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RESEARCH ARTICLE

MicroRNA Gene Signature for Predicting Mechanisms in Nasopharyngeal Carcinoma: A Case Study on the Potential Application of Circulating Biomarkers

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Abstract: ***Background and Aim*:** Nasopharyngeal Carcinoma (NPC) is an upper respiratory tract cancer prevalent in Southeast Asia and related to chronic EBV infection. microRNAs (miRNAs) regulate gene expression implicated in NPC's carcinogenesis. However, this circulating RNA molecule's role and clinical utility remain unknown. Therefore, this study examined the circulation of miRNAs and their association with clinical data.

***Methods*:** 160 plasma samples of NPC and 80 non-tumor samples were extracted to evaluate and validate the gene expressions. Quantification expression was performed using relative quantification of qPCR analysis level expression methods. The intrinsic cellular roles involving biological signaling in NPC's oncogenesis using Ingenuity Pathways Analysis (IPA) were also used.

***Results*:** The results of the quantification significance profiling of NPC samples revealed decreased miR-29c-3p (fold change 1.16; $p<0.05$) and increased 195-5p expression (fold change 1.157; $p<0.05$). Furthermore, the validation of hsa-miR-29c-3p expression on plasma NPC with known tumor vs. non-tumor and significant changes was also performed using a fold change of 4.45 (medians of 31.45 ± 1.868 and 24.96 ± 1.872, respectively; $p<0.0005$). miR-29c had a 2.14 fold change correlated with T primary status with a median of 31.99±1.319 and 31.35±2.412, respectively ($p<0.05$). Stage status with fold change 1.99 also had median levels of 31.98±1.105 and 31.21 ± 2.355, respectively (p-value <0.05). Furthermore, the node's status for the lower expression of miR-29c with fold change 1.17 had median levels of 32.78 ± 2.221 and 31.33 ± 1.689, respectively (p-value of 0.7). Bioinformatics analysis established the roles and functions of miR-29 in NPC progression, cell death and survival, cellular development, cellular function, and cell maintenance by inhibiting COL4A, PI3K, VEGFA, JUN, and CDK6.

***Conclusion*:** Overall, we conclude that decreased miR-29c expression is associated with poor clinical status and might inhibit NPC's five target genes.

Keywords: MicroRNA, clinical outcome, profiling, nasopharyngeal, cancer, circulating.

ARTICLE HISTORY

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1. INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial disease that is ethnically and geographically prevalent in East and South-East Asia [1]. Multiple factors associated with the pathogenesis of NPC have been implicated, including latent Epstein-Barr virus (EBV) infection, smoking habit, and dietary intake of a salted fish [2, 3]. In Indonesia, incidence and mortality rate are continuously increasing every year. It was found that the newly diagnosed NPCs per year almost reached 14.000 and primarily presented in the advanced stage [4]. Furthermore, the low recovery rate, poor prognosis, difficulty in diagnosing early, nonspecific symptoms, and small primary tumor size have become significant NPC issues.

MicroRNAs (miRNAs/miRNA) are small untranslated RNAs (18-24 nucleotides) commonly used as transcriptional regulators. The dysregulation of miRNA expression has been

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correlated to malignant transformation in various malignancies and proposed as a promising marker for cancer diagnosis and prognosis determination [5-7]. Naturally, this RNA molecule can target hundreds or even thousands of miRNA degradation and transcription inhibition. They are primarily in circulation and relatively stable in RNAse treated-body fluids in response to repetitive freeze-thawing procedures, low and high pH solutions, and room temperature for 24 hours [8-10]. In addition, they are not only expressed in cancer cells but also in the systemic circulation of normal conditions.

miRNAs have a unique pattern capable of detecting tumors, specifically among cancer subtypes and associated clinical features. This study found differential miR-29c expression and a correlation between pathological and clinical characteristics of NPC patients based on initial profiling and validation. Furthermore, the potential biological mechanisms underlying NPC carcinogenesis were investigated through expression profiles circulating miRNA.

2. MATERIALS AND METHODS

2.1. Ethics Statement

The Ethics Committee Faculty of Medicine Gadjah Mada University approved the sample collection procedure for this study (KE/FK/898/EC/2016). Furthermore, all participants were given an informed consent form that entails using samples, acquisition, and clinical data. We declared that all human subjects in the study had followed the Helsinki Declaration procedure.

2.2. Sample Collection

This study enrolled patients with nasopharyngeal carcinoma and non-tumor. Participants in this study were patients who had a histopathological diagnosis and had not received therapy. The peripheral blood sample was collected from diagnosed NPC (n=160) and non-tumor (n=80) patients at Dharmais National Cancer Hospital in Jakarta and RSUP Dr. Sardjito in Yogyakarta. The sample size was estimated using Cohen's technique with an effect size of 0.5, a significance level of α=0.05, and a statistical power of 0.9, resulting in 49 patients being required.

A sample of NPC plasma was collected and stored until analysis. Furthermore, the EBV-associated markers were tested in all samples. Patients and participants with a history of another malignancy and previously received therapy were excluded. Table **1** summarizes the clinical histopathology characteristics of the NPC patient, including staging status, lymph node involvement, tumor size, and local and distant metastasis.

2.3. miRNA Extraction and Synthesis cDNA

RNA was extracted from 200 plasma using miRCURY-Biofluids, Exiqon Denmark (Cat no. 30112), cDNA synthesis, and 8-64 rxns using universal cDNA synthesis kit II (Cat No. 203301, Exiqon). The cDNA synthesis was carried out using a thermal cycle bio rad C1000, set to 42°C for 60 minutes, 95°C for 5 minutes, and 4°C. All conditions and procedures were carried out following the manufacturer's recommendations.

Table 1. Clinical pathology data of the subject involved in this study.

Characteristic	N = 240	Percent (%)
Non-tumor	160	33%
Nasopharyngeal Carcinoma	80	67%
Age	-	-
Median	47	
Range	14-68	
Sex	-	-
Male	34	66%
Female	18	34%
Histology		
WHO I	0	0%
WHO II	24	15%
WHO III	49	31%
WHO IV	87	54%
N Lymph Node	-	-
N0	10	6%

(Table 1) contd….

