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DNA Methylation in Acute Leukemia

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1. Introduction

After birth, all blood cells are produced in the bone marrow by a process known as hematopoiesis. The basic biological process of hematopoiesis is the differentiation of hematopoietic stem cells (HSCs) to generate different types of mature blood cells (Kawamoto et al., 2010). In an adult, approximately 10^{12} blood cells are produced daily and released into the circulating peripheral blood. As new cells are released, some old cells undergo apoptosis in tissues or cleaned by spleen in order to maintain a homeostatic level of blood cells. Hematopoiesis is highly regulated through the interaction between hematopoietic cells and the bone marrow microenvironment. Many cytokines or growth factors and extracellular matrix molecules that are secreted either from stromal cells or from hematopoietic cells, as well as nutrients and vitamins, provide a favorable microenvironment for hematopoiesis to occur (Metcalf, 2008). Under the influence of specific growth factors such as c-KIT ligand and FLT-3 ligand, after rounds of asymmetric divisions of hematopoietic stem cells (HSCs), some daughter cells participate in lineage-commitment differentiation to become either lymphoid or myeloid progenitors. After several rounds of proliferation, these progenitor cells undergo terminal differentiation becoming mature lymphocytes (B-cells, T-cells and NK-cells) or myeloid cells (granulocytes, monocytes, red blood cells, mast cells and platelets). Generation of lymphocytes and myeloid cells in bone marrow during hematopoiesis is termed as lymphopoiesis and myelopoiesis, respectively. Disruption of these normal physiological processes at the stages of HSCs and/or progenitors may initiate leukemogenesis, a neoplastic transformation, and result in leukemia. Leukemia is classified as acute or chronic leukemia based on the clinical presentation and pathophysiological features. Acute leukemia is classified as acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) based on the cell lineages. Therefore, ALL may be B-cell, T-cell, or NK-cell in origin, while AML may be a granulocytic, monocytic, erythroid, or megakaryocytic subtype (Swerdlow et al., 2008; Vardiman et al., 2009).

2. Clinical aspects of acute leukemia

ALL and AML are the most common leukemias in children and adults, respectively (Siegel et al., 2011). Both diseases are characterized by acute onset and rapid accumulation of immature leukemic cells (blasts) in the bone marrow and blood (>20% of nucleated cells). Leukemic blasts are abnormal because they remain immature and do not function like

mature white blood cells. ALL occurs mainly in children with peak prevalence between the ages of 2 and 5 years. Approximately 3,200 new ALL cases in childhood are diagnosed in the United States each year and two thirds of these cases are the B-cell subtype (Pui et al. 2004). Worldwide, ALL occurs approximately 5 cases per 100,000 populations per year. In contrast, AML occurs mainly in adults aged 65 years or older with a median age of 60 years (Löwenberg et al., 1999; Estey & Döhner, 2006). Approximately 13,400 new AML cases are diagnosed in the United States each year. Incidence of AML is about twice that of ALL worldwide. The prognosis is much poorer for AML than ALL, especially in elderly patients (Estey & Döhner, 2006).

Both ALL and AML are clonal disorders of hematopoietic stem cells (HSCs) or progenitor cells characterized by loss of normal maturation and gain of capacity of uncontrolled proliferation (Pui et al. 2004; Estey & Döhner, 2006; Becker et al. 2010). Leukemic blasts accumulate in blood and bone marrow and replace normal hematopoietic elements in the bone marrow. Furthermore, the blasts can infiltrate other organs and tissues including spleen, liver, lymph nodes and even skin. Most patients with acute leukemia present with the consequences of bone marrow failure presenting as anemia (decreased red blood cells), neutropenia (decreased neutrophils), and thrombocytopenia (decreased platelets). Pancytopenia (decreased all types of blood cells) with circulating blasts is the strong evidence for the diagnosis of acute leukemia. After diagnosis of a specific subtype of acute leukemia with advanced and integrated approaches including clinical information, laboratory data, morphology, immunophenotype (flow cytometry or immunohistochemistry), and genetic tests (cytogenetic karyotype and molecular genetic analysis), an effective chemotherapy regimen must be immediately initiated. The goal of treatment is to eliminate the leukemic blasts, preserve and restore normal hematopoiesis, and to prevent relapse (Bassan & Hoelzer, 2011; Burnett et al., 2011). To avoid the toxicity of chemotherapy, the patients are stratified based on the biological features of leukemia blasts and risk factors of the individual patient (host). In addition to clinical factors (age, gender, leukocyte count, etc.), specific genetic abnormalities, such as chromosomal translocations and/or gene mutations, are the most important factors in determining risk stratification in modern treatment of the leukemia patients (Pui et al., 2011). Although the treatment regimens are different in ALL and AML, a standard protocol typically consists of three phases: a remission-induction phase, an intensification (or consolidation) phase, and a continuation (maintenance) phase (Bassan & Hoelzer, 2011; Burnett et al., 2011). The goal of remission-induction treatment is to eradicate more than 99% of the initial leukemic cell burden and to restore normal hematopoiesis. The intensification treatment is generally aimed to eradicate drug-resistant residual leukemic cells including leukemia stem cells (Becker & Jordan, 2010). The last phase is maintenance chemotherapy for an additional 2.0-2.5 years to reduce the risk of relapse. The regimens usually include several drugs that have different pharmacological mechanisms of action to have maximal efficacy. In addition to routine chemotherapy, allogeneic hematopoietic stem-cell transplantation is the most intensive form of treatment for high risk acute leukemia (Gupta et al., 2010). Compared to various solid tumors, outcomes are excellent for treatment of acute leukemia, especially in young patients. In most large clinical trials, the cure rates are more than 80% and 60% in ALL and AML, respectively (Pui et al., 2011).

3. Genetic alterations

Like all other malignancies, acute leukemia is a genetic disease. Specific genetic alterations including chromosomal translocation, deletion, addition, and gene mutations (point mutation, copy number change) have been identified in all ALL and AML cases. These genetic alterations are widely utilized for diagnosis, risk stratification, prediction of response to chemotherapeutic reagents, prognosis and detection of minimal residual disease (Estey & Döhner, 2006; Swerdlow et al., 2008; Pui et al., 2008).

3.1 Genetic alterations in ALL

At the genetic level, ALL is a group of heterogeneous diseases. Standard cytogenetic analyses can detect primary chromosomal abnormalities in more than 70% of ALL cases (Mrózek et al., 2009). Using higher resolution and/or high throughput molecular methods, genetic alterations can be identified in virtually all cases of ALL tested (Mullighan et al., 2007). These alterations include gene rearrangements, gene copy number changes (deletions or duplications) and genomic sequence point mutations. Some of these changes are directly linked with leukemogenesis and affect important cellular pathways in cell differentiation, cell cycle regulation, tumor suppression, and apoptosis. For instance, PAX5, a B-cell specific transcription factor important for B-cell differentiation, was found frequently deleted or mutated in B-ALL (Mullighan et al., 2007). Other changes may be merely "passengers" and irrelevant to the biological properties and leukemogenesis of ALL.

