Detection of Mutant *NPM1* mRNA in Acute Myeloid Leukemia Using Custom Gene Expression Arrays

Martin H. van Vliet,¹ Belinda Dumeé,¹ Erik Simons,¹ Lars Bullinger,² Konstanze Döhner,² Hartmut Döhner,² Henk Viëtor,¹ Bob Löwenberg,³ Peter J.M. Valk,³ and Erik H. van Beers¹

Mutations in the gene encoding nucleophosmin (*NPM1*) carry a prognostic value for patients with acute myeloid leukemia (AML). Various techniques are currently being used to detect these mutations in routine molecular diagnostics. Incorporation of accurate *NPM1* mutation detection on a gene expression platform would enable simultaneous detection with various other expression biomarkers. Here we present an array-based mutation detection using custom probes for *NPM1* WT mRNA and *NPM1* type A, B, and D mutant mRNA. This method was 100% accurate on a training cohort of 505 newly diagnosed unselected AML cases. Validation on an independent cohort of 143 normal-karyotype AML cases revealed no false-negative results, and one false positive (sensitivity 100.0% and specificity 98.7%). Based on this, we conclude that this method provides a reliable method for *NPM1* mutation detection. The method can be applied to other genes/mutations as long as the mutant alleles are sufficiently highly expressed.

Introduction

ACUTE MYELOID LEUKEMIA (AML) is characterized by an accumulation of immature myeloid cells in the bone marrow (Löwenberg *et al.*, 1999). The genetic background of AML is highly heterogeneous, and cytogenetic analysis is commonly used to separate patients with AML into favorable, intermediate, and unfavorable prognostic subtypes (Grimwade *et al.*, 1998). Further stratifications can be made based on the presence or absence of particular gene mutations (Theilgaard-Mönch *et al.*, 2011) such as *NPM1* (Falini *et al.*, 2005), *FLT3*-ITD (Kottaridis *et al.*, 2001), *CEBPA* (Wouters *et al.*, 2009), and *DNMT3A* (Ribeiro *et al.*, 2012), or changes in gene expression (Bullinger *et al.*, 2004; Valk *et al.*, 2004).

Cytoplasmic *NPM1* protein was detected in 35% of AML cases (Falini *et al.*, 2005), and was linked with insertions (often 4 base pairs) at position 960 (in exon 12) causing a frameshift in the nuclear localization signal domain (Falini *et al.*, 2005). In cytogenetically normal AML, 50%–60% of cases were found to harbor *NPM1* mutations (Boissel *et al.*, 2005; Döhner *et al.*, 2005; Schnittger *et al.*, 2005; Suzuki *et al.*, 2005; Thiede *et al.*, 2006). The *NPM1* mutations are always heterozygous, and the most frequently occurring have been named type A (TCTG, 80%), type B (CATG, 10%), and type D (CCTG, 5%) (Falini *et al.*, 2005). The remaining *NPM1* mutations are comprised of at least 20 other 4-bp insertions (including type C) at the same position, and a minority of insertions at other locations in

exon 12 (Falini *et al.*, 2005; Thiede *et al.*, 2006). The combination of having an *NPM1* mutation and absence of *FLT3*-ITD mutations was found to confer a favorable risk (Döhner *et al.*, 2005; Gale *et al.*, 2005; Verhaak *et al.*, 2005; Schneider *et al.*, 2012).

Several methods can be employed to detect *NPM1* mutations. Immunostaining led to the initial discovery of excess abundance of *NPM1* protein in the cytoplasm (Falini *et al.*, 2005), but has been shown to be not fully concordant with the mutant *NPM1* status as determined by sequencing (Mattsson *et al.*, 2010). Alternatively, *NPM1* mutations can be screened for using fluorescence resonance energy transfer (Scholl *et al.*, 2007), denaturing high-performance liquid chromatography (dHPLC) (Ammatuna *et al.*, 2005), allele-specific Q-PCR (Gorello *et al.*, 2006), or gene expression profiling (Verhaak *et al.*, 2005). Most of these methods are not standardized, not for *in vitro* diagnostic (IVD) use, and none of them is integrated into an AML diagnostic platform that also detects several other relevant markers.

