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(54) Title: EPIGENETIC STEM CELL COMMITMENT-ASSOCIATED SIGNATURE

(57) Abstract: Methods for determining the prognosis of a subject having acute myeloid leukemia (AML) as well as methods of treating AML subjects depending on prognosis.

EPIGENETIC STEM CELL COMMITMENT-ASSOCIATED SIGNATURE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 61/932,973, filed January 29, 2014, the contents of which are hereby incorporated by reference.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant number R00CA131503 awarded by the National Cancer Institute. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] The disclosures of all patents, patent application publications and publications referred to in this application, including those cited to by number in parentheses, are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

[0004] In the pathogenesis of acute myeloid leukemia (AML), genes encoding epigenetic modifiers are frequently mutated (1, 2). Some of these mutations have been attributed prognostic value in AML (3). Additionally, aberrant DNA cytosine methylation in AML blasts has led to the identification of novel AML subtypes, independent of features usually associated with AML (4). Differentiation of murine HSC to progenitor cells is associated with distinct changes in DNA cytosine methylation (5-7). In turn, targeted disruption of DNA cytosine methylation patterns disturbs regulation of differentiation of murine hematopoietic stem and progenitor cells (HSPC), and affects HSPC function (8-10). This suggests that methylation plays an active role in the differentiation program.

[0005] In the murine hematopoietic system, dynamic changes of DNA methylation have been described during multipotent progenitor cell differentiation (5) and hematopoietic stem cell commitment (7), with pronounced demethylation in erythroid progenitors during differentiation (6, 7). Severely perturbed hematopoiesis (8-11) and myeloid transformation (12-14) are common hallmarks of mouse models with targeted disruptions in a growing number of enzymes known to contribute to the homeostasis of DNA cytosine methylation. However, little is known about changes in DNA cytosine methylation during early human

hematopoiesis. Identification of stage- and locus-specific epigenetic mechanisms of leukemic transformation would require a detailed genome wide map of DNA cytosine methylation patterns and dynamics during the step-wise maturation of hematopoietic stem cells (HSC). Currently there are no identified stage- specific and locus-specific epigenetic mechanisms of leukemic transformation.

[0006] The present invention provides a stem cell commitment-associated methylome that is independently prognostic of poorer overall survival in AML.

SUMMARY OF THE INVENTION

[0007] This invention provides a method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising

a) quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2;

b) determining a methylation score from the methylation determined in step a);

c) comparing the methylation score of DNA of the sample of blood cells obtained from the subject with AML with a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and

d) assigning a prognosis to the subject,

wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject,

and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

[0008] Also provided is a method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising

quantifying methylation of DNA of a sample comprising blood cells obtained from a subject with AML at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as demethylated at STHSC-CMP transition and/or at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as methylated at STHSC-CMP transition,

comparing the extent of the methylation with the methylation of DNA at the same plurality or pluralities of chromosome loci, or nearest associated gene, in a sample of blood cells obtained from a subject without AML, and

assigning a prognosis to the subject,

wherein demethylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject,

and wherein demethylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject.

[0009] Also provided is a method for treating a subject having acute myeloid leukemia (AML) comprising determining the prognosis of the subject, by any of the methods described herein, as positive or negative, and treating the subject with a chemotherapy if the subject has a positive prognosis or treating the subject with a non-chemotherapeutic method if the subject has a negative prognosis.

[0010] Also provided is a microarray comprising a nucleic acid probe for all, or for less than all, of the 561 loci or nearest associated genes listed in Table 2.

[0011]

[0012] Also provided is a kit for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising

a) reagents for quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2;

b) written instructions for determining a methylation score from the methylation determined with the reagents in a);

c) written instructions for a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and for assigning a prognosis to the subject based on the methylation score compared to the predetermined reference amount,

wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject,

and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1A-1D. Hypomethylation during HSC commitment to hematopoietic progenitors - A: Genome-wide changes in DNA methylation during HSC commitment. Red dots represent loci with significantly lower methylation at the developmentally later stage, i.e. loci demethylated during the respective transition ($p<0.05$, t-test). B: Significant changes in DNA cytosine methylation at the transition from LTHSC to STHSC (outer circle), STHSC to CMP (middle circle), and CMP to MEP (inner circle) (LTHSC = long-term HSC; STHSC = short-term HSC; CMP = common myeloid progenitors; MEP = megakaryocyte-erythrocyte progenitors) are plotted in relation to the genomic position. Chromosomes are plotted along the ideogram. Red bars denote significantly demethylated loci, green bars denote significant increase in methylation at the respective locus. C: SAM was used to define an epigenetic signature based on loci which undergo the most significant methylation changes during HSPC differentiation. The epigenetic signature (561 loci) distinguishes HSPC subsets in hierarchical clustering analysis. Log2-transformed HpaII/MspI ratios (color code next to the heat map) of all 561 loci are shown for the four analyzed differentiation stages (indicated above the heat map) from three healthy human individuals. Trees result from Euclidean clustering of this signature. Associated genes are listed in Table 2. D: Ingenuity pathway analysis highlights functional implications of gene enrichment analysis of the epigenetic stem cell commitment-associated signature. 62 genes significantly associated ($p<0.05$ after Benjamini-Hochberg-correction) with Ingenuity Top Bio Functions were entered into pathway generation. Top five canonical pathways ("AML Signaling", "Molecular Mechanisms of Cancer", "Glioblastoma Multiforme Signaling", "Pancreatic Adenocarcinoma Signaling", "Glucocorticoid Receptor Signaling") and the top three characteristics of function and disease ("Differentiation of Blood Cells" ($p=8.39E-43$), "Lymphohematopoietic Cancer" ($p=3.2E-12$), "AML" ($p=2.47E-06$), and "Cell Transformation" ($p=1.41E-12$)) are depicted.

[0014] Figure 2A-2H. Stem cell commitment-associated epigenetic signature is prognostic for overall survival in AML - Application of the epigenetic signature to three

independent published sets of patients with AML (4, 39-42). A, B: Analysis of patients with AML who received standard chemotherapy. C, D: Analysis of patients with AML who received chemotherapy with a higher dose of daunorubicin (DNR). E: Analysis of the combined cohort of AML patients receiving standard or higher doses of daunorubicin (41). H: Analysis of a third independent cohort of AML patients (39, 40). A, C, G: Heat map of the respective patients (horizontal order) and the 561 loci (vertical order). Patients are ranked in descending order based on the signature score. The score was calculated by summing absolute values of the median-centered methylation values (log2[HpaII/MspI]) of the 561 signature loci for each patient sample. Patients with high signature score are indicated by a green bar, patients with a low signature score by a black bar above the median-centered methylation heat map. B, D, E: Kaplan-Meier survival curves of OS of patients with AML are plotted. Green solid lines represent OS of patients with a high signature score, black solid lines represent OS of patients with a low signature score. F: Overlay of survival curves from Figure 2B, D. Black/red lines: patients with a low epigenetic stem cell commitment-associated signature score receiving standard or high dose daunorubicin treatment. Green/blue lines: patients with a high epigenetic stem cell commitment-associated signature score receiving standard or high dose daunorubicin treatment.

[0015] Figure 3A-3D. Lower prognostic power of gene expression signature - A: Generation of a gene expression signature based on 455 gene expression probes that undergo significant changes between the four measured differentiation stages using SAM. Heat map of log2-transformed expression values of this signature is shown. B-E: Application of the stem cell commitment-associated gene expression signature to patients with AML. B, D: Heat maps of median-centered expression of the probes corresponding to the commitment-associated gene expression signature in patients with AML who received standard chemotherapy (B), or chemotherapy with a higher dose of daunorubicin (D). C, E: Kaplan-Meier curves of OS of patients with AML are plotted; green lines represent OS of patients with a high expression signature score, black lines represent OS of patients with a low expression signature score.

[0016] Figure 4. Correlation of epigenetic signature's constituents with expression of closest mappable gene product - Changes of the 561 constituents of the epigenetic signature during transition from STHSC to CMP are aligned with significant changes in gene expression at mappable loci nearby. Red represents demethylated loci, green methylated

loci during STHSC to CMP transition, yellow represents increased and blue decreased gene expression at associated loci.

[0017] Figure 5. Correlation between methylation and expression of genes in the commitment-associated signatures - Changes in DNA cytosine methylation and gene expression between the indicated differentiation stages were calculated (mean methylation in later vs. earlier compartment, and mean expression in later vs. earlier compartment) and plotted in the graphs. The black line represents the linear model of these data points, and the p-value for correlation is indicated. A: Using the stem cell commitment-associated 561 probe epigenetic signature, 530 pairs of methylation probe with adjacent transcript were mapped and their correlation is shown. B: Similarly, the commitment-associated 455 probe expression signature was used to derive 283 pairs of transcripts associated with nearby or overlapping methylation probes, which are plotted in the figure.

DETAILED DESCRIPTION OF THE INVENTION

[0018] This invention provides a method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising

a) quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2;

b) determining a methylation score from the methylation determined in step a);

c) comparing the methylation score of DNA of the sample of blood cells obtained from the subject with AML with a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and

d) assigning a prognosis to the subject,

wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject,

and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

[0019] In an embodiment, the sample comprises blood cells. In an embodiment, the sample comprises bone marrow cells.

[0020] In an embodiment, the methylation score comprises a direct or indirect measurement of the ratio of demethylated CpG residues/methylated + demethylated CpG

residues of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

[0021] In an embodiment, the methylation is determined by a isoschizomer enzyme pair method, and wherein the methylation score is obtained by summing absolute values of the median-centered methylation values (log2[methylation sensitive enzyme measured fragments/ methylation insensitive enzyme measured fragments]) of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

[0022] In an embodiment, the isoschizomer enzyme pair is HpaII and MspI.

[0023] In an embodiment, the HELP assay is used to determine the methylation of the DNA.

[0024] In one embodiment, the blood or bone marrow sample has previously been obtained from the subject. Also provided is a method as described hereinabove but further comprising the step of obtaining the blood or bone marrow sample from the subject.

[0025] Also provided is a method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising

quantifying methylation of DNA of a sample comprising blood cells obtained from a subject with AML at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as demethylated at STHSC-CMP transition and/or at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as methylated at STHSC-CMP transition,

comparing the extent of the methylation with the methylation of DNA at the same plurality or pluralities of chromosome loci, or nearest associated gene, in a sample of blood cells obtained from a subject without AML, and

assigning a prognosis to the subject,

wherein demethylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject,

and wherein demethylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates

a negative prognosis for the subject, and wherein methylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject.

[0026] In an embodiment, quantifying methylation is effected by recovering DNA from the blood cells digesting a first portion of the DNA with a methylation-sensitive restriction enzyme and a second portion of the DNA with a methylation-insensitive restriction enzyme, and hybridizing to a HELP microarray.

[0027] In an embodiment, quantifying methylation is effected using HpaII tiny fragment Enrichment by Ligation-mediated PCR.

[0028] In an embodiment, quantifying methylation is effected by contacting a first portion of the DNA with sodium bisulfite under conditions permitting conversion of cytosine residues of the DNA into uracils, sequencing the DNA of the first portion and of a second portion untreated with sodium bisulfite, and aligning the resultant sequences of the two portions and comparing the sequences so as to determine the extent and position of methylated nucleotides in the DNA.

[0029] In an embodiment, the methods further comprising PCR amplifying the DNA after contacting with sodium bisulfite but prior to sequencing.

[0030] In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 comprises at least 5 loci or nearest associated genes. In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 comprises at least 10 loci or nearest associated genes. In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 comprises at least 100 loci or nearest associated genes. In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 comprises at least 500 loci or nearest associated genes. In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 5 loci or nearest associated genes. In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 100 loci or nearest associated genes. In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 200 loci or nearest associated genes. In an embodiment, the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 500 loci or nearest associated genes.

[0031] In an embodiment, the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 5 loci or nearest associated genes. In an embodiment, the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 10 loci or nearest associated genes. In an embodiment, the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 20 loci or nearest associated genes. In an embodiment, the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 30 loci or nearest associated genes. In an embodiment, the methylation is quantified as DNA cytosine methylation.

