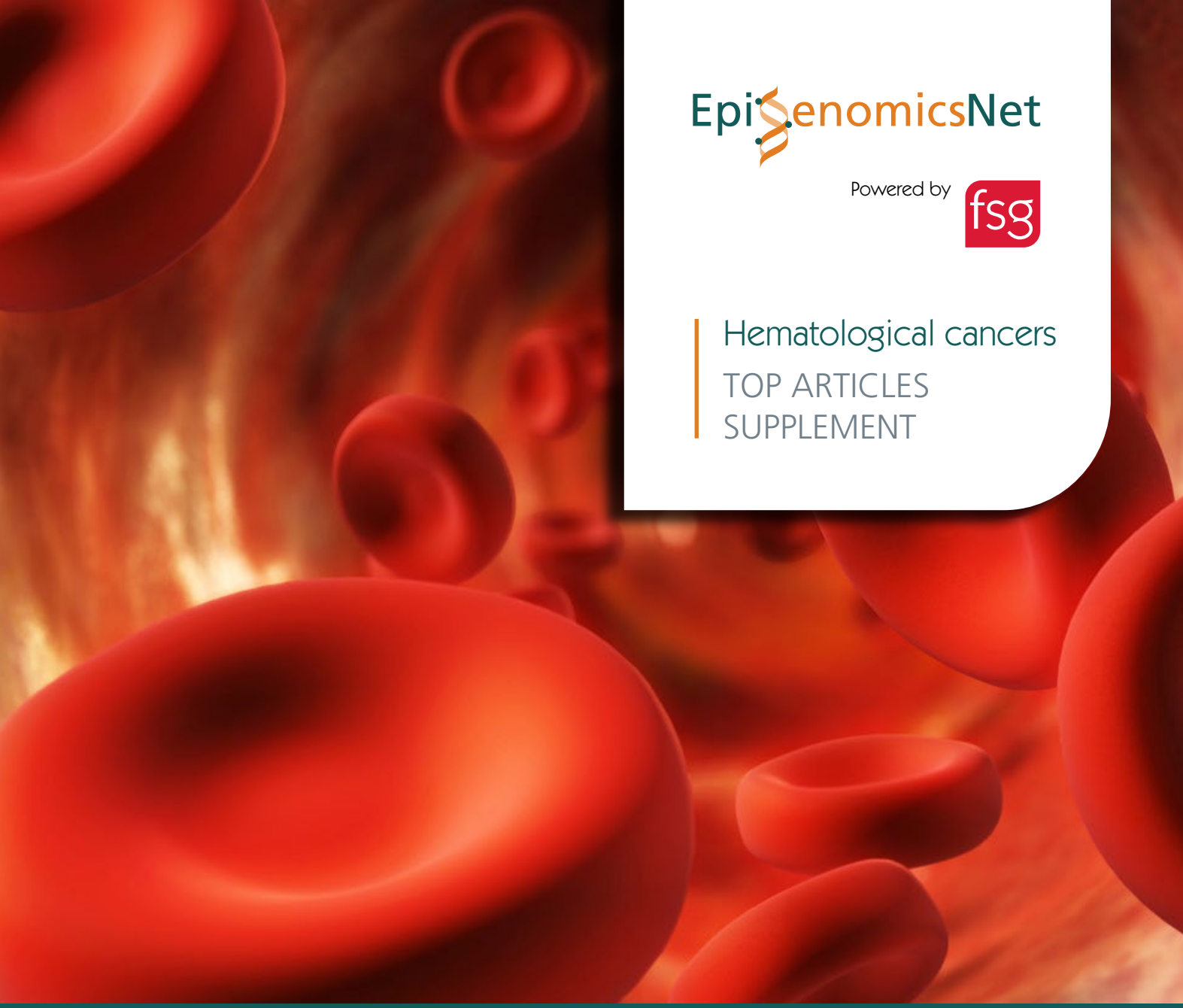

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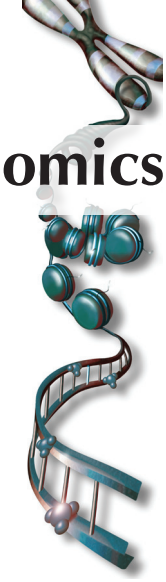

DNA methylation as a potential mediator of environmental risks in the development of childhood acute lymphoblastic leukemia

5-year survival rate for childhood acute lymphoblastic leukemia (ALL) has risen to approximately 90%, yet the causal disease pathway is still poorly understood. Evidence suggests multiple 'hits' are required for disease progression; an initial genetic abnormality followed by additional secondary 'hits'. It is plausible that environmental influences may trigger these secondary hits, and with the peak incidence of diagnosis between 2 and 5 years of age, early life exposures are likely to be key. DNA methylation can be modified by many environmental exposures and is dramatically altered in cancers, including childhood ALL. Here we explore the potential that DNA methylation may be involved in the causal pathway toward disease by acting as a mediator between established environmental factors and childhood ALL development.

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Keywords: acute lymphoblastic leukemia • alcohol • birth weight • caffeine • developmental programming • DNA methylation • environment • folate • iron • smoking

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Genetics of acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common form of childhood leukemia. ALL is a cancer of the blood and bone marrow which arises from genetic abnormalities which can occur *in utero*. These abnormalities lead to the malignant transformation of lymphocyte progenitor cells into leukemic cells of the B-cell and T-cell lineages [1]. Childhood ALL is a heterogeneous disease. Most cases are definable by large-scale chromosomal translocations/aberrations, resulting in distinct biological subtypes, each with individual characteristics. Patients are usually organized into subtypes depending on their cellular immunophenotype and recurrent cytogenetic aberrations. The most prognostically important subtypes include T-ALL, high hyperdiploidy (51–65 chromosomes), *E2A–PBX1*, *BCR–ABL*, *ETV6–RUNX1* (*TEL–AML1*) and *MLL* rearrangements [2]. The time frame between the emergence of genetic abnormalities and disease onset, and the frequency of children born with abnormalities compared with the number of children who go on to develop ALL, suggests multiple 'hits' are required for the manifestation of disease [3]. A recognized leukemic clone with the *TEL–AML1* fusion gene has been found in 1% of newborn babies by screening neonatal blood spots [4]. This frequency is a 100-times higher than the incidence of ALL defined with this fusion gene later in childhood [5]. Diagnosis of ALL is variable with peak incidences between 2 and 5 years of age [4], suggesting an undefined period of latency whereby additional abnormalities are acquired for malignant transformation to occur. Furthermore, identical twins with the *TEL–AML1* fusion gene showed only a 10% concordance rate further supporting the concept that additional events or 'hits' are needed for the full transformation to leukemia, or specifically ALL development [4].

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DNA methylation & ALL

DNA methylation was first reported as a regulatory mechanism influencing gene expression in 1975 by Holliday and Pugh, and Riggs [6]. Methylation of cytosine residues located within CpG dinucleotides across the genome is an epigenetic modification which plays a crucial role in the creation of cellular identity by influencing gene expression during early development [7]. Methyl groups are added to DNA by *DNMT*s. This group of enzymes was responsible for the establishment of methylation patterns during development (*DNMT3A*, *DNMT3B* and their co-factor *DNMT3*-like) and the maintenance of methylation during replication (*DNMT1*) [8]. The majority of mammalian CpG sites are methylated but CpG islands (CGIs) significantly deviate from the regular genomic pattern; CGIs are GC and CpG rich, and predominantly non-methylated [9]. Hyper-methylation of CpG sites occurs in a nonrandom tissue specific manner, and when this occurs at promoter sites it can block gene transcription. This mechanism of gene regulation plays an important role in tissue differentiation, X-chromosome inactivation, genomic imprinting and suppression of transposable elements [1].

Cancer is now recognized as an epigenetic disease [10]. The cancer cell genome undergoes dramatic shifts in the pattern of genomic methylation, including genome wide hypomethylation in conjunction with local areas of hypermethylation, often centered on promoter associated CGIs. One known disruptive mechanism in cancer is the silencing of tumor suppressor genes, such as $p16^{INK4a}$, via hypermethylation of their promotor associated CGIs [10]. Aberrant CpG methylation in cancer cells can have extensive effects on gene regulation, which can affect cell function and lead to adverse outcomes if genes such as tumor suppressors and others involved in important cellular processes (DNA repair, apoptosis, drug-detoxification and many more critical genes) are inactivated.

One of the first studies to investigate the DNA methylome of childhood ALL focused on the most common subtypes, *ETV6*/*RUNX1* and high hyperdiploidy [11]. A microarray platform comprising 28226 CGIs, cDNA microarrays and array comprehensive genomic hybridization was used to investigate altered global methylation, correlation between hypermethylation and gene expression, and detection of genomic imbalance, respectively. The three most common groups of genes which displayed hypermethylation and resultant under-expression were transcription regulators, regulators of apoptosis and cell signaling genes, all possible targets for future treatments.

An array designed to investigate DNA methylation patterns of 416 genes in cells from 401 diagnostic ALL samples included CpG sites 2 kb upstream and 1 kb downstream of transcription start sites [12]. CpG sites located outside of CGIs showed a greater variation and overall methylation compared with CpG sites located within CGIs. These sites may offer an alternate transcription start site, and therefore alterations in DNA methylation at these CpG sites may affect gene expression. This notion was supported by a notable inverse correlation between CpG site methylation and gene expression. Methylation profiles of forty genes were also identified as consistently and accurately discriminating between four subtypes of B-cell precursor ALL, and DNA methylation levels of 20 individual genes predicted relapse.

Integrated genetic and epigenetic analysis of childhood ALL has provided evidence for alterations in DNA methylation playing a key role in leukemogenesis [13]. Figueroa *et al.* [13] analyzed 137 B-cell and 30 T-cell lineage childhood ALL samples, and 19 B-cell samples from healthy children. Genome-wide cytosine methylation profiling analysis was carried out using the HpaII tiny fragments enrichment by ligation-mediated PCR (HELP) assay. A high degree of agreement was found between epigenetic subtypes and genetic subtypes. Furthermore a core set of epigenetically deregulated genes were common to all cases, suggesting their involvement in a central role in the initiation or maintenance of lymphoid transformation.

Nordlund *et al.* [1] used the Infinium HumanMethylation450 Beadchip (450K BeadChip) to investigate genome wide DNA methylation signatures in pediatric ALL, and were able to characterize different groups through methylation pattern changes and these corresponded closely to the known cytogenetic subtypes. They found that the methylomes of ALL cells shared 9402 predominantly hypermethylated CpG sites compared with controls, these observations were seen across all ALL subtypes. A unique set of hypo/hyper-methylated CpGs were identified for each cytogenetic subtype. Subtype specific altered methylation strongly associated to gene expression in the promotor and enhancer regions. Six thousand six hundred and twelve CpG sites were also found predominantly hypermethylated in ALL cells at relapse compared with matched samples at diagnosis. Although a significant association between methylation at diagnosis and subsequent relapse was only seen in the *ETV6*/*RUNX1* subtype. These findings were followed up by developing methodology for DNA methylation profiling for pediatric ALL [14], enabling the clarification of a heterogeneous group of cytogenetically undefined ALL patients. Gabriel *et al.* [15] were unable to predict ALL patient relapse using the 450K BeadChip or to replicate the *ETV6*/*RUNX1* relapse signature identified

by Nordlund *et al.* However, this study did reproduce the correlation Nordlund *et al.* [1] identified between genome wide DNA methylation pattern and the different cytogenetic subgroups, and validated many of the cytogenetic specific markers. This indicates that subtype specific patterns of altered methylation are consistent across different cohorts.

Epigenetic remodeling of pediatric B-cell ALL (B-ALL) has been investigated in reference to normal B-cell precursors [16] using whole-genome bisulfite sequencing and 450K BeadChips. The analysis of 227 B-ALL patients from the California Childhood Cancer Study revealed two tracks of epigenetic alterations. *De novo* methylation occurred at small functional compartments, for example, CGIs, promotors, TF-binding sites and DNase hyper sensitive sites. While demethylation in subsets of leukemia was apparent in large inter-compartmental backbones, although this change was subtle. CGIs were *de novo* methylated throughout promotors and bodies rather than gene bodies, a suggested crude yet potent way of gene silencing. In regions with hypermethylation there was an enrichment of *CTBP2* sites, suggesting that *CTBP2* may recruit factors which drive the observed hypermethylation.

MIRA-seq has also been utilized to identify differentially methylation regions (DMR) in ALL compared with healthy precursor B cells isolated from cord blood, with a total of 15,492 regions losing methylation, and 9790 regions gaining methylation [17]. The majority of DMRs associated with a CGI were hypermethylated, although roughly 80% of the total DMRs were identified in intronic or intergenic regions. Functional gene analysis revealed that 70% of the intergenic DMRs were associated with functional regulatory elements. Genes which are regulated by DNA methylation and provide a selective growth advantage to cancer have been named epi-driver genes [18], and provide insight into the progression of cancer as well as being valuable therapeutic targets. These findings elaborate and strengthen previous research proving evidence for alterations in DNA methylation in ALL and their possible implications in the causal pathway, disease progression and relapse [1,12,19,20]. These alterations in DNA methylation are an invariable feature of development of ALL and environmental factors that disrupt DNA methylation patterns could lead to increased risk to all subtypes of ALL, or increased risk of specific subtypes depending on the genomic regions affected.

DNA methylation & the environment

DNA methylation is susceptible to change through environmental influences [9], and it has been suggested that it may provide a lifetime record of a person's exposure to environmental exposures [21]. Reports from the literature provide evidence that a range of environmental exposures can influence DNA methylation [22,23]. There are several avenues through which environment has the potential to influence methylation patterns, illustrated in Figure 1.

In the early stages of life, epigenetic marks undergo radical changes, and there appears to be two main cycles of reprogramming in mammalian embryos. The first cycle occurs following fertilization, when the DNA methylation marks of the parental gametes are erased in two waves of demethylation. This is followed by a second cycle of remethylation during germ cell development, creating a more developmentally restricted epiblast [24,25]. As this developmental stage is therefore key in the establishment of epigenetic marks that will be passed on through the life course during mitosis, early life environmental exposures which may influence the establishment of these marks have the potential to affect gene expression in later life [26]. Indeed mouse studies which have utilized the *Agouti variable yellow* (A^{vy}) metastable epiallele as an epigenetic biosensor for environmental effects on the fetus have demonstrated that maternal supplementation during pregnancy with methyl donors, genistein and ethanol can influence methylation at this gene locus. This change in methylation was concomitant with altered phenotypic outcomes [27–29], providing evidence supporting the hypothesis that environmental influences on the epigenome during early life can affect adult phenotype. Waterland *et al.* [30] identified metastable epiallele loci in the human genome and subsequently observed elevated DNA methylation of those loci in individuals conceived during the nutritionally challenged rainy season in rural Gambia. Maternal aflatoxin B1 exposure has also been observed to alter DNA methylation of 71 loci when measured utilizing 450K BeadChip platform in white blood cells from infants in the Gambia [31], which the authors suggest may be relevant to aflatoxin-related child stunting. Furthermore, the effect of dietary exposure during gestation was evaluated using a quasi-experimental setting of the Dutch Famine of 1944–1945 [32]. Methylation levels were measured using the 450K BeadChip on whole blood samples from 422 individuals roughly aged 59 years. Famine exposure during the first 10 weeks of gestation was associated with altered methylation at various CpG sites linked to genes which are involved in growth, development and metabolism. Within the Dutch Famine cohort increased coronary heart disease, raised lipids, altered clotting and more obesity have been reported in association with exposure to famine in early gestation [33]. Taken together this may suggest a critical time window of susceptibility to change in DNA methylation via environmental influences.

