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ORIGINAL RESEARCH

Tumor-educated platelet miR-34c-3p and miR-18a-5p as potential liquid biopsy biomarkers for nasopharyngeal carcinoma diagnosis

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Background: Nasopharyngeal carcinoma (NPC) is the common malignant tumor of nasopharynx in southern China and other southeastern Asian countries. MicroRNAs (miRNAs) have been shown to play important roles in carcinogenesis. Recently, miR-34c-3p and miR-18a-5p have been found to be involved in carcinogenesis of NPC. Furthermore, platelets in NPC patients may acquire RNAs from NPC cells and turn into "tumor-educated platelet (TEP)", which may serve as potential biomarkers for a diagnosis of NPC. However, the expression profiles of TEP miR-34c-3p and miR-18a-5p in NPC patients and their diagnostic values are yet to be determined.

Aims: To investigate expression levels of TEP miR-34c-3p and miR-18a-5p and determine their diagnostic values for NPC.

Materials and methods: Relative quantitative real-time PCR was used to determine the expression levels of TEP miR-34c-3p and miR-18a-5p in NPC patients (n=54) as compared to normal subjects (n=36). The receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic values of TEP miR-34c-3p and miR-18a-5p for NPC.

Results: The expression levels of TEP miR-34c-3p and miR-18a-5p were significantly higher in NPC patients as compared to healthy subjects. The ROC analysis showed that the area under the ROC curve (AUC), sensitivity, specificity and accuracy for TEP miR-34c-3p, miR-18a-5p, or a combination of both miRNAs for NPC diagnostic tests were 0.952, 94.44%, 86.11%, 91.11%, or 0.884, 85.19%, 86.11%, 85.55%, or 0.954, 92.59%, 86.11%, 90.00%, respectively. No correlation was found between expression levels of TEP miR-34c-3p or miR-18a-5p and patients' demographic variables and their NPC tumor/node/metastasis stages. The positive rates of TEP miR-34c-3p and miR-18a-5p for NPC diagnosis were 93.8% and 87.5%, respectively, which were significantly higher than Epstein-Barr virus DNA with a positive rate of 66.7%.

Conclusion: The expression levels of TEP miR-34c-3p and miR-18a-5p are upregulated in NPC, rendering a significant clinical value for NPC diagnosis. The TEP miRNAs might serve as a novel type of liquid biopsies for NPC diagnosis.

Keywords: tumor-educated platelet, miRNA, liquid biopsy, diagnostic value, nasopharyngeal carcinoma

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Introduction

Nasopharyngeal carcinoma (NPC) is the common malignant tumor of nasopharynx with an annual incidence from 15 to 50 cases per 100,000 in southern China and other southeastern Asian countries.[1] Many factors contribute to the tumorigenesis

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of NPC, including viral infection, environmental influences and genetic susceptibility.[2] Early diagnosis of NPC is a clinical challenge since patients are usually diagnosed at a late stage due to ambiguous early symptoms.[3] The peripheral blood levels of Epstein-Barr virus (EBV) DNA and antibodies against EBV are among the current laboratory tests for diagnosis of NPC, but the sensitivity and specificity of these methods are not satisfied for many patients.[4] Therefore, there is an urgent need to define novel biomarkers which can determine the pathological progression of NPC for an early diagnosis.

Platelets are abundant cell fragments in circulation which execute multifunctional actions including forming thrombi, monitoring pathogens in peripheral blood, interacting with immune cells, etc. More importantly, they can be "educated" by cancer cells. They can modulate the splicing of their pre-mRNAs in response to signals from cancer cells, resulting in changes in their transcriptome and molecular profiles which reflect pathological progressions.[5] Therefore, platelets can acquire tumor-derived RNAs after interactions with tumor cells. These tumor-educated platelet (TEP) RNAs have been emerging as potential novel serum biomarkers for disease diagnosis, prognosis and prediction.[6] There are many advantages of TEP RNAs as biomarkers for cancer diagnosis: 1) platelets contain no nuclei thus interference from genomic DNA is very minimal; 2) platelets are easy to be collected, isolated and analyzed, which are ready to be standardized in general clinical laboratories; and 3) TEP RNA profiles reflect the pathological progression of cancer cells, which potentially render a better sensitivity for an early detection of cancer cells.

