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Expression of Micro RNA-21 and 29c in Blood Plasma of Patients with Nasopharyngeal Carcinoma

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MicroRNA (miRNA) are small non-coding RNA molecules that work in post-transcription and can cause degradation and cell damage through the targeted gene. Damage to these cells influences the process of carcinogenesis. One of them is in nasopharyngeal carcinoma (NPC). Among families of miRNA that play a role in the pathogenesis of NPC that miRNA-21, miRNA-29c and which is closely associated with the progression, invasion and tumor metastasis. The purpose of this study was to determine the expression of miRNA-21 and miRNA-29c. Knowledge of these miRNA could help in the development of targeted therapy in carcinoma, as a biomarker diagnostic and clinical predictive and would be useful in therapeutic decision making. The research design was observational analytic study carried out in Wahidin Sudirohusodo Makassar, January-June, 2017. The collected blood plasma was isolated and then synthesized to cDNA and calculated quantitatively with qRT-PCR and analyzed using CFX manager 96. There were 52 samples consisting of 27 plasma NPC and 25 controls. From the plasma detected miRNA-21 and miRNA-29c. Expression of miRNA-21 increased 4.47795 fold compared with controls ($p<0.05$)) but its expression can not describe the progression of the tumor at a later stadium ($p<0.05$). In contrast, the expression of miRNA-29c decreased -119.317 fold compared with control, its regulation at a later stadium did not change significantly. Both miRNA-21 and miRNA-29c in blood plasma can use for NPC detection but a single microRNA examination is less able to provide an overview of progressivity.

Key words: Nasopharyngeal carcinoma, miRNA, miRNA-21, miRNA-29c, qRT-PCR

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the most common epithelial malignancies in the head neck on the upper aerodigestive tract. It was estimated that there are approximately 80,000 new cases each year, with the highest incidence reported in the South China there are about 2500 cases per year. The highest incidence of NPC found in the Mongoloid race with a fairly high frequency in South China, Hong Kong, Vietnam, Thailand, Malaysia, Singapore and Indonesia[1].

In Indonesia, the NPC reported ranks 4th among all diseases carcinoma after carcinoma of the uterus, breast and skin, with incidence of 4.7/100,000 population. In the RSU Dadi and RS. Dr.Wahidin Sudirohusodo Hospital during the 10 year period (1990- 1999) found 274 (47.98%) cases of NPC from malignant head and neck tumors in the ratio between male and female were 2.6:1. From the nasopharyngeal carcinoma profile data at RSUP Dr. Wahidin Sudirohusodo, Makassar, South Sulawesi from January, 2004 to June, 2007 NPC was 33% of malignancy in ear, nose and throat, (2000-2009) found 362 cases (57.28%) of malignant head and neck tumors[2].

Nasopharyngeal carcinoma is a carcinoma with the success of therapy is still a problem because of the delay for early detection and difficult location of anatomy. Various studies of molecular biology have been developed to facilitate early detection, i.e., the discovery of a simple examination with ELISA and Epstein-Barr viral load in nasopharyngeal carcinoma[3].

This research is now continuing to look at the role of microRNA (miRNA) against carcinogenesis. MiRNAs are small molecules of non-coding RNA that can affect cell work in transcription post, through target genes[4,5]. MiRNAs may act as tumor suppressor genes or as oncogenes. MiRNA found in tissues and circulation, are stable and not easily degradable. The existence of miRNA in serum or plasma was quite stable, easier to observe more miRNA compared with more invasive biopsy extracted tissue, knowledge of this miRNA can help in understanding the behavior of tumor. The development of targeted therapies in carcinomas, as prognostic biomarkers and clinically predictive that will be useful in therapy decision making[4,6-9].

Among the miRNA families that play a role in the pathogenesis of NPC were miRNA-21 and miRNA-29c. MiRNA-21 was closely related to the degree of progression, invasion and tumor migration. Overexpression of MiRNA-21 in cancer causes cell motility and invasion to increase. MiRNA-21 acts as an oncogene by targeting specific genes such as PDCD4, PTEN, Bcl-2, STAT3 and so on[10-12].

