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Identification of Epstein-Barr virus microRNA in nasopharyngeal carcinoma cells

Key Messages

1. The first small RNA library from Epstein-Barr virus (EBV) positive nasopharyngeal carcinoma (NPC) cells is constructed.
2. The large proportion of EBV-encoded microRNA (miRNA) compared to cellular miRNA in NPC cells underscores their significance in the establishment and/or maintenance of latent infections and pathogenesis in NPC cells.
3. The two newly identified EBV-encoded miRNAs, ebv-miR-BART-HK1 and ebv-miR-BART-HK2, are now published in www.mirbase.org and named ebv-miR-BART22 (MIMAT0010132) and ebv-miR-BART21-5p (MIMAT0010130), respectively.

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Introduction

More than 600 human microRNAs (miRNA) have been identified as involved in numerous biological processes including cell proliferation, cell death, differentiation, morphogenesis, and development. After the discovery of Epstein-Barr virus (EBV)–encoded miRNAs in lymphoma cells, viral-encoded miRNAs become increasingly recognised in the role of carcinogenesis.

Nasopharyngeal carcinoma (NPC) is prevalent in Southern China (including Hong Kong) and is associated with EBV. Successful cloning of EBV miRNA from lymphoma cell lines demonstrates that EBV has the potential to exploit RNA silencing as a convenient mechanism for the regulation of host and viral gene expression. Current data on EBV miRNA are largely derived from EBV-positive lymphoma cell lines. Data on expression of EBV miRNAs in epithelial malignancy are scanty, as are comprehensive data on the expression profile of EBV-encoded miRNAs in NPC cell lines. To enhance understanding of NPC tumourigenesis and provide new diagnostic and therapeutic prospects, we investigated the EBV miRNA expression profile in NPC cells by construction of miRNA libraries from EBV-positive NPC cell lines and xenografts.

Methods

This study was conducted from January 2007 to January 2008.

Cell lines, xenografts, and primary tumours

For library construction, an EBV-positive NPC cell line (C666-1) and a xenograft (X2117) were used. To compare the expression of novel EBV-encoded miRNAs, a panel of EBV-positive cells including four lymphoid cell lines (Raji, Namalwas, BC-1, and Akata), four NPC xenografts (X666, X1915, C15, and C17), and EBV-negative immortalised normal nasopharyngeal epithelial cells (NP460 and NP69) were included. In all, 23 primary NPC biopsies were obtained from our Department of Anatomical and Cellular Pathology.

Cloning of miRNAs

Enriched small-RNA fractions for library construction were collected with the miVana miRNA Isolation Kit (Ambion). Cloning miRNAs mainly followed the manufacturer's protocol for miRCat™ small RNA cloning kit (Integrated DNA Technologies, Coralville, IA, USA). In brief, the 3' and 5' cloning linkers were ligated to the small RNA, and cDNA was synthesised and amplified. After concaternisation, the fragment was cloned and sequenced using the TOPO TA Cloning Kit (Invitrogen).

Bioinformatic analysis

The extracted small RNA fragments ≥18 nt were annotated to the genome. The known human and EBV miRNAs were identified by blasting the sequences to miRBase. The remaining sequences were individually blasted to NCBI databases. Cloned sequences that matched the wild-type EBV genome (NC_007605) were examined. The putative precursor sequence of 50 nt 5' and 3' of the clone sequences were extracted from the EBV genome for prediction of fold-back structure by MFOLD.

Detection of miRNA expression by Northern Blot and real-time RT-PCR

For Northern Blot analysis, 10 μg of total RNA were separated in 12% urea-PAGE, electroblotted onto Nytran Supercharge membrane (Schleicher & Schuell, Germany) and fixed by UV-crosslinking. Oligodeoxynucleotide probes were end-labelled with [γ-32P]-ATP and hybridised at 28ºC to 32ºC overnight. The membrane was then exposed on X-ray film after several washes. For miRNA quantification, SYBR green quantitative RT-PCR assay was used. RNA was polyadenylated and reverse transcribed. SYBR RT-PCR with the iScript Universal primer and miRNA-specific primers were performed in the ABI PRISM 7500 Fast Real-time PCR system (Applied Biosystems).

