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Chromosome abnormalities in advanced stage T-cell lymphoblastic lymphoma of children and adolescents: a report from Japanese Paediatric Leukaemia/Lymphoma Study Group (JPLSG) and review of the literature

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Summary

T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL) are combined into one category as T lymphoblastic leukaemia/lymphoma in the current World Health Organization (WHO) classification. However, there is still ongoing discussion on whether T-ALL and T-LBL are two separate entities or represent two variant phenotypes of the same disease. Cytogenetic analysis has been used to identify the molecular background of haematological malignancies. To compare the distribution of chromosomal abnormalities of T-ALL and T-LBL, large series of cytogenetic data are required, but are absent in T-LBL in contrast to the abundant data in T-ALL. Among 111 T-LBL cases in our clinical trial, we obtained complete cytogenetic data from 56 patients. The comparison between our cytogenetic findings and those from three published T-LBL studies revealed no significant difference. However, meta-analysis showed that translocations involving chromosome region 9q34 were significantly more common in T-LBL than in T-ALL. In particular, four out of the 92 T-LBL cases, but none of the 523 paediatric T-ALL cases, showed translocation t(9;17)(q34;q22–23) ($P = 0{\cdot}0004$). Further studies are needed for the possible linkage between abnormal expression of genes located at 9q34 and/or 17q22–23 and the unique 'lymphoma phenotype' of T-LBL.

Keywords: T-cell lymphoma, child, non-Hodgkin lymphoma, cancer cytogenetics, leukaemia.

In children and adolescents, precursor T lymphoblastic neoplasms have been classified into two diseases: T-cell acute lymphoblastic leukaemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL). Although the current World Health Organization (WHO) classification designates both malignancies as T lymphoblastic leukaemia/lymphoma (Borowitz & Chan, 2008), there is continuing discussion on whether T-ALL and T-LBL are two separate entities or whether they represent two different clinical presentations of the same disease. They show overlapping clinical, pathological and immunophenotypic features. In general, the word 'lymphoma' is used if there is a bulky mass in the mediastinum or elsewhere, with less peripheral blood and bone marrow (BM) involvement. Most study groups distinguish between leukaemia and lymphoma on the basis of the extent of BM involvement: patients with <25% lymphoblasts in the BM are diagnosed with lymphoblastic lymphoma; in cases

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of 25% or more BM blasts, the diagnosis is leukaemia. While this distinction may appear somewhat arbitrary, a notable observation is that T-LBL patients with large mediastinal masses frequently exhibit little, if any, evidence of tumour dissemination and BM involvement, but the molecular background for this difference is unknown.

Chromosomal analysis has been widely used as a primary step that is required to narrow down the responsible genes that define a disease entity. For instance, discovery of Ph chromosome led to the identification of the chimeric *BCR/ABL1* gene, which is responsible for and defines chronic myeloid leukaemia. Compared with T-ALL, chromosomal abnormalities in T-LBL are not well defined. Reports in the literature and current textbooks claim that the typical chromosomal aberrations reported in T-ALL can also be found in T-LBL (Borowitz & Chan, 2008). However, there are no large series of cytogenetic data on T-LBL (Burkhardt, 2010).

This study aimed to fill the gap regarding cytogenetic data in T-LBL and compare the cytogenetic findings of T-ALL and T-LBL, which may lead to identification of the molecular background behind phenotypical differences between the two disease entities.

Study patients

From November 2004 to October 2010, 154 eligible children (aged 1–18 years) with newly diagnosed advanced stage LBL (Murphy stages III and IV) (Murphy, 1980) were entered in the Japanese Paediatric Leukaemia/Lymphoma Study Group (JPLSG) ALB-NHL03 study (UMIN000002212, http://www.umin.ac.jp/ctr/index-j.htm). Patients with primary immunodeficiencies, Down syndrome and T-cell diseases as second malignancies were excluded. The ethics committee of each participating institute approved the study protocol.

Cytogenetic analysis

Cytogenetic analysis was performed on cell suspensions obtained from 31 tumour/lymph nodes, 19 pleural effusions and six bone marrow samples. The methods of chromosome preparation for cytogenetic analysis are described elsewhere (Sanger *et al*, 1987; Horsman *et al*, 2001). Karyotypes are described according to the International System for Human Cytogenetic Nomenclature (ISCN) (Shaffer & Tommerup, 2005). Only those cases with abnormal cytogenetic study results, defined as two or more cells with the same structural abnormality or the same numerical gain, three or more cells with the same numerical loss or isolated cells with disease-associated abnormalities, were eligible for inclusion in this study.

Statistical methods

Two-tailed Fisher's exact test was used to analyse the patients' characteristics and the frequency of each chromosome abnormality. Significant differences in the analysis of he frequency of each chromosome abnormality were determined by the two-tailed Fisher's exact test with Bonferroni correction comparison. The P value threshold for inclusion of a new variable was chosen to be $P < 0{\cdot}003$ in this analysis (0·05/17, after Bonferroni correction). A review of T-LBL and T-ALL karyotypes reported in the literature was obtained from a PubMed search and information on chromosome abnormalities and gene fusions was obtained from Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

Results

Patient characteristics

A total of 154 children were enrolled on JPLSG ALB-NHL03 protocols; 111 cases were T-LBL. Among 111 T-LBL cases, the study population for the current analysis included 56 patients for whom complete cytogenetic data were obtained. With respect to presenting features, patients with reviewed and accepted cytogenetic data were similar to both those without accepted cytogenetic data and the entire cohort of concurrently enrolled T-lineage LBL patients (Table S1).

Frequency of chromosomal abnormalities

Multiple chromosome abnormalities were identified in 31 patients (45%). Structural chromosome abnormalities were identified in 29 patients (52%), and numerical chromosome abnormalities were identified in 18 patients (32%). Ploidy results included pseudodiploid in 14 patients (25%), hypodiploid in three patients (5%), hyperdiploid with 47–50 chromosomes in 10 patients (18%), hyperdiploid with more than 50 chromosomes in four patients (7%) and diploid in 25 patients (45%) (Table S2).

All of the hypodiploid cases had 43–45 chromosomes; none had a near-haploid karyotype. Of the four cases with more than 50 chromosomes, two had near-tetraploid karyotypes. The frequencies of ploidy groups in this series are compared with those reported in other series of karyotyped T-LBL patients and paediatric T-ALL (Table S2). Structural chromosome abnormalities were identified in 29 patients (52%). In the current study, seven patients (13% of those with abnormal karyotypes) exhibited a rearrangement at one or more of the chromosome bands (7p15, 7q32–36 and/or 14q11–13) that are the locations of T-cell receptor chain genes. Rearrangements in the 14q11–13 region, in which the T-cell receptor α/δ chain genes are located, were present in three patients (5%) of the karyotypically abnormal cases in this series (Table S2). Structural abnormalities involving chromosome region 9q34 were identified in nine patients (16%). Translocations involving chromosome region 9q34 were identified in three patients (5%) (t(9;17)(q34;q22), t(7;9)(q34;q34) and t(2;9)(q23;q34)). In comparison between cytogenetic findings in the current data and combined data of three published reports (Burkhardt

et al, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007; Table S1), the frequencies of numerical and structural cytogenetic abnormalities in T-LBL and T-ALL had no significant difference (Table S2).

We compared the cytogenetic findings in the current study with the published reports from the three largest-scale studies on T-LBL (Burkhardt *et al*, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007; Table S3) and those from the two largest-scale studies on T-ALL combined (Heerema *et al*, 1998; Schneider *et al*, 2000; Table S3) (Table I). The frequencies of almost all of the cytogenetic abnormalities in T-LBL and T-ALL had no significant difference, but translocation involving chromosome region 9q34 was significantly more common in T-LBL than in T-ALL (P = 0·0004, Table S3) and translocation t(9;17) was also more common in T-LBL (4%, 4/92) than in T-ALL (0%, 0/523, P = 0·0004) (Table I).

The current study included a patient with translocation t(9;17)(q34;q22). As far as we could tell from the consulted published reports, all T-LBL patients with translocation t(9;17) presented with a mediastinal mass and without any bone marrow involvement (Kaneko *et al*, 1988; Shikano *et al*, 1992) (Table II).

Table I. Comparison of cytogenetic findings between T-LBL and T-ALL.

	T-LBL		T-ALL		
	n	%	*n*	%	*P* value
Total	92		523		
Normal karyotype†	36	39	219	42	0·6478
Abnormal karyotype	56	61	304	58	0·6478
Hypodiploid	4	4	20	4	0·9999
Pseudodiploid	30	33	204	39	0·2000
Hyperdiploid(47–50)	18	20	64	12	0·0328
Hyperdiploid(>50)	4	4	16	3	0·5217
Any translocation	26	28	177	34	0·3367
Any del chrome.	19	21	160	31	0·0328
Any der chrome.	4	4	58	11	0·0583
del(6q)	6	7	69	13	0·0833
Loss of 9p	10	11	44	8	0·5487
Any 14q11–13 abnormality	10	11	72	14	0·5100
Any 7q32–36 abnormality	7	8	35	7	0·8220
Any translocation including 9q34	8	9	7	1	0·0004*
t(7;10)	1	1	2	0	0·3855
t(10;11)	1	1	8	2	0·9999
t(9;17)	4	4	0	0	0·0004*

†Includes one Klinefelter syndrome, and one inv(9) without other abnormality in current report.

The P value threshold for inclusion of a new variable was chosen to be 0·003 (0·05/17, after Bonferroni correction). $^{*}P < 0·003$.

T-LBL: current study (JPLSG ALB-NHL03) combined with three published reports(Burkhardt *et al*, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007).

T-ALL: combined two published reports (Heerema *et al*, 1998; Schneider *et al*, 2000).

Discussion

This is the largest study involving cytogenetic analysis of T-LBL and the first study to directly compare cytogenetic findings of T-LBL and T-ALL. The frequencies of almost all of the cytogenetic abnormalities in both entities were found to have no significant difference, but translocation involving chromosome region 9q34 was significantly more common in T-LBL than in T-ALL. The current study included a patient with unique translocation t(9;17)(q34;q22). Interestingly, four out of the 92 T-LBL cases, but none of the 523 paediatric T-ALL cases, showed this translocation (P = 0·0004) (Table I). Translocation t(9;17) has been reported in several haematological diseases, such as precursor B-cell ALL (Coyaud *et al*, 2010), acute myeloid leukaemia (Mrózek *et al*, 2001), chronic myeloid leukaemia (DeAngelo *et al*, 2004), chronic lymphocytic leukaemia (Michaux *et al*, 2005), diffuse large B-cell lymphoma (Hammond *et al*, 1992) and follicular lymphoma (Aamot *et al*, 2007), but these breakpoints, 9q34 and 17q22–23, are limited in the cases of T-LBL (http://cgap.nci.nih.gov/Chromosomes/Mitelman). These results imply a linkage between abnormal expression of genes located at 9q34 and/or 17q22–23 and the unique phenotypes of the T-LBL mentioned above.

Cytogenetic analysis has been used to identify the molecular background of haematological malignancies. To compare the distribution of chromosomal abnormalities of T-ALL and T-LBL, large series of cytogenetic data are required, but are absent in T-LBL in contrast to the abundant data in T-ALL. Three recent series of cytogenetic data on paediatric T-LBL have been published, reporting the cytogenetic findings in 13, 11 and 12 paediatric T-LBL cases (Burkhardt *et al*, 2006; Lones *et al*, 2007; Uyttebroeck *et al*, 2007). Thus, this study can play a role to fill the gap of cytogenetic data on T-LBL.

Translocation involving chromosome region 9q34 was found to be significantly more common in T-LBL than in T-ALL (Table I). Among genes located in the 9q34 region, *SET, PKN3, ABL1, NUP214* and *NOTCH1* have previously been implicated in malignancy, with *SET, ABL1, NUP214* and *NOTCH1* being implicated in leukemogenesis (Ellisen *et al*, 1991; van Vlierberghe *et al*, 2008; Hagemeijer & Graux, 2010).

An oncogenic *SET-NUP214* fusion gene has been reported in a case of acute undifferentiated leukaemia with a reciprocal translocation t(9;9)(q34; q34) (von Lindern *et al*, 1992) and NK adult acute myeloid leukaemia as a result of a cryptic deletion of 9q34 (Rosati *et al*, 2007). van Vlierberghe *et al* (2008) identified the *SET-NUP214* fusion gene in three patient samples out of 92 paediatric cases of T-cell leukaemia. *SET-NUP214* may contribute to T-ALL pathogenesis by inhibition of T-cell maturation through the transcriptional activation of the *HOXA* genes (van Vlierberghe *et al*, 2008). However, the frequency of this mutation in T-LBL is unknown.

NOTCH1, previously termed *TAN1*, was discovered as a partner gene in T-ALL with a translocation t(7;9)(q34;q34.3), and was found in <1% of T-ALLs (Ellisen *et al*, 1991). Several

Table II. Clinical characteristics and detailed karyotype data in T-LBL patients with t(9;17).

