

# 論 文 内 容 要 旨

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

(次世代シーケンスを用いた神経芽腫の血漿マイクロRNA解析)

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## Introduction

In Japan, neuroblastoma (NB) occupies 8 % of pediatric malignancies and 150 -200 patients were diagnosed each year. Many patients come in advanced stage of disease. In NB, the stages of disease and tumor biology affect the outcome. About 20-30% of NB patients have *MYCN* amplification, which was detected from surgical biopsy of tumor tissues. The *MYCN* amplification is already known to correlate with poor outcome, and advanced stage of disease. The recent advances in cancer diagnosis is inclination toward liquid biopsy method. With its relatively less invasive properties, liquid biopsy is a promising diagnosis method of cancer. One method of liquid biopsy is by analyzing the level of exosomal miRNA in peripheral blood samples of NB patients. Our study aimed to find differentially expressed miRNAs between plasma and tissue which may have potential to be used as biomarkers, particularly for unfavorable NB patients, using Next Generation Sequencing (NGS) platform.

## Materials and Methods

We extracted exosomal miRNAs from 31 plasma and 37 tissue samples of untreated NB patients diagnosed in Hiroshima University Hospital and its affiliated hospitals. We made NGS libraries from those samples, and sequenced them using MiSeq, a NGS machine. Raw counts of miRNA, obtained after sequencing, were normalized using 6 miRNAs (miR-24, miR-484, miR-93-5p, miR-191-5p, miR-126-3p and miR-16-5p) which has been reported to have consistent expression. We then analyzed the correlation between plasma or tissue expression levels of miRNAs and the clinical factors including staging by International Neuroblastoma Risk Group Staging System (INRGSS), outcome, and *MYCN* status. In INRGSS staging, we divided into M stage (n = 11 (plasma), 15 (tissue)) and non M stage cases (n = 20 (plasma), 22 (tissue)). In outcome analysis group, we divided into deceased cases (n = 10 (plasma), 16 (tissue)) and alive with disease-free cases (n = 21 (plasma), 21 (tissue)). In *MYCN* analysis group, we divided into non-amplified cases (n = 25 (plasma), 26 (tissue)) and amplified cases (n = 6 (plasma), 11 (tissue)). We chose statistically significant miRNAs with similar expression pattern in both plasma and tissue samples in each of the 3 analyses groups above. We combined those miRNAs and obtained the most significant combination of miRNAs that might be useful as biomarkers.

## Results

In the correlation to outcomes, miRNA-92a-3p was upregulated significantly in deceased cases both in plasma (p = 0.017) and tissue samples. Average expression levels in plasma of miRNA-92a-3p were 1069.1 & 594.3 in deceased and alive cases, respectively. Focusing more on significance of miR-92a-3p to outcome, we classified its expression levels into two category based on the average level (732.15) of miRNA-92a-3p expressions in all cases of outcome analyses group. Those cases whose miRNA-92a-3p expression levels were less than 732.15, showed favorable outcome, with 5-years overall survival rate was 89%. On the other hand, the overall survival rate of cases whose expression levels were more than 732.15, was only 45 %. In correlation to INRGSS staging, miRNA-375 was upregulated significantly in M stage cases both in plasma (p = 0.002) and tissue samples (p = 0.008). Average expression levels in plasma of miRNA-375 were 509 and 16.3 in M and non M stage cases, respectively. In correlation to *MYCN* status, miRNA 92a-3p and miR-99a-5p were upregulated significantly in *MYCN* amplified cases both in plasma (p = 0.007 & 0.006) and tissue samples (p = 0.001 & 0.001). Average expression levels in plasma samples for miR-92a-3p were 1296.7 and 596.6 in amplified and non-amplified cases, respectively. While average miR-99a-5p expression levels in plasma were 5.6 and 0.7 in amplified and non-amplified cases, respectively. We found that combination of miR-92a-3p, miR-375 & miR-99a-5p has higher stratifying indicator than any other combination of significant miRNAs found in our study (AUC = 0.726, p = 0.001, 95 % CI = 0.612-0.841, sensitivity = 77 %, specificity = 56.7 %).

## Discussion

Micro RNA 92a-3p was reported as an oncomir which targets cadherin 1 (CDH1) genes in negative relationship, subsequently affected  $\beta$ -catenin signaling pathway in glioma. Expression of miRNA-375 might be linked to tumorigenic neuroblastic cell phenotype. The N-Myc protein (product of *MYCN* gene) may possibly upregulate miR-375 and suppress expression of HuD, a neuronal-specific RNA-binding protein which affects neuronal differentiation. This will result in inhibition of neuronal differentiation of neuroblastic cells. Finally miR-99a-5p was reported as a tumor-suppressor miRNA in other cancer such as breast cancer and small cell lung cancer. Limitation of our study is the quality of the original sample. Hemolysis and blood coagulation were known to affect exosome yield because blood cells contains a lot of exosome and miRNAs. There might be a possibility that this was also happened in our study even though we used hemolysis-free samples. Therefore, careful attention should be paid to serum or plasma samples preparation for subsequent exosomal miRNA sequencing studies.

## Conclusion

We found that combination of miR-92a3p, miR-375 & miR-99a-5p may be used as biomarker particularly for diagnosis of unfavorable NB. We realized that further validation step needs to be done on larger number of NB patients.