患者の同意には、個別の遺伝子解析研究に限定したものと、班研究で行われるすべての遺伝子解析研究に対する包括的同意がある。患者担当施設では、その旨を登録票に記載し、患者登録事務局ならびに検体集積施設は患者の意思に従い、それらの検体を別個に保管する。
個別の研究の同意のみ得られた検体は、そのすべてを該当研究実施施設に供与し、各研究施設では研究終了後、検体を適切な方法で廃棄するとともに、別紙5の検体廃棄届を検体集積事業事務局に提出する。
遺伝子解析研究に対する包括的同意が得られた患者検体は、各遺伝子研究実施施設に必要量を送付し、残りは検体保管期限まで検体集積施設で保存する。各研究施設は、解析終了後の残余検体を検体集積施設に別紙6の残余検体送付書とともに返却する。
本事業・研究実施期間中に、患者もしくはその代諾者より本事業・研究協力への同意撤回の申し出でがあった際には、患者担当医師は事業・研究協力への同意撤回書（別紙9）への記載を依頼し、その写しの患者名を登録UPNに置き換えた後、検体集積事業総括責任者に送付する。検体集積事業総括責任者は検体集積施設管理責任者に該当検体の廃棄を依頼する。

7、遺伝子情報の開示

本事業・研究の結果判明した遺伝子解析結果の多くは、長期にわたる検討・解析の後にはじめて患者の状態を評価することに役立つものと予想され、そのような解析結果は提供者が特に希望されない限りは開示の対象とならない。しかし、病態を左右する遺伝子・治療法に影響を与える遺伝子が特定された場合は、前もって確認された希望に応じてその結果を開示する。

8、遺伝カウンセリング

患者担当医師は、患者およびその血縁者に遺伝カウンセリングの利用に関する情報を含めて説明するとともに、必要に応じて遺伝カウンセリングの機会を提供する。遺伝カウンセリングは、遺伝医学に関する十分な知識を有し、遺伝カウンセリングに習熟した医師、医療従事者が協力して実施する。実施体制については、各実施医療機関のカウンセラーが行うこととするが、当該実施医療機関において体制整備がなされていない場合には、実施医療機関の長が遺伝カウンセリングの適切な施設を紹介することとする。

9、説明と同意

担当医師は本研究の開始にあたって、下記の内容につき口頭と文書で説明する。説明・同意文書（別紙 7、8）は説明の前、または説明時に患者本人に手渡す。

1) 本研究は厚生労働科学研究費補助金・難治性疾患克服事業の一部として行われること
2) 検体集積事業で集積された検体は遺伝子解析研究に用いられること
3) 現在施行の決まっている研究は「造血器腫瘍における遺伝子異常の網羅的解析」、「骨髄異形成症候群の分子病態の解析と層別化治療の確立」、「骨髄異形成症候群のSPARC(secreted protein acidic and rich in cysteine)発現ネットワーク解析」および「骨髄不全症候群の酸化ストレス系遺伝子の発現ネットワーク解析」であるが、「不応性貧血の治癒率向上を目指した分子・免疫病態研究」および「特発性造血障害に関する調査研究班」であらたな研究計画が承認、決定される可能性があること
4) 研究への参加は個別研究への参加、もしくは今後予定される遺伝子研究を含めたもののいずれかであること
5) 本研究参加に対する経済的負担、もしくは報酬はないこと
6) 本研究参加による健康被害は生じないこと

7) 参加の同意が得られなくても不利益を受けないこと
8) 同意した後に随時これを撤回できること
9) プライバシーは守秘されること
10) 遺伝子解析結果は原則として希望に応じて開示できること、また必要に応じてカウンセリングを受けることができること
ただし、得られた情報の意義が直ちに提供者の状態を理解するのに役立つとは考えられないものもあり、そのような場合は遺伝子解析結果の開示が行われないこともある。

患者本人が本研究の参加に同意した場合は、添付同意書に自署による署名を得る。患者が未成年の場合には患者と法定代理人の双方より同意と自署による署名を得る。同意書は2部作成し、1部はカルテに、残り1部は患者もしくは家族が保管するものとする。

10、研究資金

本研究のうち検体集積事業は厚生労働科学研究費補助金により、遺伝子解析研究は個別遺伝子解析研究の研究責任者の獲得した研究資金により行われる。

11、結果の公表

本事業により集積された検体を用いた研究により得られた成果の公表に際して、個別遺伝子解析研究の研究責任者は、本事業・研究の研究代表者、ならびに「再生不良性貧血と骨髄異形成症候群の前方視症例登録・セントラルレビュー・追跡調査研究」の研究代表者から事前に承諾を得る。また、個別遺伝子解析研究の終了時に際して、研究責任者は本事業・研究の研究代表者に研究結果の概要を文書で報告する。