Characteristic	N = 240	Percent (%)
N1	43	27%
N2	58	36%
N3	49	31%
T Classification	-	-
T1	6	4%
T2	71	44%
T3	37	23%
T4	46	29%
Clinical Stages	-	-
I	0	0%
II	25	16%
III	49	31%
IV	86	54%
Pathology Anatomy	-	-
Undifferentiated	31	19%
Non-Keratin, Undif Sub Type	117	73%
Non-Keratin, Differentiated	9	6%
Keratin	3	2%
EBV - EA	-	-
Positive	132	83%
Negative	28	18%
EBV - EBNA	-	-
Positive	111	69%
Negative	49	31%
EBV - VCA	-	-
Positive	145	91%
Negative	15	9%

2.4. Quantitative PCR (q-PCR)

The microRNA profiling was performed using cancer focus qPCR Panels (Exiqon, Denmark). Using Exilent SYBR Green master mix 2.5 mL (cat no. 203402, Exiqon Denmark) and 196 microRNA primer targets were performed. miR-29c validation was performed with a LNATM PCR Primer set UniRT (Exiqon Cat no. 204729). qPCR was performed using CFX96 Thermocycler (Bio-Rad) with the following conditions, namely 95°C for 10 minutes and 10 seconds, 60°C, and 1-minute ramp-rate 1.6°C/s. All protocols were in line with the manufacturer's recommendation.

2.5. Data Analysis

This was performed using Genex Pro with Exiqon qPCR wizard, qPCR analysis software, and perpetual license for academic use (Cat No. 207006, Exiqon). Data expression was performed using relative expression data and Software GenEx 6 Multi analysis, Livak's methods [11]. Statistical analysis was also used to determine the differences in expression for both groups and clinical status. Furthermore, a potential mechanism impact of alterations in the expression of miRNA was performed using Ingenuity Pathway Analysis (IPA). All statistical tests with 2-sided and a p-value <0.05 indicated statistical significance. The graphical evaluation was performed using GraphPad Prism 6 (La Jolla CA, USA).

2.6. Functional Enrichment Analysis of miR-29c Targets

Analysis of the relationship between target miRNA-mRNA was performed using the miRNAs database, namely miRBase (http://www.miRbase.org/) [12], and The Diana

Tools-miRPath v.3 (http://diana.tips.athena-innovation.gr/DianaTools/index.php) [13] was explicitly used to find out predictive algorithms based on the introduction of miRNA Recognition Elements (MREs) at 3'UTR and CDS regions. An illustration of the mechanism of miR-29c on cancer was presented using the Biorender application (https://app.biorender.com/) [14].

3. RESULTS

3.1. Patient Characteristics

160 patients were used as subjects, and 80 non-tumor consisting of 3 subtypes with the cross-sectional study were collected based on WHO characteristics. The clinical characteristics of the samples are listed in Table **1**. Patient characteristics data consisting of clinical histopathology data and EBV titer examination were used as standard procedural diagnoses in the incidence of NPC. Furthermore, the EBV EA, VCA, and EBNA titers were measured using Elisa. The titer results in the value of EBV EA, VCA, and EBNA were between 50.84 to 294.9, 27.60 to 126.2, and 6.93 to 41.8 Elisa units

3.2. Identification of Differential Expression microRNAs

The differential expression of miRNA in detecting tumors was carried out using profiling plate qPCR. Data analysis consisting of processing stages determined the target miRNA expression's quantity and quality. Table **2** shows the analysis of expression profiles in determining the signature of deregulated miRNA in NPC. Test quality control performed on each expression analysis with qPCR was used to determine the confidence interval of data obtained with 95% of the value of Cq. Furthermore, the T-test analysis was used to determine the significant expression between cancer and non-tumor samples using relative quantification by Livak's methods [11]. Based on the statistical profiling data analysis, two alterations of miRNAs expression with fold change (FC) log2 and a high significance *p*-value <0.05 were found. Over-expression miR-195-5p and down-expression miR-29c-3p were the most significant deregulation of NPC plasma (Fig. **1**).

Table 2. Summary of differential circulation of profile expression in NPC with different type samples between tumor and non-tumor, ↓: Down expression, ↑: Overexpression.

No.	miRNA	Expression Level	Fold Change	*p*-Value
1	hsa-miR-195-5p	↑	1.157836	0.04194
2	hsa-miR-149-3p	↑	1.069721	0.24031
3	hsa-miR-155-5p	↑	1.170565	0.28618
4	hsa-miR-10a-5p	↑	1.098633	0.31343
5	hsa-miR-106a-5p	↑	1.467512	0.35354
6	hsa-miR-1	↑	1.173713	0.37783
7	hsa-miR-29c-3p	↓	-1.161949	0.03281
8	hsa-miR-7-5p	↓	-1.063892	0.07502
9	hsa-miR-125b-5p	↓	-1.041707	0.08152
10	hsa-miR-182-5p	↓	-1.165714	0.10429
11	hsa-miR-26a-5p	↓	-1.475091	0.13213
12	hsa-miR-19a-5p	↓	-1.097614	0.15679
13	hsa-miR-101-3p	↓	-1.103286	0.17735
14	hsa-miR-200b-3p	↓	-1.029434	0.18256
15	hsa-miR-22-3p	↓	-1.260699	0.20561
16	hsa-let-7e-5p	↓	-1.158974	0.23375
17	hsa-miR-20a-5p	↓	-1.098586	0.23863
18	hsa-miR-210	↓	-1.132135	0.24619
19	hsa-miR-222-3p	↓	-1.136764	0.27400
20	hsa-miR-423-5p	↓	-1.150846	0.32001

(Table 2) contd….