Another miRNA family in NPC was miRNA-29c which serves as a tumor suppressor gene. The expression of microRNA-29c was identified as a potential biomarker especially in terms of evaluation of treatment outcomes[5,13]. MiRNA-29c can inhibit some targets that affect the synthesis and function of extracellular matrix. In addition miRNA-29c also mediates the effects of tumor suppression through inhibition of T cell lymphoma and metastasis 1 (TIAM1) gene, which supports the occurrence of migration and invasion of nasopharyngeal carcinoma cells[14].

Research by Zhang *et al*.[14] shows a decrease in the expression of miRNA-29c associated with resistance of nasopharyngeal carcinoma to chemotherapy and radiotherapy through experiments *in vivo* and *in vitro*. Through mediation of target proteins MCl-1 (myeloid cell leukemia 1) and BCL-2 (B cell lymphoma 2) which was antiapoptotic[13].

Research on microRNA-21 and microRNA-29c on NPC was still very rare. Therefore, the study relate to the expression of circulating miRNA-21 and miRNA-29c in the blood plasma of nasopharyngeal carcinoma patients between NPC and controls as well as between the stadium was highly required.

MATERIALS AND METHODS

This research is a cross sectional study that is analytic observational.

Research location: The study was conducted in Otolaryngology Outpatient Clinic Dr.Wahidin Sudirohusodo Hospital during the period of January-June 2017. Dr. Wahidin Sudirohusodo Hospital chosen because it is a reference center for Eastern Indonesia, especially for cases of carcinoma. Therefore the data obtained is a representation of various layers of society and tribes in Eastern Indonesia.

Inclusion criteria were NPC patients aged 20-70 years old, there were no tumors / malignancies in other organs, do not have a history of hemostatic disorders, willing to as research sample. Exclusion criteria were patients with NPC, who have received prior chemotherapy, radiotherapy or chemo-radiotherapy.

In undertaking this study, every action carried out with the permission of the patient/parents of patients with informed consent sheet and otherwise meets the requirements of conduct for implementation of Biomedical Research Ethics Commission on Human Faculty of Medicine Hasanuddin University.

Data collection: Blood samples were collected in tubes containing EDTA 10% (vaculab®) for commercial grade, as much as ±3-5 mL. Afterwards centrifuged at 3000 rpm for 10 min. Plasma was taken, labeled and stored in a refrigerator at a temperature of -80°C. After collected samples RNA isolation was performed with miRCURY RNA Isolation Kit-Biofluid Exiqon (Cat No.300112, Exiqon) then synthesized into cDNA by universal cDNA Synthesis kit II (Cat No. 203301, Exiqon). Then quantitative quantities are

Table 1: Comparison of miRNA-21 and 29c expression between T, N and M in NPC patients

		MiRNA-21			MiRNA-29c		
NPC	Numbers	Mean	SD	p-value	Mean	SD	p-value
T1	4	25.66	1.03	0.128	28.51	1.66	0.095
T2	10	26.22	0.95		30.29	1.14	
T3	7	27.82	2.09		20.98	1.17	
T4	6	27.18	2.15		31.13	2.67	
N0	9	26.28	1.72	0.594	29.99	2.14	0.659
N1	5	27.49	2.50		29.95	1.33	
N2	3	27.39	1.85		31.22	2.87	
N3	10	26.66	1.32		30.72	1.41	
M0	24	26.84	1.72	0.553	30.29	1.81	0.407
M1	3	26.20	2.02		31.22	1.71	

One-way ANOVA (p>0.05), T: Tumor, N: Nodules, M: Metastasis

measured with qRT-PCR (Bio-rad inc, C1000). The material used was Exilent SYBR Green master mix. 2.5 mL (Exiqon, Denmark), primer set miRCURY LNA hsa-miR-21-5p (cat no. 204230) with primary sequence UAGCUUAUCAGACUGAUGUUGA, hsa-miR-29c-3p (Cat no. 204729) with primary sequence UAGCACCAUUUGAAAUCGGUUA and hsa-miR-16-5p as reference gene (Cat no. 205702) with primary sequence UAGCAGCACGUAAAUAUUGGCG.

