the Cancer Genomics Consortium (CGC) Myeloid Neoplasms Working Group

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Highlights:

- Genome-wide assessment for copy number aberrations (CNAs) and copy-neutral loss-of-heterozygosity (CN-LOH) allows better diagnostic precision, detects prognostic markers and informs treatment decisions for acute myeloid leukemia (AML).
- Chromosomal microarray (CMA) currently represents a clinically applicable and widely available assay that allows genome-wide assessment for CNAs and CN-LOH with increased detection rate in AML patients compared with conventional cytogenetic testing including karyotype and Fluorescence *in situ* hybridization (FISH).
- Evidence from published research and clinical studies supports the use of CMA testing for patients with AML negative for cytogenetic and molecular high-risk markers (intermediate risk), AML with unobtainable or inadequate cytogenetic results, AML with unusual morphologic and immunophenotypic features, and refractory and relapsed AML.

Abstract

Structural genomic abnormalities, including balanced chromosomal rearrangements, copy number gains and losses and copy-neutral loss-of-heterozygosity (CN-LOH) represent an important category of diagnostic, prognostic and therapeutic markers in acute myeloid leukemia (AML). Genome-wide evaluation for copy number aberrations (CNAs) is at present performed by karyotype analysis which has low resolution and is unobtainable in a subset of cases. Furthermore, examination for possible CN-LOH in leukemia cells is at present not routinely performed in the clinical setting. Chromosomal microarray (CMA) analysis is a widely available assay for CNAs and CN-LOH in diagnostic laboratories, but there are currently no guidelines how to best incorporate this technology into clinical testing algorithms for neoplastic diseases including AML. The Cancer Genomics Consortium Working Group for Myeloid Neoplasms performed an extensive review of peer-reviewed publications focused on CMA analysis in AML. Here we summarize evidence regarding clinical utility of CMA analysis in AML extracted from published data, and provide recommendations for optimal utilization of CMA testing in the diagnostic workup. In addition, we provide a list of CNAs and CN-LOH regions which have documented clinical significance in diagnosis, prognosis and treatment decisions in AML.

1. Introduction/background

Significance of diagnostic, prognostic and predictive genetic markers in AML

Acute myeloid leukemia (AML) encompasses a heterogeneous group of hematopoietic neoplasms involving precursor cells which are committed to myeloid development and differentiation into granulocytic, monocytic, erythroid or megakaryocytic elements [1]. AML is the most common acute leukemia in adults, with an incidence of 3 to 5 cases per 100,000 individuals, but it accounts for less than 10 percent of acute leukemia in children under 10 years of age [2, 3].

A definitive diagnosis of AML is made based on combined results of morphologic, immunophenotypic, cytogenetic and molecular studies, which should be performed in every case to obtain information necessary for accurate subclassification of the disease [4]. It has been recognized for decades that specific cytogenetic abnormalities in AML closely, and sometimes pathognomonically, correlate with morphologically and clinically distinct subsets of the disease [5]. More recently, a similar correlation with specific pathologic and clinical features of AML has been recognized for sequence abnormalities (molecular mutations) in certain genes. The 2017 WHO categorization recognizes nine chromosomal and molecular abnormalities that define specific AML subtypes: t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), inv(16)(p13.1q22) or t(16;16)(p13.1;q22) (*CBFB-MYH11*), acute promyelocytic leukemia (APL) with *PML-RARA*, t(9;11)(p21.3;q23.3) (*MLLT3-KMT2A*), t(6;9)(p23;q34.1) (*DEK-NUP214*), inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) (*GATA2,MECOM*), t(1;22)(p13.3;q13.3) (*RBM15-MKL1*), *BCR-ABL1*, mutated *NPM1*, bi-allelic mutation of *CEBPA* and mutated *RUNX1*. The nine subtypes defined by specific chromosomal and molecular abnormalities account for approximately 20 to 30 percent of AML cases [1, 6].

In addition to their use in disease classification, cytogenetic abnormalities and molecular mutations are critical prognostic markers in AML [7]. Broadly accepted prognostic classification systems based on genetic abnormalities were put forward by the National Comprehensive Cancer Network (NCCN) and European LeukemiaNet (ELN), and both risk stratification schemes integrate similar cytogenetic and molecular features to divide AML into favorable, intermediate and poor risk prognostic groups [8, 9].

With new information on the genomic landscape of AML and the continual identification of potential therapeutic targets, the understanding of the prognostic implications of specific cytogenetic and molecular findings is also rapidly evolving. While it is likely that prognostic categorization of AML will become even more complex in the future, it will continue to center on genetic abnormalities in leukemic cells, with different prognostic subgroups defined based on combinations of chromosomal aberrations (including balanced rearrangements, gains, losses, and amplifications), molecular mono and bi-allelic mutations and over/under-expression of different genes. Routine genetic profiling on all newly diagnosed AML patients is currently standard of care, and its importance will likely continue to increase in the future.

Current testing methods for identification of genetic markers in AML

Conventional karyotype analysis remains mandatory in the evaluation of suspected AML, and its critical role is highlighted in recent practice guidelines by professional organizations and expert groups [4, 9]. Using standard banding techniques, an abnormal karyotype can be detected in 50 to 60 percent of patients with *de novo* AML [10], and conventional karyotype analysis remains the most broadly available and clinically applicable genome-wide test for detecting both numerical and structural chromosomal abnormalities. Cell-based analysis makes karyotyping suitable for detecting clonal heterogeneity and evolution, and for detecting abnormal clones in the presence of normal dividing bone marrow cells. However, karyotype analysis has limitations (including low resolution, the requirement for viable cells, reliance on the ability of leukemic cells to divide in culture, need for highly trained and experienced laboratory staff and limited possibilities for automation), which continue to drive development and implementation of complementary testing methodologies.

Fluorescence *in situ* hybridization (FISH) is a valuable targeted assay for detecting diagnostically and/or prognostically important abnormalities in AML, such as *RUNX1-RUNX1T1*, *CBFB-MYH11*, *KMT2A* (*MLL*) rearrangements, *NUP98-NSD1* and loss of chromosome 5q, 7q, or 17p material. In the clinical setting, G-banded karyotype analysis is the test of choice at the time of diagnosis; however, if karyotype is normal or unobtainable, FISH can be used as a more sensitive, higher resolution method independent on availability of dividing cells, to evaluate for specific AML-associated abnormalities and thus support

the clinical and morphologic diagnosis. FISH testing for abnormalities detected at diagnosis can also be helpful during follow-up, to evaluate for residual disease or detect relapse.

Growing recognition of the diagnostic and prognostic importance of specific molecular mutations in AML resulted in a standard practice to combine cytogenetic results with targeted testing for mutations in *FLT3, NPM1, CEBPA,* and *KIT* in order to determine the diagnostic and prognostic subgroup of the disease. Based on the recent updates in WHO classification and risk stratifications guidelines, routine testing for molecular mutations should likely also include those in *RUNX1, TP53* and *ASXL1*. Finally, development of targeting agents that show efficacy in AML cases with *IDH1*, and *IDH2* mutations may soon mandate inclusion of these genetic markers into the first line testing for AML. Rather than performing individual assays for mutations in clinically important genes, diagnostic laboratories increasingly offer simultaneous evaluation of multiple AML-associated genes using next-generation sequencing (NGS) technologies.