No	miRNA	Expression Level	Fold Change	*p*-Value
21	hsa-miR-133a	↓	-1.241809	0.33092
22	hsa-miR-200a-3p	↓	-1.059794	0.33232
23	hsa-miR-181b-5p	↓	-1.077098	0.33368
24	hsa-miR-19b-3p	↓	-1.105635	0.33675
25	hsa-miR-148a-3p	↓	-1.132502	0.34118
26	hsa-miR-106a-5p	↓	-1.167916	0.34157
27	hsa-miR-92b-3p	↓	-1.137464	0.34207
28	hsa-miR-29a-3p	↓	-1.108886	0.36469
29	hsa-miR-30d-5p	↓	-1.132282	0.36790
30	hsa-miR-27a-3p	↓	-1.163402	0.38292

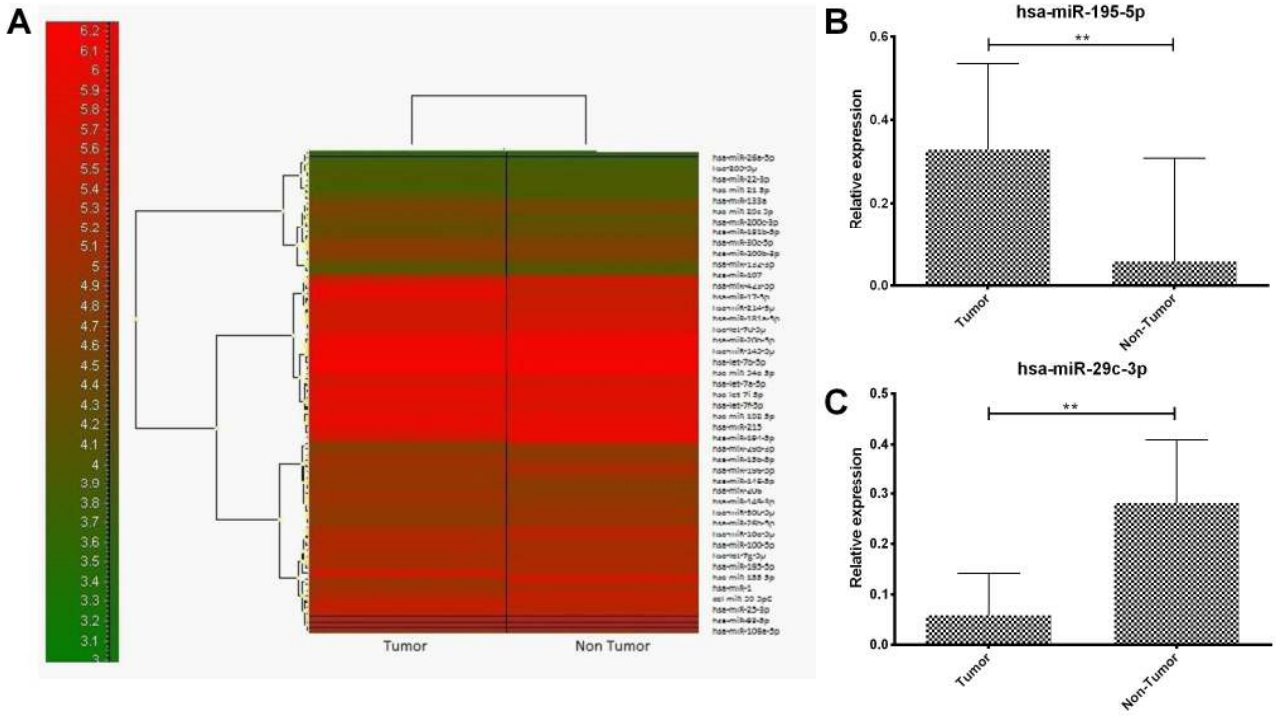

Fig. (1). microRNA (miRNA) expression signature distinguish non-tumour and NPC plasma selected two miRNA with high significance *p*-value: miR-29c-3p and 195-5p. All data analyzed with duplicate (**A**) deregulation expression of miRNAs on plasma NPC. Hierarchical clustering of 196 miRNAs target, differential expression based on different colour: high expression (red) – low expression (green). The analysis method used relative quantification normalized with the reference gene. Quantification data analysis used GenEx-6 Multid Software. (**B**) The over-expression of has-miR-195-5p significantly increased plasma NPC by a fold change of 1.157 (*p*-value 0.05). (**C**) Down-expression of miR-29c-3p quantification analyzed on NPC with fold change 1.16 (*p*-value <0.05). (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

The expression of miR-195-5p was observed with FC 1.157 (*p*-value <0.05) and miR-29c with FC 1.16 (*p*-value <0.05). Furthermore, 30 miRNAs were found with differential expressions with FC susceptibility between 1.1 to -1.1 (Table **2**). The miR-29c was used for validation based on several suggestions. Previous studies also reported that it correlated with poor prognosis through the extracellular matrix [15-17]. Secondly, in a previous study, miR-29c indicated down-expression in several types of cancer and the ability to be used as a candidate biomarker. However, it shows the overexpression of miR-195 contrary to previous studies. It also demonstrated that decreased miR-195 expression might

be used as a biomarker in lung, breast, and colorectal cancers [15, 18, 19].

3.3. Validating Specific Target miR-29c

Investigating the expression of miR-29c was important to evaluate the malignancy level expression. The expression of miR-29c was quantified from liquid biopsy using qPCR with sample tumor (n=160) and non-tumor (n=80). Furthermore, the qPCR analysis of miR-29c expression was stable and generally distributed in NPC patients' plasma (Figs. **2A-E**). Primer development of LNA Exiqon may also influence the stability of its expression. The value of qPCR was calculated to know the expression of the targeted gene. The Livak analysis method was also used to determine the differences in expression for clinical status groups (Tumor, Staging, T-primary tumor, and N-lymph node).