	Age (years)	Sex	Tumour site	Stage	BM blast %	Karyotype
Kaneko *et al* (1988)	14	F	Mediastinum	III	0	46,XX,t(9;17)(q34;q23)
	15	M	Mediastinum	III	0	46,XY,-9,del(6)(q13q21),t(9;17)(q34;q23),+der(9)t(9;17)(q34;q23)
	10	M	Mediastinum	III	0	47,XY,+19,t(9;17)(q34;q23)
Shikano *et al* (1992)	14	F	Mediastinum	III	0	46,XX,t(9;17)(q34;q23)
	7	M	Mediastinum	III	0	49,XY-l,+der(l)t(l;?)(p36;?),t(9;17)(q34;q23),+14,+marl,+mar2
	5	F	Mediastinum	III	0	47,XX,t(9;17)(q34;q23),+der(17)t(9;17)(q34;q23)
Burkhardt *et al* (2006)	ND	ND	ND	ND	ND	46,XX,del(6)(q1?2q1?6),t(9;17)(q34;q22)
	ND	ND	ND	ND	ND	47,XX,t(9;17)(q34;q22),+20
Lones *et al* (2007)	8	M	Mediastinum	III	0	47,XY,t(9;17)(q3?4;q2?3),+20
Current study	7	M	Mediastinum	III	0	46,XY,t(9;17)(q34;q22)

ND, no data available.

study groups reported *NOTCH1* mutations in 31–62% of T-ALL patients (Weng *et al*, 2004; Breit *et al*, 2006; van Grotel *et al*, 2006; Zhu *et al*, 2006; Malyukova *et al*, 2007; Asnafi *et al*, 2009; Gedman *et al*, 2009; Park *et al*, 2009). In contrast, only two studies reported *NOTCH1* mutation analyses in T-LBL: Park *et al* (2009) reported *NOTCH1* mutations in six out of 14 paediatric T-LBL patients (43%), and Baleydier *et al* (2008) reported mutations in six out of nine paediatric T-LBL (66%), with 32 adult patients with *NOTCH1* mutations in 16 cases (54% in all patients) (Baleydier *et al*, 2008). According to these reports, the frequencies of *NOTCH1* mutation were not significantly different between T-LBL and T-ALL.

ABL1 fusion genes have been identified that provide proliferation and survival advantage to lymphoblasts. *NUP214-ABL1*, *EML1-ABL1*, *BCR-ABL1* and *ETV6-ABL1* chimeric genes have been reported. The most frequent one in T-ALL is the *NUP214-ABL*1 fusion gene, which has been identified in 6% of cases, in both children and adults (Graux *et al*, 2009). In addition, using an oligonucleotide microarray, *ABL1* overexpression was identified in 8% of cases in T-ALL (Chiaretti *et al*, 2007). Our review of these published reports indicated that the frequency of ABL1 mutation in T-LBL is unknown.

Raetz *et al* (2006) analysed the gene expression profiles of ten T-ALL BM samples and nine T-LBL samples using a microarray. They identified 133 genes for which the expression levels differed between T-LBL and T-ALL. *ZNF79* (encoding zinc finger protein 79) and *ABL1*, both located in chromosome region 9q34, were included in these genes and showed at least twofold higher overexpression in T-LBL than that in T-ALL. Additionally, *MED13* (previously termed *THRAP1*), which is located in 17q22-q23, also showed at least twofold higher overexpression in T-LBL than that in T-ALL (Raetz *et al*, 2006). Taking these findings together, it is possible that *ZNF79*, *ABL1* or *THRAP1* as well as other genes at 9q34 and 17q22–23 are involved in the 'lymphoma phenotype' such as a bulky mass in the mediastinum and minimal BM involvement. These findings need further study to determine if this linkage constitutes a unique 'lymphoma phenotype'.

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Authorship

MS designed the study, prepared the data file, performed the analysis, interpreted data and wrote the manuscript. SS is a lead principal investigator for the JPLSG ALB-NHL03 study. AN contributed to pathological diagnosis. YH contributed to chromosome analysis. YO is a principal investigator contributing a patient to this study. AMS contributed to statistical analysis. KH received a research grant from the Ministry of Health, Labour and Welfare of Japan. MT is a chairperson of JPLSG. TM is a chairperson of JPLSG lymphoma committee. SS, KH, MT and TM were primarily responsible for the study design, data analysis and interpretation of the data. All authors approved the final manuscript.

Disclosure

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Respective clinical characteristics with and without karyotype data in 111 T-LBL patients in the current study.

Table S2. Comparison of cytogenetic findings in T-LBL between current study and combined data of three published reports.

Table S3. Published data of cytogenetic findings in T-LBL and T-ALL.

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References

Aamot, H.V., Torlakovic, E.E., Eide, M.B., Holte, H. & Heim, S. (2007) Non-Hodgkin lymphoma with t(14;18): clonal evolution patterns and cytogenetic-pathologic-clinical correlations. *Journal of Cancer Research and Clinical Oncology*, **133**, 455–470.

Asnafi, V., Buzyn, A., Le Noir, S., Baleydier, F., Simon, A., Beldjord, K., Reman, O., Witz, F., Fagot, T., Tavernier, E., Turlure, P., Leguay, T., Huguet, F., Vernant, J.P., Daniel, F., Bene, M.C., Ifrah, N., Thomas, X., Dombret, H. & Macintyre, E. (2009) NOTCH1/FBXW7 mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): a Group for Research on Adult Acute Lymphoblastic leukemia (GRAALL) study. *Blood*, **113**, 3918–3924.

Baleydier, F., Decouvelaere, A.V., Bergeron, J., Gaulard, P., Canioni, D., Bertrand, Y., Lepretre, S., Petit, B., Dombret, H., Beldjord, K., Molina, T., Asnafi, V. & Macintyre, E. (2008) T cell receptor genotyping and HOXA/TLX1 expression define three T lymphoblastic lymphoma subsets which might affect clinical outcome. *Clinical Cancer Research*, **14**, 692–700.

Borowitz, M. & Chan, J. (2008) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. In: T lymphoblatic leukaemia/lymphoma (ed. by S. Swerdlow, E. Campo, N. Harris, E. Jaffe, S. Pileri, H. Stein, J. Thiele & J. Vardiman), pp. 176–178. Internationa Agency for Resarchon Cancer, Lyon.

Breit, S., Stanulla, M., Flohr, T., Schrappe, M., Ludwig, W.D., Tolle, G., Happich, M., Muckenthaler, M.U. & Kulozik, A.E. (2006) Activating NOTCH1 mutations predict favorable early treatment response and long term outcome in child-hood precursor T-cell lymphoblastic leukemia. *Blood*, **108**, 1151–1157.

Burkhardt, B. (2010) Paediatric lymphoblastic T-cell leukaemia and lymphoma: one or two diseases? *British Journal of Haematology*, **149**, 653–668.

Burkhardt, B., Bruch, J., Zimmermann, M., Strauch, K., Parwaresch, R., Ludwig, W.D., Harder, L., Schlegelberger, B., Mueller, F., Harbott, J. & Reiter, A. (2006) Loss of heterozygosity on chromosome 6q14-q24 is associated with poor outcome in children and adolescents with T-cell lymphoblastic lymphoma. *Leukemia*, **20**, 1422–1429.

Chiaretti, S., Tavolaro, S., Ghia, E.M., Ariola, C., Matteucci, C., Elia, L., Maggio, R., Messina, M., Ricciardi, M.R., Vitale, A., Ritz, J., Mecucci, C., Guarini, A. & Foa, R. (2007) Characterization of ABL1 expression in adult T-cell acute lymphoblastic leukemia by oligonucleotide array analysis. *Haematologica*, **92**, 619–626.

Coyaud, E., Struski, S., Prade, N., Familiades, J., Eichner, R., Quelen, C., Bousquet, M., Mugneret, F., Talmant, P., Pages, M.P., Lefebvre, C., Penther, D., Lippert, E., Nadal, N., Taviaux, S., Poppe, B., Luquet, I., Baranger, L., Eclache, V., Radford, I., Barin, C., Mozziconacci, M.J., Lafage-Pochitaloff, M., Antoine-Poirel, H., Charrin, C., Perot, C., Terre, C., Brousset, P., Dastugue, N. & Broccardo, C. (2010) Wide diversity of PAX5 alterations in B-ALL: a Groupe Francophone de Cytogenetique Hematologique Study. *Blood*, **115**, 3089–3097.

DeAngelo, D.J., Hochberg, E.P., Alyea, E.P., Longtine, J., Lee, S., Galinsky, I., Parekkedon, B., Ritz, J., Antin, J.H., Stone, R.M. & Soiffer, R.J. (2004) Extended follow-up of patients treated with imatinib mesylate (gleevec) for chronic myelogenous leukemia relapse after allogeneic transplantation: durable cytogenetic remission and conversion to complete donor chimerism without graft-versus-host disease. *Clinical Cancer Research*, **10**, 5065–5071.

Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D. & Sklar, J. (1991) TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell*, **66**, 649–661.

Gedman, A.L., Chen, Q., Kugel Desmoulin, S., Ge, Y., Lafiura, K., Haska, C.L., Cherian, C., Devidas, M., Linda, S.B., Taub, J.W. & Matherly, L.H. (2009) The impact of NOTCH1, FBW7 and PTEN mutations on prognosis and downstream signaling in paediatric T-cell acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Leukemia*, **23**, 1417–1425.

Graux, C., Stevens-Kroef, M., Lafage, M., Dastugue, N., Harrison, C.J., Mugneret, F., Bahloula, K., Struski, S., Gregoire, M.J., Nadal, N., Lippert, E., Taviaux, S., Simons, A., Kuiper, R.P., Moorman, A.V., Barber, K., Bosly, A., Michaux, L., Vandenberghe, P., Lahortiga, I., de Keersmaecker, K., Wlodarska, I., Cools, J., Hagemeijer, A. & Poirel, H.A. (2009) Heterogeneous patterns of amplification of the NUP214-ABL1 fusion gene in T-cell acute lymphoblastic leukemia. *Leukemia*, **23**, 125–133.

van Grotel, M., Meijerink, J.P., Beverloo, H.B., Langerak, A.W., Buys- Gladdines, J.G., Schneider, P., Poulsen, T.S., den Boer, M.L., Horstmann, M., Kamps, W.A., Veerman, A.J., van Wering, E.R., van Noesel, M.M. & Pieters, R. (2006) The outcome of molecularcytogenetic subgroups in pediatric T-cell acute lymphoblastic leukemia: a retrospective study of patients treated according to DCOG or COALL protocols. *Haematologica*, **91**, 1212–1221.

Hagemeijer, A. & Graux, C. (2010) ABL1 rearrangements in T-cell acute lymphoblastic leukemia. *Genes, Chromsomes & Cancer*, **59**, 299.

Hammond, D.W., Goepel, J.R., Aitken, M., Hancock, B.W., Potter, A.M. & Goyns, M.H. (1992) Cytogenetic analysis of a United Kingdom series of non-Hodgkins lymphomas. *Cancer Genetics and Cytogenetics*, **61**, 31–38.

Heerema, N.A., Sather, H.N., Sensel, M.G., Kraft, P., Nachman, J.B., Steinherz, P.G., Lange, B.J., Hutchinson, R.S., Reaman, G.H., Trigg, M.E., Arthur, D.C., Gaynon, P.S. & Uckun, F.M. (1998) Frequency and clinical significance of cytogenetic abnormalities in pediatric T-lineage acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Journal of Clinical Oncology*, **16**, 1270–1278.

Horsman, D.E., Connors, J.M., Pantzar, T. & Gascoyne, R.D. (2001) Analysis of secondary chromosomal alterations in 165 cases of follicular lymphoma with t(14;18). *Genes, Chromosomes and Cancer*, **30**, 375–382.

Kaneko, Y., Frizzera, G., Maseki, N., Sakurai, M., Komada, Y., Hiyoshi, Y., Nakadate, H. & Takeda, T. (1988) A novel translocation, t(9;17)(q34;q23), in aggressive childhood lymphoblastic lymphoma. *Leukemia*, **2**, 745–748.

von Lindern, M., Breems, D., van Baal, S., Adriaansen, H. & Grosveld, G. (1992) Characterization of the translocation breakpoint sequences of two DEK-CAN fusion genes present in t(6;9) acute myeloid leukaemia and a SET-CAN fusion gene found in a case of acute undifferentiated leukemia. *Genes, Chromosomes and Cancer*, **5**, 227–234.

Lones, M.A., Heerema, N.A., Le Beau, M.M., Sposto, R., Perkins, S.L., Kadin, M.E., Kjeldsberg, C.R., Meadows, A., Siegel, S., Buckley, J., Abromowitch, M., Kersey, J., Bergeron, S., Cairo, M.S. & Sanger, W.G. (2007) Chromosome abnormalities in advanced stage lymphoblastic lymphoma of children and adolescents: a report from CCG-E08. *Cancer Genetics and Cytogenetics*, **172**, 1–11.

Malyukova, A., Dohda, T., von der Lehr, N., Akhoondi, S., Corcoran, M., Heyman, M., Spruck, C., Grander, D., Lendahl, U. & Sangfelt, O. (2007) The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. *Cancer Research*, **67**, 5611–5616.

Michaux, L., Wlodarska, I., Rack, K., Stul, M., Criel, A., Maerevoet, M., Marichal, S., Demuynck, H., Mineur, P., Kargar Samani, K., Van Hoof, A., Ferrant, A., Marynen, P. & Hagemeijer, A. (2005) Translocation t(1;6)(p35.3;p25.2): a new recurrent aberration in "unmutated" B-CLL. *Leukemia*, **19**, 77–82.