MicroRNAs (miRNAs) are small noncoding RNA molecules containing about 18–24 nucleotides. The function of miRNAs includes development, cell differentiation, and regulation of cell cycle and apoptosis.[7] miRNAs are mainly located within cells while they can also be found in circulatory system and extracellular environment. The circulating miRNAs can serve as liquid biopsies and have become a growing research hotspot for their potential diagnostic values for an early detection of tumors.[8] Particularly, Wan found that miR-34c (including miR-34c-3p, miR-34c-5p) and miR-18a (including miR-18a-3p, miR-18a-5p) were dysregulated in NPC using microarrays.[9] The miRNA-34 family contributes to the anti-proliferation and pro-apoptosis of cancer cells functioning as a p53 effector.[10,11] The miR-18a-5p belongs to the miRNA 17–92 family, locating at 13q31.3.[12] Both miR-34c-3p and miR-18a-5p have been found to play roles in tumorigenesis and metastasis. However, their roles in development, progression and diagnosis of NPC remain to be determined. In this study, we attempted to determine the expression levels of TEP miR-34c-3p and miR-18a-5p in NPC as compared with normal subjects as well as evaluate their diagnostic values for NPC. We found that the expression levels of both TEP miRNAs are significantly upregulated in NPC and they provide a good specificity and sensitivity for NPC diagnosis.

Methods and materials

Sample collection

The study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (No: IORG0003571). The clinically left-over, de-identified whole blood samples (2 mL) were collected from NPC patients (n=54) and healthy subjects (n=36) from March to April 2018 at the Cancer Center of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was obtained by the patients. The inclusion criteria of NPC subjects were as follows: 18–85 years old, newly diagnosed with a NPC, no radiotherapy or chemotherapy being received, and no other primary tumors. The samples were subsequently subject to platelet isolation within 3 hrs after blood collection. The healthy subjects were independent to the NPC group with matching age ranges.

Platelet isolation

The whole blood (in EDTAK2) (2 mL) was centrifuged at 120 g for 20 mins at room temperature. The platelet-enriched top-layer (~600 μL) was collected and subjected to spinning at 360 g for 20 mins to precipitate the platelets. The platelets were washed twice by phosphate buffer saline. The number of platelets was counted by hemocytometer after Crystal Violet Staining. A high purity platelet preparation was determined by a ratio of <5 karyocytes per 10^7 platelets.[13] The quality and quantity of PLT were controlled and counted to be the same for each sample in the subsequent experiments.

RNA extraction and relative real-time quantitative PCR (qPCR) analysis

The total RNA was extracted from isolated platelets or blood plasma using a Liquid Total RNA Isolation Kit (RP4002,

BioTeke, Beijing, China). The quantity and quality of RNAs were determined by Nanodrop 2000 spectrophotometer and analyzed by an agarose gel electrophoresis. In total, 2 μg of RNAs were used for cDNA synthesis using a PrimeScript™RT reagent Kit with gDNA Eraser (RR047A, TAKARA, Dalian, China). The qPCR was performed using iQ™ SYBR® Green Supermix (BIO-RAD, CA, USA) on a BIO-RAD CFX96 system. The U6 was used as the internal amplification control. The primers used for amplification of miR-34c-3p, miR-18a-5p and U6 are listed in Table 1. The URP was universal amplification primer, which was used in pairs with hsa-miR-34c-3p-S and hsa-miR-18a-5p-S. Relative quantification of RT-PCR was based upon the amplification efficiency of the target and reference genes, and the cycle number at which fluorescence crossed a prescribed background level cycle threshold (Ct) as we described previously.[14] Specifically, the relative expression levels of miRNAs were calculated by $2^{-\triangle CT}$ method; while $\triangle CT = CT_{miRNAs} - CT_{U6.}$

EBV DNA levels from lymphocyte and plasma were determined using EB viral nucleic acid quantitative fluorescent probe PCR assay (Shengxiang Biotechnology, Hunan, China) on the Stratagene Mx3000P system (Agilent Technologies, CA, USA) at Department of Laboratory Medicine of Union Hospital.

Data analysis

SPSS19.0 software was used for data analysis. The Mann–Whitney U test was used to compare the differential expression levels of miR-34c-3p and miR-18a-5p among NPC, normal groups and subgroups with different NPC tumor/node/metastasis (TNM) stages. The logistic regression was used to access the joint-probability of TEP miR-34c-3p and TEP miR-18a-5p in diagnosis of NPC. The independent-samples *t*-test and Fisher test were used to determine the correlations between expression levels of miR-34c-3p or miR-18a-5p and various clinical parameters of subjects. The McNemar and Kappa tests were used to compare the positive rates of miR-34c-3p or miR-18a-5p with EBV DNA for NPC diagnosis. A two-sided test was performed in all statistical analyses. Statistically, significant difference was set at $p<0.05$.

Results

Upregulation of miR-34c-3p and miR-18a-5p in NPC platelets

The expression levels of TEP miR-34c-3p and miR-18a-5p were significantly higher in NPC patients as compared to healthy control subjects ($p<0.001$, Figure 1, Figure S1). There was no significant difference in expression levels of both miRNAs in plasma from subjects in both groups ($p=0.352$ and 0.401, respectively, Figure 1).

