Plasma EBV miRNAs Profiles Reveal Potential Biomarkers for clinical prognosis of Acquired Immune Deficiency Syndrome-related Lymphoma

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Plasma EBV miRNAs Profiles Reveal Potential Biomarkers for clinical prognosis of Acquired Immune Deficiency Syndrome-related Lymphoma

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Runing Tile: EBV variation in patients with HIV in eastern China

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Abstract

Background : Acquired immune deficiency syndrome-related lymphoma (ARL) is closely related to Epstein-Barr virus (EBV) infection. However, there are few studies on the occurrence and development of EBV microRNAs (miRNAs) in ARL patients.

Methods : The plasma of 5 EBV-infected ARL patients and 8 EBV-infected HIV patients were screened for EBV miRNAs differentially expressed between the two groups through a customized EBV microRNA quantification chip. The plasma of 35 EBV-infected ARL patients and 20 EBV-infected HIV patients was verified by qRT-PCR expanded samples. And we gave a further analysis of the correlation between differentially expressed EBV miRNAs and clinical indicators in ARL patients.

Results : 1. There were differential expressions of EBV miRNAs in the plasma of EBV-infected ARL patients and EBV-infected HIV patients. It was found that ebv-miR-BART2-5p, ebv-miR-BART8-3p, ebv-miR-BART15, ebv-miR-BART19-5p expression was significantly up-regulated and ebv-miR-BART9-5p expression was significantly down-regulated; 2. EBV miRNAs with significantly different expressions in the screening results were expanded and verified by qRT-PCR, and the differential expression was found to be consistent with the screening results; 3. The relative expression levels of ebv-miR-BART8-3p,ebv-miR-BART19-5p and ebv-miR-BART9-5p in plasma were positive correlated with the international prognostic index (IPI) score of Lymphoma, eastern cooperative oncology group (ECOG) scores are the incidence of lymphoma B symptoms (P = 0.03, 0.01, and 0.04, respectively);4. EBV miRNAs could be used as biomarkers for ARL prognosis evaluation.

Conclusions : The expression of ebv-miR-BART2-5p, ebv-miR-BART8-3p, ebv-miR-BART15,ebv-miR-BART19-5p,ebv-miR-BART9-5p and ebv-miR-BART6-3p in ARL patients were highly different; and ebv-miR-BART8-3p, ebv-miR-BART19-5p, ebv-miR-BART9-5p could be used as the biomarkers of the prognosis of ARL evaluation.

Key words: EBV; microRNA; lymphoma; HIV; Prognosis

Background

Epstein-Barr virus (EBV) is the first human tumor virus to be discovered [1]. EBV mainly establishes infection in two types of cells, lymphocytes and epithelial cells, and exists for a long time in the host， which is the cause of many malignancies, including nasopharyngeal carcinoma (NPC), EBV-associated gastric cancer (EBVaGC) as well as acquired immune deficiency syndrome-related lymphoma (ARL) and post-transplant lymphoproliferative disease (PTLD) [1， 2]. EBV has two infection states in the human body, namely latent infection and lytic infection. The latent infection period can be divided into 4 types: latent period 0, I, II and III [3]. EBV infections in different periods have been proved to promote the occurrence and development of EBV-related tumors[1， 4]. MicroRNAs (miRNAs) are a group of non-coding small RNAs, consisting of 19-25 nucleotides, which directly bind to the 3'-untranslated region (3'-UTR) of mRNA, promote mRNA degradation and inhibit its translation gene regulation [2]. EBV was the first virus found to encode miRNAs [5]. So far, previous studies have found that there were 44 mature miRNAs encoded by EBV: 40 mature miRNAs encoded by 22 miRNA precursors (miR-BART1-22) in BamHI-A region right transcript (BART) and 4 mature miRNAs produced by 3 miRNA precursors (miR-BHRF1-1, -2, -3) expressesed in BamHI fragment H opens to the right Reading frame 1 (BHRF1) [5， 6]. EBV miRNAs are expressed differently during different infection periods. Among them, miR-BARTs are expressed in all types of incubation periods, especially in incubation periods I and II, while miR-BHRF1s are more common in cells infected in incubation period III and lysis periods[7， 8]. At present, there are many studies on the occurrence and development of EBV miRNAs in NPC, but such studies are few in ARL patients. Studies have reported that compared with non-HIV-infected patients with EBV-positive diffuse large B-cell Lymphoma (DLBCL), the expression of the three miR-BHRF1s is higher in patients with AIDS and DLBCL[6].

In this study, we firstly performed differential expression analysis of EBV miRNAs in 13 blood samples of patients with ARL. Then, these results were verified by 55 patients.

Whether these EBV miRNAs could be the biomaker for clinical prognosis were be tested by ROC analysis.

Methods

Research Subjects

The study included 55 HIV-1 patients with EBV infection admitted to the AIDS ward of the First Affiliated Hospital of Zhejiang University School of Medicine. The average age was 47 years old, 48 male patients and 7 female patients. Of these, 35 were lymphoma patients (including 1 Hodgkin lymphoma patient, 34 non-Hodgkin lymphoma patients, 1 non-Hodgkin lymphoma patient, 1 plasmablastoma patient, 7 Burkitt lymphoma Tumor patients, 7 patients with high-grade B-cell lymphoma, 19 patients with diffuse large B-lymphoma). Twenty patients were non-lymphoma patients as a control group. Other basic information is shown in Table 1. The study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Approved No. of ethic committee: 2018764), and all patients signed an informed consent. Collect the whole blood specimens of 55 patients, routinely separate the plasma, and store in the refrigerator at -80 ℃ for future use.

