**Figure 3.** Detection of aUPD in SNP-A karyotyping. (a) aUPD or CN-LOH refers to the allelic status caused by loss of one of the two parental alleles and duplication of the remaining allele. (b) In SNP-A karyotyping, aUPD is detected by significant dissociation of AsCNs (red arrows) or by the reduction of the number of heterozygous SNP calls (blue arrow). (c) Sensitive detection of aUPD using AsCN analysis was evaluated using intentionally mixed tumor and normal cells at the indicated tumor proportions. The reduction of heterozygous SNP calles (green bars) in the aUPD(+) region is obscured with less than 40% of tumor content, whereas the dissociation of AsCNs (green and red lines) clearly indicates the presence of aUPD even with. 20% of tumor content. (d) AsCN-based detection of aUPD (orange) outperforms that relying on the reduced heterozygous SNP calls (blue) in sensitivity. The gray line indicates numbers of heterozygous SNP calls within the target region with aUPD. (e) aUPD is generated as a result of somatic recombination between sister chromatids or deletion of a chromosome segment and duplication of the remaining allele, rendering a mutated allele homozygous. (e) Disappearance of heterozygous SNP calls are also caused by inheritance of identical IBD alleles from parents. Reflecting multiple meiotic recombinations within the parents' gametes, they usually appear as multiple segments with loss of heterozygous SNP calls intervened with heterozygous diploid segments.

Homozygous deletions are less common than simple deletions in cancer genomes, but they provide an important clue to the identification of tumor-suppressor genes, since the typical size of homozygous deletions is less than 1 Mb. Taking advantage of their high resolution of analysis, the SNP-A platforms enable genome-wide detection of these lesions and have contributed to the discovery of novel tumor-suppressor genes.[29,30] While in primary tumor specimens, the presence of normal cells frequently prevents detection of homozygous deletions by polymerase chain reaction (PCR), such deletions could be detected as a biallelic reduction in AsCNs in SNP-A karyotyping even with low tumor contents (Figure 2d).

## DETECTION OF CN-LOH OR UPD

The other target of SNP-A karyotyping is CN-LOH. CN-LOH has been the focus of recent attention in cancer genetics, especially hematopoietic neoplasms. It represents an abnormal allelic status, in which both of the two existing alleles have a single parental origin, and thus it is also called "uniparental" disomy (UPD) (Figure 3a). In SNP-A karyotyping, UPD is detected as a significant dissociation in AsCN plots, where higher and lower copy number plots indicate the duplicated and missing alleles, respectively (Figure 3b). When the proportion of UPD-positive tumor components exceed 70% in the specimen, the frequency of heterozygous SNP calls is significantly reduced, indicating the presence of LOH. However, detection of LOH relying on heterozygous SNP calls is much less sensitive compared to the AsCN-based detection; with less than 70% of UPD-positive tumor cells, no significant reduction of heterozygous SNP calls is observed (Figure 3c). Although the size of the dissociation in AsCN plots varies depending on the proportion of the tumor components having UPD within the specimen, as few as 20% of UPD-positive components can be detected by SNP-A karyotyping[25] (Figure 3d).

UPD may occur as an inborn error in congenital disorders, including Beckwith-Wiedemann syndrome (UPD in 11p), and Angelman syndrome and Prader-Willi syndrome (UPD in 15q), where the consequent abnormal imprinting status of the involved chromosomes is implicated in their pathogenesis.[31-33] However, recent studies using SNP-A karyotyping indicate that UPD is more commonly found in cancers as an acquired abnormality (acquired UPD [aUPD]).[34] Several mechanisms have been implicated in the generation of aUPD during the development of cancer (Figure 3e). For example, mis-segregation of a chromosome with total or partial deletion of the other allele is thought to be a common mechanism of aUPD among cancers, especially those showing hyperploidy, leading to aUPD of whole chromosomes or aUPD plus trisomy of the surrounding chromosomal segments. On the other

hand, in many hematopoietic neoplasms aUPDs frequently involve the telomere end of affected chromosomal arms, suggesting that they are generated by somatic recombinations between sister chromatids. These aUPDs should be strictly discriminated from identity-by-descent (IBD) alleles, which are not uniparental but inherited from both parents by varying degrees of consanguinity between close kin. For example, one sixteenth of the total genome is expected to consist of IBD alleles in children born to marriage between cousins. Thus, IBD alleles are more common in older individuals, reflecting higher frequencies of consanguinity in the past. Usually, IBD alleles tend to be found in the middle of diploid regions and involve multiple chromosomal sites (Figure 3f).[35,36] Unfortunately, however, discrimination between aUPD and IBD alleles is difficult in some cases, especially when they occur in mostly diploid genome and involved chromosomal ends.

In cancer genetics, aUPD has been established as one of the common mechanisms for biallelic inactivation of tumor-suppressor genes, by which the intact allele is lost and replaced by the mutant allele.[37] However, the precise incidence of aUPD among human cancers has not been fully evaluated until recently, when the genome-wide detection of this abnormality has been enabled by the advent of the SNP-A karyotyping technology. aUPD has been shown to frequently occur in human cancers, including hematopoietic neoplasms. aUPDs are found in 20% of acute myeloid leukemia (AML), 30% of myelodysplastic syndromes (MDS), and related disorders, and more than 80% of malignant lymphomas.[30,35,38-45] As expected, these UPDs are shown to be tightly associated with homozygous mutations of known tumor-suppressor genes, including *TET2* in 4q, *CDKN2A/B* in 9p, *TP53* in 17p, *NF1* in 17q, *Rb* in 13q, *CEBPA* in 19q, and *RUNX1* in 21q[28,35,37,40-42,44] (Figure 4a). Moreover, recent evidence suggests that aUPD may accompany not only loss-of-function alleles of tumor-suppressor genes but also gain-of-function alleles of oncogenes. This was first demonstrated for 9pUPD causing homozygous *JAK2* V617F mutations in polycythemia vera (PV), as well as other myeloproliferative neoplasms (MPN), and to a lesser extent in MDS.[46-48] Thereafter, the association between aUPDs and oncogenic mutations was further confirmed for oncogenes in a variety of hematopoietic neoplasms.[35,49-51] Common examples include homozygous mutations of *c-MPL* or *NRAS*, *c-CBL*, and *FLT3*, which are caused by aUPDs in 1p, 4q, 7q, 11q, and 13q, in a variety of myeloid neoplasms, respectively (Figure 4b).

## SENSITIVITY OF SNP-A KARYOTYPING

The sensitivity to detect particular genetic lesions in SNP-A karyotyping depends on the size of genetic le-
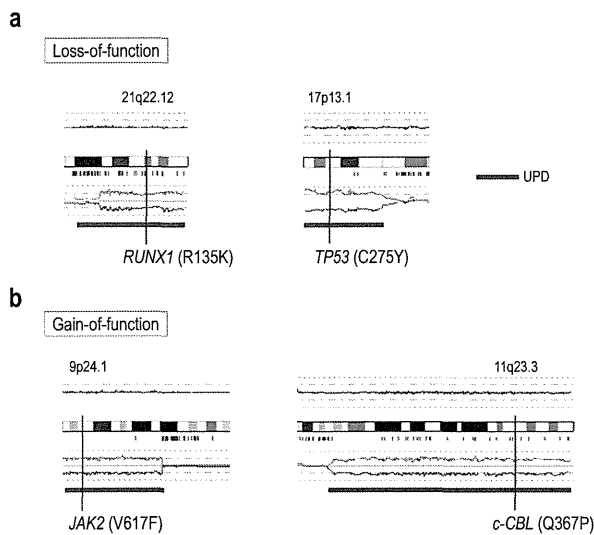
**Figure 4.** Recurrent aUPDs and their gene targets in hematopoietic neoplasms. Recurrent aUPD found in hematopoietic neoplasms are frequently associated with loss-of-function mutations of tumor-suppressor genes (a) or gain-of-function oncogenic mutations (b).

sions and the tumor contents within the samples, and also on the algorithm with which they are detected. These computer-assisted algorithms, as well as other bioinformatics tools for SNP-A karyotyping, are especially useful to detect complex genetic lesions objectively and to summarize them for a large number of specimens, facilitating identification of genetic targets. A number of algorithms for computer-assisted detection/inference of genetic lesions in SNP-A karyotyping have been developed, among which hidden Markov model (HMM)-based algorithms and those using circular binary segmentation (CBS) are widely applied by researchers.[52-54] Regardless of algorithms, to detect copy number changes the size of the measured copy number changes ($\Delta$) needs to be significantly larger than the mean size of measurement errors, eg, standard deviation of measured copy numbers in diploid genome ($SD^{dip}$). Because the relative intensity of probe-specific signals to the background signals is substantially weaker in Affymetrix GeneChip than in CGH arrays, the mean log2 ratio of haploid to diploid signals remains approximately 0.5 (Nsp250K arrays) rather than achieves the theoretically expected value ($\sim$1.0) obtained in CGH arrays. In addition, SNP-A tends to show significantly higher $SD^{dip}$ values than array CGH systems. As a result, SNP array-based copy number detection is more prone to loss of sensitivity with low tumor cell components than CGH-based copy number analysis. In typical SNP-A analyses, approximately 20% to 30% of tumor contents are required for detection of abnormalities in large chromosomal segments. In contrast, similar genetic lesions could be successfully captured even with less than 10% of tumor contents in

metaphase karyotyping and typical CGH arrays (BAC array and Agilent 224K), although metaphase karyotyping depends on viable cells capable of cell division for analysis.

