Review Article

Tumor Heterogeneity Makes AML a "Moving Target" for Detection of Residual Disease

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Detection of minimal residual disease is recognized as an important post-therapy risk factor in acute myeloid leukemia patients. Two most commonly used methods for residual disease monitoring are real-time quantitative polymerase chain reaction and multiparameter flow cytometry. The results so far are very promising, whereby it is likely that minimal residual disease results will enable to guide future post-remission treatment strategies. However, the leukemic clone may change between diagnosis and relapse due to the instability of the tumor cells. This instability may already be evident at diagnosis if different subpopulations of tumor cells coexist. Such tumor heterogeneity, which may be reflected by immunophenotypic, molecular, and/or cytogenetic changes, can have important consequences for minimal residual disease detection, since false-negative results can be expected to be the result of losses of aberrancies used as minimal residual disease markers.

In this review the role of such changes in minimal residual disease monitoring is explored. Furthermore, possible causes of tumor instability are discussed, whereby the concept of clonal selection and expansion of a chemotherapy-resistant subpopulation is highlighted. Accordingly, detailed knowledge of the process of clonal evolution is required to improve both minimal residual disease risk stratification and patient outcome. © **2013 International Clinical Cytometry Society**

Key words: acute myeloid leukemia; minimal residual disease; phenotypic changes; clonal evolution; flow cytometry; quantitative polymerase chain reaction

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INTRODUCTION

With current treatment strategies, almost 80% of adult acute myeloid leukemia (AML) patients (<60 years) will achieve complete remission (CR). However, about 40% of these patients will experience a relapse, and five-year survival rates are only around 35–40% (1). Hence, there is a definite need to improve risk stratification and to develop novel therapies for AML patients. Currently, the standard method for evaluating therapy response and defining remission status is morphologic assessment of the bone marrow (BM). CR is thereby defined as less than 5% blast cells present in the BM, concurrent with evidence of normal erythropoiesis, granulopoiesis, and megakaryopoiesis.

Nowadays, the so-called minimal residual disease (MRD) quantitation offers a more sensitive method for the evaluation of therapy response. This term refers to a small number of leukemic cells that persist after treatment, in the absence of clinical or hematological signs of disease. A major advantage of using MRD as prognostic factor (2–9) is that it is the resultant of all diagnostic factors that affect prognosis, as well as post-diagnostic factors such as unfavorable drug kinetics (10). As such, MRD levels should be able to predict both forthcoming relapses and continuing complete remissions. Methods for MRD detection are multiparameter flow cytometry (MFC) and real-time quantitative polymerase chain reaction (RQ-PCR). Of these methods, RQ-PCR is in general

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the most sensitive technique, with sensitivities reported in the range of 10^{-3} to 10^{-6} (11–15). Common targets for molecular MRD monitoring include fusion genes, overexpressed genes, and gene mutations. Often a threshold is set to classify MRD results, based on, for instance, a 2 or 3 log reduction in the level of the molecular target after therapy as compared to the level at diagnosis (14,16,17). However, because not all AML patients have a specific molecular aberrancy, molecular MRD detection is applicable in only approximately half of AML patients.

MRD detection through MFC reportedly has sensitivities in the range of 10^{-3} to 10^{-5} (2,4–6). For MRD detection by MFC, often so-called leukemia associated [immuno]phenotypes (LA[I]Ps) are determined at AML diagnosis. Such LAPs consist of a normal progenitor antigen (CD34, CD117, or CD133), typically combined with HLA-DR and/or a myeloid marker (CD13 or CD33) and an aberrantly expressed marker. Preferentially, a LAP consists of cell surface markers, and they can be grouped into (i) cross-lineage antigen expression (e.g., expression of lymphoid markers on myeloid blasts), (ii) asynchronous antigen expression (co-expression of antigens that are not concomitantly present during normal differentiation), (iii) lack of antigen expression, and (iv) antigen overexpression (18–22). Such aberrancies can subsequently be used to detect MRD during treatment. Often a threshold is set to define MRD positivity (high levels of MRD) and MRD negativity (low levels of MRD). Most important cut-offs for positivity after second induction and consolidation therapy, used in different studies, range from 0.03 to 0.1% (10).

An alternative flow cytometric method to detect residual leukemic cells is the "different-from-normal" approach. Using this approach, expression patterns of all cells is compared to expression patterns during normal differentiation. Cells that cluster, in specific antigen combination plots, in sites where normal cells are absent (the so-called empty spaces) are consequently defined as "residual leukemic cells" irrespective of the diagnostic phenotype of the leukemia (18,20,23–25). In this review, focus will be primarily on the commonly used LAP approach, while the "different-from-normal" approach is further discussed in the perspectives section. A major advantage of MRD detection by MFC is its broad applicability, as 80–100% of AML patients have "aberrant" expression of antigens usable for MRD detection (2–5,7,9,20). Many retrospective studies have demonstrated the prognostic value of MRD quantitation by MFC in patients who ultimately achieved complete remission (2–9). These results emphasize the importance of frequency assessment of malignant cells for future clinical decision making. However, acute myeloid leukemia is a heterogeneous disease. Tumor heterogeneity may contribute to the occurrence of changes in the main tumor cell population. Therefore, immunophenotypic and/or molecular changes may impede proper use of MRD as a biomarker for response. These changes, occurring between diagnosis and relapse, may at least in part account for the emergence of relapses in patients with apparently low levels or absence of MRD. This situation implies that these patients, at least based on aberrancies present at diagnosis, have disease which is not or not adequately detected during follow-up. To give more insight into this phenomenon, we review here the literature on immunophenotypic and molecular/cytogenetic changes occurring from diagnosis to relapse in adult AML patients. Both disappearing and emerging aberrancies are discussed. In addition, correlations between molecular and immunophenotypic changes are reviewed. Finally, strategies to overcome false-negative MRD results, including the "different-from-normal" approach, will be outlined.

