TABLE 2. Individual Characteristics of the *NUP98-NSD1* Signature-Positive Patients

ID	Age (y)	Sex	FAB	Cytogenetic aberrations	*NUP98-NSD1*	Risk	CR	Relapse	SCT	Class I mutations	Class II mutations	Other mutations	Survival (m)
A106	5	F	M1	50,XX,+6,+8,del (9q?),+21,+22	+	Off study	+	–	CR 1	*FLT3*-ITD	–	–	63+
A188	10	M	U/C	Normal	+	High	+	+	CR 2	*FLT3*-ITD	–	*WT1*	21
A282	2	M	M5	46,XY,del(9)(q13q22)	+	Intermediate	+	–	CR 1	*FLT3*-ITD	–	–	43+
A325	5	M	M5	Normal	+	High	+	+	CR 2	*FLT3*-ITD	–	–	15
A333	15	M	M4	Normal	+	Intermediate	+	+	–	*NRAS*	–	–	31
A335	6	M	M4	Normal	+	Intermediate	+	–	CR 1	*KRAS*+*KIT*	–	*WT1*	7[a]
A044	6	M	M2	Normal	–	Intermediate	+	+	–	–	–	–	30
A059	12	F	M7	Normal	–	Intermediate	+	+	CR 2	*FLT3*-ITD	–	*WT1*	16
A089	9	F	M4	Normal	–	Off study	–	–	Non-CR	*FLT3*-ITD	*MLL*-PTD	–	25
A154	8	F	M0	Normal	–	High	+	+	CR 2	*FLT3*-ITD	–	–	20
A167	2	M	M5	Normal	–	High	+	–	CR 1	–	*MLL*-PTD	–	55+
A171	12	M	M1	46,XY,t(1;7;11) (q32;p15;p15)	–	Intermediate	+	–	CR 1	–	*MLL*-PTD	*WT1*	55+
A173	13	M	M1	Normal	–	Intermediate	+	–	CR 1	*FLT3*-ITD +*KIT*	*MLL*-PTD	*WT1*	54+
A199	10	F	M5	46,XX,add(10)(p11.2), del(11)(q13q23)	–	Intermediate	+	+	–	*KIT*+*KRAS*	–	–	41
A202	15	F	M4	Normal	–	Intermediate	+	–	–	–	*NPM1*	–	50+
A211	14	F	M1	47,XX,+8	+[b]	Intermediate	+	+	CR 2	*FLT3*-ITD	*MLL*-PTD	–	14
A234	13	M	M4	46,XY, t(6;11) (q27;q23)	–	Intermediate	+	+	–	*KRAS*	*MLL*-PTD	–	29
A243	5	F	M1	Normal	–	Intermediate	+	+	–	*FLT3*-ITD	–	–	8
A245	11	F	M4	Normal	–	Intermediate	+	–	–	*FLT3*-TK (D835)	*NPM1*	–	46+
A249	0	M	M4	46,XY,t(5;6) (q33;q22)	–	Low	+	–	–	–	–	–	46+
A259	5	M	M5	46,XY,t(6;9) (p23;q34)	–	Intermediate	+	–	CR 1	*FLT3*-TK (D835) + *KRAS*	–	–	48+
A297	6	M	M5	Normal	–	Off study	–	–	–	*FLT3*-ITD	*NPM1*	–	6
A299	13	F	M4	Normal	–	Intermediate	+	+	–	–	*MLL*-PTD	–	27
A355	13	M	M2	46,XY,t(15;17) (q13;q11)	–	Intermediate	+	+	–	*FLT3*-ITD	–	–	15

Abbreviations: y, years; F, female; M, male; FAB, French-American-British subtype; U/C, unclassified; CR, complete remission; SCT, stem cell transplantation; CR 1, first CR; CR 2, second CR; m, month; +, alive. The *NUP98-NSD1*-positive cases are indicated in bold type.

[a]A335 died of severe GVHD and acute pneumonia.

[b]Another type of *NUP98-NSD1* fusion transcript was identified by additional RT-PCR using another forward primer.

TABLE 3. Clinical and Molecular Characteristics of the *NUP98-NSD1* Signature Positive and Negative Cases

	NUP98-NSD1 signature (+)	*NUP98-NSD1* signature (−)	*P*-value
Total ($n = 124$)	24	100	
Age (range), y	8.8 (0–15)	6.7 (0–15)	0.069
Mean WBC (-10^9/l) (range)	74.4 (2.3–329.0)	49.7 (1.0–440.0)	0.025
Gender			1.00
Male	14 (58.3%)	57 (57.0%)	
Female	10 (41.7%)	43 (43.0%)	
FAB subtype[a]			
M0	1 (4.2%)	3 (3.0%)	
M1	5 (20.8%)	14 (14.0%)	
M2	2 (8.3%)	39 (39.0%)	
M3	0 (0.0%)	10 (10.0%)	
M4	8 (33.3%)	9 (9.0%)	
M5	6 (25.0%)	11 (11.0%)	
M6	0 (0.0%)	1 (1.0%)	
M7	1(4.2%)	12 (12.0%)	
Unclassified	1 (4.2%)	1 (1.0%)	
Cytogenetics			
Normal	15 (62.5%)	11 (11.0%)	<0.001
t(8;21)(q22;q22)	0 (0.0%)	41 (41.0%)	<0.001
t(15;17)(q22;q12)	0 (0.0%)	10 (10.0%)	0.21
inv(16)(p13q22)	0 (0.0%)	6 (6.0%)	0.60
abnormal 11q23	2 (8.3%)	9 (9.0%)	1.00
del(9q)	2 (8.3%)	0 (0.0%)	0.036
Others	5 (20.8%)	23 (23.0%)	1.00
Gene mutations			
FLT3-ITD	12 (50.0%)	6 (6.0%)	<0.001
DNMT3A	0 (0.0%)	0 (0.0%)	1.00
NPM1	3 (12.5%)	0 (0.0%)	0.007
KIT	3 (12.5%)	12 (12.0%)	1.00
NRAS	1 (4.2%)	9 (9.0%)	0.69
KRAS	4 (16.7%)	9 (9.0%)	0.28
WT1	5 (20.8%)	6 (6.0%)	0.037
MLL-PTD	7 (29.2%)	14 (14.0%)	0.12

[a]*NUP98-NSD1* signature-positive patients were significantly associated with the M4 and M5 subtypes (14/24; $P < 0.001$) when compared with *NUP98-NSD1* signature-negative patients.

patients (4-year OS: 25% vs. 50%), although the difference was not significant ($P = 0.400$).

We further analyzed the prognostic significance of *NUP98-NSD1*-positive patients and *NUP98-NSD1*-like patients other than those with t(15;17) and Down syndrome because they represent distinct AML entities. All 10 patients with t(15;17) and all six patients with Down syndrome were *NUP98-NSD1* signature negative. The outcome of the six patients with *NUP98-NSD1* gene fusion was significantly worse than that of the *NUP98-NSD1* signature-negative patients in OS ($P < 0.001$; 4-year OS: 33.3% vs. 85.7%; Fig. 2C) and in EFS ($P = 0.022$; 4-year EFS: 33.3% versus 70.2%; Fig. 2D). Furthermore, the outcome of the 18 *NUP98-NSD1*-like AML patients was significantly worse than that of the *NUP98-NSD1* signature-negative patients in OS ($P < 0.001$; 4-year OS: 38.9% versus 85.7%; Fig. 2C) and in EFS ($P = 0.002$; 4-year EFS: 38.9% versus 70.2%; Fig. 2D).

DISCUSSION

In this study, we found 24 patients with *NUP98-NSD1*-related gene expression signature, including six with the *NUP98-NSD1* gene fusion (*NUP98-NSD1*-positive) and 18 without (*NUP98-NSD1*-like). This signature represented 19% (24/124) of all pediatric AML patients and 58% (15/26) of all cytogenetically normal cases (Fig. 3). Our results also revealed that the *NUP98-NSD1* signature, irrespective of the presence of the *NUP98-NSD1* fusion, is a novel poor prognostic factor in AML.

The relationship between *NUP98-NSD1*-positive AML and *NUP98-NSD1*-like AML resembles that of *BCR-ABL*-positive acute lymphoblastic

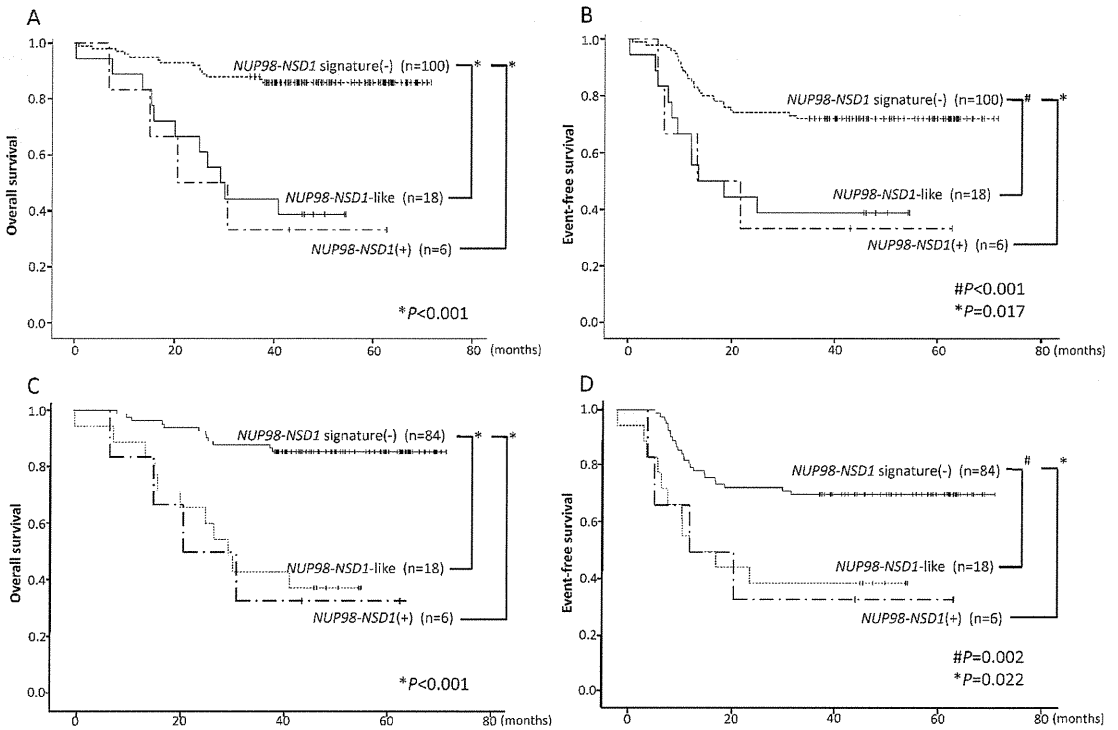

Figure 2. Survival based on *NUP98-NSD1* status by Kaplan-Meier method. Comparison of *NUP98-NSD1*-positive, *NUP98-NSD1*-like, and *NUP98-NSD1* signature-negative patients as regards OS (A) and EFS (B). Comparison of *NUP98-NSD1*-positive, *NUP98-NSD1*-like patients, and *NUP98-NSD1* signature-negative patients other than t(15;17) and Down syndrome, as regards OS (C) and EFS (D).

TABLE 4. Multivariate Analysis of OS of the Pediatric AML Patients

	P-value	SE	HR	Lower CI	Upper CI
NUP98-NSD1 signature	0.005	0.38	2.89	1.38	6.02
FLT3-ITD	0.005	0.40	3.06	1.41	6.63
WT1	0.20	0.54	0.50	0.18	1.43
NPM1	0.49	1.05	0.48	0.06	3.77
t(8;21)	0.18	0.46	0.54	0.22	1.32
del(9q)	0.97	371.69	<0.001	<0.001	–
Initial WBC	0.10	0.002	1.00	1.00	1.01

Abbreviations: SE, standard error; HR, hazard ratio; CI, confidence interval.

leukemia (ALL) and *BCR-ABL*-like ALL (Den Boer et al., 2009; Mullighan et al., 2009). In their gene expression analyses, a significant number of genetically unclassified B-cell precursor ALL patients clustered together with *BCR-ABL*-positive patients, and these *BCR-ABL*-like patients had a poor prognosis, similar to *BCR-ABL*-positive patients. Recently, it was reported that some *BCR-ABL*-like patients express tyrosine kinase/cytokine receptor gene-related fusion genes, such as *NUP214-ABL1*, *EBF1-PDGFRB*, *BCR-JAK2*, and *STRN3-JAK2* (Roberts et al., 2012). Thus, as potentially important fusions might be detected in *NUP98-NSD1*-like patients, we performed RT-PCR using various primer sets for detecting *NUP98-HOXA9*, *NUP98-HOXA11*, *NUP98-HOXA13*, *NUP98-TOP1*, *NUP98-PRRX1*, *NUP98-DDX10*, *NUP98-MLL*, *NUP98-NSD3*, *DEK-NUP214*, *MLL-MLLT4*, and other junction points of *NUP98-NSD1* (Supporting Information Table S2). As a result, t(6;9)/*DEK-NUP214*, t(1;7;11)/*NUP98-HOXA13*, and t(6;11)/*MLL-MLLT4*, which are well-known poor prognostic markers, were found in some *NUP98-NSD1*-like patients in our study (Table 2). Furthermore, another type of *NUP98-NSD1* fusion transcript was identified in a *NUP98-NSD1*-like patient (Supporting Information Fig. S4). This fusion transcript has previously been reported in an adult patient with refractory anemia with excess blasts (La Starza et al., 2004). It is likely that other *NUP98-NSD1*-like patients also have unknown fusion genes with the same functions as *NUP98-NSD1* gene fusion.