[0032] In one embodiment, the blood sample has previously been obtained from the subject. Also provided is a method as described hereinabove but further comprising the step of obtaining the blood sample from the subject.

[0033] Also provided is a method for treating a subject having acute myeloid leukemia (AML) comprising determining the prognosis of the subject, by any of the methods described herein, as positive or negative, and treating the subject with a chemotherapy if the subject has a positive prognosis or treating the subject with a non-chemotherapeutic method if the subject has a negative prognosis.

[0034] In an embodiment, the chemotherapy comprises administering an anthracycline and/or cytarabine and/or a demethylating agent, and or/a TKI. In an embodiment, the anthracycline is daunorubicin. In an embodiment, the non-chemotherapeutic method comprises an allogeneic stem cell transplantation into the subject.

[0035] In an embodiment, the non-chemotherapeutic treatment comprises all-trans-retinoic acid (ATRA), optionally with arsenic trioxide.

[0036] The practice of the present invention can employ, unless otherwise indicated, conventional techniques of molecular biology, such as PCR, e.g. see PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994).

[0037] In some embodiments, the subject involved in methods of the invention is considered to be at risk for AML relapse. “At risk” is an art-recognized term in the medical literature. A subject who has had a remission of AML may be at risk of a relapse as determined by duration of first complete remission, adverse cytogenetics, age and FLT3 mutation status.

[0038] Further examples of isoschizomer enzyme pairs that may be used in an embodiment of the invention are the methylation sensitive and insensitive enzyme pairs listed in Table 1 of US Patent Application Publication 2010-0267021 A1, published October 21, 2010, hereby incorporated by reference.

[0039] Also provided is a microarray comprising a nucleic acid probe for all, or for less than all, of the 561 loci or nearest associated genes listed in Table 2. Also provided is a kit comprising the microarray and instructions for use in determining the prognosis of an AML patient from a blood or bone marrow sample from the patient. In an embodiment, the kit further comprises reagents comprising an isoschizomer enzyme pairs having a methylation sensitive and insensitive enzyme pair.

[0040] Also provided is a kit for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising

a) reagents for quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2;

b) written instructions for determining a methylation score from the methylation determined with the reagents in a);

c) written instructions for a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and for assigning a prognosis to the subject based on the methylation score compared to the predetermined reference amount,

wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject,

and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

[0041] In an embodiment of the kit, the methylation score comprises a direct or indirect measurement of the ratio of demethylated CpG residues/methylated + demethylated CpG residues of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

[0042] In an embodiment of the kit, the methylation is determined by a isoschizomer enzyme pair method, and wherein the kit comprises an isoschizomer enzyme pair, and wherein the methylation score is obtained by summing absolute values of the median-centered methylation values (log2[methylation sensitive enzyme measured fragments/

methylation insensitive enzyme measured fragments]) of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

[0043] In an embodiment of the kit, the isoschizomer enzyme pair is HpaII and MspI.

[0044] In an embodiment of the kit, the HELP assay is used to determine the methylation of the DNA.

[0045] The phrase "and/or" as used herein, with option A and/or option B for example, encompasses the individual embodiments of (i) option A, (ii) option B, and (iii) option A plus option B.

[0046] It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0047] Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but each member of the group subjectly and all possible subgroups of the main group, but also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

[0048] All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0049] In the event that one or more of the literature and similar materials incorporated by reference herein differs from or contradicts this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls.

[0050] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Introduction

[0051] Acute myeloid leukemia (AML) is characterized by disruption of HSC and progenitor cell differentiation. Frequently, AML is associated with mutations in genes

encoding epigenetic modifiers. It was not previously known or proposed whether analysis of alterations in DNA methylation patterns during healthy HSC commitment and differentiation would yield epigenetic signatures that could be used to identify stage-specific prognostic subgroups of AML. In one embodiment a method is disclosed comprising using a nano HpaII-tiny-fragment-enrichment-by-ligation-mediated-PCR (nanoHELP) assay to compare genome-wide cytosine methylation profiles between highly purified human long-term HSC, short-term HSC, common myeloid progenitors, and megakaryocyte-erythrocyte progenitors. It was observed that the most striking epigenetic changes occurred during the commitment of short-term HSC to common myeloid progenitors, and these alterations were predominantly characterized by loss of methylation. A metric of the HSC commitment-associated methylation pattern was developed that proved to be highly prognostic of overall survival in three independent large AML patient cohorts, regardless of patient treatment and epigenetic mutations. Application of the epigenetic signature metric for AML prognosis was superior to evaluation of commitment-based gene expression signatures. Together, the data define a stem cell commitment-associated methylome that is independently prognostic of poorer overall survival in AML. The epigenetic signature is enriched for binding sites of known hematopoietic transcription factors and microRNA loci.

Experiments

[0052] Most DNA cytosines are methylated in human HSPC - To characterize DNA cytosine methylation in early human hematopoiesis, the distribution of and changes in methylation was studied during *in vivo* physiologic differentiation from LTHSC, immunophenotypically characterized as Lineage (Lin)-, CD34+, CD38-, CD90+, to STHSC (Lin-, CD34+, CD38-, CD90-), to CMP (Lin-, CD34+, CD38+, CD123+, CD45RA-) to MEP (Lin-, CD34+, CD38+, CD123-, CD45RA-) (15-21). A novel method combining eight-parameter high-speed fluorescence-activated cell sorting (FACS) of primary human bone marrow cells with an optimized HpaII-tiny-fragment-Enrichment-by-Ligation-mediated PCR (nano-HELP) assay (22-26) was used. This approach permitted examination of single individuals' stem cells isolated as biological replicates, i.e. without pooling of samples prior to analysis. It was possible to analyze DNA cytosine methylation in rare, highly purified human HSPC populations. Globally, it was found that the majority of DNA cytosines in human LTHSC, STHSC, CMP, and MEP (76%-81% of loci) from healthy individuals were methylated. Methylation was quantitatively compared across all loci

between the stages of differentiation. Interestingly, it was found that there was a highly significant reduction in median DNA cytosine methylation specifically at the stem cell to progenitor (STHSC to CMP) transition ($p<2.2x10-16$, Mann-Whitney test).

[0053] Dynamic changes in DNA cytosine methylation during HSC commitment - To characterize the dynamics of cytosine methylation during HSC commitment, changes in the methylation status at the level of individual loci were investigated and methylation in LTHSC was compared to methylation in STHSC, STHSC to CMP, and CMP to MEP. The comparison between LTHSC and STHSC showed 509 significantly differentially methylated loci ($p<0.05$). Demethylation was observed in 40% (205/509) of these loci during transition from the LTHSC to the STHSC compartment, whereas 60% (304/509) were more methylated in STHSC compared to LTHSC. At the transition from STHSC to CMP, the step of definitive commitment of HSC, a total of 793 differentially methylated loci were observed. However, in stark contrast to the nearly balanced hypo- and hypermethylation of loci between LTHSC and STHSC, 95% (757/793) of differentially methylated loci in STHSC were more methylated than in CMP, whereas only 5% (36/793) were less methylated. The transition from CMP to MEP was accompanied by balanced hypo- and hypermethylation with 127 (52%) loci showing higher and 116 (48%) loci showing lower methylation in the CMP compartment (Figure 1A). Changes occur without apparent focus throughout the genome (Figure 1B). The findings show that in human HSC demethylation particularly occurs at the commitment step from STHSC to CMP. This had not been described thus far.

[0054] A stem cell commitment-associated epigenetic signature distinguishes human HSC and progenitor cell subsets - To identify loci with most significant methylation changes across the assessed differentiation stages, significance analysis for microarrays (SAM) was performed on loci that showed differentiation-specific methylation changes independent of the variation between biological replicates, in analogy to a recently published approach (7). This resulted in a set of 561 loci that distinguished between the four investigated stages of human HSPC development (Figure 1C). The most prominent distinction was observed at the transition from stem cells (STHSC) to progenitor cells (CMP), consistent with the analysis of changes in DNA cytosine methylation during stem cell commitment described in Figure 1A. The signature mainly consisted of loci that were significantly demethylated during commitment from STHSC to CMP (516/561 loci, 92.0%). Interestingly, this stem cell commitment-associated epigenetic signature was

enriched in loci associated with several genes that are commonly implicated not only in human HSC function and commitment but also in leukemogenesis, such as CEBPA (27-29), E2F1 (30), KRAS (31, 32), WEE1 (33), as well as a non-coding transcript, MIRLET7B (34) (Table 2). Given emerging evidence that microRNAs play an essential role in both normal hematopoiesis and leukemogenesis (35-38) additional microRNA transcripts were assessed in the vicinity of the methylation probes on the array. Using 'miRBase' it was found that a number of microRNAs that were associated with significant epigenetic changes (Table 2). Ingenuity pathway analysis using the significant constituents of this methylation signature revealed an enrichment of genes involved in the function and disease characteristics "Differentiation of Blood Cells" (p=8.39E-43), "Lymphohematopoietic Cancer" (p=3.2E-12), specifically "AML" (p=2.47E-06), and "Cell Transformation" (p=1.41E-12). The top five canonical pathways were "AML Signaling", "Molecular Mechanisms of Cancer", "Glioblastoma Multiforme Signaling", "Pancreatic Adenocarcinoma Signaling", and "Glucocorticoid Receptor Signaling" (Figure 1D). Taken together, significant changes in DNA cytosine methylation during human HSC commitment occur at genomic loci involved in hematopoietic differentiation and in hematological malignancies.

[0055] Stem cell commitment-associated epigenetic signature is prognostic for overall survival in AML - Pathway analysis of the epigenetic signature showed an enrichment of genes implicated in systemic disorders of hematopoietic development. It was sought to determine whether the methylation status of this set of 561 stem cell commitment-associated loci derived from healthy human HSPC was affected in AML, a disease associated with epigenetic deregulation in HSPC (1). A signature score was developed based on the methylation of the 561 loci defined by the stem cell commitment-associated epigenetic signature. Additionally, data from clinical trials of patients with AML were analyzed. Three published independent cohorts of patients were identified for which DNA methylation data, gene expression data, as well as data on overall survival (OS) and mutational characteristics were available (4, 39-42). To assess the prognostic impact of this epigenetic signature was developed, we associated OS of patients with their score. This approach was tested on one cohort from a prospective randomized clinical trial that compared two different doses of daunorubicin (41). In the cohort receiving the standard, lower dose daunorubicin, a low stem cell commitment-associated epigenetic signature score was associated with increased OS (HR=1.537, 95%CI=1.086-2.245, p=0.0165, log-rank test, Figure 2A, B). Patients in the group with lower epigenetic signature scores showed a

median OS of 19.0 months, compared to 10.8 months in the group with higher epigenetic scores. Next, the stem cell commitment-associated epigenetic signature score was applied to the group of patients that received a higher dose of daunorubicin (41). The association of the stem cell commitment-associated epigenetic signature score with OS was also observed in this cohort of patients (HR=1.691, 95%CI=1.169-2.552, p=0.0062, Figures 2C, D). Median OS in the group with low epigenetic signature score was 25.4 months, compared to 13.2 months in the high scoring group. Of note, the significant association of high epigenetic signature score with poor outcome persisted upon combination of the two treatment arms of this trial (Figure 2E, median OS 11.1 months for patients with a high versus 22.8 months for patients with a low score, HR=1.609, 95%CI=1.258-2.143, p=0.0003).

[0056] To independently assess the association of the loci from the stem cell commitment-associated epigenetic signature with clinical outcome, Globaltest analysis was performed (43), using these loci as covariates. This confirmed the significant association of the 561-loci-classifier with OS (p=0.000697). In a multivariate Cox proportional hazard regression analysis (44) which included the epigenetic score in addition to the well-established factors cytogenetic and molecular risk stratification (3) and age, the epigenetic score remained independently and significantly associated with OS (Table 1). As depicted in the overlay of the survival curves from Figure 2B and D, patients with a low epigenetic signature score receiving high levels of daunorubicin had a significantly better OS than patients from the other groups (Figure 2F, p=0.0005), whereas patients in the three remaining groups did not show a statistically significant difference in their respective OS.

