



# Low Multiplication Value of Absolute Monocyte Count and Absolute Lymphocyte Count at Diagnosis May Predict Poor Prognosis in Neuroblastoma

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Despite the growing evidences that immune dysfunction contributes to tumor progression, the prognostic value in patients with neuroblastoma regarding circulating immune blood cell counts has not been well characterized. To answer this, we conducted a retrospective study to evaluate the prognostic value of the circulating immune cell counts at diagnosis in a cohort of 55 patients with neuroblastoma. Based on a novel index by multiplying the absolute monocyte count (AMC)/ $\mu\text{l}$  and absolute lymphocyte count (ALC)/ $\mu\text{l}$ , we sub-grouped patients with  $\text{AMC} \times \text{ALC} \geq 1 \times 10^6$  ( $/\mu\text{l}$ )<sup>2</sup> as high group and patients with  $\text{AMC} \times \text{ALC} < 1 \times 10^6$  ( $/\mu\text{l}$ )<sup>2</sup> as low group. In the entire cohort, the 4-year progression-free survival (PFS), and overall survival (OS) for high group ( $n = 38$ ) vs low group ( $n = 17$ ) was 81.7% (95%CI; 63.6–91.3%) and 90.7% (95%CI; 73.8–96.9%) vs 31.7% (11.6–54.1%) and 56.5% (29.7–76.4%;  $p < 0.001$  for PFS and  $p = 0.015$  for OS), respectively, suggesting that a low AMC  $\times$  ALC is associated with poor prognosis. In the subgroup analysis for high-risk patients, the 4-year PFS and OS for high group ( $n = 17$ ) vs low group ( $n = 13$ ) was 59.8% (31.2–79.7%) and 79.8% (49.4–93.0%) vs 8.5% (0.5–31.7%) and 42.0% (15.4–66.8%;  $p < 0.001$  for PFS and  $p = 0.089$  for OS), respectively. Our data demonstrate that AMC  $\times$  ALC at diagnosis is a cost-effective and easily measurable biomarker for predicting prognosis in neuroblastoma.

**Keywords:** Neuroblastoma, monocyte, lymphocyte, Blood cell counts, prognosis

## INTRODUCTION

Neuroblastoma is the most common type of extracranial solid tumor and is the leading cause of cancer-related death in children (1). Despite recent therapeutic advances, the poor prognosis of high-risk neuroblastoma has not been improved (2). Given the accumulating clinical evidences that impaired immune function contributes to tumor progression, we believe that understanding

**TABLE 1** | Baseline laboratory data of the patients in each risk groups.

	High risk (n = 30)	Intermediate risk (n = 6)	Low risk (n = 19)	p value
WBC (/μl)	9,097 ± 3,637	9,100 ± 2,875	9,326 ± 2,991	0.956
AMC (/μl)	524 ± 350	602 ± 347	608 ± 435	0.596
ALC (/μl)	3,447 ± 1,814	5,034 ± 1,721	4,595 ± 1,641	0.042
ANC (/μl)	4,973 ± 2,866	3,202 ± 1,928	3,695 ± 2,002	0.131
Hb (g/dl)	9.5 ± 2.1	10.8 ± 1.4	12.4 ± 2.1	< 0.001
Plt (× 10 <sup>4</sup> /μl)	31.2 ± 14.8	36.2 ± 11.5	38.9 ± 11.0	0.052
NSE (ng/ml)	452 ± 388	80 ± 33	53 ± 72	< 0.001
VMA (μg/mg Cre)	130 ± 144	194 ± 122	153 ± 302	0.163
HVA (μg/mg Cre)	174 ± 131	171 ± 91	122 ± 214	0.007
LDH (U/l)	1,488 ± 1,466	318 ± 64	323 ± 150	< 0.001
CRP (mg/dl)	5.6 ± 7.0	0.1 ± 0.1	0.3 ± 0.1	< 0.001
Ferritin (ng/ml)	244 ± 208	33 ± 26	65 ± 69	< 0.001

ALC, absolute lymphocyte counts; AMC, absolute monocyte count; ANC, absolute neutrophil count; Cre, creatinine; CRP, C-reactive protein; Hb, hemoglobin; HVA, homovanillic acid; LDH, lactate dehydrogenase; NSE, neuron-specific enolase; Plt, platelet count; VMA, vanillylmandelic acid; and WBC, white blood cell count.

the immunological mechanisms behind this phenomenon may provide clues to new treatment strategies for high-risk neuroblastoma (3).

