Prognostic value of minimal disseminated disease assessed using digital polymerase chain reaction for 3' *ALK* assays in pediatric anaplastic lymphoma kinase-positive anaplastic large cell lymphoma

Approximately 90% of pediatric and 50% of adult anaplastic large cell lymphoma (ALCL) cases are anaplastic lymphoma kinase (ALK)-positive,[1,2] with >80% of these cases carrying the *NPM::ALK* variant.[3] In ALK-positive ALCL, minimal disseminated disease (MDD) has mainly been quantified using quantitative real-time polymerase chain reaction (qPCR) for *NPM::ALK* transcripts in the bone marrow or peripheral blood at diagnosis and is associated with a poor prognosis.[4-6] MDD assessment is highly promising as a prognostic tool for ALK-positive ALCL; however, comparing the results of MDD assessment using qPCR between laboratories is difficult because qPCR requires standard curve calibration. Although MDD assessment using qualitative reverse-transcriptase PCR (RT-PCR) for *NPM::ALK* has been established and is used internationally for patient stratification, it is challenging to interpret the results of borderline cases.[4,7,8] Recently, the efficacy of MDD assessment using digital PCR (dPCR) for *NPM::ALK* has been reported.[7,9] dPCR does not require standard curve calibration and its reproducibility may be better than that of qPCR.[7] dPCR can be used for MDD assessment; however, it requires the reference *ABL* gene assay to verify the quality of a given cDNA, as well as appropriate positive and negative controls to guarantee its performance for measurements of clinical samples.

Although the efficacy of MDD has been established for the *NPM:ALK* variant, this is not currently the case for other *ALK* variants. A previous report[10] revealed that a 3′*ALK* assay using a universal *ALK* probe works the same as an *NPM::ALK* assay; however, the correlation between MDD assessment using the 3′*ALK* assay and prognosis has not yet been evaluated. We, therefore, examined the prognostic value of MDD assessed using dPCR to establish a 3′*ALK* MDD assay as a prognostic tool for future clinical trials.

Patients aged <20 years were enrolled in the ALCL99 trial in Japan between January 2000 and April 2012. Bone marrow and/or peripheral blood samples collected from these patients at diagnosis were used. ALK-positive ALCL was confirmed using the pathological review system of the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) for all patients. This study was approved by the institutional ethics committee of Yamaguchi University Hospital and the National Hospital Organization Nagoya Medical Center.

RNA was isolated from mononuclear cells using Trizol Reagent (Sigma, St. Louis, MO, USA). The protocols applied for cDNA synthesis and quantification of *NPM::ALK* were as previously reported.[4,6] cDNA synthesis was performed using SuperScript II (Invitrogen Corporation, Carlsbad, CA, USA) with 1 μg of RNA per 20 μL of reaction volume with random primers (Invitrogen).

dPCR assays for 3′*ALK* were performed according to a previous report.[10] The primers and probe were designed to amplify the 3′ part of *ALK* and included the exon 20/21 boundary to avoid genomic amplification. Furthermore, we performed dPCR assays for *NPM::ALK* according to the previous report for patients who were confirmed as being positive for *NPM::ALK* using genetic or immunohistological findings.[9] The copy numbers of 3′*ALK* and *NPM::ALK* were calculated per 10,000 copies of *ABL* and are reported as normalized copy number (NCN). The dPCR details for 3′*ALK* and *NPM::ALK*, including primer and probe sequences, are shown in *Online Supplementary Table S1*.

Differences in the distribution of variables were assessed using the χ^2 test. Survival rates were estimated with the Kaplan-Meier method, and differences were compared using the log-rank test. Progression-free survival was estimated from the date of diagnosis until the last follow-up visit or the date of documented failure (progression, relapse, or death). The quantification of 3′*ALK* dPCR and *NPM::ALK* dPCR was compared using Spearman correlation. The prognostic effects of variables were compared by Cox regression analysis. All analyses were performed using SPSS Statistics 27 software (IBM, NY, USA).

We collected 50 bone marrow and 52 peripheral blood samples from 61 patients. We have previously reported the *NPM::ALK* MDD qPCR assay results of 34 patients.[6] The ALK-staining patterns were “cytoplasmic and nuclear”, and confirmed as *NPM::ALK* variants, in 50 patients, while other patterns were observed in four patients. As with the other *ALK* variants, the *MYH9::ALK* fusion variant was confirmed using karyotyping in one patient. For six of the 61 patients, ALK-staining patterns or karyotyping data were not available. When both bone marrow and peripheral blood samples were available for a patient, we used the MDD results for the sample with a higher NCN in the analysis of prognosis. The 3-year progression-free and overall survival rates of the 61 patients analyzed for the 3′*ALK* MDD assay were 83.5% (95% confidence interval [95% CI]: 75.528-91.821) and 96.6% (95% CI: 94.368-99.149), respectively. The clinical and biological characteristics of the patients who underwent the 3′*ALK* MDD assay are shown in Table 1.