In addition to chromosomal abnormalities resulting in abnormal gene fusions, specific copy number aberrations (CNAs) (-5/5q-, -7/7q-, del(17p)) are well recognized as important prognostic markers in AML [8, 9]. Furthermore, the prognostic relevance of copy neutral loss of heterozygosity (CN-LOH) involving specific chromosomes and chromosomal regions has also been documented [11]. Genome-wide evaluation for CNAs) is at present performed in AML by conventional karyotype analysis with all its well-known limitations, while CN-LOH is not evaluated routinely by any clinically utilized testing methodology. Chromosomal microarray (CMA) analysis allows non-targeted (genome-wide) detection of CNAs with very high resolution; additionally, many clinically used CMA platforms include single nucleotide polymorphism (SNP) probes and can also detect CN-LOH [12]. CMA analysis is used as a routine diagnostic test for constitutional CNAs [13] and is emerging as a preferred assay for formalin fixed paraffin embedded samples from brain tumors and melanomas [14-20], however, it is still not incorporated into standard testing algorithms for the majority of neoplastic diseases including AML. Recently updated ENL Recommendations for Diagnosis and Management of AML in Adults [9] and College of American Pathologists (CAP) and American Society of Hematology (ASH) Guidelines for Initial Diagnostic Workup of Acute Leukemia [4] do not discuss the role of CMA analysis in genetic evaluation of AML. To address this gap, the Cancer Genomics Consortium Working Group for Myeloid Neoplasms undertook a project to systematically review peer-reviewed published literature, summarize evidence regarding clinical utility of CNA and CN-LOH evaluation in AML, and develop an evidence-based proposal for optimal genome-wide AML testing by CMA and other established and emerging genomic technologies.

2. Methods

A systematic literature search was performed for peer-reviewed manuscripts focusing on CNA and CN-LOH testing in AML published between 2001 and 2017. The workgroup members studied in detail 70 peer- reviewed papers. Many of those publications did not focus specifically on AML, but also included other myeloid malignancies for which there are significant overlaps in the spectrum of genetic aberrations. Two types of data were extracted from the reviewed manuscripts: 1) diagnostic yield of CMA testing for CNAs and CN-LOH in AML as compared to conventional cytogenetics (including the total number and percent of cases abnormal by CMA in each study, the number and percent of cases abnormal by CMA but normal by conventional cytogenetics, the number and type of abnormalities detected by CMA but undetected by karyotype and number of abnormalities missed by CMA but detected by karyotype), and 2) list of detected recurrent CNAs and CN-LOH loci. For aberrations that are shared between AML and other myeloid malignancies, including -5/del(5q), -7/del(7q), -17/del(17p), and complex karyotype, the articles were searched for information regarding their importance in AML. The significance of these overlapping aberrations in other myeloid neoplasms is reviewed in a separate manuscript by the CGC Myeloid Malignancies Working Group (Kanagal-Shamanna et al.).

Recurrent CNAs from individual manuscripts were selected for further evaluation based on the following criteria: they had to be observed in at least 3% of AML patients in a large cohort (at least 100 patients) or in at least 2 AML patients in a small cohort (less than 100 patients). Since some of the early studies, in particular for CN-LOH, include false discoveries which represent benign copy number variants or constitutional CN-LOH regions [21], expert review was performed by working group members to ensure that only disease associated, acquired aberrations were included for further consideration [22]. A CNA was characterized as possibly being clinically significant if a study had evidence that: 1) a CNA was either specifically associated with AML and/or an AML sub-type, or it represented a recurrent finding that supported the diagnosis of AML (diagnostic significance-D), 2) a CNA showed clear statistically significant association with a superior or inferior outcome (prognostic significance-P) or 3) a CNA showed significant association with a response or a resistance to an existing drug (therapeutic significance-T). The initial broad list of potentially clinically significant and/or recurrent CNAs and CN-LOH regions was further refined to include CNAs and CN-LOH which were identified as clinically significant and/or recurrent by at least two independent studies. The strength of evidence supporting clinical significance of a particular CNV or CN-LOH locus in AML was evaluated using a classification scheme adapted from the Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer [23]. Briefly, supporting evidence was considered to be strong if a variant was included in WHO classification and professional practice guidelines (Level 1) or if its diagnostic,

prognostic and/or therapeutic significance was supported by well-powered studies with consensus from experts in the field (Level 2). The evidence was considered adequate (good) if it was based on the results of multiple small studies without any contradicting data (Level 3). The list of clinically significant and/or recurrent CNAs selected and evaluated based on this process is provided in table 1.

Focused literature review was conducted to investigate clinical utility of CMA testing in AML cases which are currently classified as having an intermediate prognosis (including normal karyotype (NK) AML and AML with non-specific chromosome abnormalities) and in AML cases for which karyotype could not be obtained. In AML with abnormal karyotype, the literature was surveyed for studies focusing on the role of CMA in better characterization of non-specific karyotype abnormalities (defined as abnormalities that have not been previously reported in association with AML and other myeloid neoplasms, and for which their clinical significance is unknown) and karyotype abnormalities that could not be fully elucidated due to poor chromosome morphology.

3. Results

Detection Rate for CNAs

The reviewed studies have documented higher detection yield for CNAs by CMA as compared to conventional karyotyping (Table 2), with an additional CMA advantage of enabling more precise CNA characterization (including determination of the exact size, breakpoints and gene content) [12, 24, 25]. As expected, balanced rearrangements, which are the driver genetic abnormalities in approximately 20% of AML patients, were typically only observed by karyotyping and FISH, although in cases with apparently balanced rearrangements CMA frequently detected cryptic CNAs at the breakpoints [12, 24, 26, 27]. Overall, karyotyping and FISH detects genetic abnormalities including CNAs and balanced rearrangements in 55% of AML patients, while CMA detects CNAs and CN-LOH in approximately 50% of AML patients (Table 2). A relatively low overall detection rate by CMA can be accounted for by a substantial proportion of AML cases that only have balanced chromosome rearrangements and sequence mutations as main oncogenic drivers. Some studies also pointed to lower sensitivity of CMA for low level mosaicism compared to karyotype and particularly FISH, suggesting that CMA shows its utility at the time of diagnosis and relapse, and has limited value for residual disease detection [12, 25, 28].

Prognostic Significance of CNA Detection

Approximately 40-50% of AML patients have no detectable abnormalities by conventional karyotype analysis and by commonly used AML FISH probes (inv(16), t(8;21), 11q23 (*KMT2A*), t(15;17), etc.). An additional 20-30% of the patients may have non-specific clonal abnormalities that do not allow classifying them into either a favorable or adverse risk group. According to the risk stratification in 2017 NCCN AML guideline and 2017 ELN recommendations, NK-AML cases are classified into intermediate risk category [8, 9]. However, these patients are prognostically heterogeneous. Molecular testing by conventional Sanger sequencing or NGS may identify an underlying driver mutation or prognostically important abnormalities in a subset of these cases. Nevertheless, a large proportion will remain classified as having an intermediate risk. CMA has clinical utility in these patients with its ability to detect cytogenetically cryptic CNAs and CN-LOH of prognostic significance.

CMA detects genetic lesions including cryptic CNAs and CN-LOH in 32-68% of NK-AML patients (table 2), and several studies suggest that detection of these aberrations may allow improved prognostic stratification in AML [28-31]. Statistically significant association of CMA abnormalities with adverse outcome in AML with both normal and abnormal karyotype has been shown in studies by Parkin et al. [25] and Tiu et al [24]. Yi et al focused specifically on the prognostic role of CMA testing in NK-AML with normal FISH testing; the authors showed in multivariate analyses that the abnormalities detected by

CMA had unfavorable prognostic significance in regards to overall survival [29]. Prognostic significance of CMA abnormalities has also been noted in pediatric AML. In the recent comprehensive analysis which included 446 pediatric patients enrolled in several Children's Oncology Group trials, Vujkovic et al observed that the presence of CNAs detectable by CMA testing was significantly associated with decreased overall survival and event-free survival in pediatric patients with standard risk AML [32].

CMA abnormalities have been demonstrated to add negative prognostic information to knowledge of mutations in *AML*-associated genes, suggesting that integration of CMA results with multi-gene sequencing panel testing may increase the precision of prognostic classification [33, 34]. Furthermore, the utility of CMA when combined with conventional karyotype has also been described. Tiu et al looked at the ability of CMA to complement the results of conventional cytogenetics, and showed that the patients with abnormalities detected by either karyotyping or CMA had worse overall survival, event-free survival and progression-free survival than patients with no abnormalities detected by either technique; furthermore, patients with additional lesions detected by CMA regardless of karyotyping results showed worse outcome than patients with unchanged findings [35].