The WHO classification of tumors of hematopoietic and lymphoid tissues (Swerdlow et al., 2008) designates ALL as B- or T-lymphoblastic leukemia/lymphoma based on cell lineages and percentage of the blasts in bone marrow. Each lineage group is further categorized as ALL with recurrent genetic abnormalities and not otherwise specified (NOS) based on identifiable chromosomal abnormalities by routine cytogenetic analysis (Figure 1). B-ALLs with recurrent genetic abnormalities include B-ALL with t(9;22)(q34;q11.2); *BCR-ABL1*, B-ALL with t(v;11q23); *MLL* rearranged, B-ALL with t(12;21)(p13;q22) *TEL-AML1(ETV6-RUNX1)*, B-ALL with hyperdiploidy, B-ALL with hypodiploidy, B-ALL with t(5;14)(q31;q32) *IL3-IGH*, B-ALL with t(1;19)(q23;p13.3); *E2A-PBX1* (*TCF3-PBX1)*. Each designation contains the chromosomes involved, chromosomal loci, and the genes as well as alternative gene names. This subgroup accounts for 60% to 80% of B-ALL cases with distinct biologic and pharmacologic features that are important in diagnosis and risk stratification. The remaining B-ALL cases with no identifiable chromosomal abnormalities are characterized on the basis of morphologic and immunophenotypic features Figure 1A. Fifty to seventy percent of T-ALL patients demonstrate abnormal karyotypes. The most common recurrent abnormalities are translocations that involve the alpha and delta T-cell receptor loci at 14q11.2, the beta locus at 7q35, or the gamma locus at 7p14-15, and many partner genes (Pui et al., 2004; Giroux et al., 2006; Swerdlow et al., 2008). The pathological significance of these abnormalities is not as clearly understood as those that are associated with B-ALL.

3.2 Genetic alterations in AML

More than 90% of AML cases have at least one known genomic alteration as demonstrated by current routine cytogenetic or molecular analysis (Löwenberg et al., 1999) Figure 1B.

Using high resolution and high throughput methods, virtually all AML cases are identified as having distinct genetic mutations (Estey & Döhner, 2006; Godley et al., 2011).

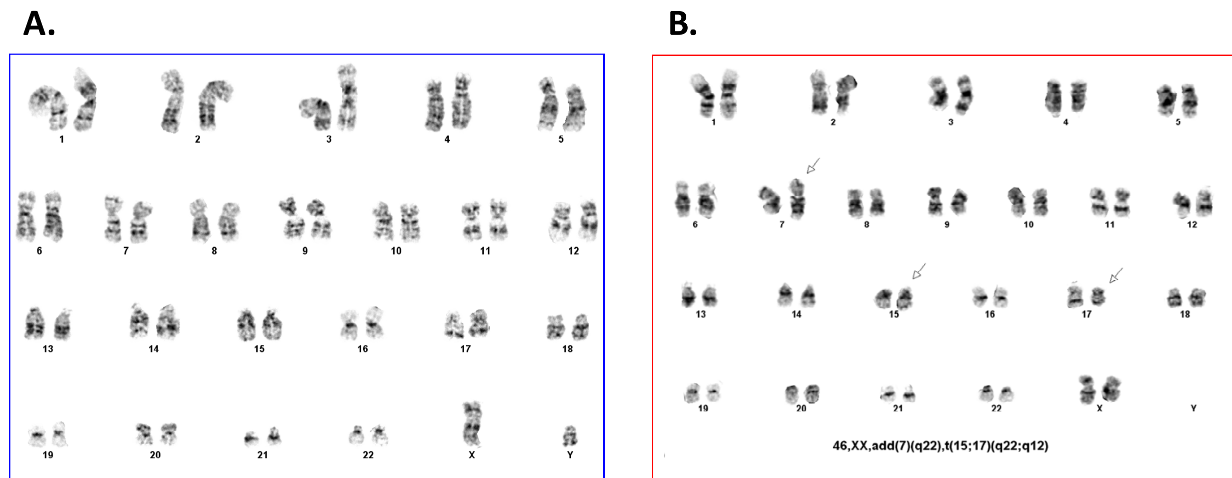

Fig. 1. Genetic alterations in ALL and AML. Genetic alterations can occur at either chromosomal level or DNA sequence level. Although some acute leukemia cases have a normal karyotype (A, an ALL patient), there are many mutations detectible at the DNA level. Most acute leukemia cases demonstrate specific chromosomal abnormalities that defined a distinct subtype of ALL or AML with important diagnostic and prognostic applications. The right panel (B) shows a case of acute promyelocytic leukemia (APL) with t(15;17)(q22;q12); *PML-RARA* (with addition at chromosome 7 in this case). APL represents a cytogenetically defined AML that can be treated with less toxic all-*trans* retinoid acid, a metabolite of vitamin A, with a favorable prognosis.

AML is a group of extremely heterogeneous diseases at the genetic level. Based on cytogenetic features and cell lineages, the WHO classification of tumors of hematopoietic and lymphoid tissues classifies AML into seven cytogenetic types with specific genetic abnormalities and nine not otherwise specified (NOS) types based on distinct morphology, cytochemistry and immunophenotype (Figure 1B). Furthermore, two provisional subtypes have been proposed based on point mutations in *NPM1* and *CEBPA* genes (Swerdlow et al., 2008, Vardiman et al., 2009; Döhner, 2010). AML with recurrent genetic abnormalities include AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*, AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*, APL with t(15;17)(q22;q12); *PML-RARA* (Figure 1B), AML with t(9;11)(p22;q23); *MLLT3-MLL*, AML with t(6;9)(p23;q34); *DEK-NUP214*, AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*, AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*. The chromosomes, loci and the genes are indicated in each specific designation. Many of these chromosomal translocations result in fusion proteins with altered functions of transcription factors critical for normal hematopoiesis and myeloid differentiation (Sternberg & Gilliland, 2004). These cytogenetic abnormalities also have important diagnostic and therapeutic implications. In contrast, the cases with mutations in nucleophosmin (*NPM1*), CCAAT/enhancer binding protein α (*CEBPA*), Fms-like tyrosine kinase (*FLIT3*) and *KIT* genes have been associated with prognosis. More than half of these cases with the gene mutations have normal karyotypes (Marcucci et al., 2005; Foran, 2010).

New approaches such as whole-genome sequencing, gene expression and microRNA profiling, proteomics and genome-wide DNA methylation profiling will not only identify all known mutations and epigenetic alterations, but can also potentially identify novel mutations and epigenetic lesions in biological pathways at the individual level (Boehm & Hahn, 2011). Deciphering the interactions between genetic and epigenetic alterations and expression profile will further provide a comprehensive and high resolution blueprint of leukemogenesis. That information, in turn, will be applied in clinical management of AML patients in the future.

Over the past few years, high-throughput next generation DNA sequencing (NGS) technologies have revolutionized the field of cancer genomics including leukemia (Metzker, 2010). Recently, Link and colleagues performed whole-genome sequencing on AML leukemic cells from a 37 year old woman with suspected cancer susceptibility syndrome (Link et al., 2011). The patient developed a therapy-related acute myeloid leukemia (t-AML) after chemotherapy for her breast cancer and ovarian cancer. Whole-genome sequencing revealed a novel, heterozygous 3-kilobase deletion removing exons 7-9 of *TP53* gene in germline DNA of patient normal skin cells. The deletion became homozygous in the leukemia DNA as a result of uniparental disomic recombination. Additionally, a total of 28 somatic single-nucleotide variations in coding regions, 8 somatic structural variants, and 12 somatic copy number alterations were identified in the patient's leukemia genome. Using a similar approach, Welch and colleagues performed whole-genome sequencing on a 39-year-old woman with a diagnosis of acute promyelocytic leukemia (APL) with unusual genetic lesion (Welch et al., 2011). The sequencing identified a novel cryptic insertion of *PML* gene into chromosome 17 that produced a classic pathogenic *PML/RARA* gene fusion figure 1B. This type of genetic event could not have been identified with routine cytogenetic and FISH techniques. The results led to a change in therapy using a less toxic targeted reagent, retinoic acid, rather than using the high risk procedure of allogeneic bone marrow transplantation.