For 3′*ALK* analysis, we initially determined whether the 3′*ALK* universal probe and primers could work using *NPM::ALK* samples and *MYH9::ALK* variants. For *NPM::ALK* variants, copy number amplifications were observed in both *NPM::ALK* and 3′*ALK* assays. However, only the 3′*ALK* assay could detect copy number amplification for *MYH9::ALK* variants (Figure 1A, B). Low-amplitude droplets, differentiated from high-amplitude positive droplets, were observed around an amplitude of 2,000 in the 3′*ALK* assays. These low-amplitude droplets were also observed in negative control samples from HL-60 cells and in peripheral blood from healthy controls (6 samples), and did not exceed an amplitude of 2,500. We considered these low-amplitude droplets to be non-specific amplifications caused by the 3′*ALK* probe and set the threshold at an amplitude of 2,500. Subsequently, we assessed the correlation between the NCN of the *NPM::ALK* dPCR and 3′*ALK* dPCR assays for 87 samples from 50 patients with an *NPM::ALK* fusion variant confirmed by their ALK-staining pattern. Forty-four samples were negative for *NPM::ALK*. Of these 44 samples, 15 were negative for *NPM::ALK* and 3′*ALK* assays whereas 29 were negative for the *NPM::ALK* assay and showed <10 NCN for 3′*ALK*. Of all 87 samples, only one showed >30 and ≤30 NCN for the *NPM::ALK* and 3′*ALK* assays, respectively. The NCN of the *NPM::ALK* and 3′*ALK* assays were highly concordant (r=0.855) (Figure 1C). Next, we analyzed the correlation between MDD and patients' survival. As for the *NPM::ALK* MDD dPCR assay, we defined a 30 NCN cutoff for the 3′*ALK* MDD dPCR assay.[9] The 3-year progression-free survival rates of the 21 patients with a NCN >30 and 40 patients with a NCN ≤30 were 56.7% (95% CI: 38.812-72.117) and 97.5% (95% CI: 91.322-100.128), respectively (*P*<0.001) (Figure 1D). The 3-year overall survival rate of patients with a NCN >30 was not significantly different from that of patients with a NCN ≤30 (100% *vs*. 100%, *P*=0.168) (Figure 1E).

MDD with NCN >30 alone exhibited a significant prognostic value in both univariate (*P*=0.004) and multivariate analyses (*P*=0.024) (Table 2). The stage at diagnosis and histological subtype of an "uncommon" component tended to have prognostic value in the univariate analysis; however, these two features showed no significant influence in the multivariate analysis.

In our study, we confirmed that the 3′*ALK* MDD assay using dPCR can be a prognostic tool for newly diagnosed ALK-positive ALCL. We determined a NCN cutoff of 30 based on previous reports on the *NPM::ALK* dPCR assay.[7,9] Non-specific amplification was a concern in the 3′*ALK* MDD assay as the probe is not designed for the fusion gene part; however, we confirmed that the NCN cutoff of 30 is appropriate for this assay. On the 3′*ALK* MDD assay using dPCR, we found that 3-year progression-free survival rates were significantly lower in patients with MDD showing a NCN >30 than in those with a NCN ≤30, consistent with the previous report on the *NPM::ALK* MDD assay using qPCR.[4-6] The 3-year progression-free survival rates for patients with NCN >30 and ≤30 using the 3′*ALK* dPCR assay in our study were higher than those reported in a previous study on MDD using the *NPM::ALK* dPCR assay (NCN >30 and ≤30: 57% and 98% *vs*. 33% and 79%, respectively).[9] This difference could be attributed to the differences in 3-year progression-free survival rates for the entire cohort between our study (84%) and the previous *NPM::ALK* dPCR report (74%).[9]

It has been reported that there is complete concordance between the ALK staining pattern and *ALK* fusion partner variant.[11,12] ALK is expressed in the nucleus in *NPM::ALK*-positive ALCL, and is absent in ALCL with other *ALK* fusion variants. The presence of an *NPM::ALK* fusion transcript

Table 1. Patients' characteristics according to minimal disseminated disease in the 3′*ALK* droplet polymerase chain reaction assay.