12、個別遺伝子解析研究の中止

個別遺伝子解析研究の研究責任者は、研究の継続が困難となる事由が発生したとき、すみやかに本事業・研究の研究代表者に報告するとともに、残余検体をすみやかに検体集積施設に送付する。研究代表者は本事業・研究参加施設にその旨を報告し、参加施設の責任者は各自医療機関内の倫理審査委員会に対し、速やかにその旨を文書で通知する。

13、研究組織

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「骨髄異形成症候群の分子病態の解析と層別化治療の確立」

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「骨髄異形成症候群の SPARC(secreted protein acidic and rich in cysteine)発現ネットワーク解析」

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「骨髄不全症候群の酸化ストレス系遺伝子の発現ネットワーク解析」

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# Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
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# Ⅳ．研究成果の刊行物・別刷

*Molecular Pathways*

Clinical Cancer Research

# Deregulated Intracellular Signaling by Mutated c-CBL in Myeloid Neoplasms

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## 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.* 

## 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),

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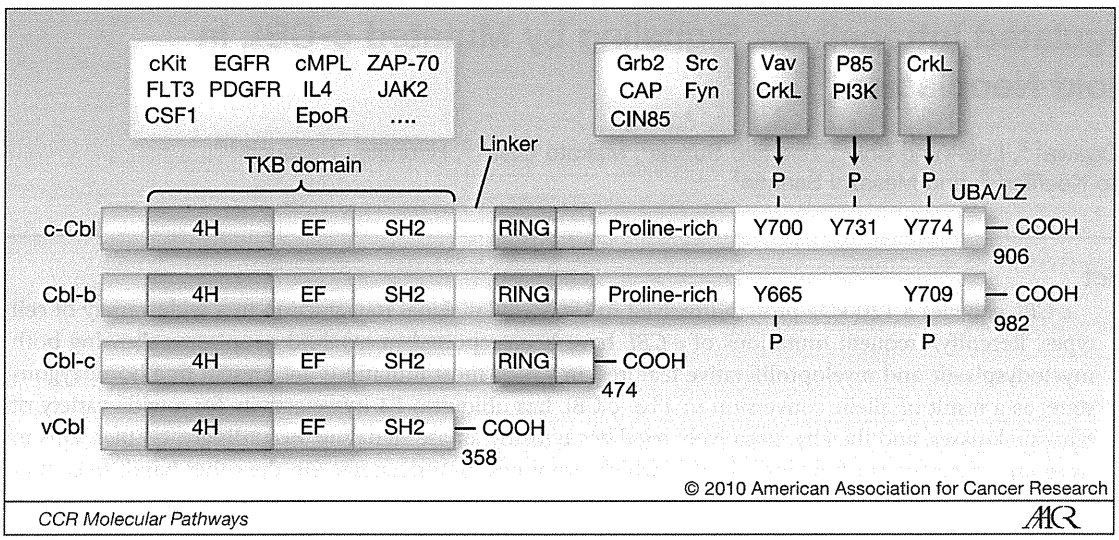


**Fig. 1.** Structure of CBL family proteins. Domain structures of CBL family proteins are depicted. Major tyrosine phosphorylation sites in c-CBL are indicated. Molecular interactions of c-CBL with cytokine receptors and other signaling molecules are also shown on top.