Mrózek, K., Prior, T.W., Edwards, C., Marcucci, G., Carroll, A.J., Snyder, P.J., Koduru, P.R.K., Theil, K.S., Pettenati, M.J., Archer, K.J., Caligiuri, M.A., Vardiman, J.W., Kolitz, J.E., Larson, R.A. & Bloomfield, C.D. (2001) Comparison of cytogenetic and molecular genetic detection of t(8;21)

and inv(16) in a prospective series of adults with de novo acute myeloid leukaemia: a Cancer and leukemia Group B study. *Journal of Clinical Oncology*, **19**, 2482–2492.

Murphy, S. (1980) Classification, staging, and end results of treatment of childhood non-Hodgkin's lymphomas: dissimilarities from lymphomas in adults. *Seminars in Oncology*, **7**, 332–339.

Park, M.J., Taki, T., Oda, M., Watanabe, T., Yumura-Yagi, K., Kobayashi, R., Suzuki, N., Hara, J., Horibe, K. & Hayashi, Y. (2009) FBXW7 and NOTCH1 mutations in childhood T cell acute lymphoblastic leukaemia and T cell non-Hodgkin lymphoma. *British Journal of Haematology*, **145**, 198–206.

Raetz, E.A., Perkins, S.L., Bhojwani, D., Smock, K., Philip, M., Carroll, W.L. & Min, D.J. (2006) Gene expression profiling reveals intrinsic differences between T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma. *Pediatric Blood and Cancer*, **47**, 130–140.

Rosati, R., La Starza, R., Barba, G., Gorello, P., Pierini, V., Matteucci, C., Roti, G., Crescenzi, B., Aloisi, T., Aversa, F., Martelli, M.F. & Mecucci, C. (2007) Cryptic chromosome 9q34 deletion generates TAF-Iα/CAN and TAF-Iβ/CAN fusion transcripts in acute myeloid leukemia. *Haematologica*, **92**, 232–235.

Sanger, W.G., Armitage, J.O., Bridge, J., Weisenburger, D.D., Fordyce, R. & Purtilo, D.T. (1987) Initial and subsequent cytogenetic studies in malignant lymphoma. *Cancer*, **60**, 3014–3019.

Schneider, N.R., Carroll, A.J., Shuster, J.J., Pullen, D.J., Link, M.P., Borowitz, M.J., Camitta, B.M., Katz, J.A. & Amylon, M.D. (2000) New recurring cytogenetic abnormalities and association of blast cell karyotypes with prognosis in childhood T-cell acute lymphoblastic leukemia: a pediatric oncology group report of 343 cases. *Blood*, **96**, 2543–2549.

Shaffer, L.G. & Tommerup, N. (2005) *ISCN (2005) an International System for Human Cytogenetic Nomenclature*. S. Karger, Basel.

Shikano, T., Ishikawa, Y., Naito, H., Kobayashi, R., Nakadate, H., Hatae, Y. & Takeda, T. (1992) Cytogenetic characteristics of childhood non-Hodgkin lymphoma. *Cancer*, **70**, 714–719.

Uyttebroeck, A., Vanhentenrijk, V., Hagemeijer, A., Boeckx, N., Renard, M., Wlodarska, I., Vandenberghe, P., Depaepe, P. & de Wolf-Peeters, C. (2007) Is there a difference in childhood T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma? *Leukemia & Lymphoma*, **48**, 1745–1754.

van Vlierberghe, P., van Grotel, M., Tchinda, J., Lee, C., Beverloo, H.B., van der Spek, P.J., Stubbs, A., Cools, J., Nagata, K., Fornerod, M., Buijs-Gladdines, J., Horstmann, M., van Wering, E.R., Soulier, J., Pieters, R. & Meijerink, J.P. (2008) The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. *Blood*, **111**, 4668–4680.

Weng, A.P., Ferrando, A.A., Lee, W., Morris, J.P., Silverman, L.B., Sanchez-Irizarry, C., Blacklow, S.C., Look, A.T. & Aster, J.C. (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*, **306**, 269–271.

Zhu, Y.M., Zhao, W.L., Fu, J.F., Shi, J.Y., Pan, Q., Hu, J., Gao, X.D., Chen, B., Li, J.M., Xiong, S.M., Gu, L.J., Tang, J.Y., Liang, H., Jiang, H., Xue, Y.Q., Shen, Z.X., Chen, Z. & Chen, S.J. (2006) NOTCH1 mutations in T-cell acute lymphoblastic leukaemia: prognostic significance and implication in multifactorial leukemogenesis. *Clinical Cancer Research*, **12**, 3043–3049.

DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia

Acute myeloid leukaemia (AML) is a complex disease caused by mutations and deregulated gene expression, leading to increased proliferation and decreased differentiation of haematopoietic progenitor cells. Contemporary treatments have resulted in 5-year event-free survival rates of almost 60% for paediatric AML (Pui *et al*, 2011).

Recently, a whole genome sequencing study of AML uncovered recurrent mutations of an epigenetic regulator, the *DNA methyltransferase 3A* (*DNMT3A*) gene, in approximately 20% of adult AML patients (Ley *et al*, 2010; Yamashita *et al*, 2010; Yan *et al*, 2011). In these studies, *DNMT3A* mutations were frequently associated with *FLT3*-internal tandem duplication (ITD), *nucleophosmin 1* (*NPM1*) and *isocitrate dehydrogenase 1* (*IDH1*) mutations (Ley *et al*, 2010; Yan *et al*, 2011). *DNMT3A* mutations were also found in adult myelodysplastic syndromes (MDS) (8%, 12/150) (Walter *et al*, 2011), AML secondary to myeloproliferative neoplasms (MPNs) (14%, 5/35), myelofibrosis (15%, 3/20) and polycythaemia vera (7%, 2/30) (Stegelmann *et al*, 2011).

DNMT3A is involved in epigenetic regulation of genes by enzymatic de novo addition of methyl groups to the cytosine residue of CpG dinucleotides. *DNMT3A* mutations were significantly enriched with a cytogenetic profile associated with intermediate risk, including a normal cytogenetic profile, as well as the M4 and M5 subtypes, according to the French-American-British (FAB) classification system (Ley *et al*, 2010; Yan *et al*, 2011). In AML patients with a normal karyotype and *FLT3*-ITD, patients with *DNMT3A* gene mutations showed a worse prognosis than those without *DNMT3A* gene mutations (Ley *et al*, 2010; Yan *et al*, 2011); however, the frequency and clinical impact of *DNMT3A* gene mutations in paediatric AML and myeloproliferative neoplasms (MPN) remain uncertain. We searched for *DNMT3A* gene mutations in 149 AMLs who were treated on the Japanese Childhood AML Cooperative protocol, AML 99 (range: 0–15 years old, M0: 5, M1: 23, M2: 44, M3: 13, M4: 22, M5: 21, M6: 1, M7: 17, unclassified: three patients), 40 juvenile myelomonocytic leukaemias (JMMLs; range: 2 months to 8 years), 24 myelodysplastic syndromes (MDSs) and 20 paediatric therapy-related leukaemia/MDSs (t-Leuk/MDSs, range: 1–17 years). *FLT3*-ITD and *NPM1* gene alterations have been reported in these 149 AML patients (Shimada *et al*, 2007, 2008).

Total RNA extracted from the bone marrow or peripheral blood samples at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *DNMT3A* mutations were thus far reported to be almost exclusively involved in exons 16-23 (especially codon R882 in exon 23) (Ley *et al*, 2010; Yamashita *et al*, 2010; Stegelmann *et al*, 2011; Walter *et al*, 2011; Yan *et al*, 2011); thus, we confined our analysis to these exons. cDNA was amplified using the following primers: *DNMT3A* cDNA 15F, 5′-CAGGTGCTTTTGCGTGGAGTGT-3′ and 19R, 5′-ATGC AGGAGGCGGTAGAACTCA-3′, 17F, 5′-AAGATCATGTAC-GTCGGGGA-3′ and 22R, 5′-CTTTGCCCTGCTTTA TG-GAG-3′ and 20F, 5′-CCCTGTGATGATTGATGCCA-3′ and 23R, 5′-GTATTTCCGCCTCTGTG-GTT-3′ for AML samples. For JMML, MDS and t-Leuk/MDS, we confined our analysis to exon 23, including the hotspot of codon R882, of the *DNMT3A* gene using the following primers: *DNMT3A* DNA 23F, 5′-AGAACTAAGCAGGGCC-TCAGAGGA-3′ and 23R, 5′-GTATTTCCGCCTCTGTGGTT-3′. Subsequently, direct sequencing was performed on a DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). The study adhered to the principles of the Helsinki Declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Centre.

No *DNMT3A* mutations were detected in any AML patients in our study. Recently, *DNMT3A* mutations have been reported in paediatric AML patients (Ho *et al*, 2011; Thol *et al*, 2011). Only two patients were identified (both 15 years old). Combined with these and our data, the frequency of *DNMT3A* mutations is extremely rare (2/524, 0·4%) in childhood AML. Furthermore, we did not identify *DNMT3A* mutations in MDS, JMML or paediatric t-Leuk/MDS. These findings were not compatible with those of adult MDS and MPN, suggesting that the frequency of *DNMT3A* gene mutations depends on age.

On the other hand, we found *FLT3*-ITD in 20 (13%) of 149 AML patients; however, no *NPM1* mutations were found (Shimada *et al*, 2007, 2008). Nine AML patients with *FLT3*-ITD were found to lack *DNMT3A* mutation. *DNMT3A* mutations have been correlated with *FLT3*-ITD and *NPM1* in adult AML, but not in paediatric AML. Although patients with *DNMT3A* mutations have been associated with FAB-M4, M5, especially *MLL*-negative M5, no mutations in these paediatric M4/M5 patients were found in this study. *DNMT3A* mutations have not been detected in any adult AML with favourable cytogenetics, including *t*(8;21) and inv(16) (Ley *et al*, 2010; Yan *et al*, 2011). Higher frequencies of *t*(8;21) and inv(16) in

paediatric than in adult AML patients may be associated with rare *DNMT3A* mutations in paediatric AML. These data suggest that the pathology of paediatric AML may be different from that of adult AML. We concluded that *DNMT3A* mutations, as well as *NPM1* mutations, may be infrequent in paediatric AML and MDS patients, especially those <15 years old.

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Authorship

TT and YH designed the study. AS, MS, SA, AT, KH and MT provided critical reagents and samples. NS and MP performed the experiments. RH, IT and HA supervised the work. NS and MP analysed the results. NS, TT, and YH wrote the paper and all the authors critically reviewed and revised it.

Conflict of interest

The authors declare no conflicts of interest.

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References

Ho, P.A., Kutny, M.A., Alonzo, T.A., Gerbing, R.B., Joaquin, J., Raimondi, S.C., Gamis, A.S. & Meshinchi, S. (2011) Leukemic mutations in the methylation-associated genes DNMT3A and IDH2 are rare events in pediatric AML: a report from the Children's Oncology Group. *Pediatric Blood & Cancer*, **57**, 204–209.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., DiPersio, J.F., Mardis, E.R. & Wilson, R.K. (2010) DNMT3A mutations in acute myeloid leukemia. *New England Journal of Medicine*, **363**, 2424–2433.

Pui, C.H., Carroll, W.L., Meshinchi, S. & Arceci, R.J. (2011) Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *Journal of Clinical Oncology*, **29**, 551–565.

Shimada, A., Taki, T., Kubota, C., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I. & Hayashi, Y. (2007) No nucleophosmin mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group. *Leukemia*, **21**, 1307.

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. *Pediatric Blood & Cancer*, **50**, 264–269.

Stegelmann, F., Bullinger, L., Schlenk, R.F., Paschka, P., Griesshammer, M., Blersch, C., Kuhn, S., Schauer, S., Döhner, H. & Döhner, K. (2011) DNMT3A mutations in myeloproliferative neoplasms. *Leukemia*, **25**, 1217–1219.

Thol, F., Heuser, M., Damm, F., Klusmann, J.H., Reinhardt, K. & Reinhardt, D. (2011) DNMT3A mutations are rare in childhood acute myeloid leukemia. *Haematologica*, **96**, 1238–1240.

Walter, M.J., Ding, L., Shen, D., Shao, J., Grillot, M., McLellan, M., Fulton, R., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Kandoth, C., Baty, J., Westervelt, P., Dipersio, J.F., Mardis, E.R., Wilson, R.K., Ley, T.J. & Graubert, T.A. (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. *Leukemia*, **25**, 1153–1158.

Yamashita, Y., Yuan, J., Suetake, I., Suzuki, H., Ishikawa, Y., Choi, Y.L., Ueno, T., Soda, M., Hamada, T., Haruta, H., Takada, S., Miyazaki, Y., Kiyoi, H., Ito, E., Naoe, T., Tomonaga, M., Toyota, M., Tajima, S., Iwama, A. & Mano, H. (2010) Array-based genomic resequencing of human leukemia. *Oncogene*, **29**, 3723–3731.

Yan, X.J., Xu, J., Gu, Z.H., Pan, C.M., Lu, G., Shen, Y., Shi, J.Y., Zhu, Y.M., Tang, L., Zhang, X.W., Liang, W.X., Mi, J.Q., Song, H.D., Li, K.Q., Chen, Z. & Chen, S.J. (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature Genetics*, **43**, 309–315.