While the clinical impact of T-cell dysfunction has been widely examined, numerous studies examining the effect of circulating blood cell counts, including lymphocytes, neutrophils, and monocytes, showed that their results varied widely depending on the types and cohorts of cancers (4–9).

Recent clinical data suggest that monocytes may also play an essential role in controlling cancer progression (10, 11).

However, the impact of the composition of circulating immune cells on the prognosis has not been elucidated in neuroblastoma. In this study, we aimed to clarify the impact of the patient’s immune status on the prognosis of pediatric neuroblastoma by retrospectively comparing circulating immune cells at the time of the initial presentation and clinical outcomes.

## MATERIALS AND METHODS

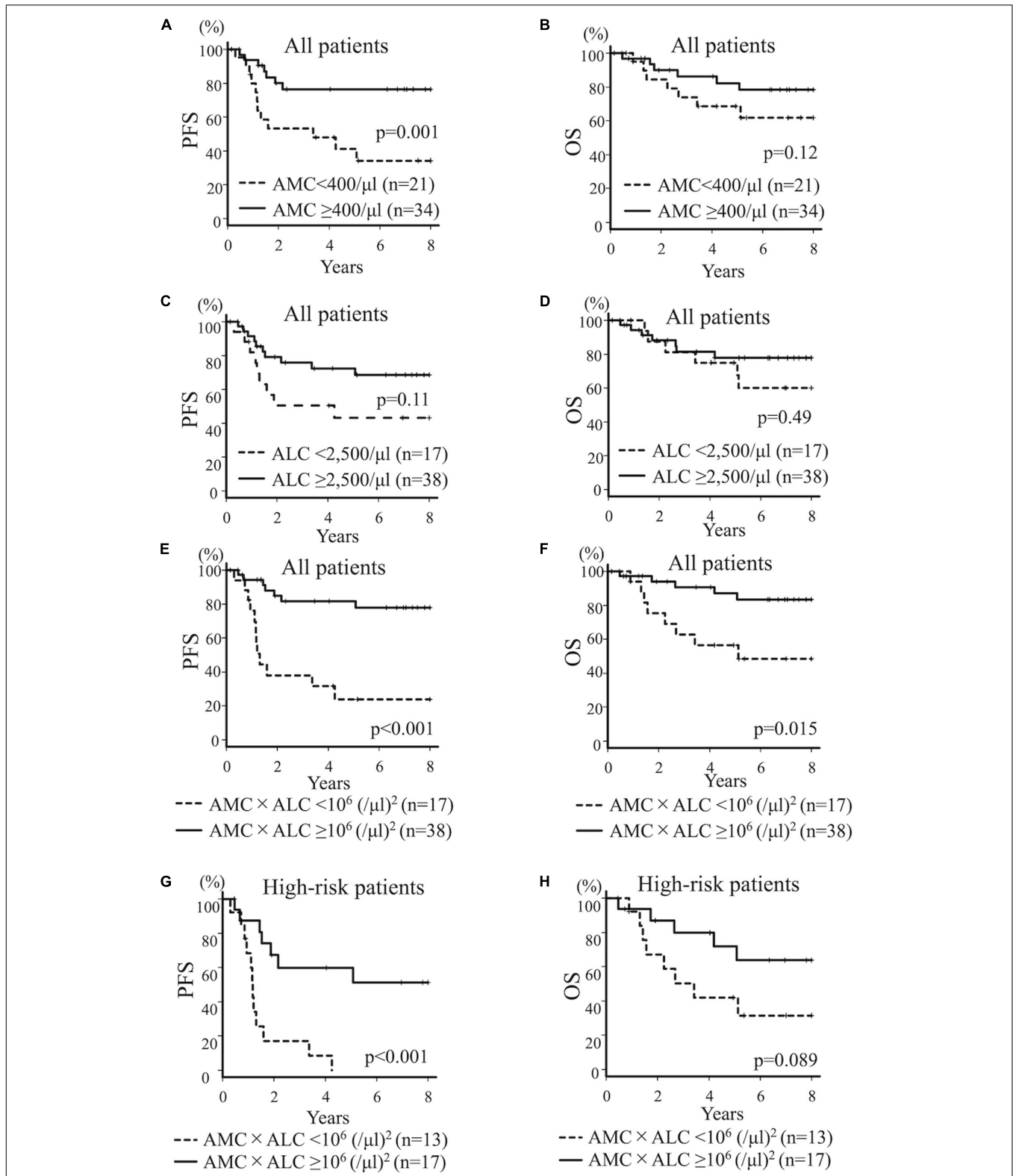
We retrospectively analyzed the prognostic value of the circulating immune cell counts at the initial presentation in a cohort of 55 patients with neuroblastoma diagnosed at Kobe Children’s Hospital (Kobe, Japan) between January 2006 and January 2020. Complete blood counts were obtained using an automatic blood analyzer (XN-2000 or XS-500i, Sysmex, Kobe, Japan), and a differential count was performed manually in most (52 of 55) cases. All complete blood counts were performed prior to the initiation of surgical resection or chemotherapy. Continuous variables were dichotomized based on the optimized cut-off values determined by receiver operating characteristic curve (ROC) analysis to predict progression-free survival (PFS). We created a novel index by multiplying the

