
59	121	ENSG00000114098	armadillo repeat containing 8 isoform 3	-21.59
60	121	ENSG00000160408	ST6	-21.51
61	120	ENSG00000153391	Uncharacterized protein C18orf37	-25.88
62	120	ENSG00000128710	Homeobox protein Hox-D10	-24.58
63	120	ENSG00000033011	Chitobiosyldiphosphodolichol beta-mannosyltransferase	-23.7
64	120	ENSG00000081277	Plakophilin-1	-23.46
65	120	ENSG00000188120	Deleted in azoospermia protein 4	-23.39
66	120	ENSG00000205944	Deleted in azoospermia protein 2	-23.39
67	120	ENSG00000187191	deleted in azoospermia 3	-23.39
68	120	ENSG00000205916	deleted in azoospermia 4 isoform 2	-23.39
69	120	ENSG00000129250	Kinesin-like protein KIF1C	-22.18

70	120	ENSG00000105974	Caveolin-1.	-21.85
71	120	ENSG00000014824	solute carrier family 30	-21.52
72	120	ENSG00000005249	cAMP-dependent protein kinase type II-beta regulatory subunit	-20.32
73	119	ENSG00000142556	Zinc finger protein 614	-26.6
74	119	ENSG00000109685	Wolf-Hirschhorn syndrome candidate 1 protein isoform 5	-24.66
75	119	ENSG00000180773	solute carrier family 36	-23.96
76	119	ENSG00000065413	Ankyrin repeat domain-containing protein 44	-23.94
77	119	ENSG00000163071	spermatogenesis associated 18 homolog	-23.72
78	119	ENSG00000164305	Caspase-3 precursor	-23.62
79	119	ENSG00000167778	SPRY domain-containing protein 3	-22.87
80	119	ENSG00000149575	Sodium channel beta-2 subunit precursor	-22.34
81	119	ENSG00000097033	SH3 domain GRB2-like protein B1	-21.84
82	119	ENSG00000115966	Cyclic AMP-dependent transcription factor ATF-2	-21.8
83	119	ENSG00000123700	Inward rectifier potassium channel 2	-21.54
84	119	ENSG00000109445	Zinc finger protein 330	-20.89
85	119	ENSG00000153914	Splicing factor arginine/serine-rich 12	-20.51
86	118	ENSG00000171444	Colorectal mutant cancer protein	-23.75
87	118	ENSG00000112245	Protein tyrosine phosphatase type IVA protein 1	-22.62
88	118	ENSG00000151882	Small inducible cytokine A28 precursor	-22.57
89	118	ENSG00000085063	CD59 glycoprotein precursor	-22.39
90	118	ENSG00000134247	Prostaglandin F2 receptor negative regulator precursor	-22.09
91	118	ENSG00000165138	Ankyrin repeat and SAM domain-containing protein 6	-22
92	118	ENSG00000119487	Stress-activated map kinase-interacting protein 1	-21.82
93	118	ENSG00000168214	Recombining binding protein suppressor of hairless	-21.79
94	118	ENSG00000115758	Ornithine decarboxylase	-21.32

95	118	ENSG00000158467	Putative adenosylhomocysteinase 3	-21.22
96	118	ENSG00000132434	LanC-like protein 2	-21.04
97	118	ENSG00000112276	Blood vessel epicardial substance	-20.91
98	118	ENSG00000169813	Heterogeneous nuclear ribonucleoprotein F	-20.69
99	118	ENSG00000147180	zinc finger protein 6	-20.24
100	117	ENSG00000106460	Transmembrane protein 106B	-25.62

Rank, score and miminum free energy (MFE) were determined by miRanda.

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