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Aberrations of *NEGR1* on 1p31 and *MYEOV* on 11q13 in neuroblastoma

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MYEOV and NEGR1 are novel candidate gene targets in neuroblastoma that were identified by chromosomal gain in 11q13 and loss in 1p31, respectively, through single nucleotide polymorphism array analysis. In the present study, to assess the involvement of MYEOV and NEGR1 in the pathogenesis of neuroblastoma, we analyzed their mutation status and/or expression profiles in a panel of 55 neuroblastoma samples, including 25 cell lines, followed by additional functional studies. No tumor-specific mutations of MYEOV or NEGR1 were identified in our case series. Expression of MYEOV was upregulated in 11 of 25 cell lines (44%) and in seven of 20 fresh tumors (35%). The siRNA-mediated knockdown of MYEOV in NB-19 cells, which exhibit high expression of MYEOV, resulted in a significant decrease in cell proliferation ($P = 0.0027$). Conversely, expression studies of NEGR1 revealed significantly lower expression of this gene in neuroblastomas at an advanced stage of the disease. Exogenous NEGR1 expression in neuroblastoma cells induced significant inhibition of cell growth ($P = 0.019$). The results of these studies provide supporting evidence for MYEOV and NEGR1 as gene targets of 11q13 gains and 1p31 deletions in a neuroblastoma subset. In addition, the findings suggest a possible prognostic value for NEGR1 in neuroblastoma. (*Cancer Sci* 2011; 102: 1645–1650)

Neuroblastoma is one of the most common forms of solid tumors in childhood and accounts for approximately 15% of all pediatric cancer deaths.(1) Despite recent advances in chemoradiotherapy, the prognosis for advanced neuroblastoma remains poor, with an approximate 40% 5-year survival, underscoring the importance of developing novel therapeutic modalities on the basis of an understanding of the pathogenesis of neuroblastoma.(1) Conversely, knowledge of the molecular pathogenesis of neuroblastoma is largely limited in terms of targets, except for the role of *MYCN* amplifications in advanced neuroblastoma.(2) Thus, the recent discovery of *ALK* mutations/amplifications in 6–8% of neuroblastomas(3–6) represents a major development in neuroblastoma research because it not only unravels a novel molecular mechanism involved in neuroblastoma development, but could also a basis for the development of molecular-targeted therapies using ALK inhibitors.(3–6) Similar to a number of novel genetic targets discovered recently in other human cancers, *ALK* mutations were identified thorough genome-wide analyses of copy numbers using high-throughput technologies, including high-density single nucleotide polymorphism (SNP) genotyping microarrays.(3–6) A number of recurrent copy number changes other than those of the *ALK* locus have been identified by genome-wide copy number analysis of neuroblastoma, including losses of 1p31, 3q13, 9p24, 15q11, and 16p13, and high-grade amplifications of 1p36, 7q21, 7q31, 11q13, and 15q13,(3) which may provide important clues for the identification of novel target genes. In fact, several candidate target genes of these common deletions and amplifications have been identified, including *MYEOV* as the target of gains/amplifications in 11q13(7) and *NEGR1* as a candidate tumor suppressor in 1p31 deletions.(8) Previously, *MYEOV* was reported as a putative transforming gene within the 11q13 amplicons in multiple myeloma,(9) whereas *NEGR1* was described as a member of the IgLON (limbic system-associated membrane protein [LAMP]/opioid-binding cell adhesion molecule [OBCAM]/neurotrimin subgroup of the immunoglobulin superfamily) family of cell adhesion molecules.(8) However, the involvement of these genes aberrations in the pathogenesis of neuroblastoma remains unknown. Therefore, in the present study we focused on the abnormalities in both genes and assessed their role, both genetically and functionally, in the pathogenesis of neuroblastoma.

Materials and Methods

Specimens. Primary neuroblastoma specimens were obtained at the time of surgery or biopsy from patients who had been diagnosed with neuroblastoma and had been admitted to Tokyo University Hospital, Saitama Children's Medical Center, or various other hospitals between November 1993 and October 2006. Patients were staged according to the International Neuroblastoma Staging System,(10) with five patients classified as Stage 3 and 25 classified as Stage 4. The clinicopathological findings for all patients are listed in Table 1. Twenty-five neuroblastoma cell lines were also used in the present study (Table 2). The SCMC-N2 series was established in our laboratory;(11) the SJNB series and UTP-N-1(12) were generous gifts from Drs A.T. Look (Department of Pediatric Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, USA) and A. Inoue (Department of Molecular Biology, Toho University School of Medicine, Tokyo, Japan), respectively; all other cell lines were obtained from the Japanese Cancer Resource Cell Bank (http://cellbank.nibio.go.jp/wwwjcrbj.htm, accessed 7 Sep 2008). All cells were maintained in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C.

Semi-quantitative RT-PCR. Total RNA was extracted from the 25 cell lines and 20 frozen stocked tumors using Isogen reagent (Nippon Gene, Osaka, Japan) according to the manufacturer's instructions and was subjected to reverse-transcription reactions to synthesize cDNA using the SuperScript Preamplification System for First Strand cDNA synthesis (Life Technologies, Rockville, MD, USA). Semi-quantitative RT-PCR analysis for *MYEOV*, *CCND1*, and *NEGR1* gene expression was performed as described previously(13) using the primer sets listed in Table S1, available as an accessory publication to this paper. The concentration of the cDNA was normalized against that of *β-actin*, used as an internal control. The signal intensity of *MYEOV* and *CCND1* expression was estimated using NIH

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Table 1. Clinical data for the neuroblastoma cases in the present study

Case no.	Age	Stage	Diagnosis	Histology	*MYCN* amplification	Outcome
1	4 years 2 months	4	C	NBL poorly dif.	+	Alive
2	2 years 4 months	4	C	NBL poorly dif.	+	Alive
3	4 years	4	C	NBL poorly dif.	+	Alive
4	3 years	4	C	NBL poorly dif.	–	Alive
5	1 year 5 months	4	C	NBL poorly dif.	–	Alive
6	10 day	3	C	NBL dif.	–	Alive
7	4 years	4	C	NBL poorly dif.	–	Dead
8	4 years 2 months	4	C	NBL poorly dif.	–	Alive
9	2 years	3	C	GNB well dif.	–	Alive
10	10 years	4	C	NBL	–	Dead
11	4 years	4	C	NBL	+	Dead
12	3 years	3	C	NBL	+	Alive
13	11 years 9 months	4	C	NBL poorly dif.	–	Alive
14	6 months	3	MS	GNB	–	Alive
15	7 months	4	MS	NBL poorly dif.	–	Dead
16	4 years	4	C	NBL	+	Dead
17	4 years 9 months	4	C	NBL	–	Dead
18	7 months	4	MS	NBL	–	Alive
19	2 years	4	C	NBL poorly dif.	+	Alive
20	3 years	4	C	NBL	+	Dead
21	8 years	4	C	NBL poorly dif.	–	Alive
22	2 years 3 months	4	C	NBL	+	Alive
23	4 years	4	C	NBL	+	Dead
24	5 months	4	C	NBL	–	Alive
25	5 years	4	C	NBL	–	Dead
26	4 years 10 months	4	C	NBL	–	Alive
27	7 years	4	C	NBL poorly dif.	+	Dead
28	1 year 6 months	3	C	NBL	–	Alive
29	1 year 8 months	4	C	NBL	–	Alive
30	8 months	4	C	NBL	–	Alive

C, clinical; MS, mass screening program; NBL, neuroblastoma; NBL poorly dif., poorly differentiated neuroblastoma; GNB, ganglioneuroblastoma; GNB well dif., well-differentiated ganglioneuroblastoma.

Table 2. Neuroblastoma cell lines used in the present study

Cell line	MYCN amplification
CHP-134	–
GOTO	+
IMR-32	+
LAN-1	+
LAN-2	+
LAN-5	+
NB-1	–
NB-16	+
NB-19	+
NB-69	–
NH-12	+
SCMC-N2	+
SCMC-N4	+
SCMC-N5	+
SJNB-1	–
SJNB-2	+
SJNB-3	–
SJNB-4	+
SJNB-5	+
SJNB-6	+
SJNB-7	+
SJNB-8	+
SK-N-SH	–
TGW	+
UTP-N-1	+

Image 1.61 software (Wayne Rasband; National Institutes of Health, Bethesda, MD, USA).

Quantitative RT-PCR. To quantify the expression levels of *NEGR1*, real-time PCR (RQ-PCR) analysis was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Tokyo, Japan) with an iCycler iQ real-time PCR detection system (Bio-Rad Japan, Tokyo, Japan). The primer sets used for the RQ-PCR are listed in Table S1 and the PCR conditions were as described previously.[13] For the purpose of normalization, relative expression levels were calculated by dividing the expression level of the respective gene by that of *β-actin*.

Mutational analysis of *MYEOV* and *NEGR1* genes. Genetic screening for *MYEOV* and *NERG1* genes in 25 cell lines was performed by denaturing HPLC (DHPLC) using the WAVE System Model 4500 (Transgenomic, Omaha, NE, USA), as described previously.[14] The primer sets used in the present study are listed in Table S1.

Bisulfate modification and methylation-specific PCR. Bisulfate modification of genomic DNA was performed as described previously.[15] For methylation-specific PCR (MSP), approximately 10 ng bisulfite-treated DNA was amplified with primers for both the methylated and unmethylated sequences. Reaction products were separated by electorophoresis on a 2.0% agarose gel. The primer sets for methylation-specific PCR analysis are listed in Table S1.

Knockdown of *MYEOV* using siRNA. The functional roles of the *MYEOV* gene in neuroblastoma cells was assessed using gene knockdown with siRNA.[16] The siRNA was designed and synthesized for silencing *MYEOV* (Invitrogen, Carlsbad, CA,

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USA). The siRNA duplex had the following sequences: 1132 sense, 5′-UCA ACG CCC ACU CUA AAG GCU UCU C-3′; and 1132 antisense, 5′-GAG AAG CCU UUA GAG UGG GCG UUG A-3′. A chemically synthesized non-silencing siRNA duplex that had no known homology to any mammalian gene was used as a control for non-specific silencing events and had the following sequences: sense, 5′-UUC UCC GAA CGU GUC ACG UdT dT-3′; and antisense, 5′-ACG UGA CAC GUU CGG AGA AdT dT-3′. Gene knockdown was achieved in NB-19, CHP-134 and PF-SK-1 cells using HiPerFect transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Transient transfection. The expression vector (pME18S) containing the full-length *Eco*RI-*Xba*I fragment of the *NEGR1* cDNA was transfected into NB-19, SJNB-7, and PF-SK-1 cells using the lipofection method according to the manufacturer's instructions (Qiagen).[11] Briefly, 1.5×10^5 cells were seeded in a six-well plate and incubated in 1.6 mL RPMI 1640 (Gibco-BRL) with 10 µL Effectance reagent (Qiagen), 3.2 µL Enhancer (1:8) (Qiagen), 10 µL Effectene (Qiagen), and 0.4 µg expression vector. Cells were counted 72 h after transfection.

Statistical analysis. Expression of the *NEGR1* gene was compared between favorable and unfavorable cases of neuroblastomas using the Mann–Whitney *U*-test. Exact 95% confidence intervals (CI) of the proportions were calculated on the basis of binomial distribution. The Kruskal–Wallis test was used to compare the functional effects of *MYEOV* inhibition and *NEGR1* expression in neuroblastoma cells.

Results

Gain and high-grade amplification of 11q13 involving the *MYEOV* locus in neuroblastoma. In the present series, gains of chromosome 11q13 were detected in multiple neuroblastoma cases.[3] Within this gain, a high-grade amplification was found in a single case with Stage 4 disease (Case 22; Fig. 1a). The critical amplicon that had minimum overlapping amplification/gain was found in a 340-kb region exclusively containing *MYEOV*, located 360 kb upstream from the *CCND1* locus[7] (Fig. 1a). Previously, *MYEOV* had been identified as a putative transforming gene based on the NIH/3T3 tumorigenicity assay[9] and was shown to be highly expressed in a subset of multiple myelomas harboring t(11;14)(q13;q32).[7] We further examined the expression patterns of *MYEOV* in a total of 45 neuroblastoma samples using semi-quantitative RT-PCR analysis, in which 11 of 25 cell lines (44%) and seven of 20 fresh tumors (35%) showed higher expression levels of *MYEOV* compared with the median expression level (*MYEOV*/*β-actin* signal intensity = 1.4; Fig. 1b). Although most tumors exhibited increased expression of both *CCND1* and *MYEOV*, Case 22 showed high expression of *MYEOV* but not *CCND1* (Fig. 1c). Mutational analysis of the coding region of *MYEOV* was also performed in 25 cell lines, but no tumor-specific mutations were detected.