Diagnostic value of TEP miR-34c-3p and miR-18a-5p in NPC

The area under the receiver operating characteristic curve (AUC) of TEP miR-34c-3p in the NPC group was 0.952 (Figure 2A). The maximum value of the Youden index was 0.8056 and the criterion was >3.4074. The sensitivity, specificity and accuracy of TEP miR-34c-3p for a NPC diagnostic test were 94.44%, 86.11% and 91.11%, respectively (Table 4). The AUC of TEP miR-18a-5p in NPC was 0.884 (Figure 2B). The maximum value of the Youden index was 0.7130 and the criterion was >0.0602. The sensitivity, specificity and accuracy of TEP miR-18a-5p for a NPC diagnostic test were 85.19%, 86.11% and 85.55%, respectively (Table 4). The AUC of the joint-probability of TEP miR-34c-3p and TEP miR-18a-5p in NPC was 0.954 (Figure 2C). When the joint-probability >0.2783, the corresponding values of TEP miR-34c-3p and TEP miR-18a-5p were >3.4074 and >0.2020, respectively. The sensitivity, specificity and accuracy of the joint-probability of TEP miR-34c-3p and TEP miR-18a-5p for a NPC diagnostic test were 92.59%, 86.11% and 90.00%, respectively (Table 4).

Table 1 Primers used for cDNA synthesis and real-time PCR

Primers	Sequence (5′~3′)
hsa-miR-34c-3p-S	ACACTCCAGCTGGGAATCACTAACCACACG
hsa-miR-34c-3p-RT[a]	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCTGGCCG
hsa-miR-18a-5p-S	ACACTCCAGCTGGGTAAGGTGCATCTAGTGC
hsa-miR-18a-5p-RT[a]	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTATCTGC
URP	TGGTGTCGTGGAGTCG
U6-F	CTCGCTTCGGCAGCACA
U6-RT*	AACGCTTCACGAATTTGCGT

Note: [a]cDNA synthesis primers.