Results

Two individual libraries were constructed from two NPC cell samples: C666-1 NPC cell line and X2117 NPC xenograft. Of 1813 clones in the C666-1 small RNA library, 615 (34%) sequences matched known EBV miRNAs and 277 (15%) matched known human miRNAs in miRBase. In addition to the known miRNAs and virus-encoded miRNAs, 811 (45%) clones were human sequences and 33 (2%) were EBV sequences that showed no matches to any known miRNAs. Most of the human sequence clones were rRNA and tRNA fragments, and the EBV sequence clones were fragments of EBV-encoded small RNAs (EBER1 and EBER2). The remaining 4% showed no matches to any known RNAs (Fig 1a).

A similar distribution was found in the X2117 small RNA library, with both EBV and human miRNAs enriched to 45% and 39%, respectively, out of 1115 clones (Fig 1b). The EBV-encoded miRNAs showed differences in cloning frequencies (Table). In these two libraries, the most abundantly cloned EBV-encoded miRNAs were EBV-miR-BART7 (109 hits), miR-BART9 (467 hits), and miR-BART10 (251 hits) [Table 1]. EBV-miR-BART2, 11, 12, 13, 14, 16, 17-5p, 18, 19 were of low abundance, whereas EBV-miR-BART1, 3, 4, 5, 6, 8, 17-3p showed moderate copy numbers in the libraries. Not all the reported EBV-