Homozygous deletion on 1p31 detected in neuroblastoma. Detection of homozygous deletions was also of interest because they provide an important clue in pinpointing tumor suppressor loci. In an allele-specific copy number analyzer for GeneChip (CNAG) and allele-specific copy number analysis using anonymous references (AsCNAR), homozygous deletions could be identified as the loss of both parental alleles, even in the presence of significant components of normal tissues.[3] In the present study, 70 homozygous deletions were identified at 50 independent loci in the neuroblastoma samples. Unfortunately, we were not able to completely exclude the possibility that some may represent copy number variations (CNV) rather than real homozygous deletions, because paired DNA was available only in four primary neuroblastoma cases and many homozygous deletions were found in established neuroblastoma cell

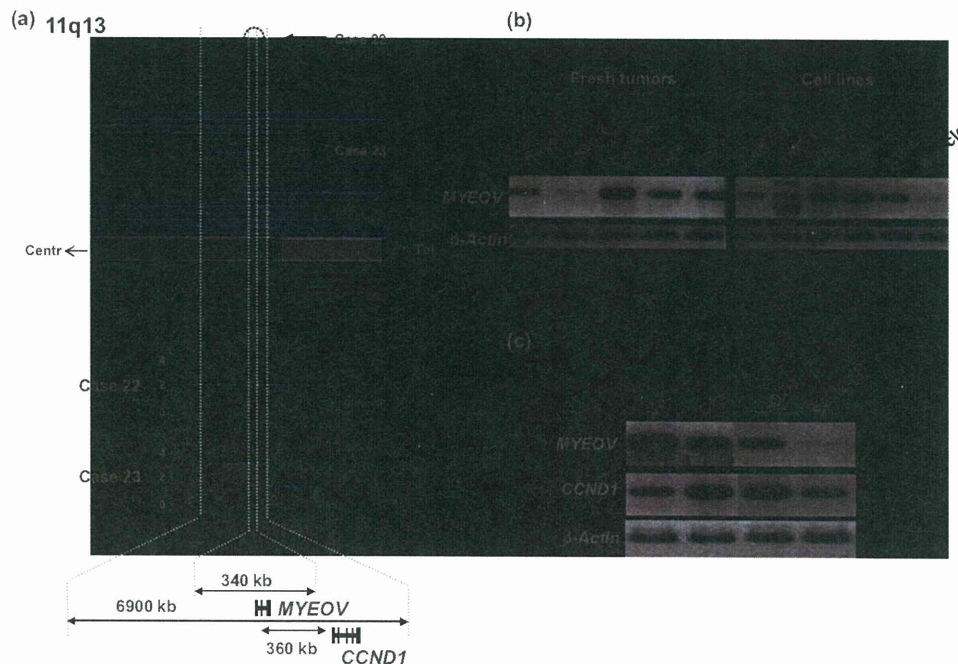

Fig. 1. Gains and high-grade amplification on chromosome 11q13 in neuroblastoma. (a) A common 340-kb region having copy number (CN) gains contains a single known gene, *MYEOV*. In addition, *CCND1* is frequently contained in CN gains at 11q13, but mapped outside the minimum region of common CN gains. Red bars, gains (3 < CN < 5); green bars, losses (CN = 1); light red bar (circled), high-grade amplification (CN ≥ 5). (b) Representative results of *MYEOV* expression in fresh tumors and cell lines (RNA from normal muscle was used as a control). (c) Expression of *MYEOV* and *CCND1* in Cases 22 and 23 (RNA from normal muscle was used as a control). The expression of *MYEOV* in Case 22 tended to be higher than that in Case 23. tel, telomere; centr, centromere.

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lines. Complete loss of genetic material at eight loci was confirmed by genomic PCR (data not shown).

Of the 70 homozygous deletions identified, we focused on a homozygous deletion involving a 370-kb region at 1p31 in NB-19. This region contains a part of *NEGR* (exon 1 and a part of intron 1), a unique candidate target gene, which was also disrupted by a translocation in another cell line, namely SJNB-6 (Fig. 2a,b). Because *NEGR1* encodes a member of the IgLON family of cell adhesion molecule sand has been reported to be a putative tumor suppressor gene in ovarian cancer,[8] we examined its expression in neuroblastoma cases in the present study to evaluate the clinical impact of *NEGR1* expression. As shown in Figure 2(c), *NEGR1* expression was absent or very low in 10 of 25 (40%) cell lines, as determined by semi-quantitative RT-PCR (Fig. 2c). In quantitative RT-PCR analysis using fresh tumor samples (20 fresh advanced-stage tumors and an additional 20 cases of early stage tumors), the expression of the *NEGR1* gene was significantly lower in advanced-stage tumors compared with early stage tumors ($P = 0.0041$; Fig. 2c). Similarly, the expression of the *NEGR1* gene was significantly lower in patients who died compared with patients who survived ($P = 0.018$; Fig. S1). Mutation analysis was also performed in neuroblastoma cell lines, but no tumor-specific mutations were detected. Methylation analysis of the promoter region of *NEGR1* using 10 neuroblastoma cell lines without *NEGR1* expression did not reveal any tumor-specific methylation pattern in neuroblastoma cell lines or fresh neuroblastoma samples (data not shown).

Functional analyses of *MYEOV* and *NEGR1* in neuroblastoma cell lines. We further evaluated the oncogenic potential of *MYEOV* using siRNA-mediated gene knockdown in the NB-19 cell line, which highly expresses *MYEOV*. As shown in Figure 3(a,b), When *MYEOV* expression was suppressed by siRNA, NB-19 cells exhibited retarded growth compared with the growth of control cells ($P = 0.0027$), indicating that MYEOV positively regulates cell proliferation (Fig. 3a,b). Similar results were obtained in CHP-134 and PF-SK-1 cells (Fig. S2). To assess the tumor suppressor function of *NEGR1* in neuroblastoma cells, we generated an *NEGR1* expression vector that was transiently transfected into NB-19 cells, in which *NEGR1* is homozygously deleted. Expression of *NEGR1* significantly suppressed the proliferation of NB-19 cells compared with mock transfection ($P = 0.019$; Fig. 3c,d). In addition, the *NEGR1* expression vector was transiently transfected into SJNB-7 and PF-SK-1 cells, in which *NEGR1* expression is absent. Following transfection into these cell lines, profound inhibition of cell proliferation was observed for both SJNB-7 and PF-SK-1 cells expressing *NEGR1* (Fig. S3).

Discussion

In the present study, we showed that *MYEOV* and *NEGR1* are candidate gene targets of 11q13 gain and 1p31 deletion, respectively, in a neuroblastoma subset. To our knowledge, this is the first report to describe aberrations of *MYEOV* and *NEGR1* in neuroblastoma.

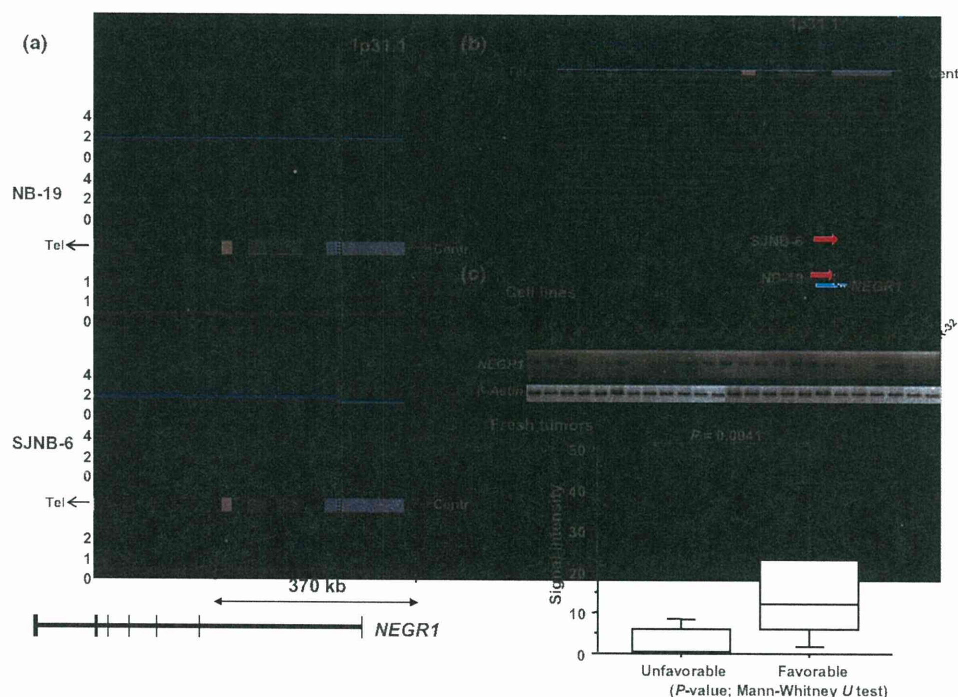

Fig. 2. *NEGR1* as a candidate tumor suppressor gene in neuroblastoma. (a) Deletion mapping of 1p31.1 disclosed a homozygous deletion spanning a 370-kb region in the NB-19 cell line, which contains part of *NEGR1* as the only structural gene. The *NEGR1* gene is also disrupted in intron 1 by the breakpoint of a segmental duplication at 1p31.1 in another neuroblastoma cell line (SJNB-6). For each panel, total copy numbers (tCN; red dots), moving averages of tCN for five consecutive single nucleotide polymorphisms (SNP; blue line), an ideogram of the relevant chromosome, the location of heterozygous SNP calls (green bars), and allele-specific copy numbers (AsCN) averaged for five consecutive SNP (red and green lines for larger and smaller alleles, respectively) are plotted. Note that the CN are expressed in terms of "observed" signal ratios between tumor and reference samples, where the baseline is adjusted to 2 for tCN plots and to 1 for AsCN. (b) Summary of CN abnormalities of 1p31.1 in neuroblastoma. Red bars, gains (3 < CN < 5); green bars, losses (CN = 1); light green bar (circled), homozygous deletion (CN = 0). A homozygous deletion detected in NB-19 and a chromosomal rearrangement detected in SJNB-6 are indicated by the red arrows. The location of *NEGR1* is shown by the blue line. (c) *NEGR1* expression in neuroblastoma. Top panel: representative result of *NEGR1* expression in neuroblastoma cell lines showing frequently reduced expression levels in a subset of neuroblastoma cell lines. Bottom graph: expression of the *NEGR1* gene as measured by quantitative PCR was significantly lower in tumors with an unfavorable outcome than in tumors with a favorable outcome ($P = 0.0041$, Mann–Whitney *U*-test). tel, telomere; centr, centromere.

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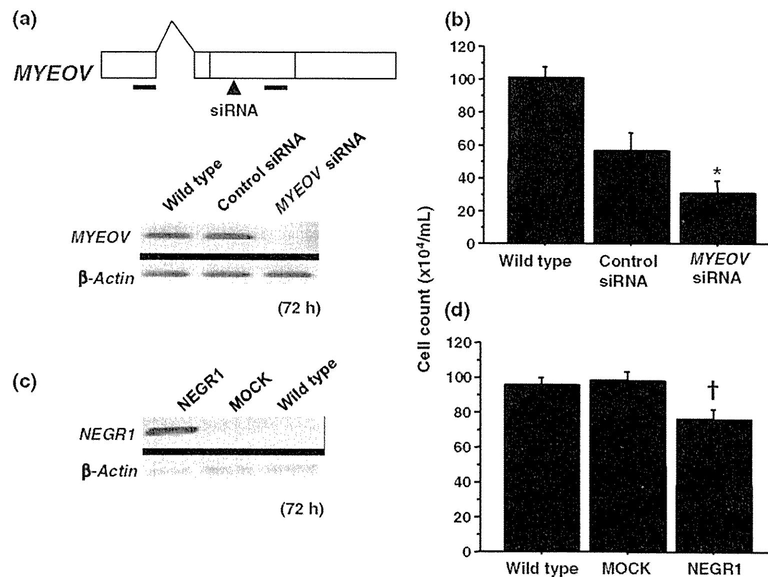

Fig. 3. Effect of *MYEOV* inhibition by siRNA on cell growth and effect of *NEGR1* on cell growth in neuroblastoma cells. (a) Confirmation of siRNA-mediated gene knockdown using semi-quantitative RT-PCR analysis. Following siRNA treatment, *MYEOV* mRNA was absent in treated cells; however, abundant *MYEOV* expression was detected in wild-type and control cells. (b) Effect of *MYEOV* inhibition by siRNA transfected into NB-19 cells on cell growth. Cell growth was impaired cell growth in siRNA-transfected cells compared with that of control cells (*$P = 0.0027$, Kruskal–Wallis test). (c) Analysis (RT-PCR) of NB-19 cells transfected with the pME18S vector. Mock-transfected and wild-type cells were used as controls. (d) The growth of cells transiently expressing *NEGR1* was impaired compared with that of mock-transfected and wild-type cells (†$P = 0.019$, Kruskal–Wallis test).

