suggest the distinct molecular basis between *NUP98*-related leukemias having *FLT3*-ITD and those having *RAS* mutations.

The relationships between clinical features and gene mutations were described in Table 1. In our study, male patients were more likely than female patients to have *FLT3*-ITD ($P=0.01$) and patients with *FLT3*-ITD have leukocytosis ($P=0.08$) more than those without *FLT3*-ITD. Patients with *RAS* mutations were significantly younger than those without the mutations (median age of 15 vs 56 years; $P=0.04$). In total, 9 (64.3%) of the 14 patients who achieved complete remission relapsed, and 9 (60.0%) of the 15 patients whose data were available died, although they were treated by different protocols (Table 1). All three patients who had both *FLT3*-ITD and *KIT* mutations, and five (83.3%) of the six patients who had both *FLT3*-ITD and *WT1* aberrations, died. Many studies have shown that *FLT3*-ITD is related to a poor prognosis in AML patients,[1] and that *KIT* mutations are associated with a worse outcome in CBF-leukemia patients.[1] *WT1* mutations are also reported to be a poor prognostic factor in adult AML patients with normal karyotypes.[7] These results suggest that simultaneous occurrence of *FLT3*-ITD, *KIT* mutations and *WT1* aberrations in *NUP98*-related leukemia may be associated with poor prognosis.

FLT3-ITD, *KIT* and *RAS* mutations lead to constitutive activation of downstream pathway, resulting in acquirement of a proliferative advantage.[1] In a mouse model, *FLT3*-ITD alone does not induce AML, and *RAS* mutations can induce myeloid leukemia with distinct leukemogenic strengths and phenotypes.[1] *NUP98*-related fusions alone require long periods of time to induce AML, although these fusions induce MDS or MPN by impaired myeloid differentiation.[2] Cooperation between BCR-ABL (which enhances proliferation) and NUP98-fusion (which inhibits differentiation) lead to CML blast crisis.[2] Moreover, the *WT1* mutations were clustered within the DNA binding domain, and were subsequently considered to impair the ability of DNA to bind to target genes associated with apoptosis, cell cycle or cellular proliferation.[8] These results suggest that a high frequency of cell proliferation gene mutations may contribute to leukemogenesis in *NUP98*-related leukemia, and that simultaneous occurrence of *FLT3-ITD* and *WT1* aberrations may have an important role in the clinical outcome of *NUP98*-related leukemia.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by a Grant-in-Aid for Cancer Research, Research on Children and Families, Research on intractable diseases, Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, and by Grants-in-Aid for Scientific Research (B, C) or Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Drs Ryoji Hanada (Division of Hematology/Oncology, Saitama Children's Medical Center, Japan), Kazuko Hamamoto (Department of Pediatrics, Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital, Japan), Hideo Nakamura (Department of Internal Medicine, Koufudai Hospital, Nagasaki, Japan), Kazuma Ohyashiki (First Department of Internal Medicine, Division of Hematology, Tokyo Medical University, Japan), Ikuo Miura, Department of Internal Medicine (Division of Hematology and Oncology, St Marianna University School of Medicine, Japan), Keiki Kawakami (Division of Hematology, Suzuka General Hospital, Japan), Hiroshi Miwa (Department of Internal Medicine, Division of Hematology, Aichi Medical University School of Medicine, Japan), Takaharu Matsuyama (Division of Hematology and Oncology, Children's Medical Center, Japanese Red Cross Nagoya First Hospital, Japan) and Yasuhito Arai, PhD (Cancer Genome Project, National Cancer Center Research Institute, Tokyo, Japan) for providing samples and clinical data of patients with chromosomal 11p15 translocations. We also thank Mrs Shoko Sohma, Hisae Soga, Midori Furui, Mayumi Naito, Mayumi Nagase and Rie Eda for their excellent technical assistance.

T Taketani[1,2], T Taki[3], T Nakamura[4], Y Kobayashi[5], E Ito[6], S Fukuda[2], S Yamaguchi[2] and Y Hayashi[7]
[1]Division of Blood Transfusion, Shimane University Hospital, Shimane, Japan;
[2]Department of Pediatrics, Shimane University School of Medicine, Shimane, Japan;
[3]Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan;
[4]Department of Carcinogenesis, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan;
[5]Hematology Division, National Cancer Center Hospital, Tokyo, Japan;
[6]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan and
[7]Department of Hematology/Oncology, Gunma Children's Medical Center, Gunma, Japan
E-mail: hayashiy-tky@umin.ac.jp

References

1 Renneville A, Roumier C, Biggio V, Nibourel O, Boissel N, Fenaux P *et al.* Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia* 2008; **22**: 915–931.
2 Moore MA, Chung KY, Plasilova M, Schuringa JJ, Shieh JH, Zhou P *et al.* NUP98 dysregulation in myeloid leukemogenesis. *Ann N Y Acad Sci* 2007; **1106**: 114–142.
3 Ageberg M, Drott K, Olofsson T, Gullberg U, Lindmark A. Identification of a novel and myeloid specific role of the leukemia-associated fusion protein DEK-NUP214 leading to increased protein synthesis. *Genes Chromosomes Cancer* 2008; **47**: 276–287.
4 Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L *et al.* Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med* 2008; **358**: 1909–1918.
5 Chou WC, Chen CY, Hou HA, Lin LI, Tang JL, Yao M *et al.* Acute myeloid leukemia bearing t(7;11)(p15;p15) is a distinct cytogenetic entity with poor outcome and a distinct mutation profile: comparative analysis of 493 adult patients. *Leukemia* 2009; **23**: 1303–1310.
6 Slape C, Liu LY, Beachy S, Aplan PD. Leukemic transformation in mice expressing a NUP98-HOXD13 transgene is accompanied by spontaneous mutations in Nras, Kras, and Cbl. *Blood* 2008; **112**: 2017–2019.
7 Virappane P, Gale R, Hills R, Kakkas I, Summers K, Stevens J *et al.* Mutation of the wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: The United Kingdom Medical Research Council Adult Leukaemia Working Party. *J Clin Oncol* 2008; **26**: 5429–5435.
8 Ariyaratana S, Loeb DM. The role of the Wilms tumour gene (WT1) in normal and malignant haematopoiesis. *Expert Rev Mol Med* 2007; **9**: 1–17.

4 Furukawa Y, Vu HA, Akutsu M, Odgerel T, Izumi T, Tsunoda S *et al.* Divergent cytotoxic effects of PKC412 in combination with conventional antileukemic agents in FLT3 mutation-positive versus negative leukemia cell lines. *Leukemia* 2007; **21**: 1005–1014.
5 Kim H-G, Lee KW, Cho Y-Y, Kang NJ, Oh S-M, Bode AM *et al.* Mitogen- and stress-activated kinase 1-mediated histone H3 phosphorylation is crucial for cell transformation. *Cancer Res* 2008; **68**: 2538–2547.
6 Srinivasa SP, Doshi PD. Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways cooperate in mediating cytokine-induced proliferation of a leukemic cell line. *Leukemia* 2002; **16**: 244–253.
7 Parmar S, Katsoulidis E, Verma A, Li Y, Sassano A, Lal L *et al.* Role of the p38 mitogen-activated protein kinase pathway in the generation of the effects of imatinib mesylate (STI571) in BCR-ABL-expressing cells. *J Biol Chem* 2004; **279**: 25345–25352.
8 Dumka D, Puri P, Carayoi N, Balachandran H, Schuster K, Verma AK *et al.* Activation of the p38 MAP kinase pathway is essential for the antileukemic effects of dasatinib. *Leuk Lymphoma* 2009; **50**: 2017–2029.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

CBL mutations in juvenile myelomonocytic leukemia and pediatric myelodysplastic syndrome

Leukemia (2010) **24,** 1090–1092; doi:10.1038/leu.2010.49; published online 1 April 2010

Juvenile myelomonocytic leukemia (JMML) is a rare form of myeloproliferative disorder, which is characterized by excessive myelomonocytic proliferation.[1] Gene mutations on RAS signaling pathways are a hallmark of JMML and are thought to be central to the pathogenesis of JMML. Mutations of *NF1*, *NRAS*, *KRAS* and *PTPN11* genes are found in approximately 70% of JMML patients, and are implicated in the aberrant RAS signaling.[1] On the other hand, the remaining approximately 30% of JMML patients have no known mutations.

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because these *CBL* mutations were shown to result in aberrant tyrosine kinase signaling, which would lead also to activation of RAS signaling pathways.[2–4] We[2] and others[3,4] reported that *CBL* mutations occurred in a variety of myeloid neoplasms in adults, especially in chronic myelomonocytic leukemia,[2–4] which prompted us to search for possible *CBL* mutations in JMML cases as well as pediatric myelodysplastic syndrome (MDS) patients.

In total, 40 primary JMML and 24 pediatric MDS specimens were examined for CBL mutations. The median age at diagnosis of JMML was 1 year and 10 months (range, 2 months to 8 years and 4 months), and the study included 25 male and 15 female patients. MDS patients included 9 patients with refractory anemia, 14 with refractory anemia with excess of blasts and 1 with secondary MDS. As *CBL* mutations thus far reported almost exclusively involved exons 7–9 that encode linker/RING finger domains,[2–4] we confined our analysis to these exons using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Branchburg, NJ, USA). Of the 40 JMML samples, 24 were also analyzed using Affymetrix GeneChip 250K *Nsp*I (Santa Clara, CA, USA).[5] Genome-wide detection of copy number abnormalities or allelic imbalances was performed using CNAG/AsCNAR software (http://www.genome.umin.jp),[5] which enabled sensitive detection of copy number neutral loss of heterozygosity (or acquired uniparental disomy (aUPD)).[5] Mutations of *RAS* and *PTPN11* were also examined as previously reported.[6] 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.

CBL mutations were identified in 3 out of 40 JMML samples (7.5%), which were located in the linker sequence (Y371H in UPN1) or in the RING finger domain (C416Y in UPN18 and C419R in UPN38). The origin of the mutations has not been determined owing to the lack of appropriate normal tissues. All 3 *CBL*-mutated cases accompanied 11q-aUPD, whereas heterozygosity of 11q was preserved in the remaining 21 patients with wild-type *CBL*, recapitulating a strong association of *CBL* mutations with 11q-aUPD as previously described.[2] In UPN18, the chromatogram exclusively showed a mutated sequence, indicating that the mutation (C416Y) was homozygous. In the remaining patients, chromatograms were apparently heterozygous (Figure 1). Nevertheless, this did not necessarily exclude the possibility of homozygous mutations in the aUPD-positive cells, which were found in a small fraction within these tumor samples, as estimated from allele-specific copy numbers.

Recently, Loh *et al.*[7] have reported on *CBL* mutations in 27 (17%) of 159 JMML patients, which were exclusive with regard to *RAS/PTPN11* mutations. They also showed that Y371H mutations represented nearly 50% of all *CBL* mutations in JMML,[7] which were also found in a patient in our series. Mutations of *NRAS, KRAS* and *PTPN11* genes were found in 18, 10 and 34% of patients in our series, respectively, which did not co-exist with three *CBL* mutations (Table 1). In our study, no *CBL* mutations were found in 24 pediatric MDS patients. Combining both studies, the prevalence of *CBL* mutations is expected to be significantly lower in pediatric MDS patients than in JMML patients (0/24 vs 30/199; $P=0.025$), although the number of patients is still small.