Clinically-Significant Submicroscopic CNAs Detected by CMA

Application of CMA testing to large cohorts of AML patients led to discovery of novel recurrent CNAs, which cannot be detected by conventional cytogenetics due to their small size and which are not targeted by routine AML-related FISH panels; several of these loci are either emerging or have already been established as important prognostic markers. Similar to well-known cytogenetically detected CNAs like 5q and 7q deletions, some of these novel loci (TET2 deletion on 4q), are shared between different myeloid malignancies including AML, MDS, and MPN. This is particularly true in secondary AML that evolves on the basis of other myeloid malignancies. However, a subset of novel submicroscopic CNAs are AML specific (Table 1). A comprehensive list of CNAs detectable by CMA testing that have diagnostic, prognostic and treatment implications in AML is provided in Table 1. Selected novel, clinically important CNA regions are discussed below.

4q24 deletion involving TET2 locus. The *TET2* gene encodes an enzyme belonging to the Ten-Eleven Translocation (TET) family of dioxygenases involved in the process of DNA demethylation [36]. Somatic *TET2* mutations are found in 8-27% of patients with *de novo* AML [37-42] and approximately 14% of therapy-related AML (tAML) patients [43, 44]. Mutational analysis of *TET2* is recommended as part of the molecular diagnostic workup for AML patients according to the most recent joint guideline from CAP and ASH [4]. However, in addition to sequence abnormalities, *TET2* disruption in AML can also be caused by partial or complete deletions. 4q24 deletions including *TET2* have been identified by

FISH and CMA in 5.2% of AML cases, including 4.2% primary or *de novo* AML (pAML) patients, 8.1% secondary AML (sAML) patients and 13.3% tAML patients [45]. These deletions are frequently accompanied by *TET2* mutations [42]. Bacher et al. showed that *TET2* loss-of-function mutations occur in the non-deleted *TET2* alleles in 37.5% of AML with *TET2* deletions [45]. This observation indicates that submicroscopic 4q24 deletions represent not-only an important mechanism to induce *TET2* haploinsufficiency, but also, in combination with concurrent sequence abnormalities, to cause a complete loss of *TET2* function. Bacher et al. also found that in *de novo* AML with intermediate cytogenetic risk, the presence of any *TET2* alteration including deletion and/or mutation was associated with worse median overall survival and event-free survival [45]. Weissmann et al. described that *TET2* alterations including deletion and/or mutation were associated with NK-AML, higher white blood cell count, lower platelet count and higher age. Survival analysis focusing on *de novo* NK-AML found *TET2* alteration is associated with inferior event-free survival [42]. Detecting 4q24 deletions encompassing *TET2* may thus be of prognostic importance in AML. About 32% of such 4q24 deletions are cytogenetically cryptic, and since FISH analysis using a *TET2* probe is not routinely performed in AML, 4q24 microdeletions encompassing *TET2* are most likely to be detected by CMA testing.

17q11.2 deletion including the NF1 gene. Submicroscopic 17q11.2 deletion encompassing *NF1* has been detected by CMA in 3.5-11% of AML patients including pAML and sAML [39, 40]. This deletion has also been identified in 3.8-6.7% of NK-AML. The majority of the 17q11.2 deletions are cytogenetically cryptic [24, 29, 46]. The tumor suppressor gene *NF1*, which encodes a GTPase-activating protein that negatively regulates RAS signaling, is the critical gene in this deletion interval. 20-52% of *NF1*-deleted patients carry mutations in the other *NF1* allele [47, 48]. In the study by Walter et al., the 17q11.2 deletion including the *NF1* gene was found to be associated with worse overall survival [49].

21q22 microdeletion involving RUNX1. *RUNX1* encodes one of the two subunits of the Core Binding Factor (CBF) and plays a critical role in homeostasis of hematopoietic stem and progenitor cells. Germline loss-of-function *RUNX1* mutations (including whole gene deletions) resulting in haploinsufficiency, as well as point mutations acting in a dominant-negative manner, are associated with the familial platelet disorder (FPD) with a predisposition to AML [50, 51]. Somatic mutations of *RUNX1*, mostly frameshift and nonsense mutations, are found in 6 -13% of AML patients [44, 52, 53] and are associated with lower complete remission rates, shorter disease-free survival and overall survival, and resistance to chemotherapy [52-55]. In addition, *RUNX1* is one of the most frequently translocated genes in hematological malignancies including pAML) and sAML. In the 2017 WHO classification of myeloid neoplasms and acute leukemia [1, 6], AML with mutated *RUNX1* was introduced as a novel category, since it appears to represent a biologically distinct group with a possibly worse prognosis than other AML

subtypes [1, 52, 53, 55, 56]. In addition to loss-of-function sequence mutations, submicroscopic deletions involving *RUNX1* have also been observed as somatic abnormalities in AML. Figure 1 shows a representative submicroscopic 21q deletion, encompassing the *RUNX1* gene, detected by CMA in an AML patient. Itzhar et al. reported cryptic 21q22 deletion including *RUNX1* in 4/30 tAML patients but not in 36 pAML patients [57]. *RUNX1* deletions have also been identified in AML patients both with and without concurrent *RUNX1* mutations [58, 59]. *RUNX1* deletion is expected to result in haploinsufficiency, similar to loss-of-function mutations of *RUNX1;* they are thus predicted to have comparable association with adverse outcome as *RUNX1* mutations, although additional studies will be needed to fully characterize the clinical significance of *RUNX1* deletions in AML. Depending on the clinical context, family history, size and mosaicism level of a *RUNX1* deletion in AML patients, the possibility that such deletion represents a germline abnormality should also be considered [60].

21q22 amplification including the ERG gene. ERG is a member of the *ETS* gene family and is an oncogene involved in the tumorigenesis of prostate cancer, Ewing sarcoma and leukemia. Amplifications of the *ERG* locus in 21q22 have been reported as recurrent CNAs in AML, and have been detected in approximately 6-9% of pAML cases. The majority of patients with *ERG* amplification have a complex karyotype [49, 57, 61, 62]. *ERG* amplification results in high level expression of the gene. The study by Walter et al. suggested that *ERG* amplification was associated with poor overall survival [49]. Nibourel et al. identified *ERG* amplification as a poor prognostic marker in their own cohort and then confirmed this finding by analysis of the Cancer Genome Atlas (TCGA) data. This study demonstrated that *ERG* amplification was associated with resistance to cytarabine, a cornerstone drug for AML treatment; the authors suggested that an alternative drug should be considered for AML patients with *ERG* amplification [62]. Nibourel et al. also revealed high frequency (64%) of co-occurrence of *TP53* mutation in TCGA AML patients with *ERG* amplification. Notably, the multivariate analysis using the TCGA cohort of patients older than 60 years of age identified *ERG* amplification as a better predictor of overall survival than the ELN risk classification and *TP53* mutation status. Another important finding by Nibourel et al. was the presence of *ERG* amplification in some AML patients classified as having an intermediate risk, who likely would have been more accurately classified in the high risk category [62]. The results to date suggest that *ERG* amplification should be considered for inclusion into risk-stratification algorithms as a high-risk marker for AML [62]. *ERG* amplification is cytogenetically cryptic, commonly hidden in a supernumerary abnormal chromosome 21 or marker chromosome. CMA is therefore the best testing method for *ERG* amplification.

Detection and Clinical Significance of CN-LOH in AML

Acquired CN-LOH has been recognized as a common mechanism through which cancer cells achieve a growth advantage by duplicating mutations in oncogenes or tumor suppressor genes [63]. Acquired CN-LOH regions either involve whole chromosomes if they originate through mitotic non-disjunction events, or encompass large (>15-20Mb) terminal regions of chromosome arms, consistent with the mitotic recombination mechanism [30, 63, 64]. CMA analysis currently remains the only technique that allows genome wide evaluation of CN-LOH. Overall, CN-LOH is found in approximately 8-36% of AML patients [12, 43, 58, 60]) and in 12-32% of patients with NK-AML (Table 2) [21, 23, 43, 61, 62].