absolute monocyte count (AMC)/μl and absolute lymphocyte count (ALC)/μl at the initial presentation (AMC × ALC; /μl)<sup>2</sup>. PFS was defined as the time from the date of diagnosis to the date of relapse or progression. Overall survival (OS) was defined as the time from the date of diagnosis to the date of death. PFS and OS were analyzed by the Kaplan–Meier method and log-rank test, and the observation period was censored at 8 years.

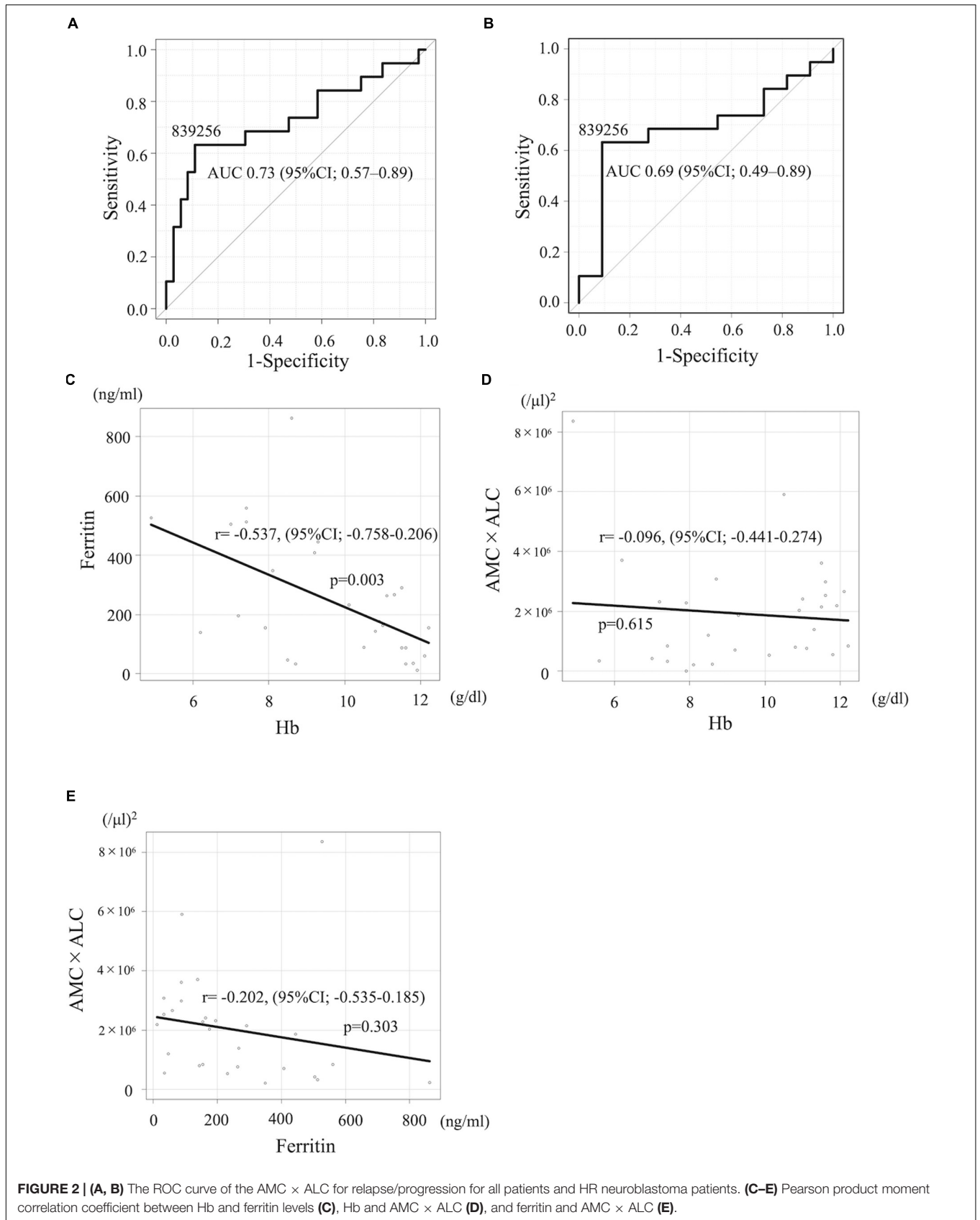
Mann–Whitney *U* test was used to compare differences between the two groups. Kruskal–Wallis test was used to compare differences in more than two groups. Fisher’s exact test was used to compare the distribution of categorical variables between groups. Univariate Cox regression model was used to estimate the hazard ratio (HR) for relapse/progression or death. Significant prognostic factors in the univariate analysis (AMC × ALC < 1 × 10<sup>6</sup> (/μl)<sup>2</sup>, Hb < 10 g/dl, and ferritin ≥ 350 ng/ml) were included in the multivariate analyses using the Cox proportional hazards model. A value *p* < 0.05 was considered as statistically significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (12). The retrospective review of medical records was approved by the institutional review board of the Kobe Children’s Hospital. This research was conducted in accordance with the Declaration of Helsinki.

