Hiroshima J. Med. Sci.
Vol. 69, No. 2, 39~45, June, 2020
HIMJ 69–6

Differential Expression Levels of Plasma microRNAs in Neuroblastoma Patients Identified by Next-Generation Sequencing

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ABSTRACT

Efforts to identify biomarkers for neuroblastoma (NB) have been ongoing, but no definite biomarker has been identified in peripheral blood. We proposed the use of plasma exosomal miRNAs as biomarkers of unfavorable NB patient outcomes. Exosomal miRNAs isolated from 31 plasma and 37 tissue samples, many from the same NB patients, were sequenced using a next-generation sequencing instrument. We analyzed the correlation between miRNA expression levels in plasma and tissue samples with International Neuroblastoma Risk Group staging system (INRGSS) outcome and *MYCN* status. We chose differentially expressed miRNAs with similar expression patterns in plasma and tissue samples in each of the three analysis groups and combined those miRNAs to find the optimal combination with the potential to be considered as a biomarker. MicroRNA-*92a-3p* was found to be significantly upregulated in deceased patients (p = 0.017), *miR-375* was upregulated in INRGSS stage M patients (p = 0.002), and plasma *miR-92a-3p* and *miR-99a-5* levels were upregulated in patients with *MYCN* amplification (p = 0.007 and 0.006). The combination of *miR-92a-3p*, *miR-375*, and *miR-99a-5p* levels was shown to be a statistically significant predictor of NB patient outcomes (AUC = 0.726, p = 0.001, 95% CI = 0.612–0.841, sensitivity = 77%, specificity = 56.7%). Thus, the combination of *miR-92a3p*, *miR-375*, and *miR-99a-5p* may potentially be used as a biomarker for unfavorable NB patient outcomes. However, further validation is required in a larger number of NB patients.

Key words: *Neuroblastoma*, *microRNA*, *Prognosis*, *NGS*

INTRODUCTION

MicroRNAs (miRNAs) are a class of small noncoding RNAs with an average length of 22 nucleotides. They play major roles in gene silencing, by binding completely or incompletely to the 3' untranslated region (UTR) of their target mRNA(s)[13,24]. Most miRNAs are located in intracellular compartments, but they can also be found in extracellular compartments, such as serum[4,10], plasma[3,5,33], breast milk[41], urine, cerebrospinal fluid, and seminal fluid[36]. These extracellular circulating miRNAs are stable and can withstand RNase activity through several mechanisms, including enclosure in membrane vesicles (exosomes, apoptotic bodies, and microparticles)[10,11,22,32], association with proteins[3,10], or enclosure in lipoprotein complexes[33]. The expression levels of these miRNAs are frequently dysregulated in cancer[9].

MicroRNAs, particularly exosomal miRNAs, are emerging as promising diagnostic and prognostic biomarkers in various diseases, especially cancer[16,25,38].

According to WHO, approximately 300,000 children are diagnosed with cancer every year and one of the most common solid malignant tumors is childhood neuroblastoma (NB)[23,37]. In Japan, NB occurs in 150–200 children each year, which represents 8% of all pediatric malignancies[6,23]. More than 50% of all NB patients are diagnosed with metastatic disease, frequently in regional lymph nodes, bone marrow, bone, liver and skin. Patient outcomes depend on the tumor biology and disease stage. The outcome of patients whose tumors are classified as high risk based on factors such as *MYCN* amplification or DNA ploidy, remains poor and further development of innovative therapeutic tools is required. Several factors contributing to outcome have been identified in NB patients. Approximately 20–30% of patients with NB present with amplification of the *MYCN* locus on chromosome 2p24, and *MYCN* of amplification strongly correlates with poor outcome, advanced disease stage, and unfavorable biological features[6,23]. Recent advances in liquid biopsy techniques for human cancer allow the analysis of tumor-free DNA and RNA isolated from plasma[1,20]. Several markers in plasma, including neuron specific enolase (NSE) and lactic dehydrogenase (LDH)