CHANGES IN THE IMMUNOPHENOTYPE OF AML

Although many studies have demonstrated the prognostic value of MRD after both induction and consolidation therapy, relapses still occur in patients with apparently low levels of MRD. Almost all flow cytometric MRD studies so far have reported that 20–40% of patients with low levels of MRD (range 3.5×10^{-4} to 5×10^{-3}) nevertheless relapse (2,5–9). There are three major explanations for this. One is that the sensitivity of the immunophenotypic assessment is not sufficient to detect MRD in an early stage. Especially with fast relapses, an increased MRD level occurring between the MRD negative time point and relapse may have been missed here. A second explanation would be that the initially present immunophenotypic aberrancies disappear, due to the loss of individual antigens or simultaneous loss of multiple antigens, while the leukemic cell population still exists (false negativity). A third explanation would be that the population completely disappears (leading to MRD negativity but at the same time new aberrancies emerge, leading to relapse.

Immunophenotypic changes (losses or gains) are not well defined: many research groups use different cut-off levels to define the relevance of a change in expression. In some studies there is even no quantitative measure of antigen positivity and immunophenotypic changes (4,26,27). In general, positivity of at least 20% of all cells is most commonly used to define antigen positivity (28–31). An immunophenotypic change is then defined as a switch from $<20\%$ to $\geq 20\%$ expression or vice versa. As a variation, the 20% cut-off maybe used but with subdivision of expression in "dimly positive" (20–40%) and "positive" ($\geq 40\%$). In that study (31), not only a switch from negative to dimly positive or positive but also a switch from dimly positive to positive expression was classified as a change. Alternatively, antigen or LAP positivity was considered positive if expressed on more than 10% of the leukemic cells (5,32), while other investigators used a cut-off level as low as 1% to define a switch from LAP positive ($\geq 1\%$ on BM cells) to LAP negative ($<1\%$) or vice versa (33). Although most researchers use a cut-off based on a defined percentage of positivity, it is important to emphasize the limitations of this approach. The main drawback, when for instance a 20% cut-off is

Table 1
Overview of Studies Describing Reduction or Loss of Antigen Expression

	Patients			Antigens				
	Total number	No. with a change	No. with a loss	Total number	No. of changes	No. of losses	Frequent losses	Ref.
Baer et al.	136	124 (91)	na	2211	252 (11)	109 (5)	CD11b; CD14; CD56; CD64	(32)
Dimov et al.	9	8 (89)	na	na	na	na	CD13	(27)
Feller et al.	30	3 (10)	3 (10)	47[a]	5 (11)	5 (11)	na	(5)
Hur et al.	46	23 (50)	na	na	na	na	na	(30)
Kern et al.	11	3 (27)	1 (9)	na	na	na	na	(4)
Langebrake et al.	48	42 (88)	32 (67)	428	114 (27)	60 (14)	CD13; CD15	(31)
Li et al.	12	na	na	122	43 (35)	21 (17)	CD4; CD11b; CD15	(28)
Macedo et al.	16	10 (63)	6 (38)	197	17 (9)	10 (5)	CD11b; CD14; CD15; HLA-DR	(29)
v.d. Velden et al.	27	21 (78)	na	na	na	na	CD11b; CD14; CD15	(26)
Voskova et al.	49	22 (45)	20 (41)	110[a]	41 (37)	30 (27)	na[a]	(33)
Zeijlemaker et al.[b]	33	14 (42)	10 (30)	125	18 (14)	11 (9)	CD7; CD19; CD34	

Numbers of patients or antigens are presented. Proportions are listed between brackets. na, not available.
[a]Number of LAPs, no data on individual antigens.
[b]Unpublished data.

used, is that a small subpopulation of tumor cells with less than 20% aberrant antigen expression is incorrectly determined as "negative." In addition, entire cell populations may only show dim antigen expression and in this way falsely be classified as negative. The potential impact of minor subpopulations and dim antigen expression is outlined further below.

Reduction or Loss of Antigen Expression

Several studies have been performed to investigate antigen losses during disease or treatment. An overview is shown in Table 1. In general, losses of antigen expression were frequently reported (range 5–27%) in AML patients (4,28,29,31,32). Also, in acute promyelocytic leukemia (APL), a distinct subset of AML patients, immunophenotypic losses occur frequently (27). Interesting results have been reported by Baer and colleagues (24) who distinguished AML cases in which either (i) all progenitor or myeloid antigens of a leukemic population were lost or gained between diagnosis and relapse; (ii) isolated changes in antigen expression occurred on an otherwise stable leukemic population; or (iii) both types of changes occurred. Cases in which at least one stable population was present were also analyzed for changes in individual antigens that were not expressed by the initial population of leukemic cells. A change in expression of isolated antigens was most frequently found (82/124 patients). A further 26 patients showed changes of both the leukemic population and isolated changes in antigen expression. The remaining 16 patients showed a change of the leukemic population only (32). These groups were not further subdivided into patients with gains or losses of expression of specific antigens. We anticipate that in some patients with large changes in antigen expression, estimation of MRD levels during follow-up may have been underestimated. Studies that have investigated stability of entire LAPs instead of individual antigens revealed that losses of LAPs may also occur (Table 1). In one of these studies (5), three patients were observed with loss of a LAP, but MRD detection remained successful in two of them, as multiple LAPs were present at diagnosis and part of these were stable. Here, the disappearance of LAPs during disease would have resulted in only one false-negative MRD result (5). We have extended this patient cohort to examine individual antigen changes (Zeijlemaker et al, unpublished; Table 1). Here, we found loss of antigen expression in 9% of the investigated antigens (11/125). The other LAP stability study (33) subdivided 22 patients with a change in LAP expression (Table 1) into one group (n = 10) where MRD detection remained successful due to the presence of one or more stable LAPs. The second group (n = 12) lost all LAPs that were initially present at diagnosis and here, false-negative MRD results are likely to occur (33).