[0057] Additionally, the power of the epigenetic score in a third, independent cohort of patients with AML was validated. For this, published clinical and methylation data from patients from four clinical trials included in a study from the Dutch-Belgian Cooperative Trial Group for Hematology Oncology (HOVON) group (4, 39, 40) were analyzed. In this study, patients with a low epigenetic score again had a significantly better OS than those with a high score (median survival 28.1 months versus 14.9 months; HR=1.390, 95%CI=1.069-1.838, p=0.0150) (Figure 2G and H). Globaltest analysis (43) of this cohort independently confirmed significant association of the signature score with survival (p=0.000335).

[0058] Taken together, the methylation status of the commitment-associated loci identified in human HSPC from healthy individuals showed independent prognostic power in human AML in a total of 688 patients.

[0059] Low correlation of commitment-associated gene expression signature with AML patient outcome - Previous studies have defined gene expression signatures predictive of OS of patients with AML (45-47). Therefore, it was sought to determine whether a gene expression signature constructed in analogy to the epigenetic signature had comparable prognostic potential in the AML cohorts studied. It was first determined whether differentiation-specific gene expression changes were independent of the variation between biological replicates by SAM. Expression of the identified transcripts distinguished between the four investigated stages of human HSPC development (Figure 3A). The approach chosen to associate the epigenetic signature with OS was repeated and applied to this gene expression signature to the AML patient cohorts. The signature consisted of 530 genes that were differentially expressed in the analyzed stem and progenitor cells from healthy human individuals (Figure 3B, D). No significant correlation of the stem cell commitment-associated gene expression signature with OS was observed in either AML treatment group (Figure 3C, E). Association of gene expression signatures with outcome using globaltest as an alternative algorithm revealed a significant association of these genes with OS only in the combined Eastern Cooperative Oncology Group (ECOG) cohort ($p=0.00168$) but not in the HOVON cohort ($p=0.363$). While a published HSC gene expression signature (46) was associated with OS in the ECOG cohort ($p=0.00202$, globaltest), the association of a leukemia stem cell gene expression signature (46) with OS missed significance ($p=0.0821$, globaltest) as did an additional leukemic stem cell gene expression signature (45) ($p=0.257$, globaltest). These findings suggest that the stem cell commitment-associated epigenetic signature is a more robust indicator of OS than a stem cell-commitment-associated gene expression signature obtained in an identical, unbiased fashion.

[0060] Correlation of methylation and gene expression changes between stages of human hematopoietic stem cell commitment - DNA cytosine methylation has been associated with regulation of transcription. Promoters of developmental genes, as well as promoters of housekeeping genes can be silenced by hypermethylation (48) while gene bodies have been reported to be methylated following increased transcription of the respective gene (49). Methylation and gene expression were correlated during the respective HSPC transitions. Besides locus-specific inverse correlation between decreasing

methylation and increasing gene expression (Figure 4, Figure 5A upper right quadrant), increasing methylation and decreasing gene expression (Figure 5A, lower left quadrant) loci were found with a positive correlation between decreasing methylation and decreasing gene expression (Figure 5A, upper left quadrant), and increasing methylation and increasing gene expression (Figure 5A, lower right quadrant). Conversely, a significant correlation between decrease of cytosine methylation and increase in gene expression at the STHSC to CMP transition appeared when correlating the commitment-associated gene expression signature with nearby CpG loci (Figure 5B). Changes in methylation at an earlier transition did not significantly associate with changes in gene expression at a later transition (e.g. methylation during transition from LTHSC to STHSC compared to gene expression during transition from STHSC to CMP, data not shown). Taken together, the epigenetic signature is not universally correlated with gene expression, although there are certain loci that show correlation or inverse correlation. Yet, at the STHSC to CMP transition an inverse correlation between gene expression and associated methylation changes can be observed. Changes in expression of the genes associated with the epigenetic stem cell-commitment associated signature were not prognostic for outcome in AML patients ($p=0.133$, ECOG cohort, Globaltest). Of note, mutations of genes known to directly affect DNA methylation, such as IDH1, IDH2, TET2, and DNMT3A, were not enriched in either the high or low scoring group. Finally, it was investigated whether specific DNA motifs were enriched around the constituents of the epigenetic signature, which could provide mechanistic insights into the regulation of these loci. Using HOMER transcription binding site analysis (50), a significant enrichment was observed of consensus binding sites for several essential transcription factors involved in hematopoietic differentiation (most notably GATA transcription factors, Maf family members, KLF4, and Smad2 (51-53)) in the epigenetic signature.

Discussion

[0061] Perturbed epigenetic regulation of differentiation from HSC to mature blood cells can result in a block in cellular differentiation, clinically apparent in hematopoietic malignancies such as AML (1). To study epigenetic regulation during earliest human hematopoiesis, the status of and changes in DNA cytosine methylation during *in vivo* differentiation of human HSC was analyzed. To this end, a novel technique was developed that enabled characterization of DNA cytosine methylation from prospectively isolated

highly enriched human HSC from single individuals in small numbers. Prospective isolation of human HSPC was coupled with a modified HELP assay, the so-called nano-HELP (22-26). It was found that most DNA cytosines in human LTHSC, STHSC, CMP, and MEP are methylated, in agreement with findings in other vertebrate somatic stem cells and differentiated tissues (5-7, 54). The findings show that, while mean methylation levels are comparable to those found in murine HSC (7), in human HSC demethylation particularly occurs at the commitment step from STHSC to CMP (Figure 1A). This has not been described thus far. Furthermore, our data define specific loci with dynamic changes in methylation during human HSPC differentiation. These loci represent a stem cell commitment-associated epigenetic signature that clusters the subsequent stages of HSC differentiation (Figure 1C), and is enriched in genes associated with hematopoietic development and also leukemogenesis, particularly AML (Figure 1D). Therefore, it was assessed whether the methylation status at these loci would have clinical implications in human AML. Indeed, it was found that this signature was able to classify three independent cohorts of patients with AML from prospective clinical trials into groups with superior or significantly inferior OS. Patients treated with standard chemotherapy with a low stem cell commitment-associated epigenetic signature score reached significantly longer OS than patients with a high score. The power of this score was assessed using data from a second cohort of AML patients treated with an experimental approach (41) and an even stronger distinction was found between the groups (Figure 2). This is in contrast to some currently used mutational markers (3), and suggests a high degree of robustness of the prognostic value of the stem cell commitment-associated epigenetic signature. Multivariate analysis demonstrated an independent association of the epigenetic score with OS, and no enrichment of mutations of known modifiers of DNA methylation was detected in either the high or low scoring group. The overlay of survival curves from the different clinical cohorts (Figure 2F) suggests that the epigenetic signature might serve as a predictor for OS particularly in AML patients receiving higher doses of daunorubicin. A third independent cohort of patients with AML studied by the HOVON group (39, 40) also segregated into better and worse prognosis on the basis of the epigenetic stem cell commitment-associated score, further demonstrating the robustness and prognostic potential of this score. Taken together, the epigenetic stem cell commitment signature was validated in three independent cohorts of AML patients, with a total of 688 patients. Of note, in each of these cohorts median survival was approximately doubled in patients with low signature score, even in

the cohort that was treated with higher dose daunorubicin, indicating the robustness of the prognostic value of this signature. Similarly derived gene expression signatures were not able to achieve the robustness that was observed using the epigenetic signature.

[0062] Recent studies have linked changes in methylation to the regulation of microRNAs, and one microRNA transcript, MIRLET7, was identified in the signature; in addition, several other microRNA genes were located adjacent to the differentially methylated region (DMR).

[0063] Sequence analysis of the DMR regions revealed a significant enrichment of motifs for transcription factors that were previously shown to be implicated in hematopoietic differentiation and leukemogenesis, such as GATA factors, MAFF and KLF4. For instance, it was recently shown that erythroid differentiation is accompanied by functional demethylation of essential erythropoietic genes, including GATA1 (6, 55). In addition, maintenance of hematopoietic stem cell programs and prevention of activation of differentiation programs are controlled by DNA methylation (8).

[0064] Analyses were performed on DNA from highly enriched HSPC, thus avoiding the measurement of DNA cytosine methylation and gene expression from heterogeneous cell populations. In addition, analyzing cells from single donors, as opposed to pooling cells from multiple donors, permitted derivation of changes propagated through various differentiation stages in individuals, in addition to changes that occurred in a stage-specific manner across all individuals studied. Furthermore, an exhaustive high quality dataset that included both data on DNA cytosine methylation in leukemic blasts and clinical data including a detailed description of risk groups and overall survival from a prospective randomized clinical trial were accessed (41). These data have been the basis for numerous analyses (3, 42, 56). The HELP assay has a bias towards CpG-rich sites, in effect concentrating on promoter regions. The performance of the HELP assay in CpG-poor regions is reduced compared to bisulfite conversion based methods.

[0065] In summary, the findings presented here identify a large fraction of CpG dinucleotides in human HSC as methylated, show a human-specific methylation decrease specifically during STHSC to CMP commitment, and reveal a stem cell commitment-associated epigenetic signature as robustly and independently prognostically significant for OS of AML patients.

Methods and Materials

[0066] Bone marrow samples: Bone marrow samples from healthy individuals were obtained from AllCells LLC.

[0067] High-speed multi-parameter fluorescence-activated cell sorting (FACS): FACS of human HSPC populations was performed as described before (15-17, 19-21, 25). Mononuclear cells from bone marrow aspirates were isolated by density gradient centrifugation. CD34+ cells were enriched by immunomagnetic beads (Miltenyi Biotech). The resulting cells were lineage depleted (Lin-) using PE-Cy5 (Tricolor)-conjugated monoclonal antibodies against CD2, CD3, CD4, CD7, CD10, CD11b, CD14, CD15, CD19, CD20, CD56, and Glycophorin A (all BD Biosciences). Further distinction into HSPC subsets was performed using fluorochrome-conjugated antibodies against CD34, CD38, CD90, CD45RA, and CD123 (all eBioscience). LTHSC (Lin-, CD34+, CD38-, CD90+), STHSC (Lin-, CD34+, CD38-, CD90-), CMP (Lin-, CD34+, CD38+, CD123+, CD45RA-), and MEP (Lin-, CD34+, CD38+, CD123-, CD45RA-) were sorted into RLT extraction buffer (Qiagen). Flow cytometric analysis and cell separation were performed on a FACSAriaII special order system (Becton Dickinson) equipped with 4 lasers (407nm, 488nm, 561/568nm, 633/647nm).

[0068] Preparation of nucleic acids: After sorting into RLT buffer (Qiagen), homogenization of the cells was achieved by passing the cells five times through a needle. Simultaneous harvest of RNA and genomic DNA was achieved with the AllPrep kit (Qiagen) following the instructions of the manufacturer. Total RNA was linearly amplified and transcribed with the MessageAmp Kit AM1751 (Ambion/ Life Technologies) prior to microarray gene expression analysis following the NimbleGen Arrays User's Guide (NimbleGen). Integrity of RNA and cDNA was verified at each step of amplification using the Agilent Bioanalyzer 2100 (Agilent).

[0069] DNA methylation analysis by nano-HELP: Methylation analysis by the HELP assay (22, 57-59) and a modified protocol to successfully work with low genomic DNA yield from low numbers of sorted stem and progenitor cells have been described (24, 25). Integrity of genomic DNA of high molecular weight was assured by electrophoresis for all samples used. HpaII or MspI (NEB) digestions of genomic DNA were performed overnight prior to overnight ligation of the HpaII adapter with T4 ligase. PCR amplified adapter-ligated HpaII or MspI fragments were submitted to Roche-NimbleGen. Labeling and DNA hybridization onto a human hg17 custom designed oligonucleotide array (50mers) was carried out. The 2005-07-20_HG17_HELP_Promoter array covers 25,626 HpaII amplifiable

fragments (HAF) at gene promoters, defined as regions 2 kb upstream and downstream of transcriptional start sites (TSS). EpiTyper by MassArray (Sequenom) was used to confirm methylation of selected loci as described (23, 60).