Initially, *MYEOV* was reported as a gene that was possibly co-overexpressed with *CCND1* in some cases of multiple myeloma with t(11;14)(q13;q32); later, it was shown to be co-amplified and co-overexpressed with *CCND1* in a subset of esophageal squamous cell carcinomas, breast cancers, gastric cancers, and colorectal cancers.[7,17–19] Although the major genetic targets of these rearrangements and amplifications have been shown to be *CCND1*, in some breast cancer cases the 11q13 amplicon exclusively contained *MYEOV* and not *CCDN1*, suggesting a *CCDN1*-independent oncogenic role for *MYEOV*.[7] The oncogenic role of *MYEOV* has also been investigated in functional studies, showing that *in vitro* siRNA-mediated knockdown of *MYEOV* resulted in inhibition of proliferation, invasion, and migration of colorectal cancer cell lines.[19] In our neuroblastoma cases, *MYEOV* was overexpressed in approximately 30% of primary neuroblastoma cases, with seven cases showing gain/amplification of MYEOV. We also confirmed that *MYEOV* was the only gene found in the common gain/amplicon at 11q13 and that proliferation of neuroblastoma cell lines was inhibited by siRNA-mediated *MYEOV* knockdown, supporting an oncogenic role for *MYEOV* in some neuroblastoma cases. Although several studies have revealed that *MYEOV* amplification is associated with poor prognosis in multiple myeloma, esophageal squamous cell carcinoma, and breast cancer,[7,18,20] the clinical impact of *MYEOV* gain/amplification or overexpression in neuroblastoma is unclear and requires further evaluation.

The *NEGR1* gene is a single gene found in one of the recurrent deletions at 1p31. Although the *NEGR1* locus is known as one of the most common CNV regions,[21] we also identified a neuroblastoma cell line in which *NEGR1* was disrupted in by gene rearrangement, supporting the fact that *NEGR1* is one of the target genes in neuroblastoma. In ovarian cancer, *NEGR1* is a putative tumor suppressor gene encoding one of the IgLON cell adhesion family members, namely OPCML, and it plays a central role in the establishment and remodeling of the central nervous system.[22] Notably, OPCML has been shown to exhibit functional characteristics of a tumor suppressor gene in epithelial ovarian cancer.[23] In our analysis, expression of *NEGR1* was substantially reduced in 43% of advanced-stage tumors without 1p31 deletions/rearrangement. In addition, re-expression of *NEGR1* in the NB-19 cell line with homozygous deletion of *NEGR1*, as well as in other neuroblastoma cell lines that did not express *NEGR1*, resulted in the inhibition of cell growth, suggesting that *NEGR1* is a candidate tumor suppressor gene in neuroblastoma and may have possible prognostic value. Although expression of OPCML in ovarian cancers is suppressed or reduced mainly through epigenetic mechanisms,[23] tumor-specific methylation was not detected in neuroblastoma cells in the present study. The mechanisms for the absence of *NEGR1* in the tumors without homozygous deletion, mutation, and methylation were not clear in the present study. We cannot role out the possibility that mutations are harbored in the promoter region of *NEGR1* with consequent gene inactivation. Furthermore, *NEGR1* was often heterozygously deleted, but not mutated or methylated, in neuroblastoma; most deletions occur in tumors at advanced stages, suggesting that *NEGR1* has haploinsufficient effects on advanced disease in neuroblastoma.

In conclusion, the results of the present study suggest that *MYEOV* at 11q13 and *NEGR1* at 1p31 are functional gene targets in a subset of neuroblastoma. Further studies on both genes will expand these pathways and provide insights into the progression of neuroblastoma, as well as possibly enabling the development of novel therapeutics based on targeting MYEOV and NEGR1 in neuroblastoma.

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Disclosure Statement

The authors have no conflicts of interest.

References

1 Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; **3**: 203–16.
2 Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984; **224**: 1121–4.
3 Chen Y, Takita J, Choi YL *et al.* Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 2008; **455**: 971–4.
4 Mosse YP, Laudenslager M, Longo L *et al.* Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008; **455**: 930–5.
5 Janoueix-Lerosey I, Lequin D, Brugieres L *et al.* Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008; **455**: 967–70.
6 George RE, Sanda T, Hanna M *et al.* Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 2008; **455**: 975–8.
7 Janssen JW, Cuny M, Orsetti B *et al.* MYEOV: a candidate gene for DNA amplification events occurring centromeric to CCND1 in breast cancer. *Int J Cancer* 2001; **102**: 608–14.
8 Ntougkos E, Rush R, Scott D *et al.* The IgLON family in epithelial ovarian cancer: expression profiles and clinicopathologic correlates. *Clin Cancer Res* 2005; **11**: 5764–8.
9 Brecht M, Steenvoorden AC, Luf S, Bartram CR, Janssen JW. Rearrangement and expression of myeov and hst in NIH/3T3 transfectants: a caveat for the interpretation of DNA transfection analyses. *Oncol Rep* 2007; **17**: 1127–31.
10 Smith EI, Haase GM, Seeger RC, Brodeur GM. A surgical perspective on the current staging in neuroblastoma: the International Neuroblastoma Staging System proposal. *J Pediatr Surg* 1989; **24**: 386–90.
11 Takita J, Hayashi Y, Nakajima T *et al.* The p16 (CDKN2A) gene is involved in the growth of neuroblastoma cells and its expression is associated with prognosis of neuroblastoma patients. *Oncogene* 1998; **17**: 3137–43.
12 Inoue HK, Shirao T. Neurite formation induced in neuroblastoma cells and genetically altered non-neuronal cells. *J Electron Microsc* 1997; **46**: 497–502.
13 Takita J, Ishii M, Tsutsumi S *et al.* Gene expression profiling and identification of novel prognostic marker genes in neuroblastoma. *Genes Chromosom Cancer* 2004; **40**: 120–32.
14 Donohoe TJ, Sintim HO, Sisangia L *et al.* Utility of the ammonia-free Birch reduction of electron-deficient pyrroles: total synthesis of the 20s proteasome inhibitor, clasto-lactacystin beta-lactone. *Chemistry* 2005; **11**: 4227–38.
15 Takita J, Yang HW, Chen YY *et al.* Allelic imbalance on chromosome 2q and alterations of the caspase 8 gene in neuroblastoma. *Oncogene* 2001; **20**: 4424–32.
16 Moss AC, Lawlor G, Murray D *et al.* ETV4 and Myeov knockdown impairs colon cancer cell line proliferation and invasion. *Biochem Biophys Res Commun* 2006; **345**: 216–21.
17 Janssen JW, Vaandrager JW, Heuser T *et al.* Concurrent activation of a novel putative transforming gene, myeov, and cyclin D1 in a subset of multiple myeloma cell lines with t(11;14)(q13;q32). *Blood* 2000; **95**: 2691–8.
18 Carneiro A, Isinger A, Karlsson A *et al.* Prognostic impact of array-based genomic profiles in esophageal squamous cell cancer. *BMC Cancer* 2008; **8**: 98.
19 Lawlor G, Doran PP, MacMathuna P, Murray DW. MYEOV (myeloma overexpressed gene) drives colon cancer cell migration and is regulated by PGE_2. *J Exp Clin Cancer Res* 2010; **29**: 81.
20 Moreaux J, Hose D, Bonnefond A *et al.* MYEOV is a prognostic factor in multiple myeloma. *Exp Hematol* 2010; **38**: 1189–98.
21 Jarick I, Vogel CI, Scherag S *et al.* Novel common copy number variation for early onset extreme obesity on chromosome 11q11 identified by a genome-wide analysis. *Hum Mol Genet* 2011; **20**: 840–52.
22 Funatsu N, Miyata S, Kumanogoh H *et al.* Characterization of a novel rat brain glycosylphosphatidylinositol-anchored protein (Kilon), a member of the IgLON cell adhesion molecule family. *J Biol Chem* 1999; **274**: 8224–30.
23 Sellar GC, Watt KP, Rabiasz GJ *et al.* OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. *Nat Genet* 2003; **34**: 337–43.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *NEGR1* expression in 30 neuroblastoma cases.

Fig. S2. Effect of siRNA inhibition of *MYEOV* on cell growth in CHP-134 and PF-SK-1 cells.

Fig. S3. Effect of *NEGR1* on cell growth in the neuroblastoma cell lines PF-SK-1 and SJNB-7.

Table S1. Primer sequences used in the present study.

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of departure from Hardy–Weinberg equilibrium ($P=0.06$), because the variant G allele is significantly more prevalent among Whites than non-Whites with the allele frequency of 0.24 versus 0.073, respectively ($P=0.0003$). Still, the observed associations retained significance in analyses restricted only to Whites. The G allele was associated with better EFS and OS in univariate analyses ($P=0.0173$ and 0.035, respectively, data not shown) and in multivariable analyses ($P=0.023$ and 0.005, respectively, data not shown).

We also observed that the variant A allele of FKBP5 SNP rs7755289 (T>A; intron 8) was significantly associated with worse EFS ($P=0.014$, hazard ratio = 3.193, 95% CI = 1.258–8.104, Figure 1c) and OS ($P=0.0036$, hazard ratio = 4.846, 95% CI = 1.68–14, Figure 1d). In addition, A allele was associated with increased day 22 MRD ($P=0.017$), increased cumulative incidence of relapse ($P=0.045$, hazard ratio = 3.4, 95% CI = 1.03–11.22) and an increased cumulative incidence of treatment-related mortality ($P=0.012$, hazard ratio = 5.57, 95% CI = 1.44–21.47). However, as this SNP occurred with the allele frequency of only ~0.2, the low sample size restricted us from performing further analysis. Although the above mentioned SNPs were the most interesting SNPs, we also observed association of SNP rs16878591 ($P=0.011$) with day 22 MRD levels and SNPs within LD block-2 with *in vitro* ara-C LC_{50} values ($P=0.03$; Table 1).

In previous reports, FKBP5 expression has been shown to positively influence response to cytarabine and gemcitabine. More recently, FKBP5 has been identified as scaffolding protein that facilitates PHLPP-mediated dephosphorylation of AKT-Ser473, thus indicating that higher expression of FKBP5 might contribute to enhanced chemosensitivity.[3–5]siRNA-mediated FKBP5 knockdown increases the resistance to cytarabine and other agents as etoposide, paclitaxel and doxetaxel.[1,3–5] Thus, FKBP5 SNPs may also be associated with response to other agents used in combination with cytarabine in AML patients. In conclusion, our preliminary results suggest that the FKBP5 polymorphisms mentioned above may also be relevant for AML treatment response. These results should be confirmed with functional studies and independent clinical studies. Identification of pharmacogenetic markers of response, such as FKBP5 SNP such as rs3798346, might help in further understanding inter-patient variation in response to chemotherapy.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Li L, Fridley B, Kalari K, Jenkins G, Batzler A, Safgren S *et al.* Gemcitabine and cytosine arabinoside cytotoxicity: association with lymphoblastoid cell expression. *Cancer Res* 2008; **68**: 7050–7058.
2 Jinwal UK, Koren III J, Borysov SI, Schmid AB, Abisambra JF, Blair LJ *et al.* The Hsp90 cochaperone, FKBP51, increases Tau stability and polymerizes microtubules. *J Neurosci* 2010; **30**: 591–599.
3 Li L, Lou Z, Wang L. The role of FKBP5 in cancer aetiology and chemoresistance. *Br J Cancer* 2011; **104**: 19–23.
4 Pei H, Li L, Fridley BL, Jenkins GD, Kalari KR, Lingle W *et al.* FKBP51 affects cancer cell response to chemotherapy by negatively regulating Akt. *Cancer Cell* 2009; **16**: 259–266.
5 Pei H, Lou Z, Wang L. Emerging role of FKBP51 in AKT kinase/protein kinase B signaling. *Cell Cycle* 2010; **9**: 6–7.
6 Rubnitz JE, Inaba H, Dahl G, Ribeiro RC, Bowman WP, Taub J *et al.* Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial. *Lancet Oncol* 2010; **11**: 543–552.
7 Lamba JK, Crews K, Pounds SB, Cao X, Gandhi V, Plunkett W *et al.* Identification of predictive markers of cytarabine response in acute myeloid leukemia by integrative analysis of gene-expression profiles with multiple phenotypes. *Pharmacogenomics* 2011; **12**: 327–239.
8 Benjamini YaH, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc* 1995. Series B. **57**: 289–300.

CBL mutation in childhood therapy-related leukemia

Leukemia (2011) **25,** 1356–1358; doi:10.1038/leu.2011.75; published online 15 April 2011

Therapy-related leukemia and myelodysplastic syndrome (t-Leuk/MDS) are mainly caused by topoisomerase II inhibitors that cause acute myeloid leukemia (AML) with an 11q23 translocation or by alkylating agents that induce MDS/AML with an *AML1* mutation and monosomy 7.[1,2] Two types of t-Leuk/MDS can be distinguished, one of which has a long latency (⩾5–7 years) and is seen following alkylating agents, frequently with an preleukemic phase.[1] The other has a short latency period (1–3 years), no preleukemic phase, and is strongly associated with the administration of topoisomerase II inhibitors and chromosomal abnormalities involving 11q23 translocation/*MLL* rearrangement (*MLL*-R).[2] Repair of etoposide (VP-16)-stabilized DNA topoisomerase II covalent complexes may initiate *MLL*-R observed in patients.[3]

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because

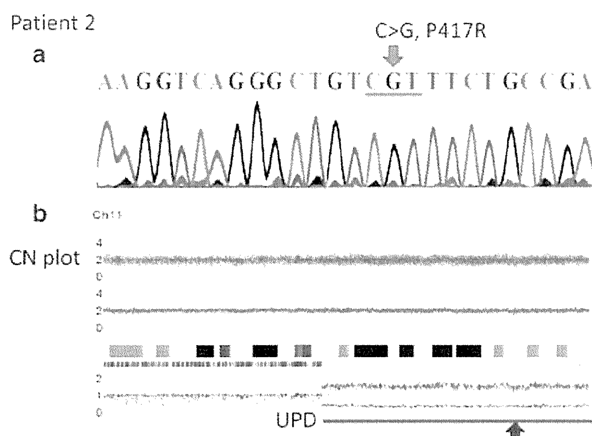

Figure 1 Identification of acquired isodisomy of 11q and *CBL* mutation in therapy-related leukemia. (**a**) Homozygous mutation of the *CBL* gene was identified in patient 2. (**b**) Copy number (CN) analysis for the gene chip output for therapy-related leukemia in patient 2. Total CNs (red plot) are shown above the cytoband, and the result of allele-specific CN analysis with anonymous references plots are shown below the cytoband. Larger allele is presented in red line, and smaller allele is presented in green line. Allele-specific analysis showed 11q-aUPD (blue line), which contained *CBL* region (black arrow).

these *CBL* mutations were shown to result in aberrant tyrosine kinase signaling, which would lead also to activation of RAS signaling pathways. We and others reported that *CBL* mutations occurred in a variety of myeloid neoplasms, including *de novo* AML,[4] MDS[4] and myeloproliferative neoplasm,[4,5] especially in chronic myelomonocytic leukemia[5] and juvenile myelomonocytic leukemia.[6] The importance of *CBL* mutations concerning about leukemogenesis is substantially increased. This prompted us to search for possible *CBL* mutations in pediatric t-Leuk/MDS.