CBL is a negative modulator of tyrosine kinase signaling and, as such, was demonstrated to act functionally and genetically as a tumor suppressor. On the other hand, CBL mutants in myeloid neoplasms have clear oncogenic properties, because they strongly transform fibroblasts. These CBL mutants inhibit the E3 ubiquitin ligase activity of both wild-type CBL and CBL-B, leading to prolonged tyrosine kinase activity after cytokine stimulation and hypersensitive proliferative responses of hematopoietic progenitors to a wide variety of cytokines.[8]

We have recently shown that the biological consequence of *CBL* mutations is prolonged activation of tyrosine kinases after cytokine/growth factor stimulations, which is further augmented under the *CBL*-null background as caused by aUPD.[2] Thus, *CBL* mutations associated with 11q-aUPD in JMML could provide a feasible explanation to the hypersensitivity to GM-CSF, which is

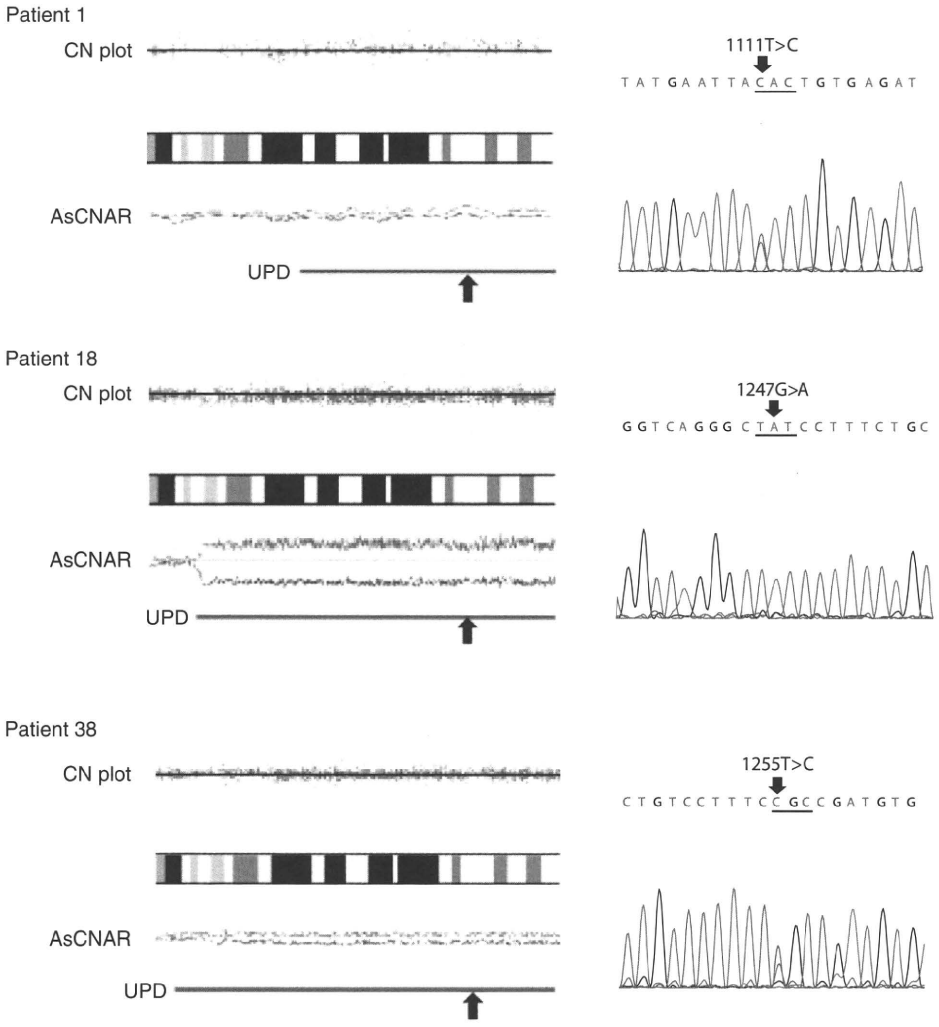

Figure 1 Identification of acquired isodisomy of 11q in JMML and *CBL* mutations. CNAG output for JMML UPN 1, UPN 18 and UPN 38. Left: Total copy numbers (tCNs) (red plot) are shown above the cytoband, and the result of allele-specific copy number analysis with anonymous reference (AsCNAR) plots are shown below the cytoband. The larger allele is presented in red line, and the smaller allele is presented in blue line. Allele-specific analysis showed 11q-aUPD (blue line), which contained the *CBL* region (arrow). Right: All samples of JMML with 11q-aUPD carried mutated *CBL*.

Table 1 Characteristics of three JMML patients with *CBL* mutations

Patient no.	*Age/sex*	*Karyotype*	*Location*	*Nucleotide change*[a]	*Amino-acid change*	*PTPN11*	*RAS*	*SCT*	*Survival*
UPN 1	3 m/F	46, XX, 5q-	Exon 8	1111T>C	371Tyr>His	—	—	—	+12 m
UPN 18	2 m/M	46, XY	Exon 9	1247G>A	416Cys>Tyr	—	—	—	+68 m
UPN 38	2 m/M	46, XY	Exon 9	1255T>C	419Cys>Arg	—	—	U-BMT	32 m[b]

Abbreviations: F, female; m, month; M, male; SCT, stem cell transplantation; U-BMT, unrelated-bone marrow transplantation; UPN, unique patient number; +, alive.
[a]GenBank accession number of CBL for nucleotide change: NM_005188.
[b]Died due to chronic graft versus host disease.

a cardinal feature of JMML. Although *NF1*, *RAS* or *PTPN11* mutations lead to deregulated RAS signaling, the outcome of *CBL* mutations could be more profound, also activating other downstream pathways of tyrosine kinases, including STAT5 and PI3K. Our data suggest that *CBL* is mutated in a subset of JMML patients without *RAS/PTPN11* mutations. Our findings suggest that 11q-aUPD-associated *CBL* mutations may define a unique subset of JMML.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Shinji Mochizuki, MD, Division of Hematology/Oncology, Saitama Children's Medical Center, and Junko

Takita, MD, Kohmei Ida, MD, Department of Pediatrics, Graduate School of Medicine, University of Tokyo, and Kazuko Kudo, Department of Hematology/Oncology, Shizuoka Children's Hospital, for providing the JMML samples. We also thank Mrs Chisato Murata and Miss Sayaka Takeuchi for their excellent technical assistance. This work was supported by a grant for Cancer Research, and a grant for Research on Children and Families from the Ministry of Health, Labor, and Welfare of Japan, a Grant-in-Aid for Scientific Research (B, C) and Exploratory Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a Research grant for Gunma Prefectural Hospitals.

N Shiba[1,2], M Kato[3], M-j Park[2], M Sanada[3], E Ito[4], K Fukushima[5], M Sako[6], H Arakawa[1], S Ogawa[3] and Y Hayashi[2]
[1]*Department of Pediatrics, Gunma University Graduate School of Medicine, Gunma, Japan;*
[2]*Department of Hematology/Oncology, Gunma Children's Medical Center, Gunma, Japan;*
[3]*Cancer Genomics Project, Graduate School of Medicine, University of Tokyo, Tokyo, Japan;*
[4]*Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan;*
[5]*Department of Pediatrics, Dokkyo Medical University School of Medicine, Mibu, Japan and*
[6]*Department of Pediatrics Hematology/Oncology, Osaka City General Hospital, Osaka, Japan*
E-mail: hayashiy-tky@umin.ac.jp

References

1 Niemeyer CM, Kratz CP. Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukaemia: molecular classification and treatment options. *Br J Haematol* 2008; **140**: 610–624.
2 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.
3 Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H *et al.* 250 K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Res* 2008; **68**: 10349–10357.
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 Yamamoto G, Nannya Y, Kato M, Sanada M, Levine RL, Kawamata N *et al.* Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. *Am J Hum Genet* 2007; **81**: 114–126.
6 Chen Y, Takita J, Hiwatari M, Igarashi T, Hanada R, Kikuchi A *et al.* Mutations of the PTPN11 and RAS genes in rhabdomyosarcoma and pediatric hematological malignancies. *Genes Chromosomes Cancer* 2006; **45**: 583–591.
7 Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S *et al.* Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. *Blood* 2009; **114**: 1859–1863.
8 Thien CB, Langdon WY. Tyrosine kinase activity of the EGF receptor is enhanced by the expression of oncogenic 70Z-Cbl. *Oncogene* 1997; **15**: 2909–2919.

Trisomy 11: prevalence among 22 403 unique patient cytogenetic studies and clinical correlates

Leukemia (2010) **24**, 1092–1094; doi:10.1038/leu.2010.51; published online 1 April 2010

Trisomy 11 is a rare cytogenetic abnormality and is yet reported to be one of the most frequent autosomal trisomies in acute myeloid leukemia (AML).[1] In a Cancer and Leukemia Group B study, isolated trisomy 11 was identified in 13 cases (0.9%) among 1496 consecutive adult patients with AML.[2] The majority of the patients with isolated trisomy 11 were older than 60 years and 46% achieved a complete remission after induction chemotherapy.[2] However, only one patient remained in first complete remission after undergoing allogeneic bone marrow transplantation. In a recent *Leukemia* paper, Wang *et al.*[3] identified 42 cases (0.008%) with trisomy 11 among ~5000 patients with myelodysplastic syndrome (MDS) or MDS with myeloproliferative features. Seventeen of the 42 patients (median age, 75 years) had trisomy 11 as a sole abnormality ($n=10$) or together with one or two additional abnormalities. Specific diagnoses in these 17 patients were refractory anemia with excess of blasts (RAEB)-2 in 8 patients, RAEB-1 in 5, refractory cytopenia with multilineage dysplasia in 1, therapy-related MDS in 1 and chronic myelomonocytic leukemia-2 in 1; bone marrow was not available for review in the remaining 1 patient. The authors compared their trisomy 11 MDS patients with historical controls and found their survival to be similar to that of high-risk MDS patients. Accordingly, they concluded that trisomy 11 should be considered a high-risk cytogenetic abnormality in MDS.

In the current study, we sought to clarify the prevalence of trisomy 11 in an unselected series of cytogenetic studies performed at the Mayo Clinic over the last 20 years and describe their clinical and pathological features. Between January 1988 and December 2008, unique patient cytogenetic studies were performed in 22 403 adults (age ⩾18 years). Among them, we identified 19 patients (~0.08%) with abnormalities that included trisomy 11; WHO (World Health Organization)-defined[4] clinical diagnosis at the first sighting of trisomy 11 was AML in 14 patients and MDS in 5 (Table 1). Among the former, 10 cases constituted *de novo* AML and 4 constituted relapsed or secondary AML. Among the five MDS patients, three had RAEB-2 and two had RAEB-1. Trisomy 11 occurred as a sole abnormality in 10 patients with AML, but in only one patient with MDS (RAEB-2).

The median age at detection of trisomy 11 in AML was 71 years and in MDS was 67 years (range, 64–86). Median (range) values in AML included 8.7 g/100 ml (6.5–11.8) for hemoglobin, 6×10^9/l (1.2–123) for leukocytes and 96×10^9/l (12–444) for platelets. The corresponding values in MDS were 9.2 g/100 ml (8.1–11.3), 1.8×10^9/l (1.1–3.2) and 129×10^9/l (78–199), respectively. Approximately 50% of the AML patients were exposed to either cytotoxic or radiation therapy before the detection of trisomy 11 (Table 1). AML transformation was documented in one patient with RAEB-2 after 23 months of follow-up. In all the patients with relapsed AML and in one

Int J Hematol (2010) 91:831–837
DOI 10.1007/s12185-010-0585-x

ORIGINAL ARTICLE

Prognostic significance of the *BAALC* isoform pattern and *CEBPA* mutations in pediatric acute myeloid leukemia with normal karyotype: a study by the Japanese Childhood AML Cooperative Study Group

Yasuhiro Mizushima · Tomohiko Taki · Akira Shimada · Yoshihiro Yui · Yoshimi Hiraumi · Hiroshi Matsubara · Motonobu Watanabe · Ken-ichiro Watanabe · Yuri Kamitsuji · Yasuhide Hayashi · Ichiro Tsukimoto · Ryoji Kobayashi · Keizo Horibe · Akio Tawa · Tatsutoshi Nakahata · Souichi Adachi

Received: 8 December 2009 / Revised: 20 April 2010 / Accepted: 21 April 2010 / Published online: 22 May 2010

Abstract High *BAALC* (brain and acute leukemia, cytoplasmic) gene expression may indicate an adverse prognosis for adults who have acute myeloid leukemia (AML) and a normal karyotype, but its prognostic significance for pediatric AML cases is unclear. Whether different *BAALC* isoform patterns are of prognostic significance is also unclear. Newly diagnosed AML patients with normal karyotype who were treated by the Japanese Childhood AML Cooperative Treatment Protocol AML 99 were analyzed in terms of their *BAALC* expression levels ($n = 29$), *BAALC* isoforms ($n = 29$), and *CEBPA* mutations ($n = 49$). Eleven and 18 patients exhibited high and low *BAALC* expression, respectively, but these groups did not differ significantly in terms of overall survival (54.6 vs. 61.1%, $P = 0.55$) or event-free survival (61.4 vs. 50.0%, $P = 0.82$). Three of these 29 patients (10.3%) expressed the exon 1-5-6-8 *BAALC* isoform along with the expected 1-6-8 isoform and had adverse clinical outcomes. Novel

Electronic supplementary material The online version of this article (doi:10.1007/s12185-010-0585-x) contains supplementary material, which is available to authorized users.

Y. Mizushima · Y. Yui · Y. Hiraumi · H. Matsubara · M. Watanabe · K. Watanabe · T. Nakahata · S. Adachi
Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto 606-8507, Japan

T. Taki
Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

A. Shimada · Y. Hayashi
Department of Hematology and Oncology, Gunma Children's Medical Center, 779 Shimohakoda, Hokkitsu, Shibukawa, Gunma 377-8577, Japan

Y. Kamitsuji
Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, 54 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto 606-8507, Japan

I. Tsukimoto
Department of the First Pediatrics, Toho University School of Medicine, 6-11-1 Omori-nishi, Otaku, Tokyo 143-8541, Japan

R. Kobayashi
Department of Pediatrics, Graduate School of Medicine, Hokkaido University, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan

K. Horibe
Clinical Research Center, National Hospital Organization Nagoya Medical Center, 4-1-1 Sannomaru, Nakaku, Nagoya, Aichi 460-0001, Japan

A. Tawa
Department of Pediatrics, National Hospital Organization Osaka National Hospital, 2-1-14 Hoenzaka, Chuoku, Osaka 540-0006, Japan

S. Adachi (✉)
Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, 53 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: adachiso@kuhp.kyoto-u.ac.jp

CEBPA mutations were also identified in four of 49 patients (8.2%). All four patients have maintained complete remission for at least 5 years. Thus, 1-5-6-8 isoform expression may be associated with an adverse prognosis in pediatric AML with normal karyotype. *CEBPA* mutations may indicate a favorable prognosis.