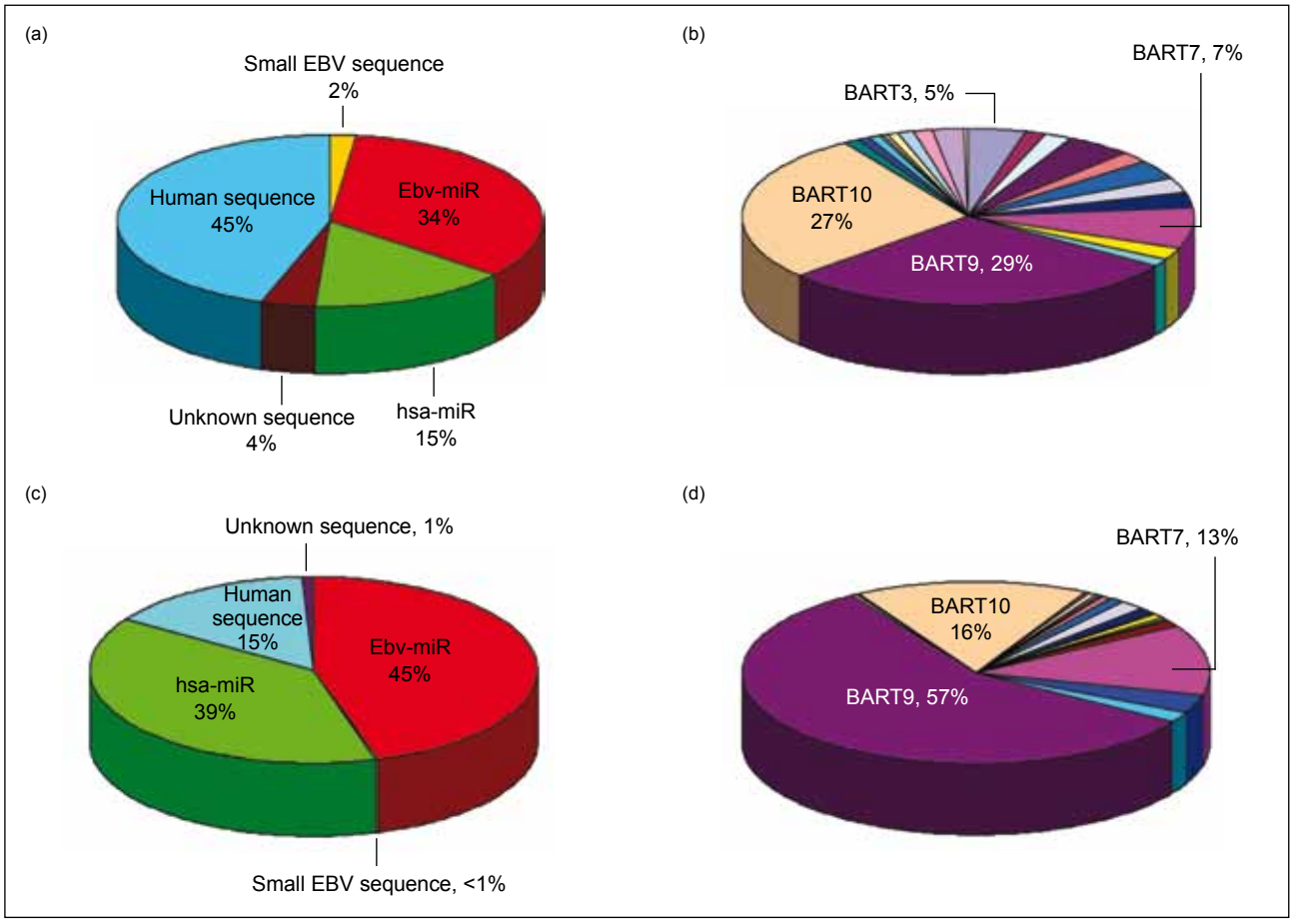

Fig 1. Distribution of the small RNAs from libraries

(a) A total of 1813 sequences were cloned from C666-1 cells. Among them, 34% corresponded to known Epstein-Barr virus (EBV) microRNAs (miRNAs) and 2% corresponded to the small EBV fragments. The distribution of the known EBV-miRNAs from C666-1 library (615 clones) was shown and the most frequently cloned miRNAs were listed. (b) A total of 1113 sequences were cloned from X2117. Among them, 45% corresponded to known EBV miRNAs and <1% was the other small EBV fragments. The distribution of the known EBV-miRNAs from X2117 library (500 clones) was shown and the frequently cloned miRNAs were listed

Table. Cloning frequency of the known Epstein-Barr virus (EBV) microRNAs (miRNAs)

EBV-miRNAs	No. of hits		Total
	C666-1 library	X2117 library	
BHRF1-1	0	0	0
BHRF1-2	0	0	0
BHRF1-3	0	0	0
BART1-5p	25	3	28
BART1-3p	9	2	11
BART2-5p	1	0	1
BART2-3p	0	0	0
BART3	30	6	36
BART3*	11	1	12
BART4	11	2	13
BART5	22	4	26
BART6-5p	18	0	18
BART6-3p	17	0	17
BART7	45	64	109
BART7*	0	0	0
BART8	15	16	31
BART8*	7	10	17
BART9	179	288	467
BART9*	0	0	0
BART10	169	82	251
BART10*	0	0	0
BART11-5p	7	0	7
BART11-3p	4	0	4
BART12	6	1	7
BART13	1	3	4
BART13*	0	1	1
BART14	5	0	5
BART14*	2	2	4
BART15	0	0	0
BART16	7	0	7
BART17-5p	9	0	9
BART17-3p	14	6	20
BART18-5p	1	0	1
BART18-3p	0	0	0
BART19-5p	0	1	1
BART19-3p	0	8	8
BART20-5p	0	0	0
BART20-3p	0	0	0
Total	615	500	1115

encoded miRNAs were present in our library. EBV-miR-BART2-3p, 7*, 9*, 10*, 15, 18-3p, 20, and all the EBV-miR-BHRFs were not detected.

A small portion of EBV small RNA fragments that showed no matches to the known EBV-encoded miRNAs were identified from our libraries. They accounted for 1.2% (36 clones) of total reads. Sequence analysis of these clones identified two novel EBV-encoded miRNAs, which were temporarily designated as EBV-miR-BART-HK1 and EBV-miR-BART-HK2, respectively. The former was located between EBV-miR-BART9 and EBV-miR-BART10 in the EBV BART region. It has been cloned 20 times in C666-1 and once in X2117. In contrast, the latter was located upstream to BART cluster 2. It was cloned only once in the C666-1 library but not detected in X2117 library. Both miRNAs demonstrated stable hairpin structures with long paired stems by MFOLD.

By Northern blot analysis, both EBV-miR-BART-HKs were expressed in EBV-positive cell lines, xenografts and primary tumour samples, irrespective of their tissue origin (Fig 2). Expression of EBV-miR-BART-HK1 was greater in NPC cell lines, xenografts, and primary NPC samples than in lymphoid cell lines (Fig 2a). Expression of EBV-miR-BART-HK2 could also be detected in C666-1 and X2117, but the expression level was lower than EBV-miR-BART-HK1 in the same samples (Fig 2b).