Analysis of *CBL* gene was carried out in 20 pediatric t-Leuk/MDSs, including 15 AMLs (range: 1 year and 10 months to 17 years; 8 males and 7 females), 4 MDSs (range: 7 years to 14 years; 4 males) and 1 acute lymphoblastic leukemia (4 years and 2 months; 1 male). Median age at diagnosis was 8 years and 1 months (range: 1 year and 10 months to 17 years; 13 males and 7 females). Rearrangements of *MLL* gene were found in 17 patients (85%), including 15 of 16 who received VP-16 (Sugita *et al.*[7]), and 2 of 4 who did not receive it. An initial diagnosis was made as non-Hodgkin's lymphoma in seven patients, neuroblastoma in five, acute lymphoblastic leukemia in five, AML in two and juvenile myelomonocytic leukemia in one.

Because *CBL* mutations thus far reported almost exclusively involved exons 8–9 that encode linker/RING finger domains,[4–6] we confined our mutation analysis to these exons, in which PCR-amplified exons 8–9 were subjected to direct sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Branchburg, NJ, USA). The study adhered to the principles of the Helsinki declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Center.

Homozygous mutation of the *CBL* gene was identified in 1 out of the 20 t-Leuk/MDS cases (5%), which were located in the RING finger domain (P417R in patient 2). As the frequency of 11q-acquired uniparental disomy (11q-aUPD) was reported ~85–90% in *CBL* mutations,[4–6] we analyzed his sample using Affymetrix GeneChip 250K *Nsp*I array (Affymetrix, Santa Clara, CA, USA), and found the presence of 11q-aUPD, which was the sole abnormality seen by single-nucleotide polymorphism array (Figure 1), confirming a strong association of *CBL* mutations with 11q-aUPD as previously described.[4–6] Furthermore, we examined *NRAS* and *KRAS* mutations in these patients whose samples were available and found *KRAS* mutation in one patient with t-Leuk (acute monocytic leukemia having t(9;11)(p21;q23) after B-cell precursor acute lymphoblastic leukemia having 6p−, 7q+, 9q+ and 12q−).

CBL mutation was detected in MDS cells from the patient with t-MDS after malignant lymphoma. The patient was initially diagnosed as having diffuse large T-cell type malignant lymphoma, whose biopsied specimen of the buccal lymph node showed MT1(+), MB1(−) and UCHL1(+), when he was 5 years old. He subsequently was treated with chemotherapy according to T-8801 protocol including VP-16 (200 mg/m^2) given twice weekly,[7] and obtained a complete remission. However, at 7 months after diagnosis, tumor appeared in the right maxilla, and was diagnosed as the relapsed lymphoma, then, he received local irradiation (30 Gy) and chemotherapy including ifosfamide, vincristine, THP-adriamycin and L-asparaginase. At 4 months later, enlarged spleen was resected, and the infiltrated tumor cells were microscopically seen in the tumor sections. At 6 months later, 19 months after initial diagnosis, blast cells appeared in peripheral blood. His laboratory data revealed leukocytosis (14 700/μl with 18% blast cells) and an elevated serum lactate dehydrogenase level (1458 U/l). Bone marrow aspiration revealed 9.8% blasts, which were positive for cytoplasmic myeloperoxidase, suggesting MDS. Surface marker analysis showed that the leukemic blasts in the bone marrow were positive for CD33. Chromosomal analysis of bone marrow cells revealed t(5;11)(q21;q23) in 11 of 20 cells. Rearrangement of *MLL* gene of these cells was identified by Southern blotting, however, no known chimeric mRNA with *MLL*, such as *MLL-AF5q31* and *MLL-GRAF* in t(5;11)(q31;q23), could be detected. These suggested that the gene at 5q21 was a novel partner gene of *MLL*. Although another chemotherapy for AML was performed, his blast cells increased >30% blasts in bone marrow at 25 months after initial diagnosis. Therefore, he was diagnosed as having t-Leuk resembling acute monoblastic leukemia due to VP-16. He died of mycotic infection at 35 months after initial diagnosis.

No *CBL* mutations were found in his lymphoma sample at diagnosis and in tumor cells in the enlarged spleen. We also perfomed tissue-fluorescence *in situ* hybridization analysis with *MLL* probe on paraffin-embedded tissue sections of the tumor cells in the enlarged spleen, however, no evaluable results could be detected because of poor quality of samples. No initial samples for tissue-fluorescence *in situ* hybridization analysis could be obtained.

The 11q23 translocation/*MLL*-R in t-Leuk/MDS was considered to be induced by VP-16,[3] however, gene alterations in addition to *MLL*-R have rarely reported. Recently, *CBL* mutations were found in a variety of myeloid neoplasms.[4–6] Among 2000 samples from the patients with myeloid neoplasms, *CBL* mutations have been found in ~5% samples, including AML transformed from MDS, but not *de novo* or therapy-related acute leukemia with 11q23 translocation/*MLL*-R. To our knowledge, this is the first t-Leuk/MDS patient with 11q23 translocation/*MLL*-R and *CBL* mutation. Interestingly, a *de novo* AML case with *MLL–CBL* fusion gene has also been reported.[8] These findings suggest that alterations of *CBL* gene and 11q23 translocation/*MLL*-R may cooperate in the pathogenesis of a subtype of t-Leuk/MDS and *de novo* leukemia.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Tucker MA, Meadows AT, Boice Jr JD, Stovall M, Oberlin O, Stone BJ *et al.* Leukemia after therapy with alkylating agents for childhood cancer. *J Natl Cancer Inst* 1987; **78**: 459–464.
2 Pui CH, Ribeiro RC, Hancock ML, Rivera GK, Evans WE, Raimondi SC *et al.* Acute myeloid leukemias in children treated with epipodophyllotoxins for acute lymphoblastic leukemia. *N Engl J Med* 1991; **325**: 1682–1687.
3 Nakada S, Katsuki Y, Imoto I, Yokoyama T, Nagasawa M, Inazawa J *et al.* Early G2/M checkpoint failure as a molecular mechanism underlying etoposide-induced chromosomal aberrations. *J Clin Invest* 2006; **116**: 80–89.
4 Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C *et al.* Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. *Blood* 2009; **113**: 6182–6192.
5 Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S *et al.* Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature* 2009; **460**: 904–908.
6 Shiba N, Kato M, Park MJ, Sanada M, Ito E, Fukushima K *et al.* CBL mutations in juvenile myelomonocytic leukemia and pediatric myelodysplastic syndrome. *Leukemia* 2010; **24**: 1090–1092.
7 Sugita K, Furukawa T, Tsuchida M, Okawa Y, Nakazawa S, Akatsuka J *et al.* High frequency of etoposide (VP-16)-related secondary leukemia in children with non-Hodgkin's lymphoma. *Am J Pediatr Hematol Oncol* 1993; **15**: 99–104.
8 Fu JF, Hsu JJ, Tang TC, Shih LY. Identification of CBL, a proto-oncogene at 11q23.3, as a novel MLL fusion partner in a patient with *de novo* acute myeloid leukemia. *Genes Chromosomes Cancer* 2003; **37**: 214–219.

SNP array analysis of leukemic relapse samples after allogeneic hematopoietic stem cell transplantation with a sibling donor identifies meiotic recombination spots and reveals possible correlation with the breakpoints of acquired genetic aberrations

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Allogeneic hematopoietic stem cell transplantation (HSCT) with a sibling donor is commonly used for treating hematologic malignancies.[1] Although this procedure is frequently curative, a proportion of the patients eventually suffers a relapse of the original malignancy.[1] Leukemogenesis is associated with acquired genetic aberrations caused by various mechanisms including induction of double-stranded DNA breaks by DNA toposiomerase II poisons followed by non-homologous end joining, recombination between homologous sequences and illegitimate V(D)J recombination.[2] It has been hypothesized that neoplasia-associated breakpoints may correlate with the break-points of meiotic events, that is, some parts of the genome are more prone to both meiotic and somatic rearrangements; however, this remains controversial.[3–5]

During the last five years, numerous studies have used single-nucleotide polymorphism (SNP) array analysis to investigate genetic abnormalities in hematologic malignancies, including paired diagnostic and relapse samples.[6] To the best of our knowledge, however, the particular scenario of a relapse occurring after allogeneic HSCT with a sibling donor has not been addressed with this technique. In such cases, the bone marrow consists of a mixture of the patient-derived leukemic cells and the donor-derived normal hematopoietic cells, displaying different degrees of chimerism depending on the proportion of leukemic cells. In the present study, we have investigated hematologic malignancies that relapsed after allogeneic HSCT with a sibling donor, and we here provide examples and discuss the particular properties of these samples in terms of SNP array analysis. Furthermore, we have, for the first time, investigated whether the breakpoints of acquired leukemia-associated genetic abnormalities and meiotic recom-bination events are correlated in a single individual genome.

The study included six cases of relapsed hematologic malignancies after HSCT with a sibling donor, comprising one acute myeloid leukemia M0, two acute myeloid leukemia M5, two myelodysplastic syndromes and one chronic myeloid leukemia. DNA was extracted according to standard methods from bone marrow samples obtained at relapse. In addition, a dilution series of a mixture of peripheral blood samples from two unrelated healthy individuals was prepared in ratios of 1:9, 2:8, 3:7, 4:6 and 5:5. SNP array analysis was performed using the Illumina 1M-duo bead Infinium BD BeadChip platform (Illumina, San Diego, CA, USA) as previously described.[7] Expected B-allele frequency (BAF) values for each combination of genotypes in two mixed cell populations were calculated using the formula $BAF_{exp} = [B_1p + B_2(1-p)]/[L_1p + L_2(1-p)]$, where B is the number of B alleles in the respective cell population, p is the frequency of cell population 1, and L is the

3 Takeda J, Miyata T, Kawagoe K, Iida Y, Endo Y, Fujita T *et al.* Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell* 1993; **73**: 703–711.
4 Maciejewski JP, Mufti GJ. Whole genome scanning as a cytogenetic tool in hematologic malignancies. *Blood* 2008; **112**: 965–974.
5 Gondek LP, Tiu R, Haddad AS, O'Keefe CL, Sekeres MA, Theil KS *et al.* Single nucleotide polymorphism arrays complement metaphase cytogenetics in detection of new chromosomal lesions in MDS. *Leukemia* 2007; **21**: 2058–2061.
6 Bessler M, Mason P, Hillmen P, Luzzatto L. Somatic mutations and cellular selection in paroxysmal nocturnal haemoglobinuria. *Lancet* 1994; **343**: 951–953.
7 Endo M, Ware RE, Vreeke TM, Singh SP, Howard TA, Tomita A *et al.* Molecular basis of the heterogeneity of expression of glycosyl phosphatidylinositol anchored proteins in paroxysmal nocturnal hemoglobinuria. *Blood* 1996; **87**: 2546–2557.
8 Young NS, Maciejewski JP. Genetic and environmental effects in paroxysmal nocturnal hemoglobinuria: this little PIG-A goes 'Why? Why? Why?'. *J Clin Invest* 2000; **106**: 637–641.