Keywords Pediatric AML · Normal karyotype · *BAALC* · *CEBPA*

1 Introduction

Cytogenetic abnormalities in acute myeloid leukemia (AML) that are detected at the time of diagnosis are important prognostic factors that help to determine the clinical outcome. However, 10–20% of pediatric AML cases lack known genetic abnormalities that can be used to predict clinical outcome [1]. For example, while tandem duplications of mixed lineage leukemia gene (*MLL*) and fms-like tyrosine kinase-3 (*FLT3*) correlate with a poor prognosis in pediatric AML [2], internal tandem duplications (ITD) of *FLT3* occur much more rarely in pediatric AML than in adult AML patients; indeed, there is an age-associated increase in this mutation (from 1.5% in infants to nearly 20% in teenage patients) [3]. Thus, most normal karyotype pediatric AML patients lack known markers that are of prognostic significance. To improve the prognostic stratification of this heterogeneous group of patients, novel markers should be identified.

The *BAALC* (brain and acute leukemia, cytoplasmic) gene is believed to participate in the development of AML and chronic myelogenous leukemia in blast crisis [5]. Previous studies have also reported that high *BAALC* expression levels reflect an adverse prognosis for adult AML with a normal karyotype [6–10]. However, how *BAALC* expression levels relate to the clinical outcome of pediatric AML remains unclear.

CEBPA is a transcription factor that coordinates the granulocytic differentiation of common myeloid progenitors. *CEBPA* mutations have been detected in 7–15% of adult patients with AML and are most frequently found in the AML M1 and M2 subtypes [French–American–British (FAB) classification] [10, 11]. Previous reports indicate that *CEBPA* mutations reflect a favorable prognosis in adult AML with normal karyotype [12, 13]. However, it is unclear whether the same relationship exists between *CEBPA* mutations and pediatric AML.

While previous studies have mainly examined various prognostic factors in terms of gene mutations [9–14] and changes in gene expression [15–20], some recent studies have also reported the prognostic significance of the expression of different isoforms in leukemia [21–25]. Consequently, in the present study, we investigated the prognostic relevance of high *BAALC* expression, *BAALC* isoform patterns, and *CEBPA* mutations in pediatric AML with normal karyotype. This study was performed by the Japanese Childhood AML Cooperative Study Group, which employed the AML 99 protocol [2, 4, 26, 27].

2 Patients and methods

2.1 Patients

This study included 124 of the 241 pediatric patients who were newly diagnosed with de novo AML from January 2000 to December 2002. The 241 patients included 52 patients with a normal karyotype and 49 of those were recruited into the 124-patient group. None of these 49 patients had AML-M3 or Down syndrome. AML was diagnosed according to the FAB classification, and a routine G-banding method was used for cytogenetic analysis. Of the 124 cases, 104 were subjected to *BAALC* expression analysis; of these 104 subjects, 29 had a normal karyotype. These 29 normal karyotype cases were also subjected to *BAALC* isoform analysis. All 49 normal karyotype cases were subjected to *CEBPA* mutation analysis. The characteristics of the patients subjected to *BAALC* isoform analysis and *CEBPA* mutation analysis are shown in Table 1a. The 29 normal karyotype patients who were subjected to *BAALC* isoform and expression analyses did not differ significantly in age, white blood cell (WBC) counts at diagnosis, remission rates or overall survival (OS) from the remaining 20 normal karyotype patients who were not analyzed for *BAALC* isoform and expression (Table 1b). In accordance with the Declaration of Helsinki and upon approval from the Ethics Committees of Kyoto University, informed consent was obtained from each patient or the patient's parents before entering this study.

2.2 *BAALC* expression analysis

Comparative real-time RT-PCR assays were performed and *BAALC* expression levels were measured as previously reported [5, 6]. The *BAALC* expression values of the patient group were divided at the median value (0.57) and patients were said to have a low and high *BAALC* expression if they had expression values within the lower and upper 50% of values, respectively [7].

2.3 *BAALC* isoform analysis

BAALC isoform analysis was performed by RT-PCR followed by direct sequencing. For this, the PCR product was cut out of the gel, purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA), and sequenced by the

Table 1 Characteristics of AML patients subjected to *BAALC* expression, *BAALC* isoform, and *CEBPA* mutation analyses

	BAALC		*CEBPA*
	All (n = 104)	Normal karyotype (n = 29)	Normal karyotype (n = 49)
(a)			
FAB			
M0	4	2	4
M1	18	2	6
M2	36	9	12
M4	18	8	12
M5	19	7	12
M7	7	1	3
UN	2	0	0
Age	4 days to 15 years	3 months to 15 years	3 months to 15 years
Sex			
Male	60	16	27
Female	44	13	22
Risk group			
Low	45	0	0
Intermediate	51	29	49
High	8	0	0

	BAALC		*P* value
	Analyzed patients (n = 29)	Non-analyzed patients (n = 20)	
(b)			
Age			
Median	8 years	8 years	
Range	3 months to 15 years	7 months to 15 years	0.591
WBC at diagnosis ($\times 10^9$/L)			
Median	53.09	15.20	
Range	2.30–343.40	1.20–236.90	0.275
Remission rates	89.7%	100%	0.083
Overall survival	58.6%	65.2%	0.639

UN undifferentiated

dideoxynucleotide termination method with ABI 3100 (Applied Biosystems, Foster City, CA). The primers used are shown in supplementary Table 1. The conditions for the PCR reactions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 7 min.

2.4 *CEBPA* mutation analysis

The entire coding region of the gene was amplified using overlapping PCR primer pairs followed by direct sequencing as previously described [28].

2.5 Statistical analysis

Survival distributions were estimated using the Kaplan–Meier method and the differences were compared using the log-rank test. OS and event-free survival (EFS) were defined as the time from diagnosis to death from any cause or the last follow-up and the time from diagnosis to event (relapse or death from any cause), respectively.

3 Results

3.1 *BAALC* expression levels

High *BAALC* expression was associated with M0, M1, and M2 FAB subtypes, while M4 and M5 FAB subtypes correlated with low *BAALC* expression (Fig. 1). Healthy volunteers (I) have remarkably small range of *BAALC* expression levels compared to AML patients (II, III), as the previous study was reported [15]. We did not observe significant differences between normal karyotype patients

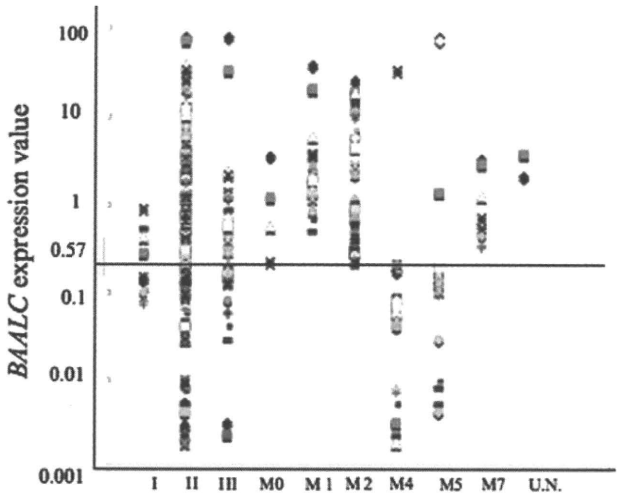

Fig. 1 *BAALC* expression levels in pediatric AML with normal karyotype and FAB subtype patients. The *dot plot* indicates the individual *BAALC* expression levels of healthy volunteers (I, $n = 9$), all AML patients (II, $n = 104$), AML patients with normal karyotype (III, $n = 29$), and the M0 ($n = 4$), M1 ($n = 18$), M2 ($n = 36$), M4 ($n = 18$), M5 ($n = 19$), M7 ($n = 7$), and undifferentiated (U.N., $n = 2$) FAB subtype patients

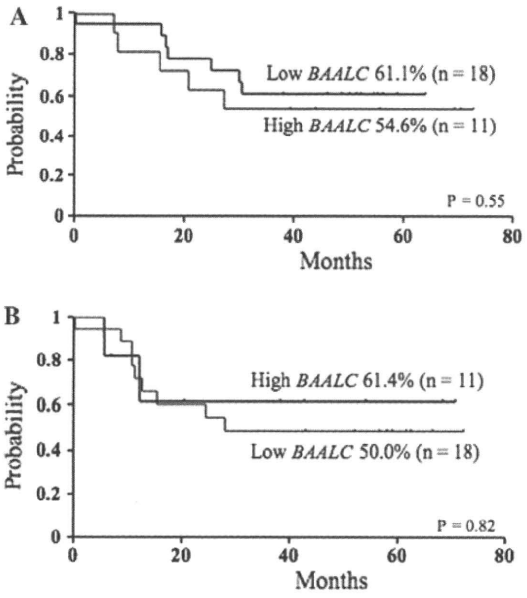

Fig. 2 Kaplan–Meier analysis of the overall survival (OS) and event-free survival (EFS) of pediatric AML patients with normal karyotype who express *BAALC* at high and low levels. The OS (**a**) and EFS (**b**) of the high and low *BAALC*-expressing pediatric AML with normal karyotype patients did not differ significantly

with high ($n = 11$) and low ($n = 18$) *BAALC* expression with regard to their OS (54.6 vs. 61.1%, $P = 0.55$, Fig. 2a) or EFS (61.4 vs. 50.0%, $P = 0.82$, Fig. 2b).

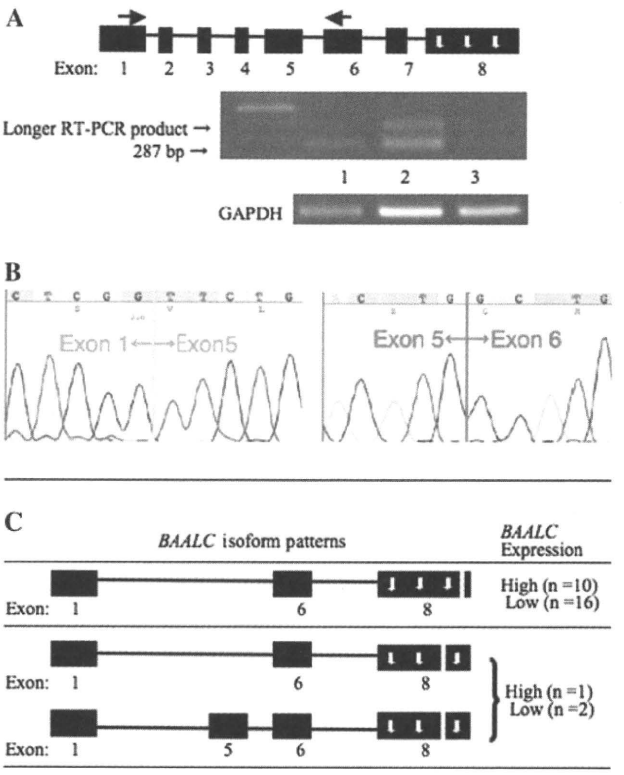

Fig. 3 **a** Schematic depiction of the *BAALC* gene, which consists of eight exons (indicated by *black boxes*). The three polyadenylation signals in the 3′ untranslated region (UTR) of exon 8 that lead to three differently sized transcripts are indicated by *down arrows*. Comparative RT-PCR using primers in exons 1 and 6 revealed that three samples had a longer RT-PCR product in addition to the expected 287 bp product. **b** A partial sequence trace of exons 1, 5, and 6 in the longer RT-PCR product. **c** Schematic depiction of the relationship between *BAALC* isoform patterns and expression levels. Three (10.3%) of the 29 cases had the 1-5-6-8 isoform

3.2 *BAALC* isoform pattern and its relationship to *BAALC* expression levels

The *BAALC* gene consists of eight exons and its transcription followed by alternative splicing yields several different transcripts. Five stable isoforms have been detected in leukemic blasts, namely 1-8, 1-6-8, 1-5-6-8, 1-4-5-6-8, and 1-5-6-7-8 [5]. To investigate the prognostic relevance of *BAALC* isoform patterns for pediatric AML patients, we subjected 29 pediatric AML patients with normal karyotype to RT-PCR and direct sequencing. All samples had the predicted product, which consisted of exon 1, 6, and 8 sequences (1-6-8). However, three samples (10.3%) also had a longer RT-PCR product that contained in addition the exon 5 sequence (1-5-6-8) (Fig. 3a, b). How these isoform patterns relate to the *BAALC* expression levels of the 29 patients is depicted schematically in Fig. 3c. One of three patients with the

Table 2 Characteristics of patients with the 1-5-6-8 *BAALC* isoform

Case no.	*BAALC* expression value	Relapse	Clinical outcome	FAB classification	Age (years)	Sex
1	High (2.43)	+	Dead	M4	13	Female
2	Low (0.39)	−	Dead	M5a	6	Male
3	Low (0.21)	+	Dead	M4	15	Male

Table 3 *CEBPA* mutations

FAB subtype	All ($n = 49$)	*CEBPA* mutations
M0	4	0
M1	6	2 c. 1074_1075insAGA c. 1092_1093insCAC
M2	12	2 c. 214_224delCCCCGCACGCG c. 212_213insC and c. 720_726insCGCACC
M4	12	0
M5	12	0
M7	3	0
Total	49	4

Sequence numbering is according to GenBank accession number U34070

1-5-6-8 isoform had high *BAALC* expression. Of the 26 patients with the 1-6-8 isoform only, 10 and 16 showed high and low *BAALC* expression, respectively. The three patients with the 1-5-6-8 isoform had a poor prognosis (Table 2).