[0070] Microarray quality control: Uniformity of hybridization was evaluated by adapting a published algorithm (61) for the NimbleGen platform. Hybridizations with strong regional artifacts were discarded and repeated. Normalized signal intensities from each array were compared with a 20% trimmed mean of signal intensities across all arrays in that experiment. Arrays with significant intensity bias that could not be explained by the biology of the sample were excluded.

[0071] HELP data processing: Signal intensities at each HAF were calculated as 25% trimmed mean of their component probe-level signal intensities. Any fragments found within the level of background MspI signal intensity (equaling 2.5 mean-absolute-difference, MAD) above the median of random probe signals were regarded "failed". These "failed" loci represent the population of fragments that did not amplify by PCR. Loci were designated "methylated" when the level of HpaII signal intensity was indistinguishable from background as described for MspI. Fragments successfully amplified by PCR, i.e. distinguishable above background, were subjected to normalization. For this, an intra-array quantile approach was used: HpaII/MspI ratios are aligned across density-dependent sliding windows of fragment size-sorted data. The log2(HpaII/MspI) was used as a representative for methylation and analyzed as a continuous variable. If the centered log2(HpaII/MspI) ratio was <0, the corresponding fragment was considered methylated. It was considered hypomethylated in cases where log2(HpaII/MspI) was >0.

[0072] Gene expression profiling: Gene expression profiling was performed on NimbleGen HG18 arrays (design name 2006-08-03_HG18_60mer_expr, Roche-NimbleGen). Profiling was performed by the Epigenomics Shared Facility, Albert Einstein College of Medicine.

[0073] Meta-analysis of the GSE24505 AML data set: Previously published data for gene expression (Nimblegen 2005-04-20_Human_60mer_1in2 arrays), and DNA methylation (2005-07-20_HG17_HELP_Promoter arrays) were retrieved from the GEO server (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24505). Additional annotations were extracted from these files. The methylation status of respective loci could be directly compared between the data describing human HSPC that we analyzed and the published GSE24505 AML data due to identical platforms.

[0074] Statistical analysis: HELP loci were annotated using UCSC annotations for hg17. Means of locus-specific methylation between consecutive HSPC subsets were compared using Student's two-sided t-test for unpaired samples. Significance was assumed when $p<0.05$. Significance analysis for microarrays (SAM) was performed using Multiple Experiment Viewer as was supervised clustering using Euclidean distance correlation with complete linkage. SAM ($q<0.015$) was performed on the values of the 4 cell populations that remained significant after an initial SAM had filtered probes in which the difference between replicates was more significant than the difference between stages of differentiation. A similar approach to account for variability in analyses of DNA cytosine methylation has recently been published (7). Survival data and corresponding methylation values have previously been published (41, 42). An epigenetic score was calculated by summing absolute values of the median-centered methylation values (log2[HpaII/MspI]) of the 561 signature loci for each patient sample. Samples from ECOG (GSE24505) and HOVON (GSE18700) studies (4, 42) were ranked and uniformly dichotomized according to the 55th percentile into patients with a low and those with a high signature score. An association of this score with Kaplan-Meier-survival estimates (62) was probed by the log rank test and assumed to be statistically significant when $p<0.05$. The association of individual methylation loci and genes in this set of patient samples was probed by globaltest (43) after linear transformation to obtain positive values, similarly to a recently published analysis (63). Gene expression analysis was performed in an identical fashion, with $q<0.2$. Ingenuity (Ingenuity Systems) was used for pathway analysis. After Benjamini-Hochberg-correction, Top Bio Functions that were significantly ($p<0.05$) associated with the 561 constituents of the epigenetic commitment-associated stem cell signature were entered into a pathway generator. The top five canonical pathways and the top three characteristics in function and disease were chosen for display. Circos plots were prepared following the instruction at http://circos.ca. To perform correlation analyses between methylation probes and gene expression changes, as well as globaltest analyses of gene expression signatures from various microarray platforms, all probes were remapped to hg19 using liftOver (genome.ucsc.edu/cgi-bin/hgLiftOver), and remapped probes were associated with overlapping hg19 RefSeq genes (retrieved from UCSC table browser genome.ucsc.edu/cgi-bin/hgTables, refGene table, retrieved 2012/09/18)) using bedtools intersect, and closest non-overlapping genes were associated using bedtools closest. Additional identifiers of these genes were retrieved from ENSEMBL BioMart using biomaRt in R/Bioconductor to

match probe identifiers across various microarray platforms (Nimblegen HG18 for the ECOG data set (42), Nimblegen HG17 for healthy human HSPC, Affymetrix U133plus2.0 for the signatures published by Eppert et al. (46), Entrez IDs for those published by Gentles et al. (45)). Collapsing of multiple probes, where necessary, was performed using the collapseRows function in the R/Bioconductor WGCNA package. Genomic coordinates of pre-microRNA in the hg19 genome were retrieved from miRBase (mirbase.org/pub/mirbase/20/genomes/hsa.gff2), miRBase v20, date: 2013-05-24 , genome build: GRCh37.p5, NCBI_Assembly:GCA_000001405.6).

[0075] Data were compared by 2-sided t test for unpaired samples, or by significance analysis for microarrays (SAM) using Multiple Experiment Viewer (version 4.8) and q-value thresholds as indicated. To determine the association of DNA methylation or RNA expression signatures with overall survival, Kaplan-Meier survival analysis was performed and survival differences between groups were assessed with the log-rank test. Alternatively, globaltest analysis was performed. Univariate and multivariate analyses of hazard ratios were performed using the Cox proportional hazards model. Survival analyses were performed with R/Bioconductor software and the packages globaltest, survival, eha, and MASS, or with GraphPad Prism software (version 6). P-values < 0.05 were considered significant.

[0076] Table 1: Multivariate analysis using the epigenetic stem cell commitment signature score, cytogenetic and molecular risk stratification, age, and treatment of the patients from GSE24505 as covariates. Score, risk and treatment were considered as categorical values. Survival analysis was performed using a Cox proportional hazards model.

	HR (95%CI), p-value	GSE24505
epigenetic score (high=1)	0.6856 (0.5247-0.8957), 0.0056	
intermediate risk	2.0328 (1.3415-3.0803), 0.0008	
unfavorable risk	4.2794 (2.8662-6.3893), 1.17e-12	
age (>=46=1)	0.6905 (0.5267-0.9052), 0.0073	
treatment arm (A=1)	0.7682 (0.5889-1.0021), 0.0518	

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[0077] Table 2:

ID	chromosome	start	end	nearest associated gene	demethylated at STHSC-CMP transition	methylated at STHSC-CMP transition	associated mirBase miRNA
NR_027693	chr1	957906	958178	C1orf170	•		
NM_003327	chr1	1189309	1189551	TNFRSF4	•		
NM_003327	chr1	1189552	1190026	TNFRSF4	•		
NM_058167	chr1	1237557	1237908	UBE2J2	•		
NM_058167	chr1	1249687	1250185	UBE2J2	•		
NM_024848	chr1	2354082	2354504	MORN1	•		
NR_033711	chr1	3687181	3688411	TP73-AS1	•		
NM_024654	chr1	6549100	6549357	NOL9	•		
NM_015164	chr1	15754221	15755055	PLEKHM2	•		
NM_014424	chr1	16090875	16091622	HSPB7	•		
NR_027504	chr1	16716927	16717382	MST1P2	•		
NM_003689	chr1	19382942	19383319	AKR7A2	•		
NM_032236	chr1	21855465	21856098	USP48	•		
NM_005529	chr1	22010022	22010782	HSPG2	•		
NM_030634	chr1	23440314	23440761	ZNF436	•		
NM_004091	chr1	23603359	23604042	E2F2	•		
NM_003680	chr1	32953197	32954032	YARS	•		
NM_003680	chr1	32954033	32954680	YARS	•		
NM_145047	chr1	36585404	36586033	OSCP1	•		
NR_024270	chr1	45438795	45439054	LOC400752	•		
NR_038953	chr1	53416058	53416293	LOC100507564		•	
NM_016126	chr1	54122232	54122724	HSPB11	•		
NR_026782	chr1	54817457	54819004	HEATR8	•		
NM_001902	chr1	70589060	70590030	CTH	•		
NM_198549	chr1	77956930	77957172	FAM73A	•		
NM_006536	chr1	86599385	86600099	CLCA2	•		

NR_026988	chr1	87307708	87308405	LOC339524	•			
NR_026988	chr1	87308406	87308954	LOC339524	•			
NR_026987	chr1	87308955	87309663	LOC339524	•			
NM_144988	chr1	95250505	95251771	ALG14		•		
NM_001144822	chr1	116826399	116827849	CD58	•			
NM_005105	chr1	142995970	142996399	RBM8A	•			
NM_178348	chr1	149612211	149613164	LCE1A		•		
NM_006556	chr1	151723132	151723767	PMVK	•			
NM_001018016	chr1	151975007	151975698	MUC1	•			
NM_001162384	chr1	152761420	152761690	ARHGEF2	•			
NM_024540	chr1	153527535	153527805	MRPL24	•			
NM_004833	chr1	155859524	155860191	AIM2		•		
NM_020335	chr1	157197685	157197955	VANGL2	•			
NM_005099	chr1	157980315	157980942	ADAMTS4	•			
NM_053053	chr1	163577801	163578340	TADA1	•			
NR_037845	chr1	170176093	170177170	LOC100506023	•			
NM_198493	chr1	170370693	170372081	ANKRD45	•			
NM_001200050	chr1	179490846	179491482	NPL	•			
NM_007212	chr1	181744853	181745145	RNF2	•			
NM_007212	chr1	181745146	181745897	RNF2	•			
NM_014388	chr1	206388104	206389080	DIEXF	•			
NM_001164688	chr1	208053353	208053680	RD3	•			
NM_001164688	chr1	208053681	208054061	RD3	•			
NR_024485	chr1	224224936	224225388	LOC100130093	•			
NM_145257	chr1	225785590	225785825	C1orf96	•			
NM_004481	chr1	226508139	226509317	GALNT2	•			
NM_004485	chr1	232140050	232141799	GNG4	•			
NM_019891	chr1	232771598	232772990	ERO1LB		•		
NR_026833	chr2	6072507	6073401	LOC400940		•		
NM_003597	chr2	10097327	10097750	KLF11	•			
NM_002539	chr2	10539882	10540677	ODC1	•			

NM_001006946	chr2	20347390	20348252	SDC1	•			
NM_175629	chr2	25506723	25507149	DNMT3A	•			
NM_004802	chr2	26590216	26591090	OTOF	•			
NM_001168364	chr2	27575821	27576237	KRTCAP3	•			
NM_001168364	chr2	27576238	27576601	KRTCAP3	•			
NM_001199139	chr2	32402061	32402439	NLRC4	•			
NM_144736	chr2	37370960	37371797	C2orf56	•			
NM_001001521	chr2	63979435	63979829	UGP2	•			
NM_181784	chr2	65571224	65571496	SPRED2	•			
NM_181784	chr2	65575553	65576585	SPRED2	•			
NM_001190265	chr2	68201971	68202668	C1D	•			
NM_001111101	chr2	68458347	68458608	CNRIP1	•			
NM_017880	chr2	70329727	70330679	C2orf42		•		
NM_001206840	chr2	85467468	85467949	TGOLN2	•			
NM_017750	chr2	85492695	85492916	RETSAT	•			
NM_012483	chr2	85831349	85832243	GNLY	•			
NM_001079	chr2	97787264	97787854	ZAP70	•			
NM_014044	chr2	98684341	98684820	UNC50	•			
NM_032411	chr2	106139577	106140059	C2orf40	•			
NM_006343	chr2	112370475	112371817	MERTK	•			
NM_173843	chr2	113590796	113591607	IL1RN	•			
NM_001105198	chr2	120151906	120152205	TMEM177	•			
NM_005291	chr2	128119819	128120032	GPR17	•			
NM_012290	chr2	171843202	171844410	TLK1	•			
NM_001193528	chr2	175086383	175086752	SCRN3	•			
NR_026966	chr2	178082238	178082772	LOC100130691	•			
NM_001128144	chr2	190472769	190473769	PMS1	•			
NM_199440	chr2	198191088	198191715	HSPD1	•			
NM_021824	chr2	201579689	201580307	NIF3L1	•			
NM_018571	chr2	202142471	202143236	STRADB	•			
NM_173511	chr2	203323728	203324851	FAM117B	•			

