Validation of DNA Methylation Biomarkers for Diagnosis of Acute Lymphoblastic Leukemia

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BACKGROUND: DNA methylation biomarkers capable of diagnosis and subtyping have been found for many cancers. Fifteen such markers have previously been identified for pediatric acute lymphoblastic leukemia (ALL). Validation of these markers is necessary to assess their clinical utility for molecular diagnostics. Substantial efficiencies could be achieved with these DNA methylation markers for disease tracking with potential to replace patient-specific genetic testing.

METHODS: We evaluated DNA methylation of promoter regions of TLX3 (T-cell leukemia homeobox) and FOXE3 (forkhead box E3) in bone marrow biopsies from 197 patients classified as leukemic (n = 95) or clear of the disease (n = 102) by MALDI-TOF. Using a single nucleotide extension assay (methylSABER), we tested 10 bone marrow biopsies collected throughout the course of patient chemotherapy. Using reference materials, diagnostic thresholds and limits of detection were characterized for both methods.

RESULTS: Reliable detection of DNA methylation of TLX3 and FOXE3 segregated ALL from those clear of disease with minimal false-negative and false-positive results. The limit of detection with MALDI-TOF was 1000–5000 copies of methylated allele. For methylSABER, the limit of detection was 10 copies of methylated TLX3, which enabled monitoring of minimal residual disease in ALL patients.

CONCLUSIONS: Mass spectrometry procedures can be used to regionally multiplex and detect rare DNA methylation events, establish DNA methylation loci as clinically applicable biomarkers for disease diagnosis, and track pediatric ALL.

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disease markers to define clinically useful disease thresholds for DNA methylation biomarkers.

MALDI-TOF mass spectrometry technology is a useful and commonly used tool for measuring DNA methylation in research (8, 12, 13). Here we used synthetic DNA methylation reference materials that enabled us to determine the diagnostic thresholds and LOD of novel and established MALDI-TOF MS techniques. As a logical step toward developing this methodology, we targeted 2 gene promoters, TLX3 (T-cell leukemia homeobox 3) and FOXE3 (forkhead box E3), which displayed the largest differential methylation between pediatric ALL patients and nonleukemic controls in validation experiments (8). Two complementary techniques were used to interrogate TLX3 and FOXE3 promoter methylation within pediatric ALL patient samples (8).

Materials and Methods

We collected patient samples (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue7) according to Royal Children’s Hospital Human Research Ethics Committee Approval #27138F. Additionally, we prepared reference materials for the promoter regions of TLX3 and FOXE3 by PCR amplification of FOXE3 and TLX3 promoter regions using human genomic DNA (Sigma) followed by in vitro methylation using M.SssI and serial dilution of the unmethylated and in vitro methylated amplicons. DNA extraction methods for patient samples and bisulfite conversion methods for patient samples and reference materials are described in the online Supplemental Methods file.

Three different schemes, all based on detection by MALDI-TOF mass spectrometry, were used for evaluation of the methylation level of the promoter regions of TLX3 and FOXE3. The standard, commercially available, EpiTYPERM scheme was used for analysis of 197 bone marrow smears from patients classified as leukemic (95 patients) or clear of the disease (88 remission, 12 follow-up, and 2 donors, 102 in total). This measuring system gave signals for both methylated and unmethylated target regions, so results were commonly reported as a ratio of the methylated signal to the sum of methylated and unmethylated signals. During this work, we found that this measuring system was not able to detect changes in methylation levels during patient treatment, and so 2 different primer extension schemes were investigated.

First, the standard, commercially available procedure iPLEX (Sequenom Bioscience) was evaluated, but was also found to be unable to detect changes in methylation levels during patient treatment. The results are reported in the online Supplemental Methods. Second, the commercially available procedure, SABER (Sequenom Bioscience), was adapted for methylation analysis, resulting in an improved LOD. The methylSABER measuring system detects only methylated target regions and, therefore, to normalize the MALDI-TOF output, a ratio of the methylated signal to the sum of signals from extended (methylated) primer to the unextended primer was calculated.