3.3 *CEBPA* mutation

CEBPA mutations were detected in four of the 49 AML with normal karyotype patients (8.2%). Two of these belonged to the M1 subset, and the remaining two belonged to the M2 subset. N-terminal frameshift mutations and in-frame insertions in the basic-leucine zipper (bZIP) domain were detected (Table 3). Novel mutations were identified, namely, c. 212_213insC, c. 214_224delCCCCGCACGCG, c. 720_726insCGCACC, c. 1074_1075insAGA, and c. 1092_1093insCAC. One patient had biallelic mutations in both the N-terminal part and the bZIP domain of *CEBPA*.

4 Discussion

High *BAALC* expression was associated with the M2 subset and the more immature M0/M1 FAB subtypes, while the monocytic-differentiated M4 and M5 FAB subtypes correlated with low *BAALC* expression (Fig. 1). This relationship between *BAALC* expression and FAB subtypes is generally consistent with previously reported observations of adult AML cases [7], although the high *BAALC* expression in the M2 subtype cases was only observed in the pediatric AML patients. *BAALC* expression level was not associated with WBC counts at diagnosis [all AML patients ($n = 104$), $P = 0.91$; AML with normal karyotype ($n = 29$), $P = 0.97$]. The *BAALC* gene is normally expressed by neuroectoderm-derived tissues and CD34-positive hematopoietic progenitor cells and has been implicated in AML and chronic myelogenous leukemia in blast crisis [5]. Recently, quantification of *BAALC* gene expression made it possible to assess MRD in patients with CD34-positive acute leukemia [29]. Little is known about the functions of the *BAALC* gene, but it has been reported to mediate the anabolic action of PTH (parathyroid hormone) on bone cells [30]. It also serves as a marker of the mesodermal lineage (especially muscle) [31] and synaptogenesis [32], and a study on hematopoietic progenitor cells has shown that *BAALC* downregulation occurs upon cell differentiation [33]. Thus, while the functions of the *BAALC* gene remain unclear, our observations are consistent with the notion that it may be associated with monocytic cell differentiation.

We did not observe significant differences between normal karyotype patients with high ($n = 11$) and low ($n = 18$) *BAALC* expression with regard to their OS (54.6 vs. 61.1%, $P = 0.55$, Fig. 2a) or EFS (61.4 vs. 50.0%, $P = 0.82$, Fig. 2b). These results are not consistent with those of a previous study that examined the *BAALC* expression of adult normal karyotype AML patients [7]. In that study, high *BAALC* expression was significantly associated with a poor OS and a higher cumulative incidence of relapse. The discrepancy between this study and ours could reflect the fact that in the other study, the *BAALC* expression values of the patient group were divided at the median value of twelve healthy volunteers, which served as the cutoff [15]. We compared the results according to two different cutoff levels. AML samples were dichotomized at the median value (0.15) of nine healthy volunteers, but we also observed no significant differences of two expression groups (date not shown). To resolve this apparent discrepancy, a larger number of pediatric AML patients will need to be studied.

The *BAALC* gene consists of eight exons and its transcription followed by alternative splicing yields several

different transcripts in leukemic blasts, namely, 1-8, 1-6-8, 1-5-6-8, 1-4-5-6-8, and 1-5-6-7-8 [5]. In our study, all samples of 29 pediatric AML patients with normal karyotype had the *BAALC* isoform that consisted of exon 1, 6, and 8 sequences (1-6-8). However, three samples (10.3%) also had the *BAALC* isoform that contained in addition the exon 5 sequence (1-5-6-8) (Fig. 3a, b). One of the three patients with the 1-5-6-8 isoform also had high *BAALC* expression. With regard to prognosis, all three patients with the 1-5-6-8 *BAALC* isoform have died (Table 2). Two relapsed after complete remission and the third died after intracranial hemorrhage during induction therapy. Of the 29 normal karyotype patients who were subjected to *BAALC* isoform and expression analyses, *FLT3*-ITD were found in eight (27.6%), *FLT3*-D835Mt in two (6.9%), *NRAS* mutations in two (6.9%), and *KRAS* mutations in four (13.8%), but no *NPM1* gene mutations were found [4]. There are no differences of *FLT3*-ITD, *MLL*-PTD and *CEBPA* in high and low *BAALC* expression group (data not shown). Of the three 1-5-6-8 *BAALC* isoform-bearing patients, one had the *FLT3*-ITD mutation and another patient had a *RAS* mutation. A previous study did not detect a difference in outcome between cytogenetically normal adult AML patients with and without *NRAS* mutations [34]. Thus, it seems that the possession of the 1-5-6-8 *BAALC* isoform by pediatric AML patients with normal karyotype may be associated with a candidate for some adverse prognostic factor. Studies with greater patient numbers should be performed to confirm this. Recent reports have suggested that the isoform patterns of other genes (i.e., *AML1-ETO9a* [21], *WT1* [22], *PML/RARa* [23], *Ikaros* [24], and *FHIT* [25]) are of prognostic significance, which supports the significance of investigating the *BAALC* isoform patterns.

CEBPA mutations were detected in four of the 49 AML patients with normal karyotype (8.2%). Two of these belonged to the M1 subset and the remaining two belonged to the M2 subset. One patient had biallelic mutations in both the N-terminal part and bZIP domain of *CEBPA*. To date, two categories of *CEBPA* mutations have been reported: out-of-frame ins/del mutations that often occur in the N-terminal region, and in-frame ins/del mutations that often occur in the C-terminal region [12, 13]. The mutations in both the N-terminal part and bZIP domain have been described in adult AML, but the reported frequencies vary considerably, ranging between 11 and 47% [35]. In a study of pediatric AML patients, of whom six had a normal karyotype, four of the six (67%) had one or more *CEBPA* mutations [36], but the clinical outcomes associated with these mutations are unclear. Notably, in the absence of poor prognostic factors, adult patients with *CEBPA* mutations have been shown to have favorable clinical outcomes [33, 37]. In our study, none of the patients exhibiting a *CEBPA* mutation also had the *FLT3*-ITD mutation and all maintained complete remission for at least 5 years. The statistical significance was not indicated for insufficient sample numbers in AML 99 protocol between normal karyotype patients with CEBPA mutation (+) ($n = 4$) and mutation (−) ($n = 45$) with regard to their OS (100 vs. 55.4%, $P = 0.14$) or EFS (100 vs. 48.9%, $P = 0.09$) (supplementary Fig. 1). But differing from previous report about pediatric AML patients with *CEBPA* mutations, the presentation of clinical information about them may be evaluated. Thus, in the absence of other adverse factors, *CEBPA* mutations may also be suspected to favorable prognostic factors for pediatric AML with normal karyotype.

In summary, we report here for the first time that the presence of the 1-5-6-8 *BAALC* isoform may be associated with a poor prognosis for pediatric AML patients with normal karyotype. In contrast, *CEBPA* mutations are suspected to a good prognosis.

Acknowledgments The authors are grateful to all members of the Japanese Childhood AML Cooperative Study Group. We also thank to Ken Tabuchi for statistical analysis. This work was supported in part by a Grant-in-Aid for Cancer Research (16-3) from the Ministry of Health, Labor and Welfare of Japan, and in part by a Scientific Research (C) grant (17591083) from the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

References

1. Brown P, Mclntyre E, Rau R, Meshinchi S, Lacayo N, Dahl G, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007;110:979–85.
2. Shimada A, Taki T, Tabuchi K, Taketani T, Hanada R, Tawa A, et al. Tandem duplications of MLL and FLT3 are correlated with poor prognosis in pediatric acute myeloid leukemia: a study of the Japanese childhood AML Cooperative Study Group. Pediatr Blood Cancer. 2008;50:264–9.
3. Meshinchi S, Alonzo TA, Stirewalt DL, Zwaan M, Zimmerman M, Reinhardt D, et al. Clinical implications of FLT3 mutations in pediatric AML. Blood. 2006;108:3654–61.
4. Shimada A, Taki T, Kubota C, Tawa A, Horibe K, Tsuchida M, et al. No nucleophosmin mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group. Leukemia. 2007;21:1307.
5. Tanner SM, Austin JL, Leone G, Rush LJ, Plass C, Heinonen K, et al. BAALC, the human member of a novel mammalian neuroectoderm gene lineage, is implicated in hematopoiesis and acute leukemia. Proc Natl Acad Sci USA. 2001;98:13901–6.
6. Baldus CD, Tanner SM, Ruppert AS, Whitman SP, Archer KJ, Marcucci G, et al. BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: a Cancer and Leukemia Group B Study. Blood. 2003;102:1613–8.
7. Baldus CD, Thiede C, Soucek S, Bloomfield CD, Thiel E, Ehninger G, et al. BAALC expression and FLT3 internal tandem duplication mutations in acute myeloid leukemia patients with

normal cytogenetics: prognostic implications. J Clin Oncol. 2006;24:790–7.
8. Marcucci G, Mrózek K, Bloomfield CD. Molecular heterogeneity and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics. Curr Opin Hematol. 2005;12:68–75.
9. Mrózek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? Blood. 2007;109:431–48.
10. Baldus CD, Mrózek K, Marcucci G, Bloomfield CD. Clinical outcome of de novo acute myeloid leukaemia patients with normal cytogenetics is affected by molecular genetic alterations: a concise review. Br J Haematol. 2007;137:387–400.
11. Gombart AF, Hofmann WK, Kawano S, Takeuchi S, Krug U, Kwok SH, et al. Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein in myelodysplastic syndromes and acute myeloid leukemias. Blood. 2002;99:1332–40.
12. Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, et al. Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association. Blood. 2002;100:2717–23.
13. Fröhling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, et al. CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol. 2004;22:624–33.
14. Basecke J, Whelan JT, Griesinger F, Bertrand FE. The MLL partial tandem duplication in acute myeloid leukaemia. Br J Haematol. 2006;135:438–49.
15. Bienz M, Ludwig M, Leibundgut EO, Mueller BU, Ratschiller D, Solenthaler M, et al. Risk assessment in patients with acute myeloid leukemia and a normal karyotype. Clin Cancer Res. 2005;11:1416–24.
16. Marcucci G, Baldus CD, Ruppert AS, Radmacher MD, Mrózek K, Whitman SP, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. J Clin Oncol. 2005;23:9234–42.
17. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, Valk PJ, van der Poel-van de Luytgaarde S, Hack R, et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. Blood. 2003;101:837–45.
18. Damiani D, Tiribelli M, Calistri E, Geromin A, Chiarvesio A, Michelutti A, et al. The prognostic value of P-glycoprotein (ABCB) and breast cancer resistance protein (ABCG2) in adults with de novo acute myeloid leukemia with normal karyotype. Haematologica. 2006;91:825–8.
19. Heuser M, Beutel G, Krauter J, Döhner K, von Neuhoff N, Schlegelberger B, et al. High meningioma 1 (MN1) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. Blood. 2006;108:3898–905.
20. Radmacher MD, Marcucci G, Ruppert AS, Mrózek K, Whitman SP, Vardiman JW, et al. Independent confirmation of a prognostic gene-expression signature in adult acute myeloid leukemia with a normal karyotype: a Cancer and Leukemia Group B study. Blood. 2006;108:1677–83.
21. Yan M, Kanbe E, Peterson LF, Boyapati A, Miao Y, Wang Y, et al. A previous unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. Nat Med. 2006;12:945–9.
22. Hossain A, Nixon M, Kuo MT, Saunders GF. N-terminally truncated WT1 protein with oncogenic properties overexpressed in Leukemia. J Biol Chem. 2006;281:28122–30.
23. Jurcic JG, Nimer SD, Scheinberg DA, DeBlasio T, Warrell RP Jr, Miller WH Jr, et al. Prognostic significance of minimal residual disease and PML/RARa isoform type: long-term follow-up in acute promyelocytic leukemia. Blood. 2001;98:2651–6.
24. Yagi T, Hibi S, Takanashi M, Kano G, Tabata Y, Imamura T, et al. High frequency of Ikaros isoform 6 expression in acute myelomonocytic and monocytic leukemias: implications for up-regulation of the antiapoptotic protein Bcl-XL in leukemogenesis. Blood. 2002;99:1350–4.
25. Yang HW, Piao HY, Taki T, Chen T, Hashizume K, Ohnishi H, et al. Pattern of FHIT gene expression in normal and leukaemic cells. Int J Cancer. 1999;81:897–901.
26. 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;107:1806–9.
27. Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial from the Japanese Childhood AML Cooperative Study Group. J Clin Oncol. 2009;27:4007–13.
28. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, et al. Dominant-negative mutations of CEBPA, encoding CCAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet. 2001;27:263–70.
29. Najima Y, Ohashi K, Kawamura M, Onozuka Y, Yamaguchi T, Akiyama H, et al. Molecular monitoring of *BAALC* expression in patients with CD34-positive acute leukemia. Int J Hematol. 2010. doi:10.1007/s12185-010-0550-8.
30. Robinson JA, Susulic V, Liu YB, Taylor C, Hardenburg J, Gironda V, et al. Identification of a PTH regulated gene selectively induced in vivo during PTH-mediated bone formation. J Cell Biochem. 2006;98:1203–20.
31. Satoskar AA, Tanner SM, Weinstein M, Qualman SJ, de la Chapelle A. BAALC, a marker of mesoderm and muscle. Gene Expr Patterns. 2005;5:463–73.
32. Wang X, Tian QB, Okano A, Sakagami H, Moon IS, Kondo H, et al. BAALC 1-6-8 protein is targeted to postsynaptic lipid rafts by its N-terminal myristoylation and palmitoylation, and interacts with alpha, but not beta, subunit of Ca/calmodulin-dependent protein kinase II. J Neurochem. 2005;92:647–59.
33. Baldus CD, Tanner SM, Kusewitt DF, Liyanarachchi S, Choi C, Caligiuri MA, et al. BAALC, a novel marker of human hematopoietic progenitor cells. Exp Hematol. 2003;31:1051–6.
34. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. Blood. 2006;107:3847–53.
35. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. CEBPA point mutations in hematological malignancies. Leukemia. 2005;19:329–34.
36. Liang DC, Shih LY, Huang CF, Hung IJ, Yang CP, Liu HC, et al. CEBPA mutations in childhood acute myeloid leukemia. Leukemia. 2005;19:410–4.
37. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009; 113:3088–91.