NM_003872	chr2	206471042	206471328	NRP2	•				
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NM_001204527	chrX	152578988	152580122	SSR4	•		
NM_001110792	chrX	152884156	152885140	MECP2	•		

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What is claimed is:

1. A method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising
a) quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2;
b) determining a methylation score from the methylation determined in step a);
c) comparing the methylation score of DNA of the sample of blood cells obtained from the subject with AML with a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and
d) assigning a prognosis to the subject,
wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject,
and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

2. The method of Claim 1, wherein the methylation score comprises a direct or indirect measurement of the ratio of demethylated CpG residues/methylated + demethylated CpG residues of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

3. The method of Claim 1, wherein the methylation is determined by a isoschizomer enzyme pair method, and wherein the methylation score is obtained by summing absolute values of the median-centered methylation values (log2[methylation sensitive enzyme measured fragments/ methylation insensitive enzyme measured fragments]) of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

4. The method of Claim 1, 2 or 3, wherein the isoschizomer enzyme pair is HpaII and MspI.

5. The method of any of Claims 1-4, wherein the HELP assay is used to determine the methylation of the DNA.

6. A method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising

quantifying methylation of DNA of a sample comprising blood cells obtained from a subject with AML at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as demethylated at STHSC-CMP transition and/or at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as methylated at STHSC-CMP transition,

comparing the extent of the methylation with the methylation of DNA at the same plurality or pluralities of chromosome loci, or nearest associated gene, in a sample of blood cells obtained from a subject without AML, and

assigning a prognosis to the subject,

wherein demethylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject,

and wherein demethylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject.

7. The method of Claim 1 or 6, wherein quantifying methylation is effected by recovering DNA from the blood cells digesting a first portion of the DNA with a methylation-sensitive restriction enzyme and a second portion of the DNA with a methylation-insensitive restriction enzyme, and hybridizing to a HELP microarray.

8. The method of Claim 1, 2 or 6, wherein quantifying methylation is effected using HpaII tiny fragment Enrichment by Ligation-mediated PCR.

9. The method of Claim 1 or 6, wherein quantifying methylation is effected by contacting a first portion of the DNA with sodium bisulfite under conditions permitting conversion of cytosine residues of the DNA into uracils, sequencing the DNA of the first portion and of a second portion untreated with sodium bisulfite, and aligning the resultant sequences of the two portions and comparing the sequences so as to determine the extent and position of methylated nucleotides in the DNA.

10. The method of Claim 9, further comprising PCR amplifying the DNA after contacting with sodium bisulfite but prior to sequencing.

11. The method of any of Claims 1-5, wherein the plurality of loci or nearest associated gene listed in Table 2 comprises at least 5 loci or nearest associated genes.

12. The method of any of Claims 1-5, wherein the plurality of loci or nearest associated gene listed in Table 2 comprises at least 10 loci or nearest associated genes.

13. The method of any of Claims 1-5, wherein the plurality of loci or nearest associated gene listed in Table 2 comprises at least 100 loci or nearest associated genes.

14. The method of any of Claims 1-5, wherein the plurality of loci or nearest associated gene listed in Table 2 comprises at least 500 loci or nearest associated genes.

15. The method of any of Claims 6-10, wherein the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 5 loci or nearest associated genes.

16. The method of any of Claims 6-10, wherein the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 100 loci or nearest associated genes.

17. The method of any of Claims 6-10, wherein the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 200 loci or nearest associated genes.

18. The method of any of Claims 6-10, wherein the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition comprises at least 500 loci or nearest associated genes.

19. The method of any of Claims 6-10 or 15-18, wherein the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 5 loci or nearest associated genes.

20. The method of any of Claims 6-10 or 15-18, wherein the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 10 loci or nearest associated genes.

21. The method of any of Claims 6-10 or 15-18, wherein the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 20 loci or nearest associated genes.

22. The method of any of Claims 6-10 or 15-18, wherein the plurality of the loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition comprises at least 30 loci or nearest associated genes.

23. The method of any of Claims 1-22, wherein the methylation is quantified as DNA cytosine methylation.

24. A method for treating a subject having acute myeloid leukemia (AML) comprising determining the prognosis of the subject by the method of any of Claims 1-23 as positive or negative, and treating the subject with a chemotherapy if the subject has a positive prognosis or treating the subject with a non-chemotherapeutic method if the subject has a negative prognosis.

25. The method of Claim 24, wherein the chemotherapy comprises administering an anthracycline and/or cytarabine and/or a demethylating agent, and or/a TKI.

26. The method of Claim 25, wherein the anthracycline is daunorubicin.

27. The method of Claim 25, wherein the non-chemotherapeutic method comprises an allogeneic stem cell transplantation into the subject.

28. A kit for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising
a) reagents for quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2;
b) written instructions for determining a methylation score from the methylation determined with the reagents in a);
c) written instructions for a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and for assigning a prognosis to the subject based on the methylation score compared to the predetermined reference amount,
wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject,
and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

29. The kit of Claim 28, wherein the methylation score comprises a direct or indirect measurement of the ratio of demethylated CpG residues/methylated + demethylated CpG residues of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

30. The kit of Claim 28 or 29, wherein the methylation is determined by a isoschizomer enzyme pair method, and wherein the kit comprises an isoschizomer enzyme pair, and wherein the methylation score is obtained by summing absolute values of the median-centered methylation values (log2[methylation sensitive enzyme measured fragments/ methylation insensitive enzyme measured fragments]) of the plurality of the chromosome loci, or nearest associated gene for the DNA of a sample.

31. The kit of Claim 30, wherein the isoschizomer enzyme pair is HpaII and MspI.

32. The kit of any of Claims 28-31, wherein the HELP assay is used to determine the methylation of the DNA.

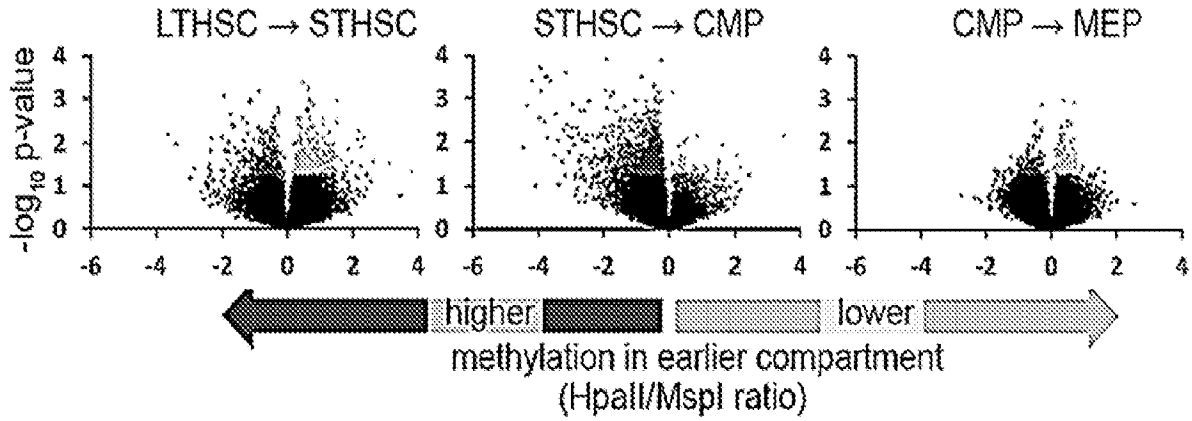

LTHSC → STHSC
STHSC → CMP
CMP → MEP
-log10 p-value
higher
lower
methylation in earlier compartment
(HpaII/MspI ratio)

FIG. 1A

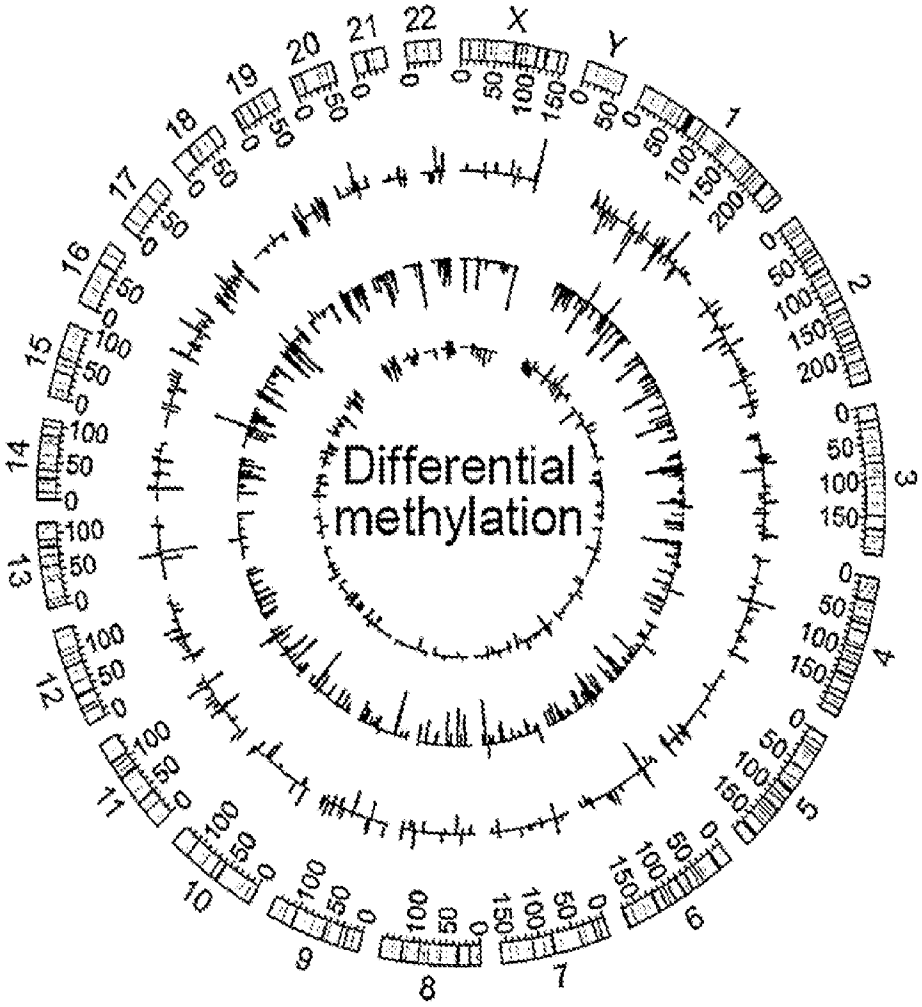

Differential methylation
Inner circle: CMP → MEP
Middle circle: STHSC → CMP
Outer circle: LTHSC → STHSC