RNA isolation: The kit used for the isolation of RNA that was miRCURY RNA Isolation Kit-Biofluid Exiqon. At first plasma thawed and then centrifuged with 3000 rpm with 11,000 g for 5 min, the supernatant at the surface then pipetted 200 μL and transferred to a new tube. Add 60 μL Lysis solution buffer then in the vortex 5 sec and incubated at room temperature for 3 min. Then add 20 μL protein precipitation solution BF, vortex for 5 sec, incubated at room temperature for 1 min, centrifugation 3000 rpm with 11,000 g, for 3 min. Move the clear supernatant into a new 2 mL tube. Add 270 μL of isopropanol, vortex for 5 sec. Plug the mini spin microRNA column into the collection tube, enter 300 μL sample, incubate for 2 min at room temperature, then centrifuged 11,000 g for 30 sec and then dispose the liquid that goes into the collection tube, then plug it back into the collection tube. Add 100 μL wash solution 1 BF to the column, centrifugation 11,000 g for 30 sec, dispose the liquid and plug the column back into the collection tube. Add 700 μL wash solution 2 BF to the column, centrifuge 11,000 g for 30 sec and dispose the liquid reinstall the column. Add 250 μL wash solution 2 BF, centrifugation 11,000 g for 2 min, dispose the liquid and reinstall the column. Add 50 μL rDNAse to the column, incubate for 15 min at room temperature. Add another 100 μL wash solution 1 BF to the column, centrifugation 11,000 g for 30 sec, dispose the liquid and put the column back into the collection tube. Add 700 μL wash solution 2 BF to the column, centrifuge 11,000 g for 30 sec, dispose the liquid reinstall the column. Add 250 μL wash solution 2 BF, centrifuge 11,000 g for 2 min, dispose the liquid reinstall the column. Replace with new 1.5 mL tube then add 25 μL RNAse free water, incubate in room temperature for 1 min and centrifugation 11,000 g for 1 min, with the same tube then repeat the treatment by adding 25 μL RNAse free water, 1 min incubation at room temperature and then centrifuged 11,000 g for 1 min. The isolated RNA can be stored in the refrigerator -20°C.

cDNA synthesis: Synthesis of cDNA was done by universal cDNA Synthesis kit II (exiqon). Remove the RNA that has been isolated in the refrigerator, lined with the ice pack. Do homogenization with vortex and spin down. Prepare reagents to be used for the making of a master mix consisting of 5x reaction buffer (2 mL), nuclease free water (4.5 mL), enzyme mix (1 μL), spike in (0.5 μL) so, that the total 8 μL. Homogenization with vortex and spin down (for mix according to the number of samples to be used), keep in containers lined with ice packs tube. Divide the master mix into the tube as much as 16 μL per reaction. Put 4 μL RNA sample into the tube then spin down. Tube inserted into a Applied Biosystems thermal cycler with the protocols in accordance with Table 1. Store the result of cDNA synthesis in refrigerator -20°C.

In Table 1, it appears that the expression of miRNA-21 and miRNA-29c between the size of tumor (T), nodules (N) and metastasis (M) was not significant (p>0.05).

Real time qPCR: The material used was Exilent SYBR Green master mix. 2.5 mL (Exiqon), primary set (forward and reverse) microRNA, pre-made cDNA. Take the cDNA from the -20^{0}C refrigerator. Place on a tube container that lined with the ice pack. Let it melt slowly. Homogenization with the vortex then spin down. Dilute cDNA with RNAse free water with 1:80 ratio, 5μL cDNA with 395 μL RNAse free water. Homogenization with a spin down vortex. Store in a tube container that lined with the ice pack. Take the SYBR Green master mix and primary set of microRNA and primary control miR-16 from the refrigerator. Primary homogenization with vortex then spin down. Create a mixed master mix of 2 pieces (miRNA 16 as reference gen and microRNA being targeted). Combine 5 μL SYBR Green master mix and 1 μL PCR primer mix (for each differentiated mixture). Homogenization with

the vortex then spin down. Divide the master mix into each of the 6 μL reaction jars. Insert 4 μL diluted cDNA samples. Set the real time qPCR Bio-Rad laboratories Inc, C1000, California) as follows: Denaturation 95°C (10 min), amplification 40 cycles, 95°C (10 sec), 60°C (1 min ramp-rate 1.6°C sec^{-1}, Optical read, Seam yield curve analysis choose yes). Analyze with CFX Manager 96 software (version 3.0, Bio-Rad Laboratories Inc, California).