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,

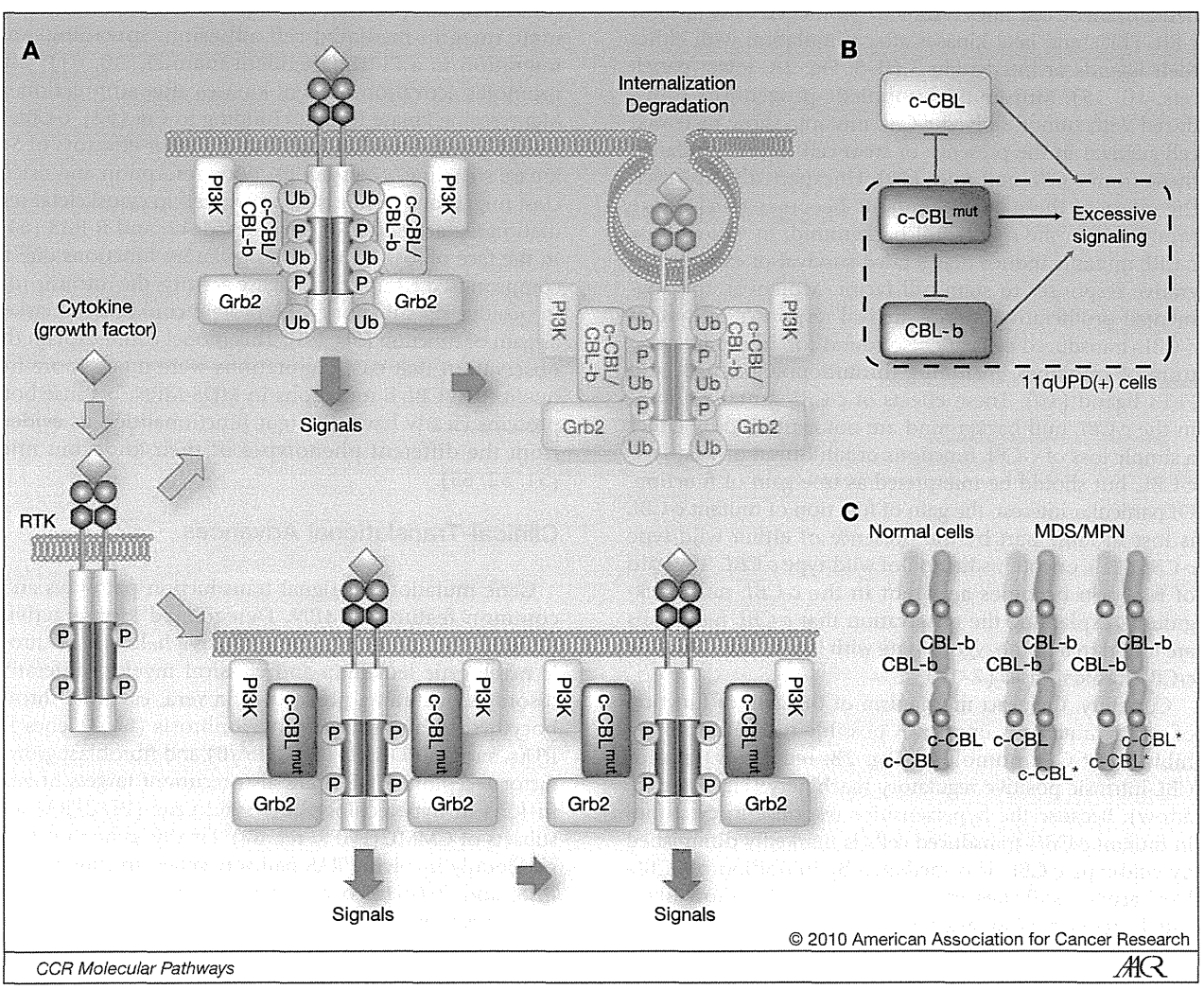


**Fig. 2.** Putative mechanism of gain of function of c-CBL mutants. A, after cytokine (growth factor) stimulation, RTKs are phosphorylated, to which c-CBL or CBL-b binds to ubiquitinate the receptors, while participating in signal transduction. Ubiquitinated RTKs are then subjected to degradation or 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).

these c-CBL mutants strongly inhibit the E3 ligase activity of wild-type c-CBL, indicating that linker-RING finger mutants act in a dominant negative manner against wild-type c-CBL (10). This finding is expected because a simple loss-of-function would not explain the dominant effect of c-CBL mutant on transforming activity in NIH3T3 cells expressing wild-type c-CBL. Interestingly, this inhibitory effect does not seem to depend on dimerization with the wild-type c-CBL, but on intact binding to phosphorylated tyrosine kinases, because a G306E mutation abolishes oncogenic capacity of these c-CBL mutants.[10] Thus, when overexpressed in EGFR-transduced NIH3T3 cells, mutant c-CBL inhibits ubiquitinylation of EGFR, leading to prolonged activation of the receptor after EGF stimulation. Similarly, transduction of c-CBL mutants into hematopoietic cell lines results in prolonged activation of c-Kit, FLT3, and Jak2 kinases after stimulation with either their ligands or interleukin 3 (IL-3; Fig. 2A, lower panel; refs. 10, 55). Murine hematopoietic progenitors transduced with tumor-derived *c-CBL* mutants show increased cell survival in the presence of stem cell factor, similar to those from *c-CBL* null mice (10). Unexpectedly, however, the effect of these c-CBL mutants becomes much more prominent in the *c-CBL* null background, in which these c-CBL mutants induce exaggerated survival or even proliferative responses to stem cell factor. Moreover, the augmented proliferative and/or survival responses of mutant c-CBL–transduced cells are also found for a broader spectrum of cytokines, including thrombopoietin, IL-3, and FLT3 ligand (10). These effects of *c-CBL* mutants found in the *c-CBL* null background are not explained by either a simple loss of c-CBL functions or inhibition of wild-type c-CBL, but should be interpreted as true gain of function. Of particular interest, the gain of function of mutant c-CBL is lost in large part by the presence of either wild-type *c-CBL* allele or cotransduction of wild-type *c-CBL*. The gain of function becomes apparent in the *c-CBL* null background, explaining the observation that *c-CBL* mutations are found in a homozygous state with loss of the wild-type c-CBL in most cases (7–10).