CN-LOH in AML exhibits non-random distribution. The most frequently involved chromosome arms include 4q, 7q, 13q, 11p, 11q, and 17p (Table 1). Although CN-LOH has been identified across all clinical and molecular subtypes and cytogenetic risk groups of AML [65], there are subtype-related differences in overall frequency and the most commonly observed CN-LOH loci. For example, 9p CN-LOH associated with *JAK2* mutations is most commonly seen in sAML arising from myeloproliferative neoplasms [66, 67]. Some of the recurrent CN-LOH loci like 7q and 17p correspond to chromosome regions which are frequently affected by CNAs. However, there are also regions including 5q and 20q that are commonly affected by deletions in myeloid malignancies, but where CN-LOH is almost never detected. Although its significance is not completely clear, this observation may suggest that CN-LOH and CNAs may sometimes contribute to leukemogenesis through shared molecular mechanisms, but in other cases work via completely different and independent pathways [63].

Correlation of CN-LOH with mutation status of candidate genes.

Since CN-LOH has been hypothesized to act as a mechanism to duplicate mutations in oncogenes or tumor suppressor genes, a candidate gene has been sought for every recurrent CN-LOH locus in AML (Table 1). Many studies correlated CN-LOH in a specific region with the presence of a pathogenic sequence change in the proposed candidate gene. Although for the majority of the loci a correlation could be established, it was not absolute. For example, a *FLT3-ITD* mutation could be identified in virtually all AML patients with 13q CN-LOH [11, 30, 65-69]. In addition, homozygous *RUNX1* mutations are detected in almost all AML patients with 21q CN-LOH [70]. In contrast, homozygous c-*CBL* mutations have been found in only half of the MDS/AML patients with CN-LOH 11q [67] and *TP53* mutations were found in approximately 60% of patients with CN-LOH 17p [71]. Furthermore, for some recurrent CN-LOH regions in AML, a plausible candidate gene has yet to be identified. Several mechanisms were put forward to explain the oncogenic role of CN-LOH in the absence of demonstrable mutation in a candidate oncogene or tumor suppressor gene. For genes that are regulated by methylation-based promoter silencing, CN-LOH could result in duplication of an aberrantly methylated or unmethylated allele, thus

leading to either effective knockout or enhanced expression. Finally, CN-LOH can become oncogenic through a duplication of a minor disease-prone allele present in the germline [63].

Clinical significance of CN-LOH in AML.

A landmark study that specifically looked into the prognostic relevance of detection of CN-LOH by CMA was reported by Gronseth et al. By testing 112 consecutive AML patients, the authors found, in multivariate analyses, that detection of CN-LOH had an independent predictive value for early disease recurrence in patients with intermediate and unfavorable cytogenetics. The prognostic effects were the most pronounced for 13q CN-LOH, which was associated with a 6.64-fold higher rate of disease recurrence and a 3.45-fold worse OS, and was enriched in cases with the *FLT3-ITD* mutation [11].

CN-LOH in 13q is one of the most common CN-LOH events in AML, found in 3.3-6.3% of the cases [11, 30, 65] (Figure 2). In addition, 13q is the region where detection of CN-LOH has the best documented prognostic implications [11]. 13q CN-LOH shows strong correlation with the presence of the *FLT3-ITD* mutation [11, 30, 63, 65-69]. In addition, practically all patients with a high *FLT3-ITD* level have CN-LOH involving 13q [65], confirming CN-LOH as the key mechanism leading to high *FLT3-ITD* allelic burden (rather than amplification of the *FLT3* locus for example). Interestingly, patients with CN-LOH of 13q rarely carry *FLT3-TKD* mutation [65].

FLT3-ITD is known to be a secondary, cooperating mutation in AML rather than an early driver. AML is a polyclonal disease, particularly at initial diagnosis, and the *FLT3* mutant-to-wild type allelic ratio is considered to be a reflection of the fraction of leukemia cells that harbor the mutation. It has been shown that at diagnosis the mutant burden estimated by PCR-based methods can range from barely detectable to nearly 100%, and that a high allelic *FLT3-ITD* burden correlates with a particularly poor outcome in *FLT3*-mutated AML [72, 73]. In fact, current risk stratification schemes for AML only include the presence of *FLT3-ITD* as a marker of adverse prognosis if there is a high allelic burden for the mutant allele [9]. *FLT3-ITD* is clinically most commonly detected by PCR amplification and fragment size analysis based methods, since NGS based approaches may fail to identify large duplications which often cannot be accurately aligned to the reference sequence by current algorithms. PCR-based approaches allow estimation of the allelic burden as the ratio between the mutant and the wild-type allele, but many laboratories currently do not provide this information. Even when the allele ratio is reported, the methods for its estimation are far from standardized, and they depend on the blast percentage of the specimen analyzed, which can be influenced by the quality of the aspirate. As a result, molecular estimations of the allelic level are unreliable, with a concern that the allelic burden may be underestimated. 13q CN-LOH is reliably detectable by CMA in samples that have ≥ 20% abnormal myeloid blasts, and has been reproducibly shown to be associated with higher rate of disease recurrence and worse OS in AML [11, 30,

65, 69]. CMA testing and detection of 13q CN-LOH is not meant to replace molecular testing for *FLT-ITD*, but can provide additional prognostically important information in *FLT3* mutated AML.

A comprehensive list of recurrent CN-LOH loci in AML and summary of their clinical significance are presented in Table 1. While most of these recurrent CN-LOH regions are shared among myeloid malignancies, 13q CN-LOH is highly specific for AML. To date, the clinical impact has been firmly established in AML only for a few recurrent areas of CN-LOH, including 9p CN-LOH in sAML evolving from *JAK2* mutated myeloproliferative neoplasms, 4q CN-LOH seen frequently with a co-occurring *TET2* mutations, and 13q CN-LOH associated with AML with *FLT3–ITD* mutation. Additionally, CN-LOH in 17p correlates frequently with the presence of a *TP53* mutation, which is a marker of a very poor prognosis in AML. If not already performed, *TP53* mutation analysis should be recommended for all AML cases with 17p CN-LOH. CN-LOH 11q in *c-CBL* mutated AML is also emerging as a potentially relevant prognostic marker [11]. Further studies will be needed to accurately assess prognostic significance of other recurrent CN-LOH loci, and confirm that, as suggested by Gronseth et al., detection of any region of CN-LOH may have prognostic implications in AML [11].

CMA testing in diagnostic cases with unobtainable karyotype

Karyotype analysis of leukemic cells at the time of diagnosis is standard of care, but cytogenetic analysis requires a viable bone marrow or leukemic blood sample which is not always available. A common reason for failure to produce metaphase cells for karyotype analysis is treatment with hydroxyurea or other cytotoxic agents prior to collection of the diagnostic bone marrow or peripheral blood sample. Karyotype analysis is not possible with these non-mitotic samples, however, they can be studied with CMA to detect CNAs and CN-LOH, and with FISH to exclude the common AML associated balanced rearrangements. Figure 3 depicts an illustrative AML case with a failed karyotype analysis and normal results of FISH testing with the D7S486 probe located in 7q31, in which CMA evaluation revealed a prognostically important 7q deletion outside of the region targeted by the FISH probe.

Extramedullary AML or myeloid sarcoma (MS), a tumor of malignant granulocytic precursor cells in anatomical sites outside of the bone marrow, is also frequently associated with lack of cytogenetic information, for reasons other than a technical failure to obtain analyzable metaphases. MS commonly develops concurrently or after the diagnosis of AML, but occasionally occurs as the initial manifestation of the disease without bone marrow infiltration [74]. In this situation, the disease may not be recognized as a hematologic neoplasm, and samples may get processed following solid tumor protocols. Consequently, fresh MS samples fail to get submitted for cytogenetic evaluation, and only formalin-fixed paraffin-embedded (FFPE) tissue is available for genetic analysis. CMA may thus be a particularly useful

diagnostic method for detecting CNAs in MS samples, because the analysis can be performed on DNA extracted from FFPE. Although only a handful of CMA studies focused on MS, they clearly demonstrated that CMA using FFPE material is a feasible and clinically applicable approach for detection of prognostically significant genomic abnormalities in MS [75, 76].

Likewise, in those AML cases in which a bone marrow sample has inadvertently not been sent for cytogenetic analysis, CMA could be performed on FFPE bone marrow aspirate clot or fresh bone marrow biopsy to obtain important information about the leukemic karyotype. Array platforms optimized for FFPE testing are now available in clinical cytogenetic laboratories for solid tumor testing, and thus can be utilized for FFPE samples from hematologic neoplasms.