RNA extraction

Remove the plasma sample, centrifuge at 3000G for 5 min after thawing, add 750 ul TRIZOL-LS (Invitrogen, NO. MAN0000806) to the supernatant, vortex for 5 s, and incubate at room temperature for 5 min. Add 0.2ml of chloroform per 1ml of homogenized sample and vortex for 15s, then incubate for 3min. Centrifuge at 13000G for 15 minutes at 4 ° C. Isopropanol was added to the aqueous phase layer, and after standing at 4 ° C for 30 minutes, it was centrifuged at 13000 G at 4 ° C for 15 minutes. Remove the supernatant and add 1ml of 75% ethanol to each 1ml of homogenized sample to wash the RNA pellet. Let stand for 10min, then centrifuge at 10000G for 5min at 4 ° C. Precipitate the RNA in the air for 5-10 min, add RNA-free water to elute the RNA, and measure the optical density (OD) at 260 nm, 260/280 ratio using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) To assess the concentration and purity of RNA, and finally stored at -80 ℃.

Synthesis of complementary DNA (cDNA)

Add 1μl of ATP (10mM) to the reaction system, 1μl of 10X A-Plus Reaction Buffer, 700ng of RNA sample, 0.5μl of A-Plus Poly (A) Polymerase (4U / μl), 0.2μl of RNase inhibitor (40u / μl), Add RNase-free water to a total volume of 10 μl. After incubating at 37 ° C for 10 minutes, add oligo dT linker primer miR-RT (5'GTCGGTGTCGTGGAGTCGTTTGCAATTGCACTGGATTTTTTTTTT TTTTTTTTV3 ') 1μl and downstream primer U6 (5'CGCTTCACGAATTTGCGTGTCAT3') 0.3μl, mix at 60 ° C for 5min, add dNTP (2.5mM) 10X RT Buffer 2μl, MMLV reverse transcriptase (10U / μl) 0.4μl, RNase inhibitor (40u / μl) 0.3μl to a total volume of 20μl. Incubate at 30 ° C for 60 minutes, incubate at 85 ° C for 5 minutes, and store at -20 ° C until use.

EBV miRNAs gene chip screening differential expression and data analysis

Through the miRBase database (version 22.1, http: // www, mirbase.org/), 44 mature miRNA sequences of EBV were retrieved. The miR-423-5p sequence was used as an internal reference. Entrusted Shanghai Kangcheng Biology to customize the chip. Plasma samples of 13 HIV-1 infected patients (5 combined lymphomas and 8 non-lymphomas) were randomly selected for high-throughput screening. Use the software attached to the PCR instrument (QuantStudio5 Real-time PCR System (Applied Biosystems)) to perform preliminary data analysis to obtain the original Ct value, and then use GenEx qPCR analysis software (www.exiqon.com/mirna-pcr-analysis) for standardization and in-depth data analysis. Finally, the expression difference of the two groups of corresponding genes was calculated by 2-ΔΔCt, and the EBV miRNAs with obvious expression differences were further verified by the following expanded sample.

qRT-PCR verification of differentially expressed EBV miRNAs

qRT PCR reaction system is 2 × Master Mix 5μl, forward and reverse miRNA primers (see Table 2) 0.5μl, add water to a total volume of 8μl, centrifuge briefly at 5000 rpm, and sequentially add to the 384-PCR well plate, each add 2 μl of cDNA to the well,

cover with sealing film and centrifuge briefly. The specific PCR reaction program is 95 ℃, 10min; 40 PCR cycles (95 ℃, 10 seconds; 62 ℃, 60 seconds).

Data analysis

The results of the differential expression levels of EBV miRNAs are expressed in standard errors. The non-parametric chi-square test is used to evaluate the difference analysis of EBV miRNAs between the ARL group and the control group; Spearman rank correlation coefficients are used to test the expression levels of EBV miRNAs and clinical indicators of lactate. Correlation between dehydrogenase (LDH), B symptoms, international prognostic index score (IPI score), Eastern Cooperative Oncology Group score (ECOG score), etc. ROC curve analysis was used to evaluate the sensitivity and specificity of EBV miRNAs as biomarkers for ARL prognosis evaluation. The study used SPSS 26.0 software for statistical analysis, $P < 0.05$ was considered statistically significant.

Results

Differential expression of EBV miRNAs in ARL patients

In this study, $2^{-\Delta\Delta Ct}$ was used to calculate the differential expression multiples of EBV miRNAs between ARL and the control group, and the P values were plotted as scatter plots, volcano plots, and histograms (Figure 1 (P value <0.05), and then screen based on the condition that the CT value is less than 35, and finally screen out ebv-miR-BART2-5p, ebv-miR-BART8-3p, ebv-miR-BART15, ebv-miR-BART19-5p ebv-miR-BART9-5p 5 EBV miRNAs. Compared with the control group, the relative expression of ebv-miR-BART19-5p in ARL was down-regulated, and the rest were up-regulated.

Expanded sample qRT-PCR verification

Further expanded sample qRT-PCR verification found that the results are consistent with the screening results (see Figure 2).

The expression of ebv-miR-BART8-3p, ebv-miR-BART19-5p, ebv-miR-BART9-5p in patients with ARL is related to clinical prognosis

We used the Spearman rank correlation coefficient for analysis and found that in ARL

patients, the expressions of ebv-miR-BART8-3p, ebv-miR-BART19-5p, ebv-miR-BART9-5p were associated with IPI score, ECOG score There is a correlation between the occurrence of B symptoms (Figure 3). We used the clinical outcome of ARL patients to achieve complete remission after clinical chemotherapy as a clinical outcome, and found that EBV miRNAs were superior to IPI scores in ARL prognosis judgment (Figure 4).