The $SD^{dip}$ values or noises in SNP-A show substantial variation depending on the experimental conditions and the algorithms with which copy numbers are calculated. In the Affymetrix platform, the genomic DNA is digested with a proper restriction enzyme and the adapter-ligated restriction fragments are subjected to PCR amplification before hybridization. Because PCR amplification assumes successful digestion of both ends of the fragments, the difference in the mean length of genomic DNA between test and reference DNA can bias copy number calculation, especially at those SNP sites on the longer DNA fragments. This causes a serious problem to analyze degraded DNA prepared from formalin-fixed paraffin-embedded (FFPE) samples, although the problem is partly circumvented at the cost of resolution by eliminating SNPs on long restriction fragments (>500 bp) from the analysis.[55] The subsequent PCR reactions also produce biases, because relative amplification efficiency among different DNA fragments could be easily affected by subtle differences in PCR conditions, including types of polymerase and thermal cyclers.[12,56] Thus, in order to obtain the best results, it is very important to perform experiments as uniformly as possible between test and reference samples. For example, it is recommended that whenever possible, array experiments should be performed with a set of normal DNA included for reference, especially in those centers with less experience in SNP-A analysis, although this leads to increased costs and reduced throughput. Using a set of array data from normal DNA as a common reference can reduce costs and increase throughput but generally results in increased $SD^{dip}$ and reduced resolution and sensitivity (Figure 5). $SD^{dip}$ values in typical experiments are between 0.15 and 0.20, while they can be controlled to less than 0.10 in well-performed experiments.

## COPY NUMBER VARIATIONS AND THE USE OF GERMLINE CONTROL

Copy number variations (CNVs) are a type of polymorphism widely found in our genomes, where the number of particular genomic segments shows variations.[57-59] Most CNVs are less than 1 Mb in length, but some CNVs span genomic segments of more than several megabases in length. While CNVs could be potential targets of SNP-A karyotyping, they may complicate the discrimination between somatic and germline events in cancer specimens, because difference in CNVs between test and references from different individuals could be erroneously detected as somatic copy number changes. Although using a germline DNA as a reference could largely circumvent the false positive
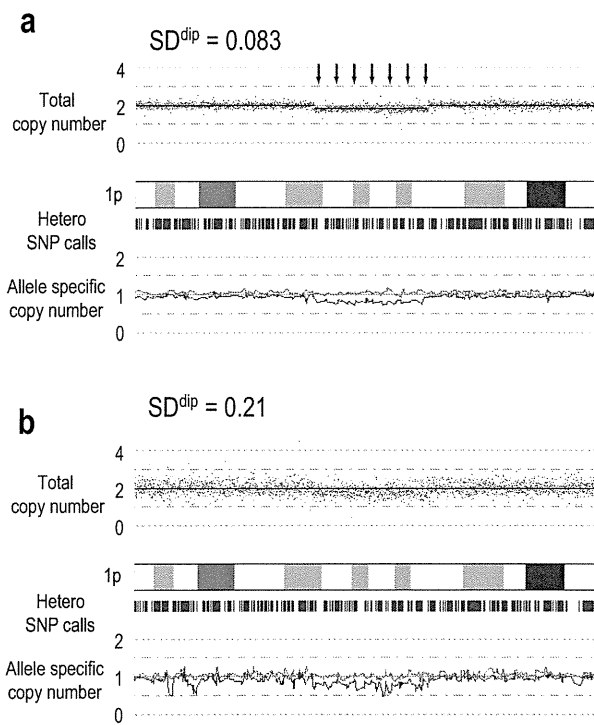
a

SD$^{dip}$ = 0.083

Total copy number

Hetero SNP calls

Allele specific copy number

b

SD$^{dip}$ = 0.21

Total copy number

Hetero SNP calls

Allele specific copy number

**Figure 5.** Effect of reference sets on detection of genetic lesions. The same array data for a tumor sample was analyzed with a set of reference array data of simultaneously processed normal diploid DNAs (a) or with a different array data set of normal diploid DNAs generated from a different set of experiments (b). The set of reference used for the analysis substantially influenced the result. The analysis in a shows a lower SD$^{dip}$ value than that in (b), enabling identification of a interstitial deletion (red arrows) that is not clear in (b).

Give their primary use for GWAS studies, processing a large number of specimens is an important pre-requisite for the development of SNP-A platforms. With simplified experimental protocols and semi-automated procedures, both SNP-A platforms achieve high-throughput sample processing, in which dozens of specimens can be analyzed within a few days in a single set of SNP array systems. This is in contrast to conventional metaphase karyotyping. Obtaining high-quality metaphases may not always be possible and, as previously mentioned, absolutely requires cell culture before analysis, precluding the analysis of archived samples. Production of a large enough number of karyograms for analysis is also time-consuming and their interpretation requires some discipline.

On the other hand, metaphase karyotyping may reveal the presence of several tumor subpopulations with different genomic profiles, as typically found in some MDS or AML M6 patients with poor prognosis, where individual metaphases show different karyotypes. DNA-based analyses including SNP-A and CGH array measure mean copy numbers among different subclones. They could infer such complexities in some cases, but generally would fail to fully dissect such complex abnormalities within each tumor subpopulation, suggesting the importance of combined used of metaphase karyotyping and array-based karyotyping technologies. Features of different platforms for detection of genetic alterations are summarized in Table 1. Apparently, what is important is the judicious use of the appropriate platforms according to the types of target genetic lesions to be detected.
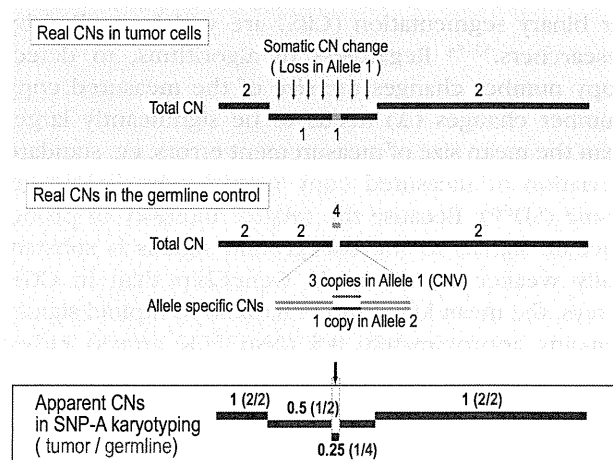
As long as the target genetic abnormalities are un-

detection of CNVs as somatic copy number changes, it does not completely avoid the problem. When a CNV is located within a segment showing an allelic imbalance and analyzed with a germline control, an apparent copy number change may appear at that CNV locus, even though it is not real (Figure 6).

## CLINICAL APPLICATIONS

Until recently, application of SNP-A karyotyping has been largely limited to exploratory research on cancer genetics. However, given its excellent performance in detecting genetic abnormalities in cancers, application of SNP-A karyotyping to clinical hematology could be a logical approach in an attempt to establish better management of cancer patients, although there remains a number of issues to be answered before its use in clinical settings. Clearly, SNP-A karyotyping does not replace the conventional metaphase karyotyping or other PCR-based detection of a variety of fusion genes, because SNP-A karyotyping cannot detect balanced translocations that are relevant to the management of a variety of hematopoietic malignancies.



**Figure 6.** False detection of copy number abnormality. In most cases, CNVs are successfully discriminated from somatic changes using a germline control. However, in some cases, the use of a germline control may lead to false detection of CNVs as somatic changes. This occurs when two parental alleles have different CNVs and that CNV site is located in a segment showing copy number gain or loss.

**Table 1. Comparison of Different Platforms**

| | Resolution | Mean Probe Interval | Dependence on Cell Division | Genome Coverage | Detection of UPD | Detection of Tumor Subclones | Sensitivity to Tumor Components | Detection of Balanced Translocation | Throughput | Cost |
|---|---|---|---|---|---|---|---|---|---|---|
| Cytogenetics | Low | >5–10Mb | + | + | – | ++ | ~10% | + | Low | Low |
| Interphase FISH | NA | NA | – | – | – | NA | <~5% | +*** | Intermediate | Low |
| aCGH | High | 1.8kb*~70kb | – | ++ | – | +/– | 10–20% | – | Intermediate | High |
| SNP-A | High | 5kb**~20kb | – | ++ | + | +/– | 20–30% | – | High | High |

*Agilent 1M.
**Affymetrix SNP6.0.
***Selected targets only.

balanced changes shared by the main tumor population, SNP-A karyotyping would be a good alternative to conventional karyotyping or could even outperform the latter, especially when no metaphases are obtained in conventional karyotyping. SNP-A karyotyping reveals comprehensive registries of genetic lesions, including copy number gains and losses, as well as UPD, in hematologic neoplasms (Figure 7). In hematopoietic neoplasms, aUPDs are found in varying frequencies depending on tumor types, ranging from approximately 20% in AML to nearly 80% in diffuse large B-cell lymphoma. While some aUPDs are closely related to particular gene mutations, the clinical significance of other aUPDs remains to be elucidated.