In addition to clinically significant submicroscopic CNAs and regions of CN-LOH discussed above, several cytogenetic prognostic markers used by broadly accepted risk stratification schemes are detectable by CMA if karyotype cannot be obtained, including genomic complexity, monosomy 7, and 5q and 17p deletions.

Multiple genomic abnormalities (genomic complexity). The presence of three or more unrelated chromosomal abnormalities (complex karyotype) represents a strong predictor of poor outcome in AML. In all currently used risk stratification schemes (ELN, NCCN), AML patients with a complex karyotype are considered 'poor risk' [8, 9]. AML with a complex karyotype is characterized by frequent loss of 5q and/or 7q and/or 17p regions, and some studies suggest that complex karyotype cases with these 'typical' abnormalities have worse prognosis than AMLs with ≥ 3 abnormalities but without the presence of any of these characteristic changes [77-79]. Abnormalities observed in AML with a complex karyotype were shown by karyotype analysis, FISH and CMA to almost invariably involve loss and gain of genomic material, making them amenable for detection and characterization by CMA. Considering that CMA routinely detects increased number of abnormalities relative to karyotype analysis, including much smaller CNAs than those visible by routine cytogenetics, it remains to be confirmed whether the definition of complexity based on conventional cytogenetic studies (3 or more unrelated abnormalities) can be directly applied to CMA. Several studies published to date help to address this question. Parkin et al showed that increased genomic complexity defined as ≥4 CMA lesions demonstrated a strong, independent negative impact on overall survival [HR = 1.98; 95% confidence interval (CI), 1.20–3.28; P < 0.01] , while ≥3 CMA lesions also trended toward an independent negative effect but did not reach clinical significance (HR = 1.57; 95% CI, 0.95–2.57; P = 0.07) [71]. Gronseth et al. found that genomic complexity defined as the presence of at least three CMA abnormalities (including both CNVs and CN-LOHs), was associated with a shorter overall survival. Notably, in the same study, the authors were able to reclassify based on CMA findings 5 out of 112 AML patients who were originally in the good or

intermediate cytogenetic risk group into the group with genomic complexity and high risk [11]. A formal definition of 'CMA-detected genomic complexity' in a large scale clinical trial would allow its incorporation into risk stratification schemes, and utilization of CMA for detection of this subset of high risk AML cases in the clinical setting.

Monosomy 7 and deletion 7q (-7/7q-) are recognized as common cytogenetic abnormalities in AML, particularly in sAML and tAML, and are associated with an adverse prognosis. Most current guidelines for clinical evaluation and management of AML include -7/7q- as key prognostic markers, and recommend testing for this and other prognostic aberrations by karyotype and/or FISH at the time of diagnosis [9]. Mapping the critical region(s)/gene(s) on 7q has been a challenge over the years, and has more recently extensively relied on the use of CMA analysis. In different studies, the commonly deleted region (CDR) has been mapped to 7q22, 7q32-33, and 7q35-36 regions [80-83]. Haploinsufficiency of multiple tumor genes in 7q, instead of inactivation of one particular candidate gene according to the Knudsen 2-hit model, has been proposed as the mechanism underlying oncogenic potential of chromosome 7 loss and 7q deletion [84]. Several candidate genes have been proposed to contribute to the oncogenic potential of -7/7q- in myeloid malignancies, including *EZH2* (7q36) [80-82], CUX1(7q22.1) [83], SAMDL9 (7q21.3) [84], MLL3 (7q36.1) [85], DOCK4 (7q31.1) [86] and LUC7L2 (7q34) [87].

The commercial FISH probes for 7q deletion testing commonly used by clinical cytogenetics laboratories typically target the 7q31 region, and would thus not detect the other CDRs mentioned above. While the majority of 7q deletions encompass multiple CDRs, smaller deletions are also observed and should be identified for accurate risk stratification. The advantage of CMA as compared to FISH testing for detection of chromosome 7 abnormalities if karyotype is unobtainable is in the ability to detect submicroscopic deletions and deletions that do not include the region targeted by the commonly used CEP7/7q31 probe set (Figure 3).

Deletion 5q (-5/5q-) is among the most common karyotypic abnormalities in myeloid neoplasms including AML; it is observed in 10–15% of patients with pAML, and in up to 40% of patients with sAML [85]. The presence of del(5q) has distinct clinical implications depending on the concurrent morphologic findings and associated cytogenetic abnormalities. Furthermore, deletions of different regions in 5q appear to have different morphologic and clinical correlations and different prognostic implications; more distal deletions involving the critical region in 5q32-33 (known as the distal region or CDR1) have an important role in the pathogenesis of the 5q- syndrome, which is a subtype of low-risk MDS, while deletions in the proximal region in 5q31 including *EGR1*, *CDC25C* and *CTNNA1* (CDR2) characterize higher-risk MDS and AML [85, 86]. Notably, recent CMA studies proposed a further refinement of the CDR boundaries, and defined two commonly retained regions (CRRs) in 5q. In contrast

to monosomy 7, which is relatively common, a complete loss of chromosome 5 is exceedingly rare in myeloid malignancies. According to recent CMA studies, the two chromosome 5 regions that are typically retained in MDS and AML are CRR1 (commonly retained region 1) extending from the centromere to 5q14.2 and CRR2 mapping between band 5q34 and the telomere [87]. In addition, patients with small interstitial deletions have been suggested to have a better outcome as compared to those with larger deletions [87].

Considering that clinical implications of 5q deletions may be different depending on the size, location of the deleted region and concurrent abnormalities, methods like CMA that provide genome-wide information but also detect and precisely map deletions in any region of chromosome 5 are superior to FISH in evaluating 5q abnormalities in AML.

17p deletion involving the *TP53* gene has long been recognized as a marker of an adverse outcome in AML patients [9], occurring in 3% of pAML and 12% of tAML cases [57]. *TP53* abnormalities, including both pathogenic sequence variants and deletions encompassing the gene, have been shown in AML to be associated with sAML [66], a complex karyotype, older age and a particularly poor outcome [66, 77]. Notably, among complex karyotype AML positive for a *TP53* abnormality, ~40% have *TP53* loss due to a deletion and the remaining 60% were found to carry a pathogenic sequence change [77]. *TP53* mutation testing is recommended as a part of the molecular diagnostic workup for AML patients according to the NCCN guidelines and the most recent guideline from the CAP and ASH [4, 8], and it is clearly important that such testing includes methods that identify *TP53* loss due to chromosomal 17p deletions. Of note, due to poor morphology and complexity of chromosomal abnormalities, such deletions may remain cryptic by karyotype analysis. In one study involving AML with a complex karyotype, CMA testing identified 17p deletions encompassing *TP53* in 6% of additional cases in which they were not detected by conventional karyotype analysis [88].

Characterization of chromosomal abnormalities by CMA

Due to low resolution of conventional cytogenetics, karyotype complexity or very poor chromosome morphology, the nature of chromosome abnormalities often cannot be completely elucidated by karyotype analysis. Accurate characterization of CNAs by CMA can thus be helpful for accurate risk stratification. For example, CMA analysis has shown that marker chromosomes seen in a significant proportion of AMLs with a complex karyotype often arise from chromothripsis, a single catastrophic event of multiple breaks and random reassembly of one or a small number of chromosomes. In the study by Bochtler et al., chromothripsis-positive AML cases were characterized by a particularly high degree of karyotype complexity, *TP53* mutations and dismal prognosis [89]. Chromosomes with additional chromosome

material of unknown origin may also harbor chromothripsis. Figure 4 depicts a chromothripsis-like pattern of CNAs on chromosome 8 detected by CMA in an AML patient with an add(8q) abnormality seen by karyotype analysis.

