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# Identification of Novel Epstein-Barr Virus MicroRNA Genes from Nasopharyngeal Carcinomas▽†

Jia Yun Zhu,[1] Thorsten Pfuhl,[2] Natalie Motsch,[2] Stephanie Barth,[2] John Nicholls,[3] Friedrich Grässer,[2]* and Gunter Meister[1]*

*Center for Integrated Protein Sciences Munich (CIPSM), Laboratory of RNA Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany[1]; Institute of Microbiology and Hygiene, Department of Virology, University of Homburg/Saar, House 47, 66421 Homburg/Saar, Germany[2]; and Department of Pathology, The University of Hong Kong, Pok Fu Lam, Hong Kong[3]*

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**MicroRNAs (miRNAs) represent a conserved class of small noncoding RNAs that are found in all higher eukaryotes as well as some DNA viruses. miRNAs are 20 to 25 nucleotides in length and have important regulatory functions in biological processes such as embryonic development, cell differentiation, hormone secretion, and metabolism. Furthermore, miRNAs have been implicated in the pathology of various diseases, including cancer. miRNA expression profiles not only classify different types of cancer but also may even help to characterize distinct tumor stages, therefore constituting a valuable tool for prognosis. Here we report the miRNA profile of Epstein-Barr virus (EBV)-positive nasopharyngeal carcinoma (NPC) tissue samples characterized by cloning and sequencing. We found that all EBV miRNAs from the BART region are expressed in NPC tissues, whereas EBV miRNAs from the BHRF1 region are not found. Moreover, we identified two novel EBV miRNA genes originating from the BART region that have not been found in other tissues or cell lines before. We also identified three new human miRNAs which might be specific for nasopharyngeal tissues. We further show that a number of different cellular miRNAs, including miR-15a and miR-16, are up- or downregulated in NPC tissues compared to control tissues. We found that the tumor suppressor BRCA-1 is a target of miR-15a as well as miR-16, suggesting a miRNA role in NPC pathogenesis.**

MicroRNAs (miRNAs) constitute a conserved class of endogenously expressed small noncoding RNAs of 20 to 25 nucleotides (nt) in size with regulatory functions in various cellular processes (1, 4, 7, 14). miRNA genes are transcribed by RNA polymerase II or III as primary transcripts that are processed to stem-loop structured precursors (pre-miRNAs) by the nuclear microprocessor complex containing the RNase III Drosha and its cofactor DGCR8 (33, 51). Pre-miRNAs are transported to the cytoplasm by the export receptor exportin-5, where the RNase III Dicer cleaves off the loop of the hairpin, thereby creating a short double-stranded RNA (1, 4, 39). Such intermediates are subsequently unwound, and one strand is incorporated as mature miRNA into a miRNA-protein complex often referred to as miRNP, while the other strand, referred to as miRNA*, is rapidly degraded (22, 39). The miRNA guides miRNPs to partially complementary sequences located in the 3′-untranslated region (UTR) of target mRNAs and regulates their expression by translational inhibition and/or mRNA destabilization (20, 23, 38, 40, 43).

Soon after the discovery of mammalian miRNAs, viruses were analyzed for miRNA expression as well (17). Indeed, DNA viruses of the herpesvirus family, including Epstein-Barr virus (EBV) (42), Kaposi's sarcoma-associated herpesvirus (9–11, 41), and human cytomegalovirus (41), encode and express miRNAs that are easily detectable in infected cells. The individual functions of the majority of the viral miRNAs are still unknown. However, some viral miRNAs have been characterized functionally in more detail. For example, human cytomegalovirus miR-UL112 targets genes of the host immune system, leading to reduced killing of natural killer cells (44). Moreover, simian virus 40 expresses a miRNA that regulates viral gene expression to reduce susceptibility to cytotoxic T cells (45). It has also been reported that Kaposi's sarcoma-associated herpesvirus miR-K12-11 functions as an ortholog of cellular miR-155 and may therefore exploit preexisting pathways in B cells (24). Very recently, it was shown that herpes simplex virus type 1 expresses miRNAs that regulate and maintain latent infection of neurons of the sensory ganglia (47).

miRNAs have been implicated in a variety of diseases, including cancer. Initially, it was reported that miR-15a and miR-16 expression is frequently downregulated or even deleted in B-cell chronic lymphocytic leukemia (13). To date, a variety of different types of cancer have been analyzed for miRNA expression (12, 19). In many cases, distinct miRNA profiles have been reported. Moreover, oncogenes such as RAS and BCL2 are controlled by the miRNA pathway, and alterations in miRNA expression result in upregulation of these oncogenes (15, 31). miRNAs can therefore function as

* Corresponding author. Mailing address for Gunter Meister: Center for Integrated Protein Sciences Munich (CIPSM), Laboratory of RNA Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany. Phone: 49 89 8578 3420. Fax: 49 8578 3430. E-mail: meister@biochem.mpg.de. Mailing address for Friedrich Grässer: Institute of Microbiology and Hygiene, Department of Virology, University of Homburg/Saar, House 47, 66421 Homburg/Saar, Germany. Phone: 4968411623983. Fax: 4968411623980. E-mail: graesser@uks.eu.

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tumor suppressors. Reciprocally, miRNAs have also been reported to function as oncogenes (12, 19, 48).

Nasopharyngeal carcinoma (NPC) is a human malignancy derived from the epithelium of the nasopharyngeal recess which shows a particularly high incidence in the area of Southeast Asia and is constantly associated with EBV infection. The full-length viral genome is contained in the nuclei of all malignant NPC cells, and its encoded viral RNAs and proteins probably contribute to the malignant phenotype (8, 50). So far, up to 23 EBV miRNAs from two distinct clusters (BHRF1 and BART) have been identified in EBV-positive cell lines and show different expression patterns depending on various infectious samples and stages (11, 26, 42).

Here we report a small RNA cloning and deep sequencing approach with different NPC samples as well as healthy tissues. In addition to three new human miRNA genes, we identify two new EBV miRNA genes from the BART region that have not been found in other tissues or cell lines before. Moreover, we demonstrate that miR-15a and miR-16 are overexpressed in NPC samples and inhibit expression of the tumor suppressor BRCA-1.