It has been reported that hematological malignancies with *NUP98*-fusion genes are strongly

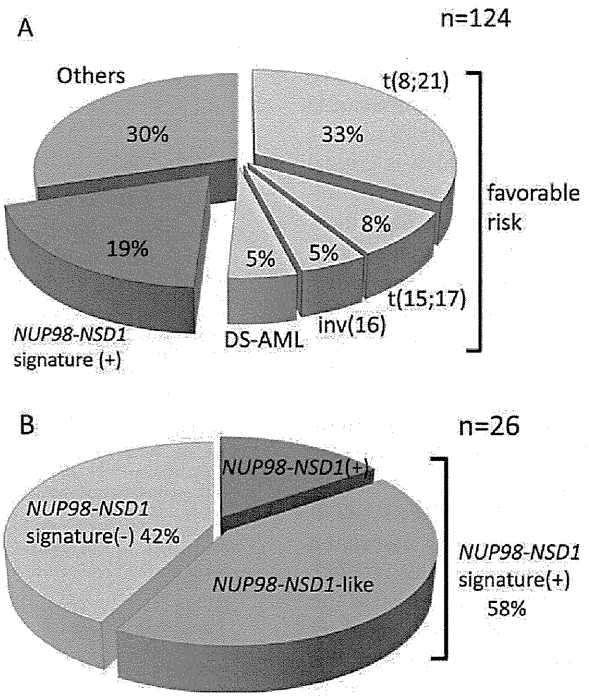

Figure 3. The *NUP98-NSD1* signature is frequent in pediatric AML. Frequencies of *NUP98-NSD1* signature-positive patients in the total pediatric AML cohort (A) and in cytogenetically normal AML (B).

associated with class I mutations (Chou et al., 2009; Taketani et al., 2010). Interestingly, *NUP98-NSD1*-positive patients had only class I aberrations, except for *WT1* mutations, although *NUP98-NSD1*-like patients frequently had Class II aberrations (*NPM1* mutations, *MLL*-PTD, and the *NUP98-HOXA13*, *MLL-MLLT4*, and *DEK-NUP214* fusion genes) in addition to class I aberrations. Because the *NUP98-NSD1* fusion is considered to act as a Class II aberration, no additional Class II aberrations might be necessary for leukemogenesis in *NUP98-NSD1*-positive patients.

The frequency of *FLT3*-ITD was higher than other mutations in both *NUP98-NSD1*-positive and *NUP98-NSD1*-like patients. Many studies have shown that *FLT3*-ITD has a negative impact on outcome in both adult and pediatric AML patients. In fact, in this study, *FLT3*-ITD was an independent poor prognostic factor in addition to the *NUP98-NSD1* signature (Table 4). This suggests that the *NUP98-NSD1* signature and *FLT3*-ITD play a key potential role in AML patients with poor prognosis.

Among the 100 *NUP98-NSD1* signature-negative patients, 23 patients relapsed and of those patients, nine died. On the other hand, of the 24 *NUP98-NSD1* signature-positive patients, 13 patients relapsed and all of those died. Except for 2 patients who did not achieve CR, 12 of the 22 *NUP98-NSD1* signature-positive patients received allo-SCT. Six of the seven patients (86%) who received allo-SCT in first CR were still alive without relapse; however, all five patients who received allo-SCT in second CR died. Only three patients were alive among the 10 patients who were treated with chemotherapy alone. Thus, allo-SCT is recommended in first CR of *NUP98-NSD1* signature-positive patients.

In the *NUP98-NSD1* signature-positive patients, the *HOXA9*, *HOXA10*, *HOXB3*, *HOXB5*, and *HOXB6* genes were up-regulated (Fig. 1). *NUP98* is frequently fused to homeobox genes, and some *NUP98*-homeobox and *NUP98*-non-homeobox fusion genes were revealed to activate *HOXA* cluster genes in hematopoietic cells (Gough et al., 2011). Overexpression of some *HOX* genes is known to enhance the self-renewal of hematopoietic stem and progenitor cells and to perturb differentiation (Grier et al., 2005). It is expected that the aberrant expression of *HOX* genes plays an important role in the leukemogenesis of AML displaying the *NUP98-NSD1* signature. In addition,

two transcription factor genes, *PRDM16* and *NKX2–3*, were markedly up-regulated (Fig. 1). *PRDM16* (also known as *MEL1*) was originally isolated as a translocated gene in t(1;3)(p36;q21) AML (Mochizuki et al., 2000) and encodes a zinc finger protein with a PR domain, which is critical for the establishment and maintenance of the hematopoietic stem cell pool (Agulio et al., 2011). *NKX2–3* is an NKX family homeobox gene (Pabst et al., 1999), whose involvement in leukemogenesis has not been reported; however, its highly homologous paralog *NKX2–5* is rearranged and ectopically expressed in T-cell ALL with t(5;14)(q35;q32) and t(5;14)(q35;q11.2) (Nagel et al., 2003; Przybylski et al., 2006). Taken together, these data suggest that both *PRDM16* and *NKX2–3* play an important role in leukemogenesis.

Our results indicate that the *NUP98-NSD1*-related gene expression signature is associated with a poor outcome in addition to the *NUP98-NSD1* gene fusion in pediatric AML. Most of the patients displaying the *NUP98-NSD1* signature were classified into an intermediate risk group, but their unfavorable outcome suggests that a high-risk group is a more suitable stratification. Although further investigations are necessary, we believe that our work contributes to improving the risk stratification of pediatric AML.

ACKNOWLEDGMENTS

The authors would like to thank the following Japanese childhood AML Cooperative Study Group institutions and investigators that participated in the study: Akira Morimoto, Department of Pediatrics, Kyoto Prefectural University of Medicine; Ryoji Kobayashi, Department of Pediatrics, Hokkaido University School of Medicine; Hiromasa Yabe, Department of Pediatrics, Tokai University School of Medicine; Kazuko Hamamoto, Department of Pediatrics, Hiroshima Red Cross Hospital; Shigeru Tsuchiya, Department of Pediatric Oncology, Institute of Development, Aging and Cancer, Tohoku University; Yuichi Akiyama, Department of Pediatrics, National Hospital Organization Kyoto Medical Center; Hisato Kigasawa, Department of Hematology, Kanagawa Children's Medical Center; Akira Ohara, Department of First Pediatrics, Toho University School of Medicine; Hideki Nakayama, Department of Pediatrics, Hamanomachi Hospital; Kazuko Kudo, Department of Pediatrics, Nagoya University Graduate School of Medicine; and Masue Imaizumi, Department of Hematology/Oncology, Miyagi Prefectural Children's Hospital.

REFERENCES

Agulio F, Avagyan S, Labar A, Sevilla A, Lee DF, Kumar P, Lemischka IR, Zhou BY, Snoeck HW. 2011. Prdm16 is a physiologic regulator of hematopoietic stem cells. Blood 117:5057–5066.

Arai Y, Hosoda F, Kobayashi H, Kawakami K, Takahara J. 1997. The inv(11)(p15q22) chromosome translocation of de novo and therapy-related myeloid malignancies results in fusion of the nucleoporin gene, NUP98, with the putative RNA helicase case, DDX10. Blood 89:3936–3944.

Brown J, Jawad M, Twigg SR, Saracoglu K, Sauerbrey A, Thomas AE, Eils R, Harbott J, Kearney L. 2002. A cryptic t(5;11)(q35;p15.5) in 2 children with acute myeloid leukemaia with apparently normal karyotypes, identified by a multiplex fluorescence in situ hybridization telomere assay. Blood 99:2526–2531.

Casas S, Aventín A, Nomdedéu J, Sierra J. 2003. Cryptic t(5;11)(q35;p15.5) in adult de novo acute myelocytic leukemia with normal karyotype. Cancer Genet Cytogenet 145:183.

Cerveira N, Correia C, Dória S, Bizarro S, Rocha P, Gomes P, Torres L, Norton L, Borges BS, Castedo S, Teixeira MR. 2003. Frequency of NUP98-NSD1 fusion transcript in childhood acute myeloid leukaemia. Leukemia 17:2244–2247.

Chou WC, Chen CY, Hou HA, Lin LI, Tang JL, Yao M, Tsay W, Ko BS, Wu SJ, Huang SY, Hsu SC, Chen YC, Huang YN, Tseng MH, Huang CF, Tien HF. 2009. Acute myeloid leukemia bearing t(7;11)(p15;p15) is a distinct cytogenetic entity with poor outcome and a distinct mutation profile: comparative analysis of 493 adult patients. Leukemia 23:1303–1310.

Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JG, Peters ST, Van Zutven LJ, Beverloo HB, Van der Spek PJ, Escherich G, Horstmann MA, Janka-Schaub GE, Kamps WA, Evans WE, Pieters R. 2009. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. Lancet Oncol 10:125–134.

Döhner K, Schlenk RF, Habdank M, Scholl C, Rücker FG, Corbacioglu A, Bullinger L, Fröhling S, Döhner H. 2005. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 106:3740–3746.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868.

Frohling S, Scholl C, Gilliland DG, Levine RL. 2005. Genetics of myeloid malignancies: Pathogenetic and clinical implications. J Clin Oncol 23:6285–6295.

Gough SM, Slape CI, Aplan PD. 2011. NUP98 gene fusions and hematopoietic malignancies: Common themes and new biological insights. Blood 118:6247–6257.

Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. 2005. The pathophysiology of HOX genes and their role in cancer. J Pathol 205:154–171.

Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Pratcorona M, Abbas S, Kuipers JE, van Galen JF, Beverloo HB, Sonneveld E, Kaspers GJ, Trka J, Baruchel A, Zimmermann M, Creutzig U, Reinhardt D, Pieters R, Valk PJ, Zwaan CM. 2011. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood 118:3645–3656.

Ichikawa H, Tanabe K, Mizushima H, Hayashi Y, Mizutani S, Ishii E, Hongo T, Kikuchi A, Satake M. 2006. Common gene expression signatures in t(8;21)- and inv(16)-acute myeloid leukemia. Br J Haematol 135:336–347.

Imaizumi M, Tawa A, Hanada R, Tsuchida M, Tabuchi K, Kigasawa H, Kobayashi R, Morimoto A, Nakayama H, Hamamoto K, Kudo K, Yabe H, Horibe K, Tsuchiya S, Tsukimoto I. 2011. Prospective study of a therapeutic regimen with all-trans retinoic acid and anthracyclines in combination of cytarabine in children with acute promyelocytic leukaemia: the Japanese childhood acute myeloid leukaemia cooperative study. Br J Haematol 152:89–98.

Jaju RJ, Fidler C, Haas OA, Strickson AJ, Watkins F, Clark K, Cross NC, Cheng JF, Aplan PD, Kearney L, Boultwood J,

Wainscoat JS. 2001. A novel gene, NSD1, is fused to NUP98 in the t(5;11)(q35;p15.5) in de novo childhood acute myeloid leukemia. Blood 98:1264–1267.

Jo A, Tsukimoto I, Ishii E, Asou N, Mitani S, Shimada A, Igarashi T, Hayashi Y, Ichikawa H. 2009. Age-associated difference in gene expression of paediatric acute myelomonocytic lineage leukaemia (FAB M4 and M5 subtypes) and its correlation with prognosis. Br J Haematol 144:917–929.

Kudo K, Kojima S, Tabuchi K, Yabe H, Tawa A, Imaizumi M, Hanada R, Hamamoto K, Kobayashi R, Morimoto A, Nakayama H, Tsuchida M, Horibe K, Kigasawa H, Tsukimoto I. 2007. Prospective study of a pirarubicin, intermediate-dose cytarabine, and etoposide regimen in children with Down syndrome and acute myeloid leukemia: The Japanese Childhood AML Cooperative Study Group. J Clin Oncol 25:5442–5447.

La Starza R, Gorello P, Rosati R, Riezzo A, Veronese A, Ferrazzi E, Martelli MF, Negrini M, Mecucci C. 2004. Cryptic insertion producing two NUP98/NSD1 chimeric transcripts in adult refractory anemia with an excess of blasts. Genes Chromosomes Cancer 41:395–399.

Marcucci G, Haferlach T, Döhner H. 2011. Molecular genetics of adult acute myeloid leukemia: Prognostic and therapeutic implications. J Clin Oncol 29:475–486.

Mochizuki N, Shimizu S, Nagasawa T, Tanaka H, Taniwaki M, Yokota J, Morishita K. 2000. A novel gene, MEL1, mapped to 1p36.3 is highly homologous to the MDS1/EVI1 gene and is transcriptionally activated in t(1;3)(p36;q21)-positive leukemia cells. Blood 96:3209–3214.

Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, Ma J, Liu W, Cheng C, Schulman BA, Harvey RC, Chen IM, Clifford RJ, Carroll WL, Reaman G, Bowman WP, Devidas M, Gerhard DS, Yang W, Relling MV, Shurtleff SA, Campana D, Borowitz MJ, Pui CH, Smith M, Hunger SP, Willman CL, Downing JR. 2009. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med 360:470–480.

Nagel S, Kaufmann M, Drexler HG, MacLeod RA. 2003. The cardiac homeobox gene NKX2–5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2). Cancer Res 63:5329–5334.

Nakamura T. 2005. NUP98 fusion in human leukemia: Dysregulation of the nuclear pore and homeodomain proteins. Int J Hematol 82:21–27.

Nakamura T, Largaespada DA, Lee MP, Johnson LA, Ohyashiki K, Toyama K, Chen SJ, Willman CL, Chen IM, Feinberg AP, Jenkins NA, Copeland NG, Shaughnessy JD Jr. 1996. Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. Nature Genet 12:154–158.

Nebral K, König M, Schmidt HH, Lutz D, Sperr WR, Kalwak K, Brugger S, Dworzak MN, Haas OA, Strehl S. 2005. Screening for NUP98 rearrangements in hematopoietic malignancies by fluorescence in situ hybridization. Haematologica 90:746–752.

Pabst O, Zweigerdt R, Arnold HH. 1999. Targeted disruption of the homeobox transcription factor Nkx2–3 in mice results in postnatal lethality and abnormal development of small intestine and spleen. Development 126:2215–2225.

Panarello C, Rosanda C, Morerio C. 2002. Cryptic translocation t(5;11)(q35;p15.5) with involvement of the NSD1 and NUP98 genes without 5q deletion in childhood acute myeloid leukemia. Genes Chromosomes Cancer 35:277–281.

Przybylski GK, Dik WA, Grabarczyk P, Wanzeck J, Chudobska P, Jankowski K, von Bergh A, van Dongen JJ, Schmidt CA, Langerak AW. 2006. The effect of a novel recombination between the homeobox gene NKX2–5 and the TRD locus in T-cell acute lymphoblastic leukemia on activation of the NKX2–5 gene. Haematologica 91:317–321.

Pui CH, Carroll WL, Meshinchi S, Arceci RJ. 2011. Biology, risk stratification, and therapy of pediatric acute leukemias: An update. J Clin Oncol 29:551–565.

Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, Chen SC, Payne-Turner D, Churchman ML, Harvey RC, Chen X, Kasap C, Yan C, Becksfort J, Finney RP, Teachey DT, Maude SL, Tse K, Moore R, Jones S, Mungall K, Birol I, Edmonson MN, Hu Y, Buetow KE, Chen IM, Carroll WL, Wei L, Ma J, Kleppe M, Levine RL, Garcia-Manero G, Larsen E, Shah NP, Devidas M, Reaman G, Smith M, Paugh SW, Evans WE, Grupp SA, Jeha S, Pui CH, Gerhard DS, Downing JR, Willman CL, Loh M, Hunger SP, Marra MA, Mullighan CG. 2012. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 22:153–166.

Romana SP, Radford-Weiss I, Ben Abdelali R, Schluth C, Petit A, Dastugue N, Talmant P, Bilhou-Nabera C, Mugneret F, Lafage-Pochitaloff M, Mozziconacci MJ, Andrieu J, Lai JL, Terre C, Rack K, Cornillet-Lefebvre P, Luquet I, Nadal N, Nguyen-Khac F, Perot C, Van den Akker J, Fert-Ferrer S, Cabrol C, Charrin C, Tigaud I, Poirel H, Vekemans M, Bernard OA, Berger R. 2006. NUP98 rearrangements in hematopoietic malignancies: a study of the Groupe Francophone de Cytogénétique Hématologique. Leukemia 20:696–706.

Sano H, Shimada A, Taki T, Murata C, Park MJ, Sotomatsu M, Tabuchi K, Tawa A, Kobayashi R, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Hayashi Y. 2012. RAS mutations are frequent in FAB type M4 and M5 of acute myeloid leukemia, and related to late relapse: A study of the Japanese Childhood AML Cooperative Study Group. Int J Hematol 95:509–515.

Shiba N, Taki T, Park MJ, Shimada A, Sotomatsu M, Adachi S, Tawa A, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Arakawa H, Hayashi Y. 2012. DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia. Br J Haematol 156:413–414.

Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Hayashi Y. 2006. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood 107:1806–1809.

Shimada A, Taki T, Tabuchi K, Taketani T, Hanada R, Tawa A, Tsuchida M, Horibe K, Tsukimoto I, Hayashi Y. 2008. Tandem duplications of MLL and FLT3 are correlated with poor prognoses in pediatric acute myeloid leukemia: a study of the Japanese childhood AML Cooperative Study Group. Pediatr Blood Cancer 50:264–269.

Taketani T, Taki T, Shibuya N, Ito E, Kitazawa J, Terui K, Hayashi Y. 2002a The HOXD11 gene is fused to the NUP98 gene in acute myeloid leukemia with t(2;11)(q31;p15). Cancer Res 62:33–37.

Taketani T, Taki T, Ono R, Kobayashi Y, Ida K, Hayashi Y. 2002b. The chromosome translocation t(7;11)(p15;p15) in acute myeloid leukemia results in fusion of the NUP98 gene with a HOXA cluster gene, HOXA13, but not HOXA9. Genes Chromosomes Cancer 34:437–443.

Taketani T, Taki T, Shibuya N, Kikuchi A, Hanada R, Hayashi Y. 2002c Novel NUP98-HOXC11 fusion gene resulted from a chromosomal break within exon 1 of HOXC11 in acute myeloid leukemia with t(11;12)(p15;q13). Cancer Res 62:4571–4574.

Taketani T, Taki T, Nakamura T, Kobayashi Y, Ito E, Fukuda S, Yamaguchi S, Hayashi Y. 2010. High frequencies of simultaneous FLT3-ITD, WT1 and KIT mutations in hematological malignancies with NUP98-fusion genes. Leukemia 24:1975–1977.

Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, Yabe H, Nakayama H, Kudo K, Kobayashi R, Hamamoto K, Imaizumi M, Morimoto A, Tsuchiya S, Hanada R. 2009. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: The AML99 trial from the Japanese Childhood AML Cooperative Study Group. J Clin Oncol 27:4007–4013.

Walter MJ, Payton JE, Ries RE, Shannon WD, Deshmukh H, Zhao Y, Baty J, Heath S, Westervelt P, Watson MA, Tomasson MH, Nagarajan R, O'Gara BP, Bloomfield CD, Mrózek K, Selzer RR, Richmond TA, Kitzman J, Geoghegan J, Eis PS, Maupin R, Fulton RS, McLellan M, Wilson RK, Mardis ER, Link DC, Graubert TA, DiPersio JF, Ley TJ. 2009. Acquired copy number alterations in adult acute myeloid leukemia genomes. Proc Natl Acad Sci USA 106:12950–12955.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2013, Article ID 238528, 9 pages
http://dx.doi.org/10.1155/2013/238528

Clinical Study

Polymorphisms of *MTHFR* Associated with Higher Relapse/Death Ratio and Delayed Weekly MTX Administration in Pediatric Lymphoid Malignancies

Hiroko Fukushima,[1] Takashi Fukushima,[1] Aiko Sakai,[2] Ryoko Suzuki,[2] Ryoko Nakajima-Yamaguchi,[2] Chie Kobayashi,[1] Atsushi Iwabuchi,[2] Makoto Saito,[1] Ai Yoshimi,[3] Tomohei Nakao,[3] Keisuke Kato,[3] Masahiro Tsuchida,[3] Hideto Takahashi,[4] Kazutoshi Koike,[3] Nobutaka Kiyokawa,[5] Emiko Noguchi,[6] and Ryo Sumazaki[1]

[1] *Department of Child Health, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[2] *Department of Pediatrics, University of Tsukuba Hospital, Japan*
[3] *Department of Hematology/Oncology, Ibaraki Children's Hospital, Japan*
[4] *Department of Epidemiology, Faculty of Medicine, University of Tsukuba, Japan*
[5] *Department of Pediatric Hematology and Oncology Research, National Research Institute for Child Health and Development, Japan*
[6] *Department of Genetics, Faculty of Medicine, University of Tsukuba, Japan*

Correspondence should be addressed to Takashi Fukushima; tksfksm@md.tsukuba.ac.jp

Received 24 July 2013; Accepted 10 October 2013

Academic Editor: Massimo Breccia

Backgrounds. Outcome of childhood malignancy has been improved mostly due to the advances in diagnostic techniques and treatment strategies. While methotrexate (MTX) related polymorphisms have been under investigation in childhood malignancies, many controversial results have been offered. *Objectives*. To evaluate associations of polymorphisms related MTX metabolisms and clinical course in childhood lymphoid malignancies. *Method*. Eighty-two acute lymphoblastic leukemia and 21 non-Hodgkin's lymphoma children were enrolled in this study. Four single nucleotide polymorphisms in 2 genes (*MTHFR* (rs1801133/c.677C>T/p.Ala222Val and rs1801131/c.1298A>C/p.Glu429Ala) and *SLCO1B1* (rs4149056/c.521T>C/p.V174A and rs11045879/c.1865+4846T>C)) were genotyped by Taqman PCR method or direct sequencing. Clinical courses were reviewed retrospectively. *Results*. No patient who had the AC/CC genotype of rs1801131 (*MTHFR*) had relapsed or died, in which distribution was statistically different among the AA genotype of rs1801131 ($P = 0.004$). Polymorphisms of *SLCO1B1* (rs11045879 and rs4149056) were not correlated with MTX concentrations, adverse events, or disease outcome. *Conclusions*. Polymorphisms of *MTHFR* (rs1801131) could be the plausive candidate for prognostic predictor in childhood lymphoid malignancies.

1. Introduction

Childhood cancer is a rare disease affecting 1 in 70,000 children aged 14 years and younger [1, 2]. Lymphoid malignancy, including leukemia and lymphoma, is the most common childhood cancer, accounting for 40% of all pediatric malignancies [2]. During the last 20 years, survival rates for pediatric acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma (NHL) have improved dramatically, mostly due to improvement of chemotherapy, allogeneic hematopoietic stem cell transplantation, and diagnostic techniques, with expected cure rates higher than 80% for pediatric lymphoid malignancy [1–3].

Methotrexate (MTX) is one of the key drugs for cancer treatment and a proven critical component for pediatric ALL and NHL [1, 4, 5]. MTX interrupts the folic acid cycle by inhibiting two enzymes (Figure 1). Firstly, as an analog of folate, MTX is a powerful competitive inhibitor of dihydrofolate reductase (DHFR) [6, 7]. DHFR is responsible for converting folates to their active form tetrahydrofolate,

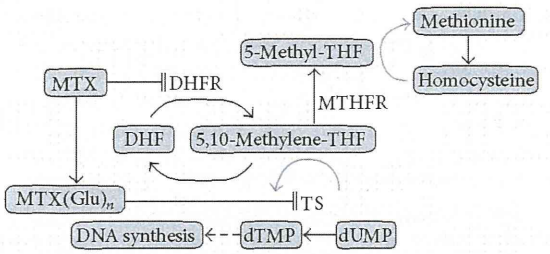

FIGURE 1: Schematic representation of methotrexate action. MTX inhibits folic metabolism through two mechanisms: MTX inhibits DHFR, which leads to the depletion of THF compounds, resulting in impairment of purine and thymidine synthesis. Polyglutamated MTX inhibits TS directly, which causes depletion of DNA synthesis. MTX: methotrexate; DHFR: dihydrofolate reductase; DHF: dihydrofolate; THF: tetrahydrofolate; TS: thymidylate synthase; MTHFR: methylenetetrahydrofolate reductase; MTX $(Glu)_n$: MTX glutamates; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate.

a substrate of thymidylate synthase (TS), to convert deoxyuridine monophosphate to deoxythymidine-5′-monophosphate resulting in DNA synthesis. Secondly, the polyglutamated forms of MTX inhibit the activity of TS directly [6, 8]. High-dose MTX (HD-MTX) treatment has been proven for its efficacy for the treatment of ALL and NHL [4, 5]. However, MTX often causes toxicity such as renal failure, hepatotoxicity, and severe mucositis requiring a dose reduction and cessation of treatment or hemodialysis, and it is well known that large interindividual MTX kinetic variability exhibits [9]. Therefore, it is beneficial to find patients with a high risk of developing adverse events before the initiation of the treatment [9, 10]. In folate metabolism, methylenetetrahydrofolate reductase (MTHFR) is a key molecule to convert 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF), and 5,10-methylene-THF is a substrate of TS [11]. There are two extensively examined *MTHFR* polymorphisms, rs1801133 (c.677C>T, p.Ala222Val) and rs1801131 (c.1298A>C, p.Glu429Ala), that have been shown to have lower enzyme activity when they carry mutant alleles [12].

A previous study reported that the T allele of *MTHFR* rs1801133 was associated with an increased risk of relapse events but not associated with MTX concentrations nor adverse events and that rs1801131 was not associated with altered risks of relapse nor toxicity in 520 pediatric ALL patients by the Children's Oncology Group [13]. Others showed conflicting results, showing that patients with the TT genotype of *MTHFR* rs1801133 resulted in a better overall survival rate in 126 Brazilian pediatric ALL patients treated with MTX [14], and others reported that patients carrying the T allele of rs1801133 and the A allele of rs1801131 (*MTHFR* c.T677A1298 haplotype) had a lower event free survival [15], and T allele of *MTHFR* rs1801133 and C allele of *MTHFR* rs1801131 had higher relapse ration [16].