These two cases represent excellent examples that whole-genome sequencing in leukemia not only detects novel genetic mutations in a cancer genome, but also directly benefits patient care. Although next generation sequencing has not been used for routine clinical diagnosis due to its high cost and long turnaround time (7 weeks in the latter case), it does provide insights for understanding the molecular mechanisms of leukemogenesis at the DNA sequence level in individual patients. Most likely, it will be used in clinical diagnosis with a "one-size-fits-all" feature in the near future.

4. Epigenetics

The completion of the human genome in 2003 held great promise for uncovering the cancer-causing genetic mutations that would allow for the development of targeted therapies and the eradication of cancer. Unfortunately, this has not come to fruition due, in large part, to the role of epigenetic modifications in the development of cancer. An individual's epigenetic makeup is much more complex than their genetic makeup. Human DNA sequence in all somatic tissue cells (except lymphocytes) is identical in a given individual. However, epigenetics presents in a developmental and tissue-specific manor. Epigenetic modifications are largely responsible for the differences in all somatic tissue cells such as brain, liver, skin

cells, hair cells and the cells that make up the human eye. As a matter of fact, epigenetic modifications are responsible for regulating the expression of genes that establish each of the tissue types in the body.

Epigenetics is defined as the change of gene expression caused other than DNA sequence change (Bird, 1980; Robertson & Jones, 2000). There are 3 key epigenetic players which work in concert to control/alter gene expression. These include DNA methylation, histone modifications and non-coding RNAs inference (Figure 2). DNA cytosine methylation occurs at the 5th position of cytosine in CpG dinucleotide. Methyl binding proteins are recruited to methylated DNA regions and can block transcription factors in the promoter of a gene. The inaccessibility of transcription factors to the DNA results in a silenced gene. There are also numerous histone modifications such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation which can be present alone or in combination and each has an impact on chromatin structure. For example, some histone modifications (histone code) are associated with open chromatin (euchromatin) and some are associated with condensed chromatin (heterochromatin). If the chromatin is open, genes are accessible and can be transcribed. Alternately if the chromatin is closed, the genes are inaccessible to transcription

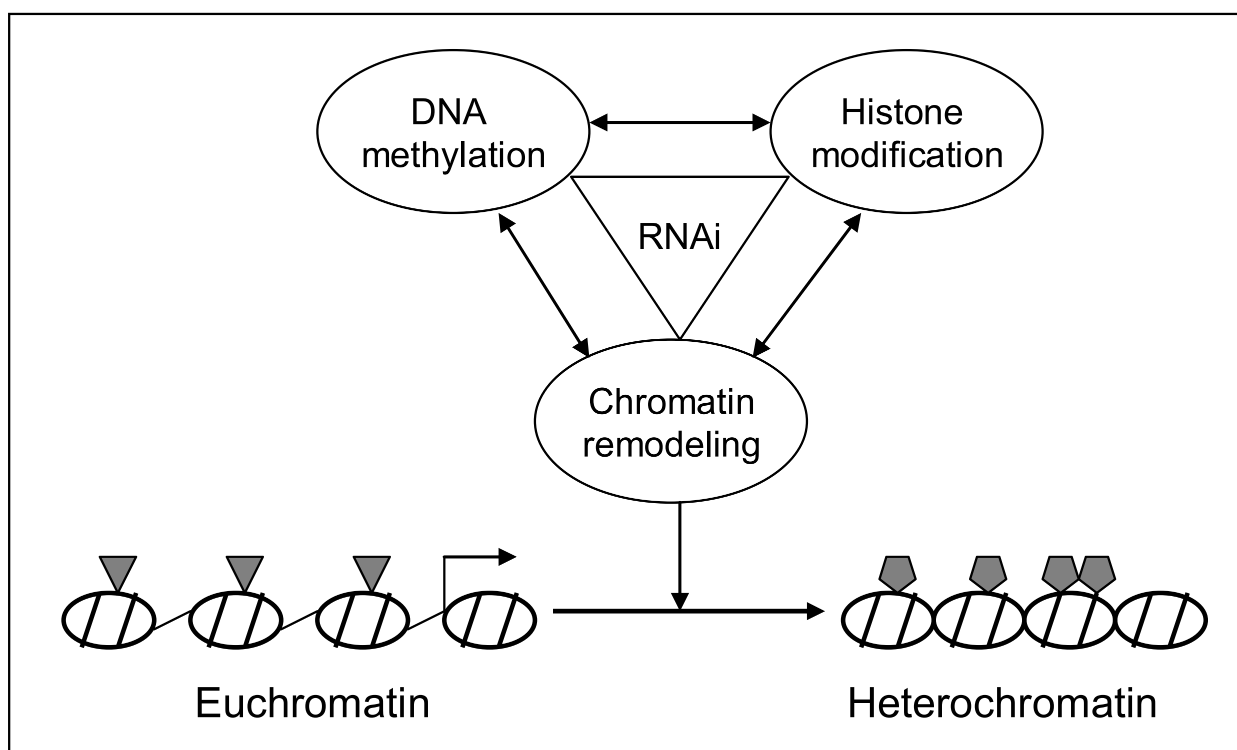

Fig. 2. Epigenetic network. Cell epigenetic network consists of DNA methylation, histone methylations (pentagon), histone acetylation (triangle), chromatin (nucleosome) remodeling, and RNA interference (RNAi) induced by microRNAs and short interfering RNAs (siRNAs). Interaction between these epigenetic components results in transition of euchromatin to heterochromatin. The transcription is inactivated (silenced) permanently when the focal open chromatin becomes a closed heterochromatin configuration (Adapted from Wang MX and Shi HD: Basics of Molecular Biology, Genetic Engineering and Metabolic Engineering. In: Fu, PC, Latterich, M, and Panke, S (Eds): Systems Biology and Synthetic Biology, John Wiley & Sons, Inc., pp.36, 2009, with permission)

factors and therefore cannot be transcribed. Finally, non-coding RNAs play a role in gene expression by binding and degrading messenger RNA and inhibiting protein assembly (Chen J et al, 2010, Melo and Esteller, 2011). While each of these modifications plays an important role in gene expression, DNA methylation has been the most widely studied epigenetic modification that is associated with the development of acute leukemia and other types of cancer.

DNA methylation occurs globally in the normal genome and is estimated to affect between 70 and 80% of all CpG dinucleotides in human cells (Bird, 1980; Robertson & Jones, 2000). These dinucleotides are not uniformly distributed across the genome but occur in clusters such as large repetitive sequences or in CG-rich DNA stretches known as CpG islands (CGIs). Normally, the majority of the CpG dinucleotides which are found in intragenic regions, including repetitive sequences such as satellite sequences and centromeric repeats, contains methylated CpG dinucleotides; while CGIs which are found preferentially in the promoter regions of genes typically contain unmethylated CpG dinucleotide (Craig & Bickmore, 1994). Some exceptions to this rule include those CGIs located on the inactive X chromosome in females (Goto & Monk,1998) and those associated with imprinted genes (genes for which only the paternally- or maternally-inherited allele is expressed) which are methylated in the normal state (Li et al., 1993; Razin & Cedar, 1994).