## RESULTS

The cohort includes a total of 55 children with neuroblastoma with a median observation period of 5.4 years (range; 0.2–8.0 years), comprising of 30 high-risk (HR), 6 intermediate-risk (IR), and 19 low-risk (LR) patients based on the risk classification of the children’s oncology group (COG) (13). To identify prognostic factors for HR patients, we compared baseline laboratory data between the patients for each COG risk classification (Table 1). The white blood cell count (WBC), AMC, and absolute neutrophil count (ANC) did not differ



**FIGURE 1** | Kaplan–Meier curves for all patients (A–F) and high-risk patients (G, H) with neuroblastoma. PFS (A) and OS (B) for all patients with neuroblastoma dichotomized by AMC at diagnosis (AMC  $\geq$  400/ $\mu$ l vs AMC < 400/ $\mu$ l). PFS (C) and OS (D) for all patients with neuroblastoma dichotomized by ALC at diagnosis (ALC  $\geq$  2,500/ $\mu$ l vs ALC < 2,500/ $\mu$ l). PFS (E) and OS (F) for all patients with neuroblastoma dichotomized by AMC  $\times$  ALC at diagnosis [AMC  $\times$  ALC  $\geq$   $1 \times 10^6$  (/ $\mu$ l)<sup>2</sup> vs AMC  $\times$  ALC <  $1 \times 10^6$  (/ $\mu$ l)<sup>2</sup>]. PFS (G) and OS (H) for patients with high-risk neuroblastoma dichotomized by AMC  $\times$  ALC at diagnosis [AMC  $\times$  ALC  $\geq$   $1 \times 10^6$  (/ $\mu$ l)<sup>2</sup> vs AMC  $\times$  ALC <  $1 \times 10^6$  (/ $\mu$ l)<sup>2</sup>].



significantly among risk groups. However, the ALC (mean ± SD) was markedly lower in HR patients (HR; 3,477 ± 1,814/μl, IR; 5,034 ± 1,721/μl, and LR; 4,595 ± 1,641/μl; *p* = 0.042), suggesting that low ALC is a potential feature for high-risk neuroblastoma patients. The hemoglobin (Hb) level was significantly lower in HR patients, while the platelet count (Plt) was not significantly affected. As expected, neuron-specific enolase (NSE), lactate dehydrogenase (LDH), C-reactive protein (CRP), and ferritin levels were higher in HR patients. 4-year PFS and OS in each COG risk group was 36.9% (95% CI; 19.3–54.7%) and 62.5% (95% CI; 41.4–77.8%) for HR, 100 and 100% for IR, and 100 and 100% for LR, respectively.

We next aimed to elucidate the association between blood cell counts and clinical outcomes. Due to the fluctuation of blood cell count after therapy, we could not identify any prognostic significance of blood cell count at any time point after treatment (data not shown).