Transporters such as adenosine triphosphate-binding cassette (ABC) transporters and organic anion transporters were also reported to act for MTX disposition [17]. Solute carrier organic anion transporter family member 1B1 (SLCO1B1) is one of the organic anion transporters and localized at the sinusoidal membrane of hepatocytes, and its transcript has been detected in enterocytes. *SLCO1B1* transfected cells were proven to uptake MTX in vitro, as well as other compounds such as estradiol, bilirubin, bile acids, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), rifampicin, angiotensin-converting enzyme inhibitors, and the active metabolite of irinotecan, SN-38 [18–20]. Tirona et al. reported *SLCO1B1* polymorphisms (rs56101265, rs5606188, rs4149056, rs55901008) altered in transport of substrates in vitro [19]. Kameyama et al. reported *SLCO1B1* polymorphisms rs4149056 (c.521T>C, p.V174A) transfected HEK cells, decreasing transporting activities [21]. Children who underwent HD-MTX for ALL who carry *SLCO1B1* polymorphism rs11045879 (c.1865+4846T>C) and rs4149056 were shown to have lower MTX elimination in a genome-wide-association-study (GWAS), whose cohort mostly consisted of Caucasians and a limited number of Asians [20, 22]. These polymorphisms in *SLCO1B1* were confirmed associations with a higher MTX concentration at 72 hrs among Spanish B-ALL children [22, 23]. Japanese patients treated with MTX due to rheumatoid arthritis showed no association between *SLCO1B1* polymorphisms (rs4149056 and rs2306283 (c.388A>G, p.N432D)) and MTX concentration nor disease status [24].

Although many studies have been conducted to investigate associations between *MTHFR* polymorphisms and MTX toxicity in pediatric ALL patients, results derived from these studies are conflicting. Study populations were mostly Caucasians and reports from Asians were limited [10, 25]. Allele frequencies of polymorphisms differ in each ethnic population, and their effects can be influenced by different chemotherapy protocols, genetic backgrounds, and others [13, 26, 27].

In the present study, we genotyped 2 polymorphisms in *MTHFR* (rs1801133 and rs1801131) and 2 more polymorphisms in *SLCO1B1* (rs4149056 and rs11045879) in Japanese ALL/NHL patients treated with HD-MTX and examined the relationship between genotypes and prognosis/adverse events.

2. Method

2.1. Objectives. To investigate associations of polymorphisms of *MTHFR* (rs1801133 and rs1801131) and *SLCO1B1* (rs4149056 and rs11045879) and prognosis or clinical course including MTX concentrations in Japanese children who developed lymphoid malignancy treated with HD-MTX.

2.2. Patients. Eighty-two acute lymphoblastic leukemia (ALL) and 21 non-Hodgkin lymphoma (NHL) children were enrolled in the present study. All patients were treated at two main and exclusively pediatric malignancy treating regional hospitals in Ibaraki prefecture (University of Tsukuba Hospital and Ibaraki Children's Hospital) between November 1993 and November 2012. Patients' characteristics are shown in Table 1.

Table 1: Patient characteristics.

Diagnosis	ALL	BCP	69
		T	9
		Other	4
	NHL	Burkitt	10
		DLBCL	2
		T	5
		Other	4
1CR/relapsed/dead			89/8/6
Sex	M : F		62 : 41
Age at diagnosis (y)			7.43 (0.2–19.2)

ALL: acute lymphoblastic leukemia; NHL: non-Hodgkin's lymphoma; BCP: B-cell precursor leukemia; T: T-cell linage; CR: complete remission.

Informed consent was obtained from each parent/guardian or patient. The study was approved by the ethics committee of the University of Tsukuba in accordance with the Ethical Guidelines for Human Genome/Gene Analysis Research of the Ministry of Health, Labor and Welfare of Japan and the Declaration of Helsinki.

2.3. Treatment, Methotrexate Concentration, and Toxicity Evaluation. All patients received intravenous MTX continuously at 3 g/m^2/12 hrs or 24 hrs, or 5 g/m^2/24 hrs with folic acid rescue following the protocol of the Tokyo Children's Cancer Study Group (TCCSG) L99-15 [28], ALL-BFM 95 [29], or NHL B9604 [30].

Monitoring of MTX concentration in plasma was carried out every day until the concentration was below 0.1 μmol/L.

Toxicity data were retrospectively collected objectively, blinded genotypes, from the patients' medical files. Toxicity was graded according to the Common Terminology Criteria for Adverse Event (CTCAE) v4.0 released from the Cancer Therapy Evaluation Program of the National Cancer Institute (http://ctep.cancer.gov/). The highest grade of toxicity observed for each patient during the MTX therapy period was recorded. Data were collected including vomiting, diarrhea, serum hepatic enzyme (ALT), serum bilirubin and renal toxicity (serum creatinine), and MTX concentrations at 48 and 72 hrs after infusion. MTX levels were considered high if the concentration was above 1.0 μmol/L at 48 hrs or 0.1 μmol/L at 72 hrs. Above 1.5 mg/dL was considered as hyperbilirubinemia.

Patients who developed at least one adverse event (maximum/base line creatinine ratio higher than 1.5, ALT elevation more than grade 1 according to the criteria of CTCAE v.4.0, T-Bil elevation ≥1.5 mg/dL) were categorized as having global toxicity.

2.4. Genotyping. DNA was extracted using Genomic DNA Isolation Kit (QiAamp DNA Blood Mini Kit, QiAamp DNA Blood Midi Kit or QIAamp DNA FFPE Tissue: Qiagen, Vealo, The Netherlands) from 0.5 to 2 mL peripheral blood, bone marrow, or paraffin-embedded bone marrow/tissue in complete remission following the manufacturer's instructions.

Polymorphisms of *MTHFR* (c.677C>T, rs1801133, c.1298A>C, rs1801131) and *SLCO1B1* (c.1865+4846T>C, rs11045879, c.521T>C, rs4149056) were genotyped using the TaqMan Assay-on-Demand SNP Typing System (Applied Bio Systems, Foster City, CA, USA) following the manufacturer's instructions. PCR was performed on a 384-well format with 5 ng of DNA each, and automatic allele calling was performed using ABI PRISM 7900HT data collection and analysis software, version 2.2.2 (Applied Biosystems).

The accuracy of the genotyping for rs1801131 in children who had relapsed or died was confirmed by direct sequence using the primers 5′-TTTGGGGAGCTGAAGGACTA-3′ (forward) and 5′-CTTTGTGACCATTCCGGTTT-3′ (reverse) as reported by Shimasaki et al. [31] and the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

2.5. Statistics. Deviation from Hardy-Weinberg expectations was examined by chi-square test. Control genotype frequencies were obtained from HapMap database (HapMap JPT, http://www.ncbi.nlm.nih.gov/) and Genome Medicine Database of Japan (GeMDBJ, https://gemdbj.nibio.go.jp/dgdb/index.do) which consisted of healthy Japanese individuals and have been genotyped using Illumina Sentrix HumanHap550 Genotyping BeadChip and InfiniumHD 610-Quad BeadChip (Illumina, San Diego, CA), and principal component analysis has been performed to remove relatives, duplications, and Han Chinese (https://gemdbj.nibio.go.jp/dgdb/web/common/Help.jsp#manual). The genetic effects of the association between the case-control status and each individual SNP were assessed by chi-square test or Fisher's exact test.

For the analysis of associations among adverse events during MTX treatment and polymorphisms, the worst value of toxicity markers (ALT elevation, bilirubin elevation, or creatinine ratio) that each patient experienced during all HD-MTX courses and polymorphisms of *MTHFR* and *SLCO1B1* were assessed by chi-square test.

To confirm the suitability of MTX concentrations as a toxicity marker, the associations between different toxicity markers (ALT elevation, bilirubin elevation, or creatinine ratio) and MTX plasma concentration at 48 and 72 hrs among evaluable 149 courses conducted for ALL in total were examined by chi-square test or Fisher's exact test.

Analysis was conducted using SPSS, version 12.0 (SPSS Inc. Chicago, IL, USA). A P value <0.05 was considered statistically significant.

3. Results

Children enrolled in our study consisted of 82 patients diagnosed as ALL (B-cell precursor 69, T-lineage 9, others 4) and 21 children diagnosed as NHL (Burkitt 10, T-lineage 5, Diffuse large B cell lymphoma 2, others 4). The number of patients who were in their first complete remission was 89, relapsed 8, and died 6. The average age at diagnosis was 7.4 (0.2–19.2) years old. There were 62 boys and 41 girls. Patients' characteristics are shown in Table 1.

Table 2 shows the genotype of *MTHFR* and *SLCO1B1* in patients and controls Hapmap JPT obtained from the NCBI

Table 2: Allele frequencies.

Gene	Reference SNP ID	Genotype	Our study (n = 103)	HWE P	HapmapJPT† (n = 172)	P	GeMDBJ‡ (*MTHFR* c.677C>T: n = 2,375 *MTHFR* c.1298A>C: n = 1,428 *SLCO1B1* c.521T>C: n = 1,427)	P
MTHFR c.677 C>T	rs1801133	CC	32		68		864	
		CT	61		84		1123	
		TT	10	0.049	20	0.442	388	0.885
MTHFR c.1298 A>C	rs1801131	AA	68		112		952	
		AC	31		56		435	
		CC	4	0.633	4	0.924	41	0.765
SLCO1B1	rs11045879	TT	42		70		n.d.	
		TC	43		74		n.d.	
		CC	18	0.495	28	0.896	n.d.	n.d.
SLCO1B1 c.521 T>C	rs4149056	TT	73		138		1048	
		TC	26		30		347	
		CC	4	0.686	4	0.066	32	0.408

Difference of each allele frequency was calculated by χ^2 test.

†NCBI Hapmap JPT: database at National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov).

The submitted SNP numbers to NCBI were ss65837366 for *MTHFR* C677T, ss76885974 for *MTHFR* A1298C, ss15510724 for *SLCO1B1* rs11045879, and ss105439952 for *SLCO1B1* T521C.

‡GeMDBJ: Genome Medicine Database of Japan (https://gemdbj.nibio.go.jp/dgdb/index.do).

Table 3: Outcome according to each genotype in all patients.

Gene	Locus or reference SNP ID		Patients number according to genotype	Relapsed/died of disease
MTHFR	C677T	CC	32	5
		CT/TT	70	8
			P	0.540
MTHFR	A1298C	AA	67	13
		AC/CC	35	0
			P	0.004
SLCO1B1	rs11045879	TT	42	6
		TC/CC	60	7
			P	0.696
SLCO1B1	T521C	TT	73	8
		TC/CC	29	5
			P	0.511
MTHFR	677CT/TT and 1298AA	no	18	0
		yes	53	8
			P	0.105

All patients who had relapsed or died of disease had *MTHFR* c.1298AA genotype.

database and GeMDBJ. Deviation from Hardy-Weinberg expectations was examined by chi-square test and was applied to each of SNPs (P = 0.049 for rs1801133, P = 0.633 for rs1801131, P = 0.495 for rs11045879, and P = 0.686 for rs4149056). Differences of allele frequencies were assessed by chi-square test and there were no differences with control data. Among Hapmap JPT, the P value for rs1801133 was 0.442, rs1801131 was 0.924, rs11045879 was 0.896, and rs4149056 was 0.066. Among GeMDBJ, the P value for rs1801133 was 0.885, rs1801131 was 0.765, and rs4149056 was 0.408.

The outcome and each of polymorphisms are shown in Table 3. Thirty-four of 89 children in their first complete remission carried at least one C allele of rs1801131 (*MTHFR* c.1298AC/CC genotype), and no children who relapsed/died carried this genotype, in which frequency was significantly different (P = 0.004). Figure 2 shows event free survival according to the genotype of *MTHFR* c.1298A>C (rs1801131).

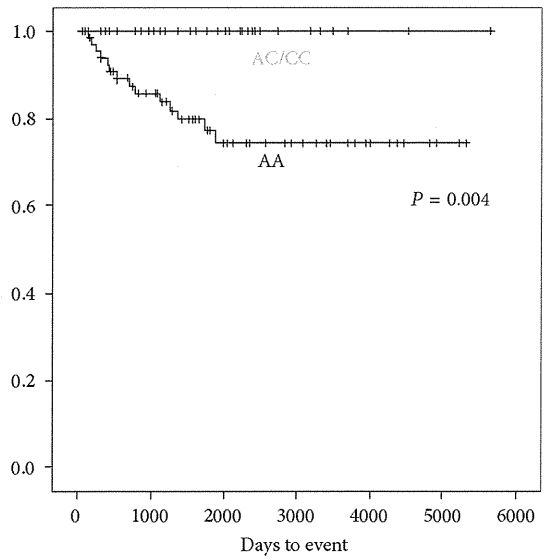

FIGURE 2: Event free survival according to *MTHFR* c.1298AA or AC/CC. Figure shows event free survival according to *MTHFR* c.1298A>C.