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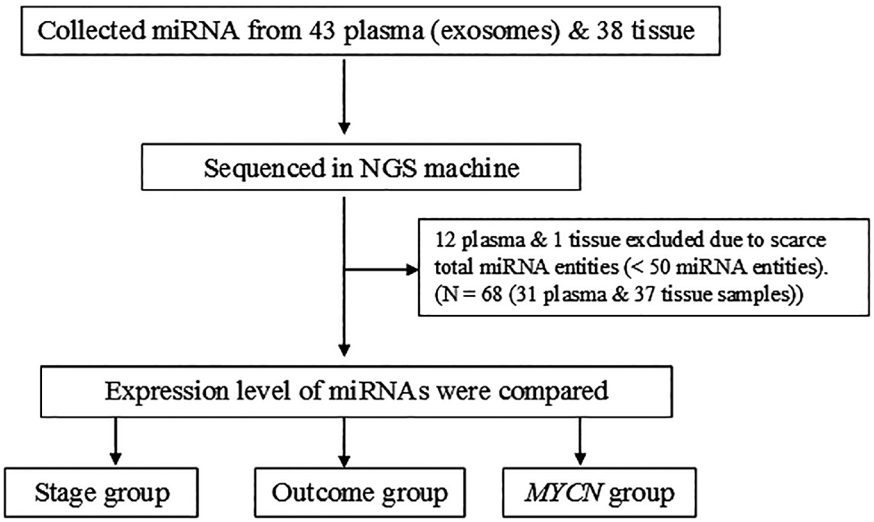

Figure 1 Workflow of the current study. Stage group: INRGSS stage M and non M. Outcome group : deceased and alive. *MYCN* group : amplified and non-amplified.

have been used, but they are not satisfactory[35]. There are also several reports of the detection of *MYCN* amplification in tumor-free DNA from NB patients[7,12]. In this study, we used next-generation sequencing (NGS) to identify differentially expressed miRNAs between plasma and tissue, which may have the potential to serve as plasma biomarkers of unfavorable NB outcomes.

MATERIALS AND METHODS

Samples

Plasma (n=31) and tissue (n=37) samples were collected from 31 untreated NB patients of various disease stages, who were admitted to Hiroshima University Hospital and its affiliated hospitals between 1983 and 2015 (Table 1). Samples were stored at –80°C at the Department of Pediatric Surgery, Hiroshima University Hospital. All participants provided informed consent before participating in this study.

Clinical stages and histological findings were assessed according to the International Neuroblastoma Risk Group staging system (INRGSS). Patients of any age with stage L1 and those less than 12 months old with stage L2 or MS were treated with either surgery or both surgery and chemotherapy. Patients 12 months of age or older with stage L2 or M disease were typically treated according to the protocol recommended by the Japanese Neuroblastoma Study Group[27]. Most patients with stage M or L1/L2 and *MYCN*-amplified tumors, with the exception of some infants, underwent myeloablative chemotherapy followed by bone marrow transplantation.

Exosomes and RNA Isolation

We isolated plasma exosomal and tissue miRNAs and prepared small RNA libraries using Invitrogen Total Exosome Isolation and Invitrogen Total Exosome RNA and Protein Isolation kits (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol, up to the small RNA enrichment steps (Figure 1). Isolated RNA samples were then stored at –80°C for further NGS library preparation.