Then, using the pre-therapeutic blood cell count, we dichotomized the entire cohort according to the levels of AMC,

ALC, and ANC at diagnosis. The cut off values (400/μl for AMC, 2,500/μl for ALC, and 4,000/μl for ANC) were derived from the ROC to predict PFS. 4-year PFS and OS for all patients with AMC ≥ 400/μl (*n* = 34) vs AMC < 400/μl (*n* = 21) was 76.4% (95% CI; 56.7–88.0%) and 86.2% (95% CI; 67.2–94.6%) vs 47.9% (95% CI; 24.9–67.8%) and 68.6% (95% CI; 43.0–84.5%; *p* = 0.001 for PFS and *p* = 0.12 for OS), respectively, suggesting that lower AMC is associated with poorer PFS (Figures 1A,B). 4-year PFS and OS for all patients with ALC ≥ 2,500/μl (*n* = 38) vs ALC < 2,500/μl (*n* = 17) was 72.4% (95% CI; 53.5–84.7%) and 81.3% (95% CI; 62.9–91.2%) vs 50.4% (95% CI; 24.9–71.4%) and 75.0% (95% CI; 46.3–89.8%; *p* = 0.11 for PFS and *p* = 0.49 for OS; Figures 1C,D). Although not statistically significant, patients with poorer prognosis tended to have a lower ALC. 4-year PFS and OS for all patients with ANC ≥ 4,000/μl (*n* = 26) vs ANC < 4,000/μl (*n* = 29) was 67.3% (95% CI; 45.1–82.2%) and 74.6% (95% CI; 51.9–87.8%) vs 63.7% (95% CI; 41.7–79.3%) and 83.7% (62.2–93.6%; *p* = 0.89 for PFS and *p* = 0.36 for OS),

**TABLE 2 |** Univariate analysis of the factors associated with prognosis in children with high-risk neuroblastoma.

Variables	Classification	N	PFS		OS	
			HR (95% CI)	<i>p</i> value	HR (95% CI)	<i>p</i> value
WBC	≥9,000/μl	15	1		1	
	<9,000/μl	15	2.01 (0.78–5.15)	0.147	1.46 (0.48–4.45)	0.512
AMC	≥400/μl	16	1		1	
	<400/μl	14	2.69 (1.05–6.88)	0.039	1.28 (0.43–3.81)	0.658
ALC	≥2,500/μl	17	1		1	
	<2,500/μl	13	1.28 (0.52–3.16)	0.591	0.94 (0.32–2.81)	0.916
ANC	≥4,000/μl	17	1		1	
	<4,000/μl	13	1.42 (0.57–3.52)	0.445	0.87 (0.29–2.59)	0.797
AMC × ALC	≥10 <sup>6</sup> (μl) <sup>2</sup>	17	1		1	
	<10 <sup>6</sup> (μl) <sup>2</sup>	13	5.18 (1.88–14.32)	<0.001	2.56 (0.83–7.88)	0.089
Hb	≥10 g/dl	15	1		1	
	<10 g/dl	15	3.02 (1.15–7.91)	0.025	3.15 (0.96–10.31)	0.057
Plt	≥34 × 10 <sup>4</sup> /μl	12	1		1	
	<34 × 10 <sup>4</sup> /μl	18	1.66 (0.63–4.39)	0.306	2.28 (0.62–8.29)	0.212
NSE	≥500 ng/ml	11	1		1	
	<500 ng/ml	19	0.90 (0.34–2.38)	0.830	0.63 (0.20–1.93)	0.416
VMA	≥120 μg/mg Cre	12	1		1	
	<120 μg/mg Cre	18	0.44 (0.18–1.10)	0.081	0.75 (0.25–2.24)	0.606
HVA	≥180 μg/mg Cre	12	1		1	
	<180 μg/mg Cre	18	0.77 (0.31–1.91)	0.579	1.16 (0.38–3.56)	0.793
LDH	≥1,200 U/l	11	1		1	
	<1,200 U/l	19	0.79 (0.31–2.03)	0.625	0.50 (0.17–1.52)	0.225
CRP	≥5 mg/dl	13	1		1	
	<5 mg/dl	17	0.60 (0.24–1.50)	0.273	0.32 (0.11–1.00)	0.050
Ferritin	<350 ng/ml	21	1		1	
	≥350 ng/ml	7	8.41 (2.24–31.57)	0.002	6.19 (1.94–19.72)	0.002
N-myc amplification	No	16	1		1	
	Yes	14	0.61 (0.24–1.55)	0.300	1.13 (0.38–3.36)	0.831

ALC, absolute lymphocyte counts; AMC, absolute monocyte count; ANC, absolute neutrophil count; CI, confidence interval; CRP, C-reactive protein; Hb, hemoglobin; HR, hazard ratio; HVA, homovanillic acid; LDH, lactate dehydrogenase; NSE, neuron-specific enolase; OS, overall survival; PFS, progression-free survival; Plt, platelet count; VMA, vanillylmandelic acid; and WBC, white blood cell count.

respectively; not statistically significant. Based on these results, we introduced a novel prognostic index by multiplying the AMC/ $\mu\text{l}$  and ALC/ $\mu\text{l}$  to further improve the prediction of prognosis, and sub-grouped patients with  $\text{AMC} \times \text{ALC} \geq 1 \times 10^6$  ( $/\mu\text{l}$ )<sup>2</sup> as high group and patients with  $\text{AMC} \times \text{ALC} < 1 \times 10^6$  ( $/\mu\text{l}$ )<sup>2</sup> as low group.