## AML AND ACUTE LYMPHOBLASTIC LEUKEMIA

In leukemias and lymphomas, a number of novel genetic targets have been identified through SNP-A karyotyping of acute lymphoblastic leukemia (ALL). SNP-A karyotyping identified recurrent deletions/translocations involving *EBF1* and *PAX5* in childhood ALL,[20,27,28] and frequent deletion of *Ikaros* in lymphoid blastic crisis of CML, as well as Ph1^{+} ALL.[29] Meanwhile, the clinically relevant disease-specific translocations are out of the scope of SNP-A karyotyping, which are among common targets in metaphase karyotyping and could be more sensitively detected by targeted approach, including interphase fluroesence in situ hybridization (FISH) and reverse transcriptase-PCR. This is a major drawback of SNP-A karyotyping. However, the excellent performance of SNP-A karyotyping in genome-wide detection of complex unbalanced lesions as well as aUPD could compensate the drawback, and add unique values to this platform in clinical setting.

## MDS AND RELATED MYELOID NEOPLASMS

MDS, MDS/MPN, and secondary AML are among the best targets of SNP-A karyotyping, in which the unbalanced genetic changes are predominant,[60,61] and these changes are directly incorporated into their prognostic scores.[62,63] It was demonstrated that SNP-A karyotyping showed a higher performance compared to metaphase karyotyping.[35,43] In our series consisting of 222 cases with MDS and related myeloid neoplasms, SNP-A karyotyping captured all the genetic lesions found in metaphase karyotyping except for four balanced translocations. Moreover, 41 of the 91 cases with normal karyotypes by metaphase cytogenetics showed one or more genetic lesions by SNP-A karyotyping. Overall, SNP-A karyotyping revealed approximately 1.5 times more genetic lesions, including -7/7q- and complex karyotypes indicating poor prognosis[35,43] (Figure 8). Assuming that the masked lesions in metaphase karyotyping are also valid in evaluating the International Prognostic Scoring System (IPSS) score,
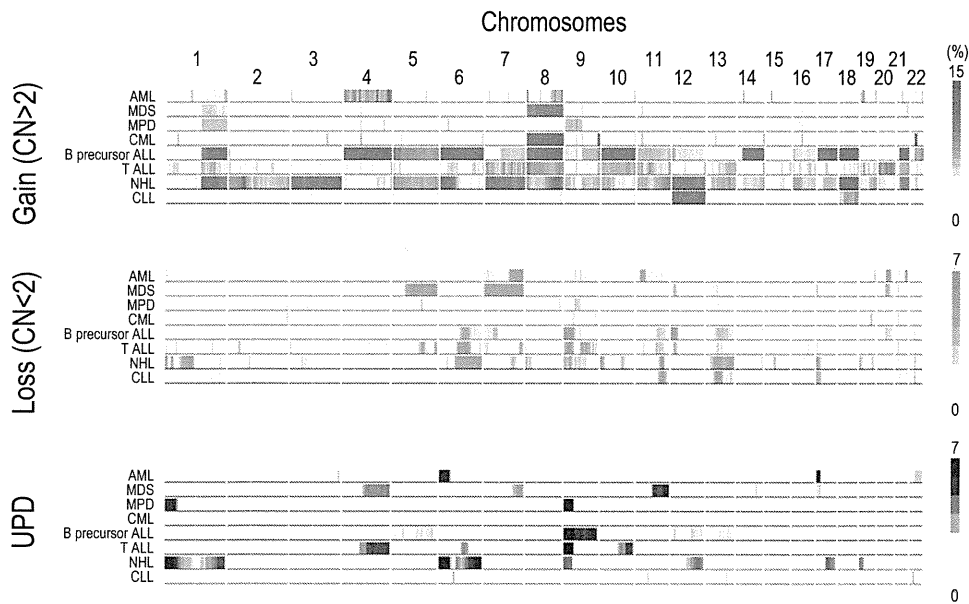
**Figure 7.** Genomic profiles of different hematopoietic neoplasms in SNP-A karyotyping. Genomic profiles revealed by SNP-A karyotyping are shown for different hematopoietic neoplasms, including AML (N = 36), MDS (N = 294), MPN (N = 57), CML (N = 51), B-precursor ALL (N = 507), T-cell ALL (N = 84), non-Hodgkin lymphoma (NHL) (N = 238), and CLL (N = 131). Frequencies of copy number gains and losses, as well as aUPDs, across the genome are color-coded in each neoplasm type as indicated. Each neoplasm type has a characteristic genomic profile of its own.

SNP-A karyotyping would be a more appropriate tool for the management of MDS and related neoplasms.

In these myeloid neoplasms, aUPDs are found in about one fourth to one third of the patients and, in some cases, represent the only genetic lesions found by SNP-A karyotyping.[35,43] These aUPDs are preferentially involved in particular chromosomal arms, such as 1p, 1q, 4q, 7q, 9p, 11p, 11q, 13q, 14q, 17p, and 21q. Importantly, recent

studies demonstrated that many of these aUPDs are tightly associated with mutations of tumor-suppressor genes or oncogenes (Table 2).[35,40,43,46,50,64,65]

## MALIGNANT LYMPHOMAS

Malignant lymphomas consist of a diversity of different histology types. This wide heterogeneity of lym-
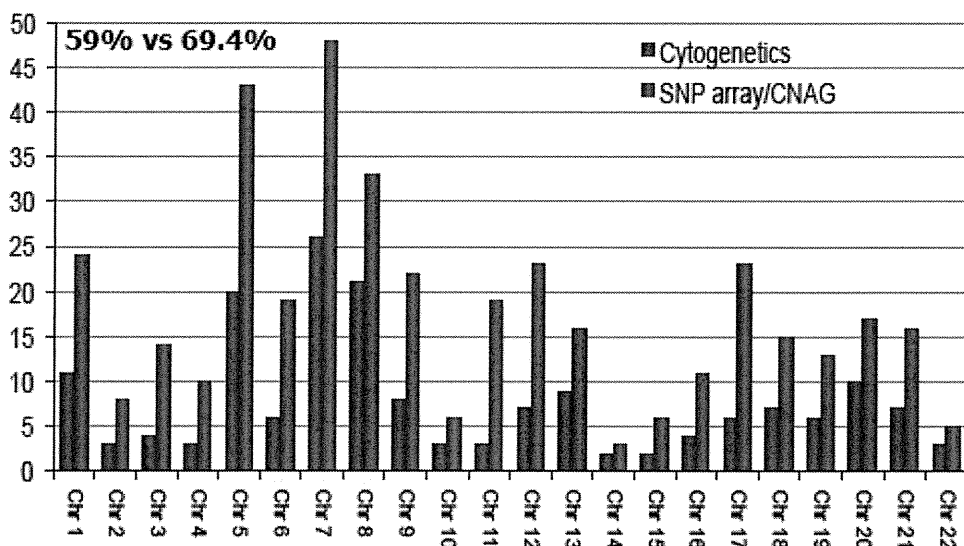


**Figure 8.** Numbers of genetic lesions in MDS and related myeloid neoplasms detected by SNP-A and metaphase karyotyping. The numbers of genetic lesions detected in a cohort of MDS, MDS/MPN, and sAML were compared between SNP-A (red bars) and metaphase karyotyping (blue bars) in each chromosome. The comparison was made among the 173 cases, in which successful metaphase karyotype data had been obtained.

**Table 2.** aUPDs and Their Gene Targets in Hematologic Neoplasms

| Chromosome | Disease(s) | Gene Target(s) | | References |
|---|---|---|---|---|
| 1p13.1 | MDS | Nras | Mutations | 35,50 |
| 1p34 | MPN, RARSt | cMPL | Mutations | 49,64,72 |
| 4q24 | MDS, MPN | TET2 | Mutations | 44 |
| 6q23 | MALT, DLBCL | A20 | Mutation or deletion | 30 |
| 7q35 | MDS, MDS/MPN | EZH2 | Mutations | 65 |
| 9p21 | ALL | CDKN2A | Deletion | 29 |
| 9p24 | MPN | JAK2 | Mutations | 25,46 |
| 11p13 | AML | WT1 | Mutations | 51 |
| 11q23.3 | MDS/MPN | c-CBL | Mutations | 35,50 |
| 13q12 | AML | FLT3 | ITD | 51 |
| 13q14.3 | CLL | miR-15a, miR-16-1 | Deletion | 69 |
| 17p13.1 | AML, MDS | TP53 | Mutations | 35,73 |
| 17q11.2 | JMML | NF1 | Mutations | 74 |
| 19q13.1 | AML | CEBPA | Mutations | 51 |
| 21q22.3 | AML, MDS | RUNX1 | Mutations | 35,51 |

Abbreviations: MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; RARSt, refractory anemia with ring sideroblasts and thrombocytosis; MALT, mucosa-associated lymphoid tissue-derived lymphoma; DLBCL, diffuse large B-cell lymphoma; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; JMML, juvenile myelomonocytic leukemia.

