Epigenetic Alterations and targeted chromatin remodeling drugs in pediatric leukemia

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Introduction
Cancer formation has long been considered the result of progressive genetic alterations such as mutations in tumor suppressor genes and oncogenes, and chromosomal abnormalities. However, cancer is also a disease of altered epigenetic mechanisms. Epigenetic changes include all mechanisms that alter the gene expression pattern without altering the genetic code. Mostly, these mechanisms are involved with the structure and conformation of the chromatin, and directly determine the level of transcriptional activity of genes. In most cancer types specific epigenetic changes occur and impose semi-permanent epigenetic ‘marks’, similar to genetic changes[1]. The best-known change is DNA hypermethylation of gene promoters and subsequent gene silencing. However, DNA methylation is only one step in chromatin remodeling and transcriptional silencing of genes. Probably equally important in determining the level of transcription are alterations in the histone proteins that package the DNA.

Here, the mechanisms of epigenetic alterations will be explained in relation to their significance in pediatric leukemia. ‘Epigenetics’ plays an important role in the tumor formation and possibly tumor progression and can increase our understanding of the malignant cells. More importantly, epigenetic changes are semi-permanent which means that they can be turned-around by so-called epigenetic drugs, leading to restoration of the proper gene transcription. The potential role of several compounds will be discussed in relation to the treatment of pediatric leukemia.

Historic overview of epigenetic research in cancer
In the 1980s, it was observed that a substantial proportion of the DNA in cancer genomes was hypomethylated in comparison to normal tissue genomes[2]. This was the first epigenetic abnormality observed in cancer cells and the loss of methylation involved every tumor type studied, both benign and malignant and even in pre-malignant adenomas. Two functional consequences have become evident: First, hypomethylation increases the transcriptional level of some genes. Examples of normally hypermethylated genes that are hypomethylated and over expressed in cancer include important genes such as cyclin D2 and Maspin in gastric cancer, MN/CA9 in renal-cell carcinoma, S100A4 metastasis-associated gene in colon cancer and HPV16 in cervical cancer (reviewed in [3]). Also viral transcripts have been detected in increasing amounts in hypomethylated cancer genomes and probably originate from the viral DNA sequences in the ‘junk DNA’ of our genome.

Second, tumor hypomethylation is linked to chromosomal instability[4]. Hypomethylation is particularly seen in pericentromeric regions. Several cancers, such as Wilms’ tumor, ovarian and breast cancers frequently contain unbalanced translocations with breakpoint in pericentromeric regions of chromosomes 1 and 16. In Wilms’ tumors, translocation t(1;16) produces Loss of Heterozygosity (LOH) for markers on chromosome 16, which are associated with anaplasia. Another example of chromosomal instability in a hypomethylated region is well illustrated by the ICF syndrome (immunodeficiency, chromosomal instability and facial abnormalities). In ICF, a loss-of-function mutation in one of the DNA methylating enzymes DNA methyltransferase 3B (DNMT3B) causes pericentromeric hypomethylation and chromosomal breakage in the same regions[5]. The causative role of hypomethylation of the genome in leukemogenic translocations or chromosomal instability needs to be proven.
In the 1990s the attention shifted to so-called ‘regional hypermethylation’ and gene silencing, especially of presumed tumor suppressor genes[6]. Regional hypermethylation has become a hallmark of cancer cells. In normal cells the majority of gene promoters contain so-called CpG-islands, regions of dense CpG- and GC-content stretching from 0.2 to 5 kb. For reasons unknown, these islands are normally protected from methylation and remain hypomethylated. However, in cancer cells, CpG-islands of specific genes can lose the protection from methylation and become regionally hypermethylated. This is strongly associated with transcriptional silencing of the gene. An abundance of studies in adult type tumors and to a lesser extend in pediatric malignancies has shown that: A. Silenced genes by hypermethylation are present in virtually all cancer types studied. B. Tumor specific methylation patterns have been recognized for different cancer types.