Cell Cycle 9:6, 1-6; March 15, 2010; © 2010 Landes Bioscience

Gain-of-function *c-CBL* mutations associated with uniparental disomy of 11q in myeloid neoplasms

Seishi Ogawa,[1,2,*] Masashi Sanada,[1] Lee-Young Shih,[4] Takahiro Suzuki,[3] Makoto Otsu,[5] Hiromitsu Nakauchi[5] and H. Philip Koeffler[6]

[1]Cancer Genomics Project; The University of Tokyo; Bunkyo-ku, Tokyo Japan; [2]Core Research for Evolutional Science and Technology; Exploratory Research for Advanced Technology; Japan Science and Technology Agency; Kawaguchi-shi, Saitama Japan; [3]Division of Hematology-Oncology; Department of Internal Medicine; Chang Gung Memorial Hospital; Chang Gung University; Taipei, Taiwan; [4]Division of Hematology; Department of Medicine; Jichi Medical University; Shimotsuke-shi, Tochigi Japan; [5]Division of Stem Cell Therapy; Center for Stem Cell and Regenerative Medicine; Institute of Medical ScienceDivision of Stem Cell Therapy; Division of Systems Biomedical Technology; Institute of Medical Science; The University of Tokyo; Minato-ku, Tokyo Japan; [6]Hematology/Oncology; Cedars-Sinai Medical Center; Los Angeles, CA USA

***c-CBL (CBL)* encodes a multifunctional protein engaged in the regulation of intracellular signaling pathways.[1,2] It was first identified as a cellular counterpart of the viral oncogene, *v-CBL*, that causes murine lymphoma.[3,4] Although no genetic evidence existed suggesting its role in human carcinogenesis, the recent discovery of c-*CBL* mutations in myeloid cancers has unveiled a unique oncogenic mechanism mediated by gain-of-function of a mutated tumor suppressor, closely associated with allelic conversion of 11q arms.[5-9] In this review, we summarize our current knowledge about *c-CBL* mutations and discuss the molecular mechanisms of their gain-of-function.**

Key words: c-CBL, 11qUPD, myeloproliferative neoplasms, gain-of-function, MDS/MPN, tyrosine kinases

Submitted: 12/21/09

Accepted: 12/23/09

Previously published online: www.landesbioscience.com/journals/cc/article/11034

*Correspondence to: Seishi Ogawa; Email: sogawa-tky@umin.ac.jp

Myeloproliferative Neoplasms and Related Disorders

Myeloproliferative neoplasms (MPNs) are a heterogeneous group of blood cancers, characterized by clonal hematopoiesis that causes excessive production of one or more components of mature blood cells with hypercellular bone marrow and extramedullary hematopoiesis.[10] Some patients also show abnormalities in cell morphology and differentiation with dysplastic bone marrow, and are classified into myelodysplastic/myeloproliferative neoplasms (MDS/MPN) in the new World Health Organization (WHO) classification.[11] A genetic hallmark of MPN and MDS/MPN is frequent mutations of genes on signal transduction pathways, which have been causally linked to hypersensitivity of neoplastic progenitors to growth factors and cytokines.[10] A notable example is *JAK2 V617F* mutations found in most cases of polycythemia vera (PV), a form of MPNs that is characterized by overproduction of mature erythrocytes together with other blood components.[12-14]

JAK2 Mutations in MPNs

These mutants encode constitutive active kinases that transmit signals from erythropoietin receptor, and induce a hypersensitive proliferative response to erythropoietin.[12] Of particular interest about *JAK2* mutations in PV is the presence of one or more subclones showing acquired uniparental disomy (aUPD) involving the 9p arm that leads to homozygous *JAK2* mutations (*JAK2*$^{mut/mut}$) by allelic conversion (**Fig. 1**).[15] One of the initial discoveries of *JAK2* mutations relied on the detailed mapping of loss of heterozygosity (LOH) caused by aUPD in 9p.[13] The consequence of 9p-aUPD is loss of wild type *JAK2* and duplication of mutated *JAK2*, but the latter seems to be more important for the clonal selection of UPD clones, because mutated *JAK2* is duplicated without loss of wild-type allele in 9p trisomy in some cases.[16] Similarly gain-of-function mutations of *cMPL* are

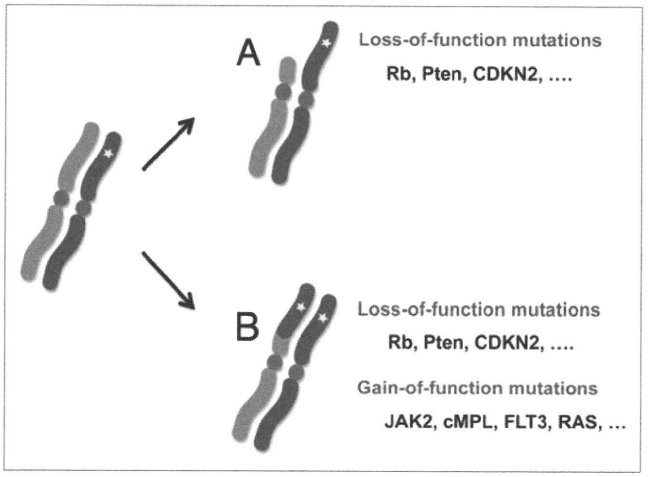

Figure 1. In cancer cells, LOH is frequently associated with a mutated tumor suppressor locus, in which a normal copy of the tumor suppressor is lost by simple allelic deletion (A), or replaced by the mutated copy through allelic conversion that leads to copy number neutral LOH or aUPD (B). In either case, the common consequence is biallelic loss-of-function of the tumor suppressor. In addition, LOH caused by aUPD is also implicated in the common mechanism of homozygous mutations of proto-oncogenes. A number of gain-of-function oncogenic mutations found in aUPD regions have been shown to exist in a homozygous state, including mutations of *JAK2* (9pUPD), *MPL* (1pUPD), *NRAS* (1pUPD), *KRAS* (12pUPD), *BRAF* and *FLT3* (13qUPD). The clonal outgrowth of aUPD-positive clones indicates that two copies of mutations confer a growth advantage to aUPD positive cells through their gain-of-function.

frequently found in primary myelofibrosis in close association with 1p-aUPD.[17] Thus, aUPD, or copy number neutral LOH, is associated not only with biallelic loss-of-function of classical tumor suppressor genes in the Knudson's paradigm,[18] but also with gain-of-function of proto-oncogenes. Moreover, genome-wide analysis of genetic imbalances in a variety of myeloid neoplasms revealed that aUPD is another genetic feature of MPNs, where 42% of chronic myelomonocytic leukemia (CMML) cases had one or more regions of aUPD and were grouped into several discrete clusters, which may or may not harbor mutations of known cancer related genes.[9] Among these one of the most prominent is the cluster that is defined by 11q-aUPD, from which mutated *c-CBL* proto-oncogene was identified.[9]

c-CBL Mutations in MDS/MPNs

Although *c-CBL* mutations have been reported in a variety of myeloid neoplasms including acute myeloid leukemia, myelodysplastic syndromes, as well as classical myeloproliferative disorders, the majority of *c-CBL*-mutated cases are MDS/MPN, including CMML (~15%), juvenile myelomonocytic leukemia (JMML) (~17%), and atypical chronic myeloid leukemia (~5%).[5-9,19,20] In most cases, *c-CBL* mutations are associated with 11q-aUPD involving *c-CBL* locus, which converts these mutations into a homozygous state. Loss of wild-type *c-CBL* is rarely caused by chromosomal deletion.[6,7,9] *c-CBL* mutations exclusively occur independent of *RAS* and *PTPN11* in CMML and JMML.[8,9] Notably, *c-CBL* mutations have a germline origin in some JMML cases.[8] Approximately half of the *CBL* mutations in JMML cases involve Y371, while mutations are widely distributed within linker/RING finger domain in other neoplasms. *c-CBL* mutants strongly transform fibroblasts and enhance proliferation of hematopoietic progenitors in methylcellulose culture.[9] These genetic and functional observations indicate that mutant *c-CBL* may have some gain-of-function, which promotes clonal evolution, especially of aUPD-positive clones carrying two copies of the mutations.

c-CBL as a Tumor Suppressor Gene

c-CBL proto-oncogene is a cellular homologue of a viral oncogene, *v-CBL*, isolated from the Casitas-NS-lymphoma virus that induces murine lymphoma.[3,4] Together with other two homologues CBL-b and CBL-c, it comprises the CBL family of proteins. All c-CBL proteins have an N-terminal domain for binding to phosphorylated tyrosine kinases (TKB domain) connected through a linker sequence to the RING finger, but CBL-c lacks most of the C-terminal domains shared by c-CBL and CBL-b (**Fig. 2A**). While c-CBL has multivalent molecular functions in signal transduction and cytoskeletal regulation, the most intensively studied-function is its role in negative regulation of receptor tyrosine kinase (RTK) signalings, which depends on the E3 ubquitin ligase activity of this molecule.[1,21] After RTKs are phosphorylated on cytokine stimulation, c-CBL binds to the phosphorylated RTKs through the TKB domain, and mono-ubiquitinates these RTKs at multiple sites in concert with the E2 conjugating enzyme, which is followed by internalization and degradation/recycling of the phosphorylated RTKs.[21] Thus, c-CBL prevents excessive RTK signaling after cytokine/growth factor stimulation and potentially acts as a tumor suppressor. *c-CBL*$^{-/-}$ mice have an enlarged thymus, splenomegaly with extramedullary hematopoiesis.[22,23] In these mice, hematopoietic progenitor pools are expanded,[9,24] and their hematopoietic progenitors exhibit hypersensitive proliferative responses to cytokine stimulations. When introduced into *BCR/ABL* transgenic mice, a *c-CBL*$^{-/-}$ allele accelerates blastic crisis.[9] Moreover, *c-CBL*$^{-/-}$ mice developed invasive cancer spontaneously (in preparation), further supporting that *c-CBL* has tumor suppressor functions.

Gain of Function of CBL Mutants

How can we reconcile with the tumor suppressor functions of c-CBL on the one hand, and the oncogenic properties of c-CBL mutants on the other? A simple explanation would be an inhibition of tumor suppressor function of wild type c-CBL by mutant c-CBL. Most *c-CBL* mutations in MPNs occur within the linker/RING finger domains, through which c-CBL binds E2 conjugating enzymes, and thus are expected to compromise the E3 ligase activity of the molecule.