Although CMA is not expected to detect balanced rearrangements, it is a useful tool for detecting cryptic rearrangements resulting in abnormal gene fusions, which may be associated with the presence of deletions and duplications at the breakpoint sites. The study on 111 pediatric AML cases by Radke et al identified 4 leukemias that expressed the t(5;11)-encoded *NUP98-NSD1* chimeric transcript, of which two had CNAs adjacent to one or both translocation partners [90]. Similarly, in a recent study which also focused on childhood AML, Vujkovic et al showed that CNAs frequently mapped at the breakpoints of rearrangements resulting in prognostically significant gene fusions like *NUP98-NSD1* and *LPP-KMT2A* [32]. Some gene fusions can only be formed by complex rearrangements due to 'incompatible' gene orientation of the partner genes on the corresponding chromosomes. The examples include the fusion between the *MLLT10* gene in 10p12.3 and *KMT2A* gene in 11q23 [94], and the fusion between the ETV6 gene in 12p13.2 and the ABL1 gene in 9q34.12 [95]. Generation of such fusions occurs through complex rearrangements with multiple breakpoints and is often accompanied by non-specific abnormalities in the karyotype; CNAs mapping to the breakpoints within the partner genes are frequent in these cases, and can be detected by CMA [26, 30, 49, 91].

The other mechanism through which CMA analysis can reveal the presence of an abnormal fusion is a gain or loss of one of the derivative chromosomes from a reciprocal translocation, which is a common secondary abnormality in AML and other cancers where an abnormal gene fusion is the main oncogenic driver. Acquisition of an extra copy of a derivative chromosome from which the fusion is expressed is a known mechanism of tumor cells to gain a selective advantage. The best known example of this phenomenon is a gain of an additional copy of the der(22)t(9;22)(q34;q11.2)-Philadelphia chromosome, which is a very frequent abnormality during progression of chronic myeloid leukemia into an accelerated phase or blast phase [92]. Both gain and loss of 'non-functional' derivative chromosomes are also frequent, and in most cases represent random events in cancer cells. If only one of the derivative chromosomes from a clinically important translocation is present in the karyotype, the abnormality may be difficult to recognize by conventional cytogenetic analysis. However, the resulting copy number imbalance with the breakpoints mapping within the fusion partner-genes make such abnormalities easily recognizable by CMA [91].

Discussion

Presented evidence supports genome-wide assessment for CNAs and CN-LOH as the best practice in diagnostic evaluation of AML. In addition to allowing better diagnostic precision, such evaluation can detect prognostic markers and inform treatment decisions. While traditionally whole genome- CNA assessment was solely performed by karyotyping, CMA currently allows increased resolution and also detects CN-LOH. That makes CMA a valuable tool in clinical diagnostics for AML at the time of diagnosis and relapse, even with its limited sensitivity to detect genetic aberrations present in a low proportion of cells in a sample and its inability to detect balanced rearrangements. Although not widely available and practical for clinical use, NGS-based approaches for genome-wide evaluation for CNAs and CN-LOH may also become feasible in the future.

Recommendations for CMA testing in AML

CMA testing identifies genetic abnormalities in a large proportion of AML cases, frequently detecting abnormalities that influence risk stratification and patient management. Even in AML with a favorable translocation, CMA may detect additional abnormalities that might predict resistance to treatment, thus warranting increased surveillance or treatment modifications. Assuming successful karyotype analysis, CMA testing may not be clinically indicated for every newly diagnosed AML patient. However, existing evidence supports clinical use of CMA testing in the following circumstances:

1. Intermediate risk AML

This includes AML cases with a normal karyotype, non-specific cytogenetic abnormalities and chromosome abnormalities associated with intermediate prognosis (like trisomy 8), in particular when sequencing based testing also does not identify molecular mutations associated with adverse prognosis. In intermediate prognosis AML, CMA analysis may identify cytogenetically cryptic and clinically important CNAs (including deletions resulting in haploinsufficiency of *EZH2*, *CUX1*, *TET2*, *TP53*, *RUNX1*, *NF1*, *WT1*, *RB1*, etc.) or prognostically significant CN-LOH regions (13q CN-LOH in AML with *FLT3-ITD*, 9p CN-LOH in sAML with *JAK2* mutation, and others) as shown in Table 1.

2. AML with unobtainable cytogenetic results

This includes cases in which hydroxyurea or other cytotoxic therapy was initiated prior to collection of the diagnostic bone marrow or peripheral blood sample, preventing the sample from producing metaphase cells for karyotype analysis. In such cases, DNA for CMA can be extracted from the fresh sample or a fixed cell pellet after culturing. In cases in which bone marrow

aspirate was not sufficient for cytogenetic analysis, CMA can be performed on DNA isolated from the FFPE bone marrow clot or fresh bone marrow biopsy. CMA can also be performed on FFPE myeloid sarcoma biopsies which were not initially recognized as hematologic tumors and fresh tissue was not submitted for cytogenetic studies.

3. AML with inadequate results of cytogenetic analysis

This includes cases where chromosome abnormalities cannot be deciphered by conventional analysis due to an insufficient number of abnormal cells, low chromosome band resolution, complexity of the rearrangements and/or suboptimal chromosome morphology. CMA in such cases is capable of detecting prognostically significant CNAs, CN-LOH or chromothripsis and often allows characterization of complex abnormalities.

4. AML with unusual morphologic and immunophenotypic findings

This includes acute leukemias that are diagnostic dilemmas with unusual or initially conflicting clinical, morphologic, immunophenotypic and/or cytogenetic findings. In such cases, comprehensive genetic analysis including both CMA and sequencing based testing can be helpful to reach a definitive diagnosis.

5. Refractory and relapsed AML

In refractory and relapsed AML, CMA analysis may detect CNAs associated with resistance to chemotherapy, including 17p deletion, *ERG* amplification, CN-LOH of 13q, chromothripsis or other unfavorable cryptic genetic findings. Additionally, CMA analysis may identify rearrangements that implicate molecular pathways targetable by existing agents (including gene amplifications, fusions involving kinase genes etc).

Even if a diagnostic or relapsed sample is not specifically collected for CMA studies, most current CMA platforms have very modest sample requirements and a sufficient amount of DNA can typically be obtained from available residual fresh specimens, fixed cell pellets, FFPE bone marrow clots and FFPE myeloid sarcoma samples. Residual DNA from molecular studies can also be used for CMA. Some laboratories systematically preserve DNA from residual PB or BM samples sent for conventional cytogenetic studies for possible CMA testing.

Future directions

CMA analysis is at present recommended as the first-tier test for detecting constitutional CNAs in individuals with developmental disabilities or congenital anomalies [13]. In addition, CMA represents a clinically applicable, reproducible and sensitive, economical and widely available approach for genome-

wide detection of CNAs and CN-LOH in cancer [12]. Because of their critical clinical significance, testing for sequence changes in AML using NGS based panels is becoming a part of routine diagnostic work up for AML. Additionally, it has been shown that NGS panels can be robust tools to detect clinically significant gene fusions in cancers including AML [93]. One approach that could be explored as an alternative for chromosomal analysis and FISH is the use of NGS for detecting sequence variants and gene fusions, in combination with CMA for genome wide detection of CNAs and CN-LOH. Clinical trials would be useful to compare the diagnostic yield, cost, turn-around-time and other clinically important parameters between the two approaches. The importance of such objective evaluation is underscored by both NGS panels and CMA analysis becoming well-established and relatively widely available in diagnostic laboratories.