Reliability and efficiency are important in clinical molecular diagnostics. Streamlining diagnostics by combining multiple tests into a single assay could contribute toward satisfying stringent quality demands. A gene expression microarray assay for AML can be used to detect t(8;21), inv(16)/t(16;16), t(15;17) (Valk *et al.*, 2004), and *CEBPA* double mutants (Wouters *et al.*, 2009) based on their typical gene expression signatures. Previous evaluation of gene expression-based

¹Skyline Diagnostics BV, Rotterdam, The Netherlands.

²Department of Internal Medicine III, University of Ulm, Ulm, Germany.

³Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands.

prediction of the *NPM1* mutation, however, showed limited specificity [82.7% specificity, (Verhaak *et al.*, 2005)]. Therefore, we set out to develop an alternative approach, yet compatible with a gene expression array. This led to the development of custom-designed probes and algorithms for the array-based mutation detection of *NPM1* mutations. The algorithm was developed on a large cohort of AML cases, and subsequently validated on an independent cohort of AML cases.

Materials and Methods

Gene expression array

A customized Affymetrix GeneChip microarray was designed (AMLprofiler™). For *NPM1* detection, seven perfect match (PM) and seven mismatch (MM) probes for the WT and three mutation types were included (type A, type B, and type D). These seven probe probes were tiled at -4, -2, -1, 0, +1, +3, and +4 nucleotides relative to the 960- (exon 12) insertion hotspot of the *NPM1* gene. The seven PM probes for the WT and type A mutant are shown in Figure 1A. This tiling setup is similar to Affymetrix single-nucleotide polymorphism (SNP) platform designs (Di *et al.*, 2005). All probes were 25-mer long, and MM probes are the same as PM probes, although with their middle position flipped. In total, 56 probes were used for *NPM1* detection [$7 \times 4 \times 2 = 56$; seven tiled probes, four variants (WT and three mutations), and both PM and MM probes].

Datasets

As a training cohort, we employed the blood or marrow specimens from 505 patients with AML, who had been enrolled in the Dutch–Belgian Hematology–Oncology Cooperative group protocols -04, -04A, -29, and -42 (HOVON-SAKK, Erasmus Medical Center, Rotterdam) (Valk *et al.*, 2004; Wouters *et al.*, 2009). Sample processing and quality control were performed as previously described (Verhaak *et al.*, 2005). Mutations in *NPM1* exon 12 were determined by cDNA amplification using polymerase chain reaction (PCR) with specific

primers. PCR products were subsequently subjected to dHPLC using a Transgenomics WAVE dHPLC system. All samples with an abnormal dHPLC profile were Sanger-sequenced to determine the type of the *NPM1* mutation (Verhaak *et al.*, 2005).

As an independent validation cohort, we employed 143 AML cases with a normal karyotype, collected by the German–Austrian AML Study Group (AMLSG; Ulm University) (Kohlmann *et al.*, 2010; Taskesen *et al.* 2011). Sample processing and quality control were performed as previously described (Kohlmann *et al.*, 2010; Taskesen *et al.* 2011). *NPM1* exon 12 was amplified using a PCR with specific primers. PCR products were subjected to Sanger sequencing, providing the mutation status (Döhner *et al.*, 2005).

Table 1 shows an overview of the *NPM1* mutation status of all samples in the training and validation cohorts. All *NPM1* mutations were found to be heterozygous. *NPM1* non-ABD indicates a mutant, but not type A, B, or D. In this study, we consider *NPM1* A, B, and D as the positive group (collectively referred to as *NPM1* ABD), and all remaining cases as negative.

Hybridization cocktails were obtained for all samples in the training and validation cohort, and were hybridized onto the AMLprofiler platform. The datasets are available at the Gene Expression Omnibus (National Center for Biotechnology Information) under accession number GSE42194 (training) and GSE42200 (validation).

All patients provided written informed consent in accordance with the Declaration of Helsinki.