Statistical analysis: The analysis was performed using CFX Manager 96 software (version 3.0 for windows, Bio-Rad Laboratories Inc, California). The step was to enter data, select tools, exiqon PCR wizard. Select CFX 96 enter data file containing panel layout. Enter the data file of each sample ct. Set interplate calibrator, internal control, validate sheet and normalize with reference gene. Data was stored in the software control panel. Analyses were performed with T-test, heat map and descriptive statistics.

RESULTS

In the study obtained total of 52 samples. The samples that met the inclusion criteria were 27 plasma NPC patients and 25 plasma controls. Based on this research the characteristic obtained was that men suffer more NPC than women with ratio of 3:1. The most aged NPC age group was in the age range 40-49 years as many as 14 people or 51.9%. The youngest age was 23 years and the oldest was 77 years old. The average age of patients in this study was 45.5 years (Table 2).

Distribution for histopathology of NPC patients according to WHO, obtained WHO type III is the most that was 51.9%, then WHO type II 40.7% and WHO type I only 7.4%. Based on stadium of NPC, according to AJCC most is stadium IVB 33.3% and most of patient come in state of later stadium 70.4%. This was due to a late diagnosis because of the location of the nasopharynx hidden behind the nasal cavity. The difficult location sometimes makes the patient aware of the disturbance after a lump appears on the neck. In this study most patients came with a lump complaint in the neck 33.3% (Table 3).

Quantifying method performed by using primary miRCURY LNA hsa-miR-21-5p (cat no. 204230) with primary sequences UAGCUUAUCAGACUGAUGUUGA, hsa-miR-29c-3p (Cat no. 204729) with primary sequences UAGCACCAUUUGAAAUCGGUUA and hsa-miR-16-5p as reference gene (Cat no. 205702) with primary sequences UAGCAGCACGUAAAUAUUGGCG. Quantifying the expression level by using miRCURRY SYBR Green Master mix (Exiqon, Denmark). qPCR Machine using Bio-Rad CFX 96 and analyzed using CFX Manager 96 and GraphPad.

Table 2: Distribution of NPC patients by age, gender and ethnicity

Variables	n = 27	Percent (%)
Age: (years)		
20-29	2	7.4
30-39	5	18.5
40-49	14	51.9
50-59	2	7.4
≥60	4	14.8
Sex:		
Male	18	66.7
Female	9	33.3

Table 3: Characteristics of NPC patients sample

Variables	n = 27	Percent (%)
Stadium NPC:		
Stadium 1	1	3.7
Stadium 2	7	25.9
Stadium 3	3	11.1
Stadium 4A	5	18.5
Stadium 4B	9	33.3
Stadium 4C	2	7.4
Staging NPC:		
Early	8	29.6
Late	19	70.4
Histopathology degrees:		
WHO Type 1	2	7.4
WHO Type 2	11	40.7
WHO Type 3	14	51.9
Main complains:		
Neck Lump	9	33.3
Epistaxis	3	11.1
Cephalalgia	6	22.2
Nasal obstruction	5	18.5
Hearing disorders	2	7.4
Blood stain rinore	2	7.4

Quantification results get 3 graphs in the form of amplification graphs, melt curve and melt peak which can be seen in (Fig. 1a-c). The third expression of miRNA determined by the value of the cycle quantification (Cq) on the amplification graph shows that the miRNA isolated from the plasma sample has been successfully quantified. While to check the specifies can be seen on the melt peak graph which shows only 1 peak on each miRNA so it can be seen that there is no inhibitor, contaminants or other intruders affecting the amplification process as shown in Fig. 2.

In addition performed a comparative analysis of the level of expression miR-21 and 29c in each sample that has been examined to find out how the pattern of expression that occurs in each sample (Fig. 3).