After the recognition of regional hypermethylation and silencing of tumor suppressor genes, again the research field shifted gears and started to explore the mechanisms of chromatin remodeling and cancer treatment. Chromatin remodeling involves not only DNA methylation, but also alterations of the histone proteins. These complex interactions between the histone proteins and the DNA determine the transcriptional activity of gene promoters. Both processes can be influenced by chemical compounds or epigenetic drugs and are potential targets for treatment of cancer patients.

Chromatin structure and the ‘histone code’

The level of gene transcription is dependent on the attraction and binding of transcription activators and repressors to gene promoters. Their binding is greatly dependent on the structure of the chromatin. Chromatin can be ‘open’ (euchromatin) and transcriptionally accessible, or ‘closed’ (heterochromatin) and transcriptionally repressive. Changing from euchromatin towards heterochromatin is a multi step process on the surface of histone proteins and the DNA. Very distinct chemical steps can be recognized:

The histone code

DNA is wound around histone complexes, which contain several histone proteins 1, 2A, 2B, 3. Especially histone 3 (H3) has lysine-rich tails that stick out of the surface. Several amino acids on the lysine tails can be altered by (de-)acetylation, (de)methylation and phosphorylation. These alterations induce conformational changes of the whole histone complex. For instance, acetylated lysine-tails ‘stick out’ more and induce a more widely spaced chromatin conformation, which is open to transcriptional activators. On the opposite, deacetylation of lysines will compact the H3 protein and heterochromatin will be formed. The combination of (de-)acetylated, (de-)methylated and phosphorylated residues on the histone tails is called the histone code. The code therefore refers to a conformational state of the chromatin, which is associated with a certain level of transcription. Only a few details of the histone code are known. For instance deacetylation of lysine (K) in position 9 of the H3 protein (H3K9) and H3K14 and the methylation of H3K9 and H3K27 are strongly associated with a repressive state, whereas methylation of H3K4 is an activating modification (reviewed in [7]). Furthermore, it is assumed that many epigenetic marks are encompassed by the histone code, for instance it probably marks regions for imprinted genes in the genome.

DNA methylation

Methylation of DNA involves the replacement of cytosine (C) by 5-methylcytosine (5mC). For chemical reasons this can only happen to cytosine in the 5’ position from a guanine, a so-called 5’-CpG -3’ dinucleotide or simply CpG. Replacing C for 5mC induces conformational changes to the chromatin and DNA methylation is strongly associated with heterochromatin and gene silencing[6]. As a result, transcriptionally repressive Methyl Binding Proteins (MBD’s) like MeCP2 and MBD2 are attracted, which eventually recruit other enzymes, like histone deacetylases (HDAC)[7]. These HDACs remove acetyl groups from lysine residues of histone H3 and H4. DNA methylation and histone deacetylation, especially of lysine 9 on histone H3 (H3K9) are strongly connected and associated with gene silencing.
Chromatin, open or closed?
From the above one can conclude on a simplified scheme of how chromatin will change from one conformation to the other. Acetylated histones are associated with euchromatin and gene transcription. If the histones become deacetylated, the conformation changes to heterochromatin and transcriptional repression. Especially H3K9 is a strong repressor and associated with methylation of DNA. Although deacetylation itself is associated with gene silencing, the additional role of DNAmethylation is important. DNA methylation is considered a semi-permanent lock into the repressor state. Acetylation and deacetylation are considered relatively ‘fluent’ states that can change from one into the other (Figure 1). Methylation of DNA represses genes long term, even over generations of cell division.

Figure 1: Chromatin remodeling. Flowchart of interactions between histone acetylation and DNA methylation. Euchromatin can be converted to heterochromatin by histone deacetylation and DNA methylation. Histone acetylation and deacetylation are fluent states. DNA methylation is semi-permanent by lack of any (known) intracellular demethylating enzym. Therefore demethylation can only be induced by demethylating drugs. DNMT: DNA methyltransferases, MBD: Methyl-binding domain proteins, HDAC: histone deacetylases, HAT: histone acetyltransferases, HDACi: HDAC inhibitor