Finally, a multiplex EpiTYPER assay was developed because we envisaged that the DNA methylation analysis of multiple genomic regions would aid patient subtyping. The multiplex EpiTYPER and methylSABER schemes are illustrated in Fig. 1. The resulting methylation levels from all of these measuring schemes are highly dependent on experimental parameters that affect DNA yield and discrimination between methylated and unmethylated DNA and thus can only be related to real DNA copy number ratios by comparison to reference materials with known values.

EpiTYPER ANALYSIS OF BISULFITE-CONVERTED DNA

Bisulfite-converted DNA was amplified by end-point PCR before analysis by the EpiTYPER procedure. Endpoint PCR primers for bisulfite-converted DNA were designed by use of the guidelines outlined in (14) and verified in silico by use of PrimerExpress 3.0 (design shown in online Supplemental Figs. 1 and 2). Converted patient DNA (1 μL, 10 ng/μL, approximately 3300 equivalent copies) was added to 7.5 μL FastStart PCR master mix (Roche), 0.2 μmol/L of each forward and reverse primer, and H2O to a final volume of 15 μL. This corresponded to an equivalent concentration of 220 copies/μL of patient DNA in the PCR reaction mixture. The cycling profile was 95 °C for 10 min, followed by 5 cycles (95 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min), followed by 40 cycles (95 °C for 20 s, 60 °C for 30 s, 72 °C for 2 min), and a final extension at 72 °C for 7 min.

Converted combined TLX3 and FOXE3 reference materials (5 μL, 20 000 copies/μL) were added to 37.5 μL FastStart PCR master mix (Roche), 0.2 μmol/L of each TLX3 and FOXE3 EpiTYPER primer (forward and reverse), and H2O to a final volume of 75 μL. This corresponded to an equivalent concentration of the TLX3 and FOXE3 reference materials of 1300 copies/μL in the reaction mixture. The cycling profile was 95 °C for 10 min, followed by 40 cycles (95 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min), and final extension at 72 °C for 7 min. The cleavage pattern for the multiplex reaction was determined in silico by use of the Seque-

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10 Human genes: TLX3, T-cell leukemia homeobox 3; FOXE3, forkhead box E3.
nom EpiTYPER input file sequence and MassArray package of the R statistical program (see online Supplemental Figs. 3 and 4) (17).

After amplification, a subsample of the reaction mixtures (5 μL) was incubated with 2 μL shrimp alkaline phosphatase (SAP) at 37 °C for 40 min followed by heat inactivation at 85 °C for 5 min to convert remaining dNTPs to the corresponding nucleosides. For EpiTYPER analysis, the mixtures were then subjected to RNA transcription and RNase cleavage before MALDI-TOF analysis following the manufacturer’s instructions. RNase cleavage produces short RNA fragments independent of the methylation pattern of the original DNA. The molecular weight of fragments that map to a DNA sequence containing CpG sites increases by 16 Da for

Fig. 1. Procedures used for DNA methylation analysis.
Bisulfite-converted DNA serves as template for the multiplex EpiTYPER and methylSABER procedures. The multiplex EpiTYPER procedure analyzes multiple genomic regions for DNA methylation by use of a multiplex PCR, in vitro transcription, and amplicon cleavage to produce unique methylated and unmethylated fragments that are interrogated by MALDI-TOF MS. The methylSABER procedure uses primer extension chemistry across methylated CpG loci to produce a G-extended assay that is interrogated by MALDI-TOF MS.
each site methylated compared to the molecular weight of the unmethylated fragment.

**MethylSABER ANALYSIS OF CONVERTED DNA**

Bisulfite-converted DNA was amplified by use of endpoint PCR before primer extension reactions. The PCR before methylSABER analysis comprised 25 μL FastStart PCR master mix (Roche), 0.2 μmol/L TLX3 forward and reverse primer used for EpiTYPER, 1 μL converted patient DNA (10 000 equivalent copies) or 5 μL TLX3 reference material (100 000 equivalent copies), and H₂O to a final volume of 50 μL. The cycling profile was 95 °C for 10 min, followed by 40 cycles (95 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min), and final extension at 72 °C for 7 min. After PCR, a 5-μL subsample of the reaction mixture was incubated with 2 μL SAP as given for EpiTYPER above.