DISCUSSION

MicroRNAs are small molecules of non-coding RNA that can target proteins that encode mRNA by affecting post transcription, translation and degradation of mRNA. MicroRNAs are reported to correlate to the process of carcinogenesis in humans and play a role in influencing the proliferation, apoptosis, migration and invasion of cancer cells.

MicroRNA can be analyzed by Real Time PCR method (qRT-PCR), microarray and next generation sequencing. However, the analysis of microRNA expression results

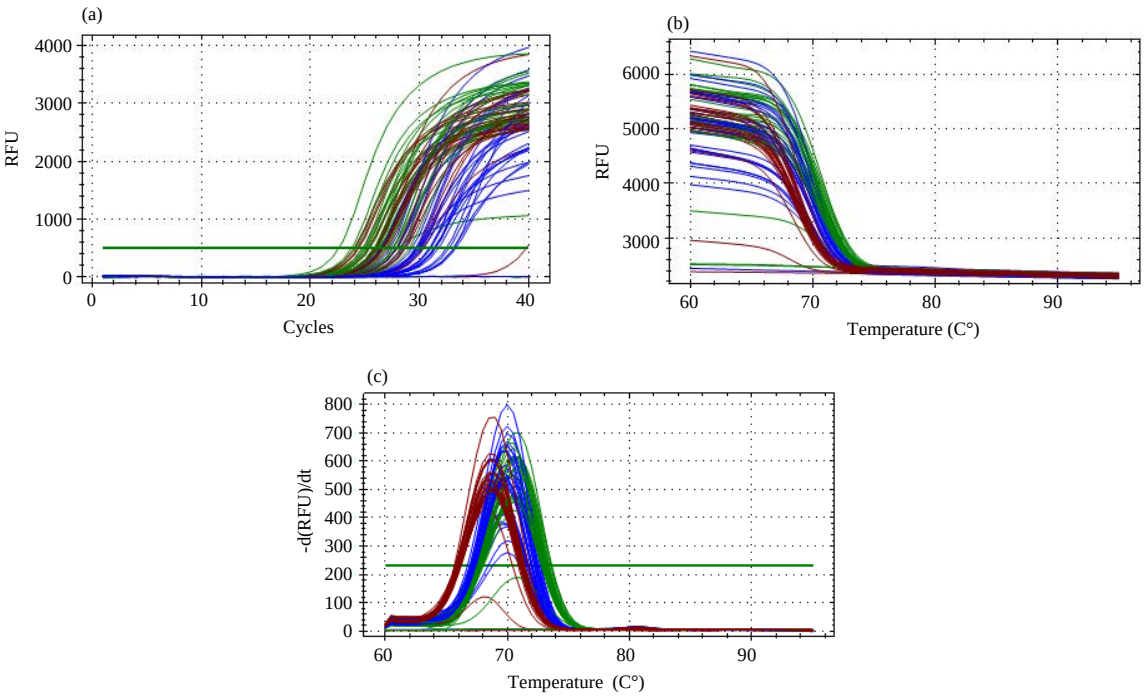

Fig. 1(a-c): Graph the results of quantification of miRNA expression levels in plasma samples of patients with nasopharyngeal carcinoma . (Red: MiR-21, Blue: MiR-29c and Green: MiR-16) (a) An amplification graph that shows the number of target amplifications performed, (b) Melt curve is a graph to know the total number of reactions that occur with the RFU unit and (c) Melt peak is a graph that can be used to view the sensitivity and specificity of amplification targets