Increment or Gain of Antigen Expression

In several of the 11 abovementioned studies, increments or gains in antigen expression were also studied (Table 2). In one of these studies (33), no LAP could be detected at diagnosis in 12 patients (24%), but 7 of them (58%) had acquired at least one new LAP upon relapse. In the studies addressing individual antigens, increments or gains in expression were also regularly observed (range 6–18%, Table 2) (28,29,31,32). Several studies revealed gains of CD117 (26,27,31) and CD33 (27,32). A variety of markers show both gains and losses, for instance CD4 (28,29,32), CD7 (31), CD11b (26,28,29,32), CD13 (27,29,31,32), CD15 (26,28,29,31), CD56 (28,32), HLA-DR (26,27,29), and CD34 (26,27,29,31,32). One of the pediatric AML studies (31) showed that more patients displayed an immature phenotype at relapse with increased expression of CD34 and/or CD117 at relapse compared to diagnosis (31).

Table 2
Overview of Studies Describing Increments or Gains of Antigen Expression

	Patients		Antigens			
	Total number	No. with a gain	Total number	No. of gains	Frequently gained	Ref.
Baer et al.	136	na	2211	143 (6)	CD4; CD13; CD33; CD34	(32)
Dimov et al.	9	na	na	na	CD33; CD34; CD117; HLA-DR	(27)
Kern et al.	11	3	na	na	na	(4)
Langebrake et al.	48	30 (63)	428	54 (13)	CD7; CD13; CD34; CD117	(31)
Li et al.	12	na	122	22 (18)	CD10; CD11b; CD56	(28)
Macedo et al.	16	5 (31)	197	7 (4)	CD4; CD11b; CD13; CD15; CD34	(29)
v.d. Velden et al.	27	na	na	na	CD34; CD117; HLA-DR	(26)
Voskova et al.	49	9 (18)	110[a]	11 (10)	na[a]	(33)
Zeijlemaker et al.[b]	33	7 (21)	125	7 (6)	CD34	

Numbers of patients or antigens are presented. Proportions are listed between brackets. na, not available.
[a]Number of LAPs, no data on individual antigens.
[b]Unpublished data.

The largest study in this respect also addressed survival; no significant difference in disease-free survival time between patients with and without immunophenotype changes was found (32).

Interim Conclusion: Immunophenotypic Changes

Despite the fact that differences in antigen expression levels between studies may be due to technical aspects, like differences in analysis strategies or sample preparation, there is convincing evidence for the occurrence of reduction or disappearance of antigen expression and increments or gains in antigen expression in AML, with possible consequences for MRD detection. Although the available data are limited and multiple antigens have been investigated, several studies have demonstrated frequent losses of CD11b (26,28,29,32), CD14 (26,29,32), and CD15 (26,28,29,31). In addition, frequent gains of CD34 (26,27,29,31,32) and CD117 (26,27,31) have been demonstrated. These results suggest that the immunophenotype at relapse might be more immature compared to diagnosis.

MOLECULAR CHANGES

Because of its sensitivity, RQ-PCR is the method of primary choice for detection of residual cells in part of the AML cases. Here, molecular MRD detection may provide prognostic information (12,16,34–36). Similar to flow cytometric MRD studies, relapses may occur in patients with low levels of molecular MRD (36,37). Knowledge of stability of such molecular aberrancies during treatment and follow-up is essential.

Losses of Cytogenetic and/or Molecular Aberrancies

Several studies have investigated cytogenetic losses during disease or treatment and found that karyotype regression can take place during disease/treatment (Table 3, range 0–11%) (22,27,32,33,38–40). This regression implies that at relapse, less aberrancies or less complex aberrancies are present compared to karyotype at diagnosis (Fig. 1). Interestingly, in a cohort of APL patients no regression of the initially aberrant karyotype was found (27). Moreover, some studies even observed both loss of a cytogenetic aberrancy and emergence of a novel cytogenetic aberrancy in the same patient (Table 3) (32,33,38,39). Furthermore, it has been demonstrated that patients with an unfavorable karyotype at diagnosis were more susceptible to a change compared to all other patients (38). Others have found that patients who

Table 3
Overview of Studies on Losses and Gains of Cytogenetic Aberrancies

	Total number of patients	No. with a change	Regression	Evolution	Regression and evolution	Distinct aberrancy[b]	Ref.
Baer et al.	72	40 (56)	6 (8)	28 (39)	5 (7)	1 (1)	(32)
Dimov et al.	10	4 (40)	0 (0)	4 (40)	0 (0)	0 (0)	(27)
Hur et al.	23	11 (48)	na	4 (17)	na	7 (30)	(30)
Kern et al.	117	44 (38)	10 (9)	29 (25)	4 (3)	1 (1)	(38)
Schmidt-Hieber et al.	18	12 (67)	2 (11)	6 (33)	4 (22)	0 (0)	(39)
Schnittger et al.	25	9 (36)	1 (4)	8 (32)	0 (0)	0 (0)	(40)
Schnittger et al.	61	16 (26)	4 (7)	8 (13)	0 (0)	4 (7)	(22)
Voskova et al.	49	10 (20)	2 (4)	7 (14)	1 (2)	0 (0)	(33)
Wang et al.	60[a]	30 (50)	na	30 (50)	na	na	(53)

Number of patients (percentage). na, not available.
[a]Normal karyotype patients.
[b]Distinct aberrancy: An abnormal karyotype found in the relapse material with completely different aberrancies as compared to the abnormal karyotype at diagnosis.