Discussion

EBV expresses high levels of miRNAs at all stages of its life cycle, indicating that these miRNAs may be involved in the interaction between EBV and the host immune system [9]. More and more studies have found that EBV miRNAs can promote cell proliferation and transformation by targeting host mRNA and inhibit cell apoptosis [10]. In addition, EBV miRNAs can also suppress the expression of viral antigens, thereby allowing infected cells to escape immune recognition [11]. More interestingly, EBV miRNAs can directly suppress the host's antiviral immunity by interfering with antigen presentation and immune cell activation [12]. In ARL patients, there are few studies on the expression characteristics and related clinical significance of EBV miRNAs.

In this study, we collected blood samples from 55 HIV-1 infected patients and found the higher expression of ebv-miR-BART2-5p, ebv-miR-BART8-3p, ebv-miR-BART15, ebv-miR-BART19-5p and lower expression of ebv-miR-BART9-5p in ARL patients compared with non-ARL.

Currently, the IPI score is the most commonly used prognostic evaluation system for patients with lymphoma [13]. Clinicians evaluate the prognosis of patients by scoring five items: patient age, lymphoma stage, ECOG score, extranodal lesions, and LDH. These projects involve a variety of blood, ultrasound, PET-CT and other tests, which consume a lot of medical resources. Our research and further analysis found that the relative expression of ebv-miR-BART8-3p, ebv-miR-BART19-5p and ebv-miR-BART9-5p in plasma is correlated with ARL's IPI score, ECOG score and the occurrence of B symptoms -miR-BART9-5p, which can be used as a biomarker for prognosis evaluation, can more easily achieve prognosis evaluation, and is better than

IPI score in prognosis judgment. ECOG as a system for evaluating the physical status of patients with lymphoma can better assess the tolerance of patients to treatment. Our research results found that the expression level of ebv-miR-BART19-5p can also be used as a reference marker for this evaluation.

Related research further studies the mechanism of EBV miRNAs in promoting tumorigenesis and development. RND3 is a miR-BART2-5p targeting gene. RND3 is related to apoptosis, cell cycle arrest and cell differentiation [14]. According to reports, in nasopharyngeal carcinoma, miR-BART2-5p has potential value in promoting nasopharyngeal carcinoma tumor metastasis and its use as a prognostic indicator or therapeutic target [15]. There are also reports that miR-BART2-5p can be used as an early detection indicator for patients with nasopharyngeal carcinoma [16]. Experiments have shown that miR-BART8-3p can regulate ataxia telangiectasia mutations / ataxia telangiectasia mutations (ATM / ATR) and Rad3 The activity of related signaling pathways promotes NPC's radiation resistance [17]. MiR-BART15 is capable of targeting nucleotide-bound oligomerization domain-like receptor family pyridine domain-containing 3 (NLRP3) with less inflammation to limit inflammation and promote EBV infection [18]. LMP1 is a transmembrane latent protein encoded by EBV, which is essential for cell proliferation and transformation [19], but overexpression of LMP1 will inhibite cell proliferation and increase the sensitivity of cells to pro-apoptotic stress [20]. According to previous reports, miR-BART19-5p can inhibit the expression of LMP1, thereby maintaining a balance between the growth-promoting effect of LMP1 and its pro-apoptotic function [10]. We infer that the EBV miRNAs found in our research may also be through similar molecular mechanisms It led to the occurrence and development of ARL and led to different clinical outcomes.

In the next stage of research, we will predict the target genes of differentially expressed EBV miRNAs and obtain relevant cell signaling pathways through further analysis to further reveal and clarify the role and mechanism of EBV miRNAs in the development and development of ARL for the prognosis of ARL Provide a basis for judging and finding new therapeutic targets.

Conclusion

This study found that the differentially expressed EBV miRNAs in ARL patients are closely related to the clinical prognostic indicators (IPI score, ECOG score, and lymphoma B symptoms) of ARL patients, and can be used as biomarkers for ARL prognosis assessment.

List of abbreviations

ARL: Acquired immune deficiency syndrome-related lymphoma; EBV: Epstein-Barr virus; miRNAs: microRNAs; IPI: international prognostic index; ECOG: eastern cooperative oncology group; NPC: nasopharyngeal carcinoma; EBVaGC: EBV-associated gastric cancer; PTLD: post-transplant lymphoproliferative disease; BHRF1: BamHI fragment H opens to the right Reading frame 1; DLBCL: diffuse large B-cell Lymphoma; OD: optical density; cDNA: complementary DNA; ATM / ATR: ataxia telangiectasia mutations / ataxia telangiectasia mutations.

DECLARATIONS

Ethics approval and consent to participate

All procedures conducted in this study involving human participants were performed in accordance with the ethical standards of the Hospital Institutional Research Council and the 1964 Helsinki Declaration and its subsequent revisions or similar ethical standards.

This study was approved by the ethics committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Approval No. 2018764), and Chinese Clinical Trial Registry (ChiCTR, ChiCTR 1900023600. Registered 3 June 2019, http://www.chictr.org.cn/usercenter.aspx).

The study has obtained informed consent from all study participants.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no potential or actual competitive interests, economic issues or personal relationships with others or organizations to influence their research work.