In leukemia, the normal pattern of methylation gets reversed. Those regions of the genome that are normally methylated (and inactivated) such as repetitive sequences lose methylation and result in genomic instability. Those regions of the genome which are typically unmethylated such as the promoters of tumor suppressor genes become methylated and gene expression is lost. A typical gene comprises a promoter region, transcriptional start site (TSS), 5′ untranslated region (UTR), exons, introns and a 3′UTR. CGIs may encompass each of these genetic regions. Typically methylation present within the gene body (exons, introns) is associated with gene expression and methylation present in gene promoters, 5′UTRs and the first exon is associated with gene silencing. The majority of genome-wide methylation studies to date have focused on CGI at the promoter regions of genes via microarray methylation analysis. Recently, it has been suggested that not only are the CGI themselves important but that the flanking genomic regions termed CpG shores are more highly correlated with gene silencing (Irizarry et al., 2009).

5. Emerging epigenetic technologies

DNA methylation plays an important role in acute leukemia. This is evidenced by the growing number of studies that have recently been published describing methylation profiles for many types and subtypes of leukemia. A number of strategies have been used to examine DNA methylation and over time these methods have progressed from small-scale candidate gene analysis to the ability to construct whole-genome methylation profiles (Laird, 2010). These can broadly be divided into three classes: those that require bisulfite conversion, those that are affinity based and those that require the use of restriction enzymes. Restriction enzyme-based methods such as DMH (Huang et al., 1999) and MCA (Huang et al., 1999; Toyota & Issa, 2002) rely on methylation-sensitive enzymes and are limited by the fact that restriction sites are not present in all possible CpG rich regions of interest. In affinity based approaches such as methylated DNA immunoprecipitation (MeDIP) (Weber et al., 2005) and methylated CpG island recovery assay (MIRA) (Rauch &

Pfeifer, 2005), fragmented DNA is immunoprecipitated using a monoclonal antibody to 5-methylcytosine or by methylated DNA binding proteins respectively. Methylated DNA is enriched in affinity based methods, however, these methods are limited by the sensitivity of the antibody or protein being used and are typically biased towards regions of the genome with a high density of methylation. Sodium bisulfite converts unmethylated cytosines to uracils in DNA strands. Once the conversion has taken place the sequence can be determined after PCR because the cytosines that were originally unmethylated will be converted to thymines and those that are methylated will remain cytosines. The net effect of the conversion process is that the complexity of the genome is reduced from 4 bases to only 3 bases. Bias may be introduced due to incomplete bisulfite conversion, destruction of DNA strands and also by the efficiency of bisulfite PCR. After the samples are prepared, they can be hybridized to microarrays or used to make sequencing libraries for next generation sequencing. The recent development of high throughput technologies holds the promise of providing biological insight and new avenues for translational research and clinical applications.

5.1 Arrays

The extent of genome coverage is primarily determined by the resolution of the utilized microarray platform which has progressed rapidly from arrays that examine the DNA methylation present in select CGI to those that examine all CGIs and/or promoters and even to those that examine the entire genome (i.e. Agilent and Nimblegen). Microarray analysis is dependent upon the sample preparation used. Those that use restriction enzymes typically involve a co-hybridization of a test (i.e. leukemia) sample and a reference sample (i.e. bone-marrow cells from a healthy donor). Differential methylation is determined by comparing the intensity of the test sample to the intensity of the reference sample at each locus represented on the array. If the DNA is prepared using an affinity method, the reference sample is usually an aliquot of the original sample DNA while the test sample is the immunoprecipitated portion derived from the original sample. Methylated sequences are detected by comparing the fluorescence signal for each probe corresponding to known genomic sequences for reference and the test samples. The loci that are enriched in the test sample are potential methylation candidates. There are also arrays that require DNA to be bisulfite converted (i.e. Illumina). These arrays are also known as SNP chips and methylated cytosines are identified by the presence of a cytosine at a particular locus as opposed to a thymine. These later arrays give site-specific methylation profiles whereas the oligonucleotide microarrays give region specific methylation profiles.

5.2 Next Generation Sequencing (NGS)

Next generation sequencing has revolutionized the ability to perform genomic research since it was introduced in 2007 (Metzker, 2010 for a review). More recently, this technology has been adapted to epigenomic research including DNA methylation analyses. NGS technologies can generate millions of sequencing reads in parallel and has led to a dramatic increase in the number of genomic and epigenomic sequences encompassing normal and diseased tissues. There are multiple sequencing platforms available and the choice of platform and methodology is dependent on the scientific application and the capacity for extensive bioinformatics analysis.

Whole genome bisulfite sequencing (WGBS) provides coverage at a single base pair resolution and is the most comprehensive NGS technology in DNA methylation analysis. WGBS provides unbiased coverage of the genome allowing for interrogation of whole regions of the genome that are often missed by other methodologies. This method requires the most extensive bioinformatics and is the most expensive because more sequences are required to cover the entire genome. Therefore, this option may not be suitable for laboratories without bioinformatics support or with a small budget.

Reduced representation bisulfite sequencing (RRBS) is a less expensive alternative to WGBS. As the name of the method implies, the genome is reduced in size using an enzyme which enriches for regions of the genome that contain CpG dinucleotides. To be more specific, coverage includes approximately 12% of all CpG dinucleotides and 84% of all CpG islands in the human genome (Smith et al., 2009). In total, about 1% of the genome is covered which greatly reduces the cost of sequencing and the resources needed for alignments while providing data for those regions of the genome that are enriched for CpG dinucleotides. RRBS is an excellent alternative to WGBS with the caveat that important genomic regions that are not enriched for CpG dinucleotides are not assayed. As an example of the consequence of this limitation, it was recently published that much of the methylation present in colon cancer occurred in CGI shores and that this methylation was highly correlated with gene expression (Irizarry et al., 2009). RRBS covers less than 50% of the CGI shores so in this particular region of the genome, important data may be lost (Gu et al., 2011). RRBS may become an optional platform for targeted DNA methylation analysis for clinical diagnostic purposes.

Affinity sequencing utilizes either an antibody against 5-methylcytosine or proteins with methyl binding domains to immunoprecipate (or enrich for) methylated DNA. This method does not require bisulfite conversion and produces sequences for regions of the genome that are methylated. However, it does not provide data for the methylation status of individual CpG dinucleotides. Bioinformatics analysis requires genome alignment and must take into account the density of CpG dinucleotides in a given region because the efficiency of anti-5′ methylcytosine and methyl binding proteins is dependent on the number of methylated cytosines in a given region (Weber et al., 2005).