Currently, the exact mechanism of the gain of function of c-CBL mutants is unclear. A possible mechanism is inhibition of CBL homologs (Fig. 2B, red arrow) and/or CBL-intrinsic positive regulatory machinery (Fig. 2B, blue arrow). Because the hypersensitive response to cytokines in mutant *c-CBL*–transduced cells is markedly diminished by wild-type c-CBL, it is mediated by inhibition of "CBL-like" activity still present in *c-CBL* null cells, most likely CBL-b. Mutant c-CBL also inhibits E3 ubiquitin ligase activity of CBL-b, which is expressed in hematopoietic progenitor cells (10). *c-CBL/CBL-b* double knockout T cells show exaggerated proliferative response to anti-CD3 stimulation and prolonged T-cell receptor signaling, as compared with *c-CBL* or *CBL-b* single knockout T cells (57). According to this model, two mutant *c-CBL* alleles could functionally titrate out two wild-type *CBL-b* alleles, whereas one mutant *c-CBL* allele might not be sufficient to overcome one wild-type *c-CBL* plus two wild-type *CBL-b* alleles (Fig. 2C).

Another possible mechanism of the gain of function of mutated c-CBL is related to its function as a multi-adaptor, which is implicated in positive regulatory functions in signal transduction (Fig. 2B, blue arrow). As an adaptor protein, kinase-bound c-CBL recruits a number of molecules involved in signal transductions and cytoskeletal regulations. For examples, upon either IL-4 or granulocyte colony-stimulating factor stimulation, c-CBL is tyrosine-phosphorylated and binds to the p85 subunit of phosphoinositide 3 kinase (PI3K) to transmit mitogenic and/or survival signals (58, 59). Similarly, CBL was shown to regulate integrin-mediated cell adhesion, spreading, and migration in a PI3K-dependent manner (60, 61). CBL promotes activation of MAP kinases after stimulation of Met tyrosine kinase through binding to Crk (62). c-CBL is one of the downstream substrates and/or effectors of Src kinase signaling, necessary for bone resorption and osteoclast migration (63). It is also involved in cytoskeletal regulation via activation of Rac1 or Cdc42, and *R*-RAS (64). In the face of loss of negative regulatory functions due to compromised E3 ubiquitin ligase activity, the intrinsic role in positive signaling of c-CBL protein could be unmasked as gain of function (Fig. 2B). This model could explain the observation that c-CBL mutations were much more frequent than CBL-b mutations in MDS-MPN, because both proteins clearly have different functionalities, as evident from the different phenotypes of their knockout mice (51, 52, 65).

## Clinical-Translational Advances

Gene mutations in signal transduction pathways are a common feature of MPN. Deregulated kinase activity caused by *bcr-abl* and mutated *JAK2* is a hallmark of chronic myelocytic leukemia and classical myeloproliferative disorders, including polycythemia vera, essential thrombocythemia, and primary myelofibrosis (66). Genes for RTKs, such as PDGFRs (PDGFRA/B) and fibroblast growth factor receptors (FGFR) are also recurrent targets of gene fusions in hypereosinophilic syndrome (PDGFRA) and subsets of CMML (FGFR; ref. 67). Finally, gene mutations commonly involving RAS pathway genes, including *NF-1*, *RAS*, and *PTPN11*, occur in more than 70% of CMML cases, responsible for their hypersensitivity to granulocyte-macrophage colony-stimulating factor (15, 67). The recent finding of frequent *c-CBL* mutations in the MDS-MPD subgroup revealed a novel mechanism for excessive cell signaling through deregulated kinase activity in MPN, especially MDS-MPN subtypes, and also provided an insight into the therapeutics of *c-CBL*–mutated myeloid neoplasms.