phomas has been confirmed by SNP-A karyotyping, in terms of the distribution of their genetic abnormalities, including copy number gains and losses, as well as aUPD, conferring unique genomic profiles to each lymphoma subtype.[30] For example, gains of both chromosomes 3 and 18, as well as focal deletions at the A20 locus, are a common feature of mucosa-associated lymphoid tissue (MALT)-derived lymphoma, while mantle cell lymphomas show recurrent deletions in the segments of 1p, 10p, and 11q, and gains of 3q, 8q, and 18q.[30] On the other hand, diffuse large B-cell lymphomas and follicular lymphoma show largely similar genomic profiles, including gains/amplifications involving the c-rel locus, and gains of 1q and chromosomes 3, 7, 12, and 18, indicating a common genetic background in both subtypes. aUPD is found in about 80% of follicular center–derived lymohomas and less frequently found in MALT and mantle cell lymphomas.[30,45,66] In follicular center–derived lymphomas, common targets of aUPD include 1p, 1q, 6p, 9p, 12q, and 17q, whereas 6qUPD is characteristic to MALT-type lymphoma.[30,45,66] Similar to aUPD in myeloid cancers, discrete gene targets have been clarified for some aUPDs in lymphomas, including HLA associated with 6pUPD, A20 with 6qUPD, and CDKN2 with 9pUPD, although the genetic targets of common aUPDs in 1p, 12q, and 17q have not been elucidated.[30]

## CHRONIC LYMPHOCYTIC LEUKEMIA AND MULTIPLE MYELOMA

Chronic lymphocytic leukemia (CLL) and multiple myeloma (MM) are also among good indications for

SNP-A karyotyping, because difficulty in obtaining metaphases frequently prevents successful conventional karyotyping. SNP-A analysis can sensitively detect genetic lesions in more than 80% of CLL cases, including frequent homozygous deletions involving the miR15a/miR16–1 locus, as well as gains of chromosome 12 associated with poor prognosis.[67–69] Other common genetic lesions in CLL detected by SNP-A karyotyping include recurrent deletions in 5q, 6q, 11q, and 17p, where the common deletion in 6q and 11q contains AIM1 and ATM, respectively. Because of a high median age of CLL cases, aUPD should be carefully discriminated from IBD alleles. After excluding suspected IBD alleles, aUPD was relatively uncommon, being found in four of 56 cases, which involved 11q, 13q, and 17p.[68]

SNP-A karyotyping also can be applied to MM, but frequent low tumor contents in myeloma specimens may compromise the sensitivity of detecting genetic lesions. To keep the sensitivity of SNP-A karyotyping, enrichment of myeloma cells has been performed by sorting CD138+ fractions.[70] As for the copy number changes, comparative results were obtained between array CGH and SNP-A karyotyping. Common genetic changes detected by SNP-A karyotyping include gains of 1q, 6p, and 11q and whole chromosomes 3, 5, 7, 9, 15, and 19, typically associated with hyperploidy, and deletions in 1p, 8p, and 16q and whole chromosomes 13 and X.[70,71] SNP-A karyotyping showed concordant results with those from FISH experiments in most cases, except for rare tetraploid samples, which were erroneously analyzed as diploid in SNP-A karyotyping.

aUPD is also common in MM with the median number of regions showing aUPD being three.[70]

## CONCLUSION

SNP-A karyotyping represents one of the recent technological advances in the field of cancer genomics. It has enabled high-throughput analysis of genetic lesions in human cancers in terms of copy number alterations and allelic imbalances, unveiling a number of novel genetic targets and mechanisms that are involved in cancer development. Given such high performance of SNP-A karyotyping, it could be potentially applicable to bedside diagnosis and the clinical management of patients. While there exist accumulating observations that suggest diagnostic and/or prognostic values of SNP-A karyotyping, they need to be confirmed through more controlled studies. For example, when evaluating those abnormalities whose clinical values have been well established, SNP-A karyotyping would complement and even outperform metaphase karyotyping. On the other hand, SNP-A karyotyping will identify large numbers of novel genetic lesions whose clinical significance needs to be clarified before their clinical use, which might not always feasible with realistic numbers of cases due to higher heterogeneity these lesions could reveal. Clearly, more works should be required to establish the clinical values of SNP-A karyotyping technologies.

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# MDS における acquired uniparental disomy と *c-CBL* 変異

真 田　昌

骨髄異形成症候群 (Myelodysplastic syndromes, MDS) においては染色体コピー数の欠失や増加などのゲノム異常が約半数の症例で観察され，重要な予後指標となっている[1,2]。これらのゲノム異常が観察される領域には MDS の病態に関連する標的遺伝子が存在すると考えられるが，その多くは同定には至っていない。近年，ゲノム解析技術は急速な進歩を遂げ，多くの腫瘍性疾患で遺伝子病態が明らかになりつつある。本稿では高密度 SNP アレイを用いた MDS の網羅的なゲノム解析と 11 番染色体長腕 aUPD の標的として同定された *c-CBL* 変異について概説する。

## 高密度 SNP アレイを用いたゲノム解析

高密度 SNP アレイは大規模 SNP（一塩基多型，single nucleotide polymorphism）タイピング用に開発されたが，マイクロアレイに搭載された各 SNP プローブのシグナル強度とその領域のコピー数には高い相関がみられることから，ゲノムのコピー数解析に広く応用されている。マイクロアレイ技術の進歩により，非常に多数のプローブを搭載することが可能となっており，染色体分析に比し高解像度なコピー数解析を網羅的に行うことができる。コピー数解析についてはアレイ CGH (comparative genomic hybridization) 法によっても同様の解析結果が得られるが，SNP アレイにおいてはアレル別のコピー数解析が可能であり，網羅的な LOH ( loss of heterozygosity) 解析も同時に行うことができる。我々が開発した CNAG・AsCNAR プログラム[3,4]は，ノイズの軽減と感度の向上が得られ，腫瘍細胞比率が 10〜20％程度しかない検体においてもゲノム異常の同定が可能で

ある。また自己正常対照がない検体においても，他人由来の最適な正常対照プールを自動的に選択させることで，同等の解析結果を得ることも可能である。これらの利点は正常細胞の混入を避けることが困難，かつ自己正常対照検体を得ることも難しい MDS 検体のゲノム解析において非常に有用な点である。染色体分析とは異なり，分裂核が得られない検体においてもゲノム解析が可能である。

## MDS における LOH

LOH は，古典的ながん抑制遺伝子の不活化メカニズムとして知られ[5]，新規がん抑制遺伝子の探索に用いられてきた。MDS においては，5 番染色体長腕 (5q) や 7 番染色体長腕 (7q)，20 番染色体長腕 (20q) などに代表される片アレルの欠失による LOH の最小共通欠失領域から同領域の標的遺伝子探索がなされ，いくつかの候補遺伝子が報告されてきたが，未だ不明な点は多い。高密度 SNP アレイによるゲノム解析では，5q や 7q の欠失など染色体分析で同定される異常の他にも多くのゲノム異常が認められ，比較的狭い領域の欠失の他にも，コピー数の変化を伴わない LOH 領域 (Copy number neutral LOH (CN-LOH)，片親性 2 倍体，Acquired uniparental disomy (aUPD)) が新たに同定される（図 1）。aUPD は，狭義の MDS において 10〜25％程度の症例に観察され，慢性骨髄単球性白血病 (chronic myelomonocytic leukemia, CMML) において，他病型に比し高頻度 (35〜40％) に認められる[6,7]。初期に報告された SNP アレイを用いた MDS のゲノム解析では SNP コールに基づく低感度な LOH 判定にも関わらず，高頻度に aUPD が検出されたとの報告[8]もあるが，両親から同じアレルを引き継いでいる領域，すなわち先天的なホモ接合もカウントされている可能性が指摘されている。MDS クローンのみに生じた後天的な変化かは，自己正
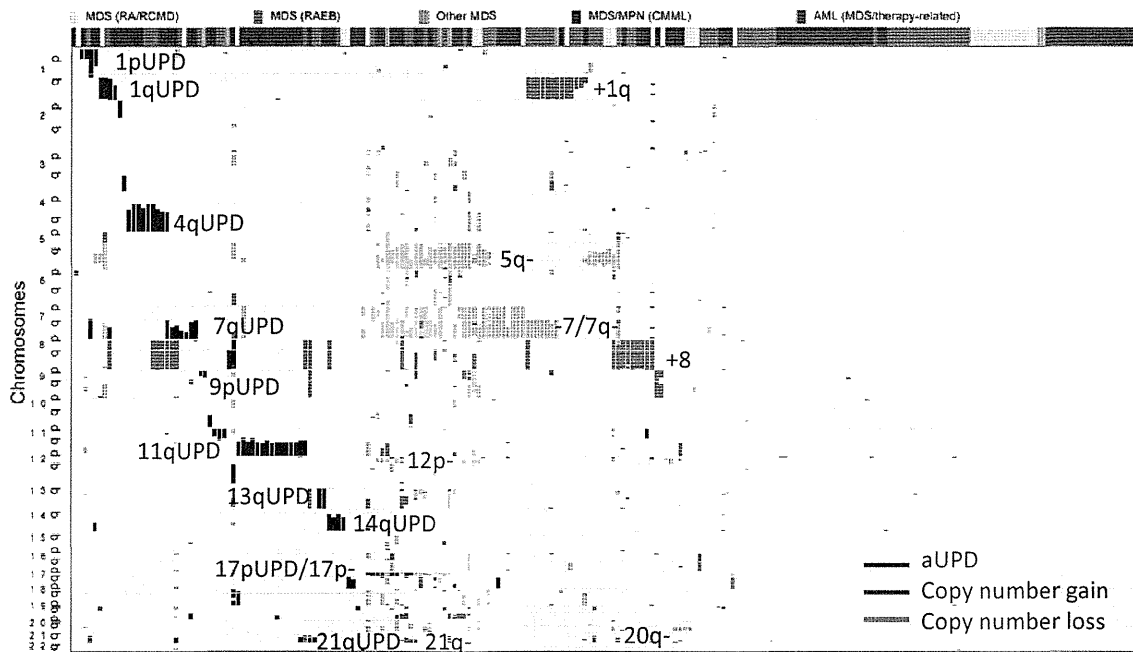
**図 1** SNP アレイによる MDS および類縁疾患のゲノム解析結果（文献 7 を改変）