IDH1 and *IDH2* mutations are rare in pediatric myeloid malignancies

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Recently, recurrent somatic missense mutations in NADP^{+}-dependent isocitrate dehydrogenase gene (*IDH1*) at codon R132, as well as *IDH2* at codon R172, have been identified in low-grade gliomas/secondary glioblastoma by high-throughput sequencing.[1] Subsequent studies also revealed that acquired somatic mutations in *IDH1* frequently occurred in adult hematological malignancies, such as acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS).[2,3] More recently, Paschka *et al.*[4]reported that not only *IDH1* but also *IDH2* mutations occurred relatively frequently in adult AML, and that these mutations were associated with older age, poor prognosis, cytogenetically normal AML (CN-AML) and the genotype of mutated *NPM1* without *FLT3*-internal tandem duplication (ITD). Exon 4 of both *IDH1* and *IDH2*, which was previously identified as a hot spot for mutations in these genes, encodes three arginine residues (R100, R109 and R132 in *IDH1* and R140, R149, and R172 in *IDH2*) that are important for protein activities.[5] Tumor-derived *IDH1* and *IDH2* mutations impair the affinity of enzymes for substrates, and dominantly inhibit wild-type *IDH1* and *IDH2* activities through the formation of catalytically inactive heterodimers.[5] Ho *et al.*[6] previously reported that *IDH1* mutations are not detected in pediatric AML; however, little is known about the incidence and prognostic values of *IDH1* and *IDH2* mutations in pediatric myeloid malignancies. Here, we analyzed mutations that involve the activation sites of *IDH1* and *IDH2* (exon 4 and exon 7 in both *IDH1* and *IDH2*) using genomic DNA-polymerase chain reaction amplification/sequencing in a total of 199 samples of pediatric myeloid malignancies, including 17 AML-derived cell lines, 115 primary cases of AML, 28 primary cases of MDS, 15 primary cases of juvenile myelomonocytic leukemia (JMML), 6 chronic myeloid leukemia (CML)-derived cell lines and 18 primary cases of CML. Moreover, to assess whether *IDH1* and *IDH2* mutations overlap with known gene abnormalities, such as *FLT3*, *c-KIT* and *NPM1* mutations, mutational analyses of *FLT3*, *c-KIT* and *NPM1* were also performed in AML samples. This study was approved by the ethics committee of the University of Tokyo (Approval Number 3043).

The common *IDH2* R140Q mutation was detected in a single AML case, whereas no *IDH1* mutation including G123E, as well as no other *IDH2* mutations, such as R172K, were detected in our study (Figure 1). The *IDH2* R140Q mutation detected in the AML case was a heterozygous substitution. No *IDH1* and *IDH2* mutations were detected in the JMML, MDS or CML samples examined. As the additional activation sites of both *IDH1* and

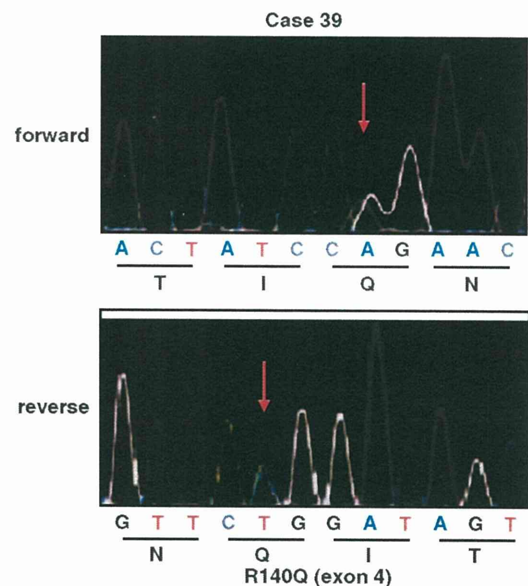

Figure 1 Sequence chromatogram of the *IDH2* mutation detected in a pediatric AML patient. A heterozygous mutation at R140 in exon 4 of *IDH2* is shown (top and bottom: forward and reverse sequencing results, respectively). Mutated nucleotides are indicated by arrows.

IDH2 are located in exon 7 of these genes, direct sequencing of exon 7 of *IDH1* and *IDH2* was also performed, but no mutations were detected in our series. Six AML samples including one cell line had *c-KIT* mutations (D816V, N822K and D419fs), and 12 AML samples had *FLT3-ITD*. The *NPM1* mutation was detected in 2 of 132 AML samples. The AML case harboring the *IDH2* mutation, case 39, showed no abnormalities of *NPM1*, *c-KIT* and *FLT3*. Case 39 was a 12-year-old boy diagnosed as AML-M2 according to the French–American–British cooperative group classification system. Bone marrow blasts obtained at initial diagnosis showed t(8;21)(q22;q22). After complete remission was achieved by the ACMP (adriamycin, cytarabine, 6-mercaptopurine, prednisolone) two-step induction therapy, the patient underwent consolidation therapy every 5 weeks, but hematological relapse occurred 11 months after the initial diagnosis. He was treated with low-dose cytarabine, but died 5 months after relapse with progressive disease. To assess the genetic mechanisms involved in the pathogenesis of the disease of this case, we further performed genome-wide copy number analysis of bone marrow blasts obtained at initial diagnosis of this case, using single-nucleotide polymorphism (SNP)-genotype microarrays (Affymetrix GeneChip Mapping 250 K *StyI* arrays,

Affymetrix, Inc., Santa Clara, CA, USA). As shown in Figure 2, complex chromosomal abnormalities, such as heterozygous deletions at chromosomes 7q11.2, 7q34-qter, 9q13-q21.33, 9q22.33, 16q23.1-q24.3 and 17q12qter, as well as gains of 4q24.3, 17q12-qter and 22q12.3-q13.33 were detected in leukemic cells of this patient (Figure 2).

To our knowledge, this is the first report to describe the *IDH2* mutation in a pediatric AML patient. In the present study, we detected the *IDH2* R140Q mutation in a single AML case out of 199 samples of pediatric myeloid malignancies, which suggests that the involvement of *IDH1* and *IDH2* mutations in the pathogenesis of pediatric AML is extremely rare compared with those in adult AML cases. Likewise, although *IDH* mutations are frequently observed in adult brain tumors, they are not observed in pediatric cases.[1] Therefore, somatically acquired *IDH1* and *IDH2* mutations may be related to an acquired neoplastic pathway exclusive to adult patients. Several groups have reported that *IDH1* and *IDH2* mutations are significantly associated with a normal karyotype in adult AML.[4,6] However, our patient with an *IDH2* mutation had t(8;21) together with complex chromosomal changes. Furthermore, a previously reported genome-wide study of pediatric AML revealed that, in contrast to our AML patients with *IDH2* mutation, pediatric *de novo* AML was characterized by a very low burden of genomic alterations.[7] These clinical and cytogenetic data suggest that pediatric AML with t(8;21) and *IDH2* mutation might be a specific subtype of AML with complex chromosomal abnormalities and poor prognosis. Thus, our result has important clinical and pathological implications regarding the role of *IDH2* mutations in the development of AML. t(8;21) is considered as a distinct AML subtype associated with characteristic morphology and a favorable prognosis.[8] Although approximately 90% of AML patients with t(8;21) achieve remission, relapse is frequent.[8] Once the disease relapses, the prognosis is poor, with an overall survival of 50% at 5 years.[8] Although the *c-KIT* mutation and *FLT3-ITD* are considered as poor prognostic factors in AML patients with t(8;21), these abnormalities occur in approximately 10% of AML patients with t(8;21).[9] Notably, *IDH1* and *IDH2* mutations constitute a poor prognostic factor in CN-AML with mutated *NPM1* without *FLT3-ITD*, which allows refined risk stratification of this AML subset.[4] Although treatment contents as well as clinical and genetic backgrounds were some of the parameters influencing the patient's outcome, our findings suggest that the *IDH2* mutation may also be related to an inferior outcome in pediatric AML patients with t(8;21) even if they lack the *c-KIT* mutation and *FLT3-ITD*. As *IDH2* mutation with t(8;21) is an extremely rare event and the prognostic values of *IDH2* mutations in AML

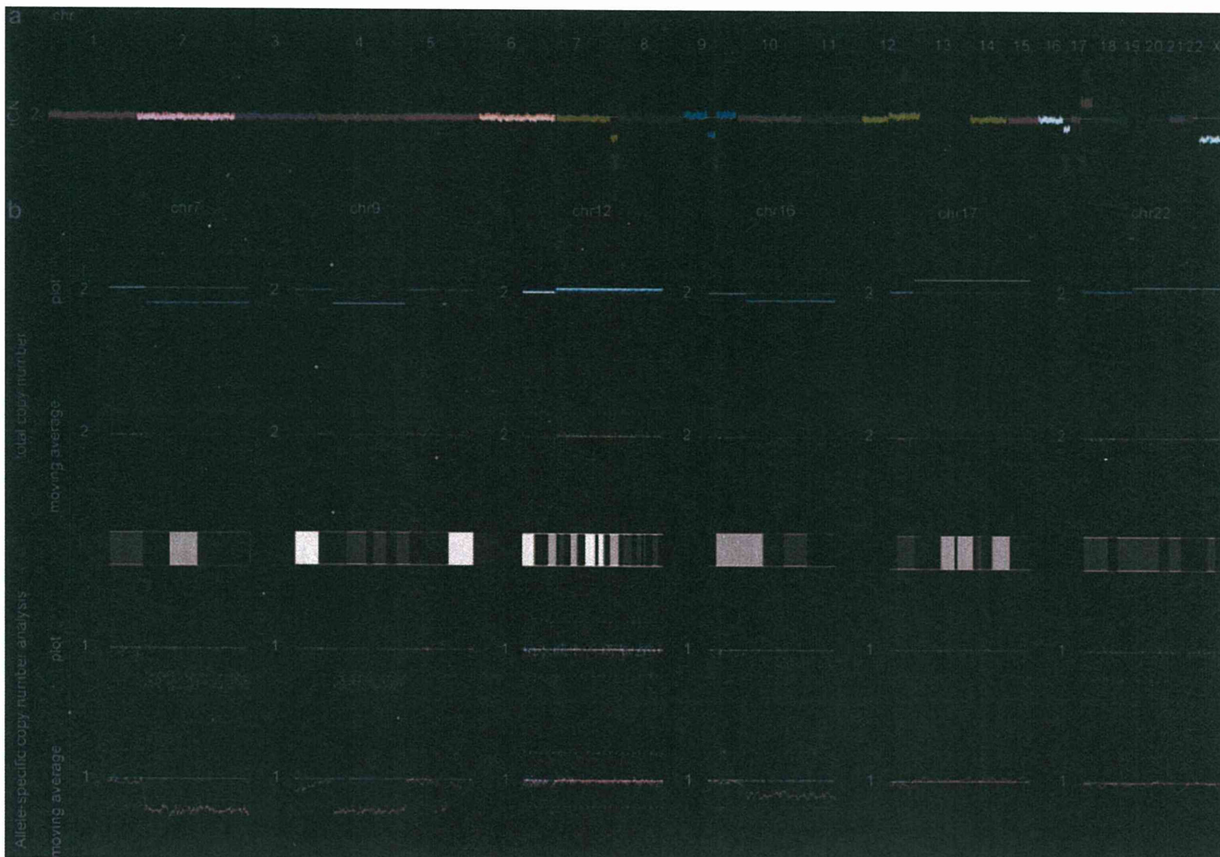

Figure 2 The result of copy number analysis using SNP-genotyping microarrays. (**a**) The moving average of the total copy number plot is presented. Each chromosome is indicated by different colors. Deletions in the regions at 7q, 9q, 16q and 17q, and gains in the region at 12q, 17q and 22q are indicated by the red arrows. (**b**) Deletions of 7q, 9q, 16q and 17q, and gains of 12q, 17q and 22q. The total copy number plot from each probe (red points) and the moving average (blue line) are shown above the cytobands. The results of the allele-specific analysis with CNAG/AsCNAR are shown below the cytobands. The larger allele is presented in red, and the smaller allele is presented in green. The numbers located at the left edge of each lane indicate a normal copy number (2 for total copy number analysis and 1 for allele-specific copy number analysis).

with t(8;21) are still unclear, further data accumulation is necessary. Although uncommon in pediatric myeloid malignancies, *IDH1* and *IDH2* mutations, particularly *IDH2* mutations, could contribute to the advanced phenotype of AML. Our findings provide additional impetus for investigating the role of *IDH1* and *IDH2* in the pathophysiology of errors of metabolism and in neoplastic disorders.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Yan H, Bigner DD, Velculescu V, Parsons DW. Mutant metabolic enzymes are at the origin of gliomas. *Cancer Res* 2009; **69**: 9157–9159.
2 Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K *et al.* Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* 2009; **361**: 1058–1066.
3 Thol F, Weissinger EM, Krauter J, Wagner K, Damm F, Wichmann M *et al. IDH1* mutations in patients with myelodysplastic syndromes are associated with an unfavorable prognosis. *Haematologica* 2010; **95**: 1668–1674.
4 Paschka P, Schlenk RF, Gaidzik VI, Habdank M, Kronke J, Bullinger L *et al. IDH1* and *IDH2* mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with *NPM1* mutation without *FLT3* internal tandem duplication. *J Clin Oncol* 2010; **28**: 3636–3643.
5 Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA *et al.* The common feature of leukemia-associated *IDH1* and *IDH2* mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* 2010; **17**: 225–234.
6 Ho PA, Alonzo TA, Kopecky KJ, Miller KL, Kuhn J, Zeng R *et al.* Molecular alterations of the *IDH1* gene in AML: a Children's Oncology Group and Southwest Oncology Group study. *Leukemia* 2010; **24**: 909–913.
7 Radtke I, Mullighan CG, Ishii M, Su X, Cheng J, Ma J *et al.* Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. *Proc Natl Acad Sci USA* 2009; **106**: 12944–12949.
8 von Neuhoff C, Reinhardt D, Sander A, Zimmermann M, Bradtke J, Betts DR *et al.* Prognostic impact of specific chromosomal aberrations in a large group of pediatric patients with acute myeloid leukemia treated uniformly according to trial AML-BFM 98. *J Clin Oncol* 2010; **28**: 2682–2689.
9 Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M *et al. 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* 2006; **107**: 1806–1809.