All assessable weekly 3 g/m^2 HD-MTX courses following the treatment protocol of TCCSG L99-15 for standard or high risk ALL patients totaled 149. For assessing the associations of adverse events during each MTX administration and each genotype, we analyzed these homogeneously treated 149 courses of weekly HD-MTX (Table 4). Courses undergone for children with the AA genotype of rs1801131 (*MTHFR* c.1298AA) tended to correlate to MTX concentration of higher than 1.0 μmol/L at 48 hrs after administration but not statistically significant (P = 0.06), and no other genotypes were correlated to MTX concentrations.

Only 9 of 49 courses undertaken for children with the CC genotype of rs1801133 (*MTHFR* c.677CC) developed Common Terminology Criteria for Adverse Event (CTCAE) v4.0 more than grade 1 ALT elevation, whereas 41 of 100 courses undertaken for children with at least one T allele of rs1801133 (*MTHFR* c.677CT/TT) developed elevated serum liver enzyme (P = 0.006). Thirty-eight of 96 courses undertaken for children with the AA genotype of rs1801131 (*MTHFR* c.1298AA) resulted in elevated serum liver enzyme, 11 of 52 with at least one C allele of rs1801131 (*MTHFR* c.1298AC/CC genotype) resulted in ALT elevation (P = 0.036). Only 8 of 32 courses conducted with patients who carried the CC genotype of rs1801133 (*MTHFR* c.677CC) resulted in the next MTX administration being delayed more than 5 days due to adverse events such as serum hepatic enzyme, renal toxicity, or infection, as 33 of 69 courses for patients who carry at least one T allele of rs1801133 (*MTHFR* c.677CT/TT genotype) resulted in the next MTX administration being delayed which had been planned to have been conducted weekly (P = 0.030).

No adverse events such as elevated MTX concentration, ALT elevation, hyperbilirubinemia, or prognosis were statistically correlated to two polymorphisms in *SLCO1B1* (rs11045879 and rs4149056) (Tables 2 and 3).

MTX concentrations and adverse events were analyzed by chi-square test (Table 5). Elevated MTX concentrations at 48 hour were associated with higher creatinine ratio (P < 0.001) and delayed weekly MTX administration (P = 0.013).

4. Discussion

High dose methotrexate is highly effective for broad cancers including lymphoid malignancies such as leukemia and lymphoma. However, its adverse events differ among each ethnicity and could significantly affect its clinical course. Previously, genetic polymorphisms of MTX pathway protein were reported as one of the predictable values.

With recent excellent advances in the prognosis among childhood lymphoid malignancy, the rate of patients who relapsed or died is very limited. It is vitally important to detect the factors that influence the prognosis in this limited number of poorer prognostic patient cohort other than factors that have already been thoroughly examined. In our study, all children who had relapsed or died carried the AA genotype of rs1801131 (P = 0.004). Previous studies using Caucasian ALL patients reported that the T allele of rs1801133 was correlated with poor prognosis, but the polymorphism of rs1801131 was not associated with the prognosis of the patients [13]. On the contrary, the TT genotype of rs1801133 and the AC genotype of rs1801131 were reported to be associated with lower overall survival in Brazilian patients with pediatric ALL [14], and the T allele of rs1801133 and the A allele of rs1801131 (*MTHFR* c.T677A1298 haplotype) had a lower event free survival among Caucasians [15].

Substitution of A to C of rs1801131 causes an amino acid change of glutamine to alanine at the position 429 in *MTHFR* and showed lower enzyme activity in vitro [12], and lymphoblasts with the AC genotype of rs1801131 required higher MTX concentrations in order to inhibit 50% of the control TS activity than those with the CC genotype of rs1801131 [32]. Our results showing the AA genotype of rs1801131 to be associated with poor prognosis might be explained by the fact that lower MTHFR enzyme activity due to substitution of A to C of rs1801131 leads to reduced conversion of 5,10-methylene-THF to 5-methyl-THF, resulting in more substrate for TS, thereby leading to more DNA synthesis and lesser MTX sensitivity as reported in a previous ex vivo study [32].

Previously, rs1801133 (*MTHFR* c.677C>T) was reported as a candidate predictor of MTX lower elimination, but its results are controversial and many different results have been reported [10, 11, 23]. Trevino et al. discovered significant associations among lower MTX elimination and polymorphisms in *SLCO1B1* (rs11045879 and rs4149056). Lopez-Lopez et al. confirmed this result in Spanish population [20, 23]. Most of those researches were conducted among Caucasians and studies among Japanese were limited [24]. In our study population, all patients consisted of Japanese, and association study among *SLCO1B1* polymorphism in pediatric lymphoid malignancies had not been conducted previously.

MTX concentration at 48 hrs was one of the strong predictors to develop a delay in the next course of MTX

Table 4: Clinical adverse event, delayed course, and polymorphisms during MTX 3 g/m^2 courses in total 149.

Gene and locus or reference SNP ID	Number of courses according to genotype		MTX concentration (μmol/L) at 48 hours ≥1.00	at 72 hours ≥0.10	Creatine elevation	ALT elevation	T Bil (mg/dL) ≥1.50	Assessable course for duration	Delayed course (more than 5 days)
MTHFR C677T	CC	49	4	17	8	9	11	32	8
	CT/TT	100	11	32	11	41	15	69	33
	P		0.774	0.742	0.360	0.006	0.272	*P*	0.030
MTHFR A1298C	AA	96	13	36	14	38	13	67	30
	AC/CC	53	2	13	5	12	13	34	11
	P		0.060	0.107	0.367	0.036	0.096	*P*	0.230
SLCO1B1 rs11045879	TT	66	6	21	9	25	11	45	18
	TC/CC	83	9	28	10	25	15	56	23
	P		0.687	0.805	0.773	0.319	0.796	*P*	0.913
SLCO1B1 T521C	TT	100	9	30	12	35	14	68	28
	TC/CC	49	6	19	7	15	12	33	13
	P		0.567	0.284	0.694	0.594	0.120	*P*	0.864
MTHFR 677CT/TT and 1298AA	no	27	1	9	3	5	27	18	5
	yes	74	10	28	9	34	73	53	27
	P		0.279	0.678	1.000	0.012	0.194	*P*	0.088

All assessable HD-MTX (3 g/m^2) courses undergone for leukemia were 149 in total. *MTHFR* c.677CT/TT and c.1298AA genotype were associated to hepatotoxicity. ALT assessed as elevated as CTCAE more than grade 1. Elevated creatinine was evaluated with increased serum creatinine more than 1.5 times compared with the value just before the MTX administration.

Table 5: MTX concentrations and ALT/Cre/T-Bil elevations evaluated for 147 courses in total.

MTX serum concentrations		Number of courses according to MTX concentrations	Creatinine ratio >1.5	ALT elevation CTCAE Grade more than 1	Bilirubin elevation >1.5 (mg/dL)	Assessable course for duration	Delayed duration (more than 5 days)
48 hour	<1.0 μmol/L	132	10	46	20	87	31
	>1.0 μmol/L	15	8	3	5	12	9
	P	0.000	0.248	0.138	*P*	0.013	
72 hours	<0.1 μmol/L	100	7	34	13	61	22
	>0.1 μmol/L	49	12	16	13	41	19
	P	0.003	0.870	0.044	*P*	0.252	

One MTX concentration less than 0.1 μmol/L at 48 hours.
Higher MTX concentration at 48 hours were associated to creatinine ratio and bilirubin elevation and MTX concentration at 72 hours were associated with creatinine elevation. No associations between MTX concentration and ALT elevations were found. One patient developed renal toxicity needing hemodialysis whose MTX concentration at 48 hours was 42.71 μmol/L, worst creatinine was 11.9 times higher than before treatment, worst serum ALT was 77 IU/L and total bilirubin was 1.7 mg/dL. This patient never underwent another HD-MTX treatment again.

administration and serum creatinine elevation (Table 5). In our study population, courses conducted for patients carrying the AA genotype of rs1801131 (*MTHFR* c.1298AA) tended to have a higher MTX concentration at 48 hrs; however, this association did not reach statistical significance. Polymorphisms in *SLCO1B1* (rs11045879 and rs4149056) were not correlated to MTX concentrations at 48 hrs ($P = 0.687$ and $P = 0.567$, resp.). The discrepancies among studies previously conducted among Caucasians might be caused by differences of genetic backgrounds, lifestyles, or treatment protocol. This is retrospective study, so in the future a prospective research for Japanese children is warranted.

Polymorphisms in *MTHFR* (rs1801133 and rs1801131) were associated with serum ALT elevation ($P = 0.006$ and $P = 0.036$, resp.). Patients who carried at least one T allele of rs1801133 (*MTHFR* c.677CT/TT genotype) were also associated with more than 5 days of delay of the next MTX administration, which was planned to undergo weekly ($P = 0.03$). Patients with adult acute lymphoblastic leukemia carrying at least one T allele of rs1801133 (*MTHFR* c.677CT/TT genotype) were associated with developing ALT elevation as CTCAE v.4.0 grade more than 1, which was the same result as reported previously [33]. However, this result is different from the two previous studies that were conducted for Japanese children with leukemia or lymphoma and did not show statistical associations between the T allele of rs1801133 and hepatic enzyme elevations [10, 31]. Chiusolo et al. reported that the AC genotype of rs1801131 (*MTHFR* c.1298 AC) showed lower adverse event [8]. de Jonge et al. reported that lymphoblast with the AC genotype of rs1801131 (*MTHFR* c.1298AC) required higher MTX concentrations in order to inhibit 50% of the control TS activity ex vivo [32]. These results suggest that the AA genotype of rs1801131 tends to develop more adverse events, which corresponds to our results.

Significant correlations of polymorphisms in *SLCO1B1* and MTX eliminations were discovered by the GWAS study, which mostly consists of ancestry of European or African descent and a small number of Asian descent [20, 23]. These polymorphisms were not correlated to MTX concentrations at 48 hrs in our study population ($P = 0.529$ for rs11045879, $P = 0.413$ for rs4149056) (Table 4). The differences might be caused from different protocols, ethnicity, or backgrounds, including supportive care or lifestyle and study design.

5. Conclusion

We conducted an association study of polymorphisms (rs1801133 and rs1801131 of *MTHFR* and rs11045879 and rs4149056 of *SLCO1B1*) and adverse events during HD-MTX treatment and prognosis in Japanese childhood lymphoid malignancies.

Patients that carried polymorphisms of rs1801131 (*MTHFR* c.1298AC/CC genotype) had not relapsed nor died. The CT/TT genotype of rs1801133 (*MTHFR* c.677CT/TT genotype) resulted in higher serum hepatic enzyme and delayed administrations during weekly high dose MTX.

Relations of *SLCO1B1* polymorphisms (rs11045879 and rs41490586) in Japanese children were assessed for the first time and found to have no correlations with any adverse events, MTX concentrations or outcome.

Conflict of Interests

The authors herein declare that there is no financial support or relationships that may pose a conflict of interests.

Acknowledgments

The authors are grateful to the Genome Medicine Database of Japan (GeMDBJ) for the approval of the usage of control genotype data. They would like to thank Mr. Charles N. Jones who provided scientific writing assistance.

References

[1] M. O'Leary, M. Krailo, J. R. Anderson, G. H. Reaman, and Children's Oncology Group, "Progress in childhood cancer: 50 years of research collaboration, a report from the children's oncology group," *Seminars in Oncology*, vol. 35, no. 5, pp. 484–493, 2008.

[2] W. T. Johnston, T. J. Lightfoot, J. Simpson, and E. Roman, "Childhood cancer survival: a report from the United Kingdom childhood cancer study," *Cancer Epidemiology*, vol. 34, no. 6, pp. 659–666, 2010.

[3] J. T. Sandlund, J. R. Downing, and W. M. Crist, "Non-Hodgkin's lymphoma in childhood," *The New England Journal of Medicine*, vol. 334, no. 19, pp. 1238–1248, 1996.

[4] B. Camitta, D. Mahoney, B. Leventhal et al., "Intensive intravenous methotrexate and mercaptopurine treatment of higher-risk non-T, non-B acute lymphocytic leukemia: a pediatric oncology group study," *Journal of Clinical Oncology*, vol. 12, no. 7, pp. 1383–1389, 1994.

[5] I. Djerassi and J. S. Kim, "Methotrexate and citrovorum factor rescue in the management of childhood lymphosarcoma and reticulum cell sarcoma (non Hodgkin's lymphomas). Prolonged unmaintained remissions," *Cancer*, vol. 38, no. 3, pp. 1043–1051, 1976.

[6] L. B. Bailey and J. F. Gregory III, "Polymorphisms of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement," *Journal of Nutrition*, vol. 129, no. 5, pp. 919–922, 1999.

[7] L. Huang, W. J. E. Tissing, R. de Jonge, B. D. van Zelst, and R. Pieters, "Polymorphisms in folate-related genes: association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia," *Leukemia*, vol. 22, no. 9, pp. 1798–1800, 2008.

[8] P. Chiusolo, S. Giammarco, S. Bellesi et al., "The role of MTHFR and RFC1 polymorphisms on toxicity and outcome of adult patients with hematological malignancies treated with high-dose methotrexate followed by leucovorin rescue," *Cancer Chemotherapy and Pharmacology*, vol. 69, no. 3, pp. 691–696, 2012.