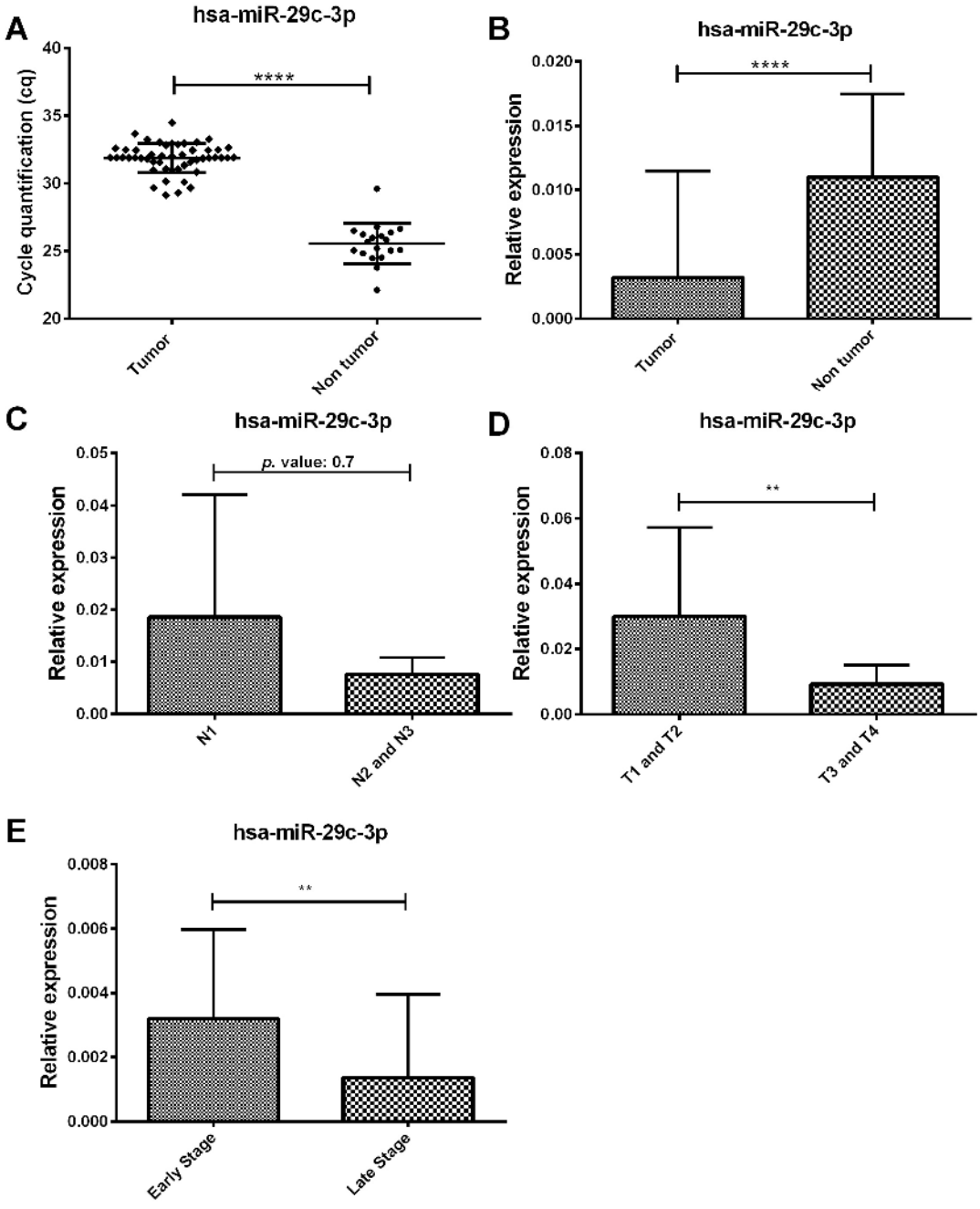

Fig. (2). Show comparison circulating expression of has-miR-29c-3p with the histological grade on nasopharyngeal carcinoma. All data analysis duplicate (**A**) distribution of cycle quantification (CQ) of miR-29c (**B**) down expression of miR-29c; fold change was 4.45, respectively; $p<0.0005$. (**C**) miR-29c with N clinical status with fold change 1.17, respectively; p: 0.7. (**D**) Down expression miR-29c with T status; fold change 2.14, respectively; $p<0,05$. (**E**) Down expression miR-29c in an advanced stage with fold change 1.99, respectively; $p<0.05$. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Generally, the quantification result of miR-29c was distributed along with the standard deviation, which was less than 2. The mean Cq value on non-tumor plasma NPC was significantly higher than the one with tumor 31.45 (± 1.868) and 24.96 (± 1.872) with a *P*-value of less than 0.05. In addition, miR-29c had a significant down expression with FC 4.45 (*p*-value <0.0005). According to histological grade, the circulation of miR-29c was significantly lower in T3 and T4 than in the T1 and T2 groups, with median expressions of 31.35 ± 2.412 and 31.99 ± 1.319 FC-2.14, respectively (*P*<0.05). Additionally, miR-29c expression in circulation was significantly lower in late-stage (grade III and IV) than in early-stage (grade I and II), with median values of 31.21 (± 2.355) and 31.98 (± 1.105), respectively, with a FC of -1.99 and *P*<0.05. However, the miR-29c expression was non-significant in N3 and N4 when compared with N1 and N2, containing median expressions of 31.33 ± 1.689 and 32.78 ± 2.221 with FC -1.17, respectively (*P*>0.05) (Table **3**).

3.4. Biomarker Circulation Performance of miR-29c

The analysis was carried out to determine the ability of miR-29c as a minimally invasive biomarker. The ROC analysis was performed on 2 groups of participants to determine the sensitivity and specificity of candidate biomarkers compared to the EBV titer, which is one indicator of NPC diagnosis. The analyzed results are shown in Figs. (**3A-D**). The AUC values for miR-29c include 0.8706 (95% confidence interval [CI]) and 0.7655 to 0.9757. These results show that miR-29c is more sensitive and specific for NPC than the EBV marker. Furthermore, the 3A-3C, AUC value for EBV EA was 0.8667 (95% CI, 0.7779 to 0.9554), EBV VCA 0.5558 (95% CI, 0.4009 to 0.7106), and EBV EBNA 0.5596 (95% CI, 0.4149 to 0.7043).

3.5. Signaling Pathways and Functionals Mechanism Prediction

The analysis regulation, gene target, and function of miRNAs profiling expression was carried out using Ingenuity Pathways Analysis (IPA). Furthermore, the miRNAs identification was performed using microRNAs Qiagen's IPA's microRNAs target filter tool. It was found that the failed expression of miR-29c significantly affects the cellular mechanism modification, development and maintenance, assembly and organization, growth and proliferation, inflammatory response, and movement pathways involved in cell death and survival (Table **4**).