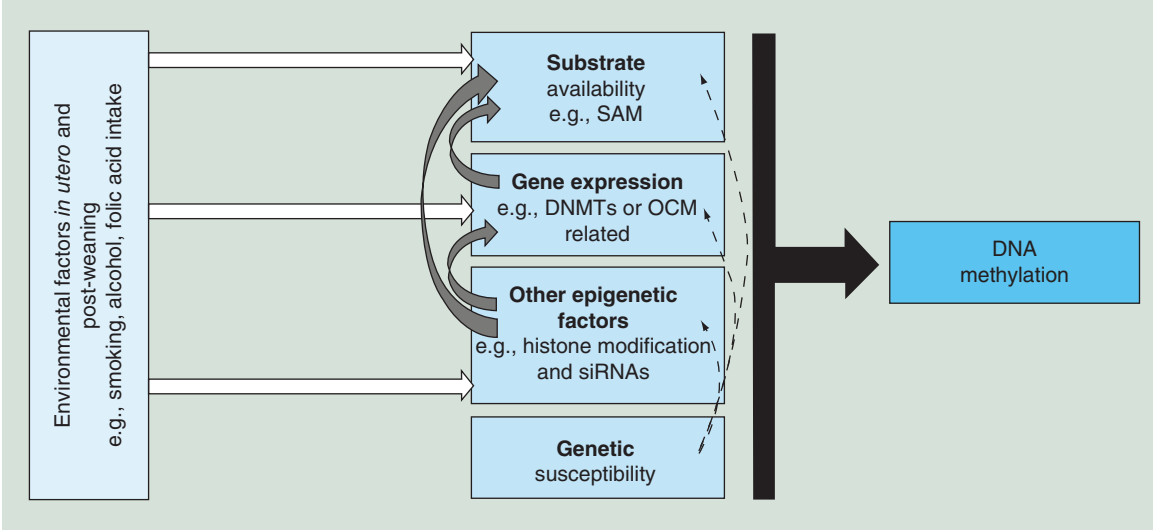

Figure 1. Overview of pathways by which environmental factors may influence DNA methylation. Environment may influence DNA methylation at any time in the life course, however, critical windows exist (i.e., during early development *in utero* and early life known as developmental programming) whereby these factors may have a more profound influence. Environmental factors may affect DNA methylation directly (white arrows) and indirectly (grey arrows). Direct pathways include altered substrate availability, in other words, of the universal methyl donor, SAM which is a substrate used to methylate DNA; altering the expression of genes responsible for maintaining or establishing methylation patterns (i.e., *DNMT*s enzymes); altering other regulatory epigenetic mechanisms which influence methylation patterns. Indirect pathways include altering expression of genes responsible for substrate availability (i.e., genes involved in OCM which is responsible for the generation of SAM); altering other epigenetic factors which may influence substrate availability through further gene expression changes. Genetic factors (indicated by dashed black arrows) are also likely to affect substrate availability, gene expression and other epigenetic factors, and may interact with environment to influence DNA methylation levels. OCM: One carbon metabolism; SAM: S-adenosylmethionine.

Identification of exposures associated with risk of developing childhood ALL

Literature searches were carried out to identify exposures associated with ALL risk (listed in Table 1) using the PubMed and ScienceDirect databases (1987–2015). An initial search was performed to identify reviews discussing ALL and possible causes or associations with an increased risk. This created a list of possible risk factors for ALL and key words (Box 1) were used for a more rigorous investigation of the literature to confirm the association between risk factor and leukemia development. There were no language restrictions imposed. In addition, the lists of references in previous studies (including reviews) were also screened to identify additional relevant studies. We subsequently investigated the literature for evidence of variation in DNA methylation in response to the exposures identified by the above analysis as having published evidence linking them to ALL risk. These further literature searches were carried out using the PubMed and ScienceDirect databases (1987–2015). The key words used were 'DNA methylation' along with key words stated for each of the exposures identified (Box 1). Each individual search produced varying amounts of literature supporting an association between an exposure and changes in methylation patterns. Duplicate publications retrieved from different databases were removed. Where large numbers of publications were found, human epidemiological studies using array based technologies to analyze DNA methylation were discussed as a priority over animal or cell line studies using site specific methods to measure DNA methylation.

Environmental factors associated with ALL risk

A number of *in utero* and early childhood exposures have been implicated in the etiology of childhood ALL [110]. These include birth weight, breast feeding, infection history, childcare/day care attendance, smoking, alcohol, caffeine, folic acid, iron, radiation, household chemicals, paints, pesticides and herbicides (see Table 1). While these listed exposures have all been observationally associated with increased risk of ALL, the weight of supporting evidence for the role of each exposure in the etiology of childhood ALL varies. While there is fairly strong evidence in support of the role of some exposures (such as day care attendance, radiation, folic acid, smoking and alcohol) for ALL risk through replication, biomarker and genetic studies, for other exposures (i.e., iron, caffeine,

pesticides/herbicides, paints and chemicals) the evidence base is much weaker. One reason for this is the lack of accurate exposure data available for such studies, as most often this is collected retrospectively and exposure measurements are often not optimal. Furthermore, given the relative rarity of the occurrence of ALL, estimating the effect of what could potentially be subtle changes in environmental exposure on risk is difficult as large numbers of cases are required to accurately assess the impact of such factors on disease risk. Therefore alternative approaches are required to strengthen the evidence for a role of environmental factors in risk of childhood ALL. As discussed, DNA methylation can be influenced by environmental exposures and is also aberrant in leukemic cells, and therefore may act as a mediator between environment and disease outcome, and as a secondary event in the multiple hit pathway to ALL (see Figure 2). The following sections summarize evidence from the current literature regarding environmental exposures and ALL risk and, to what extent these exposures have also been associated with variation in DNA methylation.

Table 1. Literature providing evidence for positive and negative associations between environmental exposures and acute lymphoblastic leukemia risk.

Environmental exposure	Ref.
Prenatal exposures	
Smoking	[34–44]
Alcohol	[35,36,45–52]
Folic acid	[40,52–61]
Caffeine	[36,42,62–65]
Iron	[66–71]
Pesticides and herbicides	[72–80]
Paints and chemicals (home/occupational use)	[76,81]
Post-natal exposures	
Infection history	[3,82–89]
Childcare and day care attendance	[3,49,84,87,88,90–104]
Radiation	[105–107]
Breast feeding	[88,94,104,105]
Birth weight	[88,108,109]

Prenatal exposures

Smoking

The knowledge of the carcinogenic properties of cigarette smoke, with an estimated 7000 chemicals affecting the body, led to studies to investigate the effect maternal smoking on ALL risk. Studies have offered conflicting evidence surrounding the association between ALL and prenatal/maternal smoking. John *et al.* [44] were one of the first studies to find an association between maternal smoking and ALL risk. A case–control study was used, and smoking data were attained by 1:1 interviews of parents from 223 cases of childhood cancer (diagnosed in Denver, Colorado 1976–1983). An increased risk of childhood cancer including ALL was observed in mothers who smoked during their first trimester of pregnancy. However, subsequent studies have found no association between maternal smoking and ALL [35–36,42]. It is pertinent to indicate that most studies utilize data collected from telephone interviews, questionnaires and 1:1 interviews with mothers. A recent review confirmed the supposition of reporting bias on self-reported smoking [111]. Previous literature was systematically reviewed and trends of underestimation were shown when the evidence is based on self-reports compared with using biomarkers. A recent meta-analysis investigated the association between childhood ALL and maternal smoking during pregnancy [43]. Data were analyzed from 21 individual studies conducted between 1999 and 2014. An association between maternal smoking and ALL was found, but the authors were not able to evaluate the effect of quantiles of cigarettes used by the women during pregnancy.

Associations have also been found between paternal smoking at home, parental smoking after birth, and number of smokers in the household and increased risk of ALL [38]. More recently, paternal smoking preconception reported through retrospective telephone questionnaires was found to be significantly associated with ALL [42]. These data were consistent with a previously published meta-analysis which used data from 18 epidemiological studies and analyzed dose-response relationship between ALL risk and smoking, finding associations with paternal smoking preconception and during pregnancy [112]. The possibility remains that this association may be confounded due to the concordance observed between maternal and paternal smoking, and thus the null associations observed between maternal smoking and ALL may be due to maternal self-report bias. Conversely, evidence from animal models suggests that paternal environmental factors can influence the sperm epigenome and pregnancy outcome [113], and influence methylation patterns of the offspring [114,115], which may suggest that paternal, as well as maternal smoking may play a role in offspring ALL risk.

While smoking clearly impacts DNA damage which is important in carcinogenesis, and therefore may influence risk of ALL [37], DNA methylation, which is also altered through smoking [34,116], may be an additional mechanism involved in the causal pathway leading to ALL. The possible long-term effects that either mater-

Box 1. Key words used to identify literature on specific environmental factors associated with acute lymphoblastic leukemia risk.

Leukemia, acute lymphoblastic, radiation, smoking, alcohol, folate, folic acid, iron, coffee, caffeine, herbicides, pesticides, household chemicals, chemicals, household paints, paints, childcare, day care, breast feeding, infection history, birth weight, infection, virus, and bacterial.

nal or paternal smoking may have on offspring DNA methylation patterns may lead to the development and progression of disease through altered gene expression.

A large epigenome-wide association study (EWAS) recently investigated extensive genome-wide changes in DNA methylation in association with current, former and never tobacco smoking in the KORA cohort (Cooperative Health Research in the Region Augsburg) [116]. Significant site-specific differences were observed in each of the 22 autosomes, identifying 187 CpG sites with differential methylation associated with smoking. Importantly it was also noted that even after participants stopped smoking there were still measured differences in methylation, showing the long term impact that smoking can have on DNA methylation [116]. While this study does not measure the effects of smoking during pregnancy and possible effect on offspring, it demonstrates that the effect of tobacco smoking on DNA methylation is evident, even long after cessation.

There is, however, evidence from the literature suggesting that maternal smoking can influence DNA methylation in offspring. Joubert *et al.* [34] used the 450K BeadChip to measure differential methylation related to maternal smoking during pregnancy in 1062 newborn cord blood samples from the Norwegian Mother and Child Cohort Study (MoBa). Maternal plasma cotinine, an objective biomarker of smoking, was measured during pregnancy and related to offspring cord blood DNA methylation [34]. In addition, maternal self-report of smoking during pregnancy was also related to offspring methylation. Twenty-six CpGs mapped to ten genes were found to be differentially methylated in association with maternal smoking, assessed by measuring plasma cotinine levels in cord blood. These included *AHRR*, *CYP1A1* and *RUNX1* (aka *AML1*). *AHRR* and *CYP1A1* are of particular interest as they encode proteins known to be involved in the detoxification of compounds from tobacco smoke (polycyclic aromatic hydrocarbons) [39]. *AHRR* codes for an evolutionary conserved bHLH-PAS (basic helix-loop-helix/Per-AHR nuclear translocator [ARNT]-Sim) protein. This protein mediates toxicity via the aryl hydrocarbon receptor signal cascade, which is also responsible for regulation of cell growth, cell differentiation and the modulation of the immune system [116]. Also of interest to this review, *RUNX1* which is involved in the development of normal hematopoiesis and leukemia. Differential methylation at the 26 CpG sites found to be related to smoking in the discovery study (MoBa) was further investigated in a replication study (using maternal self-reports). The replication population which consisted of 18 children born to smoking mothers and 18 children born to nonsmoking mothers from the US Newborn Epigenetics STudy (NEST). Of the 26 CpGs measured, 21 were found to have altered methylation in association with maternal smoking in the replication cohort. In the same cohort (MoBa), Joubert *et al.* [41] also evaluated the impact of timing of the mothers smoking. A significant association was only found with sustained smoking exposure (through at least 18 weeks gestation). *AHRR* and *RUNX1* were again highlighted as genes which had altered methylation due to smoking exposure. The most recent EWAS to date analyzing the effect of smoking on DNA methylation observed dose-response associations for 15 CpG sites in seven genes (including previously mentioned *AHRR* and *CYP1A1*) in cord blood [117]. Longitudinal analysis of the effects of smoking on DNA methylation at age 7 and 17 years demonstrated that some CpG sites methylation changes were reversed, while others such as *AHRR* and *CYP1A1* showed persistent altered patterns of methylation. This demonstrates that offspring methylation differences induced by prenatal smoking exposure persist during childhood, providing a potential mechanistic link between *in utero* exposure and later disease risk.

Altered methylation has been documented in fetal liver samples (from elective terminations between 11 and 21 weeks gestation) from mothers who smoked compared with controls [118]. For accurate classification of smoking status cotinine concentrations were measured. DNA methylation was measured at a number of regions known to be important in controlling the *IGF2*, which has been previously described as susceptible to the *in utero* environment. The male fetal liver samples showed an increase in DNA methylation at one CpG site within the *H19* imprinting control region, whereas female fetal liver samples showed a decrease in methylation at multiple CpG sites within the *IGF2* DMR associated with smoke exposure [118].

Alcohol

Studies have investigated the association between maternal alcohol drinking as a nongenetic risk factor for ALL [35]. Alcohol is recognized as being carcinogenic for humans and can affect the fetus via ethanol crossing

the placental barrier. Acetaldehyde has the ability to initiate mutagenic activity within the fetus and can be directly ingested through alcohol consumption by the mother or as a result of alcohol metabolism [48]. There is conflicting evidence with respect to the relationship of alcohol and ALL risk, some in support of an association [35,36,47], some reporting no association between alcohol and ALL risk [48,49]. In an early study, Petridou *et al.* [49] assessed the association between alcohol and childhood leukemia in a case–control study comprising 153 confirmed cases of childhood leukemia in Greece, with interviewer-administered questionnaires for exposure data collection. They found an inverse association with maternal alcohol consumption (small or moderate intake) and ALL. In another study, data collected via telephone interviews highlighted specific time frames of maternal alcohol intake that were shown to increase the risk of ALL [35], with the susceptible period being in the second or third trimester of pregnancy. Menegaux *et al.* [36] carried out a multicenter case–control study (280 incident cases and 288 hospitalized controls) in which data were collected during 1:1 interviews. Their study showed significant associations with alcoholic beverage consumption (wine, beer, spirits) during pregnancy and ALL, with a higher odds ratio for children diagnosed at less than 2 years of age. A review of data published on the effect of parental alcohol consumption and childhood cancer observed that roughly a third of the epidemiological evidence evaluated (published between 1982 and 2003) found at least one statistically significant risk increase in relation to parental drinking [47]. The first meta-analysis investigating *in utero* exposure to alcohol and its relationship with ALL found no significant association, although this report was deemed inconclusive due to the lack of appropriate published data [48]. When interpreting these data we must consider the potential problems with the method of data collection used. Reporting bias may influence the accuracy of the data as the information regarding alcohol intake during pregnancy was collected retrospectively which may introduce recall bias. Furthermore, there is also the possibility of under reporting intake during 1:1 or telephone interviews due to the stigma of alcohol drinking while pregnant.

Interactions between alcohol consumption and maternal folate intake have been reported to influence ALL risk. Several associations between genetic variations of folate pathway genes and risk of ALL have been recorded, which varied depending on levels of maternal folate and alcohol intake [52]. This provides evidence for a possible cumulative effect; in other words, being exposed to multiple ALL associated environmental exposures could create a higher risk, further strengthening the hypothesis that ALL requires multiple 'hits' to reach full disease state.

There is evidence from the literature to suggest that DNA methylation may be influenced by alcohol intake. A significant increase in DNA methylation was found in the *HERP* promoter in the blood of patients with alcohol dependence compared with controls. This was significantly associated with elevated homocysteine levels [50] (raised homocysteine levels have been found in social drinkers [51]). Since elevated homocysteine concentrations can influence genomic and gene-specific DNA methylation in peripheral blood cells [119], the elevation of homocysteine may account for the observed associations between DNA methylation and alcohol intake. Therefore alcohol consumption may induce epigenetic modification via ethanol-related dysfunction

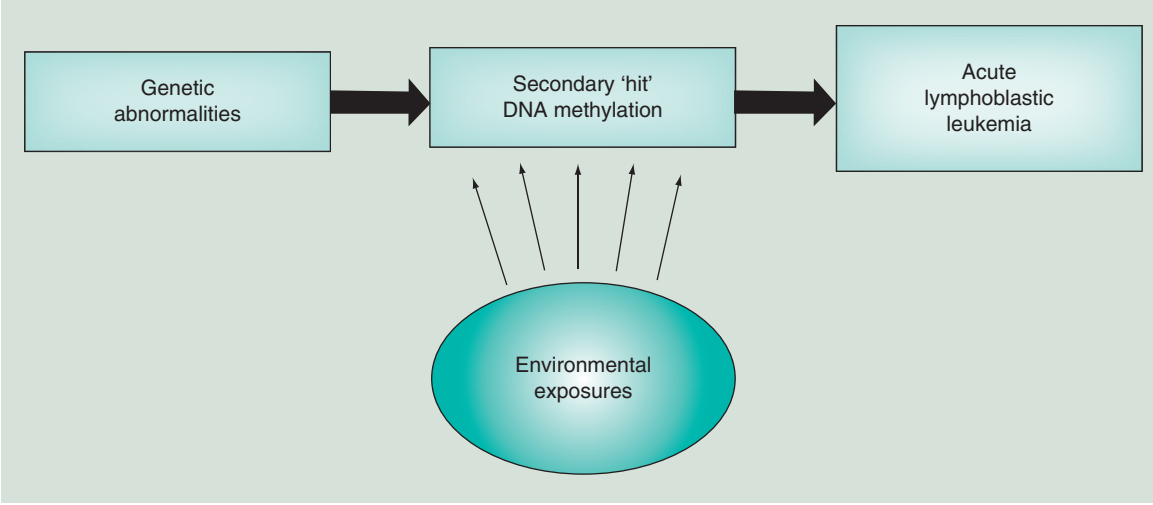

Figure 2. Plausible causal pathway to acute lymphoblastic leukemia: an initial genetic abnormality, followed by an alteration in DNA methylation influenced by an environmental exposure.

to one carbon metabolism (OCM) [45]. Furthermore, neonatal exposure to ethanol in rats resulted in global disruptions in DNA methylation [120], suggesting that maternal alcohol intake may affect epigenetic programming of offspring. Although the mechanism by which alcohol-induced methylation changes occur is unclear, one suggested pathway maybe via altered one-carbon metabolism.