[9] W. E. Evans, M. V. Relling, J. H. Rodman, W. R. Crom, J. M. Boyett, and C. Pui, "Conventional compared with individualized chemotherapy for childhood acute lymphoblastic leukemia," *The New England Journal of Medicine*, vol. 338, no. 8, pp. 499–505, 1998.

[10] H. Imanishi, N. Okamura, M. Yagi et al., "Genetic polymorphisms associated with adverse events and elimination of methotrexate in childhood acute lymphoblastic leukemia and malignant lymphoma," *Journal of Human Genetics*, vol. 52, no. 2, pp. 166–171, 2007.

[11] M. Krajinovic and A. Moghrabi, "Pharmacogenetics of methotrexate," *Pharmacogenomics*, vol. 5, no. 7, pp. 819–834, 2004.

[12] I. Weisberg, P. Tran, B. Christensen, S. Sibani, and R. Rozen, "A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity," *Molecular Genetics and Metabolism*, vol. 64, no. 3, pp. 169–172, 1998.

[13] R. Aplenc, J. Thompson, P. Han et al., "Methylenetetrahydrofolate reductase polymorphisms and therapy response in pediatric acute lymphoblastic leukemia," *Cancer Research*, vol. 65, no. 6, pp. 2482–2487, 2005.

[14] D. M. de Deus, E. L. de Lima, R. M. S. Silva, E. P. Leite, and M. T. C. Muniz, "2012Influence of Methylenetetrahydrofolate reductase C677T, A1298C, and G80A polymorphisms on the survival of pediatric patients with acute lymphoblastic leukemia," *Leukemia Research and Treatment*, vol. 2012, Article ID 292043, 6 pages, 2012.

[15] M. Krajinovic, E. Lemieux-Blanchard, S. Chiasson, M. Primeau, I. Costea, and A. Moghrabi, "Role of polymorphism in MTHFR and MTHFD1 genes in the outcome of childhood acute lymphoblastic leukemia," *Pharmacogenomics Journal*, vol. 4, no. 1, pp. 66–72, 2004.

[16] N. K. Kuzelicki, M. Milek, J. Jazbec, and I. Mlinaric-Rascan, "5,10-methylenetetrahydrofolate reductase (MTHFR) low activity genotypes reduce the risk of relapse-related acute lymphoblastic leukemia (ALL)," *Leukemia Research*, vol. 33, no. 10, pp. 1344–1348, 2009.

[17] Z. S. Chen and A. K. Tiwari, "Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases," *FEBS Journal*, vol. 278, no. 18, pp. 3226–3245, 2011.

[18] T. Abe, M. Unno, T. Onogawa et al., "LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers," *Gastroenterology*, vol. 120, no. 7, pp. 1689–1699, 2001.

[19] R. G. Tirona, B. F. Leake, G. Merino, and R. B. Kim, "Polymorphisms in *OATP-C*: identification of multiple allelic variants associated with altered transport activity among European- and African-Americans," *Journal of Biological Chemistry*, vol. 276, no. 38, pp. 35669–35675, 2001.

[20] L. R. Trevino, W. Yang, D. French et al., "Germline genomic variants associated with childhood acute lymphoblastic leukemia," *Nature Genetics*, vol. 41, no. 9, pp. 1001–1005, 2009.

[21] Y. Kameyama, K. Yamashita, K. Kobayashi, M. Hosokawa, and K. Chiba, "Functional characterization of SLCO1B1 (OATP-C) variants, SLCO1B1*5, SLCO1B1*15 and SLCO1B1*15+C1007G, by using transient expression systems of HeLa and HEK293 cells," *Pharmacogenetics and Genomics*, vol. 15, no. 7, pp. 513–522, 2005.

[22] S. Radtke, O. Zolk, B. Renner et al., "Germline genetic variations in methotrexate candidate genes are associated with pharmacokinetics, toxicity and outcome in childhood acute lymphoblastic leukemia," *Blood*, vol. 121, no. 26, pp. 5145–5153, 2013.

[23] E. Lopez-Lopez, I. Martin-Guerrero, J. Ballesteros et al., "Polymorphisms of the SLCO1B1 gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia," *Pediatric Blood and Cancer*, vol. 57, no. 4, pp. 612–619, 2011.

[24] T. Kato, A. Hamada, S. Mori, and H. Saito, "Genetic polymorphisms in metabolic and cellular transport pathway of methotrexate impact clinical outcome of methotrexate monotherapy in japanese patients with rheumatoid arthritis," *Drug Metabolism and Pharmacokinetics*, vol. 27, no. 2, pp. 192–199, 2012.

[25] N. Shimasaki, T. Mori, H. Samejima et al., "Effects of methylenetetrahydrofolate reductase and reduced folate carrier 1 polymorphisms on high-dose methotrexate-induced toxicities in children with acute lymphoblastic leukemia or lymphoma," *Journal of Pediatric Hematology/Oncology*, vol. 28, no. 2, pp. 64–68, 2006.

[26] N. S. Kadan-Lottick, K. K. Ness, S. Bhatia, and J. G. Gurney, "Survival variability by race and ethnicity in childhood acute lymphoblastic leukemia," *The Journal of the American Medical Association*, vol. 290, no. 15, pp. 2008–2014, 2003.

[27] C. H. Pui, D. Pei, A. S. Pappo et al., "Treatment outcomes in black and white children with cancer: results from the SEER database and St. Jude Children's Hospital, 1992 through 2007," *Journal of Clinical Oncology*, vol. 30, no. 16, pp. 2005–2012, 2012.

[28] A. Manabe, A. Ohara, D. Hasegawa et al., "Significance of the complete clearance of peripheral blasts after 7 days of prednisolone treatment in children with acute lymphoblastic leukemia: the Tokyo children's cancer study group study L99-15," *Haematologica*, vol. 93, no. 8, pp. 1155–1160, 2008.

[29] A. Moricke, A. Reiter, M. Zimmermann et al., "Risk-adjusted therapy of acute lymphoblastic leukemia can decrease treatment burden and improve survival: treatment results of 2169 unselected pediatric and adolescent patients enrolled in the trial ALL-BFM 95," *Blood*, vol. 111, no. 9, pp. 4477–4489, 2008.

[30] A. Kikuchi, T. Mori, J. Fujimoto et al., "Outcome of childhood B-cell non-Hodgkin lymphoma and B-cell acute lymphoblastic leukemia treated with the Tokyo children's cancer study group NHL B9604 protocol," *Leukemia and Lymphoma*, vol. 49, no. 4, pp. 757–762, 2008.

[31] N. Shimasaki, T. Mori, C. Torii et al., "Influence of MTHFR and RFC1 polymorphisms on toxicities during maintenance chemotherapy for childhood acute lymphoblastic leukemia or lymphoma," *Journal of Pediatric Hematology/Oncology*, vol. 30, no. 5, pp. 347–352, 2008.

[32] R. de Jonge, J. H. Hooijberg, B. D. van Zelst et al., "Effect of polymorphisms in folate-related genes on in vitro methotrexate sensitivity in pediatric acute lymphoblastic leukemia," *Blood*, vol. 106, no. 2, pp. 717–720, 2005.

[33] A. Ongaro, M. de Mattei, M. G. D. Porta et al., "Gene polymorphisms in folate metabolizing enzymes in adult acute lymphoblastic leukemia: effects on methotrexate-related toxicity and survival," *Haematologica*, vol. 94, no. 10, pp. 1391–1398, 2009.

Cancer Science
The official journal of the Japanese Cancer Association

Novel adaptor protein Shf interacts with ALK receptor and negatively regulates its downstream signals in neuroblastoma

Daisuke Takagi,[1,4] Yasutoshi Tatsumi,[1] Tomoki Yokochi,[1] Atsushi Takatori,[1] Miki Ohira,[2] Takehiko Kamijo,[3] Satoshi Kondo,[4] Yoshitaka Fujii[4] and Akira Nakagawara[1,5]

[1]Division of Biochemistry and Innovative Cancer Therapeutics; [2]Laboratory of Cancer Genomics; [3]Division of Molecular Carcinogenesis, Chiba Cancer Center Research Institute, Chiba; [4]Department of Oncology, Immunology and Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

(Received November 15, 2012/Revised January 17, 2013/Accepted January 22, 2012/Accepted manuscript online January 30, 2013/Article first published online March 13, 2013)

Our neuroblastoma cDNA project previously identified *Src homology 2 domain containing F* (*Shf*) as one of the genes expressed at high levels in favorable neuroblastoma. Shf is an adaptor protein containing four putative tyrosine phosphorylation sites and an SH2 domain. In this study, we found that Shf interacted with anaplastic lymphoma kinase (ALK), an oncogenic receptor tyrosine kinase in neuroblastoma. Real-time PCR analysis showed that *Shf* mRNA is highly expressed in non-metastatic neuroblastomas compared to metastatic tumor samples ($P < 0.030$, $n = 106$). Interestingly, patients showing high *ALK* and low *Shf* mRNA expressions showed poor prognosis, whereas low *ALK* and high *Shf* expressions were related to better prognosis ($P < 0.023$, $n = 38$). Overexpression of ALK and siRNA-mediated knockdown of *Shf* yielded similar results, such as an increase in cellular growth and phosphorylation of ALK, in addition to Erk1/2 and signal transducer and activator of transcription 3 (STAT3) that are downstream signals of the ALK-initiated phospho-transduction pathway. Knockdown of *Shf* also increased the cellular mobility and invasive capability of neuroblastoma cells. These results suggest that Shf interacts with ALK and negatively regulates the ALK-initiated signal transduction pathway in neuroblastoma. We thus propose that Shf inhibits phospho-transduction signals mediated by ALK, which is one of the major key players on neuroblastoma development, resulting in better prognosis of the tumor. (*Cancer Sci* 2013; 104: 563–572)

Neuroblastoma, a solid tumor that accounts for 15% of all pediatric cancer deaths, originates from the sympathoadrenal lineage derived from the neural crest. The clinical behavior of neuroblastoma is markedly heterogeneous.[1] Tumors found in patients under 1 year of age yield favorable prognosis frequently accompanied by spontaneous differentiation and regression, whereas those found in older patients grow aggressively, often resulting in fatal outcomes.[1] Despite the recent treatments and care that have been improved, neuroblastoma harboring the amplified *MYCN* oncogene in an advanced stage is closely correlated to poor outcome.[1,2]

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, originally identified as an oncogenic fusion protein nucleophosmin-ALK in anaplastic large cell lymphoma.[3–5] Such unique oncogenic fusion of the *ALK* gene due to chromosomal translocation is responsible for the activation of the ALK signaling pathway in many human cancers including non-small-cell lung cancer.[6–9] Although the expression pattern of ALK in tissues strongly suggests that ALK plays a pivotal role in normal development of the nervous system,[10–12] the molecular mechanism underlying the signal transduction pathway oriented by ALK during neural development and carcinogenesis still remains unclear. Several point mutations that activate the *ALK* gene have been studied in both familial and sporadic cases of neuroblastoma.[13–17] Frequency of point mutations activating ALK in primary neuroblastoma varied between 6% and 11% in these different studies, in which two hot spots of point mutation, *F1174* and *R1275*, were identified.[18] The *F1174* mutation in ALK was linked to a higher degree of autophosphorylation and more potent transforming capacity than the *R1275* mutant.[19] A recent study using transgenic mice indicated that ALK^{F1174L} is sufficient to facilitate neuroblastoma development.[20] In addition, ALK^{F1174L} and MYCN had synergistic effects, as double transgenic mice developed more aggressive neuroblastomas than single transgenic ones of each gene.[21]

Shf (Src homology 2 domain containing F) was originally identified as an adaptor protein homologous to Shb (Src homology 2 domain protein of beta-cells).[22] As the SH2 (Src homology 2) domain[23] at the C-termini is highly conserved among other SH2-containing proteins, they seem to comprise a subfamily of adaptor proteins.[22,24] Although the function of Shf is not fully understood, the SH2 domain is responsible for binding to the platelet-derived growth factor (PDGF)-α receptor at tyrosine 720.[22] Overexpression of Shf significantly decreases the rate of apoptosis induced by PDGF addition, suggesting that Shf is a negative regulator of a receptor-oriented signal pathway.[22]

Our neuroblastoma cDNA project previously identified *Shf* as one of the new genes differentially expressed between favorable and unfavorable subsets of neuroblastoma.[25,26] As we sought to understand how Shf participates in tumorigenesis, the functional relationship between Shf and several receptor tyrosine kinases, such as TrkA and ALK, in neuroblastoma-derived cell lines was examined. Previously, we reported physical interaction between Shf and TrkA.[27] In this work, the regulation of the signal transduction pathway managed by Shf and ALK was investigated in neuroblastoma.