We developed a more sensitive QRT-PCR assay for the detection of EBV-encoded miRNAs in biopsies. Using QRT-PCR, both novel EBV-encoded miRNAs were detected in all NPC biopsies and cell lines with relatively greater level of expression than in lymphoid cell lines. No

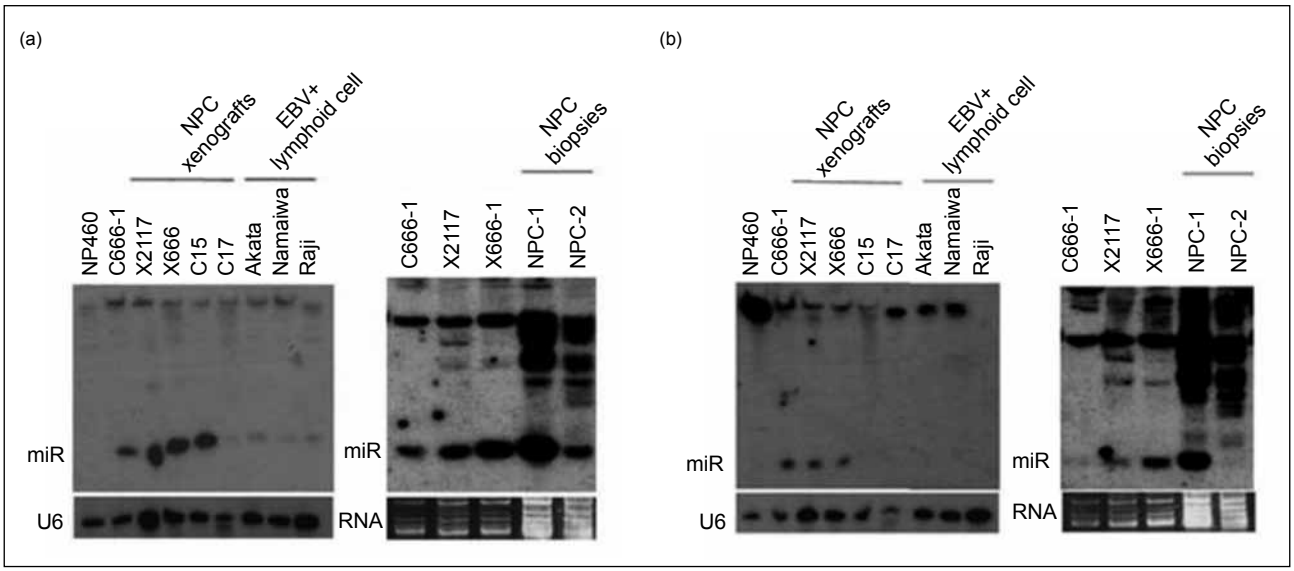

Fig 2. Northern Blot analysis was performed for the expression of (a) Epstein-Barr virus (EBV)-miR-BART-HK1 and (b) EBV-miR-BART-HK2 on EBV-positive cells lines, two NPC xenografts (X2117 and X666), and two NPC biopsies. NP460 (control) was an EBV-negative normal nasopharyngeal epithelial cell line

detectable EBV-miR-BART-HK1 or HK2 was found in the EBV-negative RNA samples. The results were consistent with the expression level as detected by Northern Blot.

Discussion

The discovery of miRNAs as key players in the micromanagement of gene expression is a remarkable breakthrough in the field of molecular biology. In case of viral infection, the successful survival of viruses depends on their ability to exploit the biosynthetic machinery of host cells and inactivate the innate defence mechanisms of the host. It has been proposed that miRNAs are generated by viruses as a convenient mechanism to regulate host and viral gene expression. Therefore, they are the potential targets for gene therapies that are designed to block tumour development or progression.