6. DNA methylation in acute leukemia

DNA methylation is an important epigenetic mechanism to control gene expression. In many cases, tumor suppressor genes (TSGs) are inactivated by somatic mutations (point mutations or deletions) as the "first hit", DNA methylation silences the gene expression of other allele as the "second hit", or in a reversed sequence. In this regards, aberrant DNA methylation plays a crucial role in leukemogenesis (Herman & Baylin, 2003; Galm et al., 2006). Using various technologies described above, many aberrant DNA methylation loci have been identified in both ALL and AML

6.1 DNA methylation in ALL

Genomic DNA methylation profile in a given cell is defined as methylome. There are numerous recurrent chromosomal and genetic abnormalities in ALL. However, these abnormalities are neither sufficient nor necessary in the development of ALL. Therefore it is

likely that epigenetic modifications also contribute to the leukemogenesis in ALL. Numerous studies have been published that have focused on a single candidate gene or groups of genes involved in important cellular processes such as cell signaling pathways, apoptosis, regulation of transcription and cell cycle control in pediatric ALL (Agirre et al., 2006; Canalli et al., 2005; Cheng et al., 2006; Corn et al., 1999; Garcia-Manero et al., 2003; Gutierrez et al., 2003; Iravani et al., 1997; Paixao et al., 2006; Roman-Gomez et al., 2002; Roman-Gomez et al., 2004; Roman-Gomez et al., 2006, 2007; Sahu & Das, 2005; Scholz et al., 2005; Stam et al., 2006; Tsellou et al., 2005; Yang et al., 2006; Zheng et al., 2004), and adult ALL (Batova et al., 1997; Chim et al., 2001; Garcia-Manero et al., 2002a; Garcia-Manero et al., 2002b; Hutter et al., 2011; Jimenez-Velasco et al., 2005; Martin et al., 2008; Roman et al., 2001; Roman-Gomez et al., 2004; Scott et al., 2004; Shteper et al., 2001; Taniguchi et al., 2008; Yang et al., 2006). A search of PubMeth (www.pubmeth.org) provides a list of 46 methylated genes that have been associated with acute lymphoblastic leukemia including *ABCB1, ABL1, ADAMTS1, ADAMTS5, AHR, APAF1, BNIP3, CDH1, CDH13, CDKN1A, CDKN1C, CDKN2A, CDKN2B, CHFR, DAPK1, DIABLO, DKK3, ESR1, EXT1, FHIT, HCK, KLK10, LATS1, LATS2, LMNA, MGMT, MME, MYOD1, NNAT, NR0B2, PARK2, PGR, PPP1R13B, PTEN, PYCARD, RARB, SFRP1, SFRP2, SFRP4, SFRP5, SYK, THBS1, THBS2, TP73, WIF1* and *WRN*.

Recent studies have increased our knowledge of aberrantly methylated loci in ALL by utilizing genome-wide technologies to construct genome-scale methylomes. These studies have shown that methylation profiles can be used in diagnosis (Davidsson et al., 2009; Dunwell et al., 2010; Milani et al., 2010; Stumpel et al., 2009; Taylor et al., 2007a; Taylor et al., 2007b; Vilas-Zornoza et al., 2011), prognosis (Davidsson et al., 2009; Hogan et al., 2011; Kuang et al., 2008; Milani et al., 2010), and in the treatment (Hogan et al., 2011; Vilas-Zornoza et al., 2011) of individuals with ALL.

As an example of the breadth and depth provided by using genome-scale technologies, Davidsson and colleagues used bacterial artificial chromosome arrays and genome-wide methylation arrays in two of the most common subtypes of pediatric ALL, t(12;21)(p13;q22) and high hyperdiploidy (Davidsson et al., 2009). The methylation microarray used covers all of the UCSC-annotated CGI and promoter regions of all RefSeq genes and contains a total of 385,000 probes. A total of 8,662 genes were identified with significant methylation present within the promoter and the 10 individuals with hyperdiploid ALL had approximately twice as many hypermethylated genes as the 10 individuals with the t(12;21) abnormality. Of particular importance is that none of the 30 genes with the highest methylation peaks in the ALL patients have previously been shown to be methylated in ALL or any other neoplasia. An additional study by Hogan and colleagues (Hogan et al., 2011) used the Infinium Human Methylation27 BeadChip to create methylation profiles for paired diagnostic/relapse samples from 33 pediatric ALL patients. This study identified over 900 genes that were preferentially methylated in relapse samples when compared to samples at diagnosis. Further combinatorial expression and copy number variation analysis identified important biological pathways such as the WNT/beta-catenin pathway and the MAPK pathway which may be implicated in the relapse of pediatric ALL.

6.2 DNA methylation in AML

Like in the case of ALL, numerous studies have been published using single-gene based methods such as methylation-specific PCR (MSP), combined bisulfite restriction analysis

(COBRA) or array to identify a single or a group of genes with abnormal DNA methylations in AML. The examples include *CDH1, ESR1, IGSF4, FHIT, p15INK4B, p21CIP1/WAF1, MEG3, SNRPN, p73, SOCS1, CALC1, HIC-1, CTNNA1, CEBPA, MLH1, MGMT, CNAML, HOXA1, MYOD, KRT13, NR2F2, PITX2, RBP1, CEBPA, BAHCC1, EVI1,* and *DAPK* genes (Ekmekci et al., 2004; Agrawal et al., 2007; Desmond et al., 2007; Glasow et al., 2008; Rosu-Myles & Wolff, 2008; Melnick, 2010; Oki & Issa, 2010; Lugthart et al., 2011; Lin et al., 2011). Often hypermethylation in these gene CpG-island-promoters results in transcriptional silencing and loss of the function in important biological pathways (Calvanese et al, 2011).

High throughput technologies such as mass spectrometry, microarray and next generation sequencing (NGS) have been used to study altered DNA methylation at the genome-wide scale. Bullinger and colleagues used a combination of base-specific cleavage biochemistry and mass spectrometry (MALDI-TOF-MS) to quantify DNA methylation in 92 selected genomic regions of 256 AML patient samples (Bullinger et al., 2010). Distinct DNA methylation patterns were identified in abnormal cytogenetic subgroups and the DNA methylation levels (CpG units) could provide independent prognostic information. Alvarez and colleagues used a bead array-based methylation assay to examine the methylation status of 1,505 CpG-sites from 807 genes on 116 de novo AML patients (Alvarez et al., 2010). They confirmed that the DNA methylation signatures were associated with the specific cytogenetic status. In addition, aberrant DNA methylation of the promoter of *DBC1* could predict the disease-free and overall survival time in normal karyotype cases. Interestingly, the aberrant DNA methylation pattern could be induced by genetic transduction of *MLL* rearrangement fusion genes in normal human hematopoietic stem/progenitor cells (HSPC), but cannot be induced by *AML1/ETO* or *CBFB/MYH11* fusion gene. This is the direct evidence of interaction between genetic and epigenetic alteration in AML. Using the same platform, Wilop and colleagues found a gain of overall methylation in 32 AML patient samples (Wilop et al., 2011). The methylation pattern was maintained at relapse with increased density and extended to addition genes, consistent with the previous studies (Agrawal et al., 2007; Kroeger et al., 2008). These observations provided a strong scientific basis for DNA methylation to be used as a biomarker for diagnosis, minimal residual disease detection and clinical follow-up in AML patients.

A more comprehensive study was conducted by Figueroa and colleagues using HpaII tiny fragment enrichment by ligation-mediated PCR (HELP), linked with a microarray platform measuring the methylation abundance of 50,000 cytosines distributed among 14,000 gene promoters in 344 AML patient samples (Figueroa et al., 2010). Based on the DNA methylation signatures, these patients could be classified into 16 epigenetically unique subtypes. Although the DNA methylation patters were different among subtypes, none of the AML subtypes were similar to any of the stages of normal myeloid maturation indicating a distinct difference between leukemia and normal myeloid cell methylomes. Furthermore, they found a set of 45 genes to be aberrantly methylated common in all AML cases, but not in normal myeloid cells. Patients with a *CEPBA* signature have markedly poor clinical outcomes. Functionally, the hypermethylated genes were down-regulated and associated with biologically relevant pathways in leukemogenesis. These genes include zinc finger transcription factors, components in retinoic acid, STAT, p53 signal pathways, DNA-damage repair, immune response and tumor suppressors (Sternberg et al, 2004). It is anticipated that a complete AML methylome with single base pair resolution by next generation sequencing such as whole genome bisulfite sequencing (WGBS) will be published soon.