The TLX3 extension primer was designed to hybridize within the TLX3 PCR amplicon by use of the TYPER 4.0 design software (Sequenom Bioscience) and adjusted manually to limit strand bias introduced by CpG sites within the assay sequence. The 7 μL PCR + SAP mixture was used as extension template. A master mix for 40 methylSABER primer extension reactions was made with each reaction mixture (2 μL) by use of 0.7 μmol/L TLX3 extension primer and 9 mmol/L of each cytosine, thymine, and guanine acyclo-nucleotide terminator (New England BioLabs), 0.7 μL H₂O, 0.2 μL extension buffer, and 0.0205 μLT enzyme (Sequenom Bioscience), giving a 9-μL total reaction volume. The cycling profile was 94 °C for 30 s, 40 cycles [94 °C for 5 s (52 °C for 5 s, 80 °C for 5 s) × 5 cycles], and 72 °C for 3 min.

Cationic resin was added to the extension products to reduce salt ions with incubation at room temperature for approximately 20 min. The product was then spotted (15 nL) onto SpectroChips with the Sequenom nanodispenser and analyzed by MALDI-TOF MS (Sequenom Bioscience).

**MALDI-TOF MS PROCESSING**

The EpiTYPER mass spectrum was processed by EpiTYPER software v1.2 (Sequenom Bioscience). The input file for the multiplex EpiTYPER reaction is given in online Supplemental Fig. 3. After base specific cleavage, each fragment containing at least 1 analyzable CpG site that could be methylated in the original genomic DNA was detected. For each analyzable fragment, the signal intensity of the peak corresponding to the methylated form over the total signal intensity of the peaks corresponding to the methylated and unmethylated forms was reported. The EpiTYPER fraction (EF) refers to the mean of these peak intensity ratios calculated by EpiTYPER software v1.2 for all analyzable (CpG-containing) fragments of an assay. The TYPER 4.0 software (Sequenom Bioscience) developed for single nucleotide polymorphisms was used to evaluate primer extension by analysis of the MALDI-TOF MS spectrum; the signal intensities corresponding to the unextended primer molecular ion and methylated (cytosine) extended primer molecular ion were used in a ratio (Eq. 1) to characterize methylation of the target CpG site within the sample.

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\text{methylated extension} = \frac{\text{cytosine extension}}{\text{UEP} + \text{cytosine extension}}
\]

**Results**

SEgregation of DISEASED AND CLEAR BONE MARROW BY USE OF EF

For patient samples, 12 fragments were measured for FOXE3 covering 19 CpG sites and 13 fragments for TLX3 covering 26 CpG sites. The EF for each target gene from the patient samples (Fig. 2) showed 2 distinct groups of results that could be segregated by use of an EF threshold of 0.20. The great majority of ALL (diseased) bone marrow biopsies had EFs >0.20 for both TLX3 and FOXE3, whereas those who were clear of disease had EFs <0.20. That is, an EF >0.20 for either FOXE3 or TLX3 was diagnostic for the presence of ALL. The exact value of the EF, once above the 0.20 threshold, was not critical for diagnosis since patients with the
disease had EFs ranging continuously from 0.20 to 0.95. This is consistent with the presence of aberrantly methylated DNA as indicative of disease, i.e., reliable detection of any level of methylated DNA may be diagnostic.

Almost 85% of pediatric ALL bone marrows that had an EF greater than the 0.20 threshold had an EF >0.50, implying that a very high proportion of the cell population present in the bone marrow aspirates contained hypermethylated CpG loci at FOXE3 and TLX3 promoters. Furthermore, a change in the magnitude of the relative standard deviation (CV) of EFs was observed for both TLX3 and FOXE3 at about the EF threshold value (Fig. 3). A CV of 33% is indicative of spectroscopy LOD, so in this case the threshold value corresponded to the LOD, supporting the observation that detection of any methylation level was diagnostic of ALL (16). A threshold of EF 0.2 was supported by CV calculations from patient samples (described above) and DNA methylation reference materials (described below).