## MATERIALS AND METHODS

**Small RNA cloning.** The small RNA fraction from tissue samples was isolated using a mirVana-microRNA isolation kit (Ambion) and separated in a denaturing 12.5% polyacrylamide (PAA) gel stained with SYBR green II. After passive elution, RNAs with a length of 15 to 30 bases were concentrated by ethanol precipitation and dissolved in water. Next, RNAs were poly(A) tailed using poly(A) polymerase, and an adapter with the following sequence was ligated to the 5′ phosphate of the miRNAs: 5′-end adapter (43 nt), 5′-GCCTCCCTCGC GCCATCAGCTNNNNGACCTTGGCTGTCACTCA-3′. NNNN represents a "bar code" sequence for the individual samples, as follows: CAAT for NPC1, ATCG for Control-1, ACTA for NPC-2, and AGGT for Control-2. Next, first-strand cDNA synthesis was performed using an oligo(dT) linker primer [3′-end oligo(dT) linker primer (61 nucleotides), 5′-GCCTTGCCAGCCCGCTCAGAC GAGACATCGCCCCGC$(T)_{25}$-3′] and Moloney murine leukemia virus RNase $H^-$ reverse transcriptase. The resulting cDNAs were PCR amplified in 22 cycles, using the high-fidelity Phusion polymerase (Finnzymes). Amplification products (120 to 135 bp) were confirmed by polyacrylamide gel electrophoresis (PAGE) analysis. All cDNA pools were mixed in equal amounts and subjected to gel fractionation. The 120- to 135-bp fraction was electroeluted from 6% PAA gels. After isolation with Nucleospin extract II (Macherey and Nagel), cDNA pools were dissolved in 5 mM Tris-HCl, pH 8.5, at a concentration of 10 ng/μl and used in single-molecule sequencing. Massively parallel sequencing was performed by 454 Life Sciences, using a Genome Sequencer 20 system.

**Oligonucleotides and plasmids.** The dual-luciferase reporter plasmid pMIR-RL was modified from the firefly luciferase reporter plasmid pMIR-REPORT (Ambion) (6, 28). The 3′ UTR of BRCA-1 mRNA was cloned via PCR amplification from cDNA libraries and ligated into the corresponding SacI and NaeI sites of pMIR-RL.

2′-*O*-Methylated miRNA (2′OMe-miRNA) inhibitors were designed as antisense oligonucleotides to the mature miRNAs according to sequences in the miRNA registry (microrna.sanger.ac.uk/sequences/index.shtml).

**Transfections and luciferase assays.** Plasmid transfections for luciferase assays were performed with 100 ng pMIR-RL and 20 pmol 2′OMe-miRNA inhibitor per $1 \times 10^4$ cells in a 96-well plate, using Lipofectamine 2000 reagent (Invitrogen) as described by the manufacturer.

Luciferase activity was measured 48 h after transfection, using a dual-luciferase reporter system as described by the manufacturer (Promega).

**Sequence analysis.** Base calling and quality trimming of sequence chromatograms were done by the publicly available Phred software (21). The adapter sequences and poly(A) tails were removed from the sequences. The remaining sequences of 15 bases or longer were subjected to later analysis. Due to the disturbing signal from poly(A) tails in 454 sequencing, the sequences were analyzed semimanually, distinguishing the false "A" signals in the sequences. The set of sequences was first compared with the mature sequences of known miRNAs from miRBase v11.0 (25). The sequences that were not perfectly matched may have been artifacts of the cloning procedure or a result of nontemplated modification of mature miRNAs (3) and were annotated according to the best BLAST hit to the database. The rest of the sequences were again compared with the hairpin sequences of known miRNAs from miRBase v11.0, and the star sequences of known miRNAs that have not been annotated in the database were identified. The sequences were then subjected to a BLAST search against noncoding RNA sequences, including rRNAs, tRNAs, snoRNAs, etc., retrieved from GenBank (http://www.ncbi.nlm.nih.gov), and the annotated sequences were discarded from the sequence set. The noncoding RNA sequences from EBV were identified by a BLAST search against the EBV genome retrieved from GenBank.

The rest of the sequences, including the unannotated sequences from the EBV and human genomes, were then subjected to a search for putative novel miRNA sequences. Genomic regions containing inserts with 100-nt flanks were retrieved from Ensembl to calculate RNA secondary structures by mfold (53). Only regions that folded into hairpins and contained an insert in one of the hairpin arms were considered putative novel miRNA sequences.

**Cell culture.** BL41, BL41/B95.8, Jijoye, and EREB2.5 cells were grown in RPMI 1640 medium with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere containing 5% $CO_2$. C666.1 cells were grown in the above-mentioned RPMI medium supplemented with 25 mM HEPES (Sigma) in flask collagen I (Biocoast BD) and digested by Acutase (PAA). HEK 293 cells were grown in Dulbecco's modified Eagle's medium with L-glutamine. MCF7 cells were grown in Dulbecco's modified Eagle's medium with L-glutamine supplemented with 18 μg/ml bovine insulin (Sigma).

**Northern blotting.** Total RNAs were isolated from BL41, BL41/B95.8, Jijoye, EREB2.5, and C666.1 cells by using Trizol reagent (Invitrogen) according to the manufacturer's manual and were separated by 15% denaturing RNA PAGE. Northern blotting against miRNAs was performed as described before (35). The probe sequences against new miRNA candidates were as follows: ebv-mir-BART-21-5P, 5′-GTTAGTTGCCTTCACTAGTGA; ebv-mir-BART-21-3P, 5′-AAACACCAGTGGGCACAACTAG; and ebv-mir-BART-22, 5′-ACTACTAG ACCATGACTTTGTA.

**Ago complex immunoprecipitation and RNA extraction.** Ago complex immunoprecipitation was performed as described previously (6). In short, HEK 293 cells were grown in 10-cm plates and transfected with 1 nmol 2′OMe-miRNA inhibitor, using RNAiMAX reagent (Invitrogen) as described by the manufacturer. Forty-eight hours later, cells were lysed and centrifuged. For immunoprecipitation, 2 ml of hybridoma supernatant from monoclonal anti-Ago1-4B8 and anti-Ago2-5D4 antibodies (6) was mixed and coupled to approximately 50 μl protein G-Sepharose (GE Healthcare). Beads were subsequently incubated with HEK 293 cell lysate for 2.5 h with constant rotation at 4°C. After incubation, the beads were washed five times with RIPA-300 buffer. Finally, the beads were washed once with phosphate-buffered saline. Coprecipitated RNA was extracted using 1 volume of phenol and subsequently precipitated from the aqueous phase using 3 volumes of ethanol. The RNA pellet was treated with DNase I (Fermentas) and used for reverse transcription.