7. Interaction between genome and epigenome in leukemia

Data from cytogenetic karyotyping, conventional sequencing, microarray and whole genome sequencing indicates that there are extensive and distinct genetic and epigenetic alterations in acute leukemia genomes. Recent studies showed that genetic and epigenetic alterations are not independent events. Specific DNA methylation patterns are identified in specific cytogenetic subgroups of ALL (Davidsson et al., 2009) and AML (Figueroa et al., 2010). Chromosomal translocations result in fusion oncoproteins that can recruit components for DNA methylation, histone deacetylation and transcriptional repressor complexes (Croce LD, 2005; Chen et al., 2010). In contrast, DNA hypomethylation may lead to abnormal microRNA expression and chromosomal instability that in turn may result in chromosomal translocations (Eden et al. 2003; Calvanese et al, 2010; Popp & Bohlander et al., 2010; Toyota et al. 2010; Melo and Esteller, 2011).

AML with t(8;21)(q22;q22) translocation results in the fusion genes of *RUNX1-RUNX1T (*or *AML1- ETO*). The oncoprotein represses the transcription of wild-type *AML1* target genes by recruiting co-repressor complexes (Ferrara & Del Vecchio, 2002). AML1 (also known as RUNX1 or CBFA) is a transcription factor containing the DNA binding domain of the α-subunit of core binding factor (CBF). Another subtype of AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22) results in *CBFB-MYH11* fusion gene that contains a β subunit of CBF. Together, α-subunit and β subunit forms a heterodimeric core binding transcription factor and plays an important role in normal hematopoiesis and myeloid differentiation (Link et al., 2010). However, fusion forms of these truncated subunits lost the ability to form core binding factor and no longer induce myeloid differentiation (Paschka, 2008). Even more, the fusion proteins actively repress the transcription of normal AML1 target genes by either recruiting histone deacetylase (HDAC) or DNA methyltransferase 1 (DNMT1) or by cooperating nuclear receptor co-repressor 1 (NCOR1), NCOR2 and sIN3A15 to form a repressor complex (Liu et al., 2005). As a result, normal myeloid differentiation mediated by CBF is disrupted and hematopoietic stem cells (HSCs) and myeloid progenitor cells cannot achieve the next mature stages, and result in accumulation of leukemia blasts in bone marrow and blood. Since AML with t(8;21) and inv(16) or t(16;16) are involved in a common pathway at the molecular level and show specific clinical features, these two genetic subtypes are called core binding factor leukemia (Ferrara & Del Vecchio, 2002). This leukemia responds well to high doses of cytorabine (HiDAC) and has a better prognosis (Dombret et al., 2009; Solis, 2011).

Acute promyelocytic leukemia (APL) is another example in which blockage of myeloid differentiation by the fusion oncoproteins is mediated by an interaction between genetic and epigenetic mechanisms. All patients with APL have the t(15;17) translocation or one of its variants t(11;17), t(5;17) (Warrell et al., 1993). Translocation t(15;17) results in a fusion protein PML-RARA typically comprised of variable portions of PML protein and all but the first 30 amino acids of retinoic acid receptor-α (RARA). Wild-type RARA protein is a transcriptional activator crucial for normal hematopoiesis and myeloid differentiation. Many RARA target genes including specific transcription factors such as *PU.1 (SPI1)* and *C/EBPβ (CEBPB*) have been identified to have RARA biding sites. The fusion protein PML-RARA functions as a transcriptional repressor, but not an activator, by binding to promoter region of RARA target genes and recruiting proteins including HDAC, NCOR1 and NCOR2

(N-CoR) complex, DNMT1, DNMT3A, repressive histone methyltransferases and polycomb group proteins (Licht et al., 2006). DNA methyltransferases (DNMT1 and DNMT3A) induce DNA methylation. Histone deacetylase (HDAC) removes acetyl group from histones. Together with other repressive proteins, the focal chromatin structure at the promoter regions of target genes are converted to a closed configuration and the transcription initiation is abolished. Promyelocytes of APL lack key transcription factors for further maturation. Accumulation of abnormal promyelocytes in bone marrow is a diagnostic feature of APL. A high dose of all-trans-retinoic acid (ATRA) relieves this repression by allowing the release of the N-CoR complex and the recruitment of a co-activator complex and it has become the cornerstone in treatment of APL by molecular targeting (Wang & Chen, 2008).

8. Molecular mechanisms of leukemogenesis

Among more than 100 types of cancer with different tissue origins, acute leukemia is a unique form that is originated from hematopoietic stem cells (HSC) or hematopoietic progenitor cells in bone marrow. In order to acquire a malignant phenotype, leukemic cells must have all the malignant biological properties including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, altered cellular metabolism, evasion of programmed cell death (apoptosis) and immunological destruction, limitless replicative proliferation and tissue invasion and metastasis (Hanahan & Weinberg, 2000; 2011). At the molecular level, the phenotype of leukemic cells represents a global change of gene expression due to irreversible genetic and epigenetic alterations. These changes affect biological pathways of cell differentiation, cell cycle regulation, tumor suppression, drug responsiveness, and apoptosis. Identification of the molecular signature of leukemia as well as the genetic background of the host individual will provide a unique biological road map for each patient that will become the foundation for personalized therapy in the future (Godley et al., 2011).

The etiology of acute leukemia is not completely clear. Some environmental risk factors for ALL including parental occupation, parental tobacco or alcohol use, prenatal vitamin use, diet, exposure to pesticides or solvents, infectious pathogens and exposure to ionizing radiation or the highest levels of residential power-frequency magnetic fields have been reported (Belson et al., 2007; Milne et al, 2010; Bailey et al., 2011). Environmental risk factors for AML include exposure to ionizing radiation and benzene (Bowen, 2006; Smith et al., 2011). The cytotoxic chemotherapy (alkylating agents and topoisomerase-II inhibitors) and/or radiotherapy for other solid tumors and pre-leukemic conditions myelodysplastic syndromes in the elderly are proven risk factors for AML (Löwenberg et al., 1999; Garcia-Manero et al., 2011).

ALL occurs exclusively in childhood although adult ALL exists. Screening of neonatal cord-blood samples has revealed several specific leukemic chromosomal translocations. One particular clone with the *TEL-AML1* fusion gene derived from chromosomal translocation t(12;21)(p13;q22) is found in 1% of newborn babies. The prevalence of B-ALL with this fusion gene is 100 times higher than those who do not have the fusion gene (Cobaleda et al., 2009; Lausten-Thomsen et al., 2011). Similarly, some leukemic translocations such as t(8;21)(q22;q22) resulting in *AML1-ETO* fusion gene can be detected in neonatal blood

samples from the teenagers diagnosed with AML (Mori et al., 2002). In addition, ALL and AML occurs in approximately 10% of identical twins with these or other karyotypes (Mori et al., 2002; Greaves et al., 2003). These observations support the hypothesis that these specific genetic alterations at the fetal stage increases the frequency of ALL and AML, but additional postnatal events, either genetic or epigenetic, are required for full leukemic transformation (Greaves & Wiemels, 2003; McHale et al., 2004; Wiemels et al., 2009).