CANDIDATE CLINICAL SENSITIVITY AND SPECIFICITY
It was possible to calculate candidate clinical sensitivity and specificity ratios by use of an EF of 0.20 as the threshold for diagnosis (Table 1). The ratios must be regarded as candidate, since samples classified as “clear” included those who subsequently relapsed and potentially had residual leukemic blasts (<5%) within the sample (see online Supplemental Table 1). It was not possible to obtain substantial numbers of healthy childhood bone marrow aspirates for this work, so true clinical sensitivity and specificity ratios could not be calculated. Notably, the 2 donor bone marrows recorded EFs ≤0.05 for both FOXE3 and TLX3.

Leukemic bone marrows recording an EF <0.20 were considered false negatives. Seven of the 10 TLX3 false-negative values and 4 of the 9 FOXE3 values were close to the 0.20 threshold. The other values were 0.05 or lower, indicating that these samples had low amounts of methylated target molecules potentially because of a different etiology for these patients. Clear bone marrows recording an EF >0.20 were considered false positives. Eight false positives were reported, recording a FOXE3 EF >0.20, and 4 patients subsequently relapsed, which may validly indicate the presence of residual leukemic cells that contribute to relapse. If this were the case, specificity calculations would not be accurate, hence the caution in the interpretation of candidate ratios, although a reduction in false positives may be anticipated.

| Table 1. Candidate clinical sensitivity and specificity using an EF of 0.20 as the diagnostic threshold. |
|------------------|------------------|------------------|------------------|
|                  | FOXE3 | TLX3 | FOXE3 and TLX3 |
| True positive    | 86    | 85   | 88              |
| False negative   | 9     | 10   | 7               |
| True negative    | 94    | 98   | 97              |
| False positive   | 8     | 4    | 5               |
| Specificity      | 0.92  | 0.96 | 0.95            |
| Sensitivity      | 0.91  | 0.90 | 0.93            |
ANALYSIS OF DNA METHYLATION REFERENCE MATERIAL BY EF

An EF threshold of 0.20 for either FOXE3 or TLX3 achieved high clinical specificity and sensitivity for ALL diagnosis, indicating that reliable detection of even low levels of methylated DNA may be diagnostic. EF was calculated from MS signals from both the methylated and unmethylated fragments, so a change in ratio may be due to either an increase in the relative amount of methylated DNA or a decrease in the relative amount of unmethylated DNA. However, because methylated DNA is the disease marker, we investigated whether this threshold may be lowered to analyze decreased relative amounts of methylated DNA that would enable monitoring of disease clearance. For this purpose, reference material mixtures with total methylated plus unmethylated amounts of 100 000 copies and with methylated template ranging from 100 000 copies to 10 copies were analyzed with the EpiTYPER system.

We designed a multiplex assay that combined TLX3 and FOXE3 in a single MALDI-TOF analysis for this work, since we envisioned that interrogation of multiple markers might be required for patient subtyping. The results (Fig. 4) showed that EF had a positive bias compared to the methylated copy number ratio, and the LOD EF threshold was similar to that found in patient samples. An EF of 0.13 (0.05 SD) for the FOXE3 1% reference material (1000 copies) and 0.15 (0.02 SD) for the TLX3 5% reference material (5000 copies) approached statistical significance from the 0% reference materials (P = 0.045 and 0.055 respectively), indicating an LOD of approximately 1%–5% (1000–5000 copies) methylated template in the reference material mixtures. Furthermore, an CV of approximately 33% was recorded in the 5% DNA methylation reference materials, again indicating technique LOD (Fig. 4B). However, although the EF threshold was similar to that from patient samples, the amount of DNA that gave these EF readings was greater than the amount of patient DNA needed for threshold detection, showing that direct calibration of patient EFs with these values was not possible.