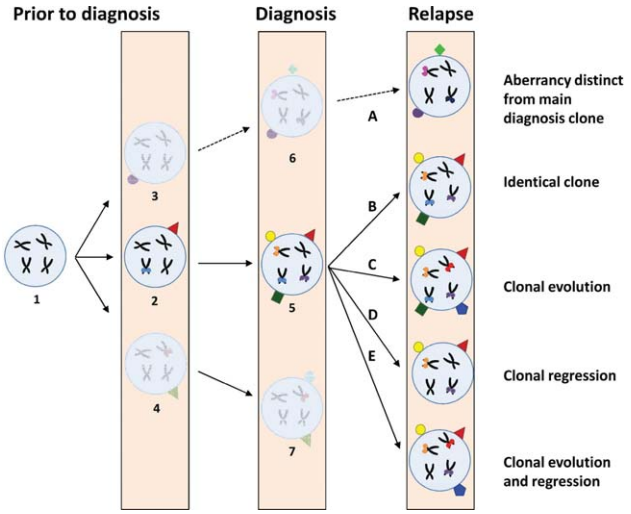

FIG. 1. Dynamics of molecular and immunophenotypic changes from diagnosis to relapse. Both the pre-leukemic (cells 2–4) and diagnostic clones (cells 5–7) consist of heterogeneous groups of cells with complex profiles of aberrancies (90). Molecular aberrancies are represented by symbols in the chromosomes and immunophenotypic aberrancies by symbols on the cell surface. Cell 5 at diagnosis represents the main diagnosis leukemic clone, which develops from a hypothetical ancestral clone without detectable abnormalities (cell 1). Often leukemic cells at relapse may be derived from this primary clone (line B). However, both clonal evolution and regression may occur during disease. In case of clonal evolution, the clone at relapse still harbors the initially present molecular aberrancies, while it has acquired new aberrancies (line C). In case of clonal regression, some of the initially present aberrancies are lost (line D), while in some relapse cases both clonal evolution and regression occur (line E). In cases C–E immunophenotypic changes may hypothetically parallel the molecular changes. In a minority of cases molecular and immunophenotypic aberrancies are detected at relapse that are distinct from the main clone at diagnosis (line A) (30,32,38). These distinct relapse specific aberrancies could have evolved from a "pre-leukemic" clone (90,92), represented here by cell 3 and may be detectable at diagnosis as a minor clone that may have undergone some additional changes (cell 6). The outgrowth of small molecularly defined clones may be accompanied by immunophenotypic aberrancies that are distinct from the primary clone at diagnosis (54). During this outgrowth (line A), such minor populations may undergo changes similar to cell 5 (for reasons of simplicity, not shown in this figure). Other minor clones (represented by cell 7) may undergo similar changes or extinct after diagnosis. Figure adapted from (93).

experienced a change in karyotype were significantly younger as compared to patients without a change (39). This suggests that younger patients may be more prone to develop karyotype changes during disease or treatment. However, in these latter analysis four B-cell precursors ALL patients were included with no possibility to exclude these from analysis and of whom three experienced karyotype changes (39). Also, this contrasts with another AML study in which no correlation between age and karyotype changes was found (38).

Besides karyotypic changes, changes in molecular aberrancies may occur (Table 4). For instance, this has been studied for *FLT3-ITD* or *FLT3* length mutations. Although initially stability has been reported (40), several research groups have subsequently demonstrated instability of *FLT3-ITD* during disease, with a considerable number of cases in which the *FLT3-ITD* mutation had changed or disappeared at relapse (16,33,41–45). Therefore, the use of *FLT3-ITD* as molecular MRD marker has serious limitations. In some studies (43,46), multiple mutations were screened for at diagnosis and relapse, and losses of various molecular aberrancies were observed (Table 4). No losses were observed for *KIT*, *WT1*, and *CEBPA* mutations (43). The results concerning *WT1* in this study are in line with a second study (47) but contradict a third study in which such losses were seen (48). Although losses of *NPM1* mutations have been described (13,43,46), most studies so far have found *NPM1* to be a stable mutation (22,49–54). Therefore, contrary to earlier described cytogenetic and molecular aberrancies, due to its stability, *NPM1* mutation, if present at diagnosis, is supposed to be a very suitable marker for MRD detection. Current thoughts are that *NPM1* is a possible driver mutation that tends to cause clonal expansion (55).

Increments in Cytogenetic and/or Molecular Aberrancies

Besides cytogenetic and molecular losses, new aberrancies can also be acquired during disease or treatment. In several studies, karyotype evolution (i.e., detection of a more complex karyotype or an additional cytogenetic aberrancy at relapse) frequently was reported during treatment and follow-up (range 13–50% between studies; Table 3) (22,30,32,33,38–40,53). In APL patients, evolution of the t(15;17)(q22;q11) karyotype was observed in 40% of the patients at first relapse (27). Besides cytogenetic evolution, the emergence of new molecular aberrancies can occur as well. In the few studies that screened for multiple mutations at diagnosis and relapse the emergence of *FLT3-ITD*, *RAS* (43,46), *FLT3-D835*, *CEBPA*, *WT1* (43), and *TP53* (46) mutations was reported. Similar results were found by others for *WT1* (47,48) and *FLT3* mutations (33,41,42,53).