FIG. 1B

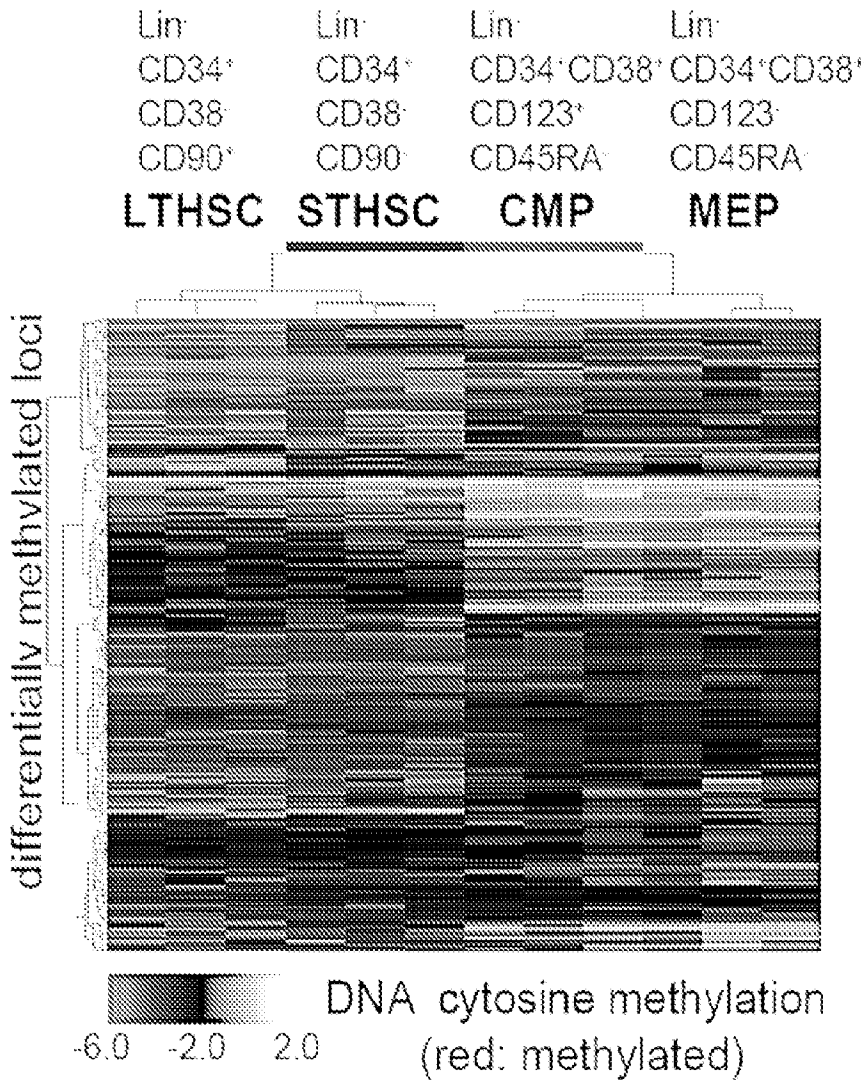

Lin-
CD34+
CD38-
CD90+
Lin-
CD34+
CD38-
CD90-
Lin-
CD34+CD38+
CD123+
CD45RA-
Lin-
CD34+CD38+
CD123-
CD45RA-
LTHSC
STHSC
CMP
MEP
differentially methylated loci
DNA cytosine methylation
(red: methylated)
-6.0
-2.0
2.0

FIG. 1C

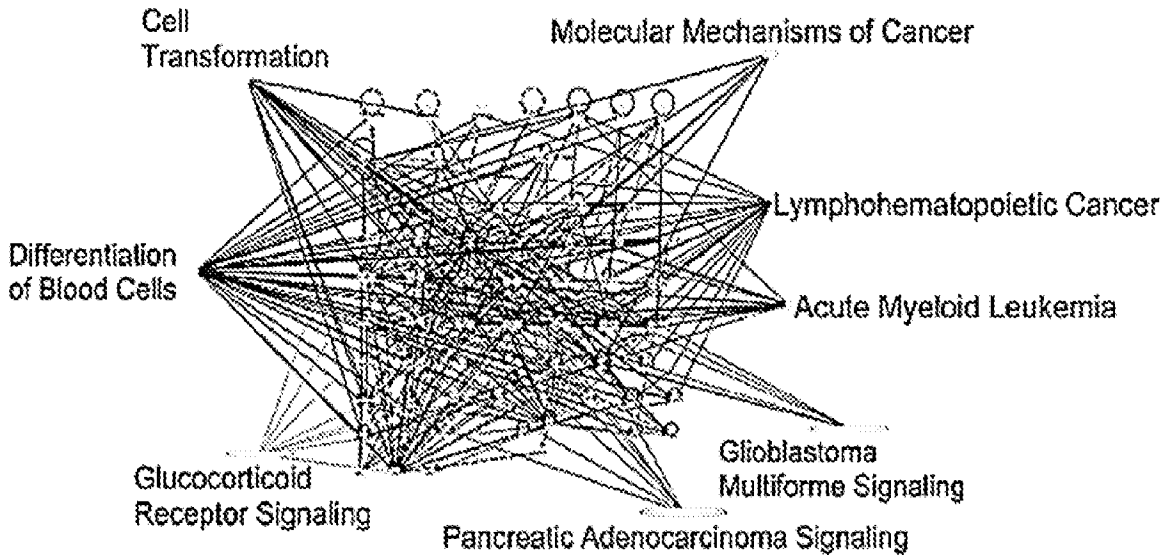

Cell
Transformation
Molecular Mechanisms of Cancer
Lymphohematopoietic Cancer
Differentiation
of Blood Cells
Acute Myeloid Leukemia
Glioblastoma
Multiforme Signaling
Glucocorticoid
Receptor Signaling
Pancreatic Adenocarcinoma Signaling