**RT and qRT-PCR.** DNase I-treated RNA was reverse transcribed using a first-strand cDNA synthesis kit (Fermentas) with $(dT)_{18}$ oligonucleotides. Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed using MESA Green qPCR Mastermix Plus for SYBR green assay with fluorescein (Eurogentec) and carried out on a MyiQ single-color real-time PCR detection system (Bio-Rad) with supplied software. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was used for normalization. The primers used were as follows: BRCA-1 for, 5′TAAGCCAGAATCCAGAAGGC; BRCA-1 rev, 5′GGGATGACCTTTCCACTCCT; GAPDH for, 5′AATGGAA ATCCCATCACCATCT; and GAPDH rev, 5′CACCCCACTTGATTTTGG.

**Western blotting.** MCF7 cells were grown in 6-cm plates and transfected with 0.5 nmol 2′OMe-miRNA inhibitor, using RNAiMAX reagent (Invitrogen). Forty-eight hours later, cells were lysed with RIPA-150 buffer. Extracted proteins were separated by 10% sodium dodecyl sulfate-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences) by semidry electroblotting. The primary antibodies used were anti-BRCA-1 (Calbiochem) and anti-β-actin (Abcam), and the secondary antibodies used were peroxidase-conjugated anti-mouse antibodies (Sigma).

**Immunohistochemistry.** Immunohistochemistry with BRCA-1 antibody was performed as follows. Eight cases of nonkeratinizing, undifferentiated NPC (according to WHO criteria) were retrieved from the files of the Department of Anatomical Pathology, Queen Mary Hospital. Five-micrometer sections were cut and then microwaved in 10 mM citrate buffer, pH 6.0, for 15 min. They were blocked with 3% $H_2O_2$ in Tris-buffered saline for 10 min, washed, blocked with an avidin-biotin blocking system (Vector Laboratories) for 15 min each with

TABLE 1. Small RNA read numbers obtained from NPC libraries and control samples

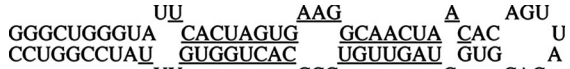
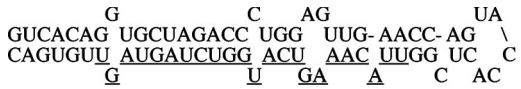
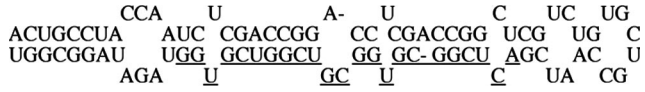
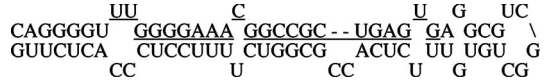
| Sample | No. of reads | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Mapped to human genome | Mapped to EBV genome | Known miRNAs | Human miRNAs | EBV miRNAs | Human ncRNAs | rRNAs | tRNAs | Other ncRNAs |
| NPC-1 | 25,223 | 21,668 | 1,014 | 20,019 | 19,262 | 757 | 2,317 | 1,294 | 156 | 867 |
| Control-1 | 16,249 | 1,874 | 34 | 1,269 | 1,242 | 27 | 616 | 462 | 90 | 64 |
| NPC-2 | 22,636 | 13,445 | 1,217 | 5,976 | 4,891 | 1,085 | 8,412 | 7,185 | 392 | 835 |
| Control-2 | 19,052 | 3,330 | 0 | 1,445 | 1,445 | 0 | 1,843 | 1,456 | 117 | 270 |

reagent A and reagent B, washed, blocked with 10% normal donkey serum for 10 min at room temperature, and then incubated with primary antibody (anti-BRCA-1; Zytomed) at 4°C overnight. The following day, they were incubated with secondary antibody at 1/100 for 30 min at room temperature, incubated with strep-ABC complex (Vector Laboratories) at 1/100 for 30 min at room temperature, and then developed with an AEC substrate kit (Vector Laboratories) at room temperature for 30 min, followed by counterstaining with Mayer's hematoxylin and mounting with Sigma Crystal Mount reagent.

## RESULTS

**Small RNA cloning and sequencing from NPC and control samples.** In order to investigate a possible contribution of cellular or EBV-derived miRNAs to NPC pathogenesis, we cloned and sequenced small RNAs from EBV-positive NPC samples as well as control tissues (GEO number GSE14738). Biopsies were taken from the nasopharynx in an area where there was clinical evidence of tumor, and control tissues were taken from clinically normal mucosa from the opposite side of the nasopharynx in the same patient. We obtained 25,223 sequence reads for NPC sample 1 (NPC-1) and 22,636 reads for NPC sample 2 (NPC-2) that were further grouped according to their origins (Table 1). For control tissues, we analyzed 16,249 (Control-1) and 19,052 (Control-2) sequences. Interestingly, only for the NPC tissues were we able to map >50% of the sequences to the human or the EBV genome. The libraries obtained from control tissues contained only a minor portion of human- or EBV-derived sequences. The majority of the sequences were derived from bacterial genomes, probably due to the bacterial flora in the throat of the patients or to contamination of the samples. About 20,000 sequences of NPC-1 and about 6,000 sequences of NPC-2 were identified as known cellular or viral miRNAs. Notably, the libraries from NPC-2 and Control-2 contained significant numbers of other noncoding RNAs, which may indicate sample-specific lower RNA quality (Table 1).