Table 1 Sample Characteristics (n = 68)

Sample		Plasma	Tissue samples (corresponding samples)[1]
Total		31	37 (27)
Age	<= 12 m	10	12 (9)
	13–17 m	4	5 (3)
	≥ 18m	17	20 (15)
Sex	Male	22	25 (19)
	Female	9	12 (8)
Stage	L1	11	12 (10)
	L2	8	8 (5)
	M	11	15 (11)
	MS	1	2 (1)
Outcome	Dead	10	16 (10)
	Alive	21	21 (17)
MYCN	Non-amplified	25	26 (21)
	Amplified	6	11 (6)

[1] ( ) = available corresponding tissue samples to plasma samples

Next Generation Sequencing

Libraries for NGS were generated using TruSeq® Small RNA Library Prep kit (Illumina, San Diego, CA, USA), in accordance with manufacturer's protocol. The libraries were then sequenced on a MiSeq NGS platform (Illumina). Post-NGS sequence modification, including adaptor sequence removal, aligning to a reference sequence, and raw count analysis were performed using Strand NGS software (version 2.8, build 230243; Strand Life Sciences, Bangalore, India).

Correlation with Clinical Factors

The correlation between miRNA expression levels in plasma and tissue samples and clinical factors (stage, outcome, and *MYCN* status) was assessed. Focusing on NB patients with unfavorable and favorable outcomes,

we compared the expression level of miRNAs in each sample type (plasma or tissue) between INRGSS M and non-M (L1, L2, MS) patients, alive and deceased patients, and patients with non-amplified copy of *MYCN* and amplified *MYCN* (≥ 10 copies). We then identified miRNAs in both plasma and tissue that showed similar expression patterns and further analyzed these miRNAs.

NGS Data Normalization Method

There is currently no single normalization method approved for miRNA sequencing analysis. As miRNA is one specific class of RNA, many different normalization methods for RNA sequencing studies have been used to analyze miRNA sequencing data[30]. There are many methods of normalization used for RNA studies[8]. We initially performed normalization using DESeq[2], a method that is widely used in RNA and miRNA NGS studies. However, the results were incomplete. Therefore, we sought another method of normalization by assessing previous methods performed prior to the availability of NGS. In this study, we normalized raw counts using six miRNAs (*miR-24*, *miR-484*, *miR-93-5p*, *miR-191-5p*, *miR-126-3p*, and *miR-16-5p*) that have frequently been used for normalization in previous qPCR-based studies of miRNA expression[14,19,29,31]. Normalization was performed using Microsoft Excel 2013. The first step was to adjust the raw counts of each of the six normalizer miRNAs across all 68 samples. The highest raw count of a normalizer miRNA in all 68 samples was revalued as 100 and the raw counts of the same normalizer miRNA in other samples were adjusted accordingly. After all raw counts of all six normalizer miRNAs across all samples were adjusted, the average adjusted raw counts of the six miRNAs were calculated in each sample. Using this method, we obtained a normalization factor for each sample, as a result of normalization across all samples and within each sample. Using the normalization factor of each sample, we normalized the raw counts of the remaining non-normalizer miRNAs in every sample.

Statistical Analyses

Spearman's correlation test was used to assess the correlation between types of samples. A Mann-Whitney U test was used to compare two different groups. Receiving operator characteristics (ROC) curve analysis was performed to generate ROC curves and calculate the area under the curve (AUC), sensitivity, specificity, 95% CI, and the optimal cut-off point for the diagnostic accuracy of the combination of miRNAs between the test groups. For all statistical analyses, a p-value < 0.05 was considered to be statistically significant. Statistical analyses and survival analysis were performed using SPSS statistical package version 22 (IBM SPSS Statistics, Armonk, NY, USA).

Ethical Approval

This study was approved by the Institutional Review Board at Hiroshima University Hospital (Hiro RIN-20). All patients provided written informed consent.