Because *c-CBL* mutations induce excessive tyrosine kinase signaling, use of tyrosine kinase inhibitors could be

[10] Unpublished data.

a logical approach to the control of *c-CBL*–mutated neoplasms. However, the broad spectrum of c-CBL–regulated tyrosine kinases may preclude the efficacy of selective kinase inhibitors, whereas the use of pan-kinase inhibitors would increase a risk of the development of unacceptable adverse effects. Otherwise, identification of functionally relevant kinases regulated by mutated c-CBL would enable efficient targeting of such inhibition. Alternatively, the downstream signaling pathways, including JAK/STAT, PI3K, as well as RAS/extracellular signal-regulated kinase (ERK) signalings, are also potential therapeutic targets for inhibition with low molecular-weight compounds.

Given the gain-of-function nature of c-CBL mutants, inhibition of these mutant proteins would be a more reasonable approach, regardless of the exact mechanism of the gain-of function. Because the oncogenic action of mutant c-CBL proteins depends on their intact binding to target kinases, inhibition of this binding would be a potential approach, especially when the inhibition could be specifically directed to mutant c-CBL, but be saved for CBL-b. Recently, piceatannol, a naturally occurring phenol stilbenenoid, was shown to induce loss of the CBL family of proteins including mutant CBL (70Z mutant; ref. 68). Piceatannol was initially isolated as an antileukemic agent from a domesticated oilseed and was shown to inhibit a broad spectrum of tyrosine kinases including Sky, Src, Lck, and FAK, as well as some serine-threonine kinases (69–72). It also induces selective loss of CBL-associated proteins; levels of PDGFRβ, c-Abl, and EGFR are reduced by piceatannol treatment, whereas those of c-Src, Lyn, Syk, and Grb2 are unaffected (68). The molecular mechanism that underlies piceatannol-induced CBL loss is still unclear. It does not depend on proteasome, lysosome, and caspase activation, but rather on reactive oxygen species, which seems to be distinct from the mechanism of inhibition of kinase activities (68). Although piceatannol shows a broad spectrum of biological activity as an anti-inflammatory, antihistamine, and general antitumor agent *in vitro* (73–75), because of its broad biochemical actions, it has not been determined if, or to what extent, the biological activities of piceatannol depend on piceatannol-induced loss of CBL proteins. Although loss of both c-CBL and CBL-b is likely to result in increased tyrosine kinase activity, it also induces CBL-associated molecules and inhibits activity of a number of kinases, actually showing general antitumor activity. Unfortunately, no information is currently available about the antitumor effect of piceatannol on c-CBL–mutated leukemia. In c-CBL–mutated leukemic cells, loss of mutant c-CBL may further augment antitumor activity of this agent.

## Conclusion

*c-CBL* mutations are tightly associated with myeloproliferative myeloid neoplasms, especially the MDS-MPD subtype. c-CBL seems to act as a tumor suppressor, but when mutated, it is converted to an oncogenic protein. Although the oncogenic potential of c-CBL mutants is thought to be related to a type of gain of function, the molecular basis of this gain of function has not been fully understood. Undoubtedly, the effect of these mutations on the E3 ubiquitin ligase activity is essential for the gain of function. What complicates the mechanism is the fact that c-CBL has dual functionalities; it can behave as a multi-adaptor signal transducer, while also terminating signals by ubiquitinylating activated tyrosine kinases. Clearly, to understand the exact oncogenic mechanism of c-CBL mutants and to develop effective therapeutics, further *in vivo* and *in vitro* analyses are required.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

# Comparative analysis of remission induction therapy for high-risk MDS and AML progressed from MDS in the MDS200 study of Japan Adult Leukemia Study Group

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**Abstract** A total of 120 patients with high-risk myelodysplastic syndrome (MDS) and AML progressed from MDS (MDS–AML) were registered in a randomized controlled study of the Japan Adult Leukemia Study Group (JALSG). Untreated adult patients with high-risk MDS and MDS–AML were randomly assigned to receive either idarubicin and cytosine arabinoside (IDR/Ara-C) (Group A) or low-dose cytosine arabinoside and aclarubicin (CA) (Group B). The remission rates were 64.7% for Group A (33 of 51 evaluable cases) and 43.9% for Group B (29 out of 66 evaluable cases). The 2-year overall survival rates and disease-free survival rates were 28.1 and 26.0% for Group A, and 32.1 and 24.8% for Group B, respectively. The duration of CR was 320.6 days for Group A and 378.7 days for Group B. There were 15 patients who lived longer than 1,000 days after diagnosis: 6 and 9 patients in Groups A and B, respectively. However, among patients enrolled in this trial, intensive chemotherapy did not produce better survival than low-dose chemotherapy. In conclusion, it is necessary to introduce the first line therapy excluding the chemotherapy that can prolong survival in patients with high-risk MDS and MDS–AML.