Folic acid

Epidemiological studies have provided evidence of the importance of folic acid in the maternal diet for fetal development [121], and suggest that it may have a protective role against some childhood cancers [122]. The protective role of folate is considered to be due to its ability to influence DNA synthesis, repair and methylation through the OCM pathway [123].

There are a number of studies which support the protective effects of maternal folic acid supplementation [54,56], and specifically for ALL [52,55,60]. One of the largest studies to date investigating maternal folic acid supplementation suggested that prenatal use of folic acid supplements reduces the risk of ALL [60]. Maternal supplementation data were obtained on 6963 ALL cases (and controls) from multiple case–control studies participating in the Childhood Leukemia International Consortium (CLIC). It was also acknowledged that the observed association varied by parental education which was used as a proxy for lifestyle and sociodemographic characteristics. Amigou *et al.* [55] also found that childhood acute leukemia was inversely associated with maternal folic acid supplements before and during pregnancy. Correlations between genetic polymorphisms of the folate metabolism pathway with ALL susceptibility were also investigated. A positive association with ALL was found with carriers of both *MTRR* variant alleles measured while being homozygous for any variant allele of the *MTHFR* polymorphisms measured. There have also been associations reported between childhood ALL and single SNPs found in *CBS* and *TYMS*, as well as haplotype blocks within *CBS*, *MTHFD1*, *MTRR* and *MTHFR*, and haplotype blocks found just outside *CBS* and *TYMS* [52]. All of these genes code for enzymes or co-factors that are involved in the OCM pathway.

Due to its important role in the biosynthesis of the universal methyl donor, S-adenosylmethionine, altered folate intake has been associated with altered DNA methylation. Indeed, DNA methylation has been reported to be decreased in cells grown in the absence of folate [53], and moderate restrictions in folate intake in human intervention studies displayed reduced genome-wide DNA methylation for women [57,58]. Amarasekera *et al.* [59] specifically investigated the effect of folic acid on regulation of fetal DNA methylation. Folate status was measured in blood samples collected in the third trimester, and mothers were split into high folate (HF) and low folate (LF) groups. The DNA methylation profiles of the offspring were analyzed in neonatal immune cells ($CD4^+$ and antigen-presenting cells) to identify the effects of folic acid. Seven folate sensitive regions were found. Hypomethylation of a CpG dense region upstream of the *ZFP57* was associated with HF. *ZFP57* controls DNA methylation during early multicellular stages of development and is required to regulate and maintain imprinting of genes [59], and has also been suggested as a novel oncogene [124]. Interestingly, Silver *et al.* [61] found that season of conception in rural Gambia affected methylation at *ZFP57,* and identified the genomic region (~3 kb upstream of *ZFP57*) as a potential metastable epiallele. This points the effect that maternal nutrition, including folate, may have on systemic methylation in offspring.

Caffeine

Caffeine has been associated with the risk of low birth weight when consumed daily by pregnant women [62]. However, the relationship between maternal caffeine intake and ALL risk has not been extensively investigated, some studies suggest that increased caffeine consumption may increase ALL risk [63], while others found no association [49]. There are a number of possible mechanisms by which caffeine could increase ALL risk. Caffeine may act as an inhibitor for DNA topoisomerase II, DNA repair or carcinogen metabolism [63]. Via the inhibition of these important cellular processes it could induce chromosomal aberrations and translocations, such as abnormalities of chromosome 11q23 which is one potential cause of ALL [63]. Caffeine may also increase ALL risk via alteration in DNA methylation.

A recent meta-analysis carried out by Cheng *et al.* [63] investigating the risk of maternal coffee consumption and risk of childhood leukemia included seven case–control studies, with a collective total of 2090 cases (AL, ALL and AML) and 3630 controls. The odds ratio for maternal coffee intake increased linearly with the amount of coffee consumed daily; compared with non/lower drinkers (≤3 cups/day) of coffee there was a 22% increased risk for ever drinkers (4–8 cups/day), and 72% increased risk for higher level coffee drinkers (<8 cups/day) and offspring acute leukemia. Findings suggest a significant association between maternal coffee drinking and childhood ALL; this association has also been found in other studies [36].

Some evidence from animal model studies suggests maternal caffeine consumption can alter DNA methylation. Ping *et al.* [62] intragastrically treated pregnant

rats with caffeine from gestational day 11–20. Caffeine treatment enhanced the expression of *DNMT1*, *DNMT3a* and *DNMT3b* genes responsible for methylating DNA. It was also associated with a notable increase in total methylation within the *SF-1* promotor, as well as increased methylation frequency at single CpG sites within the *SF-1* promotor. Buscariollo *et al.* [65] treated pregnant A1AR knockout mice with 20 mg/kg caffeine at embryonic day 8.5 and identified altered DNA methylation patterns using DNA methylation arrays. An overall decreased methylation of 26% with 7719 DMRs was observed in adult hearts of offspring exposed to caffeine during pregnancy. These data suggest that further investigations are warranted to understand the role of maternal caffeine consumption on offspring health in human studies.

Iron

Iron is an essential micronutrient required to maintain metabolic homeostasis and genome stability, it partakes in oxygen transport, mitochondrial respiration and metabolizing nucleic acids as well as being an antioxidant. Increased volemia and fetal requirement means pregnant women require more iron. The current recommended daily allowance for pregnant women is 27 mg/day. As for most nutrients, both iron deficiencies and overload are associated with health risks. Iron has the ability to damage biomolecules, which leads to the production of hydroxyl radicals and other reactive oxygen species. Iron possesses the ability to induce a wide array of DNA lesions, from base modifications to strand breaks and adducts [66].

The *HFE* gene has been identified as being associated with cancer susceptibility, including an increased risk of ALL [68,71], and these findings have been replicated [70]. Polymorphisms of the *HFE* gene are now known to have correlations with altered iron status; C282Y polymorphisms impair β2-microglobulin association and cell surface expression of the *HFE* protein, H63D leads to a loss of ability to reduce transferrin receptor affinity for its ligand. The *HFE* C282Y polymorphism has previously been reported to elevate the risk for ALL [67]. In two independent groups of patients the C282Y mutation has been shown to be associated with altered ALL risk in males specifically [68], suggesting a gender-specific increased risk for hematological malignancies according to genotype. An association with *HFE* and ALL risk appears to be heightened through an interaction with a polymorphism in the transferrin receptor gene (*TFRC*). This increased risk effect may be due to the biological interaction between *HFE* and *TFRC* genes, and iron transfer across membranes such as the placenta and intestinal mucosa [70].

Although there is limited evidence, iron has been reported to have the ability to alter DNA methylation. While iron accumulation/overload has been shown to induce DNA hypermethylation [66], a small positive association was found between *LINE-1* methylation levels in leukocyte DNA and chronic iron exposure measured in toenail clippings [125]. As there is currently very little evidence to support the relationship between iron and DNA methylation further investigations are warranted to better understand this possible interaction.

Pesticides & herbicides

Despite several pesticides and herbicides being classified as probably, possibly or carcinogenic to humans [126], we do not have a good understanding of any long-term health effects of exposures to such agents, particularly in pregnant women and their children. Past studies have revealed weak associations at best between the effects of herbicides and ALL risk [77]. However, over the last decade, a number of case–control studies have shown positive associations with home/garden use of pesticides/herbicides and an increased ALL risk in children [72–76]. All studies reported relationships between exposure and ALL outcome, with some studies observing that timing of exposure was also important. Exposure to pesticides prepregnancy, during pregnancy and early childhood, appeared to confer an increased risk of ALL compared with exposures later in life [77].

Environment, home, school and dietary intake are all possible forms of exposure to pesticides/herbicides for mothers and their children, making it hard to avoid exposure, as well as having implications for the measurement and evaluation of exposure [78]. Furthermore, for most studies data were collected through the use of self-reports which are subject to recall bias, as well as there being a lack of available information on the full range of potential active ingredients in products used. Rudant *et al.* [72] specifically investigated the effect of selection bias in previous studies of household exposure to pesticides, and found that even though selection bias was likely within these studies this still did not explain the positive correlation between the use of pesticides and increased risk of ALL. The NCCLS used several different methods to overcome previous limitations by including: quality control of self-reports; a home pesticide inventory and linkage to the Environmental Protection Agency (to obtain active ingredients data); collection and analysis of home dust samples (~600); a geographical environmental database (agricultural pesticides); and large-scale genotyping to assess the role of genes in xenobiotic pathways (transport and metabolism of pesticides) [78]. Findings from a subset (162 leukemia patients and matched controls) of

the NCCLS suggested an amplified risk of childhood leukemia when exposed to household pesticides, and further indicated that timing of exposure appeared to be important (during pregnancy and early childhood significantly increased risk) [77].

Although the evidence is limited, some animal models suggest that pesticide exposure may alter DNA methylation. An alteration in methylation patterns in the hypothalamus of rats was shown after exposure to the agricultural insecticide dichlorodiphenyltrichloroethane, whereby six CGIs were hypomethylated in dichlorodiphenyltrichloroethane exposed rats compared with controls [127]. Furthermore, Desaulniers *et al.* [79] reported that offspring DNA methylation can be altered due to maternal exposure to pesticides. Pregnant rats were exposed to high doses of organochlorine pesticides, methylmercury chloride (MeHg) or polychlorinated biphenyls (PCB) and offspring livers were collected at postnatal day 29. Gene expression levels of *DNMT1*, *DNMT3A* and *DNMT3B* were reduced with high doses of PCB, and for mRNA for *DNMT1* and *DNMT3B* with high doses of MeHg. Pyrosequencing methylation analysis revealed that high doses of PCB and MeHg were also associated with decreased methylation of the *p16* promoter region.

Home & occupational use of chemicals & paints

Evaluating the effect of household chemicals and paints used by parent's pre- and post-natally encounters the same problems as estimating the exposure of pesticides and herbicides, with the potential for recall bias and insufficient data on active ingredients in products used. Despite these difficulties, some studies have provided evidence of an association between the uses of household chemicals and paints, and an increased risk of ALL. Prolonged exposures such as occupational contact have been investigated pre and postnatally [76], with an increased risk of ALL observed in children whose fathers worked with spray paints while the mothers were pregnant, as well as working with spray paints, chlorinated solvents, dyes/pigments, methyl ethyl ketone and cutting oil once the child was born [76]. Research findings vary, with some studies finding strong associations with postnatal exposure to paints, while others, such as solvent exposure, yield inconsistent results and warrant further investigation [81]. There is also evidence that paint exposure appears to be specifically related to the ALL subtype with t(12;21)*TEL–AML1* translocations, although exactly how the exposure influences disease progression is still unclear [81].

One study investigated the influence of several chemicals in relation to epigenetic regulation and established alterations in total DNA methylation as well as at specific gene loci [128]. Chemicals investigated included bromodichloromethane, dibromochloromethane, chloroform, hydrazine, trichloroethylene, benzidine, trichloroacetic acid and di(2-ethylhexyl) (DEHP). Points of departure for cancer incidence, and change in DNA methylation were studied in laboratory animals (mice, rats and hamsters). A high degree of correlation was found between points of departure for cancer incidence and DNA methylation changes following exposure to environmental chemical carcinogens [87]. The administration of DEHP to pregnant rats and subsequent analysis of male offspring exposed a notably vulnerable epigenome during early developmental periods. This study provides evidence which supports the theory that DNA methylation may mediate the influence of chemical exposures on later cancer development. It also offers a method of testing the potentially harmful effect of chemicals on the epigenome, which could lead to better guidelines for chemical use.

Post-natal exposures

Infection, the immune system & breast feeding

Infection was one of the first suggested risk factors for ALL [82]. There are currently two main infection-based hypotheses for ALL. The Kinlen population mixing hypothesis, which states that the association is due to unusual demographic mixing of susceptible and infected individuals, this happens perinatally and is probably caused by a single novel virus. Current evidence for this hypothesis is the transiently increased incidence of ALL in several situations of population mixing or clustering [3]. The Greaves delayed infection hypothesis suggests that an abnormal immune response to delayed exposure to common infections in childhood due to a lack of early life exposure as infants increases risk of ALL. This hypothesis is supported by studies providing evidence that there is a reduced risk of ALL associated with day care attendance [3].

Greaves hypothesis concentrates on the importance of timing of exposure rather than focusing on specific agents as suggested by the Kinlen hypothesis [83]. Children with a delayed or reduced exposure to common infections at an early age will develop a less adaptive immune system. This could lead to an increased cell proliferation when later confronted with a common infection, thus an increased risk of a second mutation and the development of ALL [84]. The UK Childhood Cancer Study (UKCCS) findings support this hypothesis, showing that a dysregulated immune response to infection in the first few months of life promotes progression to ALL disease later in childhood. A higher frequency of clinically diagnosed infectious episodes was also found to be correlated to an earlier onset of

ALL [89]. Breast feeding is another proxy of early stimulation of the immune system, promoting adequate maturation of the immune system in infants, and has also been inversely associated with ALL [88].

The effect of infections on their hosts epigenetic landscape is becoming more widely discussed, with bacterial [129] and viral infections [130–132] being shown to alter the epigenetics of infected cells. The Epstein–Barr virus (EBV) has already been associated with multiple human malignancies, as well as being shown to have been of high incidence in pediatric ALL patients [133]. This virus can cause lifelong infection of resident epithelial and B cells, resulting in a distinct pattern of EBV gene expression in infected cells which is regulated via epigenetic modifications [132]. The epigenetic effect of EBV has been analyzed in an immortalized keratinocyte cell line. Global DNA methylation analysis showed over 13,000 differentially methylated CpG sites compared with controls, from this 65 genes which acquired methylation presented altered transcript levels. Birdwell *et al.* [132] suggested that the EBV virus may leave a lasting epigenetic imprint that could enhance the tumorigenic phenotype of infected cells. Parvovirus B19 (PVB19) has previously been associated with ALL [131], and a link between PVB19, DNA methylation and ALL has now also been observed. Bone marrow samples of B-cell ALL taken at diagnosis were serologically tested, revealing that samples were positive for PVB19 IgM and IgG. DNA methylation was found to be associated with a history of PVB19 infection, indicated by IgG ($p = 0.02$). This may cause increased leukemogenic potential in susceptible B-precursor cells via PVB19 driven epigenetic alterations [131].

Childcare & day care attendance

Since Greaves hypothesized that delayed exposure to common infections leads to an increased risk of ALL, a number of studies have attempted to provide evidence for this hypothesis including analysis of time spent in day care at a young age, and thus exposure time to common infections in relation to ALL onset [3]. Theoretically, attendance of day care at a young age should mean that a child is confronted with common infections at an early age. This would allow them to build a more sophisticated immune system and reduce the chances of an increased proliferation and risk of mutation if confronted with common infections at a later date [84]. A large body of evidence suggests that there is a connection between early or increased day care attendance and a reduction in the risk of ALL [49,90–103].

A recent study using the findings from the NCCLS evaluated a summarized measure of 'child-hours of exposure', allowing them to capture the variance which can be contributed by individual day care variables [84]. Non-Hispanic white children who attended more than 5000 day care hours during infancy compared with children who did not attend day care had a reduced risk of ALL [84]. Children had a 58% reduced risk for ALL (95% CI: 0.18–0.99) and a 67% reduced risk for precursor-B ALL (Burkitt's lymphoma/leukemia; 95% CI: 0.11–1.01). The trend was also observed, supporting the theory that there is a dose-response relationship. Also, as Greaves suggested, timing is important and reduction of risk was associated with attending day care during infancy, showing the importance of early life exposure [3].

When considering the effect childcare and day care attendance may have on DNA methylation one must first consider the Greaves delayed infection hypotheses [3], in other words, that childcare and day care attendance act as a proxy for exposure to infection. This appears to provide a protective effect against ALL, with timing and number of hours of care also affecting the risk. On the other hand as mentioned in the previous section infection also appears to increase the risk of ALL. It may be that more serious infections have the ability to influence the child's epigenome (discussed above) [131]. Consequently delayed or limited childcare and day care attendance could leave a child's immune system insufficiently matured and more susceptible to more serious infections.

Radiation

Potential cancer risk for children exposed to radiation is much higher than in adults as they are more radio sensitive [105]. A retrospective study found a positive association between radiation dose from CT scans and leukemia, with an almost triple risk of leukemia when children had cumulative doses of roughly 50 mGy [105]. Furthermore, while radiotherapy treatment has contributed to the improved survival rates of childhood cancer over recent decades (30–80%) [106], an investigation into secondary malignant neoplasm occurrences post-radiotherapy in children and adults revealed leukemia as one of the most prevalent secondary malignant neoplasms [107].