RESULTS

Since varied miRNA expression levels were found in the plasma and tissue samples, we searched for miRNAs with statistically significant differences in both plasma and tissue. In the analysis of correlation with outcome, *miR-92a-3p* was found to be upregulated in both plasma and tissue samples from deceased cases (p = 0.017, Figure 2a). The correlation between *miR-92a-3p* levels in plasma and tissue samples is shown in Figure 2b. In the analysis of correlation with disease stage, only *miR-375* was found to be significantly upregulated in both plasma and tissue samples at the M stage (plasma, p = 0.002; Figure 3). The correlation between *miR-375* levels in plasma and tissue samples is shown in Figure 3b. There were only two miRNAs showing statistically significant differences with respect to *MYCN* status, with similar expression patterns in both plasma and tissue samples. The plasma levels of *miR-92a-3p* and *miR-99a-5p* were significantly upregulated in *MYCN* A patients (p = 0.007 and 0.006, respectively; Figures 4a, c). The correlations between *miR-92a-3p* and *miR-99a-5p* levels in plasma and tissue samples are shown in Figures 4b and 4d. To determine their stratifying indicator for unfavorable outcome, we performed a combined analysis of the differentially expressed miRNAs. The combination of miRNAs with the greatest prognostic value was *miR-92a-3p*, *miR-375*, and *miR-99a-5p* (AUC = 0.726, p = 0.001, 95% CI = 0.612–0.841, sensitivity = 77%, specificity = 56.7%; Figure 5).

We also constructed patient survival curves of two groups (high & low) based on the average of plasma levels of *miR-92a-3p* (Figure 6). The 5-year overall survival rate was higher in patients with low *miR-92a-3p* levels (89%, SE = 0.07) than in those with high *miR-92a-3p* levels (45%, SE = 0.14).

DISCUSSION

In this study, circulating miRNAs were obtained from the plasma. Serum samples were not used, because the coagulation process may change the true circulating miRNA profile[34]. For miRNA profiling studies, data normalization is needed to minimize the effects of systematic experimental bias and technical variation. There are many ways to normalize miRNA expression data[21]. In this study, we used six miRNAs as normalizers. MicroRNA *24*, *miR-484*, *miR-93-5p*, *miR-191-5p*, *miR-126-3p*, and *miR-16-5p* have been reported to show consistent levels in the serum and plasma in qPCR-based studies[14,19,29,31].

Using ROC analysis, we found that the combination of *miR-92a-3p*, *miR-375*, and *miR-99a-5p* yielded the best prediction of NB patient outcome. These miRNAs also play roles in other types of cancers. MicroRNA *92a-3p* is known as an oncomir that targets cadherin 1 genes and negatively regulates their expression, which subsequently affects the β-catenin signaling pathway in glioma[28]. Additionally, in other types of cancer, such as colorectal cancer, *miR-92a* contributes to metastasis by

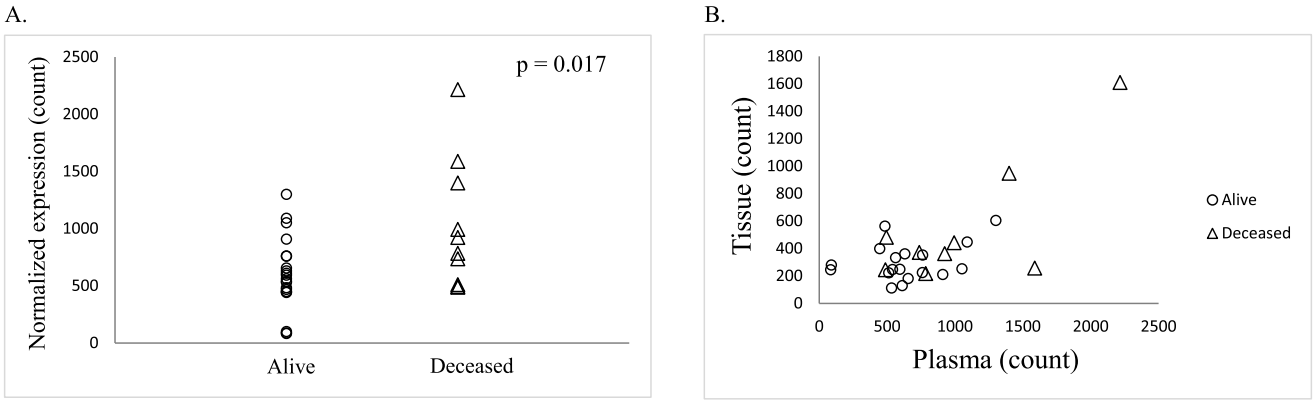

Figure 2 Results of analysis in outcome group. A. Plasma level of the miRNA significantly correlated with outcome, *miR-92a-3p*. B. Correlation plot of *miR-92a-3p* levels in plasma and tissue samples. Spearman rho = 0.308.