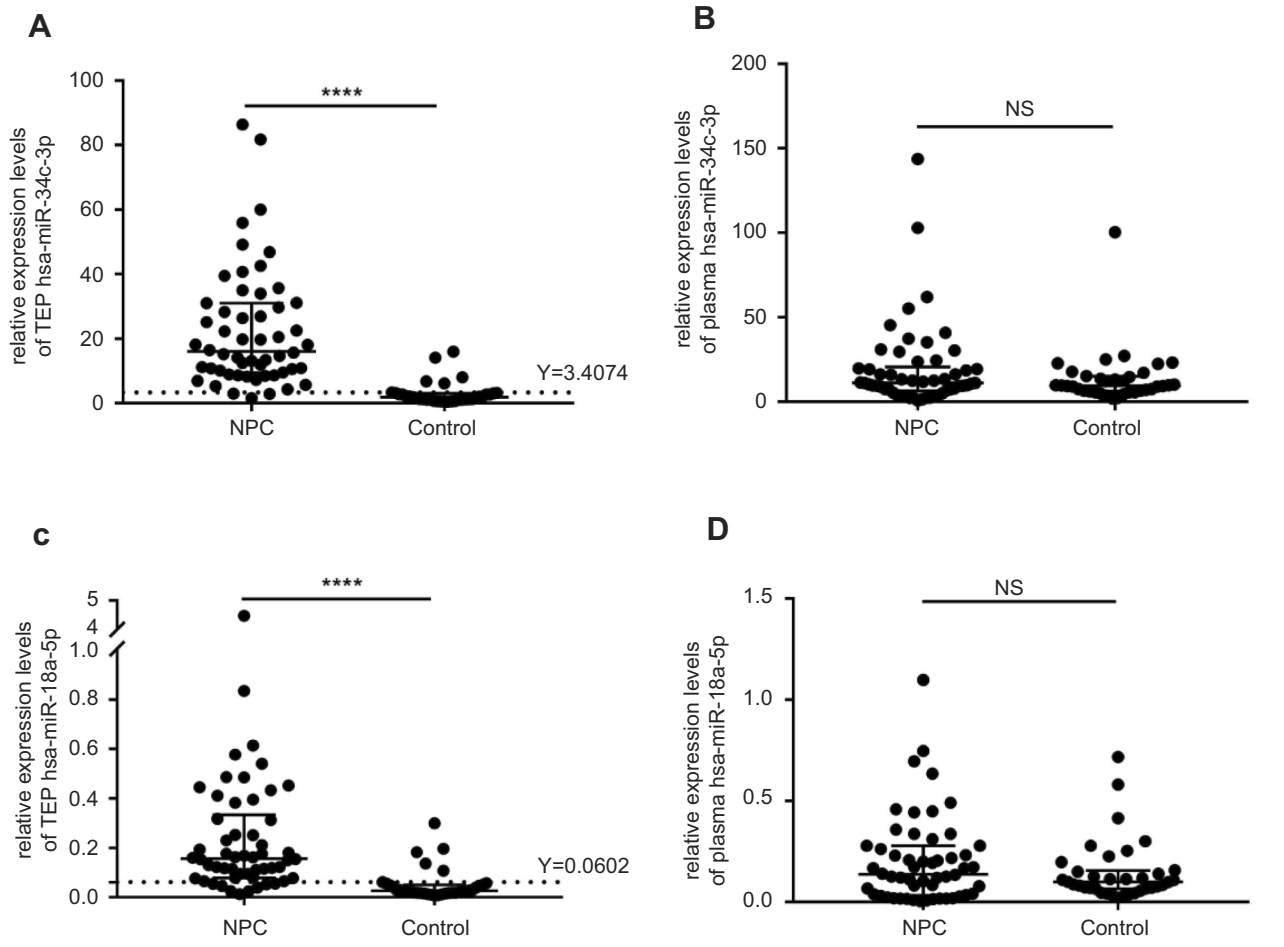


Figure 1 The relative expression levels of hsa-miR-34c-3p and hsa-miR-18a-5p in TEP and plasma from NPC patients and control subjects. (**A**) The TEP miR-34c-3p levels in NPC subjects were significantly higher than control subjects. (**B**) There was no significant difference of plasma miR-34c-3p levels between NPC and control groups. (**C**) The TEP miR-18a-5p levels in NPC subjects were significantly higher than control subjects. (**D**) There was no significant difference of plasma miR-18a-5p levels between NPC and control groups. Mann–Whitney U test was used for analysis. ****$p<0.0001$.
Abbreviations: NS, no significance; Y, cutoff point.

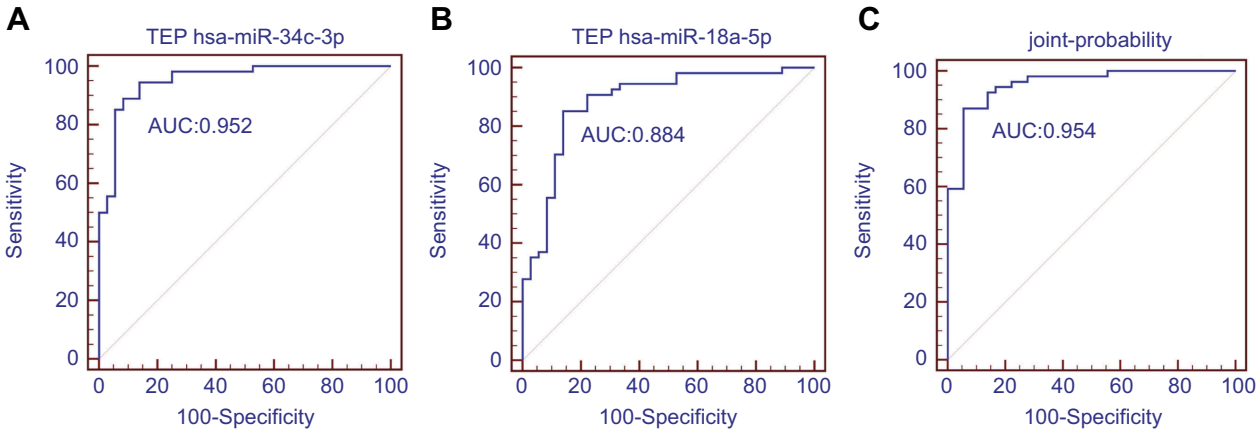


Figure 2 ROC curves of hsa-miR-34c-3p and hsa-miR-18a-5p in TEP and the joint-probability for NPC. (**A**) The AUC of TEP miR-34c-3p in NPC was 0.952. (**B**) The AUC of TEP miR-18a-5p in NPC was 0.884. (**C**) The AUC of the joint-probability of TEP miR-34c-3p and TEP miR-18a-5p in NPC was 0.954.
Abbreviations: ROC, receiver operating characteristic; AUC, area under ROC curve.