Cytogenetic instability is associated with an unfavorable outcome. Time to relapse was significantly shorter in patients with a karyotypic evolution as compared to those with a stable karyotype or with regression of an initially aberrant karyotype (38). Others found no differences in median overall survival (OS) between patients with and without karyotype changes (39). Similarly, it was found that OS and time to relapse did not differ between patients with stable normal karyotypes and those with initially normal karyotype that evolved to an aberrant one (53). However, event-free survival was significantly shorter for the latter (53). Contradictory results were also seen for instability at the molecular level: patients with a *FLT3-ITD* mutation that was already present at diagnosis or gained at relapse had a significantly shorter time to relapse than patients with $FLT3^{wt}$ at diagnosis or *FLT3-ITD* lost at relapse (43). Similar results have been found by Warren et al. (45). However, no difference in time to relapse was found by others between patients who acquired a *FLT3* mutation versus $FLT3^{wt}$ patients (42).

Interim Conclusion Molecular and/or Cytogenetic Changes

Overall, different studies have demonstrated the instability of different types of molecular and cytogenetic aberrancies. As most of the molecular MRD markers are

Table 4
Overview of Studies Concerning Losses and Gains of Molecular Aberrancies in AML Patients

	Total number	No. of patients with a change	Loss	Type	Gain	Type	Ref.
Bachas et al.	69	26	13	RAS [7] FLT3-ITD [4] FLT3-D835 NPM1	13	WT1 [5] RAS [4] FLT3-ITD [2] FLT3-D835 CEBPA	(43)
Chou et al.	22[a]	0	0		0		(49)
Chou et al.	30[b]	7	5	FLT3-ITD [5]	2	FLT3-ITD[c] [2]	(16)
Cloos et al.	80	14	4	FLT3-ITD [4]	10	FLT3-ITD [5] FLT3-ITD[c] [5]	(41)
Dvorakava et al.	10[a]	0	0		0		(51)
Haferlach et al.	31[a]	0	0		0		(50)
Hollink et al.	39	6	0		6	WT1 [6]	(47)
Hou et al.	16[d]	5	3	WT1 [3]	2	WT1 [2]	(41)
Kristensen et al.	20[e]	0	0		0		(52)
Kottaridis et al.	44	10	5	FLT3-ITD [5]	5	FLT3-ITD [2] FLT3-ITD[c] [1] FLT3-D835 [2]	(42)
Papadaki et al.	21[a]	2	2	NPM1 [2]	0		(13)
Schiller et al.	6[b]	1	0		1	FLT3-ITD[c] [1]	(44)
Schnittger et al.	25	0	0		0		(40)
Schnittger et al.	80	17	3	FLT3-LM [3]	14	FLT3-LM [14]	(22)
Suzuki et al.	39	11	6	NPM1 [2] NRAS [2] FLT3-ITD TP53	6	FLT3-ITD [4] NRAS TP53	(46)
Voskova et al.	48	7	4	FLT3-LM [2] FLT3-D835 [2]	3	FLT3-LM [3]	(33)
Warren et al.	3555	42	6	FLT3-ITD [6] FLT3-D835[1]	36	FLT3-ITD [25] FLT3-D835[18]	(45)
Wang et al.	12	2	0		2	FLT3-D835 FLT3-ITD	(53)

Number of patients and [number of cases].
[a]*NPM1* mutation.
[b]*FLT3* mutation.
[c]Original *FLT3-ITD* mutation lost and gain of a *FLT3-ITD* mutation with a different length as compared to diagnosis.
[d]*WT1* mutations.
[e]*NPM1* mutation and *WT1* overexpression.

linked to good risk AML, this also emphasizes the need for flow cytometric residual disease in poor risk patients. It is likely that whole genome sequencing will contribute to unraveling the clonal evolution process including identification of passenger and driver mutations. Furthermore, it will probably give more insight into the influence of clonal evolution and regression on clinical outcome.

CORRELATION BETWEEN IMMUNOPHENOTYPE AND MOLECULAR/CYTOGENETIC PHENOTYPE

As outlined above, both immunophenotypic and molecular or cytogenetic changes may occur during follow-up and potentially lead to false-negative MRD results when diagnosis parameters are solely used as reference. The occurrence of both immunophenotypic and molecular or cytogenetic changes between diagnosis and relapse raises the possibility of a close relationship between them. If so, immunophenotyping may be used to guide identification of molecular and cytogenetic aberrancies or vice versa. Moreover, the detection of newly arising immunophenotypes and/or molecular or cytogenetic aberrancies would contribute to more specific residual disease detection. In this section an overview of literature on correlations between immunophenotype and molecular or cytogenetic aberrancies is provided. We focused on those aberrancies that are considered most suitable for MRD detection.

t(8;21)

In translocation (8;21) the AML1 gene on chromosome 21 fuses with the MTG8(ETO) gene on chromosome 8 to produce the fusion gene *AML1-MTG8*, also called *AML1-ETO*. This phenotype is present in around 5–10% of AML patients (56,57) and is associated with a favorable prognosis (58). Higher expression of CD34, HLA-DR, and MPO, but a reduced expression of CD33, is found in t(8;21) patients as compared to those without this aberration (Table 5) (59,60). Additionally, CD15, CD19 (20,59–61), and CD56 (59–62) expression were also associated with t(8;21). The correlation between