Materials and Methods

Tumor specimens. Neuroblastoma specimens ($n = 106$) used in this study were kindly provided from various institutions and hospitals in Japan to the Chiba Cancer Center Neuroblastoma Tissue Bank (Chiba, Japan). Written informed consent was obtained at each institution or hospital. This study was approved by the Chiba Cancer Center Institutional Review Board. Tumors were classified according to the International Neuroblastoma Staging System (INSS)[28] (25 classified as

[5]To whom correspondence should be addressed.
E-mail: akiranak@chiba-cc.jp

doi: 10.1111/cas.12115

Stage 1; 13 as Stage 2; 31 as Stage 3; 33 as Stage 4; and 4 as Stage 4s). The patients were treated following the protocols proposed by the Japanese Infantile Neuroblastoma Cooperative Study and the Study Group of Japan for Treatment of Advanced Neuroblastoma.[29] Clinical information including age at diagnosis, tumor origin, Shimada histology, prognosis, and survival months of each patient was obtained and used for survival analysis. The median follow-up time for survivors was 52 months (range, 3–208 months). Cytogenetic and molecular biological analysis of all tumors was also carried out by assessing DNA ploidy, *MYCN* amplification, and *TrkA* expression.

Cell culture and transfection. Human neuroblastoma cell lines, SK-N-AS, NLF, SK-N-DZ, and SH-SY5Y were obtained from the CHOP cell line bank (Philadelphia, PA, USA) and maintained in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen), in a humidified atmosphere of 5% CO_2 at 37°C. Human embryonic kidney-derived cell line 293T cells were obtained from Riken BRC Cell Bank (Tsukuba, Japan) and were cultured in DMEM (Nissui) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. For transient expression, cells were transfected with the indicated expression plasmids using FuGene HD (Roche Applied Science, Mannheim, Germany). For knockdown of endogenous expressions, cells were transfected with 20 nmol/L of indicated siRNAs using Lipofectamine RNAiMax (Invitrogen) and On-Target plus SmartPool (Thermo Fisher Scientific, Waltham, MA, USA). The siRNAs specific to Shf (NM_138356) and ALK (NM_004304) were purchased from Dharmacon (Lafayette, CO, USA).

Cell viability, motility, and invasion assay. Transfected cells were seeded into 96-well plates at 5×10^3 cells/well. Cell viability was measured using a Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan). A BD cell culture insert (#353097) for cell motility assay, and a BD Biocoat Matrigel invasion chamber (#354480) for cell invasion assay were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Cells were seeded at 2.5×10^4 cells/well and incubated for 23 h in a migratory assay and 27 h in an invasion assay. Migratory cells that penetrated pores on the membrane were fixed with 100% methanol followed by Giemsa staining, and were counted using a conventional light microscope.

Semiquantitative RT-PCR and real-time quantitative RT-PCR. Total RNA was prepared from cultured cells and human tissues, and reverse transcribed using random primers and SuperScript II (Invitrogen), as described previously.[30] Primer sequences for human *Shf* and *GAPDH* mRNA were as follows: Shf-F, 5′-tatgagccagaggaggatgg-3′; Shf-R, 5′-ggccaaggtaggtctttgatg-3′; GAPDH-F, 5′-accacagtccatgccatcac-3′; GAPDH-R, 5′-tccaccaccctgttgctgta-3′. Expression level of *GAPDH* was used as a control. Real-time quantitative RT-PCR was carried out using an ABI PRISM 7500 System (PerkinElmer, Boston, MA, USA). TaqMan probes for Shf (Hs00403125_m1), ALK (Hs00608292_m1), and GAPDH (4310884E) were purchased from Applied Biosystems (Carlsbad, CA, USA). All reactions were carried out in triplicate experiments. The X^2 independence test was used to explore possible associations between expression levels of *Shf* and other factors. Cox regression models were used to explore associations between *Shf*, *ALK*, *TrkA*, ploidy, age, *MYCN*, and survival. $P < 0.05$ was considered significant.

Antibodies. Antibodies were as follows: rabbit anti-Shf antibody raised against SH2 domain and Anti-HA-tag antibody (#561; MBL, Aichi, Japan); human ALK antibodies (#M7195; Dako, Glostrup, Denmark) (#IM3312; Beckman Coulter, Brea, CA, USA); anti-phospho-ALK (Tyr1604) antibody (#3341), anti-Myc-tag antibody (#2276), anti-p44/p42 MAPK, Erk1/2 antibody (#9102), anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibody (#9101), anti-signal transducer and activator of transcription 3 (STAT3) antibody (#4904), and anti-phospho-STAT3 (Tyr705) antibody (#4113) (Cell Signaling Technology, Danvers, MA, USA); and anti-actin antibody (#sc-8432; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunoblotting. Cells were lysed in CHAPS cell extract buffer, separated by 10% SDS-PAGE and transferred onto PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA). Membranes were incubated with appropriate primary antibodies at room temperature for 2 h, then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Immunoreactive bands were visualized using the ECL system (GE Healthcare, Chalfont St Giles, UK). Developed signals were analyzed using a LAS-4000 imager (GE Healthcare).

Immunoprecipitation. Transfected 293T cells lysed in CHAPS cell extract buffer were mixed with indicated antibodies and rotated for 3 h at 4°C. The immune complexes were precipitated with Protein G (GE Healthcare) Sepharose beads for 1 h of incubation at 4°C by rotation. Beads were then washed with Wash buffer (50 mM PIPES, 2 mM EDTA, 150 mM NaCl, 0.1% Triton X-100); immunoprecipitated proteins were eluted from beads using 100 mM glycine (pH 2.5), boiled with SDS sample buffer, and immunoblotted.

Immunofluorescence stain. Transfected 293T cells seeded onto cover slips were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 containing PBS. Cells were then incubated with appropriate antibodies at room temperature for 2 h then incubated with goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes, Invitrogen) and goat anti-mouse IgG antibody conjugated with Alexa Fluor 546 at room temperature for 1 h in the dark. The cells were enclosed with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and observed under a Leica confocal microscope (Wetzlar, Germany).

Results

High *Shf* mRNA expression significantly associated with better prognosis in neuroblastoma. We have reported many candidate genes for novel prognostic factors of neuroblastoma[25,26] in a differential expression study using our cDNA collection prepared from the primary samples of neuroblastoma patients. Among them, *Shf* was identified as one of the possible tumor suppressor genes in neuroblastoma. Shf, a homolog of Shb, has a highly conserved SH2 domain in the C-termini, but lacks a proline-rich region and phosphotyrosine-binding (PTB) domain in the N-termini (Fig. 1a). The expression level of *Shf* was closely correlated with favorable prognosis of neuroblastoma (Fig. 1b). To further confirm the expression profile of *Shf* mRNA, 106 clinical samples were classified into two groups in regard to INSS stages (Fig. 1c). The expression level of *Shf* was higher in a non-metastatic group (INSS 1, 2, and 3) than in metastatic one (INSS 4 and 4s); the classification with favorable (INSS 1, 2, and 4s) and unfavorable groups (INSS 3 and 4) did not yield statistical significance (Fig. S1). A low level of *Shf* expression had significant correlation with poor prognostic factors, such as lower expression of *TrkA* ($P < 0.001$), DNA diploidy ($P < 0.001$), and the patients who contracted the disease after 1 year of age ($P < 0.05$), whereas no significant correlation was observed with the copy number of *MYCN* (Fig. 1d).

Another adaptor protein Shb, a homolog of Shf, interacts with several receptor tyrosine kinases and regulates such receptor-oriented signal transduction pathways. Thus, we

doi: 10.1111/cas.12115

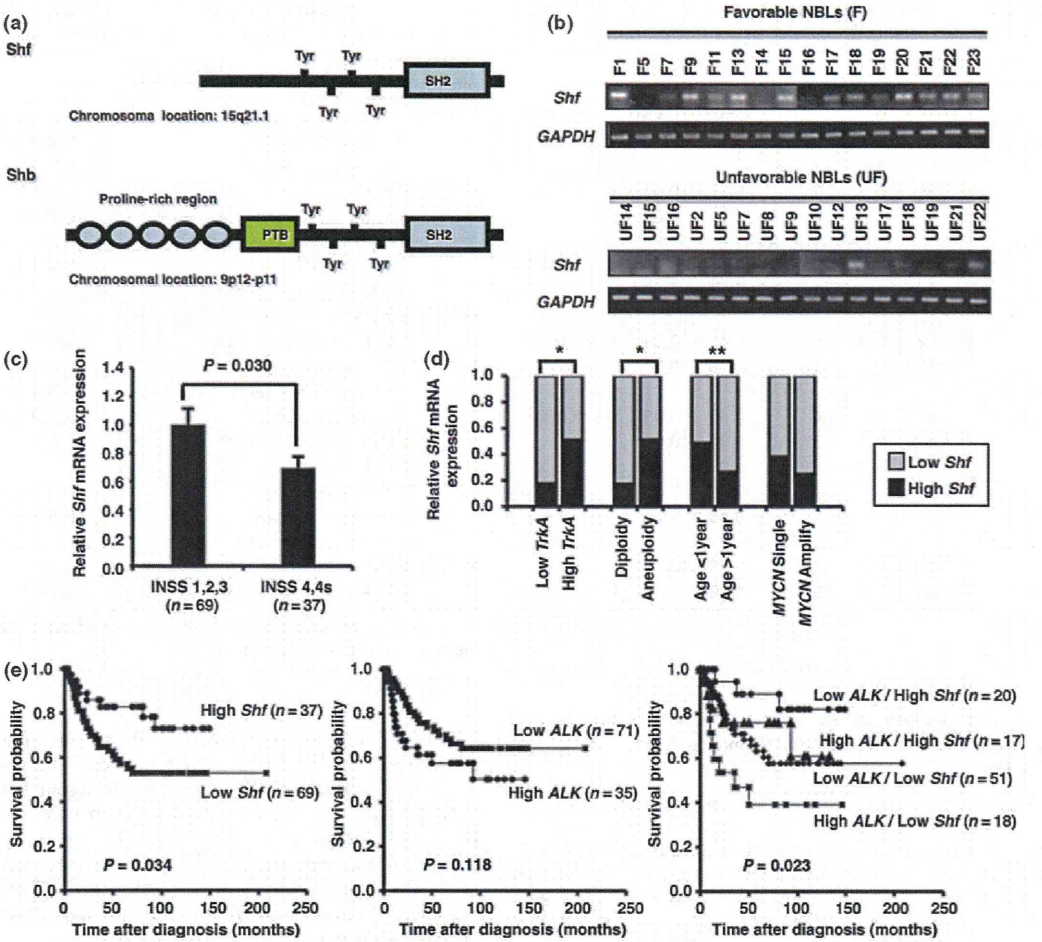

Fig. 1. Expression profiles of *Shf* mRNA in primary neuroblastoma (NBL). (a) Structural differences between Shf and Shb adaptor proteins. PTB, phosphotyrosine-binding domain; SH2, Src homology 2 domain; Tyr, tyrosine. (b) Differential expression of *Shf* in neuroblastomas with favorable (F) and unfavorable (UF) outcomes. Results of 16 representative clinical samples of each group are shown. *GAPDH* was used as a control. Favorable NBLs, stage 1 or 2, with single copy of *MYCN*. Unfavorable NBLs, stage 3 or 4, with *MYCN* amplification. (c) Relative *Shf* expression profiles regarding metastatic status in NBL specimens measured by quantitative real-time PCR. *Shf* mRNA expression was normalized to that of *GAPDH*. Values are shown as means ± SEM. Non-metastatic group, stages 1–3; metastatic group, stages 4 or 4s. (d) Correlation between *Shf* expression and other prognosis factors in NBL. The χ^2-test was used to explore possible associations. $*P < 0.001$; $**P < 0.05$. (e) Kaplan–Meier cumulative survival curves of *Shf* and anaplastic lymphoma kinase (*ALK*) expressions. High and low levels of *Shf* and *ALK* were determined based on mean values.

hypothesized that Shf also participates in the regulation of the signal pathway through its interaction with receptor tyrosine kinases including TrkA and ALK that play critical roles in the nervous system.[10,12,31] Intriguingly, *Shf* was specifically expressed in diencephalon, spinal cord, and dorsal root ganglion in mice.[27] Additionally, we showed that *Shf* was particularly expressed in human brain (Fig. S2a). Therefore, we used statistical analyses to clarify the relationship among these factors and survivability of neuroblastoma patients. The log–rank test indicated that a low level of *Shf* expression is significantly correlated to the number of deaths, as well as other prognostic factors,[32] such as low level of *TrkA* expression, DNA diploidy, age diagnosed after 1 year, and the amplification of *MYCN* copy number, whereas *ALK* expression had no significant correlation (Table 1). Univariate analysis using the Cox regression model yielded similar results (Table 2). Multivariate analysis indicated that *Shf* was not independent compared to other prognostic factors (Table 3), suggesting that *Shf* expression cannot be used as a new prognostic factor in neuroblastoma. Consistent with these statistical analyses, Kaplan–Meier cumulative survival curves indicated that higher expression of *Shf* is significantly correlated with favorable outcome (Fig. 1e). Although it is not statistically significant, higher expression of *ALK* shows some relevance to unfavorable outcome. To further confirm these results, 106 samples were classified into four groups in regard to the expression levels of *Shf* and *ALK* and the survival curves were examined. The patients with lower *Shf* and higher *ALK* were significantly associated with unfavorable outcome, whereas those with higher *Shf* and lower *ALK* yielded markedly favorable results. These results suggest that there is an inverse correlation between expression levels of *Shf* and *ALK* in terms of the clinical prognosis in neuroblastoma.