	Minimal disseminated disease			*P*
	All patients	NCN ≤30	NCN >30	
N of patients	61	40	21	-
Gender, N (%)				
Male	42	27 (68)	15 (71)	1.000
Female	19	13 (32)	6 (29)	
Age, N (%)				
<10 years	28	17 (43)	11 (52)	0.590
≥10 years	33	23 (57)	10 (48)	
Stage, N (%)				
I	7	7 (18)	0 (0)	0.004
II	18	16 (40)	2 (10)	
III	31	15 (37)	16 (76)	
IV	5	2 (5)	3 (14)	
B symptom, N (%)				
No	33	27 (68)	6 (29)	0.006
Yes	28	13 (32)	15 (71)	
Peripheral LN, N (%)				
No	14	8 (20)	6 (29)	0.527
Yes	47	32 (80)	15 (71)	
Mediastinum, N (%)				
No	41	32 (80)	9 (43)	0.005
Yes	20	8 (20)	12 (57)	
Skin, N (%)				
No	52	33 (83)	19 (90)	0.479
Yes	9	7 (17)	2 (10)	
Bone, N (%)				
No	45	31 (78)	14 (67)	0.376
Yes	16	9 (22)	7 (33)	
Bone marrow, N (%)				
No	56	38 (95)	18 (86)	0.329
Yes	5	2 (5)	3 (14)	
Visceral organs, N (%)				
No	53	36 (90)	17 (81)	0.429
Yes	8	4 (10)	4 (19)	
Histology, N (%)				
Common	40	29 (73)	11 (52)	0.199
Non-common	15	7 (17)	8 (38)	
Not available	6	4 (10)	2 (10)	

NCN: normalized copy number, LN: lymph nodes.

can also be confirmed using two-color fluorescence *in situ* hybridization (FISH) or RT-PCR; however, other ALK fusion variants cannot be evaluated for MDD using the NPM::ALK method. The 3′*ALK* MDD method can be used for all cases of ALK-positive ALCL after confirming ALK positivity by immunostaining; thus, this method is promising and suitable for MDD assessment at diagnosis. In the current study, only one of the fusion partners in ALCL with cytoplasmic ALK