MDS108 例，CMML86 例，MDS から移行した AML28 例（WHO 分類による）計 222 例の臨床検体を用いた解析結果。縦に 1 番染色体短腕～22 番染色体長腕が順に並び，横に各症例の解析結果を示す。薄灰で表示しているのが欠失領域，濃灰が増加領域，黒が aUPD 領域。MDS に特徴的とされる 5 番染色体長腕，7 番染色体長腕，17 番染色体短腕，20 番染色体長腕の欠失，1 番染色体長腕，8 番染色体の増加などが認められる。aUPD は約 30％症例で観察され，CMML で頻度が高い。4 番染色体長腕，7 番染色体長腕，9 番染色体短腕，11 番染色体長腕，13 番染色体，17 番染色体短腕，21 番染色体長腕 aUPD の標的は，それぞれ TET2, EZH2, JAK2, c-CBL, FLT3, TP53, RUNX1/AML1 変異と考えられる（表 1）。

常細胞と比較した時のみ結論付けることが可能であるが，MDS においては正常細胞の取得は困難である。Heirichs ら[9]は同一の患者から採取した頬粘膜細胞を正常対照に解析を行っているが，SNP アレイで観察されるゲノム変化の一部は，頬粘膜においても観察されており，十分な注意が必要と考えられる。これまでの観察結果から造血器腫瘍における aUPD の多くは，somatic recombination により生じ[10]，染色体断端を含む形で認められる。一方で，コピー数の変化を伴わず，かつ染色体断端を含まない比較的短い領域のホモ接合は正常細胞の解析においてもしばしば観察され，腫瘍細胞特異的に認められることは稀である。また，臨床検体においては正常細胞の混入が少なからずあり，変化したアレルとともに正常細胞由来のアレルも同時に解析されるので，多くの場合は区別をすることが可能である。

## MDS における aUPD

MDS における aUPD は 1 番染色体，4 番染色体長腕（4q），7q，11 番染色体長腕（11q），14 番染色体長腕（14q），17 番染色体短腕（17p），21 番染色体長腕（21q）

等いくつかの染色体領域に集積をする[6,7,11,12]（図 1・表 1）。7q，17p，21q は，欠失による LOH と共通して観察されるが，4q，11q，14q については，MDS において欠失例は稀である。一方，5q や 20q は欠失による LOH がしばしば認められるが，aUPD は稀である。17p，21q に aUPD を有する症例は，欠失例と同様に，それぞれ TP53 および RUNX1 遺伝子変異を高頻度に伴っており，共通した分子病態が存在すると考えられる[7]。一方，7q-aUPD の標的遺伝子変異として 7q36.1 に存在する EZH2 遺伝子変異が報告された[13]が，本変異は aUPD 例においては高頻度に変異が認められるが，7q 欠失例における変異の頻度は稀であり，これまで報告されてきた 7q における最小共通欠失領域には含まれない。5q 欠失については，最近の研究結果からは RPS14 もしくは miR-145 と miR-146a のハプロ不全により MDS 様の病態がマウスモデルで再現されており[14,15]，単一の遺伝子が標的ではないことも推測され，aUPD 例が稀であることとも一致すると考えられる。MDS および類縁疾患において高頻度に認められる aUPD 領域である 11q-aUPD の標的遺伝子として C-CBL 遺伝子変異は同定されたが，

表 1 骨髄系腫瘍における LOH と標的遺伝子変異

| position | Diseases | LOH | Mutated Gene Target |
|---|---|---|---|
| 1p13.1 | MDS | UPD | *Nras* |
| 1p34 | MPN, RARSt | UPD | *cMPL* |
| 4q24 | MDS, MPN | loss/UPD | *TET2* |
| 5q | AML, MDS | loss | |
| 7q35 | MDS, MDS/MPN | UPD | *EZH2* |
| 7q | AML, MDS | loss | |
| 9p24 | MPN | UPD | *JAK2* |
| 11p13 | AML | UPD | *WT1* |
| 11q23.3 | MDS/MPN | UPD | *c-CBL* |
| 13q12 | AML | UPD | *FLT3* |
| 14q | MDS, MDS/MPN | UPD | |
| 17p13.1 | AML, MDS | loss/UPD | *TP53* |
| 17q11.2 | JMML | UPD | *NF1* |
| 19q13.1 | AML | UPD | *CEBPA* |
| 20q | MDS, MPN | loss | |
| 21q22.3 | AML, MDS | loss/UPD | *RUNX1* |

*C-CBL* 変異は 11q-aUPD 例では高頻度に観察されるのに対し，非 aUPD 例においては頻度が低く，11q-aUPD と強い相関が認められる[7,16,17]。4q-aUPD の標的遺伝子として同定された *TET2* 変異[12,18]や 7q-aUPD の標的である *EZH2* 変異[13,19]は，aUPD が認められない症例においても，しばしば観察されるのとは対照的である。

## c-CBL

*c-CBL* は，マウスにリンパ腫や白血病を発症させる Casitas-NS-リンパ腫ウィルスから単離された v-Cbl の相同遺伝子として同定された。c-CBL は，主として RING 型 E3 ユビキチンリガーゼとして作用することが知られている[20,21]。ユビキチン化は細胞内のタンパク質の分解・機能制御において重要な翻訳後修飾であるが，c-CBL は，サイトカインなどのリガンド刺激で活性化された EGFR や FLT3，KIT などのチロシンキナーゼに TKB ドメインで結合し，E2 ユビキチン結合酵素を介し

てチロシンキナーゼをユビキチン化する。ユビキチン化されたチロシンキナーゼはプロテオソームなどで速やかに分解され，チロシンキナーゼを介したシグナルは終息する，すなわち c-CBL は同シグナルの重要な負の調整分子として機能すると考えられている。また c-CBL はチロシンキナーゼのみならず非常に多くのシグナル伝達分子と結合することが知られており，負の制御のみならずシグナル伝達においても重要な役割を担っていると推測される[20,21]。

## 骨髄系腫瘍における *c-CBL* 変異

急性骨髄性白血病 AML で *c-CBL* 変異例の報告がされ[22,23]，続いて MDS[7,16]，骨髄増殖性疾患 MPN[17]での報告もされているが，変異例の多くは MDS/MPN (myelodysplastic/myeloproliferative neoplasms) 例である。MDS/MPN は，FAB 分類においては MDS の一亜型として扱われてきたが，白血球増多や肝脾腫を認めら
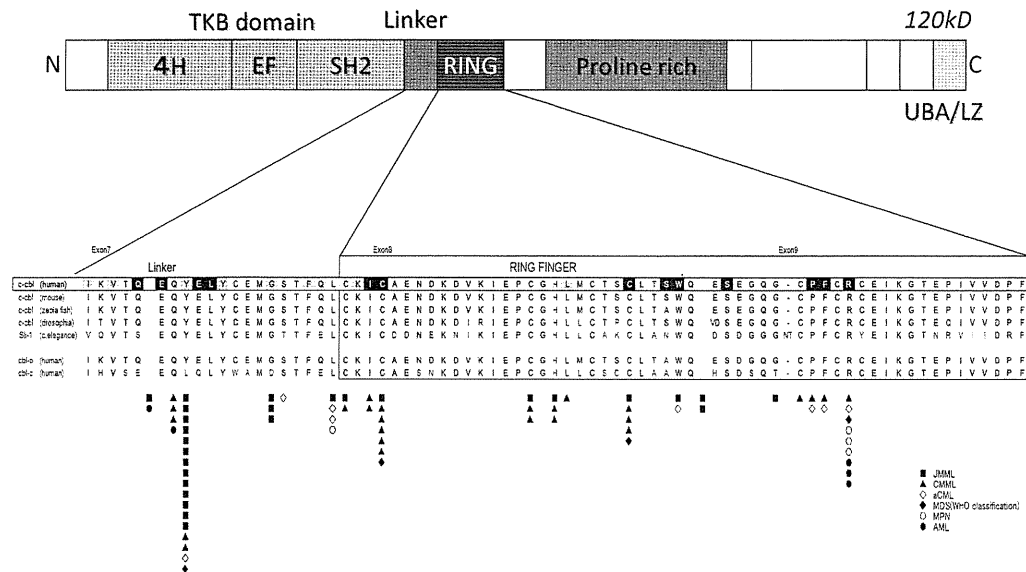
TKB domain　　　Linker　　　　　　　　　　　　　　　　*120kD*