Radiation has been observed to induce changes in DNA methylation and there is evidence to suggest that this may lead to an altered cell response to subsequent radiation exposure [134]. In nuclear power plant workers, low dose radiation was associated with DNA methylation levels [89] whereby *LINE-1* methylation levels were higher in radiation exposed-workers than controls. Associations between chromosome aberrations and radiation-induced DNA methylation were also suggested.

Birth weight

The association between birth weight and childhood cancer was originally suggested by MacMahon and Newill over 50 years ago [108]. Paltiel *et al.* [109] pooled data from six cohorts to investigate cancer incidence in relation to infant and parental characteristics, reporting a 26% increased risk of childhood cancer (including ALL) for every kilogram increment in birth weight. No association was found with prenatal overweight or pregnancy weight gain, suggesting that the component of childhood ALL risk explained by higher birth weight is not a consequence of maternal overweight or obesity but likely due to another pathway leading to fetal (over) growth.

The first large EWAS investigating the relationship between birth weight and methylation revealed methylation at 19 CpG sites to be associated with birth weight. Some of the identified CpG sites were located within genes responsible for adipogenesis and DNA repair [135]. A more recent study confirmed two CpG sites identified in the MoBa cohort as well as a further 21 CpG sites were associated with birth weight [136]. Both studies found that several of their birthweight related CpG sites were linked to genes which played an important role in development. Simpkin *et al.* [136] also acknowledged that the effect of birth weight on methylation was predominant in cord blood, and this highlights a potential critical window for the effects of prenatal and early life exposures on DNA methylation, which may impact on future disease risk.

Conclusion

ALL is the most common cancer in children [110], but the causes of this disease are still largely unknown. However, a growing amount of literature now supports the contribution of various environmental factors to risk of ALL development. Roughly 80% of cases are of precursor-B cell origin ($CD19^+$, $CD10^+$), and the incidence of this specific immuno-phenotype has increased in the Western world over the past several decades [110]. This increase may be due to changes in exposures pregnant women and young children are confronted with in modern everyday life. Indeed, this review suggests that there are a number of environmental exposures which increase the risk of ALL, and therefore warrant further investigation. Although survival rates have improved dramatically over the past few decades ALL survival is associated with a greatly increased ill health in adulthood [137]. This is due to the impact of treatment, thus prevention strategies are desirable. Therefore understanding exactly how the exposures discussed in this review are increasing the chance of ALL will be critical in understanding the causal pathway to disease. Furthermore, this will provide potential predictive disease biomarkers and plausibly may help determine appropriate and effective preventative intervention strategies.

As an epigenetic modification, DNA methylation, which plays a crucial role in forming cellular identity by influencing gene expression, is likely to be involved in the causal disease pathway of ALL. Indeed, there is a body of evidence to indicate that DNA methylation is altered in childhood ALL [1,14,138–140], but knowledge of how these alterations occur and if they could be prevented will be important in improving understanding of the underlying mechanisms of the disease. Furthermore, since DNA methylation patterns can be environmentally orchestrated, knowledge of the involvement of this mechanism in disease etiology may provide plausible and implementable intervention strategies for high risk individuals such as those with Down's syndrome or Fanconi Anemia. Here we have explored the supposition that environmental exposures associated with ALL risk have the potential to alter DNA methylation thus making DNA methylation a plausible mediator of environmental influences in the pathogenesis of ALL. We have reviewed evidence in support of this hypothesis, and conclude that evidence from the literature could suggest that several environmental exposures associated with increased childhood ALL risk, in other words, alcohol, smoking and folate are able to alter DNA methylation and therefore this may be one mechanism by which these exposures are involved in the causal pathway to disease. *AHRR* and *CYP1A1* are the two clearest genes which exhibit methylation change due to an environmental exposure (smoking), and have similarly been shown to be frequently abnormally methylated in ALL [1]. The review of the literature and crossover between aberrant methylation in response to environmental factors and in ALL is not exhaustive in the context of this review. Further rigorous investigation of the available data is required to explore further connexions, these examples add weight to the hypothesis that DNA methylation may act as a mediating mechanism in this context. Further consideration should be given to the likelihood of a cumulative effect of exposures, whereby exposure to multiple ALL risk associated factors could further increase the chance of disease development through cumulative epigenetic aberrations. Further research is therefore warranted to investigate this hypothesis in order to aid understanding of the causal pathway to disease, which is vital in facilitating new treatments, initiating preventative strategies and screening for disease.

Future perspective

To understand the pathway from the initial genetic 'hit' to a child being diagnosed with ALL, future studies will need to combine multiple investigations toward realization of the multifactorial etiology of ALL. The literature examined in this review provides evidence for a potential role of DNA methylation in ALL development through environmental exposures. Given the rarity of childhood cancers, the availability of robust exposure data and patient samples prior to diagnosis is limited, multiple complementing strategies will be required to further explore this concept. Initially, clearer evidence is required to show that ALL-associated risk exposures result in disease-associated DNA methylation changes. Global DNA methylation alterations seen in ALL are mostly seen across all subtypes [1], and thus appear to be early events in ALL development. However, the importance of these methylation changes in inducing or contributing to disease development is less clear. As environmental exposures can potentially drive these changes in methylation it is important to understand if these changes can in turn drive ALL pathogenesis. Confirmation of the establishment of aberrant methylation patterns in ALL patients prior to diagnosis will be important in determining the role of these events in the causal pathway, however, such studies may be challenging given the lack of biological material available. The utilization of neonatal blood spot samples or collective cases from multiple large cohort studies may be feasible avenues of pursuit for case–control studies in this area. However, while this may establish proof of aberrant methylation prior to diagnosis, such studies are likely to be unable (through lack of data) or underpowered to detect changes in methylation associated with environmental factors which may be associated with disease outcome. In addition to using the 'meet in the middle' approach [141] to link early initiating epigenetic events in disease to environment, Mendelian randomization approaches utilizing genetic instruments as proxy markers for environment [142], will be key where environmental data are lacking but genetic material or data are available. Data from these combined approaches would further support the evidence that environmental factors are drivers in disease progression, and provide a mechanism by which they are part of the multiple hit pathway for ALL development. Such findings may provide predictive disease biomarkers and offer insights into how preventative strategies may be introduced.

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Executive summary

DNA methylation & acute lymphoblastic leukemia

- Altered DNA methylation has been observed between acute lymphoblastic leukemia (ALL) cells and nonleukemic bone marrow, and as well as between ALL subtypes.
- Nine thousand four hundred and six CpG sites were found to be predominantly hypermethylated across all subtypes in a large scale epigenome-wide association study of ALL cells compared with controls, demonstrating a genome wide disruption of the epigenome.

Environmental exposures relationship with DNA methylation & ALL risk

- Environmental exposures have been associated with an increased risk of ALL.
- Currently there is fairly strong evidence supporting the association with exposures such as day care attendance, radiation, folic acid, smoking and alcohol.
- There is also evidence, although weaker for other exposures associated with ALL risk, in other words, iron, caffeine, pesticides/herbicides, paints and chemicals.
- There is a growing amount of supporting evidence for alterations in methylation caused due to environmental exposures that are also linked to ALL risk, especially for smoking, folic acid and infection.

Future perspective

- Further studies are warranted in order to support and strengthen the evidence for a potential mediating role of DNA methylation between risk exposures and ALL development. Multiple integrated and complementing strategies, in other words, 'meet in the middle' approaches will be required to provide evidence of this concept.

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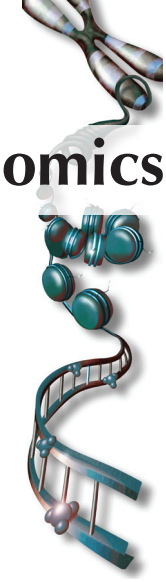

Global measures of peripheral blood-derived DNA methylation as a risk factor in the development of mature B-cell neoplasms

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Aim: To examine whether peripheral blood methylation is associated with risk of developing mature B-cell neoplasms (MBCNs). **Materials & methods:** We conducted a case–control study nested within a large prospective cohort. Peripheral blood was collected from healthy participants. Cases of MBCN were identified by linkage to cancer registries. Methylation was measured using the Infinium® HumanMethylation450. **Results:** During a median of 10.6-year follow-up, 438 MBCN cases were evaluated. Global hypomethylation was associated with increased risk of MBCN (odds ratio: 2.27, [95% CI: 1.59–3.25]). Within high CpG promoter regions, hypermethylation was associated with increased risk (odds ratio: 1.76 [95% CI: 1.25–2.48]). Promoter hypermethylation was observed in *HOXA9* and *CDH1* genes. **Conclusion:** Aberrant global DNA methylation is detectable in peripheral blood collected years before diagnosis and is associated with increased risk of MBCN, suggesting changes to DNA methylation are an early event in MBCN development.

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Background

Mature B-cell neoplasms (MBCN) are a group of hematologic malignancies originating from a mature B lymphocyte precursor and include B-cell non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL) and plasma cell neoplasms such as multiple myeloma (MM) with annual incidences of 19.7 per 100,000, 4.5 per 100,000 and 6.3 per 100,000, respectively [1,2]. Although they manifest diverse clinical features and histologic appearances, MBCN of different subtypes aggregate strongly in familial clusters [3–5], suggesting a shared genetic predisposition.

Aberrant DNA methylation is a hallmark of cancer and appears to be an important factor in the pathogenesis of MBCN [6–9]. There are several proposed mechanisms by which aberrant methylation may contribute to the neoplastic process. Hypomethylation of large regions of the genome in tumor tissue compared with normal tissue is well described in virtually all subtypes of MBCN tumor tissue [8–11]. The functional association between DNA hypomethylation and cancer is thought to be the association between reduced methylation and genomic instability, indicated by increased chromosomal rearrangements, mitotic recombination and aneuploidy [12,13]. In contrast, hypermethylation of promoter regions of tumor suppressor genes in MBCN is hypothesized to lead to downregulation and gene silencing in MBCN [14–16].

Advances in epigenetic technologies offering broad coverage of the epigenome and high-throughput capabilities raise the possibility of examining the epigenetic state at many different loci simultaneously in a large number of individuals. Such approaches examine DNA methylation across the epigenome, often utilizing array-based methylation assays in order

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to detect novel regions of differential methylation associated with a cancer phenotype. Although any tissue can be used, DNA from peripheral blood offers the advantage of being a readily available tissue from which new biomarkers for the prevention, early detection and monitoring of cancer could be identified [17]. To date, several retrospective case–control studies have demonstrated aberrant global methylation profiles in the peripheral blood of patients with colorectal cancer, breast cancer, head and neck cancers and urothelial cancers [18] but there are no published prospective studies regarding MBCN. The only published study of DNA methylation in peripheral blood in MBCN is a retrospective case–control study of follicular lymphoma measuring DNA methylation in the *DAPK1* gene only [19].

To our knowledge, ours is the first prospective to describe the importance of DNA methylation profile measured in peripheral blood to the risk of MBCN tumors. We evaluated a global measure of DNA methylation as a predictor of subsequent development of MBCN using the Infinium® HumanMethylation450 BeadChip (Infinium 450k; Illumina, CA, USA) which is suitable for epigenetic epidemiological studies due to the small amounts of DNA required, the high-throughput capability and the ability to interrogate methylation at single base pair resolution [20]. Our aims were to assess whether a global measure of DNA methylation dispersed over a large portion of the genome could be detected in DNA from peripheral blood, and whether such a measure was associated with the risk of developing MBCN.

Materials & methods

Participants

Participants were enrolled in the Melbourne Collaborative Cohort Study (MCCS), a prospective cohort study of 41,514 healthy adult volunteers (24,469 women, 17,045 men) aged between 27 and 76 years (99.3% aged 40–69 years), recruited between 1990 and 1994 [21]. Peripheral blood samples were obtained from participants at study entry (baseline). For this study, those with a history of cancer before baseline (n = 1970) or no baseline DNA sample (n = 420) were ineligible, leaving 39,124 eligible participants. Incident cases of MBCN diagnosed between baseline attendance and 31 December 2011 were identified by linkage to the Victorian Cancer Registry, which receives mandatory notification of all new cancer cases in Victoria, Australia. The diagnostic pathology reports were reviewed and classified according to the International Classification of Disease (ICD-O-3). Controls were selected using density sampling with attained age as the time scale and were individually matched to cases at a 1:1 ratio based on age at enrollment (±1 year), gender, ethnicity and DNA source (see below).

We considered baseline smoking, folate and alcohol intake and BMI as potential confounding variables. Information on smoking and alcohol consumption was obtained from an interviewer-administered questionnaire. Folate intake was assessed by a 121-item food frequency questionnaire specially designed for the MCCS. BMI was calculated from height and weight, which were measured at baseline.

Study participants provided written, informed consent. The study was approved by Cancer Council Victoria's Human Research Ethics Committee and performed in accordance with the institution's ethical guidelines.

DNA source & sample collection

Blood samples were collected at baseline entry into the study, prior to any diagnosis with cancer. For 636 samples, the DNA source was from dried blood spots (DBS). For DBS samples, whole blood was collected and transferred to Guthrie Card Diagnostic Cellulose filter paper (Whatman, Kent, UK) and stored in airtight containers at room temperature. Methylation profiles from DBS using the Infinium HumanMethylation450 BeadChip are highly reproducible between technical replicates (means correlation, r = 0.9932) and compared with methylation from buffy coat samples from the same individuals (r = 0.9932) [20]. For 234 samples, the DNA source was peripheral blood mononuclear cells and for ten samples the DNA source was buffy coats. Both had been stored at -80°C since collection. Mononuclear cells were isolated by density gradient centrifugation method using Ficoll-Paque Plus (GE Healthcare, Parramatta, Australia). Briefly, whole blood was centrifuged and plasma was removed. The red cell fraction was carefully transferred onto Ficoll and spun down for 20 min without applying brake. After the spin, the mononuclear cell layer was transferred and washed with RPMI. The mononuclear cells were stored in liquid nitrogen.

DNA extraction & bisulfite conversion

DNA was extracted from mononuclear cells and buffy coat specimens using QIAamp mini spin columns (Qiagen, Hilden, Germany). DNA was extracted from dried blood spots using a published method [20]. Briefly, 20 blood spots of 3.2 mm diameter were punched from the Guthrie card and lysed in phosphate-buffered saline using Tissue Lyser (Qiagen). The resulting supernatant was processed using Qiagen mini spin columns according to the manufacturer's protocol. The quality and quantity of DNA was assessed using the Quant-iT™ Picogreen®ds DNA assay measured on the Qubit® Fluorometer (Life Technologies, NY, USA), with a minimum of 0.3 µg

DNA considered acceptable for methylation analysis. Bisulfite conversion was performed using Zymo Gold single tube kit (EZ DNA Methylation-Gold kit, Zymo Research, CA, USA) according to the manufacturer's instructions. Postconversion quality control was performed using SYBR green-based quantitative PCR, an in-house assay, designed to determine the success of bisulfite conversion by comparing amplification of the test sample with positive control and negative controls.

DNA methylation assay

Samples were processed in batches of 96 samples per plate (8 Infinium HumanMethylation450 BeadChips per batch). In order to minimize potential batch effects, matched cases and controls were processed together and run on the same BeadChip and cancer subtypes were evenly distributed across the plates/chips. Two technical replicates and two controls (multiple myeloma cell line U266) were included on each plate. The positions for cases and controls were randomly ordered for every BeadChip to reduce any possible position effects within the chips. The Infinium HumanMethylation450 BeadChip analysis was performed according to manufacturer's instructions. A total of 200 ng of bisulfite converted DNA was whole genome amplified and hybridized onto the BeadChips. The TECAN automated liquid handler (Tecan Group Ltd, Mannedord, Switzerland) was used for the single-base extension and staining BeadChip steps. Bisulfite conversion and the Infinium 450k methylation assay were performed in accordance with the manufacturer's instructions.

Data processing

Methylation data were imported into R statistical software [22] and processed using 'minfi' bioconductor package [23]. Background correction and between-sample normalization were performed using the 'preprocessIllumina' function of minfi which is equivalent to standard normalization methods used in GenomeStudio software package provided by Illumina. Subset-quantile within array (SWAN) was performed for type I and II probe bias correction [24]. We excluded 65 CpG sites corresponding to known single nucleotide polymorphisms. Samples were excluded if >5% CpG probes (excluding chrX and chrY probes) had a detection p-value higher than 0.01, which were regarded as probes with 'missing value', while CpG sites were excluded from further analysis if they had missing values for one or more samples. ComBat normalization was applied to minimize chip and batch effects [25]. Illumina normalization to control for position effect, SWAN correction to correct for type I and II probe bias and ComBat normalization to correct for chip and batch effect have been previously used as a pipeline for analyzing such datasets [26]. Following methylation analysis, using the Infinium HumanMethylation450 BeadChip, only one pair was rejected on the basis of returning >5% of probes with detection p-value > 0.01. A total of 19,834 probes was identified as hybridizing to multiple genomic locations [27], and analysis was performed before and after exclusion in order to avoid unexpected effect of these cross-hybridizing probes on global methylation.