Sequencing *NPM1* from hybridization cocktails

For confirmation purposes, we also sequenced the *NPM1* mutation status directly from the obtained hybridization cocktails. cRNA was extracted from the hybridization cocktails using RNABee (Bio-connect), and cDNA was synthesized using Superscript II (Invitrogen) and random hexamers (Roche). The fragments were amplified with *NPM1* exon 12-specific primers. Direct sequencing was performed using Big Dye terminator chemistry ver.3.1 (Applied Biosystem). The

FIG. 1. (A) Example showing the seven 25-mer Perfect Match probes tiled across the *NPM1* insertion site (relative to nt position 960). The top shows the probes that are tiled across the *NPM1* WT sequence, whereas the bottom shows the probes tiled across the *NPM1* A variant. (B) Heatmaps showing the intensities (log2 scale) of the 56 *NPM1*-associated probes for four representative examples of an *NPM1* WT, *NPM1* A, *NPM1* B, and *NPM1* D case. Color images available online at www.liebertpub.com/gtmb

TABLE 1. OVERVIEW OF CASES IN THE TWO COHORTS, AND DISTRIBUTION OF *NPM1* MUTATIONS

<i>NPM1</i> status	Label	Training cohort		Validation cohort	
		n	%	n	%
<i>NPM1</i> WT	Neg	350	69.3%	72	50.3%
<i>NPM1</i> non-ABD	Neg	10	2.0%	5	3.5%
<i>NPM1</i> A (TCTG)	Pos	123	24.4%	56	39.2%
<i>NPM1</i> B (CATG)	Pos	15	3.0%	8	5.6%
<i>NPM1</i> D (CCTG)	Pos	7	1.4%	2	1.4%
Total		505	100.0%	143	100.0%

fragments were loaded on an Applied Biosystem 3130XL automated sequencer and analyzed with DNA Sequencing analysis (ver.5.3) and SeqScape (ver.2.6) software (Applied Biosystem). The retrieval of cRNA fragments as a substrate for reliable bi-directional Sanger sequencing had been validated using positive (*NPM1* A cell line; OCIAML3 hybridization cocktail) and negative (*NPM1* WT cell line; ME-1 hybridization cocktail) controls.

Dynamic model algorithm and data analyses

The Dynamic Model algorithm (DM, Di *et al.*, 2005) was originally developed for SNP detection purposes, and was used in the array-based mutation detection method presented in this study. The DM model considers two alleles (in this case WT and a mutant), and evaluates the possible conformations of these two alleles. To this end, probe quartets were formed consisting from all probes at the same tiling position (e.g., -4nt), that is, the WT PM, WT MM, mutant PM, and mutant MM probes. Thus, seven probe quartets were formed, one for each tiling position. For each probe quartet, separately, four models were evaluated reflecting the following four genotypes (see Fig. 2):

- Model 0: WTWT, homozygous wild type
- Model 1: WTMut, heterozygous wild type-mutant
- Model 2: MutMut, homozygous mutant
- Model -1: No call (*Null* model)

For each model, a log-likelihood was calculated using the probe intensities/standard deviations (assuming that the data are independent and identically [normal] distributed). Thus, four log-likelihoods were obtained for each of the seven probe quartets. Next, the log-likelihoods were corrected to take hybridization efficiencies and levels of nonspecific cross hybridization into account, leading to a score *S*. After that, a one-sided Wilcoxon signed-rank test was applied on the seven *S* scores for a given model, resulting in a *p*-value for each model. A low *p*-value implies that that model is highly likely, given the data observed. The model that had the lowest *p*-value was returned as the call. In case that this lowest *p*-value was larger than a

predefined threshold (default: 0.33), the call was set to -1. In such an event, none of the models was a good fit to the data.

A difference between the previous applications of the algorithm and our current use of the algorithm is that there are more than two alleles to be called, namely, WT, A, B, and D. Therefore, for each AML case, the DM algorithm was executed for the comparison WT versus A, WT versus B, and WT versus D, resulting in three genotype calls. The case was then called *NPM1* mutant if at least one model 1 (heterozygous) or model 2 (homozygous mutant) call was returned, and *NPM1* WT otherwise.

Data analysis

We employed the Affymetrix Power Tools (APT) suite (version 1.10.2) for data analysis. The APT contains the DM algorithm; and requires a CEL file (file from a scanned AMLprofiler chip) and threshold (default: 0.33) as input. For optimization purposes, we needed the *p*-values for all four models. However, the APT implementation of the DM algorithm only returns the lowest *p*-value and the associated model. The source code of the algorithm was included in the APT (C++), from which we modified the DM output routine to return the *p*-values for all four models. MatLab R2011a (www.mathworks.com) was used for all subsequent data analyses.

All performances are reported in terms of true positives, false positives (FP), true negatives, and false negatives. In addition, specificities and sensitivities are calculated along with 95% confidence lower limit and upper limit, according to CLSI EP12-A2.