3.6. Functional and Gene Target Analysis of miR-29c

The regulatory relationships between miR-29c-3p and COL4A, PI3K, VEGFA, JUN, and CDK6 signaling pathways were analyzed using (http://miRtarbase.cuhk.edu.cn/php/index.php). This was followed by determining the mechanism pathway using Diana miRPath v.3 (http://diana.tips.athena-innovation.gr/) and Biorender (https:// biorender.with/), [13, 14, 20] annotation database, visualization and analysis of the integrated biological process through David v.6.8 (https://david.ncifcrf.gov/). Analysis of the binding relationship of miRNA and mRNA based on the Minimum Free Energy (MFE) value is shown in Table **5**. The MFE value was obtained by determining the bond's stability, which is influenced by the number, composition and arrangement of the length of the RNA sequence [21].

Table 3. Fold change validation of miR-29c expression analyzed with relative expression based on clinical status.

Variables	Cycle Quantification microRNAs (Mean ± SD)		Fold Change	*P*-Value
	miR-29c	miR-16		
Cases	-	-	-	-
NPC	31.45 (±1.868)	24.22(±1.611)	-4.45	<0.0005
Non-tumor	24.96 (±1.872)	23.47 (±2.171)	-	-
N- Regional Lymph Nodes	-	-	-	-
N1 and N2	32.78 (±2.221)	25.65 (±2.156)	-1.17	0.7
N3 and N4	31.33 (±1.689)	24.44 (±1.689)	-	-
T- Primary Tumor	-	-	-	-
T1 and T2	31.99 (±1.319)	24.51 (±1.117)	-2.14	<0.05
T3 and T4	31.35 (±2.412)	25.01 (±2.473)		
Stage	-	-	-	-
Early Stage	31.98 (±1.105)	24.48 (±1.054)	-1.99	<0.05
Late Stage	31.21 (±2.355)	24.74 (±2.365)	-	-

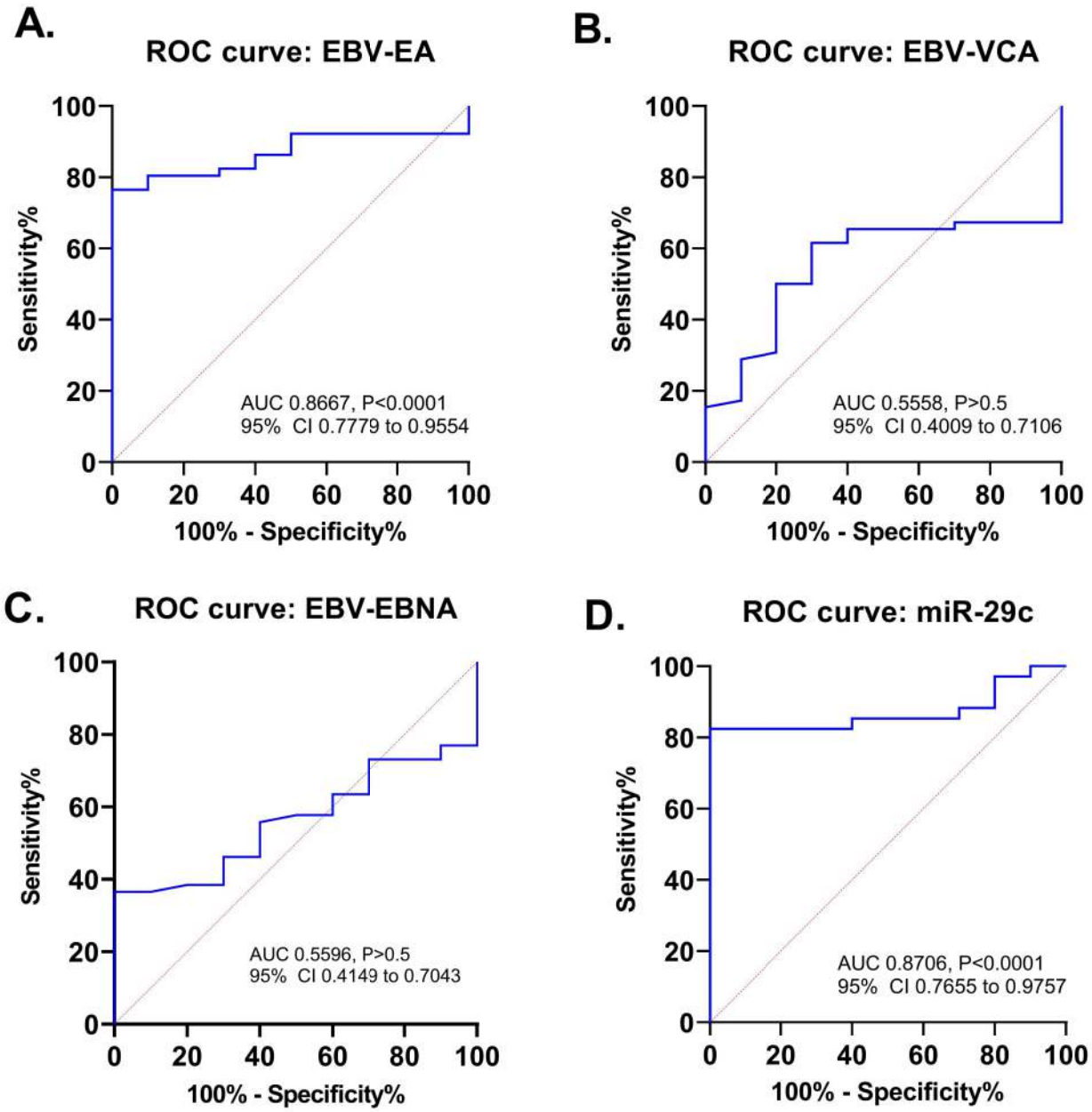

Fig. (3). Comparison of sensitivity and specificity of miR-29 expression in plasma NPC. ROC curves have been constructed to determine the potential diagnostic values of miR-29c compared to the Elisa titer EBV. (**A**) The ROC curve of EBV EA. (**B**) the ROC Curve for EBV VCA. (**C**) the ROC curve for EBV EBNA. (**D**) the ROC Curve for miR-29c. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 4. Mechanism signaling pathways of miR-29c had an impact progression of NPC.