Furthermore, with the rapid advancement of NGS technology including analytical tools, methods have been developed to detect CNAs and CN-LOH using data obtained from whole genome or exome sequencing [94-99]. However, detecting non-targeted low-level genetic abnormalities in cancer specimens by NGS remains a challenge, due to relatively low read depth in whole genome or exome sequencing. Nonetheless, it is tempting to explore the possibility of simultaneously detecting mutations, CNAs, and CN-LOH using NGS as a single platform. Shen et al. demonstrated the feasibility of detecting CNAs in targeted regions using sequencing read depth generated from a targeted NGS panel specifically designed for myeloid malignancies including AML [59]. This method has the advantage in concurrently detecting mutations and CNAs in targeted regions and is superior to CMA in identifying sub-gene level CNAs such as *KMT2A* partial tandem duplications (KMT2A-PTD); however, it does not detect CNAs outside of the targeted regions and does not identify CN-LOH. McKerrel et al. described a comprehensive genomic diagnostic platform that combines a targeted mutation and translocation panel with a genome wide SNP backbone to detect mutations, translocations, genome wide CNAs, and CN-LOH for myeloid malignancies [100]. This strategy successfully detected chromosomal CNAs in more than 20% of cells, but at the resolution and sensitivity only comparable to karyotype analysis. Most recently, Shen et al. showed that a similar approach using a genome-wide SNP sequencing backbone together with high-depth coverage of 62 genes frequently mutated in AML allows for detection of clinically significant sequence changes as well as genome-wide CNAs and CN-LOH in myeloid malignancies including AML [101]. In this study, NGS achieved concordant results with CMA in samples with >= 20% of leukemia cells, showing that a combination of SNP sequencing backbone and targeted mutation NGS panel may be a feasible strategy for more comprehensive genetic profiling of myeloid malignancies using a single assay. With the advancement of technology and decreasing cost, NGS may be able to achieve a similar resolution and sensitivity as CMA in the future. Regardless of the technologies used, detection of genome wide CNAs and CN-LOH represents a critical component in the diagnosis and prognostic stratification of

AML, and CMA is at present the optimal methodology for obtaining this clinically important genetic information.

Table 1. A comprehensive list of CNAs detectable by CMA testing with strong diagnostic, prognostic and treatment implications in AML. Clinical significance and level of evidence are defined as described in the methods.

Chromosome	AML subtype	Abnormality type (gain, loss, CN-LOH)	Region	Relevant genes (if known)	Clinical significance	Level of Evidence	
1	AML including NK-AML	CN-LOH	1p		D	3	[11, 29,
2	AML	CN-LOH	2p	*DNMT3A*	D	3	[
3	NK-AML, sAML	Loss	3p14.1	*FOXP1*	D	3	
4	sAML and pAML	CN-LOH	4q24	*TET2*	D	3	[
4	AML, NK-AML, sAML	Loss	4q24	*TET2*	D, P	3	
5	pAML, sAML	Loss	5q		D	1	[24, 33, 4 8
6	AML including NK-AML	CN-LOH	6p		D	3	[29
7	AML including NK-AML	CN-LOH	7q	*EZH2*	D	3	[
7	NK-AML, pAML, sAML	Loss	7q	*EZH2, CUX1*	D	1	[2
8	complex karyotype AML	Amplification	8q24	*MYC*	D, P	3	
9	NK-AML, sAML	CN-LOH	9p	*JAK2*	D	3	[
11*	AML w complex karyotype	Amplification	11q23	*MLL*	D, P	3	
11*	AML	CN-LOH	11p	*WT1*	D	3	[1
11	pAML, sAML, NK-AML	CN-LOH	11q	*CBL*	D	3	[1
12	AML, NK-AML, AML w complex karyotype, sAML	Loss	12p13.2	*ETV6*	D	3	[24, 30, 3 104
13*	pAML, NK-AML, *NPM1* mutated AML, *FLT3-ITD* pos AML, sAML	CN-LOH	13q	*FLT3*	D, P	2	[11, 28- 1
16	NK-AML, AML w complex karyotype, pAML, sAML	Loss	16q	*CBFB*	D	3	[2
17	AML, NK-AML,	CN-LOH	17p	*TP53*	D	3	[11, 28,

	pAML, sAML						
17	sAML, NK-AML, AML w complex karyotype, *de novo* AML	Loss	17p	*TP53*	D, P	1	[24, 28 1
17	NK-AML, pAML	Loss	17q11.2	*NF1, SUZ12*	D, P	3	[24, 28
19*	AML, NK-AML, sAML	CN-LOH	19q	*CEBPA*	D	3	[11, 29
20	sAML	Loss	20q		D	3	[24
21*	pAML, AML w complex karyotype	Amplification	21q22	*ERG, ETS2*	D, P, T	3	[49,
21*	AML, NK-AML, sAML,	CN-LOH	21q	*RUNX1*	D	3	[11, 29
21*	sAML	Loss	21q22.12	*RUNX1*	D	3	

D- diagnostic significance; P-prognostic significance; T- therapeutic significance. Classification of levels of evidence: Level 1- WHO classification or professional practice guidelines; Level 2- well-powered studies with consensus from experts in the field; Level 3- multiple small studies without any contradicting data; Level 4- individual small studies, case reports, preclinical studies.

CMA indicates chromosomal microarray; CNA indicates copy number aberration; CN-LOH indicates copy-neutral loss-of-heterozygosity; AML indicates acute myeloid leukemia; NK-AML indicates normal karyotype AML; pAML indicates primary AML; and sAML indicates secondary AML.

* indicates CNAs and CN-LOH regions that are predominantly seen in AML.

Table 2. The detection rate of CMA analysis for CNAs and CN-LOH in different AML subtypes.

Disease entity	Percentage of abnormal cases (both CNAs and CN-LOH) by CMA	Percentage of abnormal cases by karyotype	Percentage of cases with CN-LOH by CMA	Percentage of abnormal karyotype cases with additional abnormalities by CMA	Average number of CNAs per case	Average number of CN-LOH regions per case	Re
AML	50%	55%	8-36%	40-73%	2.34-5.3	1.1	[11, 31
NK-AML	32-68%	0%	12-32%	0%	1.8-2.9	1.1	[28-3
pAML	56-65%	39-59%	17-29%	NA	NA	1.1	[7, 31
sAML	77-82.6%	45-53%	23-35%	27%	NA	NA	[31, 3

CMA indicates chromosomal microarray; CNA indicates copy number aberration; CN-LOH indicates copy-neutral loss-of-heterozygosity; AML indicates acute myeloid leukemia; NK-AML indicates normal karyotype AML; pAML indicates primary AML; sAML indicates secondary AML; and NA indicates not determined.

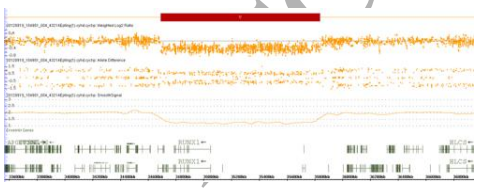

Figure 1. A 1.15 Mb deletion encompassing RUNX1, detected by CMA testing (chr21:36,059,345-37,211,393) [hg19] in a 59-year -old female patient with newly diagnosed AML. Karyotype analysis revealed a t(20;21)(p11.2;q22.1) as a sole abnormality. FISH testing using the RUNX1T1/RUNX1 dual fusion probe (Vysis, Abbott Molecular, Des Plaines, IL) was performed to determine whether the

breakpoint on chromosome 21 involves the RUNX1 locus. FISH testing was negative for a rearrangement of RUNX1, but one of the RUNX1 signals appeared to have decreased intensity. CMA testing confirmed a cryptic deletion encompassing RUNX1, which was predicted to result in RUNX1 haploinsufficiency. Loss-of-function mutations in RUNX1 are associated with an adverse prognosis in AML, and the CMA finding suggested that the patient should be considered 'high risk'. CMA results were generated using CytoScan HD arrays (Affymetrix, Santa Clara, CA). Data analysis was performed by the Chromosome Analysis Suite (ChAS) software (Affymetrix, Santa Clara, CA).

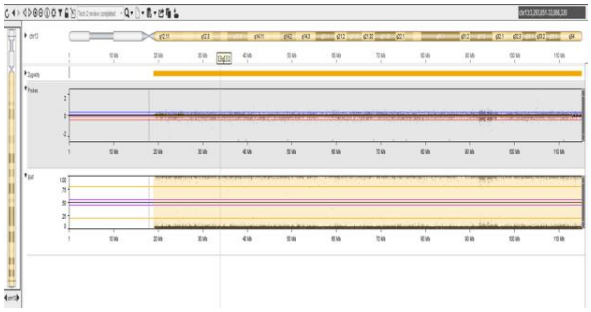

Figure 2. Copy-neutral loss-of-heterozygosity (CN-LOH) of chromosome 13. In AML with normal cytogenetics, 13q CN-LOH is associated with disease recurrence, worse overall survival and enrichment of *FLT3-ITD* mutation. The data was generated using Infinium CytoSNP-850K v1.1 BeadChip cytogenomic array (Illumina, Inc., Santa Clara, CA) and analyzed using NxClinical 3.0 analysis software (Biodiscovery, Inc., El Segundo, CA).