Four-year PFS and OS for all patients with AMC  $\times$  ALC high group ( $n = 38$ ) vs AMC  $\times$  ALC low group ( $n = 17$ ) was 81.7% (95%CI; 63.6–91.3%) and 90.7% (95%CI; 73.8–96.9%) vs 31.7% (11.6–54.1%) and 56.5% (29.7–76.4%;  $p < 0.001$  for PFS  $p = 0.015$  for OS), respectively, suggesting that low AMC  $\times$  ALC is associated with poorer prognosis (Figures 1E,F). The HR (AMC  $\times$  ALC low group compared to AMC  $\times$  ALC high group) for relapse/progression and death was 5.82 (95%CI; 2.26–14.97,  $p < 0.001$ ) and 4.02 (95%CI; 1.31–12.32,  $p = 0.015$ ) among all patients, respectively. These results demonstrate the prognostic significance of low AMC  $\times$  ALC at diagnosis for children with neuroblastoma.

The development of novel biomarkers to predict the risk of relapse before starting the therapy can lead to an optimal strategy of treatment for neuroblastoma, especially with HR patients. We

next evaluated the prognostic impact of these indices limited for HR patients. 4-year PFS and OS for high-risk patients with  $\text{AMC} \geq 400/\mu\text{l}$  ( $n = 16$ ) vs  $\text{AMC} < 400/\mu\text{l}$  ( $n = 14$ ) was 49.5% (95%CI; 22.0%–72.2%) and 71.1% (95%CI; 39.8–88.1%) vs 23.6% (95%CI; 5.7–48.2%) and 54.2% (95%CI; 25.0–76.2%;  $p = 0.032$  for PFS and  $p = 0.66$  for OS), respectively, suggesting that lower AMC is associated with poorer PFS. 4-year PFS and OS for HR patients with  $\text{ALC} \geq 2,500/\mu\text{l}$  ( $n = 17$ ) vs  $\text{ALC} < 2,500/\mu\text{l}$  ( $n = 13$ ) was 38.5% (95%CI; 14.8–62.0%) and 58.9% (95%CI; 30.2–79.2%) vs 33.8% (95%CI; 10.5–59.4%) and 66.7% (95%CI; 33.7–86.0%;  $p = 0.59$  for PFS and  $p = 0.92$  for OS), respectively; not statistically significant. 4-year PFS and OS for HR patients with AMC  $\times$  ALC high group ( $n = 17$ ) vs AMC  $\times$  ALC low group ( $n = 13$ ) was 59.8% (31.2–79.7%) and 79.8% (49.4–93.0%) vs 8.5% (0.5–31.7%) and 42.0% (15.4–66.8%;  $p < 0.001$  for PFS and  $p = 0.089$  for OS), respectively, (Figures 1G,H). The HR (AMC  $\times$  ALC low group compared to AMC  $\times$  ALC high group) for PFS and OS was 5.18 (95% CI; 1.88–14.32,  $p < 0.001$ ) and 2.56 (95% CI; 0.83–7.88,  $p = 0.089$ ) among HR patients, respectively. These results indicated that the AMC  $\times$  ALC  $< 1 \times 10^6$  ( $/\mu\text{l}$ )<sup>2</sup> at the initial presentation is associated with poorer PFS, even among HR patients, although the prognostic significance to predict poorer OS did not reach statistical significance. The area under the ROC curve of the AMC  $\times$  ALC for relapse/progression for all patients and HR patients was 0.73 (95%CI; 0.57–0.89; Figure 2A) and 0.69 (95%CI; 0.49–0.89; Figure 2B), respectively.

Next, to examine if the AMC  $\times$  ALC can work as an independent prognostic factor for high-risk neuroblastoma, we conducted univariate and multivariate analysis, including other known prognostic markers. In the univariate analysis, the  $\text{AMC} < 400/\mu\text{l}$ ,  $\text{AMC} \times \text{ALC} < 1 \times 10^6$  ( $/\mu\text{l}$ )<sup>2</sup>,  $\text{Hb} < 10$  g/dl, and ferritin  $\geq 350$  ng/ml were significant risk factors for poorer PFS among patients with high-risk neuroblastoma (Table 2). In contrast, Plt, NSE, vanillylmandelic acid (VMA), homovanillic acid (HVA), LDH, CRP, and N-myc amplification, were not associated with poorer PFS among patients with high-risk neuroblastoma.

