



# Minimal residual disease in high-risk neuroblastoma shows a dynamic and disease burden-dependent correlation between bone marrow and peripheral blood

San Lin, Kyaw

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(課程博士関係)

## 学 位 論 文 の 内 容 要 旨

Minimal residual disease in high-risk neuroblastoma shows a dynamic and disease burden-dependent correlation between bone marrow and peripheral blood

高リスク神経芽腫の微小残存病変は骨髄と末梢血の間で腫瘍量に依存した相関を示す

神戸大学大学院医学研究科 内科系講座 小児科学分野  
(指導教員：野津 寛大 教授)

Kyaw San Lin

## **【Background】**

Neuroblastoma (NB) is the most common extracranial solid tumor in children and originates from sympathoadrenal precursors or Schwann cell precursors derived from neural crest. These neural crest derivatives also constitute the hematopoietic and mesenchymal stem cells in bone marrow (BM) that is the most frequent site of metastasis and relapse. High-risk NB account for approximately half of newly diagnosed cases and their long-term survival rate remains around 40–50%. Despite extensive multimodal treatment, more than half of high-risk patients experience tumor relapse/regrowth due to chemoresistant minimal residual disease (MRD). Relapsed/regrown NB patients were rarely cured with less than 10% of long-term survival. In NB patients, MRD in BM and PB (BM-MRD and PB-MRD) can be monitored by quantitating several sets of NB-associated mRNAs (NB-mRNAs). Although previous studies have shown varying degrees of correlation between BM-MRD and PB-MRD, the underlying factors and/or mechanisms remain unknown.

## **【Purpose】**

To reveal the underlying factors and/or mechanisms influencing the correlation between BM-MRD and PB-MRD in high-risk NB patients.

## **【Patients and methods】**

*NB patients and samples:* 133 pairs of concurrently collected BM and PB samples from 19 high-risk NB patients from Kobe Children Hospital and Kobe University Hospital

*Disease evaluation:* Response was graded at every BM and PB sampling time point for computed tomography (CT)/magnetic resonance imaging (MRI), MIBG (metaiodobenzylguanidine), and BM assessments based on the available medical records, and these were combined into an overall response. The evaluation was conducted in accordance with the International neuroblastoma response criteria (INRC). Disease status for all BM and PB samples were then assigned to remission, stable, or progression according to BM and overall response.

*RNA isolation and cDNA synthesis:* Total RNA was extracted from mononucleated cells and cDNA was synthesized according to the manufacturer's instructions. All total RNA and cDNA samples were stored at -80°C.

*Droplet digital PCR (ddPCR):* ddPCR was performed using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA). To correct for differences in the amount of total RNA and efficiency of cDNA synthesis, the target copy number was normalized using hypoxanthine phosphoribosyl transferase 1 (HPRT1) as an endogenous reference. ddPCR analysis was performed in accordance with the digital MIQE guidelines.

*Level of each NB-mRNA and 7NB-mRNAs in ddPCR:* The level of each NB-mRNA (each signature) was defined as the relative copy number of each NB-mRNA (each NB-mRNA copy number divided by HPRT1 mRNA copy number and multiplied by 10,000). The level of 7NB-mRNAs (combined signature) was defined as the weighted sum of 7 relative copy numbers (level of each NB-mRNA). The reciprocal of 90 percentile in non-NB control samples was used for the weighting for each NB-mRNA.

*Statistical analysis:* The correlation of the level of 7NB-mRNAs between BM and PB samples was assessed by Spearman's rank correlation test.  $p < 0.05$  was considered statistically significant. Correlation coefficient  $r$  values were considered 0.00–0.09 as “negligible”, 0.10–0.39 as “weak”, 0.40–0.69 as “moderate”, 0.70–0.89 as “strong”, and 0.90–1.00 as “very strong”.

## **【Results】**

### **Correlation between BM-MRD and PB-MRD in overall sample pairs**

BM-MRD showed a moderate correlation with PB-MRD ( $r = 0.418$ ,  $p < 0.001$ ). However, the level of BM-MRD was approximately 10–100 times higher than that of PB-MRD.

### **Correlation between BM-MRD and PB-MRD in subgroups according to each sample evaluation**

In subgroups according to BM infiltration at sampling, the correlation between BM-MRD and PB-MRD was strong ( $r = 0.736$ ,  $p < 0.001$ ) and weak ( $r = 0.306$ ,  $p = 0.001$ ) in the positive and negative subgroups, respectively. In subgroups according to disease status, it became stronger with disease progression; a strong correlation ( $r = 0.725$ ,  $p < 0.001$ ) in the progression subgroup, a weak correlation ( $r = 0.284$ ,  $p = 0.008$ ) in the stable subgroup, and an insignificant correlation ( $p = 0.295$ ) in the remission subgroup.

## **【Discussion】**

We determined the levels of BM-MRD and PB-MRD by quantitating 7NB-mRNAs in 133 pairs of concurrently collected BM and PB samples from 19 high-risk NB patients. Overall sample pairs showed a moderate correlation. However, we found that the correlation coefficient differs drastically into strong, weak, and insignificant correlations after subgroup analysis according to clinical disease evaluation. This revealed a dynamic and disease burden-dependent correlation of MRD between BM and PB. To our knowledge, this is the first study to demonstrate that the correlation between BM-MRD and PB-MRD is associated with the disease burden in non-hematopoietic solid tumors. The present study may explain why BM is the most frequent site of metastasis and relapse in NB patients.