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Authors' contributions

Ying Chen carried out most of the experiments and wrote the manuscript. Jiangjin Hui, Xiaorong Peng, Ying Huang, Yan Xu helped with the experiments. Biao Zhu designed the experiments and revised the manuscript. All authors read and approved the final version of this manuscript.

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Table1 Description of the study population

Category	ARL（n=35）	The controls（n=20）	*P*-value
Age	45.29±11.40	50.10±15.73	0.50
Sex			0.24
male	91.43%（32/35）	80.00%（16/20）	
female	2.86%（3/35）	20.00%（4/20）	
$CD4^+$Tcell count（cells/mm3）	153.00[93.00,271.00]	260.50[115.50,422.00]	0.85
$CD4^+/CD8^+$	0.41[0.22,0.87]	0.25[0.16,0.54]	0.55
HAART			0.11
2NRTIs+NNRTIs	57.14%（20/35）	50.00%（10/20）	
2NRTIs+INSTIs	34.29%（12/35）	20.00%（4/20）	
Others	2.86%（3/35）	30.00%（6/20）	

HHART: highly effective antiretroviral therapy; NRTIs: nucleotide reverse transcriptase inhibitors; NNRTIs: non-nucleotide reverse transcriptase inhibitors; INSTIs: integrase inhibitors

Table2 qRT-PCR miRNAs primers

Gene name	Bidirectional primer sequence	Product length (bp)
hsa-miR-423-5p	F:5’ TGAGGGGCAGAGAGCGAGA3’ R:5’ GTGCGTGTCGTGGAGTCGTT3’	77
hsa-miR-191-5p	F:5’ CAACGGAATCCCAAAAGCAG3’ R:5’ GTGCGTGTCGTGGAGTCGTT3’	77
hsa-miR-93-5p	F:5’ AAAGTGCTGTTCGTGCAGGTAG3’ R:5’ GTGCGTGTCGTGGAGTCGTT3’	77
ebv-miR-BART2-5p	F:5’ TATTTTCTGCATTCGCCCTTG3’ R:5’ GTGCGTGTCGTGGAGTCGTT3’	76
ebv-miR-BART8-3p	F:5’ GGTCACAATCTATGGGGTCGTA3’ R:5’ GTGCGTGTCGTGGAGTCGTT3’	78
ebv-miR-BART9-5p	F:5’ TACTGGACCCTGAATTGGAAAC3’ R:5’ GTGCGTGTCGTGGAGTCGTT3’	76
ebv-miR-BART15	F:5’ GGTCAGTGGTTTTGTTTCCTTG3’	77