Correlations between the expression levels of TEP miR-34c-3p and miR-18a-5p and clinical parameters of NPC subjects

We next attempted to investigate whether expression levels of TEP miR-34c-3p and miR-18a-5p were associated with patients' demographic variables and their NPC TNM stages. We divided the NPC patients into a high-level group and a low-level group according to the cutoff point in the Youden index analysis, which corresponded to the values of relative expression levels of 3.4074 and 0.0602 for TEP miR-34c-3p and miR-18a-5p as compared to U6, respectively. The subjects in the NPC group with a high level of TEP miR-34c-3p were significantly older than those in the NPC group with a low level of TEP miR-34c-3p (p=0.0492, Table 2). However, the subjects in both NPC groups with a high or low level of TEP miR-18a-5p showed no age difference (p=0.4863, Table 2). There were no significant differences in expression levels of both miRNAs and fasting blood glucose levels in NPC patients (p=0.4178 and 0.5234, respectively, Table 2) or NPC differentiation status (p=0.614and 0.386, respectively, Table 2). The expression levels of TEP miR-34c-3p or miR-18a-5p showed no significant correlation to patients' gender or NPC TNM stages (p>0.05, Table 2). The NPC diagnostic positive rates of TEP miR-34c-3p and miR-18a-5p were 93.8% and 87.5%, respectively, which were significantly higher as compared to EBV DNA (p=0.004 and 0.041, respectively, Table 2). The expression levels of TEP miR-34c-3p and miR-18a-5p showed no significant differences among different TNM stages of NPC (p>0.05, Figure 3 and Table 3).

Discussion

NPC is a multifactorial disease which takes decades to develop. A majority of patients are misdiagnosed as cervical lymph node metastasis or long distant metastasis during their first clinical visits.[15,16] Therefore, it has been a major clinical barrier to determine essential biomarkers for early diagnosis of NPC. In this study, we found that the expression levels of miR-34c-3p and miR-18a-5p in TEP, but not plasma, were significantly higher in NPC patients as compared to normal subjects. Both biomarkers have shown significant clinical values by showing a high sensitivity, specificity and accuracy for NPC diagnosis. Therefore, our study has provided a molecular basis for a prospective clinical application using these miRNAs for NPC diagnosis.

There has been a growing body of evidence to reveal the roles of TEP miRNAs in various pathophysiological processes as well as cancer pathogenesis.[13] TEP miRNA signatures along with biomolecular readouts from other liquid biopsies, such as plasma RNA, cell-free DNA and circulating tumor cells, have been drawing great attention for detecting cancer-specific aberrances in genomes and transcriptomes.[8] TEP miRNAs have provided many unprecedented characteristics for detecting the status of a disease. Firstly, platelets can react to tumor growth and treatment to become TEP via various biological processes, such as regulation of RNA splicing and tumor RNA sequestration.[5] Therefore, the TEP miRNA signatures can reflect molecular profiles of tumorigenesis and cancer metastasis. Secondly, TEP miRNA signatures can pinpoint a specific tumor type with an accuracy of 71%,[17] indicating tumor type-specific TEP miRNA processing. Thirdly, TEP miRNAs respond to external stimuli rapidly, providing dynamic miRNA signatures for the progression of a cancer.[5] Finally, platelet isolation from blood samples can be easily predefined and standardized for clinical applications.

Many miRNAs have been reported to be associated with the development and progression of NPC, including miR-216b,[18] miR-218,[15] miR-26a/b,[19,20] miR-10b,[21] let-7,[22] miR-141[23] and miR-200a.[24] The functional abnormality of p53 has been involved in NPC development. Many miRNA and long non-coding RNAs have participated in modulations of p53 signaling pathways,[25] thus regulating NPC development and progression. The miR-34c-3p is one of the P53 effectors.[11] Not surprisingly, miRNAs derived from EBV can be oncogenic for NPC as well.[9,26] The functions and diagnostic values of miR-18a-5p and miR-34c-3p in NPC are yet to be determined although there are many reports showing their functions in other types of tumors and diseases. For example, upregulation of miR-18a-5p has been reported in triple-negative breast carcinoma (TNBC).[16] Inhibition of miR-18a-5p by RNA FENDRR can decrease the invasion and migration ability of prostate cancer.[27] Consistent with our data, these evidences together suggest that the upregulation of miR-18a-5p is positively associated with the cancer oncogenic process. Meanwhile, miR-34c-3p appears to have a tumor suppressive function in some cancer types. For example, miR-34c-3p can inhibit proliferation and invasion of non-small cell lung cancer by blockage of eIF4E expression and the PAC1-MAPK pathway.[28,29] Ectopic expression of miR-34c-3p inhibits migration, invasion and epithelial–mesenchymal transition in TNBC cells.[30] On the contrary, Xiao et al have reported that miR-34c-3p promotes cell proliferation and invasion in hepatocellular carcinoma[31] which aligns with our current result showing an upregulation of TEP miR-34c-3p in NPC. Therefore, the functional outputs of miR-34c-3p in

Table 2 Correlations between expression levels of target miRNAs and clinical parameters