Statistical analysis

The M-value is defined as $\log_2$ (Meth/Unmeth)], where Meth, Unmeth are the intensities of the methylated and unmethylated probes, respectively. M-values and β-values were calculated using minfi [23]. Statistical analyses were performed using M-values due to the possibility of heteroscedasticity encountered when using β-values [28]. We calculated genome-wide global DNA methylation using all 444,551 probes and global DNA methylation in specific regions of the genome. Using the annotation file provided by Illumina, CpG sites were classified according to their location in CpG islands, CpG shores or shelves or other ('open sea' locations). Promoter regions were defined as loci 1500 bp upstream of the transcription start site, within enhancer-associated regions and within the 5′UTR. Promoter regions were further divided according to their CpG content and ratio, as differential CpG content within promoter regions is known to influence methylation profile and gene expression [29,30]. High CpG promoters (HCP), intermediate CpG promoters (ICP) low CpG promoters (LCP), as described by Weber *et al.* [29], were analyzed using a published annotation file [31].

Stata Version 10 (StataCorp, TX, USA) was used for statistical analysis. For each sample, the median M-value across all probes was defined as the global measure of DNA methylation and grouped into tertiles based on the distribution in controls. Hypomethylation was defined as the lowest tertile of M-values and hypermethylation defined as the highest tertile of M-values. Conditional logistic regression was used to estimate odds ratios (OR) in relation to tertiles of global DNA methylation, with the middle tertile as the reference category. Variables adjusted for in conditional logistic regression were: gender, age at enrollment, ethnicity and DNA source. Associations were also assessed by including the median M-value as a continuous explanatory variable. p-values from the likelihood ratio test were reported. The likelihood ratio test was also used to test for subgroup heterogeneity.

The majority of blood in our study was collected and stored as whole peripheral blood in which the

proportions of leukocytes was not known. Due to the described effect of differential leukocyte cell content on DNA methylation profile [32] we sought to control for leukocyte heterogeneity. We therefore applied the algorithm of Houseman *et al.* [32] in which the cell composition of peripheral blood samples is estimated based on distinctive methylation profiles. This was performed in minfi. A paired t-test was performed comparing leukocyte proportions (B lymphocytes, T lymphocytes, natural killer (NK) cells, monocytes, granulocytes) in cases and controls. Leukocytes with significantly different proportions in cases and controls were factored into conditional logistic regression to determine whether adjustment for cell composition affected the OR.

The effect of the time interval between baseline and MBCN diagnosis on the ORs was analyzed by group-

Table 1. Characteristics of study sample.

Demographics	Controls (n = 438)	Cases (n = 438)	p-value
Age at enrollment (years):			
– Median	59	59	†
– SD	8	8	
– Range	40–70	40–70	
DNA source:			
– Dried blood spot	316	316	†
– Mononuclear cell	117	117	†
– Buffy coat	5	5	†
Age at diagnosis (years):			
– Median	69	69	
– SD	9	9	
– Range	42–87	42–87	
Tumor subtype:			
– MM		109	
– Follicular lymphoma		81	
– Low-grade NHL/CLL		136	
– High-grade NHL		112	
Ethnicity:			
– Anglo-Celtic	340	340	†
– Southern European	98	98	
BMI:‡			
– BMI ≥30	87	87	0.58
Smoking status‡:			
– Never smoked	246	241	0.53
– Former smoker	151	152	
– Current smoker	37	43	
Daily folate intake‡:			
– <320 μg/day	216	226	0.11
Daily alcohol intake‡:			
– 0 g/day	160	158	0.54
– 1–39 g/day	215	228	
– 40–59 g/day	40	37	
– ≥60 g/day	23	15	

†Variables matched for cases, controls.
‡Measured at baseline.
CLL: Chronic lymphocytic leukemia; MM: Multiple myeloma; NHL: Non-Hodgkin's lymphoma; SD: Standard deviation.

Table 2. Comparison of estimated leukocyte proportions in cases and controls.

Leukocyte breakdown	Mean		Standard error		Difference in means	95% CI	p-value
	Cases	Controls	Cases	Controls			
All samples (n = 876)							
T lymphocytes	0.286	0.287	0.005	0.005	-0.001	(-0.013–0.011)	0.863
NK cells	0.102	0.957	0.004	0.005	0.007	(-0.002–0.015)	0.127
B lymphocytes	0.107	0.081	0.005	0.002	0.026	(0.016–0.035)	<0.001
Monocytes	0.079	0.078	0.002	0.002	0.001	(-0.005–0.007)	0.786
Granulocytes	0.478	0.5	0.008	0.007	-0.022	(-0.042 to -0.002)	0.029
Dried blood spot samples (n = 632)							
T lymphocytes	0.266	0.277	0.004	0.004	-0.01	(-0.021–0.001)	0.051
NK cells	0.076	0.07	0.002	0.002	0.006	(-0.001–0.012)	0.067
B lymphocytes	0.103	0.083	0.005	0.002	0.02	(0.009–0.030)	<0.001
Monocytes	0.073	0.072	0.001	0.001	0.001	(-0.002–0.005)	0.41
Granulocytes	0.524	0.54	0.006	0.005	-0.016	(-0.031 to -0.001)	0.033
Mononuclear cell samples							
T lymphocytes	0.348	0.323	0.013	0.012	0.025	(-0.007–0.057)	0.128
NK cells	0.178	0.169	0.009	0.01	0.01	(-0.014–0.034)	0.413
B lymphocytes	0.116	0.08	0.011	0.004	0.037	(0.015–0.059)	0.001
Monocytes	0.097	0.097	0.007	0.007	<0.001	(-0.020–0.020)	1
Granulocytes	0.346	0.371	0.022	0.02	-0.05	(-0.10600.006)	0.08

Direct evaluation of leukocyte content was not available therefore leukocyte proportions were estimated by applying an algorithm based on the distinctive methylation profiles of each leukocyte. Leukocyte proportions were compared in cases and control for all sample types as well as for whole blood samples stored as dried blood spots and purified mononuclear cell samples.
Leukocyte proportions estimated according to methylation profile [32].
NK: Natural killer.

ing the interval into <5, 5–9 and >9 years. For controls, the time interval was calculated as the time between DNA collection and the date at which the matched case was diagnosed with MBCN.

DNA methylation within several target genes known to be hypermethylated in MBCN was analyzed (*HOXA9*, *CDH1*, *CDH13*, *ADAMTS18* and *PCDH10*) [33–36]. A conditional logistic regression model was used to identify CpG probes differentially methylated in cases compared with controls. We assigned a threshold of significance for differential methylation as $p < 10^{-08}$, in keeping with the cut-off used in genome-wide association studies [37]. This is a relatively strict threshold comparable to other multiple testing hypotheses such as the Bonferonni procedure and false discovery rate. Given the exploratory nature of this prospective study of methylation, we also adopted an inclusive approach and considered probes with $p < 10^{-5}$.

Results

During follow-up (median 10.6 years; range 2.4 months to 20 years), 471 MBCN cases were identified. Out of these, 24 had no suitable controls. Pairs were excluded if either the case or control had inadequate sample after DNA extraction (n = 7) or high detection p-values after data processing (n = 1), leaving 438 matched pairs for analysis.

MBCN cases were grouped into four major subtypes: MM (n = 111), follicular NHL (n = 81), high-grade NHL (n = 111), comprising diffuse large B-cell lymphoma (n = 110) and Burkitt lymphoma (n = 1) and low-grade NHL (n = 136), comprising CLL and small lymphocytic lymphoma (n = 86), splenic marginal zone lymphoma (n = 6), mantle cell lymphoma (n = 16), marginal zone lymphoma (n = 15), Waldenstrom's macroglobulinemia (n = 9) and hairy cell leukemia (n = 4). Characteristics of participants and tumors are outlined in Table 1.

The proportions of T lymphocytes, NK cells, B lymphocytes, monocytes and granulocytes in blood samples were estimated (Table 2, complete data in Supplementary Table). There were significant differences in the proportions of estimated B lymphocytes and granulocytes in cases compared with controls. Further analyses therefore included an adjustment for the

Table 3. Odds ratios for mature B-cell neoplasms in relation to hypomethylation and hypermethylation.[†]

CpG location	Global methylation level	Without cell content adjustment		With cell content adjustment	
		OR (95% CI)[‡]	Likelihood-ratio test p-value	OR (95% CI)[‡]	Likelihood-ratio test p-value
Genome-wide	Hypomethylated	2.27 (1.59–3.25)	<0.001	2.01 (1.39–2.93)	<0.001
	Hypermethylated	0.99 (0.68–1.44)		0.88 (0.60–1.30)	
Regulatory region:					
– Nonpromoter regions	Hypomethylated	1.66 (1.18–2.34)	<0.001	1.55 (1.07–2.25)	<0.001
	Hypermethylated	0.67 (0.46–1.00)		0.61 (0.41–0.92)	
– Promoter regions	Hypomethylated	1.00 (0.71–1.40)	0.01	0.92 (0.64–1.33)	<0.001
	Hypermethylated	1.54 (1.10–2.13)		1.48 (1.04–2.11)	
CpG density:					
– High CpG density	Hypomethylated	1.26 (0.90–1.78)	0.004	1.29 (0.90–1.85)	<0.001
	Hypermethylated	1.76 (1.25–2.48)		1.74 (1.21–2.48)	
– Intermediate CpG density	Hypomethylated	1.01 (0.72–1.40)	0.70	0.95 (0.65–1.39)	<0.001
	Hypermethylated	1.14 (0.81–1.61)		1.06 (0.72–1.57)	
– Low CpG density	Hypomethylated	1.89 (1.34–2.65)	<0.001	1.79 (1.23–2.58)	<0.001
	Hypermethylated	0.89 (0.61–1.29)		0.81 (0.55–1.20)	
CpG distribution:					
– CpG island	Hypomethylated	1.11 (0.78–1.59)	<0.001	1.07 (0.74–1.54)	<0.001
	Hypermethylated	1.89 (1.35–2.65)		1.82 (1.27–2.60)	
– CpG shore or shelf	Hypomethylated	2.03 (1.45–2.84)	<0.001	1.89 (1.33–2.70)	<0.001
	Hypermethylated	1.00 (0.68–1.47)		0.93 (0.63–1.38)	
– Neither island, shore or shelf	Hypomethylated	1.78 (1.26–2.52)	<0.001	1.65 (1.31–2.41)	<0.001
	Hypermethylated	0.78 (0.54–1.13)		0.69 (0.47–1.01)	

The association between methylation level and mature B-cell malignancies is reported for all CpG probes within the Infinium® HumanMethylation450 BeadChip (genome-wide) as well as for different regulatory regions and CpG density regions.
[†]OR calculated from conditional logistic regression, accounting for age, gender, ethnicity and DNA source.
[‡]OR are calculated relative to the middle tertile of methylation in the controls.
OR: Odds ratio.

B lymphocyte and granulocyte content of the sample in order to account for the potential systematic effect of different cell composition on DNA methylation.

Overall, genome-wide hypomethylation, defined as the lowest tertile of methylation values across all CpG sites analyzed was associated with increased risk of MBCN ($p = 7.20 \times 10^{-6}$; OR: 2.27 [95% CI: 1.59–3.25)] whereas genome-wide hypermethylation showed no association (OR: 0.99 [0.68–1.44]) (Table 3). Repetitive elements were analyzed, including SINE, LINE and long terminal repeat regions. Regions containing repetitive elements were associated with hypomethylation (OR: 1.84 [95% CI: 1.31–2.59]; $p < 0.001$) and persisted after correction for cell content (OR: 1.74 [95% CI: 1.20–2.52]; $p = 0.03$).

Methylation in promoter & nonpromoter regions

Within nonpromoter regions, hypomethylation was associated with increased risk of developing MBCN ($p = 3.5 \times 10^{-3}$; OR: 1.66 [95% CI: 1.18–2.34]). The OR was similar after correction for B cell and granulocyte content. Within promoter regions as a whole, higher global methylation was associated with increased risk of MBCN (OR: 1.54 [95% CI: 1.10–2.13]), and for the subgroup of high CpG promoter regions (HCPs), hypermethylation was associated with increased risk of MBCN (OR: 1.76 [95% CI: 1.25–2.48]). In contrast, for low CpG promoter regions (LCPs), lower global methylation was associated with increased risk of MBCN (OR: 1.89 [95% CI: 1.34–2.65]).

Methylation in CpG islands, shores & shelves

Within CpG Islands, where CpG content is highest, hypermethylation was associated with increased risk of developing MBCN (OR: 1.89 [95%CI: 1.35–2.65]). In contrast, within CpG shores/shelves, hypomethylation was associated with the risk of MBCN ($p = 3.3 \times 10^{-5}$, OR: 2.03 [95%CI: 1.45–2.84]). Similarly, in regions that are neither CpG islands, shores nor shelves, the 'open sea' regions, hypomethylation was associated with increased risk of MBCN (OR: 1.78 [95% CI: 1.24–2.52]).

Tumor subgroups

There was no significant heterogeneity in associations between the different tumor subgroups, with MM, follicular NHL, high-grade NHL and low-grade NHL all demonstrating similar patterns of global methylation associated with risk ($p = 0.36$).

In order to account for the known effect of some lifestyle and environmental factors on DNA methylation, a separate analysis adjusting for BMI, dietary folate intake and smoking status was performed and did not affect the magnitude of association nor p value significance levels. Analysis after adjustment for differences in B-cell and granulocyte content in blood samples was performed and made no material difference to the ORs.

Time from blood collection to diagnosis

The time from baseline blood sampling to diagnosis with MBCN was less than 5 years for 83 (20%), 5–9 years for 114 (26%) and ≥10 years for 239 (54%) cases. There was no evidence that the association between global methylation and MBCN risk was stronger when blood was sampled closer to the time of diagnosis ($p_{het} = 0.19$) (Table 4).

Methylation in promoter regions of tumor suppressor genes

We analyzed DNA methylation in CpG probes associated with *HOXA9*, *CDH1*, *CDH13*, *ADAMTS18* and *PCDH10*, genes known to exhibit promoter methylation in MBCN tumor tissue. Within the *HOXA9* gene, the Infinium HumanMethylation450 BeadChip maps 28 CpG sites; four probes demonstrated significant hypermethylation in cases compared with controls ($p < 10^{-8}$) with three probes lying in the exon region and one probe in the 5′UTR (Table 5). There are eight further hypermethylated probes within the *HOXA9* gene reaching a lower level of statistical significance ($p < 10^{-5}$) with four probes in the promoter region. In *PCDH10*, five of 24 probes within 1500 bp of the transcription start site were hypermethylated ($p < 10^{-5}$, Table 5). In *CDH1*, two of 21 probes within the gene body were hypermethylated ($p < 10^{-5}$, Table 5) and one probe was hypermethylated ($p < 10^{-5}$) in the 3′UTR. In *CDH13*, six of 64 probes within the gene body were hypomethylated ($p < 10^{-5}$)(Table 5).

Discussion

Using a case–control study nested within a prospective cohort study, and blood collected years prior to diagnosis with MBCN, we were able to demonstrate for the first time an association between genome-wide global DNA hypomethylation and the risk of developing MBCN (OR: 2.27 for lowest tertile vs middle tertile of global methylation). Further, we were able to characterize distinct patterns of global DNA methylation according to the functional status and genomic location of CpG sites. Within promoter regions containing high CpG density (HCP), hypermethylation was associated with increased risk of MBCN. HCP regions contain a high proportion of gene promoters within CpG islands and in normal tissue are overwhelmingly protected from methylation [6,29]. The loss of constitutive protection from DNA methylation within HCP promoter regions has been described in MM, follicular NHL and DLBCL tissues [11,38]. Its role in the development of MBCN has been uncertain to date, but our finding of promoter hypermethylation in pre-diagnostic blood samples suggests it is an early rather than late event.

Hypermethylation of specific gene promoters in MBCN, such as *HOXA* gene family in B-NHL, tumor suppressor genes *CDH1*, *CDH13* and *ADAMTS18* in DLBCL and mantle cell lymphoma, and *PCDH10* in NHL and MM, is described [33–36], suggesting that HCP hypermethylation, perhaps through epigenetic silencing of tumor suppressor genes, is important in MBCN pathogenesis. We confirmed that within the *HOXA9* gene, there was one CpG probe within the promoter region hypermethylated in cases compared with controls with a genome-wide level of significance ($p < 10^{-8}$). On relaxing the criteria for significance to $p < 10^{-5}$, there were four additional probes hypermethylated within the promoter region. Within the *PCDH10* gene, we confirmed there were four hypermethylated CpG probes within the promoter region ($p < 10^{-5}$). It is intriguing that we have been able to detect promoter hypermethylation in genes implicated in MBCN pathogenesis in blood samples collected many years before diagnosis with MBCN. While studies of tumor tissue commonly nominate an arbitrary cut-off for differential methylation of β(case–control) > 0.2, in our analysis, we did not apply a specific cut-off for differential methylation (some studies in tumor tissue nominate a methylation difference of β[case–

Table 4. Odds ratios for methylation and mature B-cell neoplasm risk by 5-year time intervals between baseline blood collection and diagnosis.