Thus, despite the higher amount of reference material used for the multiplexed EpiTYPER analysis, the slight decrease in LOD compared to patient samples was not sufficient to allow monitoring of disease clearance. Primer extension methods were then examined to evaluate whether improvements in LOD would be sufficient for this purpose.

MONITORING DISEASE CLEARANCE

We investigated primer extension techniques to improve LOD for application in disease monitoring. By use of published base extension chemistry (iPLEX, Sequenom Bioscience), CpG 17 of the TLX3 methylation standard was interrogated; however, the LOD was not improved over EpiTYPER chemistry (see online Supplemental Fig. 5). In genotyping experiments, Ding et al. (20) have shown that restricting the primer extension reaction toward an allele of interest by depleting the nucleotide pool markedly improved LOD. We modified the Sequenom SABER analysis by removing dATP from the primer extension mixture to give an assay that detected only methylated alleles, termed methylSABER.

Fig. 4. EpiTYPER analysis of DNA methylation reference materials.

(A), Comparison of measured EF of the DNA methylation reference material to the copy number concentration. The EF results from TLX3 and FOXE3 analyzed in multiplex show a positive bias compared to the percentage methylation in the reference materials (gray solid line). Samples were analyzed in triplicate. Error bars represent SD. (B), The relative standard deviation of the TLX3 and FOXE3 DNA methylation reference materials. An EF of 0.13 (0.05 SD) for the FOXE3 1% reference material and 0.15 (0.02 SD) for the TLX3 5% reference material is indicative of LOD, represented by a relative standard deviation of 33% (dotted line).
The results of methylSABER analysis for the TLX3 DNA methylation reference material showed that low amounts of methylated DNA were detected (Fig. 5A). This technique was most sensitive for <1000 copies/μL, since the methylated extension (ME) was maximized due to saturation of extension primers above this concentration. Because only duplicate results were available for 1 of the samples, LOD was estimated by use of the range to be a ME of 0.30. The standards were analyzed in at least triplicate with the exception of 1 duplicate (*). Error bars represent range. (B), MethylSABER measurement of TLX3 CpG 17 within patient bone marrow biopsies taken throughout treatment shows a reduction in ME as a patient proceeds through therapy.

The results of methylSABER analysis for the TLX3 DNA methylation reference material showed that low amounts of methylated DNA were detected (Fig. 5A). This technique was most sensitive for <1000 copies/μL, since the methylated extension (ME) was maximized due to saturation of extension primers above this concentration. Because only duplicate results were available for 1 of the samples, LOD was estimated by use of the range to be a ME of 0.30, corresponding to <10 copies/μL of methylated TLX3 in 100,000 copies/μL total DNA or 1 in 10^{-4}. Analysis of amounts of DNA in this range would be expected to have high variability due to stochastic effects; however, an advantage of such high analytical sensitivity may be that despite the variability in measured extension ratios, any ratio above the LOD may be interpreted as positive for diagnostic purposes.

Applying the methylSABER technique to patient samples taken throughout therapy showed a reduction in methylated DNA as a patient went through therapy (Fig. 5B). Using a threshold ME >0.30, we were able to correctly classify all 10 patient diagnostic samples. Furthermore, 2 of 3 early relapse patients recorded MEs >0.30, whereas neither of the 2 late-relapse patients and only 1 of 5 nonrelapse patients recorded an ME >0.30. Importantly, this 1 patient appeared to be responding to therapy, as evidenced by the decline in TLX3 ME between midtherapy and remission.

Discussion

Advances in genome-scale methods of DNA methylation analysis have established that cancers exhibit widespread alterations to their DNA methylation profiles (1). Such alterations represent underused biomarkers in oncology capable of discriminating cancers of different tissue types, subtyping cancers, and adding independent prognostic information to cancer diagnosis (18, 19). Here we present 2 complementary techniques that enable (a) regional multiplexing of DNA methylation (multiplexed EpiTYPER) facilitating robust diagnosis/prognosis and subtyping and (b) detection of rare DNA methylation events (methylSABER) that will facilitate disease tracking.