	R:5’ GTGCGTGTCGTGGAGTCGTT3’	
ebv-miR-BART19-5p	F:5’ CATTCCCCGCAAACATGACAT3’ R:5’ GTGCGTGTCGTGGAGTCGTT3’	76

Figure 1 Differential expression of EBV miRNAs between ARL and control group

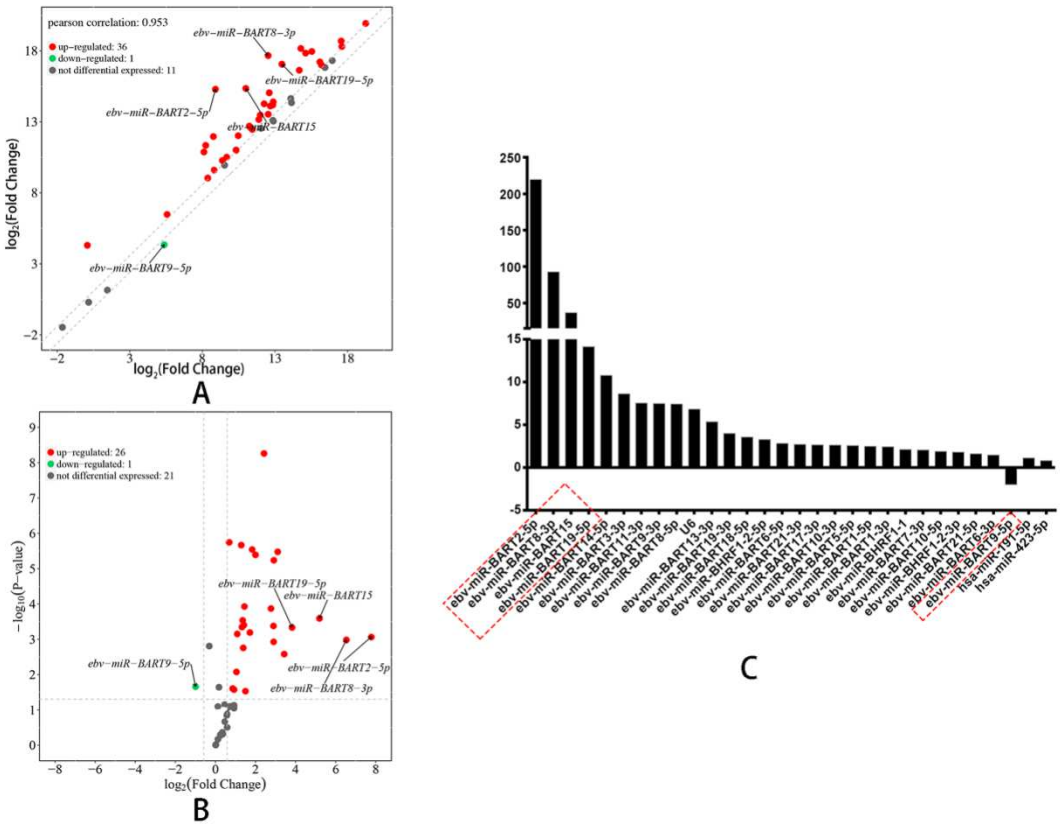
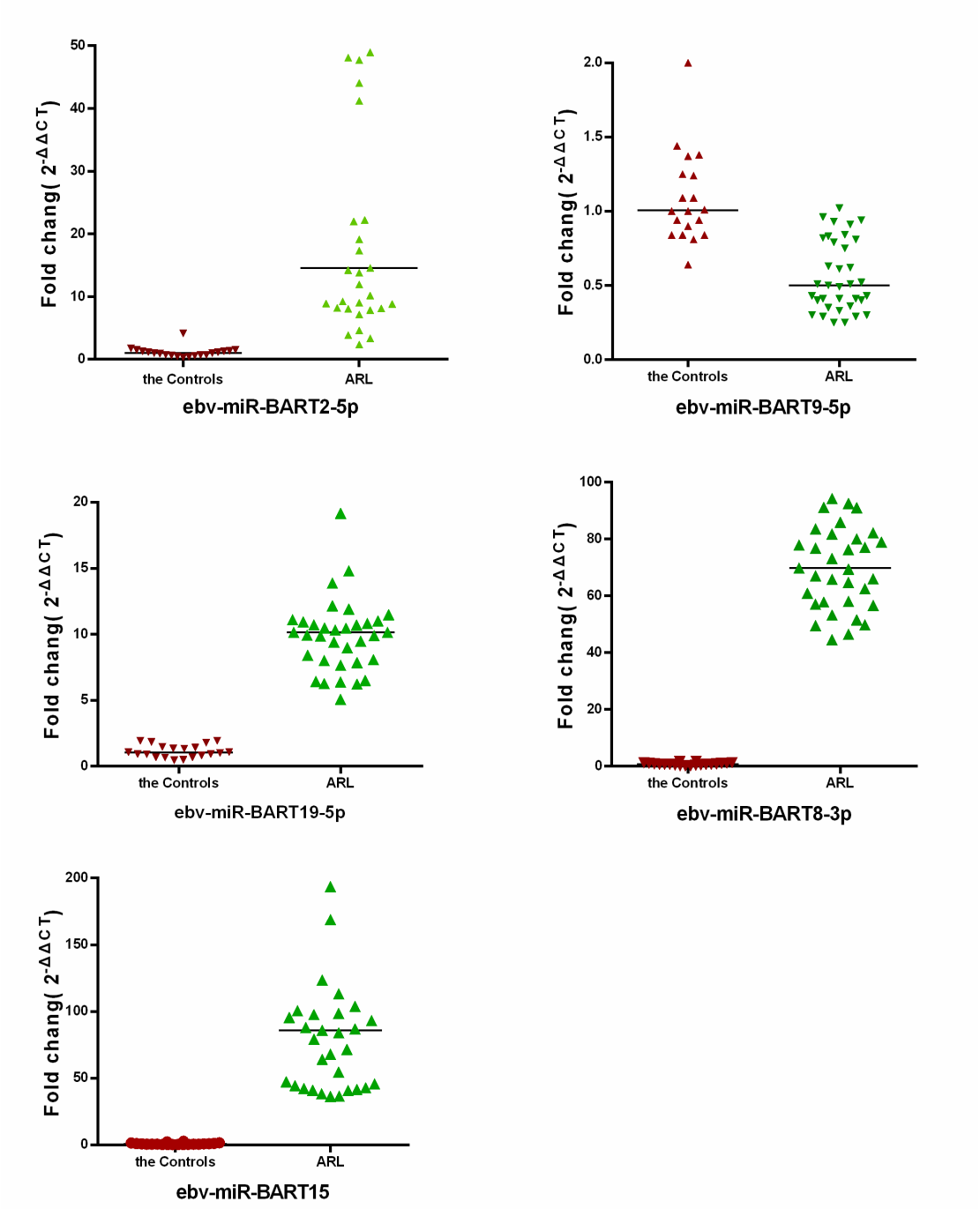


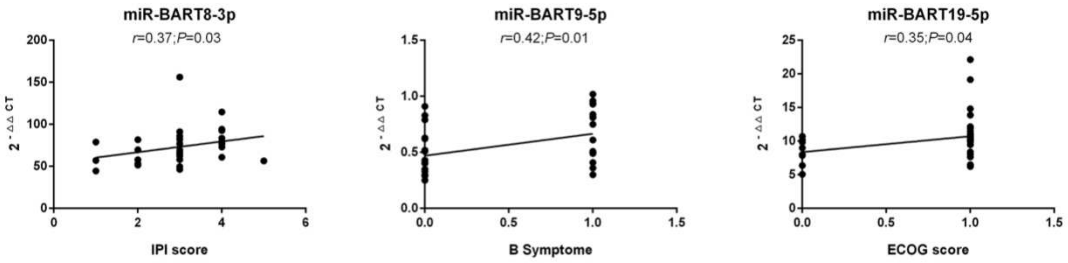
Figure A is a scatter plot; Figure B is a volcano plot; in Figure C, the P values of EBV miRNAs differentially expressed between the two groups were <0.05, U6, hsa-miR-191-5p, hsa-miR-93-5p, hsa -miR-423-5p as an internal reference.