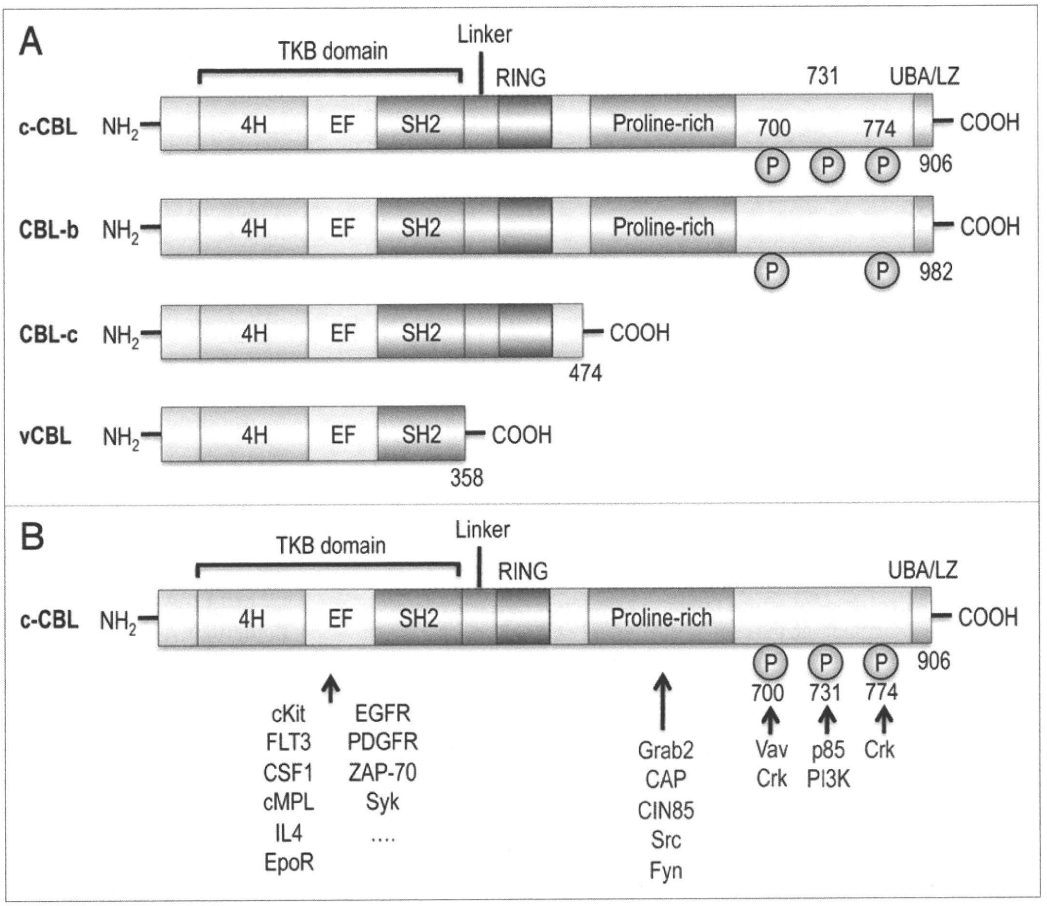

Figure 2. (A) Structure of CBL family proteins. CBL family proteins in mammals have highly conserved domains, where an N-terminal TKB domain, consisting of a four-helix bundle (4H), a Ca^{2+}-binding EF (EF), and a src-homology (SH2) domains, is connected to a RING finger domain via a linker. c-CBL and CBL-b, but not CBL-c, have a proline-rich and other C-terminal components that end with a ubiquitin-associated and leucine zipper (UBA/LZ) domain. Their viral form, v-CBL, is truncated just after its SH2 domain. (B) CBL family proteins interact with a number of signal transducing molecules. Through their TKB domain, CBL family proteins target phosphrylated tyrosine kinases, including growth factor receptors and cytokine receptors, as well as, non-receptor tyrosine kinases. Ubiquitin conjugating enzymes have contact with CBL proteins via the linker/RING finger domain, which is central to the E3 ubiquitin ligase activity. The proline-rich domain provides a binding site for SH3 domains of Grab2, CAP and Src-family kinases. The C-terminal portion contains three tyrosine residues, Y700, Y731 and Y774, which are the major phosphorylated tyrosines, and which bind to the p85 subunit of PI3 kinase (Y731), Vav (Y700) and Crk proteins (Y700 and Y774).

In fact, when expressed in fibroblasts, tumor-derived linker and RING finger mutants show severely compromised E3 ubiquitin ligase activity.[9,20] Moreover, two linker mutants (Q367P and Y371S) have been shown to inhibit the activity of wild-type c-CBL protein, although they do not make direct contact with E2 enzymes but with the TKB domain.[25] As expected from the inhibitory action of these mutants with regard to E3 ubiquitin ligase activity, transduction of the latter mutants into NIH3T3 or hematopoietic cells lead to prolonged activation of tyrosine kinases after stimulation with a variety of cytokines and growth factors, including epidermal growth factor, stem cell factor (SCF), Interleukin 3 (IL3), thrombopoietin, and FLT3 ligand.[9,20] Given the diverse spectrum of kinase targets of CBL, the enhanced sensitivity of these cells to a variety of cytokines is well expected.

Although these experimental data support a dominant negative mechanism of mutant *c-CBL*, a simple dominant negative model is defied by an experiment, in which mutant *c-CBL* was transduced into *c-CBL*$^{-/-}$ hematopoietic progenitors. Lin$^-$ Sca1$^+$cKit$^+$ (LSK) hematopoietic progenitors from *c-CBL*$^{-/-}$ mice showed enhanced survival or proliferative responses after stimulation with a variety of cytokines, including SCF, IL3, or thrombopoietin, as compared to those from *c-CBL*$^{+/+}$ mice. However, transduction of mutant *c-CBL* into *c-CBL*$^{-/-}$ progenitors dramatically augmented the responses to these cytokines and also to FLT3 ligand, while the effect of mutant *c-CBL*-transduction into *c-CBL*$^{+/+}$ progenitors was unremarkable even as compared to mock-transduced *CBL*$^{-/-}$ progenitors.[9] The augmented sensitivity to these cytokines in *c-CBL*$^{-/-}$ cells was nothing to do with the inhibition of c-CBL functions, and thus is considered to represent a true gain-of-function of the mutant c-CBL. The gain-of-function nature of *c-CBL* mutations is also predicted

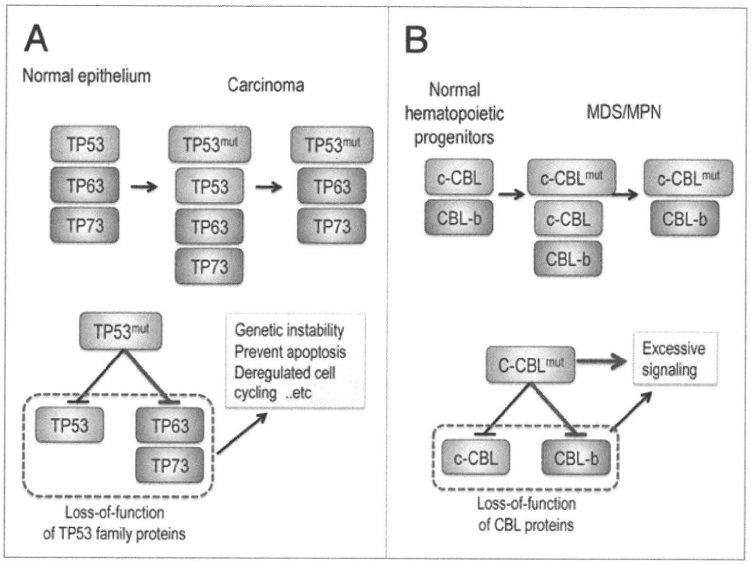

Figure 3. Possible mechanisms of gain-of-function of mutated TP53 and c-CBL. The gain-of-function of TP53 mutants is associated with their potential to induce carcinoma in mice as well as in human, which is considered to be mediated by inhibition of TP63 and TP73. TP53-deficient mice frequently develop sarcomas and lymphomas but only rarely carcinomas, which are thought to be suppressed by TP53 homologues, TP63 and TP73, in epithelial tissues, in the face of loss of TP53. Mutant TP53 inhibits tumor suppressor functions of TP63 and TP73, and compromises TP53-like activity. Similarly, the gain-of-function of CBL mutants found in MDS/MPN may be explained by the inhibition of CBL-b (red arrow), which would result in more profound defects in negative regulation of tyrosine kinase signaling compared to simple loss of c-CBL. On the other hand, c-CBL is thought to have positive regulatory functions that are not directly related to the E3 ubiquitin ligase activity and could be the source of the gain-of-function of c-CBL mutants (blue arrow).

from the fact that in myeloid neoplasms, 11qLOH is caused by aUPD in most cases and rarely accompanies 11q deletion, although in this case the target gene has tumor suppressor functions. Interestingly, the effect of the gain-of-function effect largely disappears by introducing wild type *c-CBL* or in the presence of the wild-type *c-CBL* allele,[9] which might explain the observation that the wild type *c-CBL* allele was lost in most MDS/MPN cases with *c-CBL* mutations as a result of allelic conversion or aUPD.

Origin of the Gain-of-Function of Mutant CBL

The exact mechanism through which mutant c-CBL acquires oncogenic functions even in *c-CBL*$^{-/-}$ cells is still elusive. Because the gain-of-function of mutant c-CBL is largely neutralized by the presence of wild type c-CBL, one possibility is that it could be mediated by the inhibition of some 'CBL-like' activity still present in *c-CBL*$^{-/-}$ cells, most likely CBL-b. Both c-CBL and CBL-b are expressed in immature hematopoietic progenitors, and c-CBL mutant inhibits E3 ubiquitin ligase activity of both c-CBL proteins.[9,26] Although *c-CBL/CBL-b*-double knockout mice are embryonic lethal, conditional double knockout in T cells shows hypersensitive to anti-CD3 stimulations and prolonged TCR-signaling, as compared to *c-CBL* or *CBL-b* single null T cells.[27] This reminds us of the gain-of-function of mutated TP53, which explains the difference in the phenotypes between *TP53*$^{-/-}$ and *TP53*$^{mut/-}$ mice. *TP53*$^{-/-}$ mice develop tumors at a high frequency, but they are mostly sarcomas or lymphomas and development of carcinoma is very rare, whereas *TP53*$^{mut/-}$ mice also develop carcinoma in various organs. Thus, TP53 mutant has more than null functions, which are thought to be mediated by the inhibition of its homologues, TP63 and TP73, expressed in epithelial tissues (**Fig. 3**).[28,29] Like c-CBL, TP53 tumor suppressor gene was first identified as an oncogene through its mutated, oncogenic forms in cancer cells. On the other hand, the model of gain-of-function mediated through CBL-b inhibition fails to explain why *CBL-b* mutations are extremely rare in CMML. According to this model, essentially no difference would be expected between the mutations of *c-CBL* and *CBL-b*, as long as in either case, compromised E3 ubiquitin ligase activity would result. The linker-RING finger mutants of c-CBL would be expected also to be able to inhibit E3 ubiquitin ligase activity of the wild-type c-CBL.

Another, but not necessarily exclusive, explanation of the gain-of-function of mutant c-CBL would be related to positive roles of c-CBL as a signal transducer rather than an attenuator (**Figs. 3A and 4**). c-CBL not only binds to a number of phosphorylated tyrosine kinases through its TKB domain, which is indispensable for the negative regulation of these kinases, but also interacts with more than 150 different proteins through a number of C-terminal domains and residues, and acts as a multi-domain adaptor protein, involved in signal transduction (**Fig. 2B**).[2] When recruited to phosphorylated tyrosine kinases, c-CBL is also phosphorylated at multiple tyrosine residues, and provides docking sites for the SH2 domains of Vav (pY700),[30] CrkL (pY700 and pY774)[31-34] and the p85 subunit of PI3 kinase (Y731).[35-37] c-CBL also binds to Grab2,[38-40] CAP,[41] and Src family tyrosine kinases[36] through the proline-rich domain. Several lines of evidence suggest that c-CBL positively transmits signals through these interactions. For example, c-CBL promotes cell survival and proliferation, depending on the PI3 kinase pathway,[42,43] and also enhances activation of MAP kinases after stimulation of Met tyrosine kinase.[44] c-CBL is also a key substrate/effector of Src kinase, which plays a central role in bone resorption and osteoclast migration.[45,46] It also is involved in cytoskeletal rearrangements through activation of Rac1 and Cdc42, and R-Ras.[47,48] Normally, mediated by its E3 ligase activity, kinase-bound phosphorylated c-CBL rapidly undergoes degradation,[26] by which positive signaling should be terminated. Thus, once linker/RING finger mutations abolish the E3 ligase activity of c-CBL, the consequence would be prolonged signaling due not only to loss of negative

regulation of tyrosine kinase, but also to enhanced positive regulatory functions, which should appear as gain-of-function (Fig. 4).

In contrast to the CBL-b inhibition model, the uni-laterality of c-CBL mutations could be more easily explained, because c-CBL and CBL-b have distinct biological functions, as clearly shown by the phenotypes of *c-CBL*$^{-/-}$ and *CBL-b*$^{-/-}$ mice.[22,23,49,50] For example, CBL-b lacks one of the major phosphorylated tyrosines, Y731, that provides a docking site for the p85 subunit of PI3 kinase. Although the exact molecular basis for the distinct functions between both CBL proteins remains to be elucidated, c-CBL-specific positive regulatory function in immature hematopoietic progenitors may be important for the pathogenesis of myeloid neoplasms.

Conclusion

Allelic conversion leading to aUPD is an important genetic mechanism of clonal evolution in the pathogenesis of MPN, and associated not only with loss-of-function of tumor suppressor genes, but also with gain-of-function mutations of proto-oncogenes. Homozygous *c-CBL* mutations that characterize a subset of MDS/MPD carrying 11q-aUPD, represent a unique example of gain-of-function mutations of tumor suppressor/proto-oncogene. These linker/RING finger mutations convert c-CBL, which otherwise act as a tumor suppressor, to a gain-of-function oncogenic protein. Although its exact molecular mechanism is still unknown, the gain-of-function of oncogenic c-CBL mutants seems to be related to disintegration of negative and positive regulatory machineries of normal c-CBL protein. Detailed analysis of the oncogenic mechanisms of c-CBL mutants is warranted, which should shed light on a novel aspect of physiological function of c-CBL. Considering their expression and functions in a broad spectrum of tissues, *CBL* family genes may be mutated in other human cancers.