The multiplex EpiTYPER technique analyzed fewer CpG sites than singleplex analysis owing to indistinguishable fragments (by mass) produced between the FOXE3 and TLX3 amplicons after cleavage. The multiplex EpiTYPER technique analyzed DNA methylation in 12 CpG sites within a 223-bp region of the FOXE3 promoter and 17 CpG sites within a 304-bp region of the TLX3 promoter with highly reproducible results between assays at each methylation ratio. By individually targeting the FOXE3 and TLX3 promoter DNA methylation by use of standard EpiTYPER analysis within 197 patient samples, we recorded high clinical sensitivity and specificity for the segregation of pediatric ALL bone marrow biopsies (diseased) from clear bone marrow biopsies by use of an EF threshold of 0.20 and provided LOD estimates in line with the quantitative accuracies (±5%) described previously (20).

Approximately 25% of pediatric B-cell ALL cases lack commonly assayed genetic lesions (24). We were able to correctly diagnose 18 of 20 pediatric B-cell ALL patients that lacked common genetic lesions using EpiTYPER interrogation of FOXE3 and TLX3 gene promoters and EF threshold >0.20. After induction ther-
therapy, the presence of residual leukemic blasts within bone marrow biopsies taken at remission, known as minimal residual disease (MRD), is independently predictive of relapse and directs maintenance therapy (22–24). Notably, 18 of the 100 “nonleukemic” ALL patient samples were from individuals who suffered from bone marrow relapse (18%). Of these, 4 of the 8 FOXE3 false positives were from patients that later relapsed (50%). These results suggest the presence of MRD in the original sample; however, techniques used for MRD surveillance in ALL require low LOD ($10^{-4}$), lower than Epityper capabilities (25–27).

An applicable LOD ($10^{-4}$) was established through biasing a primer extension reaction (methylSABER) toward the methylated DNA at TLX3 CpG 17. The technique does not allow accurate quantification of DNA methylation, but rather detection of rare DNA methylation events (Fig. 5A). When applied to bone marrow biopsies taken from 10 pediatric ALL patients throughout therapy, all diagnostic samples were correctly identified with a minimum ME of 0.30 (Fig. 5B). At remission, patients that relapse early (<36 months) would be expected to have a higher residual leukemic load (MRD) than those with late relapse (>36 months) and nonrelapse patients (28). Interestingly, 2 of 3 early relapse patients, none of the 2 late relapse patients, and only 1 of 5 nonrelapse patients had ME >0.30 at remission. This result raises the potential to use a single measurement for residual disease in place of developing a specific assay for each patient.

Notably, the single nonrelapse patient who did have an ME >0.3 exhibited a marked decrease in ME at remission from diagnosis/midtherapy, indicative of leukemic clearance in response to therapy. The leukemic clearance of a patient may be an important predictor of relapse and potentially explains why absolute lymphocyte count at remission is not predictive of outcome (29).

Capitalizing on the strengths of robust DNA methylation biomarkers to define disease and analytically sensitive techniques to detect DNA methylation could pave the way for new tissue sources to be used for patient diagnosis or disease tracking. For instance, high clinical sensitivity and specificity has been reported for colorectal cancer diagnosis through analysis of plasma by use of hypermethylation markers of the disease (30). In ALL, the detection of residual lymphoblasts in bone marrow is prognostic, but peripheral blood is also a source of residual leukemia (26). Considering the laborious and painful nature of bone marrow aspiration, the ability to diagnose and monitor disease by peripheral blood is appealing.

Cost-effective multiplexing of regional DNA methylation will undoubtedly reduce research validation costs and has enormous application clinically. Incorporating prognostic DNA methylation biomarkers, DNA methylation marks that are persistent in healthy individuals, as well as cell type–specific marks, will improve the diagnostic accuracy and increase the prognostic information attainable from DNA methylation analysis (31–36). Moving toward individualized therapeutics, DNA methylation biomarkers can characterize and refine disease, representing ideal next-generation biomarkers.

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