Global methylation level	Time between baseline and diagnosis					
	<5 years (n = 83)		5–9 years (n =90)		>9 years (n = 263)	
	OR (95% CI)	Likelihood ratio test p-value	OR (95% CI)	Likelihood ratio test p-value	OR (95% CI)	Likelihood ratio test p-value
Hypomethylation	2.06 (0.97–4.35)	0.002	1.43 (0.65–3.15)	0.12	2.25 (1.42–3.58)	0.001
Hypermethylation	0.41 (0.15–1.10)		0.70 (0.32–1.54)		1.30 (0.79–2.14)	

OR calculated from conditional logistic regression, accounting for age, gender, ethnicity and DNA source.
OR: Odds ratio.

control] > 0.2) as the strength of our study is comparison of a large number of matched cases and controls and rigorous control for bias by study design consisting of matched pairs and analytical methods (sample plating methods and use of normalization methods to correct for possible bias). The magnitude of methylation differences in peripheral blood collected years prior to tumor diagnosis is untested in the current literature, and we felt that the methylation thresholds from tumor tissue studies could exclude significant results. We note that the findings of promoter hypermethylation in *HOXA9* and *PCDH10* were apparent after relaxation of our criteria for genome-wide significance therefore confirmation of our findings in a similar large prospective sample is ideal.

It is increasingly recognized that regions outside CpG islands, particularly CpG shores and shelves, contain higher proportions of differentially methylated regions within tumor tissue [39,40]. We found a strong association between global DNA hypomethylation in CpG shores and shelves that was not present in CpG islands, supporting their relevance to MBCN risk.

A major strength of our study is its prospective design, with DNA collected many years before MBCN diagnosis and therefore suggesting that the aberrant methylation patterns detected are an early event. While it is possible that peripheral blood samples from cases could contain circulating tumor cells, the latency between baseline blood collection and diagnosis did not affect the risk of MBCN. Factors known to alter DNA methylation (age, gender, year of birth and ethnicity) were taken into account while lifestyle factors with the potential to affect methylation (dietary folate intake, obesity and smoking status) did not affect risk.

The interpretation of DNA methylation from peripheral blood samples requires caution due to the well-described influence of white blood cell composition on methylation [41]. While there are several published algorithms which attempt to account for differential cell composition of peripheral blood [42,43], the optimal approach remains uncertain. We used a validated algorithm to estimate B cell and granulocyte content of blood samples [32] and found there was no association between estimated cell content and measures of global methylation in our cohort. The choice of tissue in DNA methylation studies is highly likely to influence results due to tissue-specific methylation profiles. In MBCN where the cell of origin is the B lymphocyte, peripheral blood is both likely to be reflective of early changes in B lymphocytes and readily obtainable in the context of a large prospective study. The use of whole blood as opposed to purified B lymphocytes allows the potential discovery of novel germline epimutations and the potential revelation of epimutations associated with

Table 5 Differential methylation in genes associated mature B-cell neoplasms and promoter hypermethylation.

CpG probe	CHR	MAPINFO	Probes with SNPs	UCSC gene group	p-value	β(case)	β(control)	β(case–control)
HOXA9								
cg27009703	7	27204894		1stExon	2.44×10^{-09}	0.171	0.134	0.037
cg27009703	7	27204894		1stExon	2.44×10^{-09}	0.171	0.134	0.037
cg07778029	7	27205114		1stExon; 5′UTR	1.47×10^{-08}	0.063	0.047	0.016
cg26521404	7	27204981	rs11975265	1stExon	7.44×10^{-08}	0.096	0.071	0.025
cg26476852	7	27204728		1stExon	7.40×10^{-07}	0.182	0.162	0.020
cg01285501	7	27206461	rs6461991	TSS1500	2.29×10^{-06}	0.254	0.236	0.018
cg01354473	7	27204803		1stExon	3.52×10^{-06}	0.295	0.278	0.016
cg26365299	7	27205381		TSS1500	4.69×10^{-06}	0.028	0.022	0.006
cg02643054	7	27206544		TSS1500	4.82×10^{-06}	0.094	0.082	0.011
cg01381846	7	27204785		1stExon	5.16×10^{-06}	0.234	0.212	0.022
cg20399871	7	27204663		1stExon	2.15×10^{-05}	0.136	0.119	0.017
cg15506609	7	27206073	rs77936575	TSS1500	2.73×10^{-05}	0.242	0.215	0.027
PCDH10								
cg07665387	4	134069388		TSS1500	3.37×10^{-06}	0.222	0.199	0.023
cg01408654	4	134070437		TSS200	3.31×10^{-05}	0.089	0.078	0.011
cg14146100	4	134069236		TSS1500	3.55×10^{-05}	0.064	0.053	0.010
cg02043159	4	134070235		TSS1500	4.25×10^{-05}	0.171	0.161	0.010
CDH1								
cg20716119	16	68771763		Body	1.62×10^{-05}	0.217	0.204	0.013
cg01857829	16	68771498		Body	2.83×10^{-05}	0.169	0.151	0.018
cg06875305	16	68869013		3′UTR	8.93×10^{-05}	0.817	0.839	-0.022
CDH13								
cg03011928	16	83023240		Body	4.65×10^{-07}	0.694	0.716	-0.022
cg02495250	16	82735595		Body	4.27×10^{-06}	0.899	0.912	-0.013
cg01973778	16	83280511		Body	4.30×10^{-06}	0.726	0.750	-0.024
cg02168291	16	82671520		Body	3.98×10^{-05}	0.783	0.801	-0.019
cg09765297	16	83029415	rs61696909	Body	4.82×10^{-05}	0.893	0.905	-0.012
cg08856946	16	82661421		Body	6.09×10^{-05}	0.101	0.091	0.011
cg01093138	16	83374884		Body	7.10×10^{-05}	0.638	0.664	-0.026

immune regulation such as T-cell dysregulation and aberrant antigen presentation as well as changes in the microenvironment such as changes to nurse-like T cells.

The functional significance of global methylation changes in peripheral blood remains an open question. Our detection of aberrant global methylation may reflect it is an early event in MBCN pathogenesis, alternately the aberrant methylation could reflect co-incident events in the development of MBCN such as inflammatory or immune responses. The current understanding of the role of methylation in immune regulation is limited. There appears to be an association between hypomethylation of a number of genes and gene regions related to T-cell antigen presentation in autoimmune diseases such as primary Sjogrens syndrome and Bechet's disease [44,45]. In turn, autoimmune disease is associated with an increased risk of MBCN, but a role for methylation in the relationship between autoimmune disease and MBCN has not been described.

Our findings suggest that differential global methylation is detectable in DNA extracted from peripheral blood samples collected years prior to diagnosis and is strongly associated with risk of subsequent MBCN. It will be important to validate our findings in other large prospective cohorts. While this study has identified a potential marker for use in the prediction of MBCN

risk, further analyses should be conducted to detect differentially methylated regions and specific loci, particularly within HCP regions. This may identify aberrant methylation in specific regions of functional importance and refine our understanding of the role of DNA methylation in MBCN initiation and development.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi.15.97

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Executive summary

Background

- Global hypomethylation and promoter hypermethylation are recognized characteristics of tumor tissue in both solid cancer and hematological malignancies.
- To date, there have been no prospective studies examining peripheral blood methylation as a risk factor for the development of mature B-cell malignancies (MBCN).

Methods

- Participants were healthy adult volunteers who had peripheral blood samples taken at baseline enrollment into the study.
- New cases of MBCN were identified by linkage to cancer registries and subsequently matched to controls by age, gender, ethnicity and DNA source.
- DNA methylation was measured using the Infinium® HumanMethylation450 BeadChip and three tertiles of methylation were used as measures of global methylation.

Results

- After a median 10.6-year follow-up, 471 cases of MBCN were diagnosed, with 438 eligible for analysis.
- Across the entire epigenome, hypomethylation was strongly associated with an increased risk of developing MBCN.
- In contrast, within promoter regions, hypermethylation was associated with MBCN risk, particularly within promoter regions with high CpG content (OR: 1.54 [95% CI: 1.10–2.13]).
- Global hypomethylation and promoter hypermethylation was demonstrated in all tumor subgroups, with no significant tumor subgroup heterogeneity.
- Methylation status in the promoter regions of candidate genes *HOXA9*, *CDH1*, *CDH13*, *ADAMTS18* and *PCDH10*, was analyzed. Hypermethylation of CpG probes in the promoter regions of *HOXA9 and PCDH10* was associated with MBCN.
- In order to account for the known effect of leucocyte composition on methylation, we repeated the analysis after adjusting for B lymphocyte and granulocyte content and found no difference in our results.
- Further analyses were performed adjusting for potential environmental factors such as dietary folate intake, obesity and smoking with no effect on results.
- The latency between blood sampling and MBCN diagnosis did not affect results, with similar associations found for cases sampled >10 years, 5–10 years and <5 years prior to diagnosis.

Discussion

- Our findings of global hypomethylation and promoter hypermethylation in peripheral blood are concordant with the described methylation changes that occur in MBCN tumor tissue.
- This is the first time aberrant methylation patterns have been described in the peripheral blood of a prospective cohort in MBCN. Utilizing a prospective cohort with long latency between blood sampling and diagnosis (median 10.6 years) reduces the likelihood that methylation patterns are due to circulating tumor cells – a particular issue in hematological malignancies.
- The stability of our methylation findings regardless of long or short latency suggests the methylation patterns occur early in the development of MBCN.
- A further strength of our study was that we were able to correct for common confounders of methylation such as age, gender, ethnicity and lifestyle factors by the use of matched controls.

Summary

- Differential global methylation is detectable in DNA extracted from peripheral blood samples collected years prior to diagnosis, suggesting that global methylation is an early and perhaps driver event in MBCN pathogenesis.

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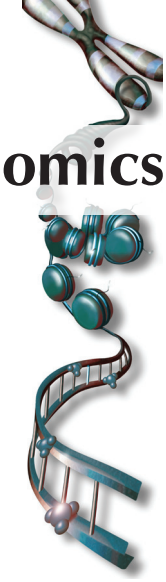

Polycomb genes are associated with response to imatinib in chronic myeloid leukemia

Aim: Imatinib is a tyrosine kinase inhibitor that has revolutionized the treatment of chronic myeloid leukemia (CML). Despite its efficacy, about a third of patients discontinue the treatment due to therapy failure or intolerance. The rational identification of patients less likely to respond to imatinib would be of paramount clinical relevance. We have shown that transmembrane transporter *hOCT1* genotyping predicts imatinib activity. In parallel, Polycomb group genes (PcGs) are epigenetic repressors implicated in CML progression and in therapy resistance. **Patients & methods:** We measured the expression of eight PcGs in paired pre- and post-imatinib bone marrow samples from 30 CML patients. **Results:** BMI1, PHC3, CBX6 and CBX7 expression was significantly increased during imatinib treatment. Post-treatment levels of CBX6 and CBX7 predicted 3-month response rate. Measurement of post-treatment BMI1 levels improved the predictive power of hOCT1 genotyping. **Conclusion:** These results suggest that the expression levels of PcGs might be useful for a more accurate risk stratification of CML patients.

Keywords: BMI1 • CBX6 • CBX7 • imatinib • pharmacoepigenetics • Polycomb

Imatinib is the first tyrosine kinase inhibitor (TKI) successfully employed in the clinical setting [1]. This small molecule inhibits the constitutive kinase activity of the BCR–ABL protein, thereby inducing apoptosis in Philadelphia chromosome-positive chronic myeloid leukemia (CML) cells. Clinical trials demonstrated that imatinib treatment was superior to conventional cytotoxic drugs as first-line therapy for CML [2]. A recent update reported an 89% overall survival rate in CML patients treated with imatinib for 5 years [3]. Despite these superlative results, objective response to imatinib is quite variable, and about 30% of patients must discontinue the treatment due to adverse events or unsatisfactory clinical outcome [1]. It has been shown that CML patients achieving a complete hematological response (CHR) within 3 months from the initiation of therapy are more likely to achieve complete cytogenetic response (CCyR) and better long-term clinical outcome [4]. The advent of second-generation TKIs provided clinicians with valuable therapeutic alternatives, thus having a positive impact on prognosis [5].

The identification of factors predicting imatinib response could support rational therapeutic decisions and foster therapeutic tailoring. For this reason, several groups have investigated the role of genetic variants in predicting CML patients' response to imatinib [6]. We have recently associated transmembrane transporter hOCT1 germline variants with imatinib pharmacokinetics and therapeutic activity [7]. Despite these promising results, no genetic factor has been conclusively linked to the clinical efficacy of imatinib.

Emerging evidence indicates that epigenetic factors could be at least as important as genetic ones in determining therapeutic responses [8]. In particular, Polycomb group genes (PcGs) are epigenetic effectors involved in the progression of both solid and hematological malignancies [9]. These proteins are

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organized in two multimeric polycomb repressive complexes (PRC1 and PRC2), which mediate gene silencing and control cell specification in physiological conditions. According to the most widely accepted molecular model, PRC2 catalyzes the trimethylation of histone H3-Lys27, and subsequently PRC1 binds to this epigenetic mark and catalyzes histone H2a monoubiquitination. These developmental regulatory mechanisms are deregulated in cancer cells, leading to the coordinated silencing of several tumor-suppressor genes. Notably, PRCs silence target *loci* in a context-specific manner that arises from their extensive combinatorial complexity. For example, a BMI1^{+}/PCGF2^{-} PRC1 drives breast cancer tumorigenesis and metastatic progression, while a BMI1^{-}/PCGF2^{+} complex exerts tumor-suppressive functions [10]. Selected PRC members have been implicated in chemotherapy resistance [11] and have been proposed as innovative therapeutic targets in hematological and solid malignancies [9].

In CML, the PRC1 member BMI1 has been identified as a prognostic marker [12] and as a downstream target of the BCR–ABL tyrosine kinase [13]. BMI1 expression is increasingly higher in peripheral blood samples from unaffected, chronic phase CML and blastic phase CML patients [13]. In addition, BMI1 expression is sufficient to reprogram B-lymphoid progenitors, thereby generating B-cell acute lymphoid leukemia-initiating cells [14]. Despite this evidence, no study has investigated the role of BMI1 or any other PRC1 member in CML patients' response to imatinib treatment.

Here, we describe pre- and post-treatment expression levels of eight PRC1 members, in 30 CML patients treated with imatinib in the context of the TIKlet study [15].

The primary aims of the present study were to demonstrate that PcG expression can be measured in bone marrow samples obtained during routine clinical procedures and PcG expression levels in bone marrow samples are associated with imatinib clinical activity. Our analysis identified the coordinated regulation of four PcGs upon TKI treatment, and suggested for the first time that post-therapeutic levels of PcGs might be used as predictors of therapeutic efficacy. We believe that our preliminary analysis paves the way for more extensive studies examining the utilization of PcGs as predictors of imatinib response in CML.

Patients & methods

Patients

Thirty consecutive CML patients observed at the Hematology Division of the Pisa University, Italy, between January 2011 and June 2013 were enrolled in the TIKlet trial [15] and then assessed for PcG expression prior to, and 3 months following, imatinib treatment. Twenty milliliter of EDTA-anticoagulated bone marrow was used for total RNA extraction after buffy coat preparation. Bone marrow was employed for cytogenetic analysis, as stated by the international guidelines [16], and for PcG expression analysis. Concomitantly, 20 ml of peripheral blood was employed for RNA extraction, banking and for analysis of the BCR–ABL1/ABL1 International Scale (IS) ratio by the GeneXpert Technology (Cepheid, Maurent-Scopont, France) after 3 and 6 months of therapy, as indicated by European guidelines [17]. For 23 patients, we obtained paired pre- and post-imatinib bone marrow samples harvested 3 months after start of imatinib treatment.

The study was approved by the Ethics Committee of Pisa University Hospital and by the University of British Columbia/British Columbia Cancer Agency REB. All patients gave informed consent to the study. Clinical outcome data were recorded throughout the whole duration of the follow-up. The clinical characteristics of the patients are reported in Supplementary Table 1 (see online at www.futuremedicine.com/doi/full/10.2217/epi.15.35).

Quantitative PCR

Gene-expression levels of PRC1 components were assayed using reverse transcribed cDNA and SYBR green primers following a previously described procedure for amplification and relative gene-expression quantification [16]. GAPDH was used as a reference gene. Expression values were calculated using the 2-ΔΔCt method and normalizing the measurement to the highest Ct in the cohort. Primers sequences are reported in Supplementary Table 2.

Statistical analyses

All statistical analyses were performed using SPSS 21.0 (SPSS Inc, Chicago, IL, USA) or GraphPad Prism 6.0 software.