Table 5
Sensitivities and Specificities in t(8;21) AML

Marker	Sensitivity (%)	Specificity (%)	Ref
$CD34^+$	100 (100)	32 (30–33)	(59,60)
$HLA\text{-}DR^+$	100 (100)	19 (18–20)	(59,60)
MPO^+	100 (100)	11 (10–11)	(59,60)
$CD33^-$	35 (31–39)	95 (94–96)	(59,60)
$CD34^+HLA\text{-}DR^+MPO^+$	100	59	(59)
$CD15^+$	89 (88–90)	34 (33–35)	(59,60)
$CD19^+$	80 (72–88)	90 (88–91)	(59,60)
$CD19^+CD34^+$	42	98	(20)
$CD15^+CD19^+$	50	97	(20)
$CD56^+$	65 (54–83)	81 (80–81)[a]	(59–62)
$CD34^+CD19^+CD56^+$	67	100	(59)

Median values (range).
[a]No specificity rates available (62).

t(8;21) and the $CD34^+CD19^+CD56^+$ immunophenotype has also been investigated and a positive predictive value of 100% and a negative predictive value of 99% were found (59). However, the sensitivity rate for this latter combination was only 67% (Table 5). Thus, the immunophenotypes $CD34^+HLA\text{-}DR^+MPO^+$ and $CD34^+CD19^+CD56^+$ are suggested to be highly predictive for t(8;21) (59).

Inv(16)/t(16;16)

The *CBFβ-MYH11* fusion gene is acquired due to an inversion of chromosome 16, inv(16)(p13q22), or a balanced translocation, (16;16](p13;q22) (63). This fusion gene is present in around 10% of AML patients and associated with good prognosis (58). The presence of inv(16)/t(16;16) is associated with aberrant positivity for CD2 (64–66). However, CD2 expression lacks specificity for inv(16)/t(16;16) as it is also present in other AML cytogenetic subgroups (20,67).

t(15;17)

In this distinct subgroup of AML patients, the fusion gene *PML-RARα* is created from part of the promyelocytic leukemia (PML) gene and the retinoic acid receptor (RAR) gene. The incidence of this fusion gene varies between 5 and 30% in AML patients, depending on geographical conditions, and is associated with favorable prognosis. It is also associated with low or absent expression of CD34, HLA-DR, CD11a, CD11b, CD15, CD18, and CD65. It has been suggested that the simple combination of HLA-DR, CD11a, and CD18 provides a reliable combination to distinguish acute promyelocytic leukemia (APL) from other AML subtypes (68,69). Furthermore, CD117 is positive in most APL cases, although with variable intensity (68). In addition, t(15;17) is characterized by high CD33 expression but variable CD13 expression (68,70,71). Lack of or low expression levels of CD34, HLA-DR, CD11a, CD11b, CD15, CD18, and CD65, but positivity for CD117 and CD33, are therefore suggested as a reliable combination to diagnose APL.

FLT3 Mutation

Although different *FLT3* mutations exist, the most common is the *FLT3*-internal tandem duplication (*ITD*). This *ITD* is thought to cause a constant activation of the tyrosine kinase receptor, resulting in increased cell survival. The *FLT3-ITD* occurs in approximately 23% of adult AML patients and is associated with poor prognosis (72,73). *FLT3-ITD* mutated AML is associated with a variable CD34 expression and high expression of the myeloid antigens HLA-DR, CD13, CD33, and MPO (74,75). In addition, expression of CD36, CD11b (75), and CD7 (74) were frequently observed in *FLT3-ITD* cases (Table 6). Furthermore, it has been reported that in T-ALL and T/myeloid mixed phenotype acute leukemia, *FLT3* mutations are associated with CD117 expression, although not very specific since CD117 is also expressed in many other cases. Moreover, in eight patients (seven mixed phenotype and one T-ALL) the $TdT^+/CD7^+/CD13^+/CD34^+/CD117^+$ immunophenotypic profile has been reported to be highly suggestive of *FLT3* mutation, with 100% sensitivity and 94% specificity (76). It has yet to be determined whether this specific phenotype is also associated with *FLT3-ITD* in AML.

NPM1 Mutation

The *NPM1* mutation, present in approximately 35% of AML patients, occurs most frequently in exon 12 of the *NPM1* gene. This results in loss of one or both C-terminal tryptophan residues and leads to an aberrant localization of the protein, i.e., in the cytoplasm instead of the nucleus. In addition, an *NPM1* mutation with concomitant absence of a *FLT3* mutation is associated with favorable prognosis. *NPM1* mutated AML is associated with low CD34 expression (sensitivity 94%, specificity 72%), high CD33 expression (50,77–79), and low HLA-DR expression (79) (Table 7). In line with that, one study reported the absence of *NPM1* mutations among $CD34^+CD33^-$ patients (79). There were no differences in CD33 expression observed between *NPM1*-mutated patients with and without concomitant *FLT3* mutations (78). However it has been demonstrated that CD56 positivity was more common in $NPM1^+FLT3^{wt}$ AML cases than in $NPM1^+FLT3\text{-}ITD^+$ cases (80). In most *NPM1*-mutated AML cases CD13, CD110 (thrombopoietin), CD117, and CD123 are found positive (80).