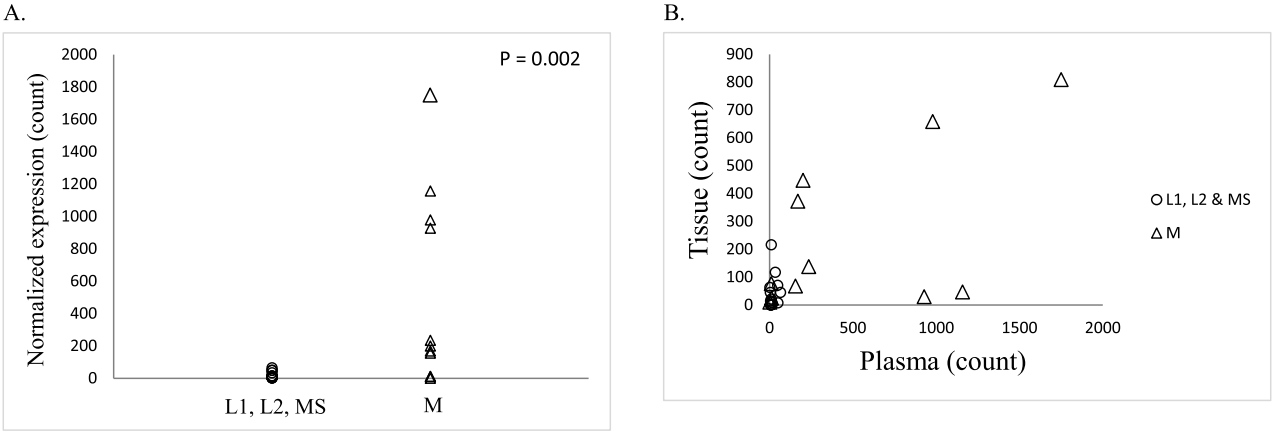

Figure 3 Results of analysis in INRGSS stage group. A. Plasma level of the miRNA correlated with INRGSS stage, *miR-375*. B. Correlation plot of *miR-375* levels in plasma and tissue samples. Spearman rho = 0.545.