Epigenetics and leukemia
Comparison of genome wide content of 5mC between normal and neoplastic hematopoietic CD34+ cells has revealed global hypomethylation only in progressive AML in adults and in CML, but not in acute leukemia’s ALL. Genome wide demethylation is considered an early event in most cancer types, contributing to chromosomal instability. However, it seems not to affect hematopoietic neoplasia, which suggests that other factors are important in chromosomal instability and induction of translocations.
A large number of genes have been studied for hypermethylation and silencing in hematopoietic neoplasia, mostly in adult patient groups. Focus has been on genes of cell cycle regulation, apoptotic pathways, genes involved in growth and differentiation, since it seemed plausible that disruption of these genes could confer neoplastic alterations. The most reported silencing is for the cyclin-dependent kinase inhibitors p15\(^{INK4B}\) and to a lesser extent for p16\(^{INK4A}\), and both genes have been found silenced in ALL and AML. In adults, the majority of AML and ALL have loss of p15 and 20-40 % loss of p16. In pediatric patients, the number of studied patients is far less, but it seems that p15 is methylated, mainly in AML in less than 50%, frequently in T-ALL, but hypermethylation of p16 does not seem important. Other genes reported hypermethylated and silenced at least 30% of samples in mostly adult studies include: Calcitonin, E-cadherin, Estrogen Receptor, MyoD, HIC1, MDR1, pTX2, SDC4, WT1 \((9-11)\). In adult studies, an unfavorable prognostic association has been observed for patients with >2 hypermethylated genes. For pediatric patients this has not been confirmed, possibly because most studies contained few patients and also hypermethylation did not seem to be as frequent in pediatric AML/ALL, at least for the genes studied \(^{12}\). Genome wide methylation studies have not been performed in pediatric AML/ALL. MLL rearranged infant ALL seems characterized by hypermethylation and silencing of the FHIT gene. FHIT had already been found genetically altered and silenced in several human malignancies. Silencing by hypermethylation was observed in 40% of ALL cell lines and 27% of pediatric ALL samples \(^{13}\), especially in hyperdiploid tumors. Stam et al\(^{14}\) found it to be specific for MLL rearranged infant ALL, since 100% of MLL+ samples were hypermethylated and silenced for FHIT versus 60% in a mixed group of MLL-infant ALL and non-infant ALL. The FHIT expression was inducible after treatment
with the demethylating agent decitabine and over expression of FHIT induced apoptosis and leukemic cell death. These data suggest that FHIT acts as a tumor suppressor gene, and may be characteristic for certain ALL subgroups such as MLL+ infant ALL.

Acute promyelocytic leukemia (APL) has long been the role model of epigenetic silencing in leukemias, although the mechanism of silencing is different. The disease is characterized by the inactivation of the retinoic acid receptor-beta (RARâ) by a HDAC repressive complex. The APL specific fusion protein PML/RARá has the ability to suppress RARâ by recruitment of a nuclear co-repressor -histone deacetylase (NCOR/HD) complex and DNMT3a, resulting in local chromatin remodeling. Treatment of cells with all trans retinoic acid (ATRA) induces demethylation of the promoter, with gene re-expression and reversal of the malignant phenotype by differentiation. In a transgenic mouse model and in clinical studies with refractory APL patients, silencing of RARâ can also be reversed by the HDAC inhibitor sodium phenyl butyrate (SPB)[15].

Epigenetic drugs

DNA methylation and chromatin remodeling are involved in important mechanisms, which are believed to contribute to cancer. Contrary to genetic alterations in cancer, DNA methylation and histone deacetylation are reversible with restoration of the normal gene transcription. This is especially applicable for transcription restoration of silenced tumor suppressor genes by regional hypermethylation. Two classes of drugs will be discussed: Demethylating agents and Histone De-Acetylase Inhibitors (HDACi):