For the Japan Adult Leukemia Study Group.

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## 1 Introduction

Myelodysplastic syndrome (MDS) is a group of disorders in which abnormalities occur at the level of hematopoietic stem cells [1], leading to disturbance in the production of blood cells characterized by ineffective hematopoiesis [2], decrease in the number of peripheral blood cells and morphological/functional abnormalities in blood cells [3]. Allogeneic hematopoietic cell transplantation (allo-HCT) is the most effective curative therapy for acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) [4]. However, for patients with high-risk MDS (those with refractory anemia with excess of blasts in transformation (RAEB)-t and some patients with RAEB) and patients with acute myeloid leukemia progressed from MDS (MDS–AML), chemotherapy aimed at remission is being used. The reasons for this are that MDS often affects elderly people [5], suitable donors are not always available at the time of disease onset, the necessity of pretransplant conditioning chemotherapy is controversial [6, 7] with a lack of sufficient evidence, and the optimal timing for transplantation varies widely depending on disease type [8].

On the other hand, reduced-intensity conditioning has extended the use of allo-HSCT to patients otherwise not eligible for this treatment due to older age or frailty [9]. However, allo-HSCT using traditional myeloablative preparative regimens is not easily tolerated by the elderly or frailer patient, and may lead to prohibitive treatment-related mortality rates. Most patients treated in the past were younger and devoid of comorbid clinical conditions. Novel reduced-intensity regimens have recently made allogeneic transplants applicable to the elderly, providing the benefit of the graft-versus-leukemia effect to a larger number of patients in need [10].

Low-dose chemotherapy, which has been used in clinical practice for 20 years, reduces the number of myeloblasts, improves pancytopenia and induces remission not only in MDS patients but also in some MDS–AML patients [11]. Common antineoplastic agents used in low-dose chemotherapy include cytosine arabinoside (Ara-C), aclarubicin (ACR), melphalan and etoposide. Nevertheless, despite improved Ara-C and regimens, the prognosis of AML in patients beyond 60 years of age remains dismal [4]. Low-dose antineoplastic drug therapy is still being used in some patients with MDS, which is common in elderly people, especially when the patient is at risk due to poor general condition or organ disorder [12].

The Japan Adult Leukemia Study Group (JALSG) previously conducted a pilot study for the treatment of high-risk MDS and MDS–AML to compare low-dose monotherapy with low-dose Ara-C plus granulocyte colony-stimulating factor (G-CSF) and multiple drug therapy with Ara-C plus Mitoxantrone plus VP-16. Later, JALSG conducted studies using a single protocol (JALSG MDS96) in 1996, in which remission induction and post-remission therapies using Ara-C and IDR in patients with high-risk MDS (RAEB-t) and in those with MDS–AML were performed, after which the efficacy and safety of these therapies were evaluated [13]. Furthermore, a randomized controlled study (JALSG MDS200) of intensive chemotherapy (IDR/Ara-C) or low-dose chemotherapy (CA) for high-risk MDS was also performed by JALSG.

Here, we present and analyze the results of the JALSG MDS200 study to assess and evaluate the validity of the MDS200 protocol for MDS treatment.

## 2 Patients and methods

### 2.1 Patient eligibility

A total of 120 patients were initially registered into the JALSG MDS200 study between June 2000 and March 2005. They were assigned into two groups, namely, Groups A and B (Table 1). Patients aged 15 years or more and diagnosed as having high-risk RAEB with high International Prognostic Scoring System score [14], RAEB-t or MDS-AML were eligible for this study. MDS–AML denotes secondary AML transformed from MDS.

Other eligibility criteria were as follows: patients with a performance status (PS) of 0–2 (ECOG); patients whose key organs other than the bone marrow retain intact function; patients who have not undergone any chemotherapy, except for pretreatment that does not affect the outcome of the main therapy; and patients who have given informed consent. Informed consent was obtained after carefully explaining the protocol and before registration.

### 2.2 Study protocol

The MDS200 protocol (Fig. 1) was designed based on the results of MDS96, and involved a dose-attenuation plan and allowed a wider range of chemotherapy. Patients were randomly assigned to either Group A or B.