Group		N	TEP hsa-miR-34c-3p			*P*-value	N	TEP hsa-miR-18a-5p			*P*-value
			Low	High	High%			Low	High	High%	
Age, mean±SD		54	60.67±5.33, n=3	48.39±1.44, n=51	-	0.0492[b]	54	51.5±4.70, n=8	48.65±1.49, n=46	-	0.4863[b]
Glucose, mean±SD		54	4.95±0.07, n=3	5.36±0.12, n=51	-	0.4178[b]	54	5.163±0.27, n=8	5.37±0.13, n=46	-	0.5234[b]
Gender	Male	35	3	32	91.4	0.5446	35	6	29	82.9	0.698
	Female	19	0	19	100		19	2	17	89.5	
Tumor type	Undifferentiated	50	3	47	94	0.614	50	8	42	84	0.386
	Differentiated	4	0	4	100		4	0	4	100	
T	T1	2	0	2	100	0.524	2	0	2	100	0.131
	T2	11	0	11	100		11	0	11	100	
	T3	15	1	14	93.3		15	4	11	73.3	
	T4	17	0	17	100		17	2	15	88.2	
N	N0~N1	12	1	11	91.7	0.271	12	1	11	91.7	0.894
	N2	22	0	22	100		22	3	19	86.4	
	N3	9	0	9	100		9	1	8	88.9	
M	M0	43	1	42	97.7	1.00	43	4	39	90.7	0.298
	M1	3	0	3	100		3	1	2	66.70	
EBV DNA	Negative	16	0	16	100	0.004[a]	16	1	15	93.8	0.041[a]
	Positive	32	3	29	90.6		32	5	27	84.4	
	Positive rate of TEP miR-34c-3p: 93.8% Positive rate of EBV DNA: 66.7%						Positive rate of TEP miR-18a-5p:87.5% Positive rate of EBV DNA: 66.7%				

Notes: [a]McNemar and Kappa tests, [b]independent-samples *t*-test, all others are Fisher test.

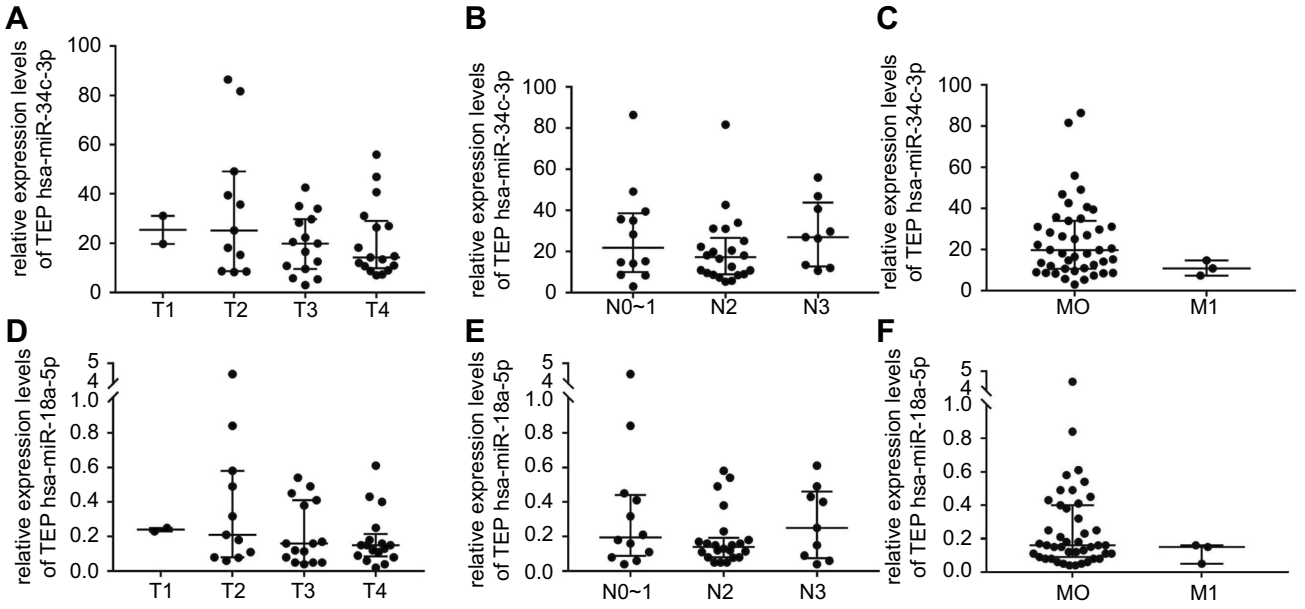


Figure 3 There was no significant change in relative expression levels of TEP hsa-miR-34c-3p or hsa-miR-18a-5p among different TNM stages of NPC. (**A**) Relative expression levels of TEP hsa-miR-34c-3p in T1-T4 stages; (**B**) Relative expression levels of TEP hsa-miR-34c-3p in N0~1, N2, and N3 stages; (**C**) Relative expression levels of TEP hsa-miR-34c-3p in M0 and M1 stages; (**D**) Relative expression levels of TEP hsa-miR-18a-5p between T1-T4 stages; (**E**) Relative expression levels of TEP hsa-miR-18a-5p in N0~1-N2, and N3 stages; (**F**) Relative expression levels of TEP hsa-miR-18a-5p in M0 and M1 stages. No significant difference was found among groups by Mann-Whitney U test.