TABLE 2. Novel miRNA genes identified from NPC samples

| Candidate miRNA[a] | Stem-loop structure of putative miRNA precursor[b] | ΔG[b] | Genomic location | Conservation of stem-loop structure[e] |
|---|---|---|---|---|
| EBV miRNAs | | | | |
| ebv-mir-BART21-5P |  | −40.4 | 145514–145534[c] | rLCV |
| ebv-mir-BART21-3P | | −40.4 | 145548–145569[c] | rLCV |
| ebv-mir-BART22 |  | −35.8 | 147203–147225[c] | |
| Human miRNAs | | | | |
| hsa-mir-1301 |  | −47.2 | 2p23.3[d] | mmu |
| hsa-mir-1307 |  | −39.6 | 10q24.33[d] | ptr, mml |
| hsa-mir-2110 |  | −34.1 | 10q25.3[d] | |

[a] Names of putative new miRNA sequences are according to the submission to miRBase (http://microrna.sanger.ac.uk/sequences/).
[b] RNA secondary structure predictions and free energy calculations were done using mfold, version 3.2. The miRNA sequences are underlined.
[c] The genomic locations of EBV miRNAs refer to the positions in the EBV genome (GenBank accession number AJ507799).
[d] The genomic locations of human miRNAs referring to the positions in human chromosomes, according to the assembly from Ensembl (http://www.ensembl.org/).
[e] rLCV, rhesus lymphocryptovirus; mmu, *Mus musculus* (mouse); ptr, *Pan troglodytes* (chimpanzee); mml, *Macaca mulatta* (monkey).

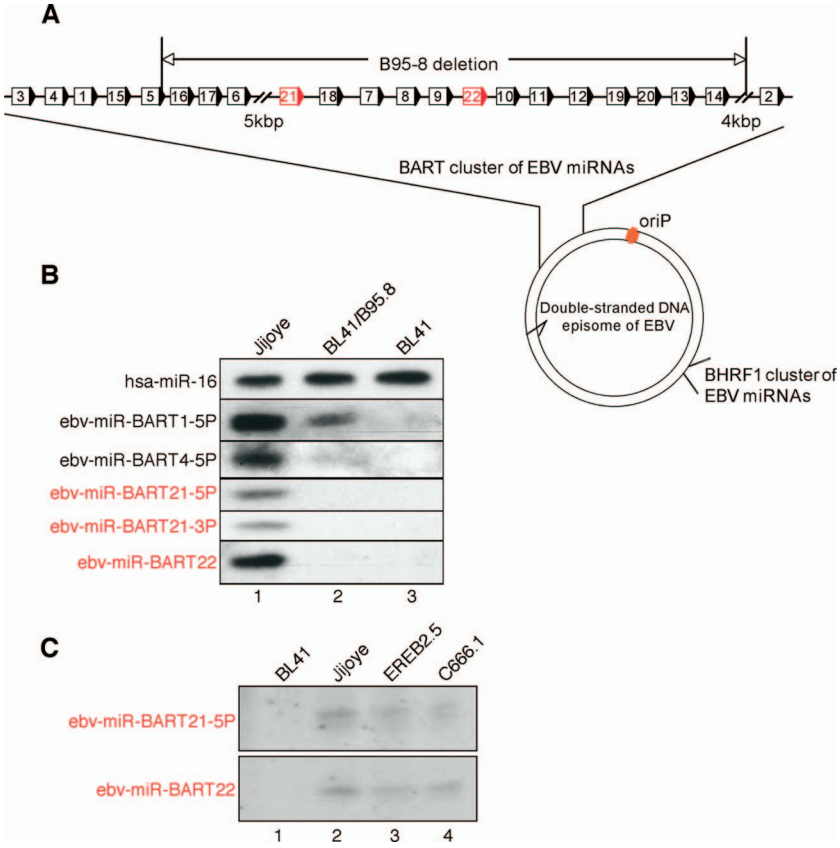



FIG. 1. (A) Schematic locations of putative new miRNAs in the BART miRNA cluster of the EBV genome. Each box with an arrow indicates an EBV miRNA precursor and its transcription direction. The putative new miRNA precursors are depicted in red. The numbers in the boxes represent the names of the BART miRNAs. The genomic localization of the BHRF1 and BART clusters are indicated schematically in the double-stranded episome. oriP, origin of replication. (B) Expression analysis of novel EBV miRNAs by Northern blotting. Total RNAs from EBV-positive Jijoye (lane 1) and BL41/B95.8 (lane 2) cells and EBV-negative BL41 cells (lane 3) were blotted onto nylon membranes and hybridized with probes complementary to miR-16, ebv-miR-BART1-5p, ebv-miR-BART4-5p, ebv-miR-BART21-5p, ebv-miR-BART21-3p, and ebv-miR-BART22. Novel EBV miRNAs are highlighted in red. (C) Total RNAs from BL41 (lane 1), Jijoye (lane 2), EREB2.5 (lane3), and C666.1 (lane 4) cells were blotted onto a nylon membrane and hybridized with probes complementary to ebv-miR-BART21-5p and ebv-miR-BART22.

**Identification of ebv-miR-BART21 and ebv-miR-BART22.** Next, we analyzed our data sets for novel miRNA candidates. First, we investigated sequences that match the EBV genome but have not yet been annotated functionally. We found three different small RNAs derived from two individual precursors that fold into hairpins and originate from the EBV BART region (Table 2; Fig. 1A). We refer to these miRNA candidates as ebv-miR-BART21 and ebv-miR-BART22. Interestingly, miR-BART21 is conserved in rhesus lymphocryptovirus, a common model system for EBV infection (Table 2). We further validated the expression levels of the novel EBV miRNA candidates by Northern blotting with the EBV-positive cell lines Jijoye, EREB2.5, C666.1, and Bl41/B95.8 as well as the EBV-negative B-cell line BL-41 (Fig. 1B and C). Probes specific to the two arms of miR-BART21 as well as miR-BART22 were readily detectable in Jijoye, EREB2.5, and C666.1 cells, whereas no signal was observed in BL41/B95.8 and BL41 cells. Notably, the BL41/B95.8 cell line carries a deletion within the BART region where the two novel miRNAs are located (Fig. 1A). The C666.1 cell line originated from an NPC, and its miRNA pattern closely resembles the EBV miRNA pattern in NPC tissues. It is therefore a model cell line for NPC pathogenesis (16). Furthermore, we detected star sequences for miR-BART4 and miR-BART5 in NPC samples, suggesting that some viral miRNA* molecules might be functional as well (Table 3). We used Target Scan to predict putative cellular mRNA targets of the new EBV miRNAs (36). Target Scan predicts several strong mRNA target candidates with multiple target sites (see Table S1 in the supplemental material).