Figure 2 QRT-PCR verification of differential expression of ARL and control EBV miRNAs



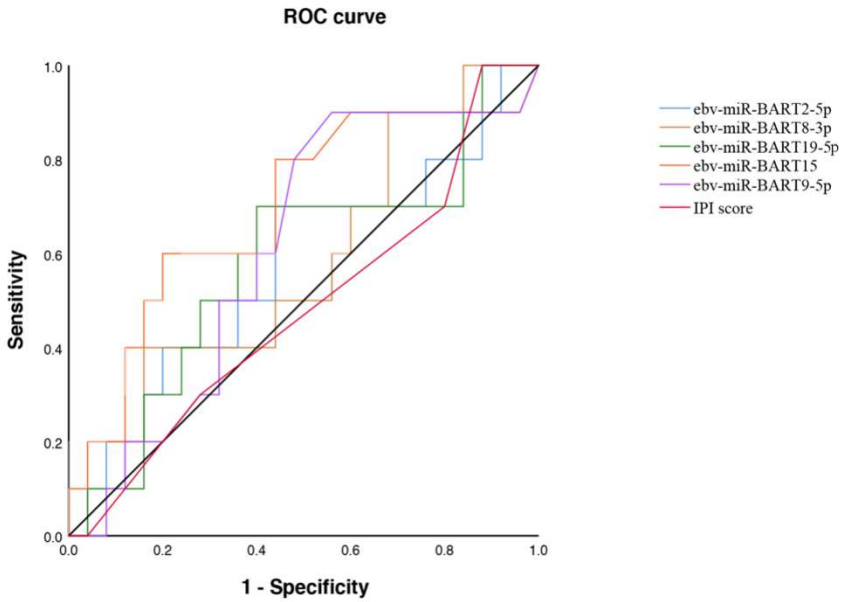
SEM indicates EBV miRNAs differential expression 2-△△ CT standard error

Figure 3 Correlation between EBV miRNAs expression and IPI score, B symptoms, ECOG score



R represents Spearman rank correlation coefficient. IPI score: The abscissas 1, 2, 3, and 4 indicate the IPI score of 0-1 (low risk), 2 (medium and low risk), 3 (high and medium risk), 4-5 (high risk); B symptoms : The abscissa 0 and 1 indicate no B symptom and B symptom respectively; ECOG score: the abscissa 0 and 1 indicate 0-1 and ≧ 2 points respectively.