The baseline characteristics including age, gender, N-myc amplification, tumor cells percentage in the bone marrow, NSE, and year of diagnosis of patients with high-risk neuroblastoma dichotomized by AMC  $\times$  ALC did not differ significantly between groups with exception of ferritin levels (Table 3). 4-year PFS and OS for high-risk patients with ferritin  $\geq 350$  ng/ml ( $n = 7$ ) vs ferritin  $< 350$  ng/ml ( $n = 21$ ) was 0% and 17.1% (95%CI; 0.8–52.6%) vs 46.7% (95%CI; 23.5–67.0%) and 78.3% (95%CI; 51.9–91.3%;  $p < 0.001$  for PFS and  $p < 0.001$  for OS). To exclude the possibility that AMC  $\times$  ALC is a confounding factor of Hb or ferritin levels, we next examined the correlations between AMC  $\times$  ALC, Hb, and ferritin levels by Pearson product-moment correlation coefficient. Hb levels were inversely correlated with ferritin levels (Figure 2C). However, AMC  $\times$  ALC was not significantly correlated with Hb or ferritin levels (Figures 2D–E). In the multivariate analysis, the AMC  $\times$  ALC  $< 1 \times 10^6$  ( $/\mu\text{l}$ )<sup>2</sup> was the only independent risk factor for poorer PFS in patients with high-risk neuroblastoma (HR 3.97, 95%CI; 1.14–13.76,  $p = 0.030$ ), in

**TABLE 3 |** Baseline characteristics of patients with high-risk neuroblastoma dichotomized by AMC  $\times$  ALC at diagnosis.

Variables	AMC $\times$ ALC $< 10^6$ ( $/\mu\text{l}$ ) <sup>2</sup> ( $n = 13$ )	AMC $\times$ ALC $\geq 10^6$ ( $/\mu\text{l}$ ) <sup>2</sup> ( $n = 17$ )	<i>p</i> value
<b>Age</b>			
<18 months	2	2	1.000
$\geq 18$ months	11	15	
<b>Gender</b>			
Male	5	13	0.061
Female	8	4	
<b>N-myc amplification</b>			
Positive	4	10	0.159
Negative	9	7	
Neuroblastoma cells percentage in the bone marrow (%)	19.2 $\pm$ 29.8	9.0 $\pm$ 21.0	0.463
<b>Biochemical markers</b>			
NSE (ng/ml)	479 $\pm$ 435	432 $\pm$ 360	0.558
VMA ( $\mu\text{g}/\text{mg}$ Cre)	155 $\pm$ 156	110 $\pm$ 135	0.341
HVA ( $\mu\text{g}/\text{mg}$ Cre)	175 $\pm$ 123	172 $\pm$ 141	0.773
Hb (g/dl)	9.0 $\pm$ 2.0	9.8 $\pm$ 2.2	0.286
LDH (U/l)	1,387 $\pm$ 1,487	1,565 $\pm$ 1,490	0.660
CRP (mg/dl)	7.3 $\pm$ 9.5	4.3 $\pm$ 3.9	0.645
Ferritin (ng/ml)	366 $\pm$ 236	165 $\pm$ 145	0.020
<b>Year of diagnosis</b>			
2006–2010	3	6	0.889
2011–2015	7	7	
2016–2020	3	4	
Cord blood transplantation	6	3	0.123

ALC, absolute lymphocyte counts; AMC, absolute monocyte count; Cre, creatinine; CRP, C-reactive protein; Hb, hemoglobin; HVA, homovanillic acid; LDH, lactate dehydrogenase; NSE, neuron-specific enolase; Plt, platelet count; and VMA, vanillylmandelic acid.