To further confirm the existence of the new EBV miRNAs in NPC samples, we measured the new miRNA levels in six undifferentiated NPC samples and one control tissue. HEK 293 cells served as a negative control (see Fig. S1 in the supplemental material). Since the amount of extracted RNA from the tissues was very low, we polyadenylated and reverse transcribed the RNA as described previously (29), generating cDNA from miRNA suitable for qRT-PCR validation. GAPDH mRNA was taken for normalization. The background signal from the control tissue sample was set to 1 for calculation. On that basis, all six NPC samples gave strong signals for one known EBV miRNA (ebv-BART1-5p) and the three new EBV miRNAs (ebv-BART21-5p, ebv-BART21-3p, and ebv-

TABLE 3. miRNAs from known novel and precursors identified from NPC samples

| Type of miRNA | Name | Sequence | Genomic location | No. of reads in libraries | | | |
|---|---|---|---|---|---|---|---|
| | | | | NPC-1 | Control-1 | NPC-2 | Control-2 |
| Novel miRNAs from new precursors | | | | | | | |
| EBV miRNA | ebv-mir-BART21-5p | UCACUAGUGAAGGCAACUAAC[a] | 145514–145534[b] | 26 | 1 | 9 | 0 |
| | ebv-mir-BART21-3p | CUAGUUGUGCCCACUGGUGUUU[a] | 145548–145569[b] | 10 | 1 | 6 | 0 |
| | ebv-mir-BART22 | UUACAAAGUCAUGGUCUAGUAGU | 147203–147225[b] | 201 | 1 | 80 | 0 |
| Human miRNA | hsa-mir-1301 | UUGCAGCUGCCUGGGAGUG[a] | 2p23.3[c] | 15 | 1 | 5 | 0 |
| | hsa-mir-1307 | ACUCGGCGUGGCGUCGGUCGUGG | 10q24.33[c] | 22 | 8 | 79 | 22 |
| | hsa-mir-2110 | UUGGGGAAACGGCCGCUGAGUG[a] | 10q25.3[c] | 3 | 1 | 2 | 3 |
| Novel miRNAs from known precursors | | | | | | | |
| EBV miRNA | ebv-mir-BART4* | CACAUCACGUAGGCACCAGGUGU | 139266–139288[b] | 6 | 0 | 9 | 0 |
| | ebv-mir-BART5* | GUGGGCCGCUGUUCACCU[a] | 139717–139734[b] | 0 | 0 | 1 | 0 |
| Human miRNA | hsa-mir-103-2* | AGCUUCUUUACAGUGCUGCCUUG | 20p13[c] | 12 | 1 | 15 | 1 |
| | hsa-mir-205* | GAUUUCAGUGGAGUGAAGUUC[a] | 1q32.2[c] | 15 | 3 | 9 | 5 |
| | hsa-mir-196b* | UCGACAGCACGACACUGCCUUC[a] | 7p15.2[c] | 0 | 1 | 0 | 0 |
| | hsa-mir-224* | AAAAUGGUGCCCUAGUGACUAC[a] | Xq23[c] | 0 | 0 | 2 | 0 |
| | hsa-mir-365-2* | AGGGACUUUCAGGGGCAGCUGU | 7q11.2[c] | 0 | 0 | 1 | 0 |
| | hsa-mir-449b* | CAGCCACAACUACCCUGCCACU | 5q11.2[c] | 1 | 0 | 0 | 0 |

[a] Due to the characteristics of 454 sequencing, the putative miRNA sequences might have an additional "A" at the end.
[b] The genomic locations of EBV miRNAs refer to the positions in the EBV genome sequence (GenBank accession number AJ507799).
[c] The genomic location of human miRNAs refer to the positions in human chromosomes, according to the assembly from Ensembl (http://www.ensembl.org/).

BART22) tested, indicating that the new EBV miRNAs can be detected in a number of NPC samples.

In order to find novel human miRNAs, we analyzed the pool of unknown sequences which match the human genome. Indeed, we identified three new miRNA genes. Two of them were recently annotated as miR-1301 and miR-1307, while this work was in progress. Using miRBase v11.0 (25), we named the third one miR-2110 (Table 2). miR-1301 is located in an intergenic region, whereas miR-1307 and miR-2110 reside in the 5′ UTRs of protein coding genes (Table 2). Additionally, so far we have found six unidentified miRNA* sequences that derive from known miRNA genes and have not been cloned before (Table 3). In summary, we have identified two new EBV miRNA genes that give rise to mature miR-BART21-5p, miR-BART21-3p, and miR-BART22. In addition, we have found three novel human miRNA genes, referred to as miR-1301, miR-1307, and miR-2110.

**miRNA expression analysis suggests an NPC-specific miRNA signature.** miRNA expression analyses revealed distinct miRNA signatures for different types of tumors (12). In order to establish a miRNA expression profile characteristic of NPCs, we examined the most abundant miRNAs in the individual libraries obtained from NPC or control tissues (Fig. 2; see Table S2 in the supplemental material). About 5% of the NPC-1 and 19% of the NPC-2 miRNAs were identified as EBV miRNAs, indicating that viral miRNAs are highly expressed in NPCs (Fig. 2A). Notably, small numbers of EBV miRNAs were present in Control-1 as well, which might be due to a minor contamination of the control tissue with tumor tissue (Table 1). Among the EBV miRNAs, we found high levels of ebv-miR-BART1, ebv-miR-BART4, ebv-miR-BART6, ebv-miR-BART7, ebv-miR-BART11, ebv-miR-BART12, and ebv-miR-BART19 as well as the new miRNAs ebv-miR-BART21 and ebv-miR-BART22. Interestingly, no EBV miRNA from the BHRF1 region was identified in our libraries, suggesting that miRNAs from this region might not be involved in NPC pathogenesis (Fig. 2B). The most abundant human miRNAs are presented in Fig. 2C. The 20 most frequently cloned miRNAs were similar between NPC and control tissues (see Table S2 in the supplemental material). However, miR-320, miR-17-5p, and miR-652 were less frequent in the libraries from the NPCs than in those from control tissues, suggesting that these miRNAs might be downregulated in NPC tissue. Furthermore, the read numbers for miR-23a/b (both miRNAs were indistinguishable in our sequencing data sets), miR-200c, and miR-27a/b were significantly increased in the NPC tumor samples, suggesting specific upregulation of these miRNAs. Consistent with the cloning data, miR-23a/b and miR-27a/b were processed from the same primary transcript.