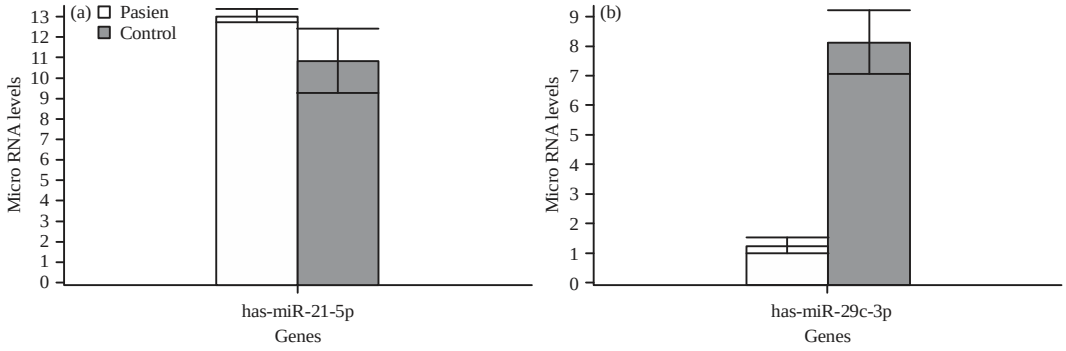

Fig. 2(a-b): Level quantification of expression level of microRNA, (a) Increased expression of hsa-miR-21-5p in NPC patients by 4.46795 ($p<0.05$) and (b) Decreased expression of hsa-miR-29c-3p in plasma of NPC patients was -119.31728 ($p<0.05$)

(miRNA) using qRT-PCR quantification method gives some advantages compared with other quantification methods such as microarray or next generation sequencing, especially at the level of sensitivity and specificity of target amplification. Using a few samples can provide a picture with high validity.

From the results of this research found that miRNA-21 can be detected in human blood plasma. The relative expression of miRNA-21 increased by 4.46795 fold compared with controls. Previous research on NPC plasma also investigated an increased expression of miRNA-21 in NPC patients compared with controls[15]. In addition, other studies conducted on tissue also confirmed an increase in the expression of miRNA-21 in NPC tissue compared with non-tumors[16-18].

In this study also found that there are significant differences between early stadium and later stadium. MiRNA expression was correlated to tumor stadium 21 but there was a decrease in the expression. In contrast to this

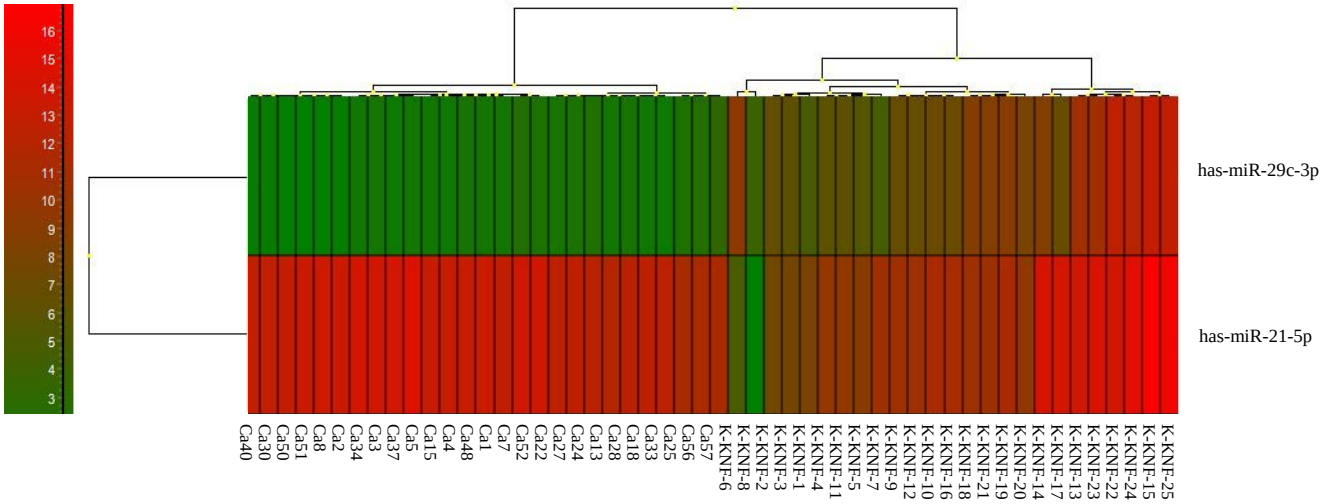

Fig. 3: Heat map expression levels miR-21 and 29c in the patient's and control samples (ca: NPC patients, K-KNF: Controls)

study, another study by Wardana *et al.*[15] declared an upregulation of 21 miRNA in plasma later stadium NPC. Research on NPC tissue by Li *et al.*[17] also showed an increase in regulation of miRNA 21 in stadium III and IV but when compared with its control. Ou *et al.*[16], which also examines the expression of miRNA in the tissues also suggests an increase in the expression of miRNA at an later stadium. In contrast, other studies on 217 plasma NPC patients conducted by Liu *et al.*[13], suggest an increase in the expression of miRNA 21 compared with controls by 5-10 fold, no specific differences between age and sex. Expression of miRNA-21 was high in the early stadium but not persistent at an later stadium. His expression was negatively correlated with different categories of cancer stadium[13]. In addition, low expression of miRNA-21 in NPC was also associated with radio resistance[19].