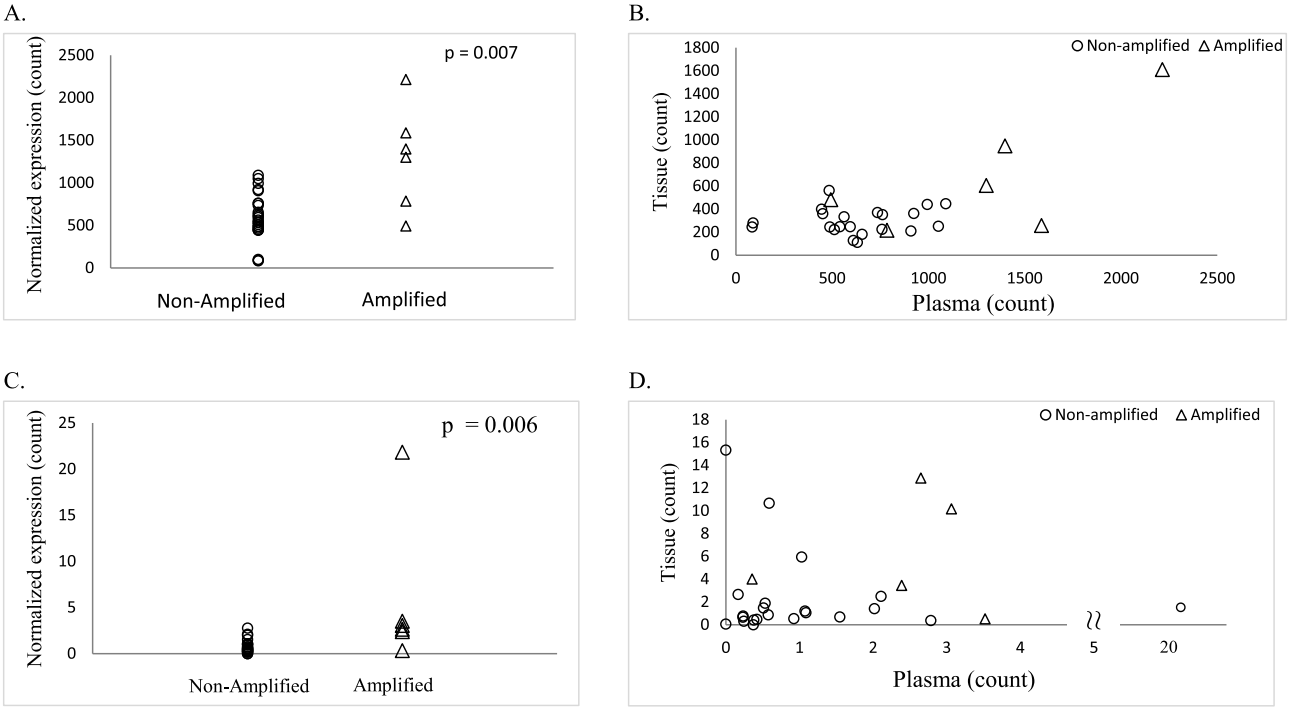

Figure 4 Plasma level of the miRNA significantly correlated with *MYCN* status, *miR-92a-3p*. B. Correlation plot of *miR-92a-3p* levels in plasma and tissue samples. Spearman rho = 0.255. C. Plasma level of the miRNA significantly correlated with *MYCN* status, *miR-99a-5p*. D. Correlation plot of *miR-99a5p* levels in plasma and tissue samples. Spearman rho = 0.212.

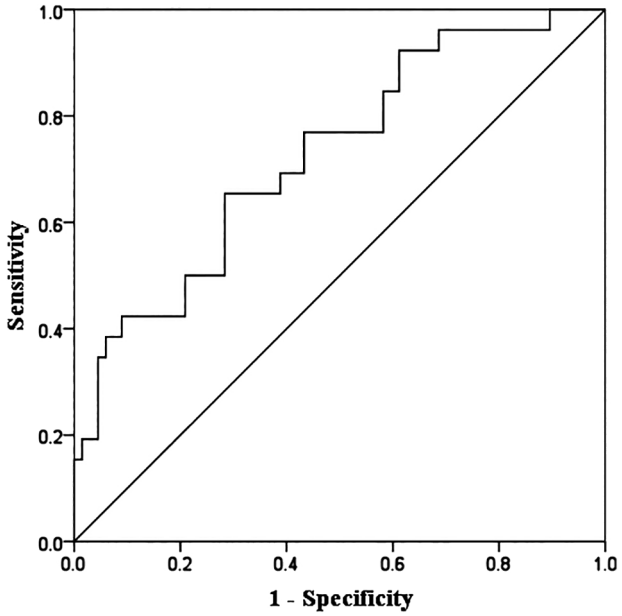

Figure 5 Receiver operating characteristic curve of the combination of *miR-92a-3p*, *miR-375*, and *miR-99a-5p* levels in plasma samples (AUC = 0.726, p = 0.001, 95% CI = 0.612–0.841, sensitivity = 77%, specificity = 56.7%)