Demethylating agents

Clinical testing of two azanucleosides already started in the 1970s. They were tested as novel cytosine analogues and at comparatively high doses seemed to have the same effect and toxicity as cytarabine/ara-C and since cytarabine was already widely used the testing of the azanucleosides was interrupted (reviewed in [16]). However, 5-aza-cytidine (Vidaza) and the more potent 5-aza-2’-deoxycytidine (Decitabine) were revived as DNA demethylating agents (figure 2). Reactivation of silenced genes by using these compounds was shown in vitro for many different situations, for instance in the hypermethylated CGG expanded repeats in the fragile X syndrome, in the silenced copy of the SNRPN and neighboring genes in cell lines of Prader-Willi patients, but also in many aberrantly hypermethylated genes in different cancer types. Clinically significant results have been obtained in myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML)[17, 18]. In MDS, demethylation of the tumor suppressor gene p15 was observed in several patients and correlated with clinical response. Vice versa, the increase in methylation of p15 in one patient correlated with disease progression. Interestingly, dose de-escalating testing showed that Decitabine was most effective in the lower or middle doses, with low or minimal non-hematologic toxicity.

Figure 2: Chemical structure of cytosine and analogues. R, Ribose; dR, deoxyribose

HDACi

Deacetylation, especially of the H3K9 is essential in the formation of heterochromatin and silencing of genes. The Histone Deacetylase enzymes (HDACs) are the driving force of deacetylation processes and they can pharmacologically be inhibited. Currently, several structurally diverse classes of HDACIs, natural and synthetic, are known to bind histone deacetylases (HDACs) and to induce histone acetylation. These include short-chain fatty acids (i.e. valproic acid, sodium butyrate, phenylbutyrate and AN-9), cyclic and non-cyclic hydroxamates (i.e. suberoyl anilide hydroxamic acid (SAHA), trichostatin A (TSA), m-Carboxycinnamic acid bishydroxamic acid (CBHA), suberic bishydroxamic acid (SBHA), oxamflatin, proxamide, NVP-LAQ824 (LAQ824), and PDX101), cyclic peptides or tetrapeptides (i.e. Depsipeptide (FK228), Trapoxin, and...
apicidin), benzamides (i.e. MS-275 and CI-994 (p-N-acetyl dinaline)), ketones (i.e. trifluoromethyl ketone and á-ketoamides), and hybrids of hydroxamic acid and cyclic tetrapeptide (i.e. CHAP). Clinical testing of HDAC inhibitors was first applied effectively in acute promyelocytic leukemia (APL), in which RARα is inactivated by an HDAC repressive complex[19]. Currently, a wide range of different HDAC inhibitors alone or in combination with demethylating agents are in pre-clinical and clinical phase I/II trials. The toxicity seems very limited, without unexpected toxicity reported, but the effectiveness has not been completely established yet.[19, 20]. In vitro, there is extensive evidence that HDACi induce cancer cell cycle arrest, growth inhibition, differentiation, and programmed cell death[21, 22].

**Combining demethylating agents and HDACi**

In a landmark paper by Cameron et al a synergistic effect of histone deacetylase inhibition and demethylation was shown[23]. TSA and decitabine were both able to reverse gene silencing of several hypermethylated genes in colon cancer cell lines. However, the combination of the two agents showed a much more significant demethylation and reactivation of the genes. This synergistic effect of a demethylating agent and an HDACi was confirmed in further studies[24, 25]. In leukemias, a phase I/II study has shown that using decitabine and valproic acid in adult patients induced responses and remissions in pre-treated and chemotherapy-naïve patients[26].

**Conclusions**

The level of transcription of genes can be altered by genetic alterations, like mutations, translocations or aneuploidy. However, epigenetic alterations play an important role in human malignancies as well. Especially regional hypermethylation and silencing of genes have become a hallmark of cancer. Also in (pediatric) leukemias, multiple presumed tumor suppressor genes have been found silenced by promoter hypermethylation. The hypermethylation is part of complex chromatin remodeling mechanisms. It has become evident that demethylating agents, HDAC inhibitors or a combination of both, can reverse both methylation and the formation of repressive chromatin. The future will teach us the value of these epigenetic drugs, since many clinical phase I/II studies are on the way in adult patients with a variety of cancer types. These data will create opportunities to design similar epigenetic drug studies in pediatric patients.

**References**