The existence of different regulation of microRNA still needs to be studied further. Ou *et al.*[16], states that STAT3 inhibition by siRNA can strongly decrease the expression of miRNA-21, otherwise activation of STAT3 increases the regulation of microRNA-21 in cells. In that study Ou *et al.*[16] also showed that STAT3 functions on transformation and affects the activation of microRNA-21[16,20]. Sun *et al.*[18] also observed a decrease in the expression of miRNA-21 inducing retained cells in the G1/S phase thus confirming the role of miRNA-21 stimulation for cell division.

Some studies indicate multiple target genes affected by miRNA-21 in causing cell proliferation and cell invasion. Among them was the research conducted by Meng *et al.*[21] showed that PTEN (Phosphatase and tensin homolog) was a direct target of microRNA-21 that contribute to cell invasion. MicroRNA-21 contributes to apoptotic and chemotherapy resistance by directly targeting PDCD4[22].

The discrepancies in some studies need to examine the factors that may affect the regulation of microRNA-21 and compares regulatory microRNA families a bigger role in the nasopharynx carcinoma.

From the results of this study also obtained miRNA-29c in blood plasma. The expression of miRNA-29c was found to decrease -119.31728 fold ($p<0.05$) statistically significantly compared with controls. As previous research have observed a decrease in the expression of miRNA-29c in blood plasma of patients with NPC[15]. Several studies on tissue also showed a decrease in the expression of microRNA-29c in tumor tissue and non-nasopharyngeal tumors[23,24].

The 29c MiRNA in this study was found to differ between the early and late stadiums but was not statistically significant. Research by Hudcova *et al.*[25], stating that miRNA-29c was correlated to tumor stadium but in the study does not explain the regulation of microRNA expression.

MiRNA-29c has been observed in some studies may act as a tumor suppressor. MiRNA-29c can suppress the progression of cancer cells by promoting apoptosis and increases sensitivity to chemotherapy drugs[14]. Expression of miRNA-29c was also associated with VEGF regulation and TIAM1[24,26]. MiRNA 29c also showed an increase during the osteoblast differentiation phase in *in vitro* experiments. Kapinas *et al.*[27] hypothesize that miR-29a and miR-29c was positively regulated by osteoblast differentiation by controlling the expression of osteonectin in the target organ. Therefore, a decrease in the expression of miRNA-29c in NPC patients contribute to the tumor characteristic[24]. Therefore, it was necessary to conduct further research on the regulation of microRNA after therapy and genes involved.

The limitations of this study are the lack of internal control and the need to determine the ratio of miRNA-21 to miRNA-29c.

CONCLUSION

MiRNA-21 and miRNA-29c can be identified in blood plasma. Expression of miRNA-21 increased when compared with control and its expression is not linear according to the stadium of tumor. MiRNA-21 in blood plasma could use for NPC detection but a single miRNA examination is less able to predict tumor progression. Beside, the expression of miRNA-29c was decreased as compare to control, the regulation was not significantly different to tumor stadium. The presence of miRNA-29c will lower the risk of getting NPC. Both of these miRNAs can be a reference to determine biomarkers and target therapy in NPCs.

SIGNIFICANCE STATEMENTS

This study discovered that the miRNA-21 in plasma NPC patient over expresses when compared with control, however, the miRNA-29c expression is lower when compared with the control, that can be a benefit to determine the biomarker in NPC. This study help the researchers to uncover the critical areas of early detection in NPC and the difference in tumor behavior that many researchers were not able to explore. Thus a new theory about plasma miRNA-21 and miRNA-29c for feature biomarker and targeted therapy model for NPC may be discovered.

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