In therapy A, the dose was adjusted according to a dose attenuation plan based on the presence of risk factors. The following 3 factors were regarded as risk factors: (1) Age ($\geq$60 years), (2) hypoplastic bone marrow and (3) PS $\geq$ 2. Patients with no risk factor received the standard dose, those with 1 risk factor received 80% of the dose and those with 2 or more risk factors received 60% of the dose (equivalent to the dose of MDS96). In therapy B, the use of

**Table 1** Characteristics of patients

| Group | A ($n = 53$) | B ($n = 67$) | $P$ value (A vs. B) |
|---|---|---|---|
| Age (range) | 63 (23–77) | 61 (32–81) | 0.505 |
| Gender | | | |
| Male | 37 | 52 | 0.332 |
| Female | 16 | 15 | |
| Disease type | | | |
| HR-RAEB | 4 | 11 | 0.269 |
| RAEB-T | 22 | 29 | |
| MDS–AML | 27 | 27 | |
| Infection | | | |
| Presence | 10 | 11 | 0.726 |
| None | 43 | 56 | |
| Karyotype[a] | | | |
| Good | 23 (44.2%) $n = 52$ | 21 (33.9%) $n = 62$ | 0.524 |
| Int | 11 (21.2%) | 15 (24.2%) | |
| Poor | 18 (34.6%) | 26 (41.9%) | |
| PB (range) | | | |
| WBC (/μL) | 2,500 (700–64,240) | 2,720 (600–43,700) | 0.665 |
| Hb (g/dL) | 8 (4.7–12.6) | 7.9 (4.4–12.7) $n = 66$ | 0.562 |
| Plt (/μL) | 5.8 (0.2–31.4) | 5.9 (0.5–36.7) | 0.363 |
| BM (range) | | | |
| Blast (%) | 30 (4–95) $n = 51$ | 24.2 (1.9–96) $n = 66$ | 0.171 |
| Biochemical data (range) | | | |
| LDH (IU/L) | 296 (132–882) | 303.5 (111–906) $n = 66$ | 0.998 |
| CRP (mg/dL) | 0.5 (0–20.2) | 0.35 (0–11.7) $n = 66$ | 0.292 |

Patients who met all of the inclusion criteria and did not meet any of the stated exclusion criteria were included the study. The disease types were classified by FAB classification

Statistical analysis between Group A and Group B was done using $\chi^2$ test or Mann–Whitney $U$-test

*MDS* myelodysplastic syndrome, *HR-RAEB* high risk-refractory anemia excess of blasts with high International Prognostic Scoring System Score, *RAEB-T* refractory anemia excess of blasts in transformation, *MDS–AML* MDS overt leukemia, *WBC* white blood cell, *Hb* hemoglobin, *Plt* platelet, *LDH* lactate dehydrogenase, *CRP* C-reactive protein, *PB* peripheral blood, *BM* bone marrow

[a] Shows IPSS risk

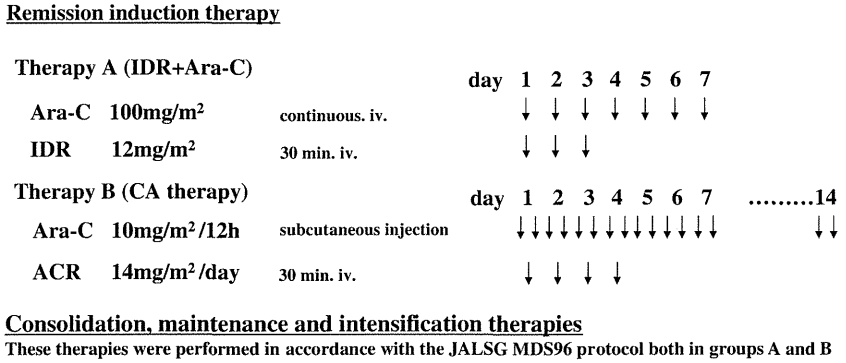


**Fig. 1** Japan Adult Leukemia Study Group—myelodysplastic syndrome (JALSG MDS200 Protocol). In therapy A, the dose was adjusted according to a dose attenuation plan based on the presence of risk factors. The following 3 factors were regarded as risk factors: (1) Age (≥60 years), (2) hypoplastic bone marrow and (3) PS ≥ 2. Patients with no risk factor received the standard dose, those with 1 risk factor received 80% of the dose, and those with 2 or more risk factors received 60% of the dose (equivalent to the dose of MDS-96). In therapy B, the use of CAG therapy involving co-administration of G-CSF was allowed. *IDR* idarubicin, *Ara-C* cytosine arabinoside, *ACR* aclarubicin, *G-CSF* granulocyte colony-stimulating factor, *iv* intravenous injection, *min* minutes

CAG therapy involving the co-administration of granulocyte colony-stimulating factor (G-CSF) was allowed.