**TABLE 4 |** Multivariate analysis of the factors associated with prognosis in children with high-risk neuroblastoma.

Variables	Classification	N	PFS		OS	
			HR (95% CI)	p value	HR (95% CI)	p value
AMC × ALC	≥10 <sup>6</sup> (/ $\mu$ l) <sup>2</sup>	17	1		1	
	<10 <sup>6</sup> (/ $\mu$ l) <sup>2</sup>	13	3.97 (1.14–13.76)	0.030	1.12 (0.27–5.31)	0.813
Hb	≥10 g/dl	15	1		1	
	<10 g/dl	15	1.76 (0.49–6.30)	0.383	1.39 (0.25–7.66)	0.709
Ferritin	<350 ng/ml	21	1		1	
	≥350 ng/ml	7	2.57 (0.52–12.78)	0.248	4.40 (0.65–29.95)	0.130

ALC, absolute lymphocyte counts; AMC, absolute monocyte count; CI, confidence interval; Hb, hemoglobin; HR, hazard ratio; OS, overall survival; and PFS, progression-free survival.

contrast to Hb < 10 g/dl (HR 1.76, 95%CI; 0.49–6.30, *p* = 0.383) and ferritin ≥ 350 ng/ml (HR 2.57, 95%CI; 0.52–12.78, and *p* = 0.248; **Table 4**).

## DISCUSSION

High-risk neuroblastoma remains a refractory disease with poor prognosis despite recent medical advances. To date, numerous prognostic markers such as elevated serum ferritin, elevated LDH, and low Hb have been reported in patients with neuroblastoma (14–18). But these alterations are presumably induced by tumor progression such as bone marrow metastasis and are unlikely to be the targets for therapeutic development. Hence, the identification of new prognostic immune biomarkers and the development of optimal treatments using them is highly desirable.

In this study, we found that the multiplication of AMC and ALC at diagnosis (AMC × ALC) is a new prognostic indicator to identify patients with poor prognosis, based on an analysis of laboratory data from the entire cohort during the neuroblastoma treatment period. Further analysis using this novel prognostic measure in the same group revealed that low AMC × ALC at the diagnosis was significantly associated with poor prognosis, even when restricted to high-risk patients. These data suggest that pre-treatment immune status may have some effect on the antitumor effects of treatment. It is also important to note that the long-term prognosis of high-risk patients with low AMC × ALC at diagnosis is exceptionally low (4-year PFS 8.5%) during a median observation period of 5.4 years.

Previous reports have already shown that abnormal balance of cytotoxic T-cells/helper T-cells or Th1/Th2 subsets contributes to some cancer progression, including breast, colon, and liver cancer (19–21). In neuroblastoma, a decrease in regulatory T cells has been noted, but its correlation with prognosis has not been identified (22). On the other hand, our finding suggests that in addition to lymphocytes, monocytes and macrophages are actively involved in the clinical prognosis of neuroblastoma. Indeed, clinical studies showed that higher infiltration of tumor-associated macrophages is associated with clinical aggressiveness in neuroblastoma (23, 24). Tumor-associated macrophages stimulate angiogenesis, increase tumor

cell invasion, and suppress antitumor immunity (25). Therefore, targeting macrophages, such as anti-CSF1R antibodies, have emerged as attractive therapeutic approaches in various types of cancer (25). In addition, recent studies that C/EBP $\beta$ -dependent non-classical monocytes regulate cancer progression also support our results (10, 11, 26–28). In this cohort, most patients did not receive anti-GD2 antibody treatment because it is not approved in Japan. As an alternative, most of the relapsed cases received killer-cell immunoglobulin-like receptor-ligand mismatch cord blood transplantation in anticipation of a graft vs tumor effect (*n* = 6 in low AMC × ALC group and *n* = 3 in high AMC × ALC group; **Table 3**) (29, 30). This fact may be the reason why the OS did not reach a significant difference despite significantly worse PFS in high-risk patients with low AMC × ALC, suggesting the importance of immune cells in neuroblastoma progression.

To our knowledge, this is the first report of a predictive immune biomarker for the prognosis of patients with neuroblastoma, especially those at high risk. However, due to the relatively small sample size and short follow-up range of this single-center study, it needs to be further validated in a larger multicenter cohort in the future. There may be a potential bias due to the circannual or seasonal rhythm of monocytes and lymphocytes (31). On the other hand, since this novel marker is a cost-effective and easily measurable method in the clinics and hospitals, it is expected to be extremely useful as a prognostic marker at the time of initial diagnosis. Besides, due to the correlation between immune cells with antitumor activity and disease prognosis, promoting basic research on the aspects of immunity, mainly monocytes and macrophages, may lead to the development of new therapies.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kobe Children’s Hospital ethics committee. Written

informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

AT designed the study methodology, collected data, performed statistical analysis, and wrote the draft. MM designed the study methodology, wrote the draft, and provided valuable discussions. SI, TM, JN, SN, AS, AK, and TI collected data and provided valuable discussions. KS supported writing the draft. DH and YK collected data and supervised the study. All authors have read and approved the final version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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