Physical interaction between Shf and ALK and their colocalization in the juxtamembrane region in 293T cells. As these statistical analyses suggested the functional relationship between Shf and ALK, we asked whether these two proteins have direct interaction *in vivo*. Toward this, we carried out

Table 1. Analysis of relationships between *Shf*, *ALK*, and *TrkA* expression and other prognostic factors in neuroblastoma patients, using the log–rank test

	No. of patients	No. of deaths	Mean ± SEM	*P*-value
Shf expression				
Low	69	29	0.53 ± 0.07	0.0345*
High	37	8	0.73 ± 0.09	
ALK expression				
Low	71	22	0.64 ± 0.06	0.1178
High	35	15	0.50 ± 0.10	
TrkA expression				
Low	52	26	0.45 ± 0.08	<0.0005*
High	51	9	0.78 ± 0.07	
DNA ploidy				
Aneuploidy	47	4	0.43 ± 0.09	<0.0001*
Diploidy	43	23	0.90 ± 0.05	
Age				
<1 year	42	5	0.88 ± 0.05	<0.0005*
>1 year	64	32	0.43 ± 0.07	
MYCN copy number				
Single	81	18	0.73 ± 0.06	<0.0001*
Amplification	25	19	0.20 ± 0.09	

*$P < 0.05$.

Table 3. Multivariate analysis of *Shf*, *ALK*, and *TrkA* expression and other prognostic factors in neuroblastoma patients using Cox regression model

Multivariate analysis		*P*-value	HR (95%CI)
A	*Shf* (low *vs* high)	0.253	1.7 (0.7–3.9)
	TrkA (low *vs* high)	0.002*	3.4 (1.5–7.5)
B	*Shf* (low *vs* high)	0.014*	2.7 (1.2–6.1)
	ALK (low *vs* high)	0.031*	2.1 (1.1–4.1)
C	*Shf* (low *vs* high)	0.260	1.9 (0.6–5.7)
	DNA ploidy (diploidy *vs* aneuploidy)	0.001*	6.3 (2.1–19.1)
D	*Shf* (low *vs* high)	0.163	1.8 (0.8–3.9)
	Age (<1 year *vs* >1 year)	0.002*	4.4 (1.7–11.4)
E	*Shf* (low *vs* high)	0.116	1.9 (0.9–4.2)
	MYCN (single *vs* amplification)	<0.001*	5.4 (2.8–10.3)
F	*Shf* (low *vs* high)	0.052	2.2 (1.0–4.8)
	Tumor origin (adrenal gland *vs* others)	0.032*	2.1 (1.1–4.2)
G	*Shf* (low *vs* high)	0.358	1.5 (0.6–3.6)
	Shimada histology (favorable *vs* unfavorable)	<0.001*	8.1 (3.1–21.5)
H	*Shf* (low *vs* high)	0.069	2.1 (0.9–4.6)
	INSS stage (1, 2, 4s *vs* 3, 4)	<0.001*	9.1 (2.8–29.7)

*$P < 0.05$. CI, confidence interval; HR, hazard ratio; INSS, International Neuroblastoma Staging System.

Table 2. Univariate analysis of *Shf*, *ALK*, and *TrkA* expression and other prognostic factors in neuroblastoma patients using Cox regression model

Univariate analysis		*n*	*P*-value	HR (95%CI)
A	*Shf* (low *vs* high)	69 *vs* 37	0.039*	2.3 (1.0–5.0)
B	*ALK* (low *vs* high)	71 *vs* 35	0.121	1.7 (0.9–3.2)
C	*TrkA* (low *vs* high)	52 *vs* 51	<0.001*	3.9 (1.8–8.4)
D	DNA ploidy (diploidy *vs* aneuploidy)	47 *vs* 43	<0.001*	7.6 (2.6–22.1)
E	Age (<1 year *vs* >1 year)	42 *vs* 64	<0.001*	4.9 (1.9–12.6)
F	*MYCN* (single *vs* amplification)	81 *vs* 25	<0.001*	5.8 (3.0–11.1)

*$P < 0.05$. CI, confidence interval; HR, hazard ratio.

immunoprecipitation using the cell lysate prepared from 293T cells in which exogenous Shf and ALK are overexpressed, and proved reciprocal interaction between ALK and Shf (Fig. 2a). To further confirm this result, we used several point mutants of ALK that were recently reported in neuroblastoma.[13,17] *F1174L* and *R1275Q* are the "hot spot" mutations in the kinase motif located in the intracellular domain of ALK, whereas the *A1099T* mutation is located in the transmembrane domain. Immunoprecipitation indicated that Shf could interact with all of these mutated constructs of ALK, as well as wild-type (Fig. 2b). There are minor differences in the binding capability of Shf to each ALK mutant, possibly suggesting that these point mutations in ALK may affect the affinity to Shf. In addition, immunofluorescence stain indicated that exogenous Shf and ALK were enriched at the cellular membrane (Fig. 2c), suggesting that two proteins colocalized at the juxtamembrane region in 293T. Taken together, we concluded that Shf binds to ALK *in vivo*.

Overexpression of ALK facilitated cellular growth. It has been reported that ALK is an oncogenic receptor tyrosine kinase that transmits survival signals in several cell lines and tissues from different origins.[33] Consistent with previous reports,[34,37] successful overexpression of ALK induced phosphorylation of Erk1/2 and STAT3 even in neuroblastoma cells, such as SK-N-DZ, SK-N-AS, and NLF, clearly suggesting that abundant ALK affects the downstream of signal transduction pathway oriented by ALK (Fig. 3a). Overexpression of ALK also increased the number of cells, indicating that ALK may play an important role during the development of neuroblastoma (Fig. 3b). In contrast, overexpression of Shf affects neither the phosphorylation of ALK (Tyr1604) and downstream factors (Fig. 3c) nor cellular growth (Fig. 3d).

Knockdown of *Shf* promoted Erk1/2 and STAT3 phosphorylation and enhanced cell growth. The results of Kaplan–Meier survival analyses suggested that *Shf* had a biological function opposite to oncogenic *ALK*. Thus, we used a knockdown strategy to investigate the cellular property of Shf in neuroblastoma. The expression of *Shf* mRNA was efficiently inhibited by siRNA transfection in three neuroblastoma cells, SK-N-DZ, SK-N-AS, and NLF (Fig. 4a), that express low levels of wild-type *ALK* (Fig. S2b). Knockdown of *Shf* accelerated phosphorylation of Erk1/2 in SK-N-DZ and SK-N-AS as well as ALK itself at tyrosine 1604. In addition, phosphorylation of STAT3 was observed by *Shf* knockdown in SK-N-DZ and NLF (Fig. 4b). Knockdown of *Shf* enhanced cell growth in these cells, which was statistically significant (Fig. 4c). Next, we used a combination of siRNAs specific to *Shf* and *ALK* in neuroblastoma cell line SH-SY5Y, in which ALK has the *F1174L* mutation (Fig. 4d) and Shf is expressed (Fig. S2b). Knockdown of *Shf* increased the growth rate of SH-SY5Y in the presence of endogenous *ALK* (Fig. 4e). However, under the experimental condition that *ALK* was suppressed by specific siRNA (Fig. 4d, lower panel), *Shf* knockdown did not facilitate cell growth (Fig. 4e). This result indicates that the acceleration of cell growth rate mediated by knockdown of *Shf* depends on *ALK*, suggesting that Shf inhibits growth signals that are downstream of the ALK-initiated signal transduction pathway in neuroblastoma.

Depletion of *Shf* facilitated cell migration and invasion of neuroblastoma cells. Various fusion proteins of ALK exert oncogenic properties (e.g. increasing migration in fibroblast and lymphoid cells)[38,39] and suppression of *Shf* might

doi: 10.1111/cas.12115

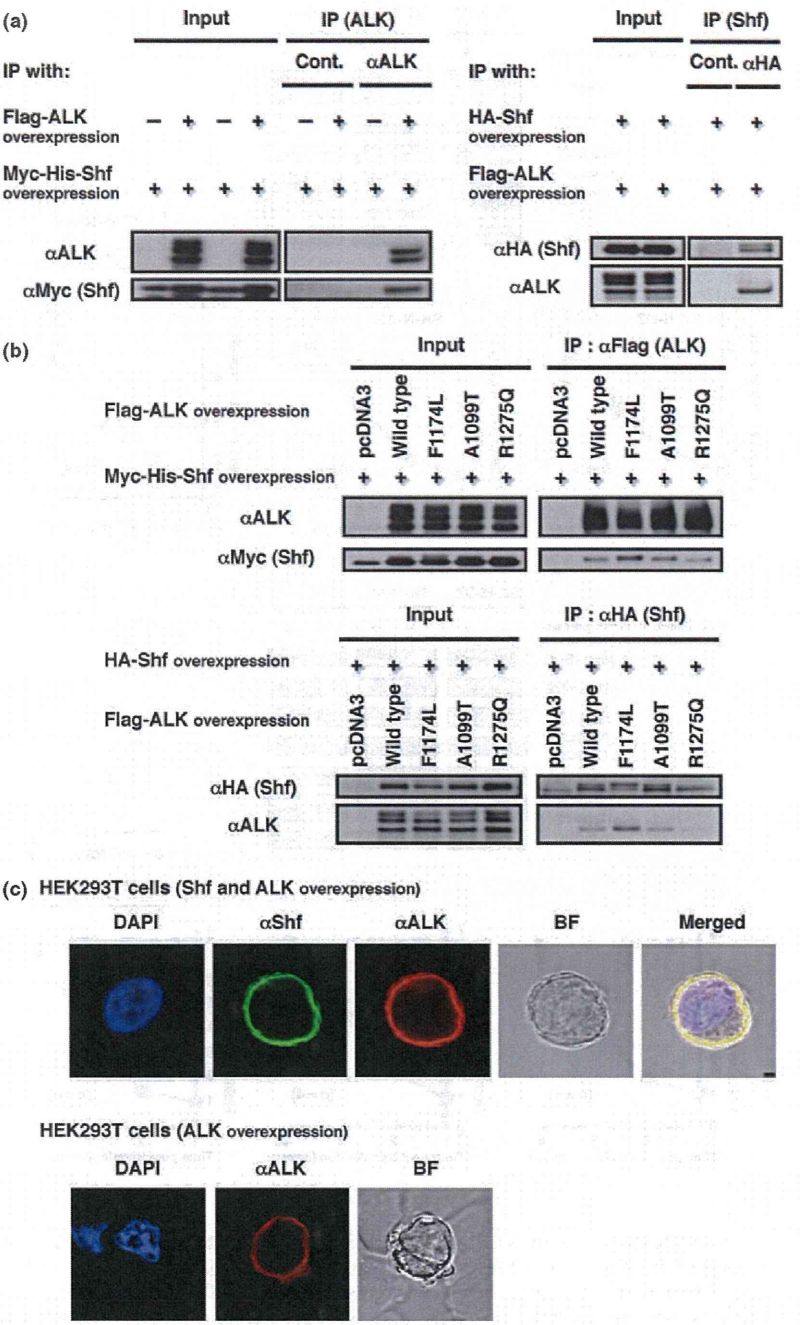

Fig. 2. Physical interaction between adaptor protein Shf and anaplastic lymphoma kinase (ALK). (a) Immunoprecipitation (IP) in 293T cells. Flag-tagged ALK and either HA-tagged or Myc-His-tagged Shf were exogenously overexpressed. Cont., control. (b) Immunoprecipitation assay under the exogenous expression of ALK mutants and Shf in 293T cells. (c) Subcellular colocalization of Shf and ALK in human embryonic kidney (HEK) 293T cells. Myc-His-Shf and Flag-ALK were overexpressed in 293T and indirect immunofluorescence staining was carried out. Upper panels: DAPI (blue), Shf (green), ALK (red), blight field (BF), and merged images. Lower panels: exogenous expression of ALK alone yielded a similar localization pattern at the juxtamembrane region, indicating that the localization of ALK was not affected by Shf overexpression.

positively affect the consequence of ALK activation. To prove this possibility, we examined the ALK-promoted cell motility and invasive ability of neuroblastoma cells under the condition that *Shf* was suppressed. Knockdown of *Shf* greatly increased the number of migrated cells in both NLF and SK-N-DZ cells, compared to the corresponding control (Fig. 5a). As well, *Shf* knockdown in NLF yielded a significant increase in the number of invasive cells. There was a mild tendency of increasing invasion in SK-N-DZ, although it was not statistically significant (Fig. 5b). These results suggest that suppression of *Shf* promotes the motility and invasive capability of neuroblastoma cells, which is consistent with our clinical data that lower expression of *Shf* was observed in metastatic primary neuroblastoma defined by INSS 4 and 4s (Fig. 1c).

Finally, we sought to confirm the biological function of Shf as a negative regulator in ALK-promoted cell mobility. Toward this, overexpression of ALK and siRNA-mediated suppression of *Shf* was carried out simultaneously. The increase of migration mediated by *Shf* knockdown was enhanced more than twofold when ALK was overexpressed (Fig. 5c). While either knockdown of Shf (Fig. 3a) or overexpression of ALK (Fig. 5d) facilitated phosphorylation of ALK, simultaneous treatment of Shf suppression and ALK overexpression further promoted the phosphorylation of ALK itself (Fig. 5d). The combination of Shf suppression and