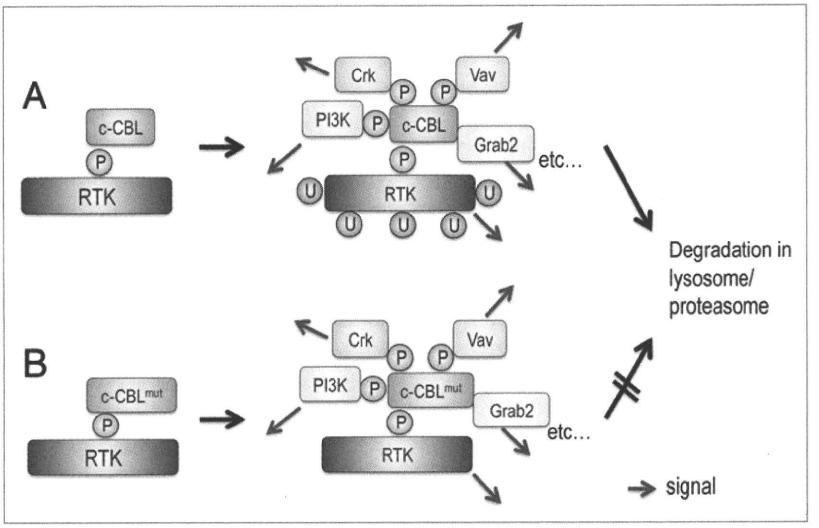

Figure 4. Positive regulation of signal transduction by c-CBL. (A) Having E3 ubiquitin ligase activity for negative regulation of signaling, c-CBL also works as an adaptor protein for multiple signal transduction molecules. When bound to phosphorylated tyrosine kinases, c-CBL is rapidly phosphorylated at multiple tyrosine residues, which in turn provide binding sites for a number of signal transduction molecules. Several lines of evidence suggest that binding to these molecules plays important roles in positive regulation of signal transduction (red arrows). Normally, phosphorylated c-CBL undergoes degradation, which is mediated by its E3 ubiquitin ligase activity. Thus, degradation of mutated c-CBL could be retarded, leading to prolonged transmission of positive signals (B).

References

1. Thien CB, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2001; 2:294-307.
2. Schmidt MH, Dikic I. The Cbl interactome and its functions. Nat Rev Mol Cell Biol 2005; 6:907-18.
3. Langdon WY, Hartley JW, Klinken SP, Ruscetti SK, Morse HC, 3rd. v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci USA 1989; 86:1168-72.
4. Blake TJ, Shapiro M, Morse HC, 3rd, Langdon WY. The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. Oncogene 1991; 6:653-7.
5. Abbas S, Rotmans G, Lowenberg B, Valk PJ. Exon 8 splice site mutations in the gene encoding the E3-ligase CBL are associated with core binding factor acute myeloid leukemias. Haematologica 2008; 93:1595-7.
6. Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res 2008; 68:10349-57.
7. 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; 113:6182-92.
8. Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, et al. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood 2009; 114:1859-63.
9. 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; 460:904-8.
10. Van Etten RA, Shannon KM. Focus on myeloproliferative diseases and myelodysplastic syndromes. Cancer Cell 2004; 6:547-52.
11. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009; 114:937-51.
12. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 2005; 434:1144-8.
13. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005; 352:1779-90.
14. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005; 7:387-97.
15. Levine RL, Pardanani A, Tefferi A, Gilliland DG. Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nat Rev Cancer 2007; 7:673-83.
16. Najfeld V, Montella L, Scalise A, Fruchtman S. Exploring polycythaemia vera with fluorescence in situ hybridization: additional cryptic 9p is the most frequent abnormality detected. Br J Haematol 2002; 119:558-66.
17. Kawamata N, Ogawa S, Yamamoto G, Lehmann S, Levine RL, Pikman Y, et al. Genetic profiling of myeloproliferative disorders by single-nucleotide polymorphism oligonucleotide microarray. Exp Hematol 2008; 36:1471-9.
18. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer 2001; 1:157-62.

19. Caligiuri MA, Briesewitz R, Yu J, Wang L, Wei M, Arnoczky KJ, et al. Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. Blood 2007; 110:1022-4.
20. Sargin B, Choudhary C, Crosetto N, Schmidt MH, Grundler R, Rensinghoff M, et al. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. Blood 2007; 110:1004-12.
21. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science 1999; 286:309-12.
22. Murphy MA, Schnall RG, Venter DJ, Barnett L, Bertoncello I, Thien CB, et al. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. Mol Cell Biol 1998; 18:4872-82.
23. Naramura M, Kole HK, Hu RJ, Gu H. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. Proc Natl Acad Sci USA 1998; 95:15547-52.
24. Rathinam C, Thien CB, Langdon WY, Gu H, Flavell RA. The E3 ubiquitin ligase c-Cbl restricts development and functions of hematopoietic stem cells. Genes Dev 2008; 22:992-7.
25. Zheng N, Wang P, Jeffrey PD, Pavletich NP. Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell 2000; 102:533-9.
26. Zeng S, Xu Z, Lipkowitz S, Longley JB. Regulation of stem cell factor receptor signaling by Cbl family proteins (Cbl-b/c-Cbl). Blood 2005; 105:226-32.
27. Naramura M, Jang IK, Kole H, Huang F, Haines D, Gu H. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. Nat Immunol 2002; 3:1192-9.
28. Lang GA, Iwakuma T, Suh YA, Liu G, Rao VA, Parant JM, et al. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell 2004; 119:861-72.
29. Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell 2004; 119:847-60.
30. Marengere LE, Mirtsos C, Kozieradzki I, Veillette A, Mak TW, Penninger JM. Proto-oncoprotein Vav interacts with c-Cbl in activated thymocytes and peripheral T cells. J Immunol 1997; 159:70-6.
31. de Jong R, ten Hoeve J, Heisterkamp N, Groffen J. Crkl is complexed with tyrosine-phosphorylated Cbl in Ph-positive leukemia. J Biol Chem 1995; 270:21468-71.
32. Buday L, Khwaja A, Sipeki S, Farago A, Downward J. Interactions of Cbl with two adapter proteins, Grb2 and Crk, upon T cell activation. J Biol Chem 1996; 271:6159-63.
33. Sattler M, Salgia R, Okuda K, Uemura N, Durstin MA, Pisick E, et al. The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway. Oncogene 1996; 12:839-46.
34. Ribon V, Hubbell S, Herrera R, Saltiel AR. The product of the cbl oncogene forms stable complexes in vivo with endogenous Crk in a tyrosine phosphorylation-dependent manner. Mol Cell Biol 1996; 16:45-52.
35. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, et al. SH2 domains recognize specific phosphopeptide sequences. Cell 1993; 72:767-78.
36. Hunter S, Burton EA, Wu SC, Anderson SM. Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. J Biol Chem 1999; 274:2097-106.
37. Hartley D, Corvera S. Formation of c-Cbl.phosphatidylinositol 3-kinase complexes on lymphocyte membranes by a p56lck-independent mechanism. J Biol Chem 1996; 271:21939-43.
38. Fukazawa T, Miyake S, Band V, Band H. Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins. J Biol Chem 1996; 271:14554-9.
39. Jiang X, Huang F, Marusyk A, Sorkin A. Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Biol Cell 2003; 14:858-70.
40. Meisner H, Czech MP. Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor. J Biol Chem 1995; 270:25332-5.
41. Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, et al. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature 2000; 407:202-7.
42. Ueno H, Sasaki K, Honda H, Nakamoto T, Yamagata T, Miyagawa K, et al. c-Cbl is tyrosine-phosphorylated by interleukin-4 and enhances mitogenic and survival signals of interleukin-4 receptor by linking with the phosphatidylinositol 3'-kinase pathway. Blood 1998; 91:46-53.
43. Grishin A, Sinha S, Roginskaya V, Boyer MJ, Gomez-Cambronero J, Zuo S, et al. Involvement of Shc and Cbl-PI 3-kinase in Lyn-dependent proliferative signaling pathways for G-CSF. Oncogene 2000; 19:97-105.
44. Garcia-Guzman M, Larsen E, Vuori K. The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. Oncogene 2000; 19:4058-65.
45. Tanaka S, Amling M, Neff L, Peyman A, Uhlmann E, Levy JB, et al. c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature 1996; 383:528-31.
46. Meng F, Lowell CA. A beta1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J 1998; 17:4391-403.
47. Scaife RM, Job D, Langdon WY. Rapid microtubule-dependent induction of neurite-like extensions in NIH 3T3 fibroblasts by inhibition of ROCK and Cbl. Mol Biol Cell 2003; 14:4605-17.
48. Gotoh T, Niino Y, Tokuda M, Hatase O, Nakamura S, Matsuda M, et al. Activation of R-Ras by Ras-guanine nucleotide-releasing factor. J Biol Chem 1997; 272:18602-7.
49. Bachmaier K, Krawczyk C, Kozieradzki I, Kong YY, Sasaki T, Oliveira-dos-Santos A, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature 2000; 403:211-6.
50. Chiang YJ, Kole HK, Brown K, Naramura M, Fukuhara S, Hu RJ, et al. Cbl-b regulates the CD28 dependence of T-cell activation. Nature 2000; 403:216-20.

Molecular Pathways

Clinical Cancer Research

Deregulated Intracellular Signaling by Mutated c-CBL in Myeloid Neoplasms

Seishi Ogawa[1,2], Lee-Yung Shih[3], Takahiro Suzuki[4], Makoto Otsu[5,6], Hiromitsu Nakauchi[5,6], H. Phillip Koeffler[7,8], and Masashi Sanada[1]

Abstract

c-CBL encodes a 120-kDa protein involved in intracellular signal transduction in a wide variety of cell types. Recently, frequent mutations of *c-CBL* have been reported in myeloid neoplasms showing both myelodysplastic and myeloproliferative features, in which most mutations are present in a homozygous state, as a result of allelic conversion in 11q. c-CBL has ubiquitin E3 ligase activity for a wide variety of tyrosine kinases, and thereby, negatively regulates tyrosine kinase signaling. Accordingly, c-CBL seems to have tumor suppressor functions, loss of which promotes tumorigenesis. On the other hand, once mutated, it is converted to an oncogenic protein and commits to myeloid leukemogenesis through a kind of gain of function causing aberrant signal transduction. The inhibition of mutant CBL protein or signaling pathways that it activates would have a role in therapeutics of myeloid neoplasms with *CBL* mutations.

Clin Cancer Res; 16(15); 3825–31. ©2010 AACR.

Background

c-CBL proto-oncogene is a cellular counterpart of a viral oncogene, *v-CBL*, isolated from a transforming retrovirus that causes B-cell lymphoma and myeloid neoplasms in mice (1). c-CBL is recognized as a 120-kDa cytoplasmic protein rapidly phosphorylated after cytokine stimulation. Interacting with a broad spectrum of signaling and cytoskeletal molecules as a multi-adaptor protein as well as an E3 ubiquitin ligase, c-CBL is thought to be involved in intracellular signaling (2, 3). Although *c-CBL* was first identified through its oncogenic versions in mice, its role in human carcinogenesis has been elusive until recently, when frequent mutations of *c-CBL* have been reported in a subset of myeloid neoplasms (4–10). Mutations of *c-CBL* are found in a variety of myeloid neoplasms, including acute myeloid leukemia and myelodysplastic syndromes (4–7, 10, 11), but they are most frequent in those subtypes of myeloid neoplasms that are now grouped into myelodysplastic-myeloproliferative neoplasms (MDS-MPN) in the World Health Organization classification (12). MDS-MPN include chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), and atypical chronic myeloid leukemia (aCML). c-CBL mutations are found in 5% of aCML (8) and up to 15% of JMML (9, 13, 14) and CMML (7, 10). They originate from immature hematopoietic progenitors and are commonly characterized by the production of dysplastic blood cells and myeloproliferative features (12, 15). Mutations seem to be somatic in most adult cases, but germline mutations were reported in some JMML cases in children (9). A conspicuous genetic feature of *c-CBL* mutations in these myeloid neoplasms is that mutations are homozygous in most cases, as a result of an allelic conversion of 11q arms that leads to duplication of the mutated parental copy of 11q and loss of the remaining wild-type allele, or "uniparental" disomy of the 11q arms. Mutations rarely accompany deletions of the wild-type allele (7–10), indicating the gain-of-function nature of the mutations rather than a simple loss-of-function (see below).