Categories	Diseases or Functions Annotation	*p*-Value	Predicted Activation State	Activation z-Score	Molecules	# Molecules
Cell Death and Survival	Apoptosis of tumor cell lines	0.000114	Increased	2.048	miR-29c-3p,let-7a-5p,miR-10a-5p,miR-122-5p,miR-132-3p,miR-145-5p,miR-146a-5p,miR-148a-3p,miR-155-5p,miR-16-5p, miR-17-5p,miR-181a-5p ,miR-21-5p ,miR-221-3p,miR-223-3p,miR-451a,miR-7a-5p	17
	Cell death of carcinoma cell lines	0.000194	-	1.801	miR-29c-3p, let-7a-5p,miR-145-5p,miR-146a-5p,miR-181a-5p,miR-21-5p,miR-221-3p,miR-223-3p	8
	Cell death of lung cancer cell lines	0.000286	-	1.564	miR-29c-3p, miR-145-5p,miR-146a-5p,miR-181a-5p,miR-21-5p,miR-221-3p,miR-223-3p	7
	Apoptosis of carcinoma cell lines	0.000409	-	1.575	miR-29c-3p, let-7a-5p,miR-145-5p,miR-181a-5p,miR-21-5p,miR-221-3p,miR-223-3p	7
	Apoptosis of lung cancer cell lines	0.00073	-	1.312	miR-29c-3p, miR-145-5p,miR-181a-5p,miR-21-5p,miR-221-3p,miR-223-3p	6
	Necrosis	0.0019	-	1.808	miR-29c-3p,let-7a-5p,miR-103-3p,miR-10a-5p,miR-122-5p,miR-132-3p,miR-142-3p,miR-145-5p,miR-146a-5p,miR-148a-3p,miR-150-5p,miR-155-5p,miR-16-5p,miR-17-5p,miR-181a-5p,miR-21-5p,miR-221-3p,miR-223-3p,miR-24-3p,miR-30c-5p,miR-320b,miR-378a-3p,miR-451a,miR-7a-5p	24
	Apoptosis of hepatoma cell lines	0.00461	-	-0.119	miR-29c-3p, let-7a-5p,miR-122-5p,miR-16-5p	4

(Table 4) contd….

Categories	Diseases or Functions Annotation	*p*-Value	Predicted Activation State	Activation z-Score	Molecules	# Molecules
-	Apoptosis	0.00914	Increased	2.208	miR-29c-3p,let-7a-5p,miR-103-3p,miR-10a-5p,miR-122-5p,miR-132-3p ,miR-145-5p,miR-146a-5p,miR-148a-3p,miR-150-5p,miR-155-5p,miR-16-5p,miR-17-5p,miR-181a-5p,miR-21-5p,miR-221-3p,miR-223-3p,miR-30c-5p,miR-320b,miR-378a-3p,miR-451a,miR-7a-5p	22
	Apoptosis of lung cell lines	0.0128	-	-	miR-29c-3p , miR-17-5p	2
	Apoptosis of leukaemia cell lines	0.0335	-	-0.605	miR-29c-3p, miR-146a-5p,miR-16-5p,miR-21-5p,	4
Cell Death and Survival, Cellular Development, Cellular Function and Maintenance	Self-renewal of breast cancer cell lines	0.0186	-	-	miR-29c-3p	1
Cellular Assembly and Organization, Cellular Function and Maintenance	Quantity of filopodia-like projection	0.0217	-	-	miR-29c-3p	1
Cellular Development, Cellular Growth and Proliferation	Cell proliferation of tumor cell lines	1.5E-12	-	-1.417	miR-29c-3p, let-7a-5p,miR-100-5p,miR-101-3p,miR-10a-5p,miR-122-5p ,miR-128-3p,miR-130a-3p,miR-132-3p,miR-133a-3p,miR-145-5p,miR-146a-5p,miR-148a-3p,miR-155-5p,miR-16-5p,miR-17-5p,miR-181a-5p,miR-18a-5p,miR-192-5p,miR-199a-5p,miR-19b-3p,miR-21-5p,miR-221-3p,miR-223-3p,miR-23a-3p,miR-24-3p,miR-27a-3p, miR-330-5p,miR-378a-3p,miR-451a,miR-708-5p,miR-7a-5p,miR-92a-3p	33

(Table 4) contd….

Categories	Diseases or Functions Annotation	*p*-Value	Predicted Activation State	Activation z-Score	Molecules	# Molecules
-	Cell proliferation of carcinoma cell lines	9.71E-06	-	-1.097	miR-29c-3p, let-7a-5p,miR-100-5p,miR-145-5p,miR-155-5p,miR-17-5pmiR-18a-5p,miR-19b-3p,miR-21-5p,miR-24-3p,miR-378a-3p	11
	Cell proliferation of hepatoma cell lines	5.48E-05	-	-0.19	miR-29c-3p, let-7a-5p,miR-100-5p,miR-122-5p,miR-155-5p,miR-16-5p,miR-223-3p	7
	Cell proliferation of breast cancer cell lines	0.000108	-	-1.387	miR-29c-3p, miR-101-3p,miR-128-3p,miR-155-5p,miR-17-5p,miR-181a-5p,miR-19b-3p,miR-21-5p,miR-27a-3p,miR-7a-5p	10
	Expansion of breast cancer cell lines	0.0155	-	-	miR-29c-3p	1
Cellular Movement	Invasion of tumor cell lines	1.86E-06	-	-1.465	miR-29c-3p, miR-10a-5p,miR-122-5p,miR-145-5p ,miR-146a-5p ,miR-151-5p ,miR-155-5p,miR-17-5p,miR-181a-5p,miR-21-5p,miR-221-3p,miR-223-3p, miR-451a,miR-483-5p,miR-7a-5p,miR-92a-3p	16
	Invasion of breast cancer cell lines	0.000088	-	-1.505	miR-29c-3p, miR-145-5p,miR-155-5p,miR-17-5p,miR-181a-5p,miR-21-5p, miR-7a-5p,miR-92a-3p	8
Inflammatory Response	Inflammation of absolute anatomical region	5.23E-16	-	-	miR-29c-3p, let-7a-5p,miR-100-5p,miR-101-3p,miR-103-3p,miR-130a-3p,miR-133a-3p,miR-140-3p,miR-140-5p,miR-142-5p,miR-150-5p,miR-16-5p,miR-17-5p,miR-181a-5p,miR-199a-5p,miR-19b-3p,miR-210-3p,miR-22-	31

(Table 4) contd….