Table 3 The *p*-values of comparisons of relative expression levels of TEP hsa-miR-34c-3p or hsa-miR-18a-5p among subgroups with different TNM stages of NPC

	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4	N0~1-N2	N0~1-N3	N2-N3	M0-M1
hsa-miR-34c-3p	>0.99	0.62	0.35	0.26	0.33	>0.99	0.38	0.75	0.09	0.15
hsa-miR-18a-5p	0.92	0.51	0.23	0.23	0.22	0.77	0.29	0.96	0.41	0.36

Table 4 Statistic data of ROC curves

	miR-34c-3p	miR-18a-5p	Joint-probability
AUC	0.952	0.884	0.954
Youden index	0.8056	0.7130	–
Criterion	3.4074	0.0602	0.2783 (3.4074 and 0.2020)
Sensitivity	94.44%	85.19%	92.59%
Specificity	86.11%	86.11%	86.11%
Accuracy	91.11%	85.55%	90.00%

Abbreviations: AUC, area under ROC curve; ROC, receiver operating characteristic.

tumorigenesis may be of great complexity and diverse and even contradict, depending on the types and progressive stages of cancer cells.

In this study, we have found that TEP miR-34c-3p and miR-18a-5p have significant clinical values for NPC diagnosis due to their high sensitivity, specificity and accuracy in the diagnostic assay. We, however, did not find such altered expression patterns in plasma miR-34c-3p and miR-18a-5p, suggesting that the aberrances of TEP miR-34c-3p and miR-18a-5p are results of an "education" from NPC to platelets. We did not find significant links between TEP miR-34c-3p or miR-18a-5p and patients' demographic variables and their NPC TNM stages. There are many possible reasons accounting for this result. First, the sample size (n=54) we used for this analysis is not big enough. Second, both miR-34c-3p and miR-18a-5p are associated with tumorigenesis but not the progression of NPC. A functional study of both miRNAs in NPC will be required to answer these questions which will be

our next research focus. EBV-DNA detection has become one of the diagnostic tests for NPC in clinics. In this study, we have shown that both TEP miR-34c-3p and miR-18a-5p render significant better positive rates for detection of NPC as compared to EBV-DNA. Our data suggest that TEP miR-34c-3p and miR-18a-5p provide potentially novel diagnostic tools which can be used along with other biomarkers, such as EBV-DNA, to facilitate the diagnosis of NPC in the future.

In our study, we used the U6 as the amplification internal control which may raise a concern of bias in results that are caused by a sole internal control. U6 is one of the most widely used internal standards for monitoring the amplification efficiency during real-time PCR due to its relative stable expression level crossing variety of tissues. More importantly, the Ct values of U6 and our target genes are relatively reasonably close and they showed similar amplification efficiencies crossing samples (Figure S1). Therefore, the potential bias in amplification efficiency by using U6 alone is minimized. Another potential concern is that how the results are not biased by amplification of non-TPE miRNAs. We prepared a high purity platelet with <5 karyocytes per 10^7 platelets for this study, which ensured to minimize the potential RNA contaminations from any types of cells. It will be interesting to know whether both miRNAs exist in serum exosomes, which will be our next research focus. It is unlikely that exosomal miRNAs have been amplified and biased the results since exosomes (50–200 nm) are too small to be prepared by the low-speed centrifugation method that is used for platelet (2–3 μm) isolation. In this study, we focus on TEP miR-34c-3p and miR-18a-5p. However, we are aware of the variations of TEP RNA profiles. The TEP RNA signatures not only reflect the RNA alterations from NPC tissues but also contain their intrinsic RNA variations corresponding to tumor development in patients. Therefore, a full characterization of the entire TEP RNAs by RNA-seq will be crucial to render a novel strategy for cancer surveillance.