N　| 4H | EF | SH2 | | RING | Proline rich | | | | |C

UBA/LZ

Exon7　　　　　　　　　　　Exon8　　　　　　　　　　　　　　　Exon9

Linker　　　　　　　　　　　　　　　　　　　RING FINGER

| | | | |
|---|---|---|---|
| c-cbl (human) | IKVTQ EQYELYCEMGSTFQL | CKICAENDKDVKIEPCGHLMCTSCLTSWQ | ESEGQG-CPFCRCEIKGTEPIVVDPF |
| c-cbl (mouse) | IKVTQ EQYELYCEMGSTFQL | CKICAENDKDVKIEPCGHLMCTSCLTSWQ | ESEGQG-CPFCRCEIKGTEPIVVDPF |
| c-cbl (zebra fish) | IKVTQ EQYELYCEMGSTFQL | CKICAENDKDVKIEPCGHLMCTSCLTAWQ | ESEGQG-CPFCRCEIKGTEPIVVDPF |
| c-cbl (drosophila) | ITVTQ EQYELYCEMGSTFQL | CKICAENDKDIRIEPCGHLLCTPCLTSWQ | VDSEGQG-CPFCRCEIKGTECIVVDPF |
| Sli-1 (c.elegance) | VQVTS EQYELYCEMGTTFEL | CKICCDNEKNIKIEPCGHLLCAKCLANWQ | DSDGGGNTCPFCRYEIKGTNRV·!DRF |
| cbl-b (human) | IKVTQ EQYELYCEMGSTFQL | CKICAENDKDVKIEPCGHLMCTSCLTAWQ | ESDGQG-CPFCRCEIKGTEPIVVDPF |
| cbl-c (human) | IHVSE EQLQLYWAMDSTFEL | CKICAESNKDVKIEPCGHLLCSCCLAAWQ | HSDSQT-CPFCRCEIKGTEPIVVDPF |

■ JMML
▲ CMML
◇ aCML
◆ MDS(WHO classification)
○ MPN
● AML

**図2　c-CBL の構造と骨髄系腫瘍で観察される CBL 変異**
c-CBL は，TKB ドメイン，リンカー，RING フィンガー・ドメイン，Proline-Rich ドメイン，UBA/LZ ドメインなどからなる。骨髄系腫瘍で同定された c-CBL 変異は，リンカーから RING フィンガー・ドメインにかけて存在する。変異の多くは，E2 ユビキチン結合酵素である UbcH7 との結合に重要とされるアミノ酸（黒・白抜き）や TKB ドメインとの立体構造形成上重要なアミノ酸（灰）に生じており，これらのアミノ酸は種を越えて保存されている。リンカーから RING フィンガーにかけての領域の欠失変異も報告されている。

れる等，骨髄増殖性疾患（myeloproliferative neoplasms, MPN）としての特徴も有していることから，WHO 分類から MDS/MPN として狭義の MDS から独立した疾患群である[24]。これまでの報告では，若年性骨髄単球性白血病（juvenile myelomonocytic leukemia, JMML）の10～17%[25, 26]，CMML の 5～25%[7, 16, 27]，atypical chronic myeloid leukemia の 5%程度[17]に c-CBL 変異は認められている。JMML においては，腫瘍細胞特異的な後天的変異のみならず，胚細胞変異例も報告されている[28~30]。Noonan 症候群は JMML の合併が認められる先天性疾患であるが，これまでに原因遺伝子と知られている *PTPN11*，*SOS1*，*RAS* などの既知の遺伝子変異を有さない Noonan 様の表現型を呈する症例の一部に *c-CBL* の胚細胞変異例があることが明らかとなっている。また *c-CBL* 変異を有する家系も報告され[28]，遺伝的に *c-CBL* 変異アレルをヘテロに有する者は心血管系の異常などを示すとともに，しばしば JMML を併発する。*c-CBL* 変異による家系内発症の JMML においても，腫瘍細胞ではしばしば 11q-aUPD によりホモ変異となるが，家系内発症例では時として自然寛解が認められることなどが特徴的である。CMML では，*RUNX1/AML1* 変異や *TET2* 変異，*JAK2V617F* 変異を伴っている症例も観察される[7, 27]。JMML においては，*RAS* や *PTPN11*

など RAS 経路の変異とは排他的に認められるとされるが[25, 26]，CMML においては *RAS* 変異との重複例も報告されている。

これまでに骨髄系腫瘍で報告されている *c-CBL* 変異は，E3 ユビキチンリガーゼとしての機能上重要である E2 との結合部位にあたるリンカーから RING フィンガー・ドメインに集積をしており（図2），その多くは生物種を越えて高度に保存されているアミノ酸に生じている点突然変異である。JMML における変異の大半はリンカードメインに存在する 371 番目のチロシンのアミノ酸置換であり[25, 26]，AML 例などでは，この領域内の部分欠失例も報告されている[7, 17, 31]。

## 変異 c-CBL の分子機構

臨床検体で認められた変異 *c-CBL* をマウスの上皮系細胞株である NIH3T3 細胞に遺伝子導入すると細胞は腫瘍化し[7, 32]，変異 c-CBL は transforming 能を有すると考えられる。またマウスの造血幹細胞に変異体を導入し，移植実験を行うと，移植されたマウスは MPN や全身性の肥満細胞腫を発症する[33]。その一方で *c-Cbl* を欠失させた遺伝子改変マウスは，慢性骨髄性白血病のモデルマウスである *BCR/ABL* 遺伝子導入マウスと交配すると欠失マウスでは急性転化が促進される。また欠失マウ
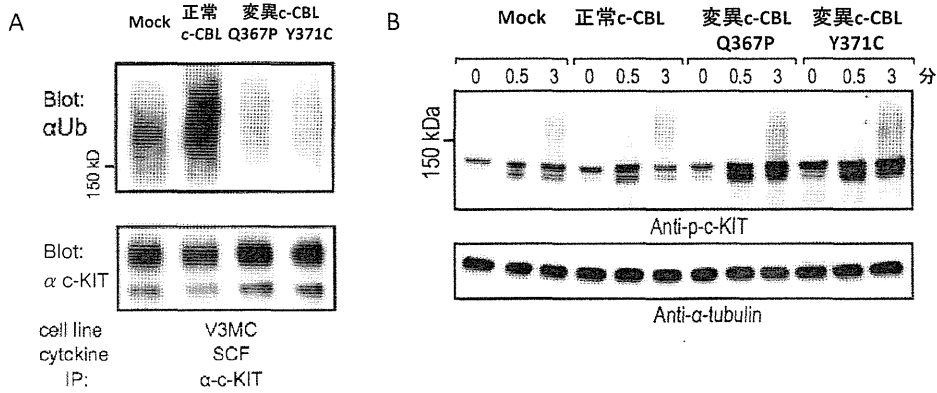
図3　変異 c-CBL とチロシンキナーゼのユビキチン化（文献 7 を改変）
　　　変異 c-CBL をマウスの細胞株 V3MC（肥満細胞・c-Kit 発現細胞）に導入し，SCF 刺激を行うと，野生型 c-CBL 導入細胞では，速やかにユビキチン化を受けるが，変異体導入細胞ではユビキチン化が観察されない（A）。野生型 c-CBL 導入細胞では，SCF 刺激 3 分後にはリン酸化 Kit の発現量が低下するのに対し，変異体導入株では Kit のリン酸化状態の遷延が観察される。

スを長期に観察すると，すべての個体に特徴的な悪性腫瘍を発症することから，c-Cbl は，個体レベルではがん抑制遺伝子として機能していると考えられる[7]。すなわち，本変異においては，変異により c-CBL のがん抑制遺伝子としての機能が失われるのみならず，より積極的に腫瘍化へ導いていることが考えられる。

　骨髄系腫瘍で観察される c-CBL 変異はユビキチンリガーゼとしての機能上重要なドメインに集積をするが，細胞株を用いた機能解析において，変異 c-CBL はユビキチンリガーゼとしての機能を喪失し（図 3A）[7, 17, 22]，変異体は正常 c-CBL のユビキチンリガーゼ作用を阻害する。その結果，変異 c-CBL 導入細胞において，サイトカイン刺激で活性化（リン酸化）されたチロシンキナーゼはユビキチン化を受けずに，リン酸化状態が遷延することが観察される（図 3B）。マウス造血前駆細胞（Lin−/Sca-1＋/c-Kit＋）に変異 c-CBL 導入すると，SCF（幹細胞因子）やトロンボポイエチンなどの広範な造血サイトカイン刺激に対し高感受性となり，過剰増殖が in-vitro で観察される。すなわち，サイトカイン刺激により活性化されたチロシンキナーゼがユビキチン化されないために，分解等を受けずに，細胞増殖シグナルが入り続けている効果と推測される。この現象は c-Cbl 欠失マウスの造血前駆細胞においても観察され，実際，c-Cbl 欠失マウスでは脾腫や造血前駆細胞の増加が個体レベルでも観察される[7, 34, 35]。変異 c-CBL 導入効果が単なるユビキチンリガーゼ機能の欠失や正常 c-CBL に対する阻害効果だけであれば，c-CBL が既に欠失した細胞には何の効果も示さないはずであるが，c-Cbl 欠失マウス由来の細胞に変異 c-CBL を導入すると，より顕著に観