FIG. 1D

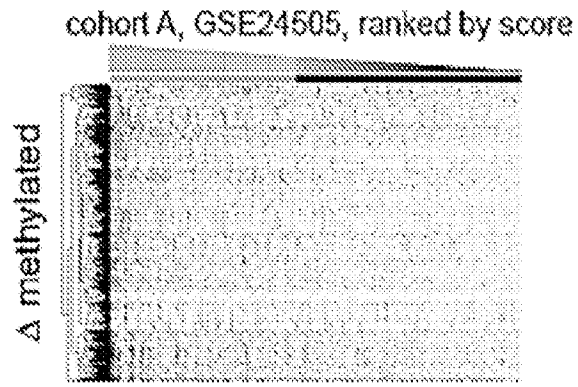


FIG. 2A

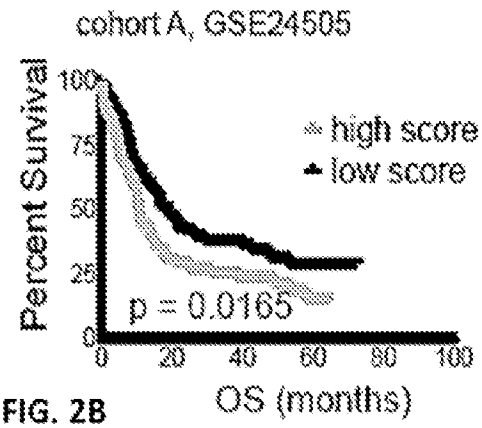


FIG. 2B

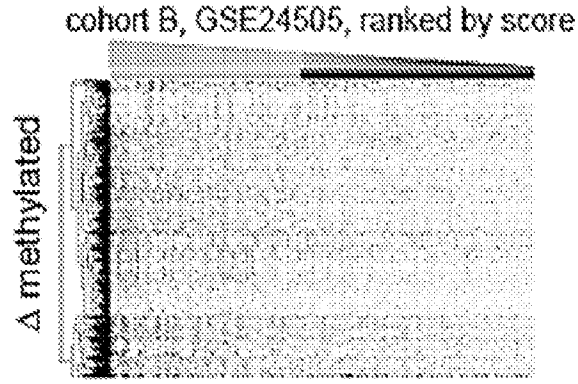


FIG. 2C

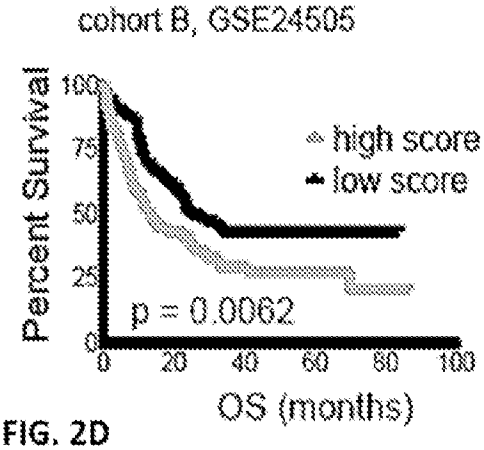


FIG. 2D

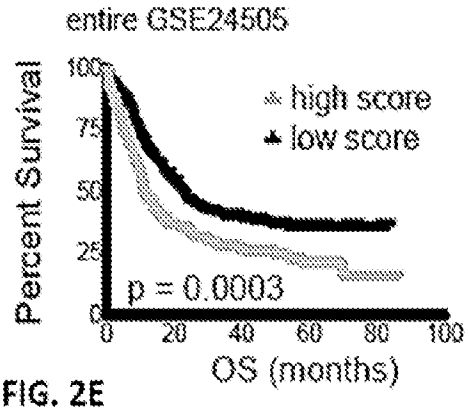


FIG. 2E

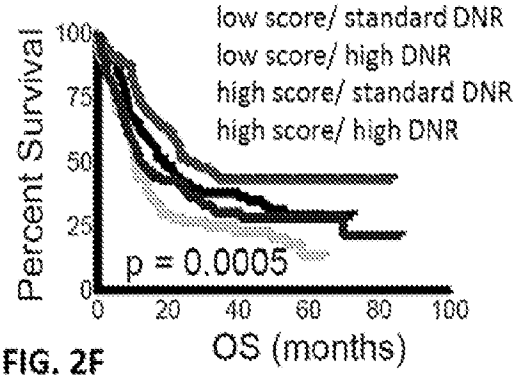


FIG. 2F

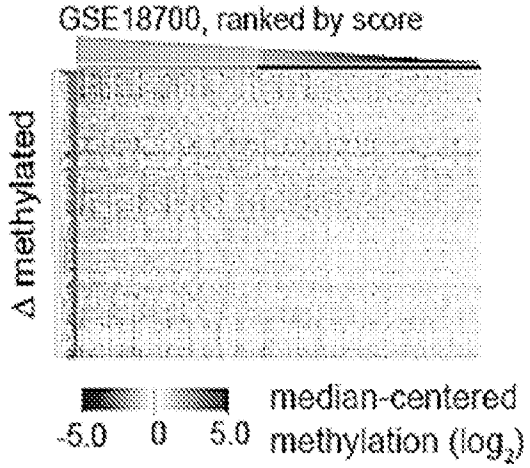


FIG. 2G

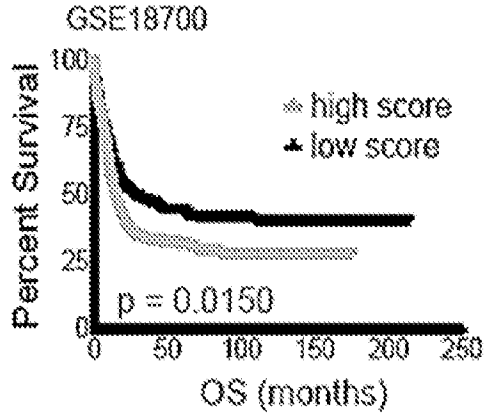


FIG. 2H

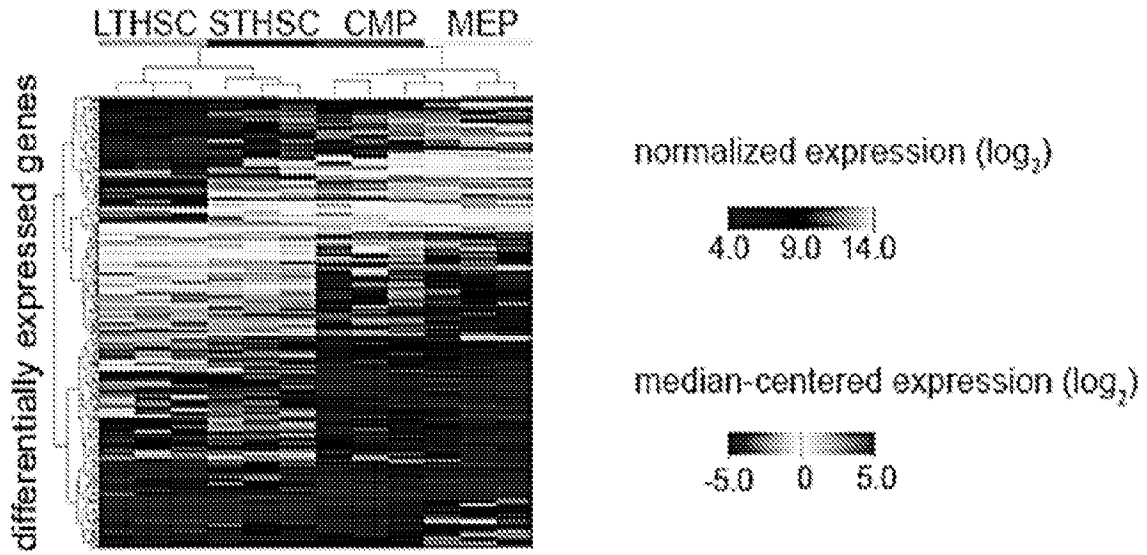


FIG. 3A

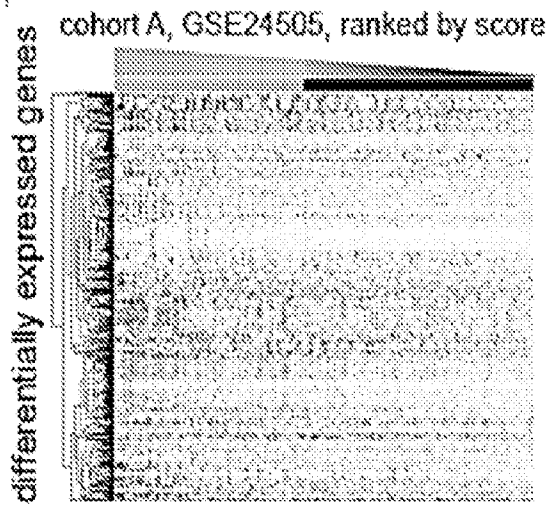


FIG. 3B

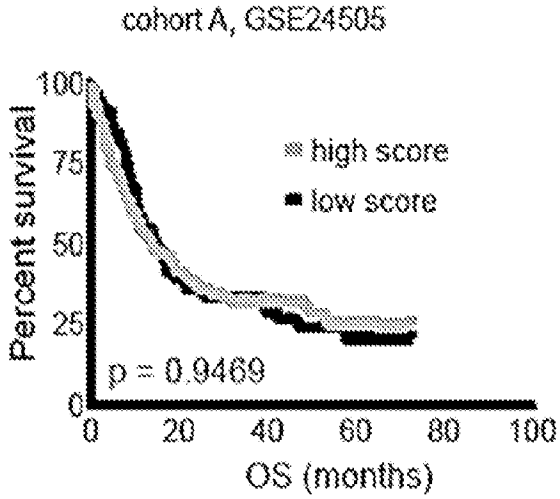


FIG. 3C

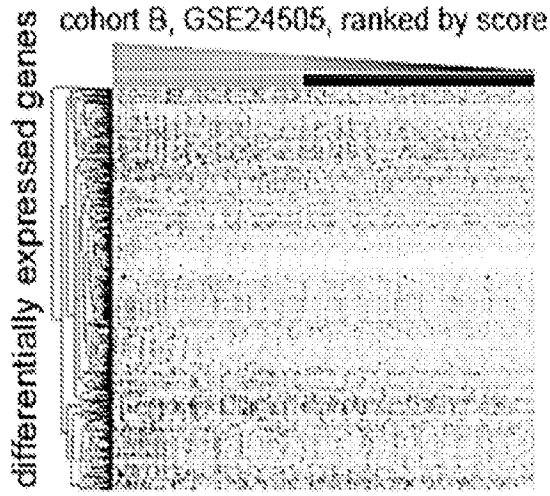


FIG. 3D

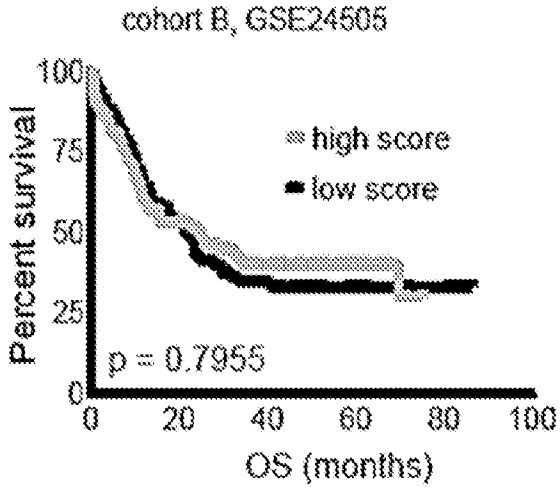


FIG. 3E

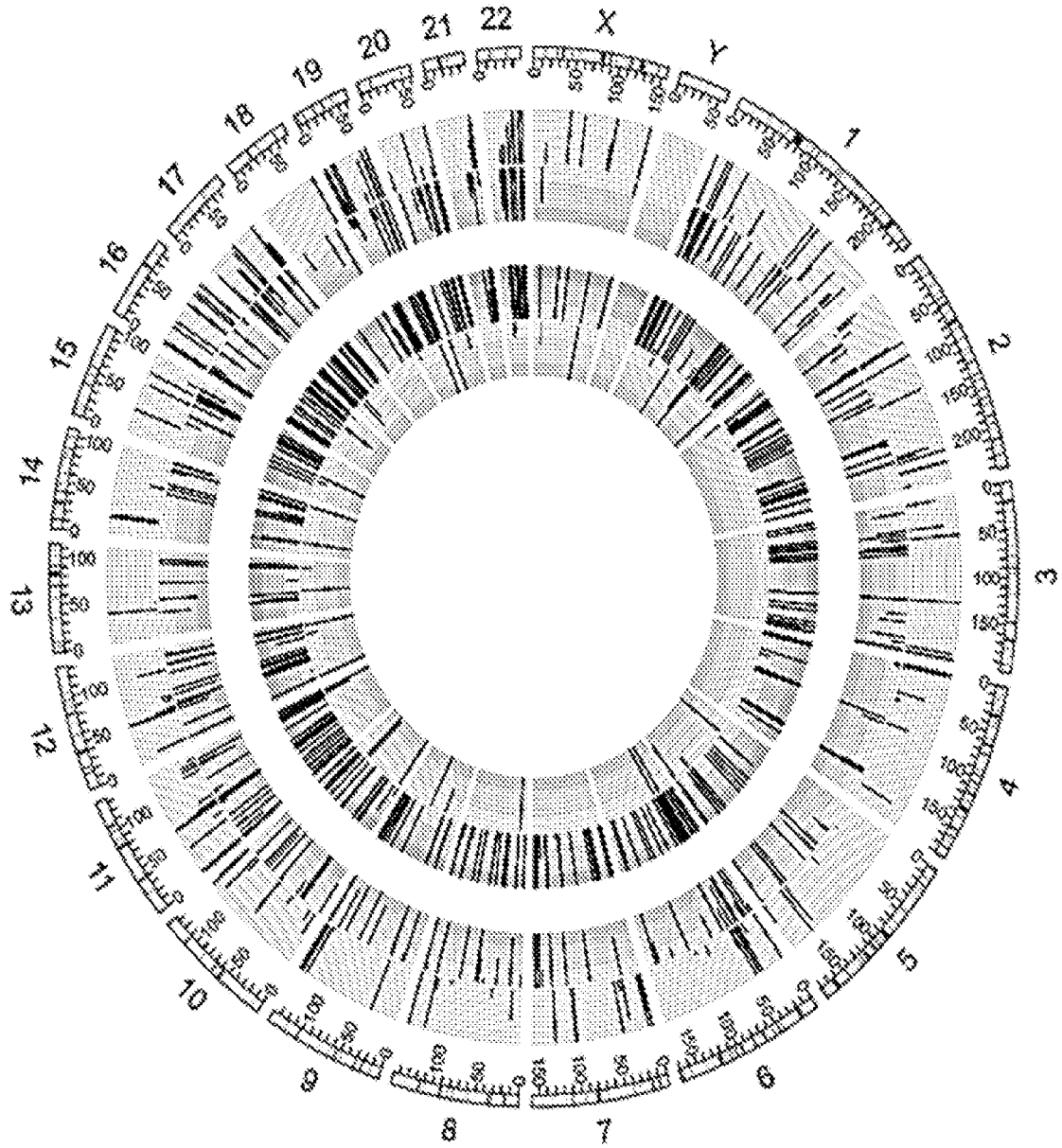

1
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10
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12
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14
15
16
17
18
19
20
21
22
X
Y
demethylated during STHSC → CMP transition
methylated during STHSC → CMP transition
gene expression increased during STHSC → CMP transition
gene expression decreased during STHSC → CMP transition

FIG. 4

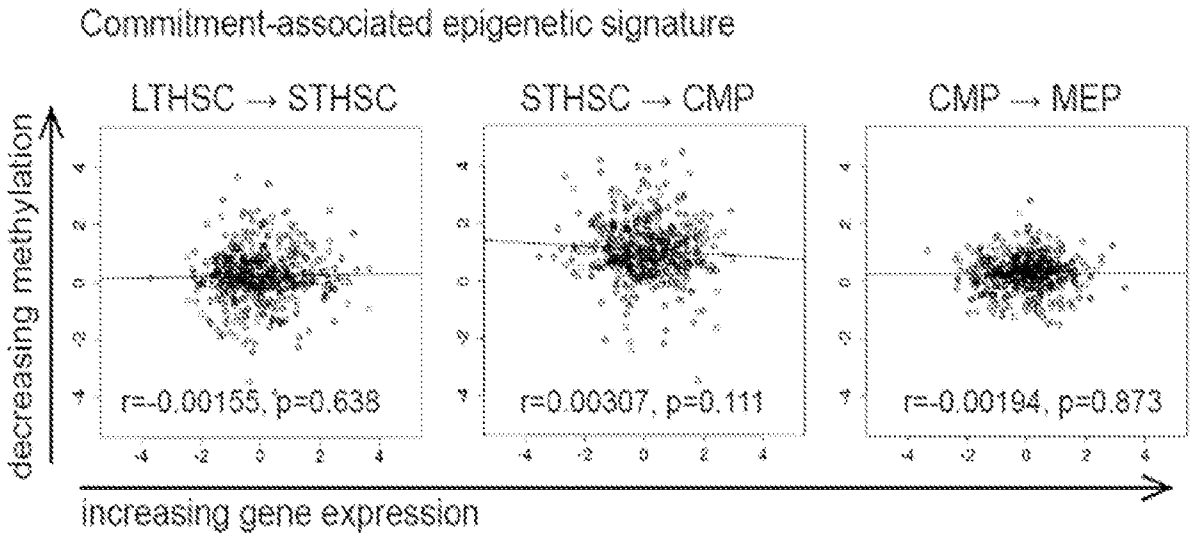

Commitment-associated epigenetic signature
LTHSC → STHSC
STHSC → CMP
CMP → MEP
decreasing methylation
r=-0.00155, p=0.638
r=0.00307, p=0.111
r=-0.00194, p=0.873
increasing gene expression