Collectively, in this study we have shown that TEP but not plasma miR-34c-3p and miR-18a-5p are upregulated in NPC patients as compared to healthy subjects. The upregulation of TEP miR-34c-3p or miR-18a-5p is not associated with patients' demographic variables and their TNM stages of NPC. Our data suggest that TEP miR-34c-3p and miR-18a-5p can potentially serve as essential biomarkers for a novel diagnostic test for NPC.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

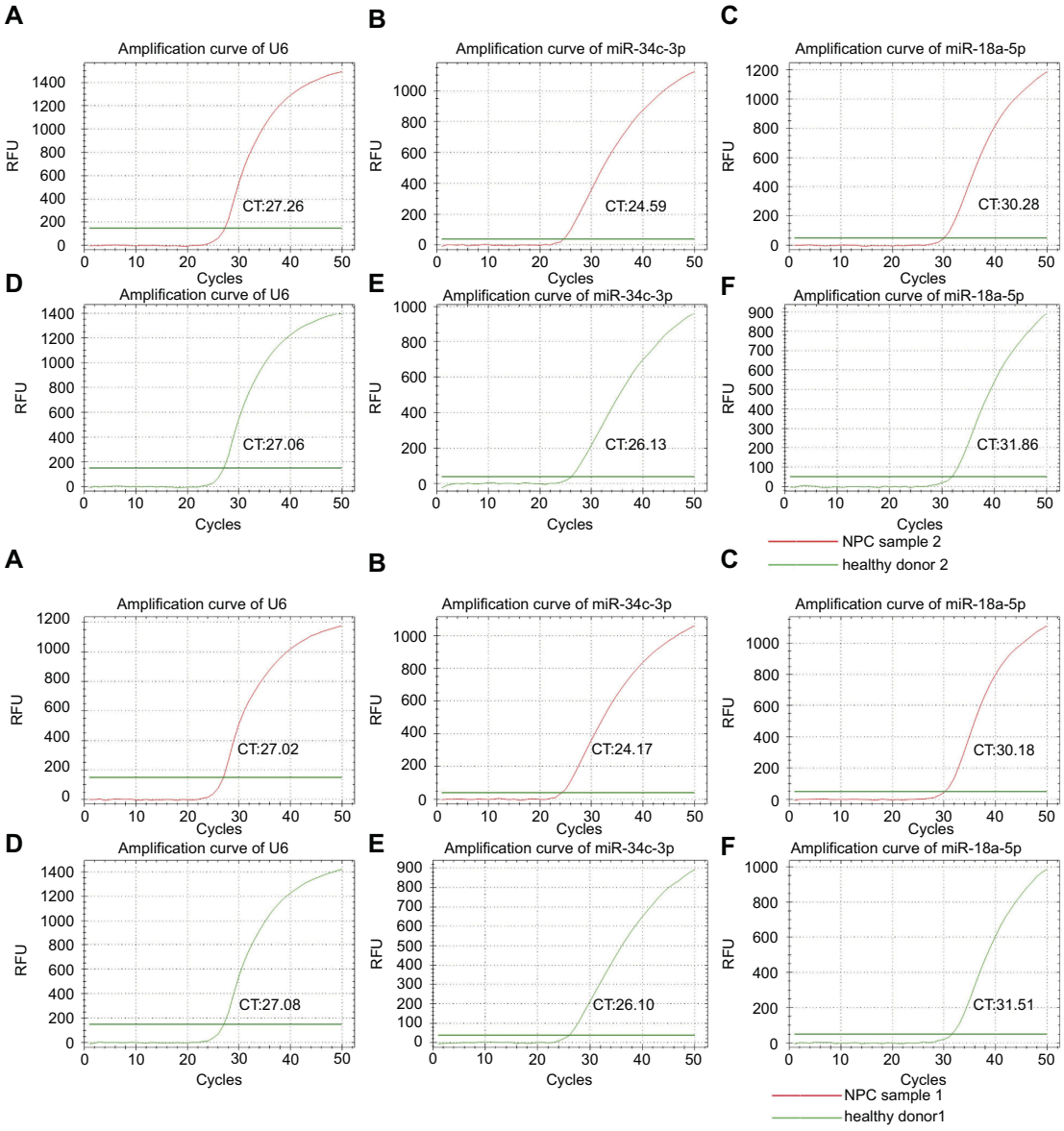


Figure S 1 The representative amplification curves of U6, miR-34c-3p and miR-18a-5p from two NPC patients and two healthy donors. (**A**) The CT values of U6 were 27.26 and 27.02 in two NPC samples, respectively. (**B**) The CT values of miR-34c-3p were 24.59 and 24.17 in two NPC samples, respectively. (**C**) The CT values of miR-18a-5p were 30.28 and 30.18 in two NPC samples, respectively. (**D**) The CT values of U6 were 27.06 and 27.08 in two healthy donors, respectively. (**E**) The CT values of miR-34c-3p were 26.13 and 26.10 in two healthy donors, respectively. (**F**) The CT values of miR-18a-5p were 31.86 and 31.51 in two healthy donors, respectively.

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