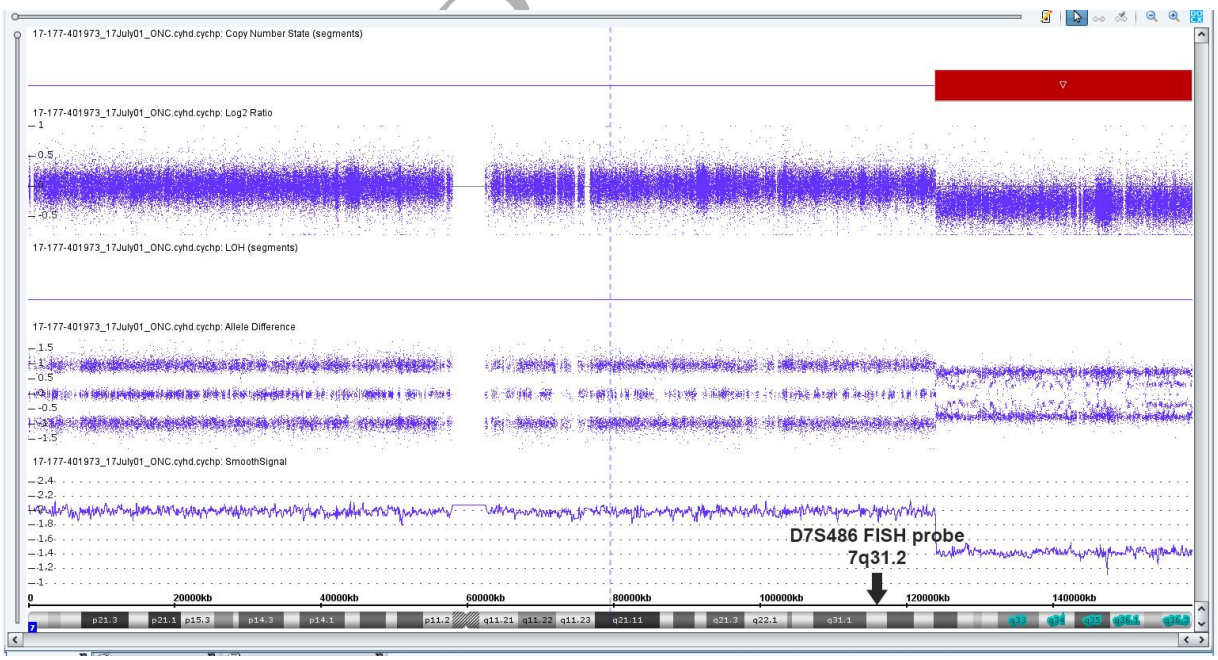

Figure 3. A 35Mb mosaic 7q terminal deletion (chr7:124,006,365-159,119,707) [hg19] in a 40-year-old AML patient with failed chromosomal analysis and normal AML FISH panel including a normal result for the D7S486 FISH probe (Vysis, Abbott Molecular, Lake Bluff, IL). The D7S486 FISH probe on 7q31.2 is located outside of the deleted region, which accounts for the discrepancy between FISH and CMA results. 7q deletion is determined as mosaic by smooth signal of 1.5 and four allele tracks. 7q deletion is associated with high risk in AML patients. CMA results were generated using CytoScan HD arrays (Affymetrix, Santa Clara, CA). Data analysis was performed by the Chromosome Analysis Suite (ChAS) software (Affymetrix, Santa Clara, CA).

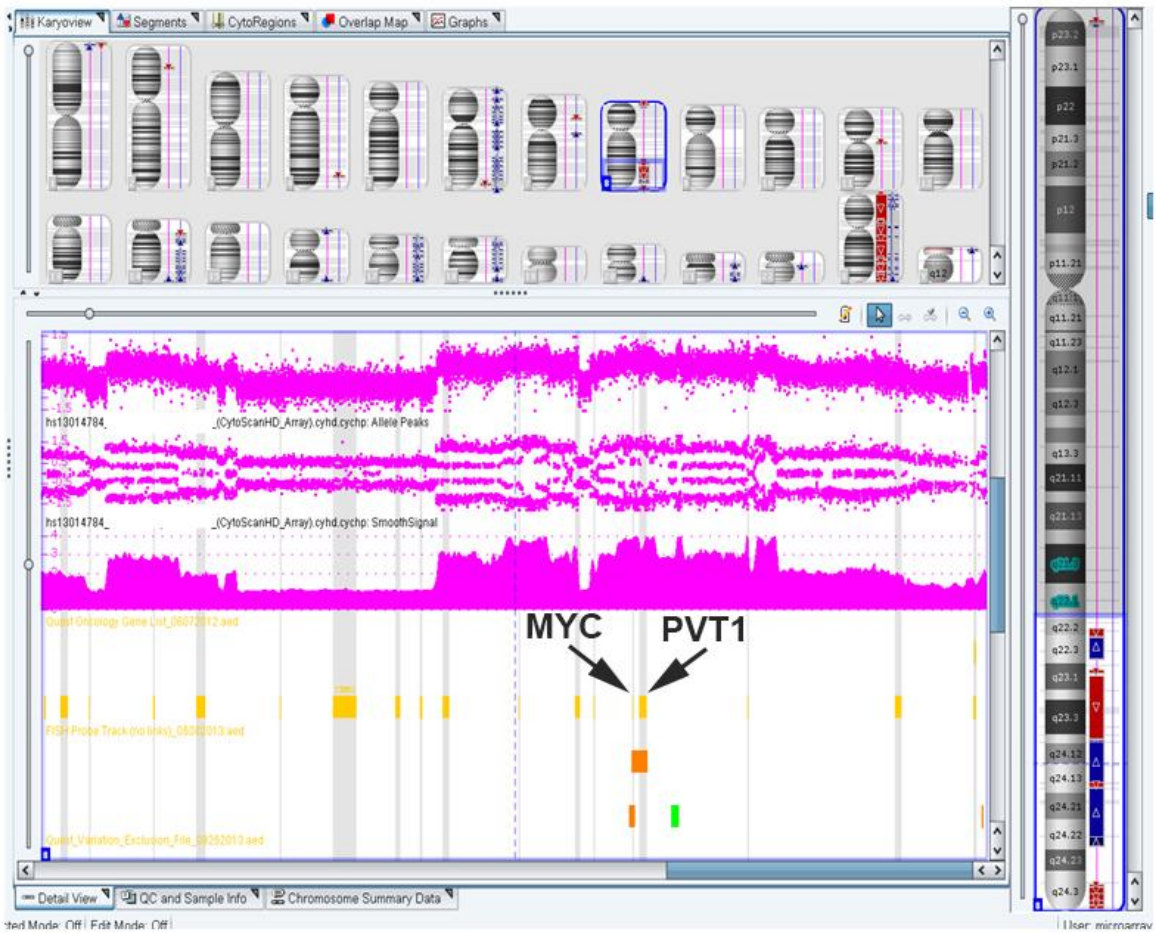

Figure 4. Chromosome 8 CNAs in a 28-year-old AML patient with bone marrow eosinophilia who failed induction chemotherapy. G-banded chromosome analysis showed a 45,X,-Y,add(8)(q22) karyotype. FISH testing using probes for *RUNX1/RUNX1T1*, *PML/RARA*, *CBFB*, *BCR/ABL1*, *KMT2A (MLL)*, *PDGFRA*, *PDGFRB* and *FGFR1* were normal. Molecular analyses for *FLT3-ITD* and TKD, as well as mutations in *NPM1* (exon 12), *KIT* (exon 8, 9, 11, 13 and 17) and *CEBPA* were normal. CMA

revealed chromothripsis-like changes involving the 8q22.2 to 8q24.3 region and including the *MYC* gene. This finding likely explains the poor response to treatment and aggressive clinical course in the patient.

CMA results were generated using CytoScan HD arrays (Affymetrix, Santa Clara, CA). Data analysis was performed by the Chromosome Analysis Suite (ChAS) software (Affymetrix, Santa Clara, CA).

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