At the third month of treatment, all cases were scored for hematological, cytogenetic and molecular response (MR). CHR was defined as white blood cells (WBC) $<10 \times 10^9$/l, platelets (PLT) $<450 \times 10^9$/l and absence of immature cells and palpable splenomegaly. Major cytogenetic response (MCyR) corresponded to <35% Philadelphia-positive metaphases, while CCyR identified cases without any Philadelphia-positive metaphases. The MR was scored according to the logarithmic reduction of the BCR–ABL1/ABL1 ratio in respect of the 100% value of the diagnosis (MR1 = reduction of 1 log; MR3 = reduction of 3 logs).

In order to ensure our measurements took into account differences in cell populations pre- and post-treatment, both basal and post-treatment PcG expression levels values have been divided by the number

of Philadelphia-positive metaphases detected by conventional cytogenetics.

Epigenetic data were analyzed to find any possible correlation with the achievement of CHR at 3 months, early molecular response (EMR; *BCR–ABL1/ABL1* ratio <10% IS at 3 months), CCyR at 6 months and MR3 (*BCR–ABL1/ABL1* ratio <0.1% IS) at 12 months. The same analyses were performed in combination with *hOCT1* genotyping, because our previous work demonstrated that the *hOCT1* c.480C>G polymorphism significantly influences both drug disposition and event-free survival (EFS) [7]. Therefore, the inclusion of these variables within the analysis could improve the identification of possible markers of clinical outcome.

The EFS was defined as time elapsed from treatment beginning and the occurrence of one of the following events: therapy discontinuation for any reason, loss of CCyR or MR3, or unacceptable toxicity. Survival curves were calculated using the Kaplan–Meier method, and statistical comparisons between curves were made using the log-rank test. The Wilcoxon matched-pairs test, chi-squared test, Fisher's exact test and Kruskal–Wallis test were used to compare variables, when appropriate. Time to CCyR and to MR3 values were not normally distributed, as demonstrated by the Shapiro–Wilk test, hence data were rank-transformed to perform a nonparametric test (Spearman's) correlation analysis.

Diagnostic accuracy of tests was evaluated by using the ROC analysis and area under curve (AUC) analyzed by a nonparametric test. All statistical comparisons were two-sided.

Results

Pre- & post-treatment expression levels of PcGs

In order to investigate whether expression of PRC1 components is affected by imatinib, we quantified pre- and post-treatment gene-expression levels on bone marrow samples. All mRNAs were reproducibly detectable (average Ct values <28) indicating that target genes were robustly expressed. Possible correlations between PcG expression values and Philadelphia metaphase percentages were explored using the Spearman's rho test; results showed that the observed variation in PcG expression was totally independent of the Philadelphia-positive residual component ($p = 0.305$). Our analysis revealed that 4/8 PcGs were significantly upregulated after 3 months of treatment (Figure 1). In particular, BMI1 was the most highly upregulated gene (fold change = 4.9; $p < 0.0001$; the Wilcoxon matched-pairs test). The median value of BMI1 expression was 21.16 (range: 1.0–55.8), while the mean value was 21.20 ± 13.98. These measurements are in line with previously reported data [13]. PHC3 and CBX6 showed greater than twofold upregulation ($p < 0.01$). Notably, no PRC1 member showed significant downregulation upon treatment and we found no significant correlation between pretreatment levels and fold-change value.

In order to investigate gene-expression correlations between each PRC1 member, we calculated Spearman's rank coefficients (Supplementary Table 3). Analysis of pretreatment levels showed relevant correlation ($R > 0.5$; $p < 0.01$) between CBX6–CBX7 and PCGF2–CBX8. Notably, after imatinib treatment, the number of significant correlations increased to 14. No significant negative correlation ($R < -0.5$) was observed either before or after treatment.

With the exception of CBX6–CBX7, the four genes that were significantly upregulated by imatinib (Figure 1) were only loosely correlated before treatment. Strikingly, the correlation of BMI1 with PHC3, CBX6 and CBX7 showed a significant increase in post-treatment samples. In keeping with this observation, the correlation between pairs of PHC3, CBX6 and CBX7 was significantly amplified upon treatment ($p < 0.01$; Spearman's correlation). When we analyzed only cases with a concomitant increase of BMI1 and CBX6 or CBX7, we observed that in about 60% of them the upregulation of BMI1 after imatinib treatment was higher than that measured for CBX6 and CBX7 ($p < 0.01$; Spearman's rho test).

Taken together, those results indicate that imatinib is able to trigger the coordinated activation of PRC1 members, with a stronger effect on BMI1, compared with the other concomitantly upregulated PcGs.

Predictive value of PcGs

In order to test the predictive value of PcGs, we investigated the correlation between their expression levels, clinical features at diagnosis and clinical outcome. The four genes, showing significant upregulation following imatinib treatment (Figure 1), were selected for this analysis. In our patient series, Sokal risk score was low in 35%, intermediate in 38% and high in 27% of cases (for further details, see Supplementary Table 1); no patient had an additional Philadelphia chromosome. The median time to CHR was 1.7 months. Within 3 months of treatment start, 76% of patients achieved CHR. At the same timepoint, 50% of patients showed a BCR–ABL1/ABL1 ratio lower than 10% IS, thereby achieving an EMR. The median time to CCyR was 8.2 months. Within 12 months of treatment start, 80% of cases achieved a CCyR. PcG expression levels (before or after treatment) were not significantly associated with sex, age or risk score (Hasford, Sokal, EUTOS). Analogously, we found no significant correlation between PcG expression values and EMR.

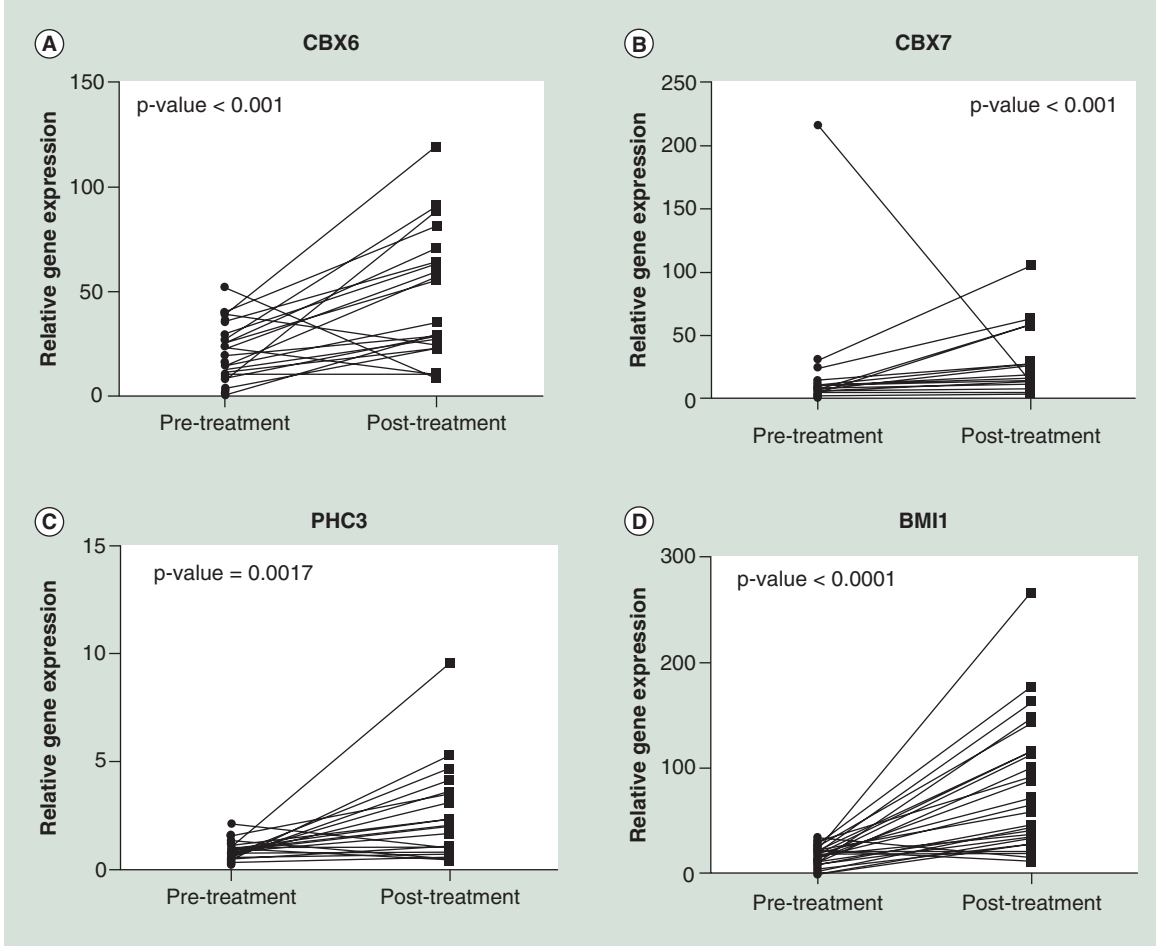

Figure 1. Modulation of Polycomb gene expression in imatinib-treated patients. Relative gene expression in **(A)** CBX6; **(B)** CBX7; **(C)** PHC3 and **(D)** BMI1. p-values refer to the Wilcoxon matched-pairs test. Mean fold changes with respect to pretreatment values: CBX6 = 2.1; CBX7 = 1.4; PHC3: 2.6; BMI1 = 4.9.

We found no correlation between pretreatment PcG mRNA levels and response to imatinib. As shown in Figure 2A & B, post-treatment levels of CBX6 and CBX7 were able to discriminate patients achieving CHR at or after 3 months of treatment (ROC AUC = 0.811 and 0.833; p = 0.029 and 0.020, respectively). Optimal cutoff values based on likelihood ratios and Youden index [18] revealed that a CBX6 value higher than 29.26 (arbitrary units, see 'Quantitative PCR' section in the 'Patients & methods' section) identified responders with 73.3% sensitivity and 100% specificity. Similarly, we found that a CBX7 value higher than 14.74 allowed us to identify responders with 73.3% sensitivity and 100% specificity. We therefore investigated if CBX6 and CBX7 modulation upon imatinib treatment was different in responders versus nonresponders (groups defined based on the achievement of CHR at or after 3 months of treatment). We found no significant differences in CBX6 modulation (data not shown). On the contrary, CBX7 modulation was sharply different in the two groups: responders elicited a significant post-treatment upregulation, while nonresponders showed a trend toward a reduced expression (Figure 2C).

When we computed PcG values after 3 months of treatment, we found that patients with higher upregulation of BMI1 during imatinib treatment required a longer time to achieve MR3 (22 vs 11 months; p = 0.06; t-test). Analogously, patients with BMI1 plus CBX6 upregulation required a significantly longer time to achieve MR3 (23.66 ± 9.77 vs 14.04 ± 7.17 months; p = 0.04; t-test). These same cases showed a lower probability of remaining free from events (disease progression, loss of CCyR and/or MR3) at 36 months (24% for patients with upregulation of BMI1 and CBX6 vs 80% for those with no or low BMI1 increase or stable levels of CBX6; p = 0.09).

Overall, these data demonstrate that upregulation of CBX6 and CBX7 predicts an optimal hematological response and that an increase of BMI1 is associated with longer time to achieve the satisfying molecular response.

To further dissect the role of BMI1 in CML progression, we analyzed three patients who switched to

second-generation TKIs upon unsatisfactory response to imatinib (Figure 2D). The first patient achieved MR3 (BCR–ABL1 transcript decreased 16-fold) after 3 months of dasatinib treatment; in this case, we measured a tenfold reduction in BMI1 levels. The second patient obtained a tenfold reduction in BCR–ABL levels after 3 months of nilotinib treatment; in this patient, post-therapy BMI1 levels halved. Finally, the third patient did not respond to second-generation TKI treatment. In this case, BMI1 downregulation was less than 1.5-fold.

When we analyzed the c.480 C>G hOCT1 polymorphism, we found that the wild-type CC genotype had a favorable impact on 36 months-EFS (87.5 vs 30% of individuals carrying the G allele; p = 0.04), hence confirming our previous results in this subset of patients [7]. In our series, 50% of cases showed a wild-type genotype. In this subset, the median EFS for cases with stable levels of BMI1 after short-term treatment was 50.40 ± 0.70 months versus 30.60 ± 8.60 months for patients with a higher increase of BMI1 and/or polymorphic genotype (p = 0.001; t-test). Unfortunately, the small sample size did not allowed us to perform further comparisons. Taken together, these results indicate that BMI1 expression profiling increases the prognostic power of hOCT1 genotyping.

Clinical significance of PcGs in myeloid leukemias

Albeit PcGs have been widely investigated in many neoplasms, evidence of their role in myeloid neopla-

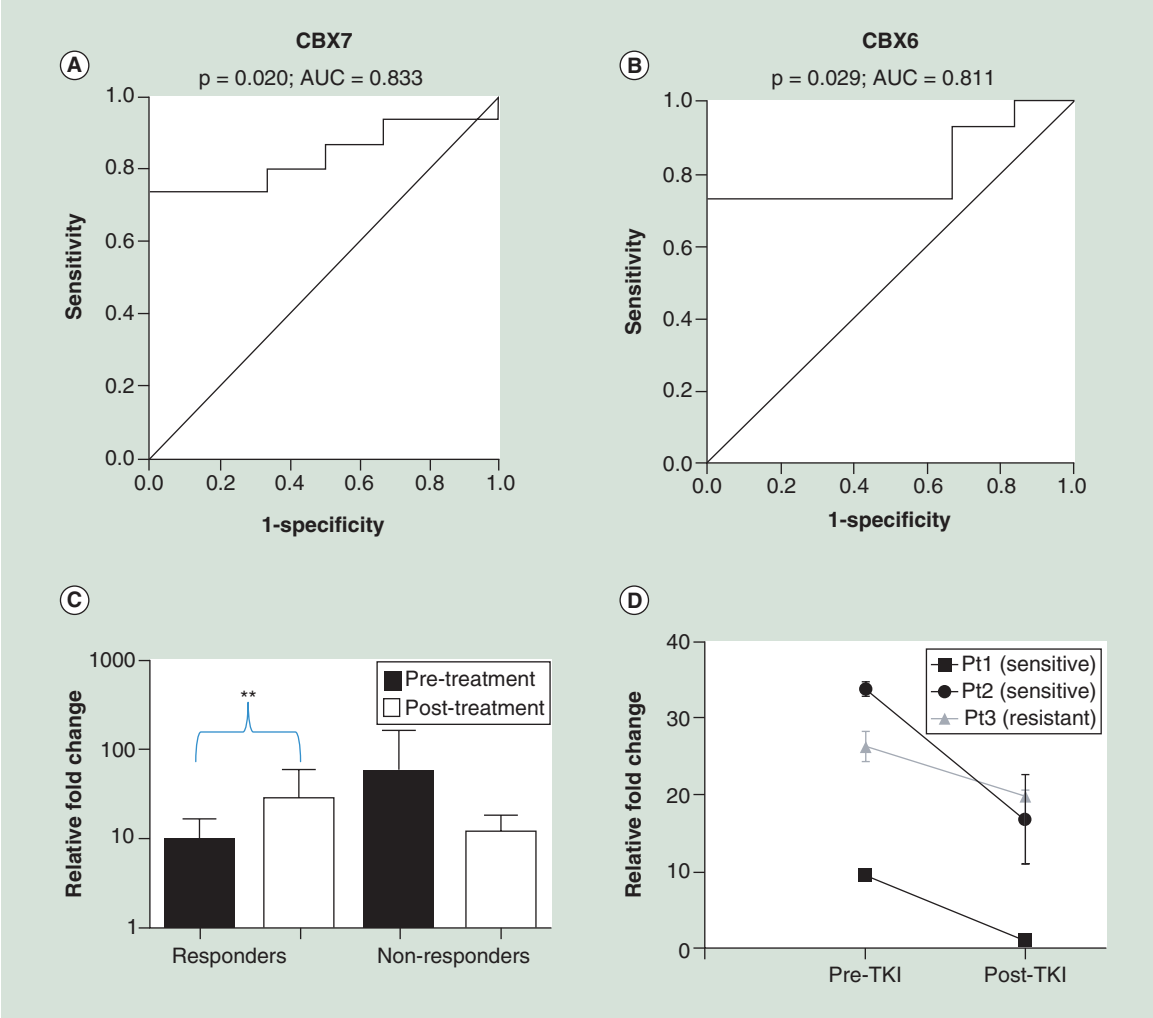

Figure 2. Clinical relevance of *Polycomb* genes in chronic myeloid leukemia patients. **(A & B)** Predictive role of post-therapy levels of CBX6 and CBX7 genes according to ROC curves. Cutoff values of 29.26 and 14.74 for CBX6 and CBX7, respectively, correspond to 73.3% sensitivity and 100% specificity for both genes. **(C)** Pre- and post-treatment levels of CBX7 in responders versus nonresponders. **p < 0.01; t-test. **(D)** Pre- and post-treatment BMI1 levels in three chronic myeloid leukemia patients treated with second-generation TKIs.
AUC: Area under the curve; TKI: Tyrosine kinase inhibitor.

sias remains scant. As mentioned in the introduction, PRC1 can play an oncogenic or oncosuppressive role, depending on its composition and cellular context. In order to elucidate the meaning of our findings in the specific context of myeloid malignancies, we analyzed the expression of CBX6, CBX7, BMI1 and PHC3 in cancer cells compared with their normal counterparts using the Oncomine database [19]. Our results indicate that BMI1 is highly upregulated, whereas CBX6 and CBX7 are downregulated, in AML samples compared with normal blood cells (Figure 3A–C). These results indicate that CBX6 and CBX7 are likely to play an oncosuppressive role in myeloid malignancies, and confirm the putative oncogenic role of BMI1 in the same context. Our results also suggest that post-therapy CBX6 and CBX7 upregulation is a partial, but not complete return to normal expression levels (average fold change [FC] in normal vs leukemic samples was 3.7–3.9; post- vs pre-therapy upregulation was 1.4–2.1-fold).