Figure 4 EBV miRNAs expression and IPI score ROC curve



Figures

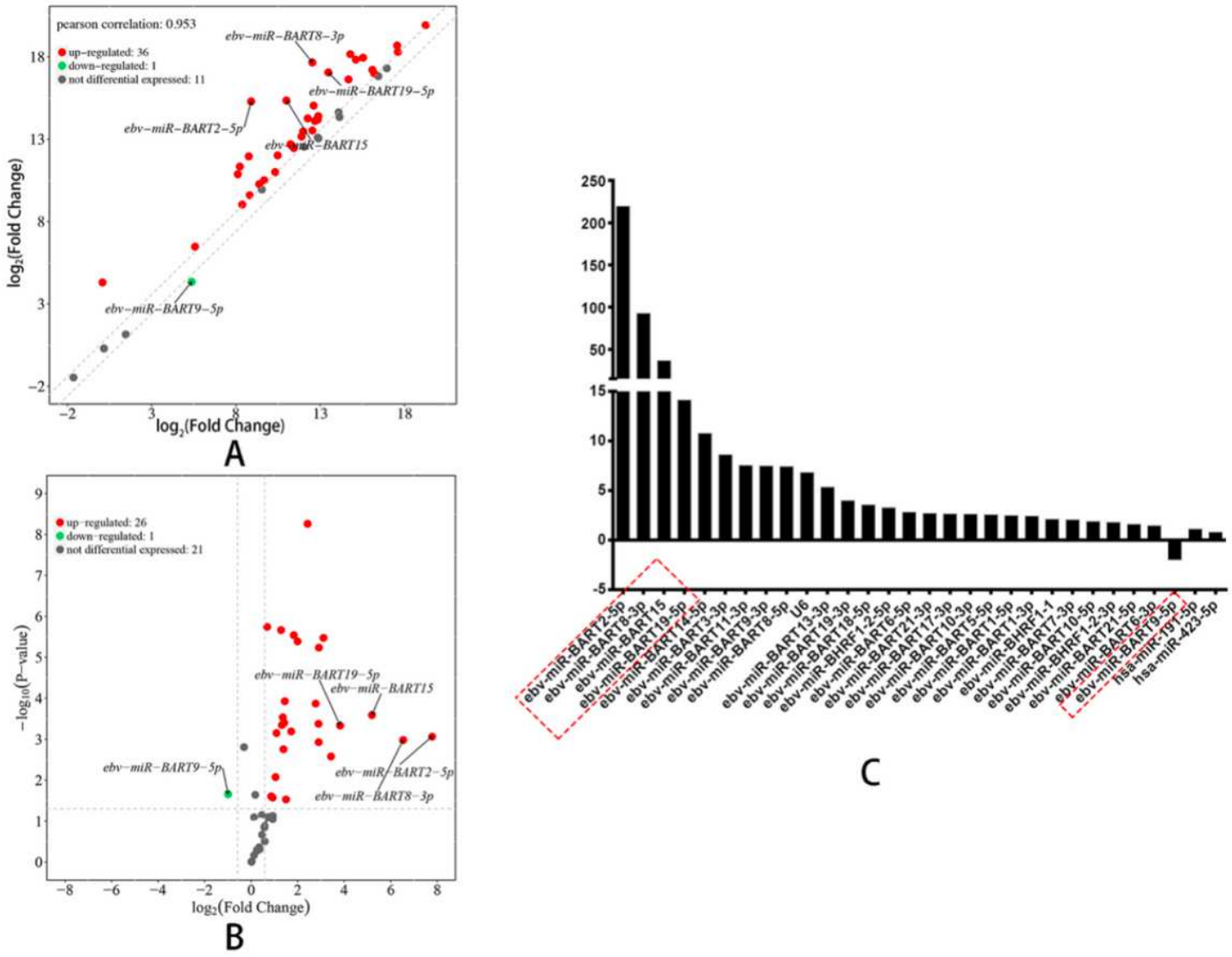


Figure 1

Differential expression of EBV miRNAs between ARL and control group. Figure A is a scatter plot; Figure B is a volcano plot; in Figure C, the P values of EBV miRNAs differentially expressed between the two groups were <0.05, U6, hsa-miR-191-5p, hsa-miR-93-5p, hsa -miR-423-5p as an internal reference.

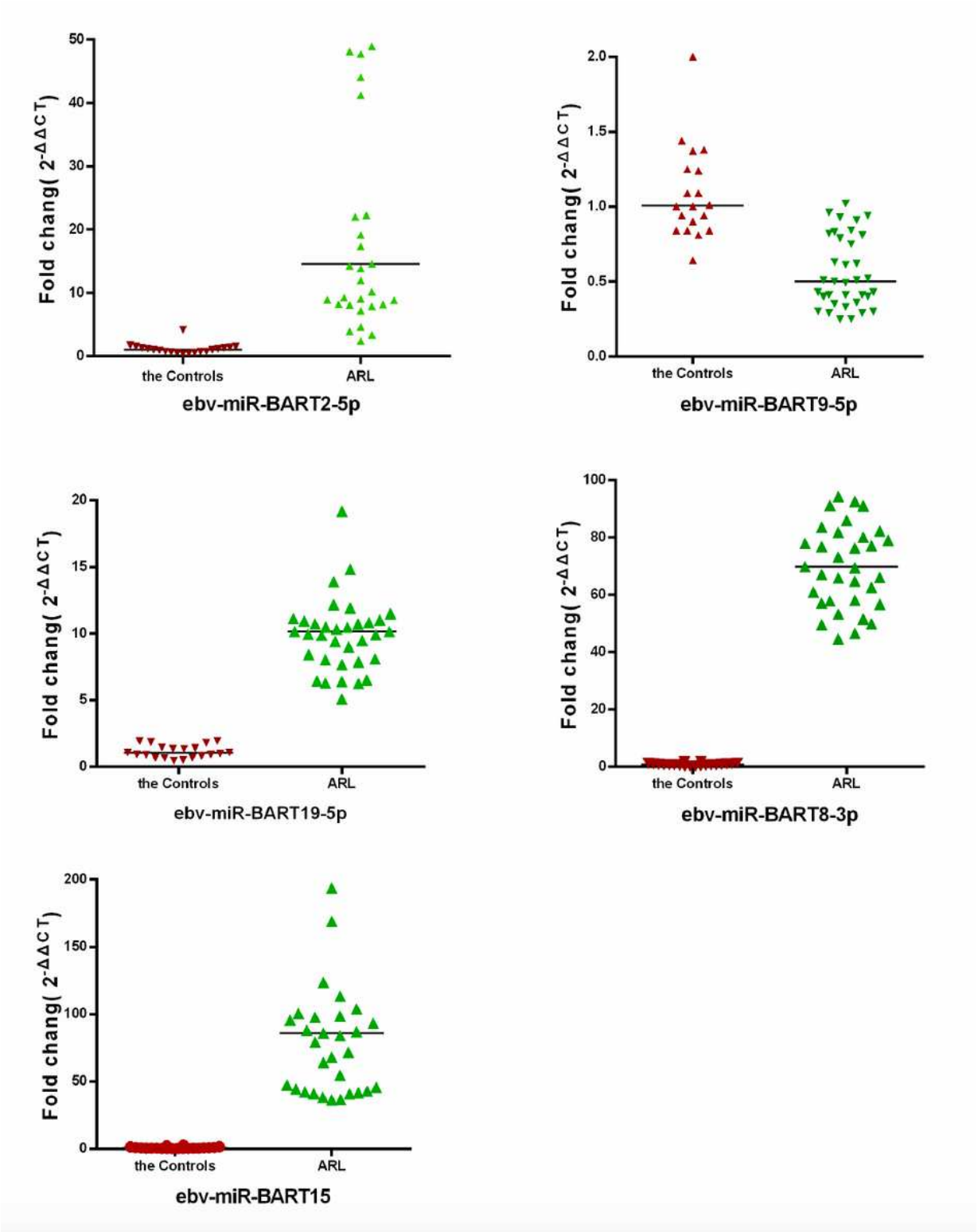


Figure 2

QRT-PCR verification of differential expression of ARL and control EBV miRNAs. SEM indicates EBV miRNAs differential expression 2-□□ CT standard error