Analysis of the miRNA sequences from our libraries indicated that EBV-encoded miRNAs accounted for over one third of the small RNA sequences detected in NPC cells. The large proportion of EBV-encoded miRNA (34% in the C666-1 library and 45% in the X2117 library) compared to cellular miRNA (45% in the C666-1 library and 39% in the X2117 library) underscored the significance of EBV-encoded miRNA in the establishment and/or maintenance of latent infections and the pathogenesis and malignant transformation in NPC cells. Greater expression of virus-encoded miRNA is common in transformed cell lines. For example, miRNA encoded by Marek's disease virus type 1 (MDV-1) and co-infected MDV-2 accounted for >60% of the 5099 sequences of the small RNA library from the MSB-1 cell.[1] miRNA-encoded by Kaposi's sarcoma-associated herpesvirus and EBV accounted for >40% of the entire miRNA pool from BC-1 cells.[2]

Not all the known EBV miRNAs were identified in our libraries (Table). We failed to clone any EBV-miR-BHRFs, as NPC cells were in EBV type II latency. The BHRF1 clusters of EBV miRNAs were selectively expressed in the lytic cycle and latency III cells, but not in cells undergoing type II or type I latency (such as NPC cells). In contrast, the viral BART miRNA cluster is highly expressed in NPC cells but barely detectable in lymphoid cells.[2] Northern analysis failed to detect EBV-miR-BHRFs in NPC cells (unpublished data). High-level expression of EBV miRNAs derived from the BART miRNA cluster was observed in the NPC cell line C666-1 and xenograft C15 without detectable expression of the miRNAs encoded within the BHRF1 cluster.[2]

Although the EBV BART miRNA cluster was highly expressed in NPC cells than in lymphoid cells, we failed to clone some of the miR-BARTs (eg EBV-miR-BART15 and EBV-miR-BART20) in our libraries. According to our previous report, miR-BART15 expression was not detected by Northern Blot in EBV-positive epithelial cells including C666-1 cell.[3] We also failed to detected several minor strands of miRNA in the BART cluster (eg miR-BART7*, miR-BART9*, miR-BART10*) despite the abundant expression of their major strands. During miRNA biogenesis, two strands of the miRNA duplex were derived. Normally only one of the dominant strands, the miRNA strand, was incorporated into the RNA-induced silencing complex and guided gene regulation. Although the non-miRNA was rapidly degraded, in many instances it was also captured during large scale cloning.[1] miR-BART7*, miR-BART9*, and miR-BART10* were identified in low abundance from a large scale cloning study that generated more that 330 000 independent small RNA sequences from 256 small RNA libraries prepared from 26 distinct organ systems and cell types of humans and rodents.[4] Not surprisingly, we were not able to detect these minor strands in our library.

Annotation of the cloned RNAs revealed two novel EBV-encoded miRNAs. EBV-miR-BART-HK1 was located at a miRNA cluster within the intron of the BART gene, as previously reported for other miR-BARTs.[2,5] The expression of EBV-miR-BART-HK1 was greater in EBV-positive NPC cells than in lymphoma cell lines using Northern blotting. EBV-miR-BART-HK1 was located within the region deleted in the B95–8 EBV strain, thus explaining the lack of detection in a study in which EBV-encoded miRNAs were isolated from the human BL cell line BL41/95, which was infected with the EBV B95–8 strain.[5] This EBV strain has an approximately12-kb deletion that removed a large part of the EBV BART gene. A relatively low level of expression of EBV-miR-BART-HK1 in lymphoma cells might be the reason for the lack of detection in another study in which EBV miRNAs were cloned from the lymphoma cell line BC-1.[2] Even in another large-scale cloning study, these two novel EBV-encoded miRNAs were also not detected.[4] The underlying explanation may require further investigation. The differential expression of EBV-miR-BART-HK1, like the BART mRNAs, may be preferentially expressed in EBV-infected epithelial cells and hence may play a particularly important role during EBV infection of this particular cell type. It will be interesting to study the biological function and target gene validation of the novel EBV miRNA EBV-miR-BART-HK1. Elucidating the contribution of EBV miRNAs to biological processes and disease will be crucial to exploit the emerging knowledge about miRNAs for the development of new human therapeutic application in EBV-related diseases.

Conclusions

We are the first group to document the EBV-encoded miRNA expression in NPC cells and identified two novel EBV-encoded miRNAs. High levels of EBV-encoded miRNA expression compared to the cellular miRNA suggest that EBV-encoded miRNA might play a significant role in the establishment and maintenance of viral latency, and probably also in EBV pathogenesis and malignant transformation. Further studies are required to reveal

the functional significance of the novel miRNA in NPC biology.

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