**【Conclusion】**

In conclusion, after analyzing 133 pairs of concurrently collected BM and PB sample pairs, we have found that BM-MRD and PB-MRD in high-risk NB show differing correlations depending on the disease burden.

論文審査の結果の要旨			
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論 文 題 目  Title of Dissertation	<p>高リスク神経芽腫の微小残存病変は骨髄と末梢血の間で腫瘍量に依存した相関を示す</p> <p>Minimal residual disease in high-risk neuroblastoma shows a dynamic and disease burden-dependent correlation between bone marrow and peripheral blood</p>		
審 査 委 員 Examiner	<p>主 査 南 博信 Chief Examiner</p> <p>副 査 尾 藤 祐 子 Vice-examiner</p> <p>副 査 青 井 貴 之 Vice-examiner</p>		

(要旨は1, 000字～2, 000字程度)

## **Introduction**

Neuroblastoma (NB) originates from sympathoadrenal precursors or Schwann cell precursors derived from neural crest. Neural crest also constitutes the hematopoietic and mesenchymal stem cells in bone marrow (BM) that is the most frequent site of metastasis and relapse. Despite extensive multimodal treatment, more than half of high-risk NB patients experience tumor relapse/regrowth due to chemoresistant minimal residual disease (MRD). Relapsed/regrown is rarely cured. In NB patients, MRD in BM and PB (BM-MRD and PB-MRD) can be monitored by quantitating several sets of NB-associated mRNAs (NB-mRNAs). Previous studies showed varying degrees of correlation between BM-MRD and PB-MRD, but the underlying mechanisms and factors influencing the relationships remain unknown. In this study, such factors were investigated in high-risk NB patients were investigated.

## **Methods**

### *NB patients and samples*

NB patients stratified into high-risk according to the Children's Oncology Group (COG) Neuroblastoma Risk Stratification System or the International Neuroblastoma Risk Group (INRG) Classification System were treated between November 2011 and August 2019 based on the JN-H-11 or JN-H-15 protocol of the Japanese Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG). All BM and PB samples were concurrently (less than 3 days apart) collected during the entire course of treatment.

### *Disease evaluation*

Disease evaluation was conducted at every collection time point in accordance with the INRC based on the available medical records. Responses were assigned to "remission" corresponding to complete response (CR) or very good partial response (VGPR), "stable" corresponding to partial response (PR), mixed response (MR), or no response (NR), or "progression" corresponding to progressive disease (PD) for all BM and PB sample pairs.

### *Sample preparation and 7NB-mRNAs ddPCR assay*

Nucleated cells were collected from BM and PB samples, and cDNA was synthesized from extracted total RNA. 7NB-mRNAs ddPCR assay measured the expression of 7 NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH) and a reference gene mRNA (HPRT1). The level of 7NB-mRNAs (combined signature) was defined as the weighted sum of 7 relative copy numbers (level of each NB-mRNA). For BM samples, the level of 7NB-mRNAs was calculated as the mean of right and left samples.

### *Statistical analysis*

Correlation of the level of 7NB-mRNAs between BM and PB samples was assessed by Spearman's rank correlation test. Correlation coefficients of 0.00–0.09 were considered as "negligible", 0.10–0.39 as "weak", 0.40–0.69 as "moderate", 0.70–0.89 as "strong", and 0.90–1.00 as "very strong". AUC was interpreted 0.50–0.69 as "low accuracy", 0.70–0.89 as "moderate accuracy", and 0.90–1.00 as "high accuracy".

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## Results

The levels of BM-MRD and PB-MRD were determined by quantitating 7NB-mRNAs in 133 pairs of concurrently collected BM and PB samples from 19 high-risk NB patients. BM-MRD showed a moderate correlation with PB-MRD ( $r = 0.418$ ,  $p < 0.001$ ). However, the level of BM-MRD was approximately 10–100 times higher than that of PB-MRD. In subgroups according to BM infiltration at sampling, the correlation between BM-MRD and PB-MRD was strong ( $r = 0.736$ ,  $p < 0.001$ ) and weak ( $r = 0.306$ ,  $p = 0.001$ ) in the positive and negative subgroups, respectively. In subgroups according to disease status, it became stronger with disease progression; a strong correlation ( $r = 0.725$ ,  $p < 0.001$ ) in the progression subgroup, a weak correlation ( $r = 0.284$ ,  $p = 0.008$ ) in the stable subgroup, and an insignificant correlation ( $p = 0.295$ ) in the remission subgroup.

## Conclusion

After analyzing 133 pairs of concurrently collected BM and PB sample pairs, BM-MRD and PB-MRD in high-risk NB showed differing correlations depending on the disease burden. The dynamic and disease burden-dependent correlations of MRD between BM and PB in solid tumors are new findings. Present study advanced the field of knowledge in the area of MRD in NB. Therefore, the candidate is certainly recognized as having qualified for the degree of Ph.D.(Medicine).

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