察される（図 4A）[7]。この結果から，変異 c-CBL は機能欠失変異であるとともに機能獲得型変異としても作用していると考えられる。このような現象は，代表的ながん抑制遺伝子である p53 における一部の変異においても観察され，p53 の相同遺伝子である p63，p73 に対する阻害効果で説明がされている[36, 37]。c-CBL においても，CBL-B，CBL-C という相同性の高い分子が存在し[20]，こと CBL-B は血球系の細胞においても広く発現しているとされ，CBL-B に対する阻害効果で説明が可能かもしれない。実際，細胞株を用いた導入実験では変異 c-CBL は CBL-B のユビキチンリガーゼ機能も阻害することが確認されている[7]。また c-Cbl と Cbl-b の両者を欠失させたマウスは胎生致死であるが，骨髄でのみ条件的に両遺伝子を欠失させたマウスにおいては，重篤な骨髄増殖性疾患を発症することが報告された[38]。これらの観察結果は c-CBL 変異体による CBL-B に対する阻害効果が c-CBL 変異例における骨髄増殖性病態に関わっている可能性を支持する。

　c-Cbl 欠失造血前駆細胞で観察される変異 c-CBL 導入によるサイトカイン高感受性は，正常 c-CBL との共導入により消失する（図 4B）[7]。この実験結果は，本異常においては変異が生ずるのみならず，正常 c-CBL アレルが失われることも重要であることが示唆される。骨髄系腫瘍において観察される c-CBL 変異例の多くがヘテロ変異ではなく，11q-aUPD により正常 c-CBL アレルを欠失していることとも一致し，発がんにおける aUPD の寄与を考える上でも興味深いと思われる。
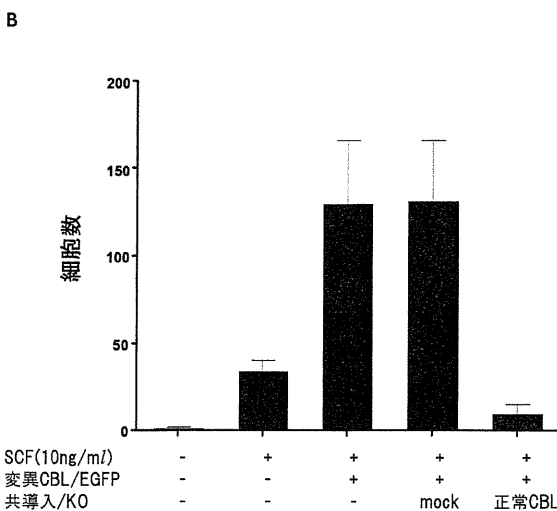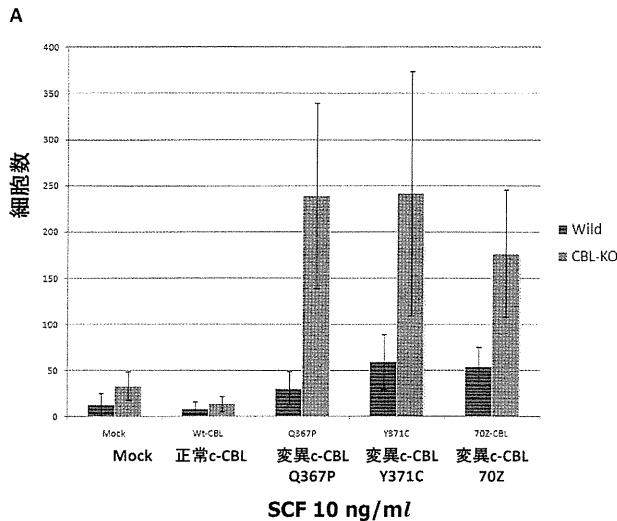
A



SCF 10 ng/ml

Mock 正常c-CBL 変異c-CBL Q367P 変異c-CBL Y371C 変異c-CBL 70Z

■ Wild
▨ CBL-KO

B



| SCF(10ng/ml) | - | + | + | + | + |
| 変異CBL/EGFP | - | - | + | + | + |
| 共導入/KO | - | - | - | mock | 正常CBL |

**図 4** 変異 c-CBL によるサイトカイン高感受性（文献 7 を改変）
マウスの造血前駆細胞（Lin-/Sca-1＋/c-Kit＋）に正常
または変異 CBL をレトロウィルスにより遺伝子導入
し，サイトカインに対する感受性を細胞数で評価し
た。（A）c-Cbl 欠失（CBL-KO）マウスの造血前駆細胞
は，SCF（幹細胞因子）刺激に対し，野生型（Wild）
マウスに比して高感受性となり過剰増殖をする。変異
c-CBL 導入によっても過剰増殖は観察されるが，この
効果は CBL-KO マウス由来の細胞おいてより顕著とな
る。同様の効果はトロンボポエチン，IL3，FLT3 リガ
ンド刺激においても認められる。（B）変異 c-CBL 導
入により観察される c-Cbl 欠失造血前駆細胞のサイト
カイン高感受性は正常 c-CBL との共導入により消失す
る。

## おわりに

これまでの知見から，c-CBL 変異を有する骨髄系腫瘍
では，チロシンキナーゼの負の制御因子である c-CBL
に変異が生じることにより，チロシンキナーゼを介した

A



正常細胞

Ligand

Degradation in
lysosome/
proteosome
⇒recycling

Signal

B

Ligand

腫瘍細胞

変異CBL

CBL-b

Prolonged transmission
of positive signal

Signal
Cell survival/ proliferation
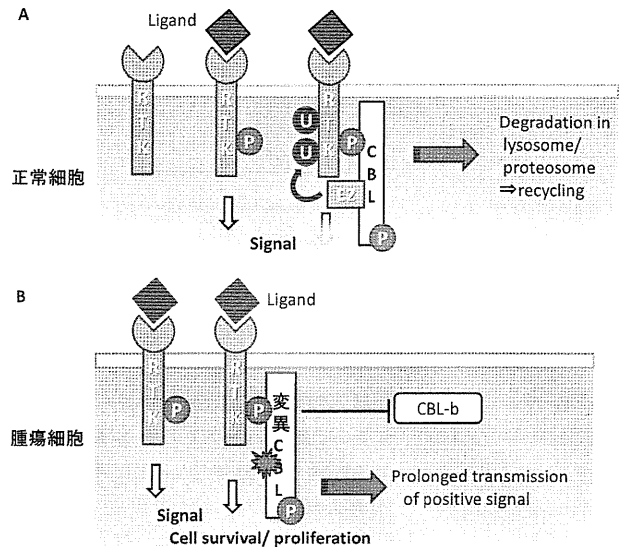
**図 5** CBL とチロシンキナーゼ・シグナル
（A）正常 c-CBL は，サイトカインなどのリガンド刺
激で活性化されたチロシンキナーゼ（RTK）に TKB
ドメインで結合し，E2 ユビキチン結合酵素を介して
チロシンキナーゼをユビキチン化する。ユビキチン化
されたチロシンキナーゼ・c-CBL 複合体はプロテオ
ソームやライソゾームで分解され，チロシンキナーゼ
を介したシグナルの重要な負の調整分子として機能す
る。（B）チロシンキナーゼの負の制御因子である c-
CBL に変異が生じることにより，チロシンキナーゼ
はユビキチン化されずに活性化（リン酸化）状態が遷
延し，シグナルが入り続けると考えられる。その結果
として，CMML や JMML で認められる異常な血球増
多が生じると推測される。変異 c-CBL はユビキチンリ
ガーゼとしての機能を喪失するのみならず，CBL-b の
ユビキチンリガーゼ能をも阻害していると考えられ
る。

造血シグナルが入り続け，骨髄増殖性の病態が生じてい
ると推測される（図 5）。発がんとチロシンキナーゼの
異常の関連は，MPN や CML 以外にも，肺がん・乳が
ん・脳腫瘍・神経芽細胞種など幅広い腫瘍性疾患で示さ
れ，近年，多くのチロシンキナーゼ阻害剤が開発，臨床
応用されている[39]。c-CBL 変異を有する骨髄系腫瘍にお
ける異常な血球増殖に対しても，チロシンキナーゼ・シ
グナルの制御が治療標的として有効であることが期待さ
れる。