**Misregulated miRNAs miR-15a and miR-16 inhibit expression of the tumor suppressor BRCA-1.** Next, we analyzed potential targets of miRNAs that are misexpressed in the two NPC libraries analyzed above. In both NPC samples, expression of miR-15a and miR-16, which are derived from one primary transcript, was upregulated, and indeed, bioinformatic predictions identified target sites for miR-15a and miR-16 in the 3′ UTR of the tumor suppressor BRCA-1 (Fig. 3A and B). Therefore, we hypothesized that high levels of miR-15a and miR-16 may lead to low levels of BRCA-1, resulting in low tumor suppressor activity and tumor growth. In order to analyze effects of miR-15a and miR-16 on BRCA-1 expression, we fused the BRCA-1 3′ UTR to a firefly luciferase reporter. The reporter plasmid was transfected into HEK 293 cells together with 2′OMe inhibitors against endogenous miR-15a or miR-16 (Fig. 3C). Indeed, luciferase expression was elevated when endogenous miR-15a or miR-16 was inhibited, suggesting that BRCA-1 is regulated by miR-15a or -16.

To further validate a functional interaction of the miRNA pathway with the BRCA-1 mRNA, we investigated binding of Ago proteins to the BRCA-1 mRNA (Fig. 3D). We and others

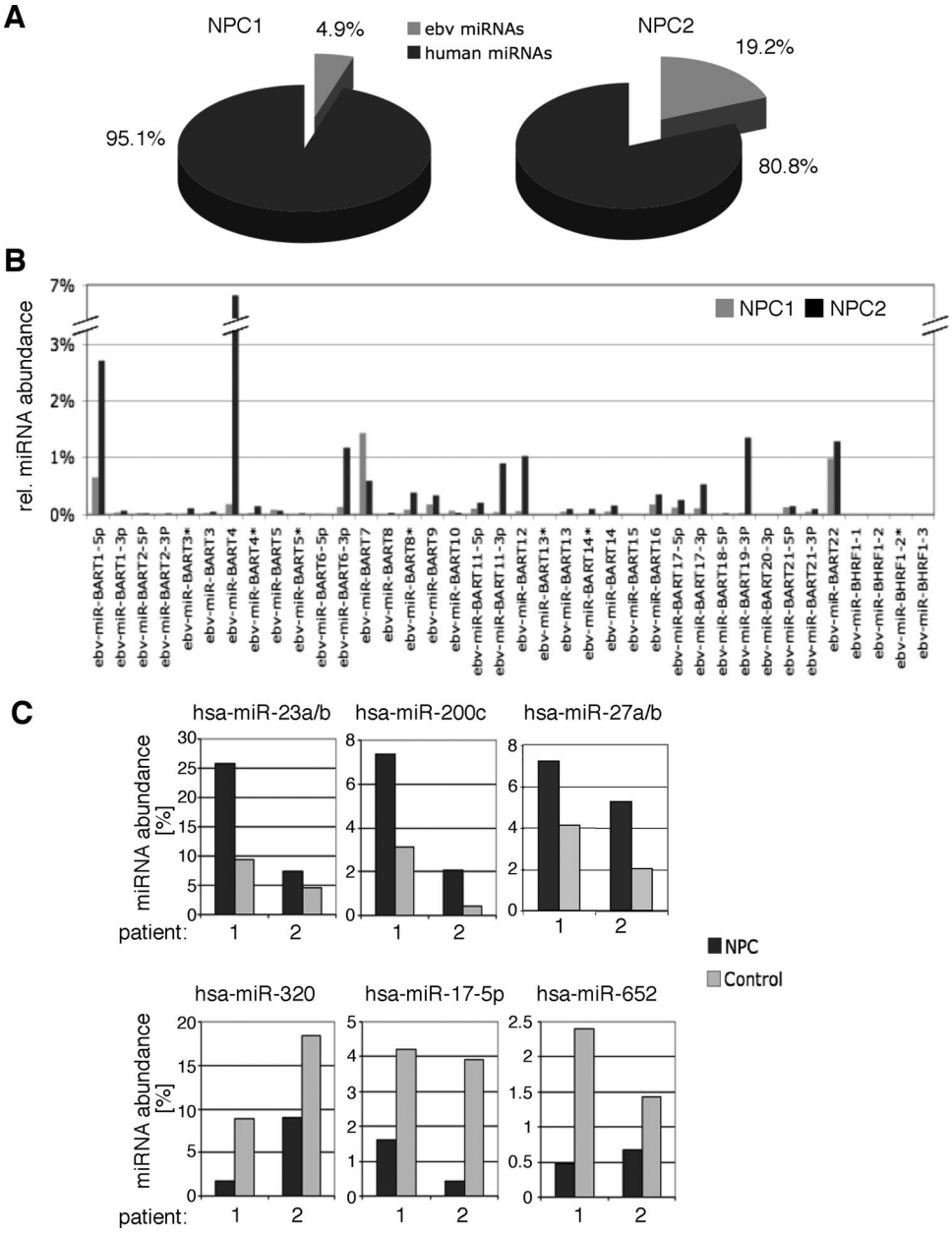



FIG. 2. miRNA expression in NPC and control tissue. (A) Schematic representation of cellular and EBV-derived miRNA fractions in NPC tissue samples. (B) Relative abundances of EBV miRNAs in the NPC-1 and NPC-2 libraries. Individual read numbers are presented as percentages of the total miRNA reads in the libraries. (C) Cellular miRNAs miR-23a/b, miR-200c, and miR-27a/b are upregulated in NPC tissues. miR-320, miR-17-5p, and miR-652 are more abundant in small RNA libraries from control tissue samples. The criteria for miRNA selection were a 1.8-fold change in both patients and at least 1% abundance in two of the four samples. miRNA read numbers are shown as percentages of the total miRNA reads in the libraries.

have shown before that miRNA target mRNAs can be immunoprecipitated and identified using antibodies against Ago proteins (6, 18, 27, 32). Endogenous Ago1 and Ago2 complexes were immunoprecipitated from a HEK 293 cell lysate by using Ago1- and Ago2-specific antibodies, and coimmunoprecipitated mRNAs were analyzed by qRT-PCR (Fig. 3D). Indeed, BRCA-1 mRNA was coimmunoprecipitated with anti-Ago1 and anti-Ago2 antibodies. Moreover, the BRCA-1 mRNA was released from Ago protein complexes when 2′OMe inhibitors complementary to miR-15a or miR-16, which interrupt miRNA-mRNA interactions, were transfected, indicating that BRCA-1 is targeted by the miRNA pathway.