Recent studies suggest that the original leukemic clone is most likely raised from hematopoietic stem cells (HSC) or lineage committed precursor cells (Clarke et al. 1987; Lapidot et al., 1994; Cox et al., 2004, 2007; Jamieson et al., 2004). Under the influence of genetic and the environmental risk factors described above, normal HSC or precursor cells undergo malignant transformation and become leukemia stem cells (LSCs) (Passegué et al, 2003). LSCs have the distinct properties with partial normal HSC and partial leukemia cell features. These cells are characterized by self-renewal, over proliferation and the capacity to develop an entire leukemic blast population (Huntly & Gilliland, 2005; Becker & Jordan, 2010). Identification of LSCs by specific biomarkers and development of specific agents to target LSCs has significant clinical implication since eradication of LSCs will prevent the relapse and cure the leukemia (Jan et al., 2011).

At the molecular level, based on the facts that chromosomal translocations and point mutations can be found in the majority of AML patients, Kelly and colleagues suggested a two-hit model that AML leukemogenesis driven by two types of gene mutations (Kelly et al., 2002). The class 1 mutations result in constitutive activation of cell-surface receptors, such as receptor tyrosine kinases, FLT3 and KIT. Through various downstream signaling pathways, constitutive activation confers proliferation and survival advantage leading to clonal expansion of the affected hematopoietic stem cell or progenitors. The class 2 mutations, exemplified by formation of fusion genes from the t(8;21) or inv(16) chromosomal translocations or overexpression of HOX genes, block myeloid differentiation. Either class 1 or class 2 lesions alone does not cause leukemia in mouse models (Downing, 2003). AML develops only when both classes of lesions are present.

This model, however, provides a less cogent explanation for AML derived from myelodysplastic syndrome and therapy-related AML (t-AML) in elderly. These AML are frequently associated with chromosomal deletion or addition (Godley & Larson, 2008). Furthermore, this model also does not fully explain the AML containing normal karyotype with multiple point mutations in *FLIT3, NPM1,* and *CEBPA* genes (Foran, 2010). The class 1 mutations in ALL have not fully established. Epigenetic factors, especially DNA hypermethylation that can inactivate various putative tumor suppressor genes, DNA-repair, cell cycle, apoptosis related genes appear to play important roles in leukemogenesis (Issa et al., 1997; Esteller, 2008; Kulis & Esteller, 2010; Deaton & Bird, 2011). An integrated model combining genetic and epigenetic factors at the individual, cellular and molecular levels for acute leukemia is proposed (Figure 3).

9. Clinical applications

Genetic and epigenetic studies from basic science have been applied to many aspects in the clinical management of acute leukemia patients. The current WHO classification of tumors of hematopoietic and lymphoid tissues has included an increasing number of clinicopathologic entities defined by chromosomal abnormalities as well as gene mutations.

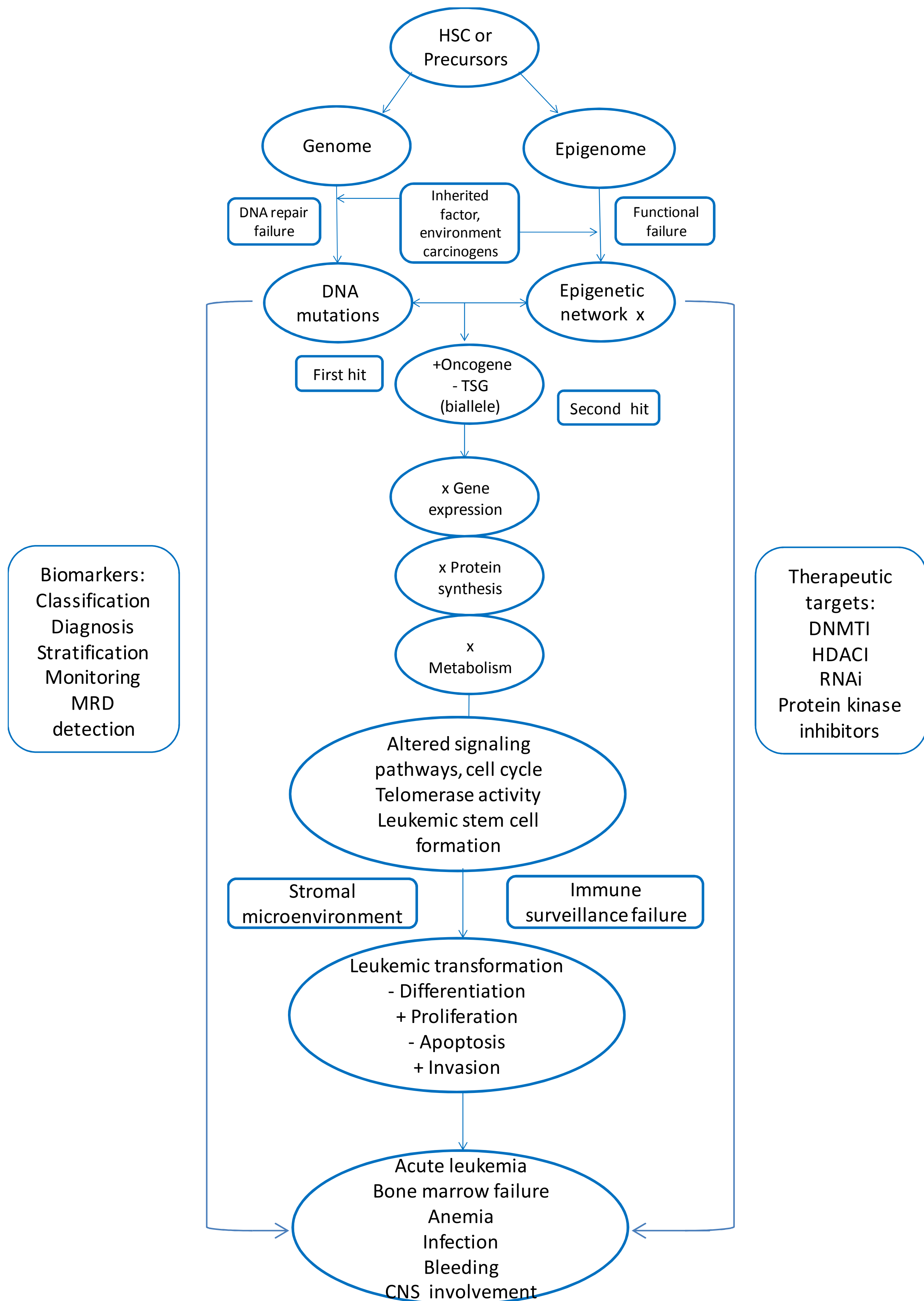
HSC or Precursors
Genome
Epigenome
DNA repair failure
Inherited factor, environment carcinogens
Functional failure
DNA mutations
Epigenetic network x
First hit
+Oncogene - TSG (biallele)
Second hit
x Gene expression
x Protein synthesis
x Metabolism
Biomarkers: Classification Diagnosis Stratification Monitoring MRD detection
Therapeutic targets: DNMTI HDACI RNAi Protein kinase inhibitors
Altered signaling pathways, cell cycle Telomerase activity Leukemic stem cell formation
Stromal microenvironment
Immune surveillance failure
Leukemic transformation - Differentiation + Proliferation - Apoptosis + Invasion
Acute leukemia Bone marrow failure Anemia Infection Bleeding CNS involvement