Creating in silico dilution series

Dilution series of samples are commonly employed to determine the limit of detection (LOD) of a test. In the current context, this would imply mixing BM from AML cases. Previously, we have performed *in vitro* mRNA mixing series and compared them with *in silico* mixing series of the AMLprofiler intensity data to show very high concordance for their results (data not shown). We rely on the validity of this approach to determine the LOD for *NPM1* *in silico* by linearly interpolating all intensities/standard deviations of all probes in steps of 1% from two CEL files, one of them *NPM1* mutant and one wild-type. In this way, we obtained 101 CEL files, the two original CEL files, and 99 interpolated variants.

Results

Results for the *NPM1* probes on the training cohort

For the 505 cases from the training cohort, we evaluated the heatmaps of the 56 probes that were tiled across the *NPM1* gene. Representative examples for a WT and the three mutants are shown in Figure 1B. As expected, the *NPM1* WT only

FIG. 2. The four models analyzed by the dynamic model algorithm, showing the assumptions of high and low intensities of a probe quartet.

FIG. 3. The number of correctly detected *NPM1* WT cases (left), and *NPM1* ABD cases (right), when varying the threshold. Results shown for the 505 cases in the training cohort.

shows signal on the *NPM1* WT PM probes. The *NPM1* A and *NPM1* B cases show signal on the *NPM1* WT PM and their corresponding mutant PM probes, owing to their heterozygote genotype. However, for *NPM1* D, the signal is observed on the *NPM1* WT PM, mutant A PM, and mutant D PM probes. Similarly, for *NPM1* A, some signal is seen on the *NPM1* D PM probes, although much lower than the *NPM1* A PM probes. Most likely, the concomitant positive signal on the *NPM1* A and *NPM1* D probes is due to cross hybridization of the *NPM1* A and D mRNA transcripts, as the difference between the A and D insertion is only one base pair.

Optimizing the DM threshold for array-based mutation detection of *NPM1* mutants

The DM algorithm was originally developed for SNP detection at the DNA level. Some assumptions that are valid at the DNA level (e.g., stoichiometry 1 to 1) might not be valid at the mRNA level. Therefore, the default threshold of 0.33 used in the DM algorithm might not be optimal for the current application. Therefore, we varied the threshold from 0.01 to 0.5 in steps of 0.01 (threshold 0 is not tested, as this would imply all cases to be predicted to be WT). At each threshold, we counted the number of correct WT and mutant calls (Fig. 3). For the WT cases, the algorithm appears robust; at all thresholds, the *NPM1* WT cases were correctly detected. For the *NPM1* ABD mutations, all but one case were correctly identified at the default threshold. Therefore, we chose a slightly higher threshold of 0.41 at which all mutants are correctly detected. Thus, using the threshold of 0.41, all 505

cases in the training cohort are correctly detected, that is, with 100% sensitivity and 100% specificity (see Table 2).

Validation of the array-based mutation detection of *NPM1* mutants

A dataset of 143 cytogenetically normal AML cases was used as the independent validation cohort. The array-based mutation detection method employing the DM algorithm with a threshold of 0.41 was applied to this cohort (Table 2). Similar to the results on the training data, high sensitivity (100.0%) and specificity (97.4%) values were achieved on the validation cohort. Nevertheless, two false-positive results were observed in the validation cohort (see Table 2). Therefore, we inspected the heatmaps of those two false-positive cases (Fig. 4) revealing a very strong (case 05PB2316) and a weak (case 46) *NPM1* A pattern. The latter also shows an atypical high intensity for *NPM1* A MM probe 4. Therefore, we hypothesized that this case is in fact a non-ABD mutant with a TCAG insertion (Fig. 1A, probe 4 with middle position flipped results in TCAG); that is, the *NPM1* A mismatch probe 4 functions as a perfect match for this particular mutation. The weak *NPM1* A pattern is most likely due to cross hybridization (similar to the cross hybridization between *NPM1* A and D). Next, we sequenced the fragmented cRNA from the hybridization cocktails of the two FP cases (see the Materials and Methods section). The sequencing confirmed the two cases to be *NPM1* A and *NPM1* non-ABD (TCAG, as hypothesized). This leaves only 1 FP (the non-ABD) relative to the definition of calling the ABD mutants the positive group, see Table 2.