Finally, we measured BRCA-1 mRNA as well as protein levels in the presence or absence of miR-15a or miR-16 inhibitor (Fig. 3E and F). It has been shown before that miRNAs not only function in translation but also have effects on mRNA stability. Endogenous BRCA-1 mRNA was robustly increased when miR-15a or miR-16 was inhibited. Notably, inhibition of let-7a, a miRNA that does not target BRCA-1, led to a decrease of BRCA-1 mRNA levels, which was most likely due to

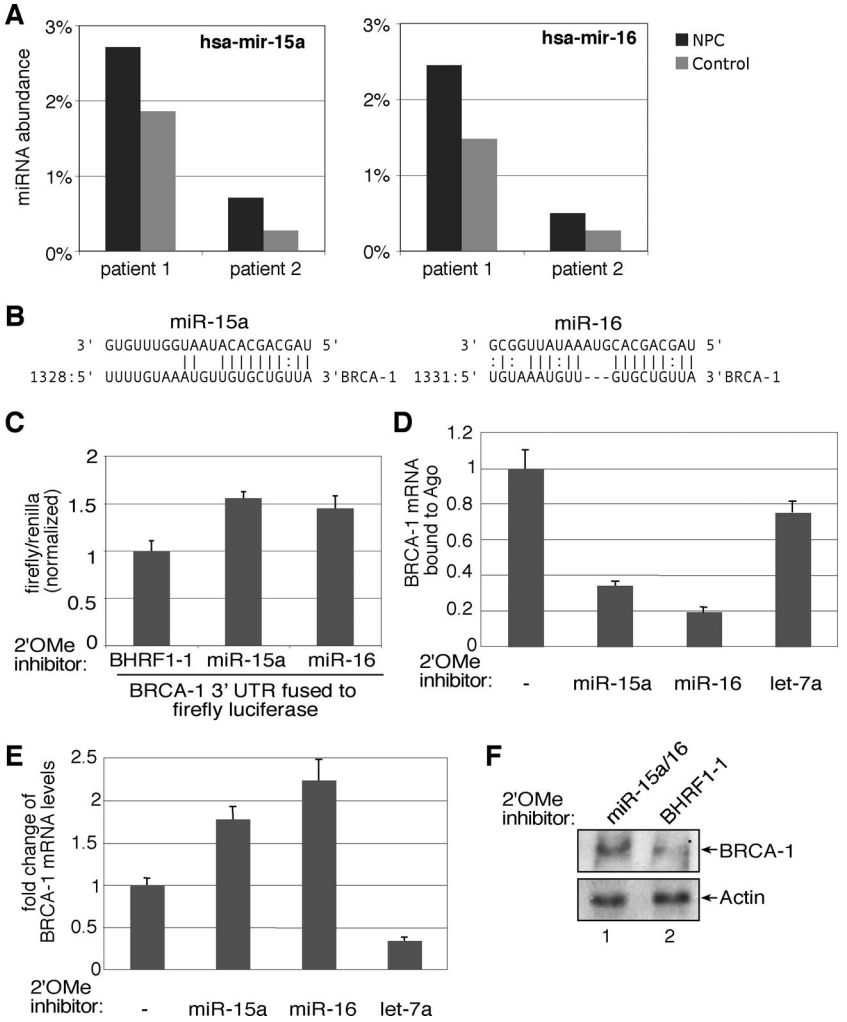



FIG. 3. miR-15a and miR-16 target the BRCA-1 mRNA. (A) Elevated miR-15a and miR-16 expression in two NPC samples compared to that in healthy tissues from the same individuals. miRNA read numbers are shown as percentages of the total miRNA reads in the libraries. (B) Putative binding sites of miR-15a and miR-16 in the BRCA-1 3′ UTR predicted by Miranda (30). (C) A firefly luciferase reporter fused to the BRCA-1 3′ UTR was cotransfected with 2′OMe antisense inhibitors against miR-15a or miR-16 into HEK 293 cells. Firefly luciferase expression was measured after 48 h and normalized to *Renilla* luciferase expression. (D) Ago1/2 complexes were immunoprecipitated from HEK 293 cell lysates transfected with miRNA inhibitors as indicated. RNAs were extracted and reverse transcribed from immunoprecipitated Ago1/2 complexes. BRCA-1 mRNA incorporated into Ago1/2 complexes was measured by qRT-PCR and normalized to the GAPDH mRNA level. (E) Total RNA was extracted and reverse transcribed from HEK 293 cells transfected with miRNA inhibitors as indicated. BRCA-1 mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA levels. (F) Analysis of BRCA-1 protein levels by Western blotting. Lysates of MCF-7 cells transfected with 2′OMe antisense inhibitors to miR-15a and miR-16 (lane 1) or to ebv-mir-BHRF1-1 as a control (lane 2) were transferred to nitrocellulose membranes and analyzed by Western blotting using anti-BRCA-1 antibodies.

unrelated, secondary let-7a effects. Consistent with the mRNA expression data, we also observed an increase in BRCA-1 protein levels when miR-15a and miR-16 were inhibited (Fig. 3F).

In summary, our data suggest that miR-15a and miR-16 are upregulated in NPC samples and target the tumor suppressor BRCA-1.

**Reduced BRCA-1 signals in NPC tissue sections.** To further support our hypothesis that BRCA-1 expression is reduced in NPC tissues, we performed immunohistochemistry experiments using paraffin-embedded tissue sections (Fig. 4). We analyzed archived tissue biopsy samples from eight NPCs and tested them for the presence or absence of BRCA-1, using a monoclonal antibody which identifies the C-terminal region of the BRCA-1 protein, which according to the manufacturer's instructions is cytoplasmic in location. In eight undifferentiated NPCs, there was strong cytoplasmic staining in two cases, medium to weak staining in four cases, and no staining in two cases (Fig. 4A). The adjacent stroma epithelium showed medium positivity, which was cytoplasmic (Fig. 4B). Thus, in six of eight NPCs, there was a degree of reduction or absence of staining with BRCA-1. Taken together, these data further support our finding that BRCA-1 expression is decreased in a number of NPC samples.