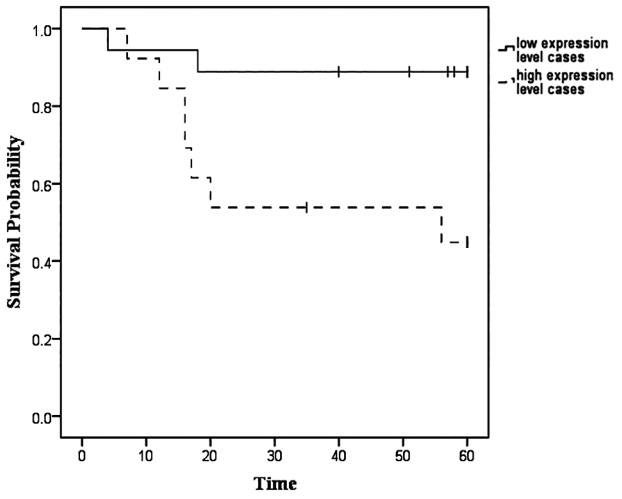

Figure 6 Kaplan-Meier plot of outcome based on *miR-92a-3p* levels. The 5-year overall survival rate was 89% for patients with low *miR-92a-3p* expression levels and 45% for patients with high *miR-92a-3p* expression levels. Low/high expression levels were defined as expression levels below or above the average of plasma *miR-92a-3p* levels in all cases (mean = 732.15 counts).

suppressing *PTEN* gene expression and activating the PI3K/AKT pathway[17]. *miR-375* expression has been linked to a tumorigenic neuroblastic cell phenotype. The N-Myc protein, which is the product of the *MYCN* gene, may upregulate *miR-375* and thus, suppress the expression of HuD, a neuronal-specific RNA-binding protein that affects neuronal differentiation, resulting in the inhibition of neuronal differentiation of neuroblastic cells[26]. Finally, *miR-99a-5p* has been shown to be a tumor-suppressor miRNA in other cancers, such as breast cancer, by targeting the mTOR/HIF-1α signaling pathway[39]. It is also a tumor-suppressor miRNA in non-small cell lung cancer, partially mediated by the AKT1 signaling pathway[40]. In our study, *miR-99a-5p* levels were increased in *MYCN*-amplified samples. In the survival analysis, patients with low levels of *miR-92a-3p* showed favorable outcomes. The exact *miR-92a-3p* expression level cut-off for the prognosis analysis of NB patients may be proposed in future studies.

As with the majority of studies, the design of the current study is subject to limitations. One factor influencing the use of exosomes or miRNAs as biomarkers is the quality of the original sample. Hemolysis and blood coagulation are known to affect exosome yield, because blood cells contain a large number of exosomes and high miRNA levels[15,18]. There is a possibility that this also occurred in our study, even though we used hemolysis-free samples. Therefore, careful attention should be paid to serum or plasma sample preparation in subsequent miRNA studies.

In conclusion, our study used NGS to identify differentially expressed miRNAs between plasma and tissue samples, for the purpose of identifying plasma biomarkers of unfavorable NB patient outcomes. We found that the combination of *miR-92a-3p*, *miR-375*, and *miR-99a-5p* may potentially be used as a biomarker of unfavorable outcomes in NB patients. We recommend further validation of this miRNA combination in a larger number of NB patients.

ACKNOWLEDGEMENTS

We thank all of the patients who were willing to participate in this study; the staff at the Department of Pediatric Surgery, Hiroshima University Hospital; and the staff at the Natural Science Center for Basic Research and Development, Hiroshima University for their technical assistance. We especially thank Mrs. Nagisa Morihara, Mrs. Fumiko Irisuna, Mrs. Shoko Hirano, and Ms. Ikuko Fukuba. Finally, we would like to thank our families and colleagues for standing by us and supporting us.

Conflict of Interest

There are no conflicts of interest to disclose.

Funding Sources

This research was partially supported by a Grant-in-Aid for Scientific Research (A) (No.19H0105600) from the Ministry of Education, Culture, Sports, Science and Technology.

(Received December 27, 2019)
(Accepted March 25, 2020)

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