Untreated adult patients (≥15 years) with MDS (RAEB, RAEB-t or MDS–AML) were randomly assigned to receive either IDR/Ara-C (Group A) or CA (Group B) [15]. Complete remission (CR) rate, CR duration, overall survival (OS) rate and disease-/relapse-free survival (DFS/RFS) rate were compared between the two groups.

Consolidation therapy and maintenance therapy were performed in accordance with JALSG MDS96 [13].

### 2.3 Evaluation of response

Response to treatment was evaluated in accordance with JALSG criteria [13]. CR was considered achieved when the following conditions remained for at least 4 weeks. For the bone marrow: blasts accounting for ≤5% of all cells; absence of blasts with Auer body; and presence of normal erythroblasts, granulocytes and megakaryocytes. For peripheral blood: absence of blasts; neutrophils ≥ 1,000/ml; platelets ≥ 100,000/μL; and no evidence of extramedullary leukemia. CR duration was defined as the duration from the day when CR is achieved to the day of relapse or death, OS or DFS as the duration from the day of initiation of treatment to the day of death and DFS as the duration in which CR patients survived without relapse. Patients who were treated with HCST were not censored at the date of transplantation. All toxicity was graded using the World Health Organization criteria [16].

### 2.4 Statistical analysis

The primary endpoint of this study is DFS. Assuming a 1-year DFS rate of 60% in the Group A and 40% in the Group B, this design required the randomization of 200 patients. Eligible patients were randomized according to age, sex and disease type. Differences in background factors (e.g., age, gender and disease type) between Groups A and B were statistically analyzed using the $\chi^2$ test or Mann–Whitney *U*-test. Probability of OS and DFS were estimated according to the method of Kaplan and Meier.

## 3 Results

### 3.1 Recruitment of patients and suspension of the study

The initially registered 120 patients were assigned into two groups, namely, Groups A and B. The clinical characteristics of the registered patients are shown in Table 1. The present protocol was originally planned to recruit 200 patients for Groups A and B within 3 years. However, the recruitment pace was slower than expected and thus the study period was extended from 3 years to 4.5 years. At the end of 2004, that is, after 4.5 years from the start of the study, the number of registered patients was only 113 in Groups A and B, which was 56.5% of the target number. At that point, the committee members discussed the progress of the MDS200 study and decided to suspend it at the end of March 2005. Since the final total number of patients did not reach the target number, we did not statistically compare DFS between Groups A and B, which was the primary endpoint of this study.

### 3.2 Characteristics of patients

There were no clear differences in the clinical characteristics of the patients between Groups A and B, such as FAB subtype, initial blood cell count, presence of infection, distribution in the karyotype group and biochemical data, as well as sex distribution (male/female ratio, 37/16 = 2.315 in Group A, and 52/15 = 3.467 in Group B).

### 3.3 Treatment outcome

The remission rates were 64.7% in Group A (33 out of 51 evaluable cases) and 43.9% in Group B (29 out of 66 evaluable cases). The 2-year overall survival (OS) rates were 28.1% in Group A and 32.1% in Group B, and the 2-year DFS rates were 26.0% in Group A and 24.8% in Group B. The mean duration of CR was 320.6 days (median: 213 days) in Group A and 378.7 days (median: 273 days) in Group B (Table 2). Reflecting the intensity of the remission induction chemotherapy, the period of WBC (<1,000/μL) after the therapy was longer in Group A than in Group B (19 days and 4 days, respectively). There were more grade 3 or 4 adverse events during the remission induction therapy in Group A (19 out of 53 evaluable patients) than in Group B (13 out of 67 evaluable patients). This difference was mostly attributable to infectious episodes (17 patients in Group A and 4 patients in Group B). In terms of bleeding episodes, 1 patient in Group A and 2 in Group B had grade 3/4 adverse events. The numbers of

**Table 2** Treatment outcome (Group A vs. B)

| | Group A ($n$ = 53) | Group B ($n$ = 67) |
|---|---|---|
| Remission rate (%) | 64.7 | 43.9 |
| Mean duration of remission (days) | 320.6 (median: 213) | 378.7 (median: 273) |
| 2-Year survival rate (%) | 28.1 | 32.1 |
| 2-Year disease-free survival rate (%) | 26.0 | 24.8 |

The remission rates, 2-year overall survival (OS) rates and 2-year disease-free survival (DFS) rates are shown as percentages