## DISCUSSION

Using small RNA cloning and sequencing, we have characterized the miRNA expression profile of NPC tissues. EBV is

A

| NPC Patient | Sex | Age | Stage | BRCA-1 Staining |
|---|---|---|---|---|
| 1 | M | 49 | T2N1 | undetectable |
| 2 | M | 45 | T1N0 | strong |
| 3 | M | 46 | T2bN2 | weak |
| 4 | M | 67 | N/A | undetectable |
| 5 | M | 20 | T3N3a | weak |
| 6 | M | 57 | T1Vb | weak |
| 7 | M | 57 | Relapse | strong |
| 8 | M | 77 | T4N1 | weak |

B

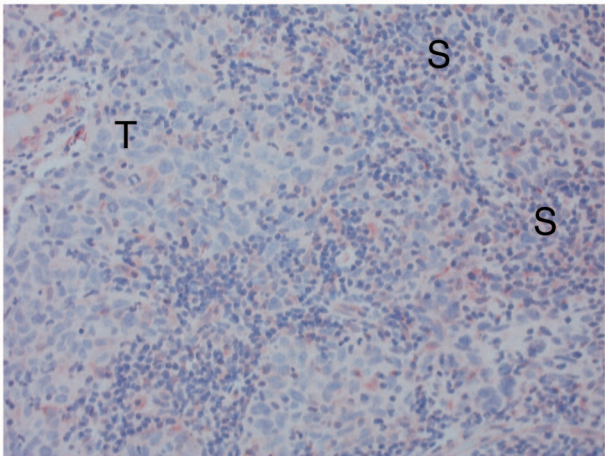



FIG. 4. Analysis of BRCA-1 protein levels in various NPC tissue sections by immunohistochemistry. Paraffin-embedded sections were stained with antibodies against BRCA-1. (A) Summary of BRCA-1 protein detection in eight different NPC samples. (B) Example of weak BRCA-1 staining in tumor tissue. S, healthy stroma tissue; T, region of tumor tissue.

reported to be present in all NPCs and can transform cells, which subsequently induces cell proliferation and tumor growth. Indeed, we find that EBV expresses all miRNAs from the BART cluster in NPC tissues, suggesting that these miRNAs may have an important function in maintaining the virus in NPC tissues. Moreover, we identified two novel and highly abundant EBV miRNA genes, namely, miR-BART21 and miR-BART22. Notably, no miRNA originating from the BHRF1 region of the EBV genome was found in our data sets, suggesting that these miRNAs might not be required for NPC pathogenesis. More provocatively, the absence of BHRF1 miRNA expression might be important for NPC pathogenesis, and only viruses with impaired BHRF1 miRNA expression might be capable of NPC tumor formation. Interestingly, BHRF1 miRNA expression was not found in EBV-positive gastric cancer samples as well (34). The detailed characterization of the small RNA profiles of other EBV-positive tumor types, such as T-cell lymphomas, B-cell lymphomas, and gastric cancer, will help to elucidate the contribution of EBV miRNAs to cancer pathogenesis.

Like other tumors, NPC samples are characterized by distinct miRNA expression profiles. In particular, we demonstrate that miR-23a/b, miR-200c, and miR-27a/b are significantly upregulated, whereas miR-320, miR-17-5p, and miR-652 are downregulated in NPC samples. Bioinformatic predictions suggest that miR-23a and -b, which are the most abundant miRNAs in NPC samples, may regulate the tumor suppressors ITGB1 (integrin-beta1), LIG4 (ATP-dependent DNA ligase IV), and SAFB (scaffold attachment factor B). It is tempting to speculate that other upregulated miRNAs might target tumor suppressors as well. Indeed, we find that the expression of miR-15a and miR-16 is elevated in NPC tissues and show that both miRNAs can target BRCA-1, a tumor suppressor gene important for breast cancer (49). Interestingly, miR-15a and miR-16 have been implicated in cancer before. In B-cell lymphocytic leukemia, miR-15a and miR-16 expression is downregulated, and it has been suggested that both miRNAs target the bcl-2 oncogene (12). Together with our data, these findings suggest that miR-15a and miR-16 can target oncogenes as well as tumor suppressors, depending on individual tissues.

BRCA-1 was shown to interact with the E6 and E7 oncoproteins of human papillomaviruses, and the association of BRCA-1 with E6 and E7 reverses the inhibition of expression of estrogen receptor alpha (52). It is thought that the growth arrest normally brought about by BRCA-1 is overcome through interaction with hypophosphorylated pRB (2). So far, no involvement of BRCA-1 in EBV-mediated induction of NPC has been reported. However, it was shown that the BRCA-1-associated ZRBK1/KAP-1 corepressor forms a tight complex with the BBLF2/3 protein of EBV (37). BBLF2/3 in turn is the bridging factor for the tripartite EBV helicase-primase complex, consisting of the helicase BBLF4, the primase BSLF1, and BBLF2/3 as the linking molecule. The ectopic expression of ZRKB1, KAP-1, or BRCA-1 stimulated oriLyt-driven replication of EBV DNA, establishing a functional link between the presence of BRCA-1 and lytic viral replication. Sera of NPC patients exhibit peculiar signs of lytic replication, while the tumor cells are conspicuously devoid of lytic viral proteins. The downregulation of BRCA-1 by miR-15a and miR-16 might inhibit the induction of lytic cycle replication in the tumor cells. This notion is in line with our previous demonstration that the EBV-encoded miRNA ebv-miR-BART2 downregulates the viral DNA polymerase BALF5, resulting in reduced virus production (5). A function for miRNAs encoded by the LAT latency transcript of herpes simplex virus in the reduction of virus production was recently demonstrated (46, 47).

The identification of cellular miRNA targets as well as the analysis of more NPC samples will help to elucidate the molecular basis of NPC pathogenesis. Our data might be a starting point for identifying and classifying NPCs, probably even at very early stages. Moreover, interfering with overrepresented miRNAs or artificial expression of underrepresented miRNAs might be useful for NPC therapy in the future.

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