FIG. 5A

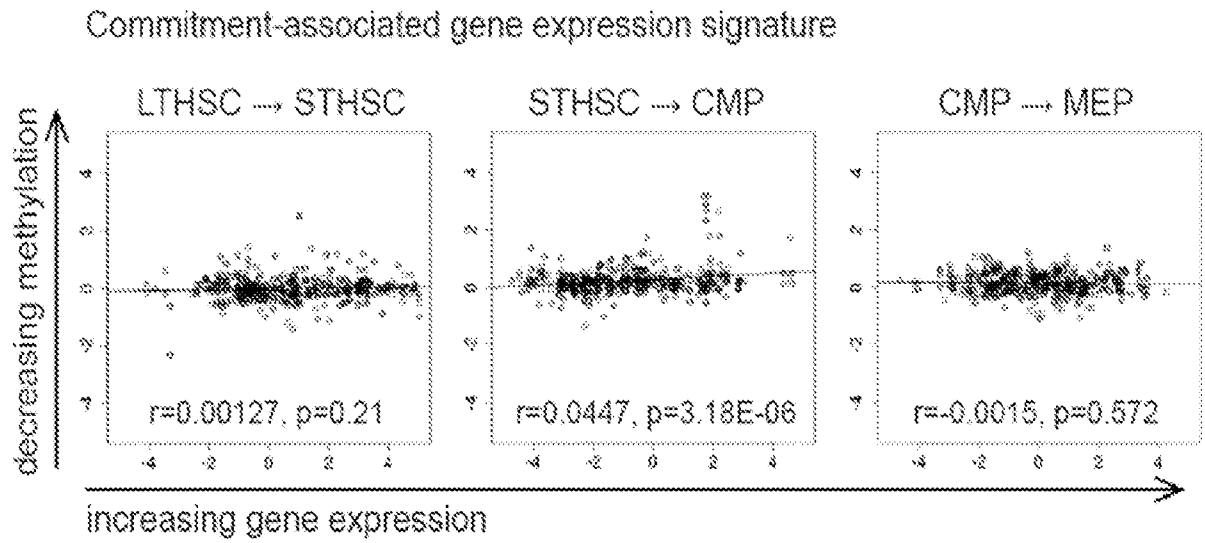

Commitment-associated gene expression signature
LTHSC → STHSC
STHSC → CMP
CMP → MEP
decreasing methylation
r=0.00127, p=0.21
r=0.0447, p=3.18E-06
r=-0.0015, p=0.572
increasing gene expression

FIG. 5B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/72474

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C40B 30/04; C12Q 1/68 (2015.01)
CPC - C12Q 2600/154, 2600/112, 1/6886
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C40B 30/04; C12Q 1/68
CPC: C12Q 2600/154, 2600/112, 1/6886, 2600/16, 1/683

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; NCBI/PubMed; Dialog ProQuest; 'acute myeloid leukemia,' 'AML,' methylation, 'CpG,' patient, subject, score, value, level, expression, prognosis, control

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0264306 A1 (CALDWELL, CW et al.); October 22, 2009; paragraphs [0018], [0021], [0027], [0028], [0042], [0067], [0079], [0104]-[0107], [0014], [0125], [0148], [0152]; Tables 3, 11	1-4, 6-10, 28-31
Y	US 2013/0261009 A1 (SINGULEX, INC.); October 03, 2013; paragraphs [0126], [0139], [0140]	1-4, 6-10, 28-31
Y	US 2010/0267021 A1 (GREALLY, JM et al.); October 21, 2010; paragraphs [0010], [0019], [0055], [0063], [0067]	3, 4/1-4/3, 7/1, 7/6, 8/1, 8/2, 8/6, 30, 31
Y	BROSKE, AM et al. DNA Methylation Protects Hematopoietic Stem Cell Multipotency From Myeloerythroid Restriction. Nat Genet. November 2009, Vol. 41, Nno. 11; pages 1207-15. DOI: 10.1038/ng.463. Epub 04 October 2009; page 1208, column 2, paragraphs 2, 3, page 1209, column 1, paragraph 2; page 1209, column 2, paragraph 2; page 1214, column 1, paragraph 2; figures 2a,b, 3b,g.	6, 7/6, 8/6, 9/6, 10/9/6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 June 2015 (04.06.2015)	08 JUL 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/72474

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+: Claims 1-4, 6-10, 28-31, gene C1orf170 (nearest associated gene loci; ID NR_027693), gene TNFRSF4 (nearest associated gene loci; ID NM_003327); Exemplary Election: gene UBE2J2 (nearest associated gene; ID: NM_058167), gene MORN1 (nearest associated gene; ID: NM_024848)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US14/72474

-***-Continued from Box III -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1-4, 6-10 and 28-31 are directed toward a method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising: quantifying methylation of DNA of a sample comprising blood cells obtained from a subject with AML at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as demethylated at STHSC-CMP transition and/or at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as methylated at STHSC-CMP transition; and a kit therefor.

The methods and kit will be searched to the extent that the plurality of chromosome loci or nearest associated genes encompass gene C10rf170 (nearest associated gene loci; ID NR_027693) and TNFRSF4 (nearest associated gene loci; ID NM_003327). It is believed that Claims 1 (in-part), 2 (in-part), 3 (in-part), 4 (in-par), 6 (in-part), 7 (in-part), 8 (in-part), 9 (in-part), 10 (in-part), 28 (in-part), 29 (in-part), 30 (in-part) and 31 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass gene C1orf170 (nearest associated gene loci; ID NR_027693) and gene TNFRSF4 (nearest associated gene loci; ID NM_003327). Applicant is invited to elect additional loci or nearest associated gene(s) to be searched. Additional loci or nearest associated gene(s) will searched upon the payment of additional fees. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: gene UBE2J2 (nearest associated gene; ID: NM_058167), gene MORN1 (nearest associated gene; ID: NM_024848).

Groups I+ share the technical features including: a method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising: a) quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2; b) determining a methylation score from the methylation determined in step a); c) comparing the methylation score of DNA of the sample of blood cells obtained from the subject with AML with a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and d) assigning a prognosis to the subject, wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject, and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject; a method for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising: quantifying methylation of DNA of a sample comprising blood cells obtained from a subject with AML at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as demethylated at STHSC-CMP transition and/or at a plurality of the chromosome loci, or nearest associated gene, listed in Table 2 as methylated at STHSC-CMP transition, comparing the extent of the methylation with the methylation of DNA at the same plurality or pluralities of chromosome loci, or nearest associated gene, in a sample of blood cells obtained from a subject without AML, and assigning a prognosis to the subject, wherein demethylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject, and wherein demethylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject; a kit for determining the prognosis of a subject having acute myeloid leukemia (AML), comprising a) reagents for quantifying the methylation of DNA of a sample comprising blood cells or bone marrow cells obtained from a subject with AML at a plurality of chromosome loci, or nearest associated gene, as listed in Table 2; b) written instructions for determining a methylation score from the methylation determined with the reagents in a); c) written instructions for a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and for assigning a prognosis to the subject based on the methylation score compared to the predetermined reference amount, wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject, and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Box III -***-

However, these shared technical features are previously disclosed by US 2009/0217801 A1 to Van Den Boom, et al. (hereinafter 'Van Den Boom') in view of the publication entitled 'Concise Review: Preleukemic Stem Cells: Molecular Biology And Clinical Implications Of The Precursors To Leukemia Stem Cells' by Pandolfi, et al. (hereinafter 'Pandolfi').

Van Den Boom discloses a method for determining the prognosis (a method for the prognosis; abstract) of a subject having acute myeloid leukemia (AML) (in patients (of a subject) with (having) acute myeloid leukemia; abstract), comprising: a) quantifying the methylation of DNA (determining the methylation state of a plurality of DNA loci (a) quantifying the methylation of DNA); paragraphs [0009], [0201]) of a sample comprising blood cells or bone marrow cells (of a nucleic acid sample from blood or bone marrow; paragraphs [0010], [0064]) obtained from a subject with AML (paragraph [0010]) at a plurality of chromosome loci (paragraphs [0010], [0201]), or nearest associated gene (or target genes; paragraph [0010]), as listed in Table 2 (including HOXA10 and CEBPA (as listed in Table 2); paragraph [0044]); b) determining a methylation score from the methylation determined in step a) (determining an analysis methylation score (determining a methylation score from the methylation determined in step a)); paragraph [0344]); c) comparing the methylation score of DNA of the sample of blood cells obtained from the subject with AML with a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene (comparing relative methylation between long and reduced survival groups; paragraphs [0010], [0320]), and d) assigning a prognosis to the subject (paragraphs [0010], [0344], [0345]), wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject (wherein a high methylation score predicts a worse outcome; paragraph [0344]), and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject (wherein low score is characterized by a good outcome; paragraph [0344]); a kit (paragraph [0037]) for determining the prognosis of a subject having acute myeloid leukemia (AML) (for determining the methylation state of target gene regions to determine the prognosis of a patient with acute myeloid leukemia; paragraphs [0010], [0037]), comprising a) reagents for quantifying the methylation of DNA of a sample (reagents for the determination the methylation state of target gene regions; paragraph [0037]) of a sample comprising blood cells or bone marrow cells obtained from a subject with AML (paragraphs [0010], [0064]) at a plurality of chromosome loci (paragraphs [0010], [0201]), or nearest associated gene (or target genes; paragraph [0010]), as listed in Table 2 (including HOXA10 and CEBPA (as listed in Table 2); paragraph [0044]); and wherein the presence or absence of one or methylated or unmethylated nucleotides may be indicative of a particular disease outcome, or prognosis thereof (wherein the presence or absence of one or methylated or unmethylated nucleotides may be indicative of a particular disease outcome, or prognosis thereof; paragraph [0293]), and wherein a relatively high number or a relatively low number of methylated nucleotide loci compared to a normal individual may be indicative of a poor prognosis (wherein a relatively high number or a relatively low number of methylated nucleotide loci compared to a normal individual may be indicative of a poor prognosis; paragraph [0293]).

Van Den Boom does not disclose wherein demethylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the majority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject, and wherein demethylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject, and wherein methylation of the minority of the plurality of loci or nearest associated gene listed in Table 2 as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject; and written instructions for determining a methylation score from the methylation determined with the reagents in a); c) written instructions for a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and for assigning a prognosis to the subject based on the methylation score compared to the predetermined reference amount, wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject, and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject.

Pandolfi discloses wherein aberrant cytosine methylation is a hallmark of patients with AML (page 145, column 2, paragraph 2), and wherein myelodysplastic syndrome (MDS) high risk patients exhibit expansion of LT-HSC while low risk patients demonstrate expansion of common myeloid progenitors (page 147, column 2, paragraph 1), and suggest wherein MDS progression to AML may be based on aberrant MDS stem cells being a pre-leukemic stem cell with regards to AML (suggest wherein MDS progression to AML may be based on aberrant MDS stem cells being a pre-leukemic stem cell with regards to AML; page 145, second column, second paragraph to page 145, second column, third paragraph; page 147, second column first paragraph to page 147, second column, second paragraph).

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-***-Continued from Box IV -***-

It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of Van Den Boom for integrating determining methylation for stem or progenitor cells at different stages, such as different HSC compartments, including LT-HSC or ST-HSC, and CMP cells, in particular between the CMP cells and their immediate precursors, ST-HSC, based on the disclosure of Pandolfi, for improving the prognosis of AML, as disclosed by Van Den Boom, by including determining the methylation levels of genes in cell type associated with better or worse prognosis of MDS, including AML, wherein demethylation of the majority of the plurality of loci or nearest associated genes as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject; wherein methylation of the majority of the plurality of loci or nearest associated genes as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject; wherein demethylation of the minority of the plurality of loci or nearest associated genes as demethylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample of the subject without AML indicates a negative prognosis for the subject; and wherein methylation of the minority of the plurality of loci or nearest associated genes as methylated at STHSC-CMP transition in the DNA of the sample from the AML subject compared to the DNA of the sample from the subject without AML indicates a negative prognosis for the subject, based on the use of a score metric and wherein a relatively high number or a relatively low number of methylated nucleotide loci compared to a normal individual may be indicative of a poor prognosis, as previously disclosed by Van Den Boom. Furthermore, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have provided appropriate written instructions or instructions displayable using a computer-based system in the kit disclosed by Van Den Boom, for implementing the determination of a methylation score from the methylation determined with the reagents in a); c) and instructions for a predetermined reference amount for the same plurality of chromosome loci, or nearest associated gene, and for assigning a prognosis to the subject based on the methylation score compared to the predetermined reference amount, wherein a methylation score at or in excess of the predetermined reference amount indicates a negative prognosis for the subject, and wherein a methylation score below the predetermined reference amount indicates a positive prognosis for the subject, for enabling a medical professional or laboratory personnel to effectively utilize the kit to perform the required measurements and prognosis.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Van Den Boom and Pandolfi references, unity of invention is lacking.