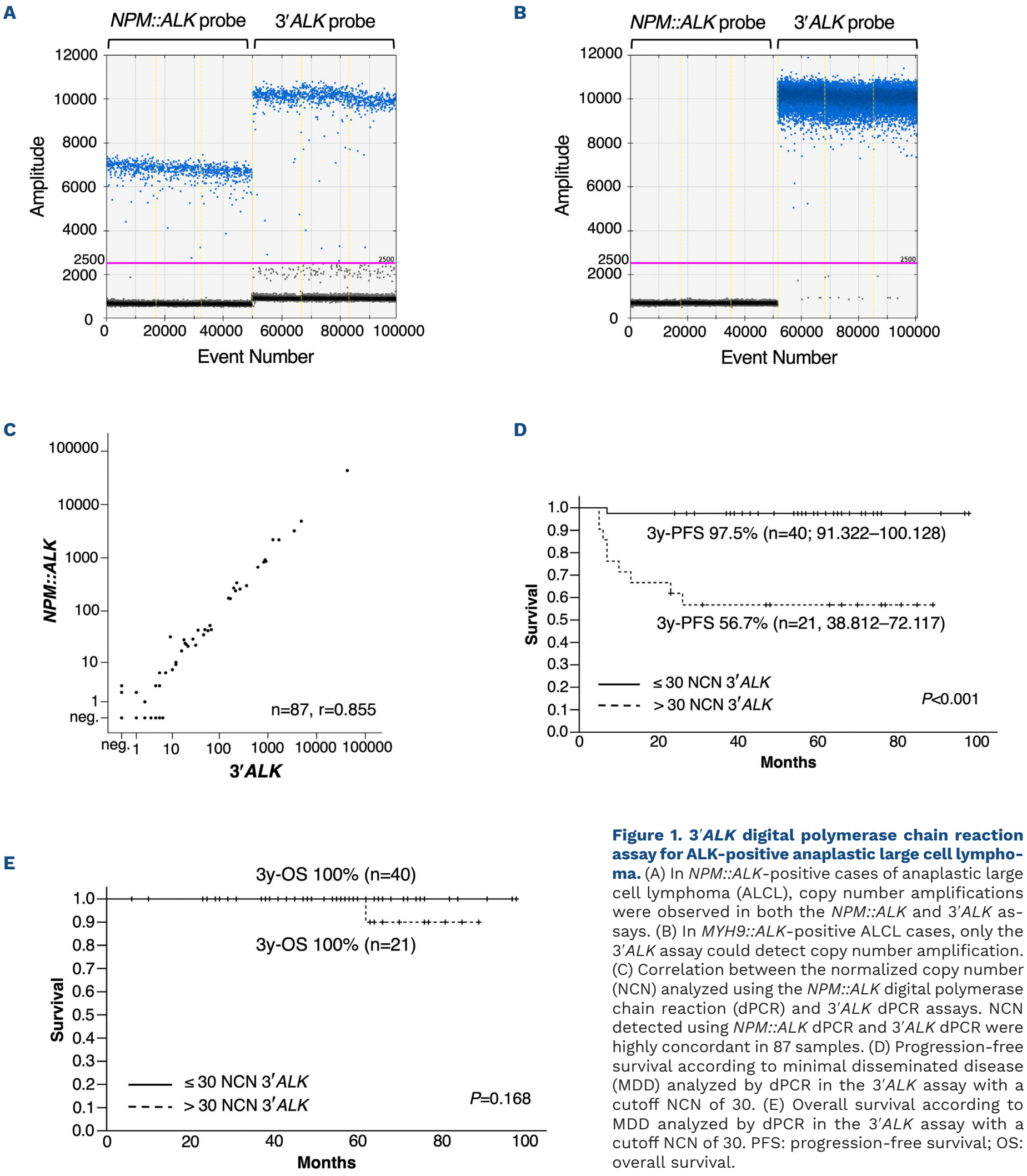

Figure 1. 3′*ALK* digital polymerase chain reaction assay for ALK-positive anaplastic large cell lymphoma. (A) In *NPM::ALK*-positive cases of anaplastic large cell lymphoma (ALCL), copy number amplifications were observed in both the *NPM::ALK* and 3′*ALK* assays. (B) In *MYH9::ALK*-positive ALCL cases, only the 3′*ALK* assay could detect copy number amplification. (C) Correlation between the normalized copy number (NCN) analyzed using the *NPM::ALK* digital polymerase chain reaction (dPCR) and 3′*ALK* dPCR assays. NCN detected using *NPM::ALK* dPCR and 3′*ALK* dPCR were highly concordant in 87 samples. (D) Progression-free survival according to minimal disseminated disease (MDD) analyzed by dPCR in the 3′*ALK* assay with a cutoff NCN of 30. (E) Overall survival according to MDD analyzed by dPCR in the 3′*ALK* assay with a cutoff NCN of 30. PFS: progression-free survival; OS: overall survival.

Table 2. Cox proportional hazard model of progression-free survival rates.

Univariate analysis	Hazard ratio	95% CI	*P*
Stage III/IV	6.883	0.872-54.346	0.067
B symptom	2.963	0.766-12.461	0.116
Mediastinum	2.139	0.619-7.393	0.229
Skin	1.484	0.315-6.995	0.618
Visceral organs	0.707	0.090-5.578	0.742
Histology: non-common type	3.612	0.969-13.464	0.056
3′*ALK* MDD NCN >30	21.160	2.675-167.411	0.004
Multivariate analysis			
Stage III/IV	1.596	0.158-16.110	0.692
Histology: non-common type	2.159	0.571-8.168	0.257
3′*ALK* MDD NCN >30	14.823	1.436-153.044	0.024

95% CI: 95% confidence interval; ALK: anaplastic lymphoma kinase; MDD: minimal disseminated disease; NCN: normalized copy number.

expression could be confirmed. To establish the efficacy of the 3′*ALK* universal probe, further evaluation of the 3′*ALK* assay for other *ALK* fusion variants is required.

Our findings suggest that 3′*ALK* MDD assessments are useful for patients with ALK-positive ALCL as 3′*ALK* MDD can be assessed without the need to confirm the *ALK* fusion partners by karyotyping, FISH, RT-PCR, or sequencing. In this era of targeted therapy, stratification of patients with ALK-positive ALCL based on MDD assessment using dPCR can help establish new standard treatments.

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Contributions

RF and YIY designed the study. RF, YIY and YS performed the experiments. RF, YIY, YS, TY, SH, and TM analyzed and interpreted the data. RF and YIY wrote the manuscript. RF, YIY, AMS, TT, MS, TM, and KH collected the clinical data and samples. HI and AN performed the pathological review. All authors read and approved the final manuscript.

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Data-sharing statement

For original data, please contact fukano.r@yamaguchi-u.ac.jp.

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