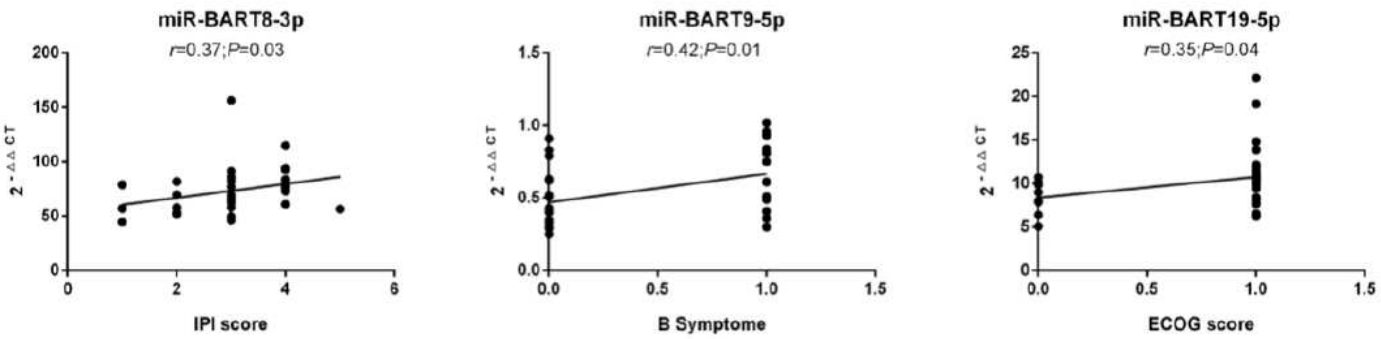


Figure 3

Correlation between EBV miRNAs expression and IPI score, B symptoms, ECOG score. R represents Spearman rank correlation coefficient. IPI score: The abscissas 1, 2, 3, and 4 indicate the IPI score of 0-1 (low risk), 2 (medium and low risk), 3 (high and medium risk), 4-5 (high risk); B symptoms : The abscissa 0 and 1 indicate no B symptom and B symptom respectively; ECOG score: the abscissa 0 and 1 indicate 0-1 and ≥ 2 points respectively.

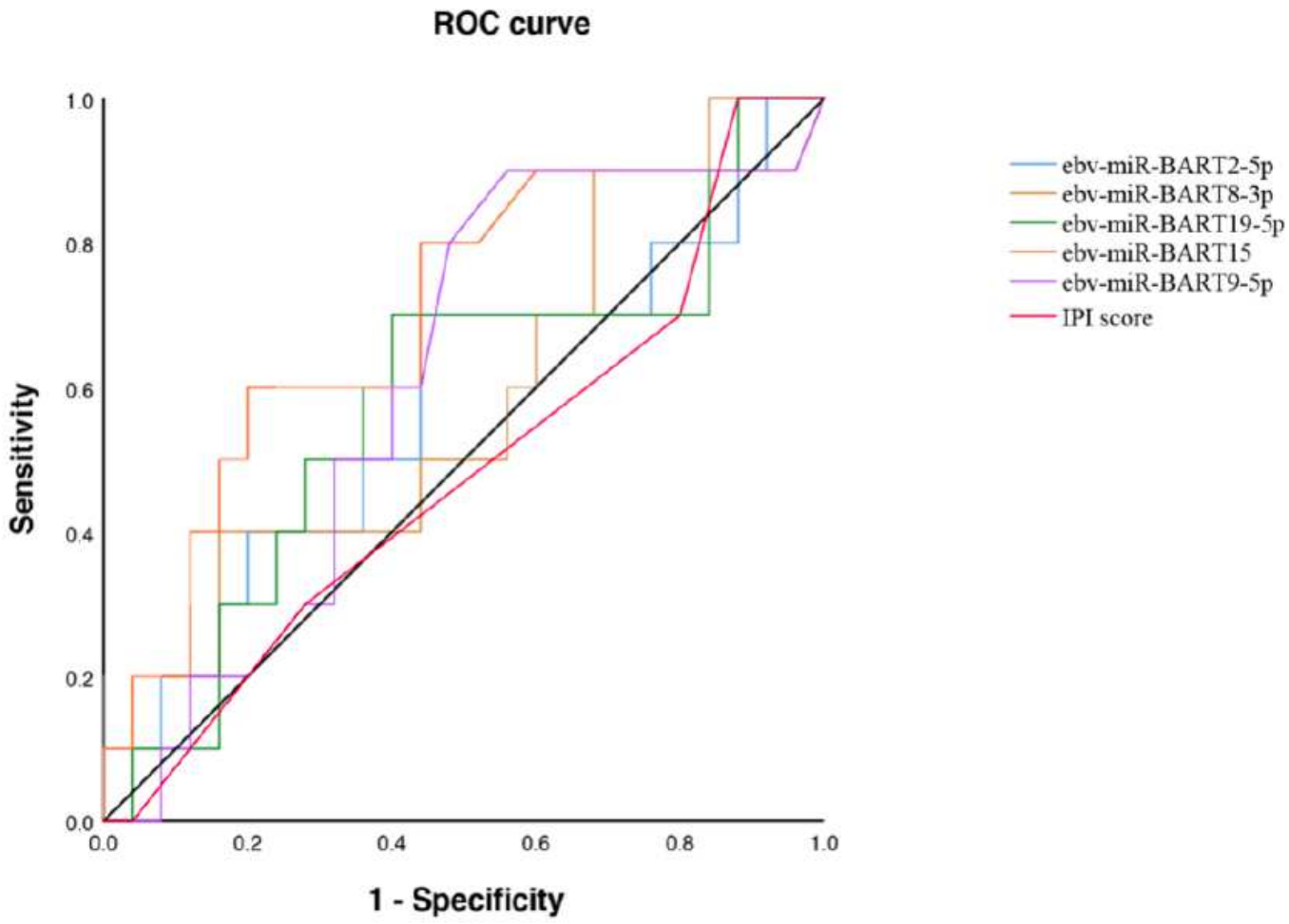


Figure 4

EBV miRNAs expression and IPI score ROC curve