SNP アレイによる網羅的なゲノム解析により同定さ
れた LOH/aUPD から骨髄系腫瘍における c-CBL，
TET2，EZH2 変異が明らかとなったが，SNP アレイに
よる解析でも全く異常が観察されない症例が，典型的な
MDS 例においても少なからず存在する。リシークエン
ス技術を代表とするゲノム解析技術は，ここ数年で革新
的な進歩を遂げており，MDS 研究においても網羅的な

変異解析などを通じて，分子病態の理解が進み，臨床応用可能な治療標的分子が明らかとなることが期待される。

## 謝　辞

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# A High Occurrence of Acquisition and/or Expansion of C-CBL Mutant Clones in the Progression of High-Risk Myelodysplastic Syndrome to Acute Myeloid Leukemia[1,2]

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## Abstract

The molecular pathogenesis of myelodysplastic syndrome (MDS) and its progression to secondary acute myeloid leukemia (sAML) remain to be explored. Somatic C-CBL mutations were recently described in MDS. Our study aimed to determine the role of C-CBL mutations in the progression of MDS to sAML and sought to correlate with clinicohematological features and outcome. Bone marrow samples from 51 patients with high-risk MDS (13 with refractory cytopenia with multilineage dysplasia, 19 with refractory anemia with excess blast 1, and 19 with refractory anemia with excess blast 2) were analyzed for C-CBL mutations at both diagnosis and sAML in the same individuals. Mutational analysis was performed for exons 7 to 9 of C-CBL gene. Of the 51 paired samples, C-CBL mutations were identified in 6 patients at the sAML phase. One patient retained the identical C-CBL mutation (G415S) at sAML evolution and exhibited clonal expansion. The other five patients acquired C-CBL mutations (Y371S, F418S, L370_Y371 ins L, L399V, and C416W) during sAML evolution. Three of the six patients harboring C-CBL mutations at sAML had additional gene mutations including $JAK2^{V617F}$, PTPN11, or N-RAS. There was no significant difference in clinicohematological features and overall survival with respect to C-CBL mutation status. Our results show that C-CBL mutation is very rare (0.6%) in MDS, but acquisition and/or expansion of C-CBL mutant clones occur in 11.8% of patients during sAML transformation. The findings suggest that C-CBL mutations play a role at least in part in a subset of MDS patients during sAML transformation.

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## Introduction

Myelodysplastic syndromes (MDSs) are hematological malignancies characterized by ineffective hematopoiesis and a high-risk transformation to secondary acute myeloid leukemia (sAML) [1]. MDSs are clonal hemopathies associated with acquired genetic aberrations. We and others have shown that genetic or epigenetic abnormalities might arise during MDS evolution or its progression to sAML [2,3]. Efforts have been made to determine the molecular pathogenesis of the progression of MDS to sAML as well as their clinical impact.

Human C-CBL gene locates on chromosome 11q23.3 and encodes a protein that contains several functional domains, including a tyrosine kinase (TK)–binding domain, a RING finger (RF) domain, a conserved Linker region between the TK-binding domain and RF in the

N-terminal portion, and a C-terminal domain with ubiquitin ligase activity [4]. The C-CBL protein has E3 ubiquitin ligase activity and is responsible for the negative regulation of activated TKs [5,6]. The importance of *C-CBL* gene in hematopoiesis has been demonstrated by knockout mice that showed prolonged activation of TKs, enhanced sensitivity to hematopoietic growth factors, expanded hematopoietic stem cell pool, and myeloproliferative features [7–10].

Loss of heterozygosity could arise either by uniparental disomy (UPD), which represent the coexistence of duplication of an entire or partial chromosome from single parent and loss of the other allele, or by hemizygous deletion. Application of single-nucleotide polymorphism (SNP) microarrays has facilitated the identifications of novel mutated tumor suppressor genes or oncogenes with loss of normal alleles [11,12]. Recently, the detection of 11q acquired UPD (aUPD) has led to the identification of *C-CBL* mutations in various myeloproliferative neoplasm or MDS subtypes [9,13–16], particularly in chronic myelomonocytic leukemia (CMML) with a frequency of 5% to 25% [9,13,17–19]. We have further demonstrated that *C-CBL* mutations result in a gain-of-function mutation if a tumor suppressor associated with 11q aUPD, which is a novel leukemogenic mechanism in a subset of CMML [9]. Of the previous studies, *C-CBL* mutations were mostly analyzed on samples either at initial diagnosis or at the time of sAML transformation. Two studies had examined paired MDS and sAML samples; however, only one case each were included in their studies [14,15]. The impact of *C-CBL* mutations on outcome of patients with MDS and their role in the progression to sAML remain to be defined. In this study, we analyzed a large cohort of matched paired bone marrow (BM) samples from 51 patients with *de novo* high-risk MDS and its corresponding sAML to determine the frequency and characters of *C-CBL* mutations at both phases of disease. The mutation status of the *C-CBL* gene at either the diagnosis of MDS or sAML was correlated with the clinicohematological features and outcome to determine its clinical and prognostic relevance.

## Design and Methods

### Patients and Materials

Between 1991 and 2010, 167 patients with the diagnosis of high-risk *de novo* MDS, including refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blast 1 (RAEB-1), and refractory anemia with excess blast 2 (RAEB-2) were followed up to observe the evolution of sAML. The morphologic subtypes of MDS were classified according to the World Health Organization's classification [20]. Patients with CMML, refractory cytopenias with unilineage dysplasia, refractory anemia with ring sideroblasts, MDS-unclassified, MDS associated with isolated del(5q), and therapy-related MDS were excluded. The cytogenetic findings according to the International Prognostic Scoring System (IPSS) were available in 138 patients and were divided into three groups: 1) good = normal, -Y, del(5q), del(20q); 2) poor = complex or chromosome 7 abnormalities; and 3) intermediate = other abnormalities [21]. Eighty-six of 167 patients progressed to sAML, of which 51 patients (13 RCMD, 19 RAEB-1, and 19 RAEB-2) had matched paired BM samples at both MDS and sAML phases available for comparative analysis. They formed the basis of this study. Forty-two patients (82.4%) in MDS phase received supportive care only, five were treated with oral chemotherapy (hydroxyurea or melphalan), one with low-dose cytarabine, and three with standard AML protocol. Of the 51 patients at sAML phase, 15 were treated with AML protocol, 2 proceeded to allogeneic hematopoietic stem cell trans-

plantation, 12 received low-dose cytarabine, 9 received oral chemotherapy, and 15 had supportive care only. The study was approved by the institutional review boards of Chang Gung Memorial Hospital, Taiwan, and the University of Tokyo, Japan.

### Cell Fractionation

The mononuclear cells were obtained from BM samples by Ficoll-Hypaque density gradient centrifugation (1.077 g/ml; Amersham Pharmacia, Buckinghamshire, United Kingdom). The BM mononuclear cells were cryopreserved in medium containing 10% dimethylsulfoxide and 20% fetal bovine serum at -70°C or in liquid nitrogen until test.

### DNA, RNA Extraction, and Complementary DNA Preparation

Genomic DNA (gDNA) and RNA were extracted from frozen BM mononuclear cells. RNA was reversely transcribed to complementary DNA (cDNA) with the SuperScript II RNase H2 Reverse Transcriptase Kit (Invitrogen Corporation, Carlsbad, CA) as described previously [22].

### C-CBL Mutation Analysis

cDNA polymerase chain reaction (PCR) assay was performed as described previously [23]. For patients with available RNA samples, the cDNA PCR products were either subjected to direct sequencing and/or screened by denaturing high-performance liquid chromatography (DHPLC; WAVE Transgenomic, Omaha, NE) system [24]. In the DHPLC assay system, we always ran a control of the patient's sample mixed with 50% wild-type DNA to distinguish homozygous mutations from wild-type. The abnormal DHPLC profiles that suggested the presence of mutations were then sequenced. For patients without RNA samples available, mutations at exon 8 were all examined by direct sequencing of gDNA PCR products, whereas mutations at exons 7 and 9 were analyzed by either direct sequencing as previously described [9] or with DHPLC system followed by sequencing for abnormal profiles obtained. The primer sequences for cDNA PCR and DHPLC analysis are listed in Tables W1 and W2. All the mutations detected were confirmed by using alternative samples and/or primers and subjected to PCR assays with sequencing again.

### Detection of Additional Gene Mutations

Mutational analysis of *FLT3*-ITD, *FLT3*-TKD, codons 12, 13, and 61 of N-*RAS* and K-*RAS*, C-*KIT*, and C-*FMS* (*CSF1R*) genes were performed as described previously [3,25–27]. *JAK2*$^{V617F}$ and *PTPN11* were analyzed according to the methods described by Baxter et al. [28] and Tartaglia et al. [29], respectively.

### SNP Microarray Analysis

High-density SNP array combined with CNAG (Copy Number Analyzed for Affymetrix GeneChip Mapping)/AsCNAR (allele-specific copy number analysis using anonymous references) software analysis was performed using Affymetrix GeneChip 50K *Xba*I, *Hind*III, or 250K *Nsp*I as described before [9], in four patients at sAML phase in which *C-CBL* mutations were detected.

### Statistical Analysis

Fisher exact test, $\chi^2$ analysis, and Wilcoxon rank sum test were used whenever appropriate to make comparisons between groups. Estimates