Categories	Diseases or Functions Annotation	*p*-Value	Predicted Activation State	Activation z-Score	Molecules	# Molecules
-	-	-	-	-	3p,miR-221-3p,miR-223-3p,miR-23a-3p,miR-27a-3p, miR-30c-5p,miR-320b,miR-328-3p,miR-338-3p,miR-344a-5p,miR-365-3p,miR-376a-3p,miR-629-5p,miR-92a-3p	-
-	The cytotoxic reaction of cells	0.0495	-	-	miR-29c-3p , miR-221-3p	2

Table 5. Characteristic interaction prediction of hsa-miR-29c-3p with human mRNA target with high complementary related tumor progression on cancer.

Hsa-miRNA	Gene	Site Type	Start of Site, nt	Region of mRNA	Minimum Free Energy (MFE)
hsa-miR-29c	COL4A1	8mer	849	13-37	-14.80
		7mer-A1		293-314	-12.30
		7mer-A1		849-876	-13.80
	PI3K	7mer-m8	438	315 - 338	-12.70
		7mer-m8		4129 - 4148	-10.30
		7mer-m8		435 - 460	-12.50
	VEGFA	8mer	278	1746 - 1765	-11.20
		8mer		278 - 299	-5.90
		7mer-A1		461 - 482	-6.90
	JUN	8mer	902	1108 - 1131	-10.30
		7mer-A1		902 - 923	-9.30
		7mer		1126 - 1156	-7.33
	CDK6	8mer	8690	8690 - 8714	-13.40
		7mer-A1		9937 - 9960	-12.50
		7mer-A1		9128 - 9145	-10.10

As illustrated in Fig. (**4**), the differential expression of miR-29c significantly affects the protein involved in the formation mechanism of cancer cells and their ability to survive. There was a strong interaction by bioinformatics analysis between COL4A, PI3K, VEGFA, JUN, CDK6 and miR-29c with a p-value <0.05. One of the ways cancer cells may survive by avoiding programmed cell death is through pathway changes by increasing the expression of proteins from COL4A1 and PI3K [22-25]. This result confirms previous research indicating that miR-29c expression is inversely correlated with target genes involved in increased ECM deposition and oncogenic metabolism, increasing cell proliferation *via* the CDK-6 target [26-29]. Furthermore, the process of metastasis and invasion and the VEGF-A expression on the VEGF and Jak-STAT signaling pathways mechanism were also used to increase the tumor progression [30, 31].

4. DISCUSSION

Late diagnosis, lack of access to treatment, and high propensity for metastases are associated with NPC's higher mortality rates in Indonesia [4, 32]. This results from the lack of in-depth understanding of molecular cancer regulation in NPC, the anatomical location and carcinoma size added to the diagnostic bridge in nasopharyngeal development. The delay in diagnosis may result in failed treatment. However, early detection of NPC has a highly successful treatment rate using radiotherapy and chemotherapy. Treatment failure was believed to be due to NPC's development. Therefore, further study needs to be carried out on its complexity and risk factors. Early detection and potential marker application may also reduce NPC's delayed recognition and mortality rates.

miRNAs are used to develop carcinoma because they play an essential role in causing repression or deleting mRNA translation, thus inhibiting protein expression. Therefore, a change in miRNA regulation causes an imbalance in the cells' mechanisms associated with tumor formation. Previous studies also suggest that it can interact with other molecules associated with the clinical manifestations of cancer. Therefore, it is necessary to estimate the prognosis and treatment planning involved in measuring such conditions.

The TNM system is clinically significant in head and neck cancer incidence, which is primarily treated non-surgically. According to the Joint Committee on Cancer of the American Medical Association (AJCC), pathological stages such as lymph nodes or microscopic ENE are uncommon. Therefore, clinical TNM status (cTNM) is required to improve treatment success rates. It is determined using clinical and pathological criteria for T, clinical N (lymph node), and metastases. Several studies have also examined the role of miRNA regulation in the incidence of NPC. MiRNA profiling is a promising approach for studying its effect on the clinical outcome of non-small cell lung cancer. Therefore, this study focused on miR-29c and 195-5p expressions with a significant *p*-value in NPC circulation. It was found that miR-29c expression was significantly correlated with clinical outcomes.