TABLE 2. PERFORMANCE OF THE ARRAY-BASED MUTATION DETECTION METHOD FOR PREDICTING *NPM1* MUTATIONS ON THE TRAINING AND VALIDATION COHORTS

Dataset	n	TP	FP	TN	FN	Sens	95% CI		Spec	95% CI	
							LL	UL		LL	UL
Training data	505	145	0	360	0	100.0	97.4	100.0	100.0	98.9	100.0
Validation data	143	66	2 ^a	75	0	100.0	94.5	100.0	97.4	91.0	99.3

^aSequencing of the hybridization cocktails confirmed one case to be *NPM1* A, and the other to be a non-ABD mutant. This results in 67 TP and 1 FP, corresponding with a sensitivity of 100.0 (95% CI 94.6–100.0) and specificity of 98.7 (95% CI 92.9–99.8).

TP, true positive; FP, false positive; TN, true negative; FN, false negative; CI, confidence interval.

FIG. 4. Heatmaps showing the intensities (log₂ scale) of the 56 *NPM1*-associated probes for the two FP (left panel: case 05PB2316, right panel: case 46) cases in the validation cohort. Color images available online at www.liebertpub.com/gtmb

LOD of the array-based detection of *NPM1* mutants

We created a dilution series of a representative heterozygous *NPM1* A and homozygous *NPM1* WT case, *in silico* in steps of 1% (see the Materials and Methods section). This resulted in *NPM1*-positive calls for samples with at least 43% *NPM1* mutant content.

Discussion

Array-based mutation detection of *NPM1* mutations at the mRNA level is possible, with a validated performance of 100.0% sensitivity and 98.7% specificity. Moreover, this test has been incorporated into an existing diagnostic chip, providing a standardized test. This is an advantage over the most screening methods such as fragment-length analysis (Scholl *et al.*, 2007) and dHPLC (Ammatuna *et al.*, 2005), which are typically not standardized across laboratories. A possible disadvantage of the array-based mutation detection, as it was employed in this study, is its limitation to only detect the *NPM1* A, B, and D mutants constituting ~95% of all *NPM1* mutant cases. Technically, it is possible to expand the chip design to include probes for the other *NPM1* mutants as well.

Typically, gene expression-derived biomarkers validate poorly across datasets without the application of some form of cohort wide corrections (e.g., GCRMA or mean variance normalization). The array-based mutation detection method does not require normalization of the data, and performs excellently across datasets, as shown on the validation dataset that was prepared in a different laboratory. This is an indication that the array-based mutation detection is robust, and is highly reproducible.

NPM1 mutations are an early event and are therefore present in all leukemic cells (Falini *et al.* 2011). Thus, the estimated LOD of 43% should be adequate.

Integration of the array-based mutation detection method for *NPM1* mutants on the AMLprofiler Affymetrix platform provides a single standardized test capable of detecting a variety of clinically relevant biomarkers in AML. Together, this enables a more efficient analysis at diagnosis, with a single sample workup that is less time consuming.

The array-based mutation detection method can be applied to other genes/mutations as well as fusion transcripts. For every gene/mutation, the threshold in the DM algorithm

should be optimized separately, as it is likely that the optimal threshold is gene/mutation specific and depends on allelic load. Probably, mutations, insertions, and deletions can be detected with high accuracy, as long as the position and type of mutation are known, and the transcript is expressed at detectable levels.

Acknowledgments

The authors would like to thank Raoul Frijters, Skyline Diagnostics, Erasmus Medical Center, for fruitful discussions on the work presented. We would also like to thank Pia Burgmer, and Linda de Quartel, Skyline Diagnostics, Erasmus Medical Center, for their assistance in the laboratory. This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine, project BioCHIP grant 03O-102.

Author Disclosure Statement

MHVV researched, designed the experiments, analyzed the data, interpreted data, and wrote the manuscript. PB, BD, ES, HV, LB, KD, and HD performed research. BL and PJMV conceptualized the project, and were involved in research, design of the experiments, data interpretation, and manuscript writing. EHVB performed research, design of experiments, data interpretation, and manuscript writing. MHVV, BD, ES, HV, EHVB, BL, and PJMV have declared ownership interests in Skyline Diagnostics.