Table 6
Sensitivities and Specificities in $FLT3\text{-}ITD^+$ *AML*

Marker	Sensitivity (%)	Specificity (%)	Ref
$CD34^+$	67 (42–73)	41 (37–44)[a]	(74,75,91)
$HLA\text{-}DR^+$	89 (89)	28[a]	(74,75)
$CD13^+$	93 (89–100)	8 (6–10)[a]	(74,75,91)
$CD33^+$	93 (93–94)	9 (0–18)[a]	(74,75,91)
MPO^+	75 (56–94)	30[a]	(74,75)
$CD7^+$	33 (11–73)	93 (92–94)[a]	(74,75,91)
$CD11b^+$	36	na	(75)
$CD36^+$	58	na	(75)

Median values (range); na, not available.
[a]No specificity rates available (75).

Table 7
Correlation between NPM1*-mutated AML and Antigen Expression*

	CD34	CD117	High expression	Weak/absent expression	Ref.
NPM1-mutated AML	–	+	CD33, CD13, CD110, CD123	HLA-DR	(50,77–80)
NPM1-mutated subgroup with limited differentiation	–	±	CD33, MPO	CD64, CD133, HLA-DR	(81,82)

Furthermore, a new subgroup of *NPM1*-mutated AML patients has been described with limited differentiation and a distinct immunophenotype (81). This subgroup was associated with lack of CD34, CD133, and HLA-DR expression and strong CD33 and MPO expression. In addition, weak expression of CD64 and dim expression of CD117 was also observed in this distinct subgroup (81,82) (Table 7).

CEBPα

CCAAT/enhancer binding protein alpha (*CEBPα*) is a transcription factor involved in the regulation of cellular growth arrest and myeloid differentiation (83). There are two major types of *CEBPA* mutations, including C-terminal mutations and N-terminal mutations. Furthermore, some patients carry biallelic mutations, whereas others are heterozygous for this mutation. *CEBPA* mutations have been reported in 8–19% of normal karyotype AML patients and are associated with favorable prognosis (84). It is associated with strong CD34, CD13, CD33, HLA-DR, CD15, and CD7 expression. Furthermore, there is also an association with low CD14 expression (85).

CORRELATION BETWEEN IMMUNOPHENOTYPIC CHANGES AND MOLECULAR OR CYTOGENETIC CHANGES

Although the actual mechanisms by which phenotype changes occur are unclear, we propose two possibilities: (i) "spontaneous" changes in the primary tumor clone(s) present at the time of diagnosis and occurring during or after therapy and (ii) selection of therapy-resistant subpopulations, already present but not routinely detected at diagnosis, in which mutations harbored in such subpopulations contribute to chemotherapy resistance and outgrowth to relapse. As particular molecular and/or cytogenetic aberrancies may correlate with particular immunophenotypes as discussed above, it may be anticipated that particular mutational "shifts" may be accompanied by specific immunophenotypic "shifts." The outgrowth of such minor subpopulation(s) to relapse with a molecular or cytogenetic character different from the predominant clone(s) at diagnosis could theoretically already be predicted at diagnosis if such a subpopulation can be identified. In a recent study of six patients that evolved from *FLT3-ITD*^{wt} at diagnosis to *FLT3-ITD*$^{+}$ at relapse, mutations could retrospectively be detected in the diagnosis material of four of these cases using patient-specific primers that increased the sensitivity of the approach (86). In another seven patients who experienced differences in mutational status between diagnosis and relapse, mutational status at diagnosis was determined in cell-sorted subfractions. Here, relapse mutations were already present in low frequencies in primitive $CD34^{+}CD38^{-/dim}$ populations (54). It has been demonstrated in an earlier study that $CD34^{+}CD38^{-}$ leukemic stem cells (LSCs) present at diagnosis proved to be relatively therapy resistant and thereby survived therapy which offered the possibility for subsequent outgrowth to relapse (87). In line with that, the frequency of LSCs within the immunophenotypically defined $CD34^{+}CD38^{-}$ stem cell compartment, both at diagnosis and in remission bone marrow, predicted clinical outcome (88). These $CD34^{+}CD38^{-}$ LSCs subpopulations seem therefore highly relevant for the identification, at diagnosis, of subpopulations surviving cytoreductive therapy.

Only few studies have investigated possible correlations between immunophenotypic and molecular/cytogenetic changes. In 36 of 40 patients who experienced a change in karyotype these changes were accompanied by immunophenotypic changes (32). However, in 29 patients immunophenotypic changes were also seen without cytogenetic changes, while molecular aberrancies were not examined. No correlations were found between particular antigen changes and specific cytogenetic changes (32). Others have examined phenotype changes in AML and demonstrated that molecular and/or cytogenetic changes were still found in patients with stable LAP expression at relapse (33). Furthermore, in a minority of patients with an immunophenotypic change, molecular and/or cytogenetic changes were observed as well. In one case (33), a gain of a *FLT3* mutation was accompanied by an increase in $CD34^{+}CD33^{+}CD7^{+}$ cells. These three antigens are all associated with the presence of a *FLT3* mutation (see previous paragraph). In a second case, the emergence of the *FLT3* mutation was accompanied by a gain in $CD34^{-}CD87^{-}CD65^{++}$ cells. No associations between these antigens and *FLT3* mutations are known so far (33). However, as most of the studies focused on the bulk of malignant cells, it can be suggested, in the light of just described explanations of phenotypic changes, that minor subpopulations harboring specific immunophenotypic, molecular and/or cytogenetic aberrancies have already been present at diagnosis to grow subsequently out to full blown relapse. Therefore, future studies should pay attention to minor subpopulations at diagnosis to elucidate the complex mechanisms of phenotypic changes in tumor cells. Possible correlations between immunophenotypic and molecular/cytogenetic changes may be influenced by the coexistence of other aberrancies, for example, *FLT3-ITD* with or without *NPM1*^{wt}, each reported to be

accompanied by specific immunophenotypic aberrancies. Therefore, future studies should focus on the parallel detection of a wide range of immunophenotypic, cytogenetic, and molecular markers, and combinations of these, to assess how these mutually affect each other.