Fig. 3. A new model of leukemogenesis integrated genetic and epigenetic mechanisms and their clinical implications. Although the inherited factors in leukemogenesis of acute leukemia is not apparent, the genetic alterations including chromosomal translocations and numerical changes such as trisomy 21 have been found at prenatal stage. The changes may be related to maternal factors such as carcinogens exposure, nutrients (including folate) and aging in pregnancy. The incidence of acute leukemia is dramatically increased (~100 times higher), but not all children will have the leukemia when carrying the specific chromosomal abnormalities at the prenatal stage. It indicates the second hit, either genetic mutations or epigenetic alterations, is required for a full leukemic transformation. With an interaction between genetic and epigenetic networks, the gene expression profile is globally changed in hematopoietic stem cell s or precursors. Corresponding functional changes including cell signalings and cell cycle control result in a malignant leukemia phenotype. These leukemia cells escape from immune surveillance and accumulate in bone marrow and blood, thus acute leukemia is developed. Clinically, genetic abnormalities have been used as biomarker for disease classification and diagnosis, while aberrant epigenetic alterations have become therapeutic targets. Note: HSC: hematopoietic stem cell; TSG: tumor suppressor gene; DNMTI; HDACI; RNAi; Epigenetic network: DNA methylation, histone modifications and microRNA. siRNAs. +: increase; -: decrease; x: disruption.

These subtypes of AML or ALL often have a distinct morphology, immunophenotype and clinical course. Some of these patients with specific genetic or epigenetic alterations may respond to specific chemotherapeutic reagents or epigenetic modifiers. Mutation status of *NPM1, CEBPA* and *FLT3* genes has been used in risk assessment, prognostic evaluation and guidance of therapy (Foran, 2010). Detection of specific fusion RNA levels using quantitative RT-PCR molecular tests in patient blood has been used routinely for therapeutic monitoring and minimal residual disease detection (Gulley et al., 2010).

Because of the genetic heterogeneity and the limited number of meaningful genetic biomarkers identified in acute leukemia, the use of aberrant epigenetic alterations, especially DNA methylation and microRNA as biomarkers, is being studied at the single gene as well as genome-wide level. Agrawal and colleagues reported that the methylation of *ERa* and *p15INK4B* genes occurred frequently and specifically in acute leukemia but not in healthy controls or in nonmalignant hematologic diseases (Agrawal et al., 2007). Aberrant DNA methylation of these two genes was detectable in >20% of leukemia patients during clinical remission. The presence of detectable methylation was correlated to minimal residual disease (MRD) and associated with subsequent relapse (Agrawal et al., 2007). Wang and colleagues demonstrated that the aberrant DNA methylation of *DLC-1, PCDHGA12* and *RPIB9* genes can be identified in over 80% of ALL patients (Wang et al., 2010). Using a single gene *DLC-1*, we could trace clinical B-ALL cases up to 10 years retrospectively and the *DLC-1* methylation is correlated with patient clinical status. Importantly, these specific DNA methylation loci are retained in leukemia cells and can be detected in relapse. Compared with primary leukemia at diagnosis, relapsed leukemia maintains the original methylation loci, yet extents methylation in addition genes (Kroeger et al., 2008; Figueroa et al., 2010). These studies indicated that the DNA methylation is a biologically stable marker that can be used for MRD detection and patient follow up in acute leukemia.

In terms of therapy, there are two groups of epigenetic agents currently in clinical use, DNA methyltransferase inhibitor (DNMTI) and histone deacetylase inhibitor (HDACI) (Peters & Schwaller, 2011). The prototypic nucleoside analogue DNMT inhibitors include 5-azacytidine (5-Aza or azacitidine) and 5-aza-2'deoxycytidine (decitabine). They exert a demethylating effect by incorporating into DNA (5-Aza is also incorporated into RNA) and form a covalent complex with the DNMT enzymes. The enzymes are trapped and eventually degraded and the newly synthesized DNA strand will not be methylated (Schoofs & Müller-Tidow, 2011). These two agents are active in a broad range of myeloid neoplasms including AML and myelodysplastic syndrome (MDS). Because of its excellent efficacy (~50% response rate) in clinical trials, both agents have been approved by the US FDA for the treatment of MDS (Silverman & Mufti, 2005). The use of these reagents in treatment of AML has been actively investigated and showed promising utility especially in elderly patients (Musolino, 2010). The second group of epigenetic therapeutic agents is histone deacetylase inhibitor (HDACI). This group consists of heterogenic compounds that may reactivate the genes that have been turned off by histone deacetylation. Particularly, HDACI has demonstrated some efficacy in treat of core binding factor (CBF) leukemia. Clinical trials have been conducted using HDACI alone or in combination with DNMTI in CBF and other subtypes of leukemia patients (Quintás-Cardama et al., 2011).

10. Conclusion

Acute leukemia (ALL and AML), like all other cancer types, is a genetic disease. DNA sequence examination in the specific loci as well as at the genome-wide level has confirmed this original hypothesis. Epigenetic alterations including DNA methylation, histone modifications and microRNA play a functional role in leukemogenesis. Interaction between genetic and epigenetic elements changes the global landscape of gene expression, protein synthesis and metabolism in hematopoietic stem cells and/or committed precursor cells which results in leukemic transformation. Systemic study at the genome level in DNA sequence and DNA methylation, gene and microRNA expression profile, proteome and metabolism not only provides the insight for understanding leukemogenesis, but also identifies biomarkers for leukemia stem cell, leukemia classification, diagnosis, risk assessment, therapy selection, response prediction, prognosis, minimal residual disease detection and other aspects of clinical decision-making and applications. Toward this end, current advanced high throughput technologies including next generation sequencing, microarray, proteomics, targeted molecular testing and bioinformatics have provided powerful tools. Well-designed clinical trials will make a clinical connection with new scientific discoveries in leukemia genome and epigenome. Assembly and synthesis of the massive amounts of new information by systems biology will generate a high resolution picture of leukemogenesis of acute leukemia. With combined efforts from bench and bedside, the ultimate goal is to eradicate all leukemic blasts including leukemic stem cells in the patients by less toxic reagents to completely cure leukemia in the future.

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12. References

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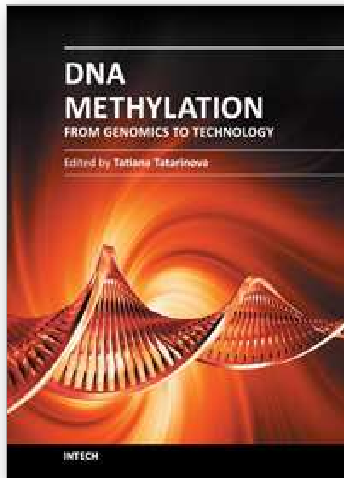

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Epigenetics is one of the most exciting and rapidly developing areas of modern genetics with applications in many disciplines from medicine to agriculture. The most common form of epigenetic modification is DNA methylation, which plays a key role in fundamental developmental processes such as embryogenesis and also in the response of organisms to a wide range of environmental stimuli. Indeed, epigenetics is increasing regarded as one of the major mechanisms used by animals and plants to modulate their genome and its expression to adapt to a wide range of environmental factors. This book brings together a group of experts at the cutting edge of research into DNA methylation and highlights recent advances in methodology and knowledge of underlying mechanisms of this most important of genetic processes. The reader will gain an understanding of the impact, significance and recent advances within the field of epigenetics with a focus on DNA methylation.

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