References

- Ammatuna E, Noguera NI, Zangrilli, *et al.* (2005) Rapid detection of nucleophosmin (*NPM1*) mutations in acute myeloid leukemia by denaturing HPLC. *Clin Chem* 51:2165–2167.
- Boissel N, Renneville A, Biggio V, *et al.* (2005) Prevalence, clinical profile, and prognosis of *NPM* mutations in AML with normal karyotype. *Blood* 106:3618–3620.
- Bullinger L, Döhner K, Bair E, *et al.* (2004) Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med* 350:1605–1616.
- Di X, Matsuzaki H, Webster TA, *et al.* (2005) Dynamic model based algorithms for screening and genotyping over 100 K SNPs on oligonucleotide microarrays. *Bioinformatics* 21:1958–1963.

- Döhner K, Schlenk RF, Habdank M, *et al.* (2005) Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood* 106:3740–3746.
- Falini B, Mecucci C, Tiacci E, *et al.* (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* 352:254–266.
- Falini B, Martelli MP, Bolli N, *et al.* (2011) Acute myeloid leukemia with mutated nucleophosmin (NPM1): is it a distinct entity? *Blood* 117:1109–1120.
- Gale RE, Hills R, Pizzey AR, *et al.* (2005) Relationship between FLT3 mutation status, biologic characteristics, and response to targeted therapy in acute promyelocytic leukemia. *Blood* 106:3768–3776.
- Gorello P, Cazzaniga G, Alberti *et al.* (2006) Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. *Leukemia* 20:1103–1110.
- Grimwade D, Walker H, Oliver F, *et al.* (1998) The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. *Blood* 92:2322–2333.
- Kohlmann A, Bullinger L, Thiede C, *et al.* (2010) Gene expression profiling in AML with normal karyotype can predict mutations for molecular markers and allows novel insights into perturbed biological pathways. *Leukemia* 24:1216–1220.
- Kottaridis PD, Gale RE, Frew ME, *et al.* (2001) The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* 98:1752–1759.
- Löwenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. *N Engl J Med* 341:1051–1062.
- Mattsson G, Turner SH, Cordell J, *et al.* (2010) Can cytoplasmic nucleophosmin be detected by immunocytochemical staining of cell smears in acute myeloid leukemia? *Haematologica* 95:670–673.
- Ribeiro AFT, Pratcorona M, Erpelinck-Verschueren C, *et al.* (2012) Mutant DNMT3A: a new marker of poor prognosis in acute myeloid leukemia. *Blood* 119:5824–5831.
- Scholl S, Mügge L-O, Landt O, *et al.* (2007) Rapid screening and sensitive detection of NPM1 (nucleophosmin) exon 12 mutations in acute myeloid leukaemia. *Leuk Res* 31:1205–1211.
- Schneider F, Hoster E, Unterhalt M, *et al.* (2012) The FLT3ITD level has a high prognostic impact in NPM1 mutated, but not NPM1 unmutated AML with a normal karyotype. *Blood* 119:4383–4386.
- Schnittger S, Schoch C, Kern W, *et al.* (2005) Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood* 106:3733–3739.
- Suzuki T, Kiyoi H, Ozeki K, *et al.* (2005) Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. *Blood* 106:2854–2861.
- Taskesen E, Bullinger L, Corbacioglu A, *et al.* (2011) Prognostic impact, concurrent genetic mutations and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML: further evidence for CEBPA double mutant AML as a distinctive disease entity. *Blood* 117:2469–2475.
- Theilgaard-Mönch K, Boultonwood J, *et al.* (2011) Gene expression profiling in MDS and AML: potential and future avenues. *Leukemia* 25:909–920.
- Thiede C, Koch S, Creutzig E, *et al.* (2006) Prevalence and prognostic impact of NPM1 mutations in 1485 adult cases with acute myeloid leukemia (AML). *Blood* 107:4011–4020.
- Valk PJM, Verhaak RGW, Beijen MA, *et al.* (2004) Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* 350:1617–1628.
- Verhaak RGW, Goudswaard CS, van Putten W, *et al.* (2005) Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood* 106: 3747–3754.
- Wouters BJ, Löwenberg B, Erpelinck-Verschueren CAJ, *et al.* (2009) Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood* 113:3088–3091.

Address correspondence to:

Erik H. van Beers, PhD

Skyline Diagnostics BV

Faculty Building–Erasmus MC

Suite Ee1983

Dr. Molewaterplein 50

Rotterdam 3015 GE

The Netherlands

E-mail: e.vanbeers@skyline-diagnostics.com