Major technological advances have now made it possible that a cancer genome can be sequenced completely, and whole genome sequencing contributes to the further unrevealing of clonal evolution and selection in AML. Recently, sequencing of eight primary AML samples and matched relapse samples has allowed to determine two different patterns of clonal evolution (89). In the first pattern, the clone that contained the bulk of primary tumor cells gained additional mutations and evolved into the relapse clone. In the second pattern, a minor subclone present at diagnosis survived therapy, gained additional mutations, and grew out to relapse. These results are in line with earlier mentioned hypotheses (54,86) for the occurrence of immunophenotype and molecular/cytogenetic changes. Obviously, it is of high importance to gain more insight into clonal evolution, not only to enable accurate quantitation of residual tumor cells but also to find targets for novel diagnosis or remission treatment strategies.

PERSPECTIVES

Losses of aberrant antigen expression, cytogenetic abnormalities, and/or molecular aberrancies are likely to occur during disease and treatment. These findings have important consequences for both immunophenotypic and molecular MRD detection, as false-negative MRD results can be expected to be the result of such losses. It is important to emphasize that possible differences in tumor instability may exist between primary AMLs and AMLs that evolved from MDS. Especially in the latter case pre-leukemic clones may have different precursor stages with different phenotypes whereby these pre-leukemic clones have the potential to evolve into a malignant population (90). However, studies performed so far do not take this variable into account, and therefore possible differences between de novo AMLs and secondary AMLs on the pattern of phenotypes changes (Fig. 1) remain unclear (4,29,32,59). False-negative results may in part be accounted for by the complete eradication of the major diagnostic population. This pattern may well be accompanied by outgrowth of minor populations present at diagnosis with emergence of "new" immunophenotypes and molecular/cytogenetic aberrancies. Also, such outgrowth may be accompanied by acquirement of new mutations probably resulting in completely new immunophenotypes. It is important to emphasize that losses of aberrant antigen expression do not necessarily result in false-negative MRD results. If, for instance, a partial loss of an MRD marker occurs, but the level is still above the cut-off value, MRD values would be underestimated, but still be defined as positive. Moreover, in case a driver mutation is present at diagnosis (e.g., *NPM1* mutation), subsequent molecular and/or cytogenetic and/or immunophenotypic changes may not affect the characteristics of the founder clone. Whether *NPM1* is indeed such a driver mutation will need verification.

In general, two major approaches to overcome these possible false-negative MRD results can be suggested. One approach would be to measure the most common immunophenotypic and molecular aberrancies both at diagnosis and follow-up instead of measuring only the initially present aberrancies. Measuring the whole panel of aberrancies during follow-up would enable the detection of emerging immunophenotypic and molecular defined subpopulations. In future, whole genome sequencing may be applied once the technique is applicable to identify low frequency tumor cells. The second approach is to use the earlier mentioned "different-from-normal" approach to determine residual disease in AML patients. A great advantage of that approach is that changes of immunophenotypes during course of the treatment can also be detected because this approach enables the identification of MRD cells irrespective of the leukemic phenotype at diagnosis. Although the "different-from-normal" approach is promising, extensive knowledge of normal expression patterns is required. Studies using the "different-from-normal" approach still report 25–30% of false-negative MRD results (23,25). Strikingly, this proportion is similar to the percentage of false-negatives found by others using the LAP approach (2,5–9). A future approach would be to try to predict which subpopulation(s) present at diagnosis is/are likely to survive therapy and grow out to relapse. To that end, putatively relevant subpopulations that are present in low frequencies at diagnosis should be investigated. It is generally thought that LSCs play a role herein, and for the prospective identification of therapy surviving cells special focus may thus be on the $CD34^{+}CD38^{-}$ LSCs subpopulations. It should be kept in mind that those LSC populations may not only play a role in selection of new clones but also in therapy selection of the main clone already present at diagnosis, thereby explaining cases in which no differences are seen between diagnosis and relapse. Furthermore, putatively relevant subpopulations other than $CD34^{+}CD38^{-}$ may prospectively be identified based on low frequency aberrant immunophenotypes at diagnosis. Early detection of an upcoming subclone would not only improve risk stratification, but it would also offer new treatment strategies. For instance, if future targeted treatments are feasible, the detection of an upcoming subclone, which harbors newly acquired mutations and lost an initially present mutation, could direct the choice for type of targeted treatment toward the upcoming clones. Therefore, present studies should be addressed to prospectively identify therapy-resistant subclones at diagnosis. It should be mentioned that this approach, whereby small aberrant subpopulations at diagnosis are identified, will not identify emerging subpopulations with new mutations that occur as a direct result of the treatment. For that approach whole genome sequencing will be necessary.

Overall, this review concludes that the bulk of AML cells frequently undergo phenotype changes and in part

of the cases these changes are likely to limit accurate immunophenotypic and molecular MRD detection. Although strategies to reduce these limitations are available, we propose that further insight in the process of preferential survival of subpopulations and clonal evolution is required to improve both risk stratification and patient outcome.

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