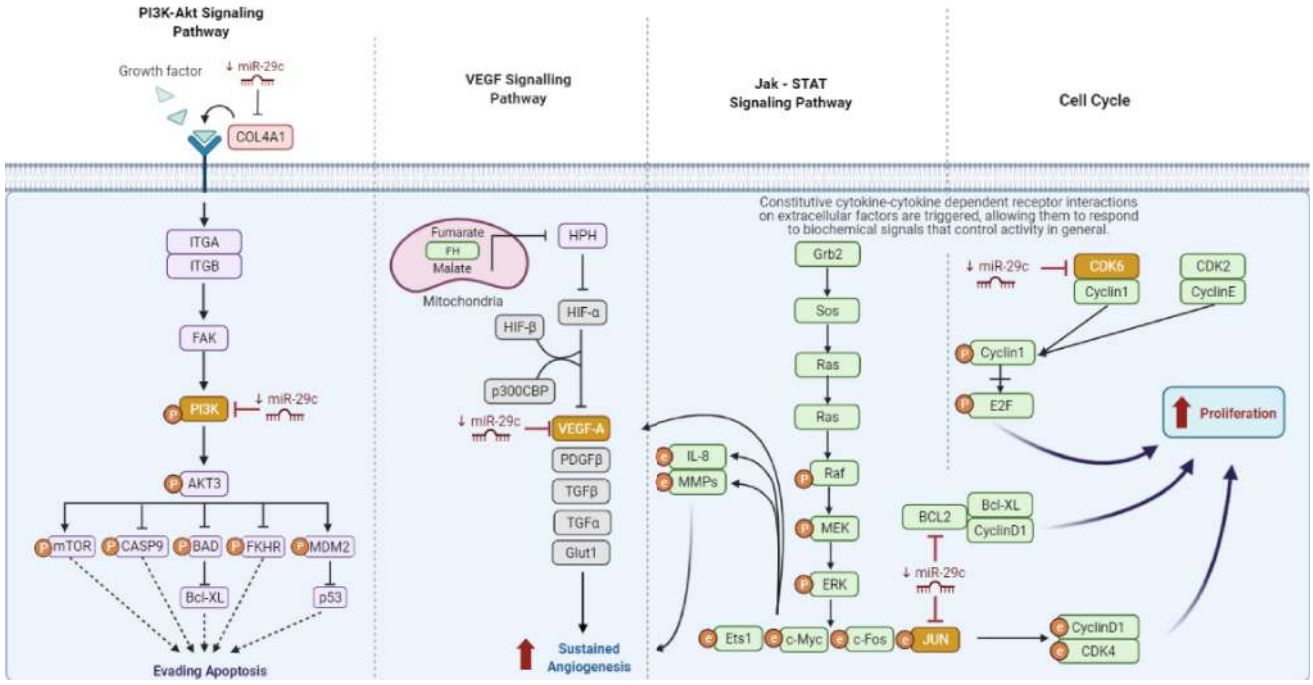

Fig. (4). The suggestion role of miR-29c in cancer as a critical regulator to inhibition through COL4A, PI3K, VEGFA, JUN and CDK6. Increased cancer cells' ability to avoid cell failure elimination mechanisms such as apoptosis, proliferation and angiogenesis through changes in cellular mechanisms. The decrease in miR-29c expression affects the increased expression of the target mRNA, altering signalling pathways and thereby disrupting the cell's normal mechanism. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Zeng *et al*. (2012) and Sengupta *et al*. (2008) reported a consistent decrease in nasopharyngeal carcinoma serum and tissue levels with high sensitivity and specificity. [16, 33] It is associated with the use of Hsa-miR-29c-3p, which is a tumor suppressor miR that targets mRNA oncogene, contributing to malignant cells from normal cells [34]. Furthermore, miR-29c has several roles in several tumor progression invasion, metastasis, and migration. Sengupta *et al*. (2007) stated that it targets the extracellular matrix involved in metastasis and invasion of nasopharyngeal carcinoma [16]. It was also confirmed that miR-29c expressions are closely correlated to poor prognosis. Therefore, the down expression impacts the stability of molecular metastases and invasion [34, 35].

A large number of studies on miRNA associated with NPC were found. However, there were no studies relating to miRNA-29c circulation and clinical manifestation. A different set of miRNAs are expressed explicitly in cell and tissue types. Therefore, miRNA has an essential role in shaping cellular identity. The miR-29 family has expression levels that are very different in nasopharyngeal carcinoma and lung cancer, targeting DNMT3A and DNMT3B in tissue cell lines associated with metastasis and invasion.

The role of miRNA in targeting hundreds or even thousands of RNA significantly impacts the molecular mechanism underlying cancer incidence. It was hypothesized that the failure of miRNA expression affects the formation and development of NPCs *via* multiple target genes. miRNA also affects various cellular processes, including cell death, survival, development, growth, function, maintenance, proliferation, cellular movement, and inflammatory response.

The miR-29 family is critical in the pathophysiological changes associated with diseases such as cardiovascular disease [36], Alzheimer's [37] and cancer [38]. In the case of cancer, miR-29c has decreased expression by regulating extracellular matrix (ECM) coding genes [39, 40], such as collagen type 4 alpha 1 (COL4A1), collagen type 3 alpha 1 (COL3A1), and collagen type 1 alpha 1 and 2 (COL1A1 and COL1A2) [41]. miR-29 inhibits CDK6, TCL1, DNMT3B, MCL1, the process of cycle cell control, DNA methylation and inhibition of apoptosis in melanoma cells and cervical cancer [42-45]. Furthermore, in NPC, high progressivity of invasion, migration, and metastasis increases angiogenesis through altered VEGFA expression [46]. VEGFA is a secreted growth factor that significantly increases cancer incidence, influenced by miR-29c regulation [47].

Despite the study's limitations, a larger number of samples and a multicenter approach were used. Also, having a comprehensive understanding of the miRNA mechanism may result in the development of biomarkers for both diagnosis and prognosis to increase patients' survival rates in the future.

CONCLUSION

This study examined the association between profile expression and clinical outcome in NPC plasma using the Exiqon profiling plate cancer panel. The results indicated that two individuals exhibited significant changes in miR-29c-3p and miR-195-5p expression. Furthermore, miR-29c was used to validate the correlation between changes in its expression and clinical manifestations. The analysis of alterations in miR-29c expression revealed a significant correlation between T-Primary tumor and staging, except for the n-lymph node. However, due to the limitations experienced in this study, it was deducted that the more significant the sample size, the greater the impact power of the research. Also, having a better understanding of the mechanism of miRNAs with clinical status may result in the development of biomarkers for diagnosis and prognosis to improve patient's survival rates in the future.

AUTHORS' CONTRIBUTIONS

TW and RO conceptualized, interpreted, and wrote the manuscript. CHM collected samples, DUP, SL, and TW analyzed the data, TA, SM, and SL, designed, advised, and conducted the study. The final manuscript was read and approved by all authors.

LIST OF ABBREVIATIONS

cDNA	=	Complementary DNA
EBV	=	Epstein Barr Virus
miRNAs	=	microRNAs
NPC	=	Nasopharyngeal Carcinoma

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics Committee Faculty of Medicine Gadjah Mada University, Indonesia approved the sample collection procedure for this study (KE/FK/898/EC/2016).

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the basis of this research. We declare that all human subjects in the study had followed the Helsinki Declaration.

CONSENT FOR PUBLICATION

All participants gave informed consent.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the study is available from the corresponding author [TW] on reasonable request.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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