In mammals, three CBL homologs, c-CBL, CBL-b, and CBL-c, exist and are grouped into the CBL family of proteins (2, 3). All three homologs have a conserved *N*-terminal domain [tyrosine kinase-binding (TKB) domain], for their binding to phosphorylated tyrosine kinases, and a RING finger domain, as well as an intervening linker sequence. c-CBL and CBL-b, but not CBL-c, have extended C-terminal structures, including a proline-rich domain, a ubiquitin-associated–leucine zipper motif at the C terminus, and several tyrosine residues that are phosphorylated upon cytokine and/or growth factor stimulation (Fig. 1). The TKB domain consists of a four-helix bundle, a Ca^{2+}-binding EF hand, and a variant Src homology 2 (SH2) domain (16),

Authors' Affiliations: [1]Cancer Genomics Project, The University of Tokyo, Tokyo, Japan; [2]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan; [3]Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Chang Gung University, Taipei, Taiwan; [4]Division of Hematology, Department of Medicine, Jichi Medical University, Tochigi, Japan; [5]Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [6]Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Saitama, Japan; [7]Hematology/Oncology, Cedars-Sinai Medical Center, Los Angeles, California; and [8]National Cancer Institute of Singapore, National University of Singapore, Singapore

Corresponding Author: Seishi Ogawa, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. Phone: 813-5800-9046; Fax: 813-5800-9047; E-mail: sogawa-tky@umin.ac.jp.

doi: 10.1158/1078-0432.CCR-09-2341

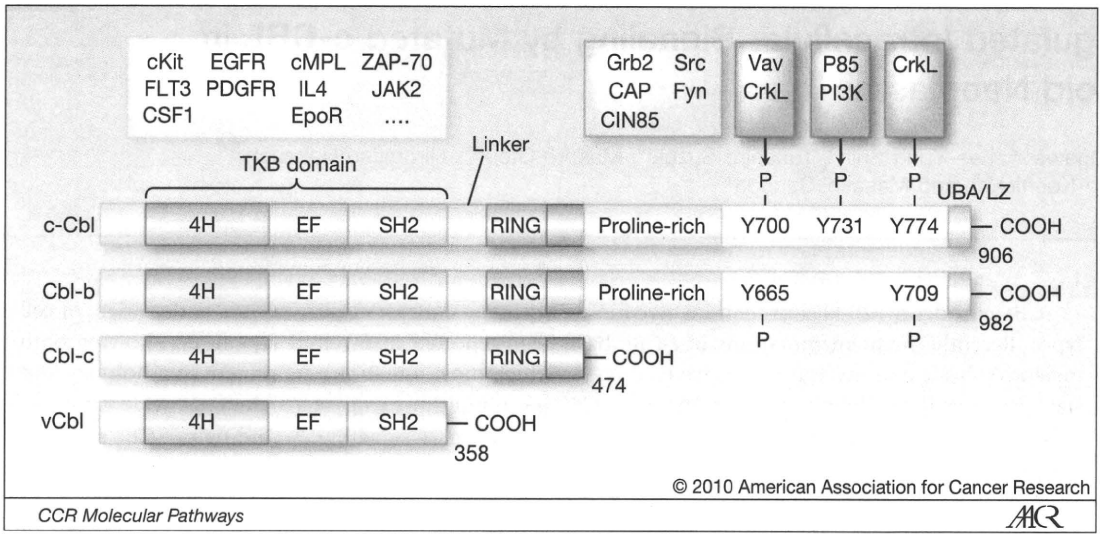

Fig. 1. Structure of CBL family proteins. Domain structures of CBL family proteins are depicted. Major tyrosine phosphorylation sites in c-CBL are indicated. Molecular interactions of c-CBL with cytokine receptors and other signaling molecules are also shown on top.

through which c-CBL binds to a phosphotyrosine-containing residue within a variety of activated tyrosine kinases. The spectrum of tyrosine kinases with which c-CBL can interact is thought to be determined by these N-terminal structures and includes receptor tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR; refs. 17–19), platelet-derived growth factor receptor (PDGFR; refs. 20–22), insulin-receptor (23, 24), c-Kit (25, 26), and FLT3 (5, 27), as well as non-RTKs (JAK2, ZAP70, and Syk; ref. 28). After being targeted to activated tyrosine kinases, c-CBL initiates a series of interactions with a variety of molecules as a multi-adaptor protein to transmit signals (Fig. 2A). First, c-CBL itself is phosphorylated at multiple tyrosine residues, to which a number of signaling molecules, including Vav (Y700; ref. 29), Crk/CrkL (Y700 and Y774; refs. 30–34), and the p85 subunit of PI3 kinase (Y731; refs. 35, 36) are recruited. The proline-rich domain provides binding sites for a variety of Src homology 3 (SH3)-containing proteins, including Grb2 (17, 18, 26, 32, 37–39) and NCK (40, 41), Src family tyrosine kinases (Fyn and Src; refs. 42–44), as well as CAP and CIN85 (45, 46). Grb2 constitutively binds to c-CBL in unstimulated cells, playing a role in recruitment of c-CBL to phosphorylated RTKs when cells are stimulated with their ligands. Src family kinases are responsible for phosphorylation of c-CBL on RTK stimulation. The long list of molecules making direct or indirect interactions with c-CBL is found in an excellent review (3), and the list is still growing. The complexity of molecular interactions of CBL comprises "the CBL interactome" and provides the basis for the diverse biological functions of c-CBL. Among these, the most extensively studied is its function as a negative regulator of tyrosine kinase signaling.

The negative regulation of tyrosine kinases by c-CBL was first implicated through genetic studies in *Caenorhabditis elegans*, in which the *c-CBL* ortholog, *sli-1*, was shown to be upstream of RAS (*let-60*) and Grb2 (*sem5*), and to suppress vulval induction that depends on *let23*, the ortholog of EGFR (47). Later, it was molecularly shown in mammalian cells that the negative regulation involves multi-ubiquitinylation of RTKs (21, 48, 49). c-CBL has E3 ubiquitin ligase activity, which is mediated by the linker-RING finger domains (50). c-CBL recruits E2 ubiquitin conjugating enzymes and ubiquitin monomers at the linker-RING finger interface and multi-ubiquitinylates the activated RTKs (Fig. 2A, upper panel). Depending on the multi-ubiquitinylation of the kinases, the kinase-c-CBL complexes are directed to endocytosis for subsequent degradation at lysosomes and/or proteasomes, or for recycling (21, 48, 49), which, in either case, limits kinase signals. Although multi-ubiquitinylation is critical for these reactions to occur, two c-CBL–bound adaptor molecules, CIN85 and CD2AP, mediate the endocytosis (45, 46). The negative regulatory roles of c-CBL in tyrosine kinase signaling suggest that the protein could have an anti-oncogenic function. In fact, *c-CBL* null mice have an enlarged thymus, expanded hematopoietic progenitor pools, splenomegaly with extramedullary hematopoiesis, as well as increased repopulating capacity of their bone marrow cells (10, 51–53). Blastic transformation of chronic myelogeneous leukemia in a bcr/abl-transgenic model is accelerated in the *c-CBL* null background (10). Finally, *c-CBL* null mice developed invasive cancers with complete penetrance.[9] Combined, these observations support that c-CBL can act as a tumor suppressor.

In contrast to the tumor suppressor function of the wild-type c-CBL, when transduced into NIH3T3 cells, *c-CBL* mutants isolated from human and murine neoplasms, as well as *v-CBL*, show clear transforming capacity in terms of

[9] Unpublished data.

anchorage-independent growth in soft agar and tumor generation in nude mice (10, 54). Bone marrow cells transduced with mutant *c-CBL* (R420Q and 70Z mutants) generate generalized mastocytosis, a myeloproliferative disease, and myeloid leukemia in lethally irradiated mice with long latency but high penetrance (55). The transforming activity of mutant c-CBL seems to be mediated by alteration of the E3 ubiquitin ligase activity. Except for rare mutations causing a premature truncation of the TKB domain, most *c-CBL* mutations in myeloid neoplasms are missense changes at highly conserved amino-acid positions within the linker and RING finger domains, or involve splice-site sequences, leading to amino acid deletions within these domains. Although the E3 ubiquitin ligase activity primarily depends on the RING finger domain, the intact linker sequence, which tightly packs with the TKB domain as well as with the E2 ligase, is also considered to be essential for efficient ubiquitinylation to occur (56). The crystal structure of the c-CBL/UBCH7 complex suggests that Y371 is important for the integrity of the linker-TKB interface (56). Thus, tumor-derived *c-CBL* mutations are expected to affect the E3 ubiquitin ligase activity. In fact, linker-RING finger mutations found in myeloid neoplasms, as well as other artificially introduced mutations within these domains, were shown to have compromised E3 ubiquitin ligase activity (5, 8, 10, 54). Moreover,

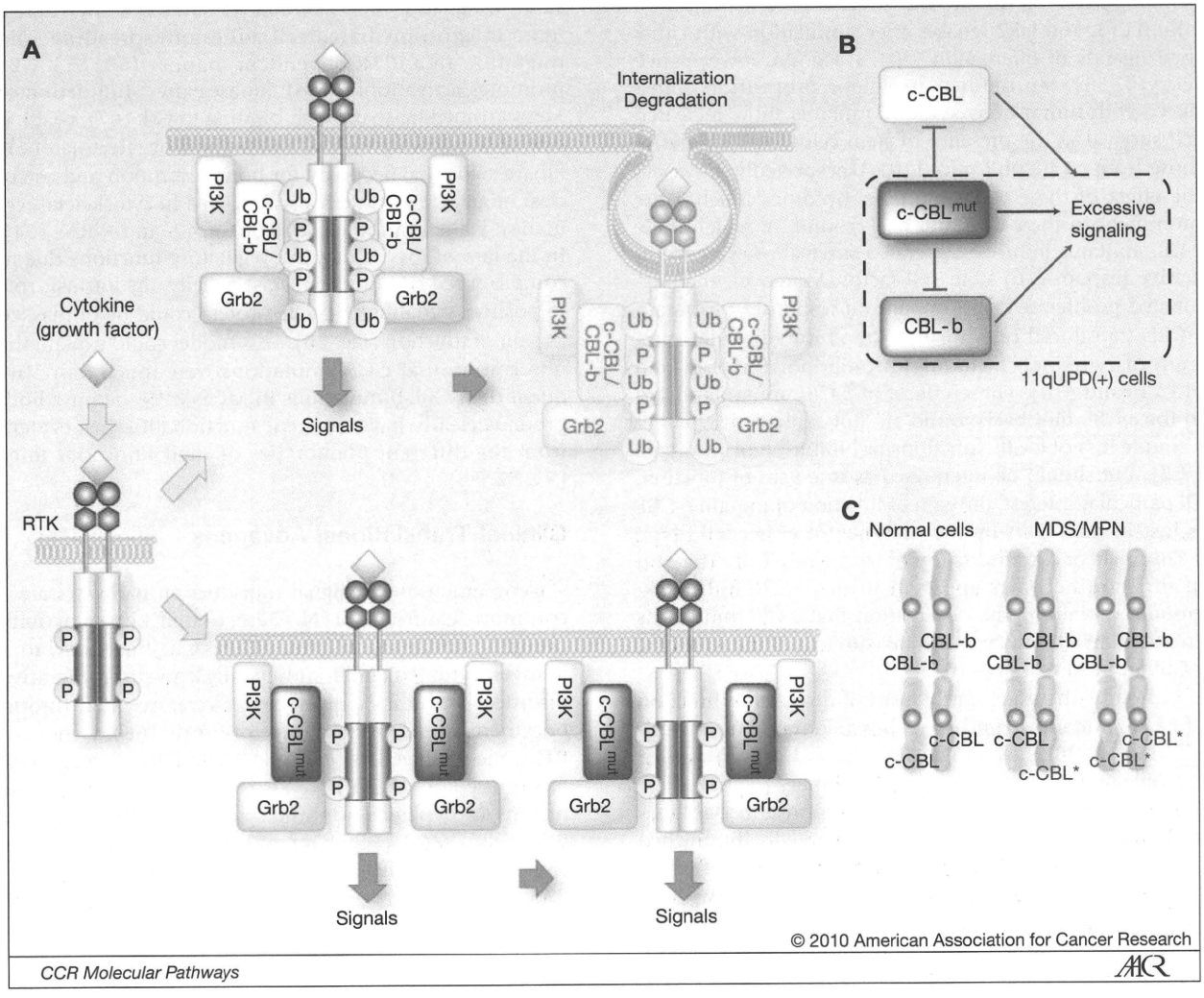

Fig. 2. Putative mechanism of gain of function of c-CBL mutants. A, after cytokine (growth factor) stimulation, RTKs are phosphorylated, to which c-CBL or CBL-b binds to ubiquitinate the receptors, while participating in signal transduction. Ubiquitinated RTKs are then subjected to degradation or recycling. On the other hand, when mutant c-CBL binds to the activated RTKs, downregulation of the RTKs is compromised, leading to prolonged signaling. B, putative mechanisms of the gain of function of c-CBL mutants; the CBL-b-inhibition model (red line) and the mechanism mediated by positive regulatory functions of c-CBL (blue line). C, in the CBL-b-inhibition model, a c-CBL mutant inhibits the E3 ubiquitin ligase activity of both c-CBL and CBL-b. In the heterozygous state, the inhibitory action of the c-CBL mutant is largely titrated out by three intact copies of c-CBL and CBL-b, leading to only modest increase in sensitivity to cytokines, as compared with the normal state (middle panel). When the mutant allele is duplicated by an allelic conversion in 11q, the mutant protein expressed from the two mutated alleles can effectively inhibit the remaining enzymatic activity from CBL-b (right panel).