The Oncomine database also included a list of genes specifically downregulated in clinical CML samples exposed to imatinib. The main pathways inactivated by imatinib involved mitochondrial function, protein synthesis and DNA replication (Supplementary Table 4). These results confirm recent preclinical findings on imatinib mechanisms of action [20]. In addition, we identified a significant overlap between PcG targets and genes downregulated by imatinib ($p < 1E^{-5}$; odds ratio: >2). The PcG target list was obtained from a genome-wide mapping study conducted on human embryonic fibroblasts [21]. These results are in line with previous evidence showing that PcGs are activated by antineoplastic therapies [9,22]. Notably, better short-term response to imatinib in CML patients was predicted by PcG target gene silencing (Figure 3D). Our results also showed that imatinib-suppressed PcG targets mediate key cellular functions, including DNA replication and energy production (Supplementary Table 5). Therefore, it is conceivable that imatinib effects are at least in part mediated by PcG activation, resulting in selected PcG target silencing.

Discussion

In this report, we show for the first time that selected PRC1 genes are coordinately upregulated in CML patients treated with the TKI imatinib. More importantly, we find significant correlations between post-treatment PcG levels and imatinib activity. In particular, higher post-treatment CBX6 and CBX7 levels are associated with faster hematological response rates. CBX6 and CBX7 bind to the PRC1 core complex, thereby directing its gene silencing activity to specific targets [23]. Both CBX6 and CBX7 have been suggested to play an oncosuppressive role in solid tumors [24] and, as shown in the Oncomine database, in myeloid leukemias (Figure 3). Notably, CBX7 oncosuppressive activity is predominant in hematologic neoplasms [25]. Taken together, this evidence suggests that CBX6 and CBX7 could play a tumor suppressive role in CML also, and that their concurrent activation upon imatinib treatment might lead to the silencing of prosurvival genes, thereby inducing neoplastic cell death and better short-term responses to imatinib.

Our analysis also indicates that the post-treatment activation of other PRC1 members is correlated with worse long-term clinical outcome. Notably, higher BMI1 post-treatment levels are associated with worse prognosis. The negative impact of increased BMI1 expression is reinforced by the observation that its upregulation could overcome the positive value of the CBX6 increase in patients that showed a BMI1 upregulation higher than that of CBX6.

Moreover, the negative predictive role of BMI1 is retained also in cases with wild-type hOCT1, which is essential for imatinib intake into the neoplastic cell. These results are in agreement with the previously described oncogenic role of BMI1 in CML [26], and with the evidence that BMI1 is able to silence proapoptotic and oncosuppressive genes, thereby mediating therapy resistance in several neoplasms [9]. It is worth noting that BMI1 expression is significantly upregulated in CML patients' $CD34^+$ cells, compared with healthy controls [13]. This finding is particularly evident during a blastic crisis [27]. Most notably, *in vitro* results demonstrated that BCR–ABL upregulation increases BMI1 expression, thus suggesting that BMI1 is positively regulated by BCR–ABL [13], and that BMI1 cooperates with BCR–ABL during disease progression [28]. These data might provide a mechanistic explanation to our clinical findings: the greater the inhibitory effect of imatinib against BCR–ABL, the lower the upregulation of BMI1, the higher the therapeutic benefit for patients.

Furthermore, our results demonstrate that a model aimed at predicting treatment efficacy can combine pharmacodynamic and pharmacokinetic biomarkers (i.e., post-therapy BMI1 gene expression and hOCT1 genotyping), thereby achieving an enhanced predictive power.

As indicated in the introduction, PRC1 can play an oncogenic or oncosuppressive role, depending on its composition and on cellular context. It has been shown that $CBX7^+/BMI1^-$ and $CBX7^-/BMI1^+$ PRC1 complexes play opposite roles in controlling embry-

onic stem cell self-renewal and differentiation [23]. Our data indicate that both oncosuppressive and oncogenic PcGs are upregulated upon imatinib treatment, but that the expression levels of the first and the latter types of genes have opposite implications on imatinib activity. A unified model to explain this phenomenon is illustrated in Figure 4. We propose that the imatinib-dependent activation of CBX7 and other oncosuppressive PcGs plays a crucial role in triggering apoptosis in most CML cells, thereby resulting in better short-term treatment response. However, a small fraction of cells survive this pro-apoptotic response due to concurrent BMI1 upregulation that sustains self-renewing capabilities [28]. This subpopulation may eventually trigger-delayed neoplastic progression. As a consequence, higher BMI1 expression is associated with worse long-term clinical outcome. The observation of a further BMI1 modulation in cases exposed to second-generation TKIs supports the hypothesis that the expression of this gene is mainly associated with neoplastic progression and BCR–ABL1 activity.

The purpose of this study was to identify an easy-to-perform molecular test to predict response to imatinib. Ideally, the test should be performed on the same biological material used for the cytogenetic analysis, thereby avoiding any further discomfort for patients. For these reasons, we could not perform sample purifications and proteomic analyses. Notwithstanding those limitations, our results suggest that post-therapy BMI1 levels can improve the predictive power of hOCT1 genotyping. Larger prospective studies are needed to confirm our preliminary findings.

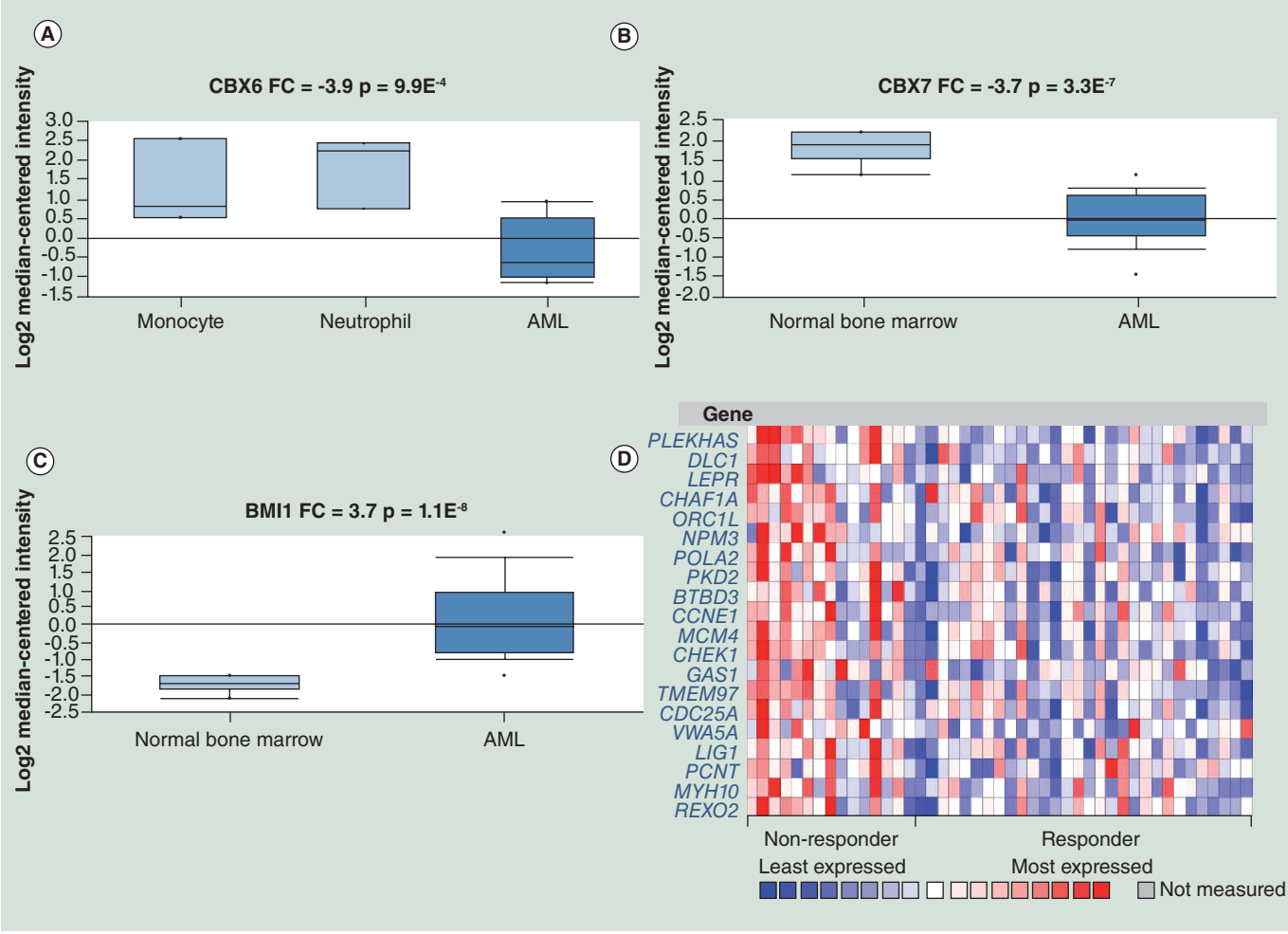

Figure 3. Expression pattern of Polycomb group genes in myeloid malignancies. Results were obtained using the following cutoff values: **(A–C)** $p < 0.01$; fold change >2; **(D)** odds ratio >2. **(D)** PcG targets are significantly downregulated in imatinib responders (30) vs nonresponders (15). Data are from 'Frank, Leukemia' study (blood or bone marrow samples obtained before the start of imatinib treatment; response was determined based on cytogenetic analysis performed on samples obtained within the first 12 months of imatinib treatment). We report noncontradictory results obtained from more than one cohort.
AML: Acute myeloid leukemia; FC: Fold change.

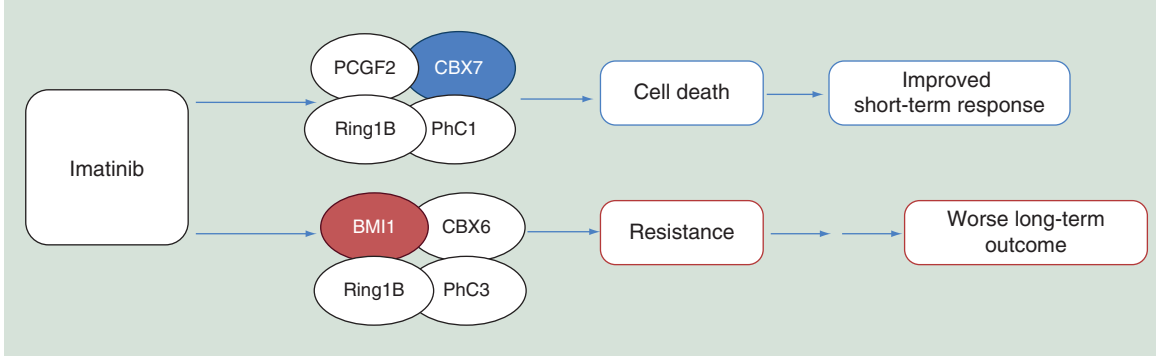

Figure 4. Proposed model of imatinib–Polycomb interaction. Imatinib is able to activate both oncosuppressive (blue) and oncogenic (red) PRC1 members. The first category triggers apoptosis on most chronic myeloid leukemia cells, thereby improving early response rate. The latter category promotes long-term survival of a fraction of chronic myeloid leukemia cells, thereby triggering delayed disease progression.
The two PRC1 multimers are adapted from [23].
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Conclusion

Results of the present study demonstrate that there is a significant correlation among PcG gene expression and clinical outcome in CML patients receiving imatinib. This correlation could be explained by an imatinib-dependent modulation of CBX6 and CBX7 genes, whose increased expression lead to an earlier achievement of MR3, whereas stable BMI1 mRNA levels anticipated a late benefit from imatinib. The latter phenomenon could be explained by a reciprocal interaction between BMI1 and BCR–ABL. Whether this approach could be extended to other BCR–ABL TKIs will be addressed in future studies.

Future perspective

Results from the present work could suggest that the adoption of a combined analysis of time-dependent events (i.e., variable expression of PRC1 genes in response to a BCR–ABL inhibitor) and fixed effects (patients' genotype with respect to transmembrane transporters) may help in the stratification of CML patients as early as possible, in order to adopt the best therapeutic strategy and possibly improve event-free-survival. In future studies, other TKIs directed against BCR–ABL should be evaluated for their role in modulating PcG gene expression, and this could pave the way to different epigenetic-based-tailored pharmacological strategies. The widespread use of high-throughput screening platforms, such as next-generation sequencing or digital droplet PCR, may accelerate further discoveries and application of biomarkers to real life.

Financial & competing interests disclosure

F Crea received one honorarium for expert opinion on histone methylation. AD Paolo received honoraria for expert opinions on CML. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Imatinib has been approved by the US FDA as first-line treatment for chronic myeloid leukemia (CML).
- Despite its efficacy, about 30% of patients experience a poor response or a frank resistance to imatinib.
- Currently, no molecular marker (other than the Philadelphia chromosome) has been associated with response to imatinib.
- We postulated that epigenetic factors could play a crucial role in imatinib response. We therefore collected RNA from 30 CML patients before and after treatment with imatinib.
- We selected Polycomb genes, since they have been implicated in CML progression and drug resistance and we studied their expression by quantitative PCR.
- Our results indicate that Polycomb group genes are coordinately upregulated upon imatinib treatment.
- BMI1 post-treatment levels were able to improve the predictive power of *hOCT1* genotyping, a pharmacogenetic biomarker that had been previously identified by our group.
- Overall, the variable BMI1, CBX6 and CBX7 gene expression after imatinib treatment and its association with clinical outcome may significantly influence the way how responders could be identified.
- The present strategy should be addressed to other BCR–ABL inhibitors that are used after imatinib failure, as well as nilotinib, dasatinib and the most recent ponatinib.

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Review

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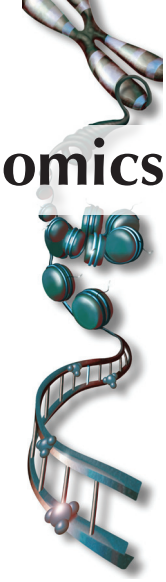
Epigenomics



DNA methylation of tumor suppressor protein-coding and non-coding genes in multiple myeloma

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Multiple myeloma is an incurable hematological malignancy arising from immortalized plasma cells in the bone marrow. DNA methylation refers to the catalytic addition of a methyl group to the cytosine ring of a CpG dinucleotide. Methylation of a promoter-associated CpG island, a cluster of CpG dinucleotides, may lead to silencing of the associated gene. In carcinogenesis, methylation of protein-coding or non-coding tumor suppressor genes/miRNAs is associated with transcriptional silencing, loss of tumor suppressor function and prognostic significance. This review first introduces pathogenesis of myeloma and DNA methylation in cancer. Then, it summarizes methylation of protein-coding tumor suppressor genes, especially, the latest genome-wide methylation studies in myeloma, followed by the latest findings of methylation of non-coding tumor suppressor miRNAs in myeloma.

Keywords: DNA methylation • multiple myeloma • tumor suppressor gene • tumor suppressor miRNA

Multiple myeloma

Multiple myeloma is a form of mature B-cell neoplasm characterized by clonal proliferation of plasma cells in the bone marrow (Figure 1) [1]. Clinically, this disease begins with immortalization of a post-germinal center B-cell, which will home to the bone marrow and presents as monoclonal gammopathy of undetermined significance (MGUS) [2]. MGUS is characterized by the presence of <3 g/dL serum monoclonal protein (M-protein), <10% clonal bone marrow plasma cells and the absence of end-organ damage, including hypercalcemia, renal failure, anemia and bone lesions (CRAB). Patients with MGUS will develop symptomatic myeloma at a rate of about 1% per year [3]. On the other hand, preceding progression to myeloma, patients with MGUS may evolve into another asymptomatic transitional stage, known as smoldering multiple myeloma (SMM) [4]. SMM is defined as ≥3 g/dl serum M-protein and/or ≥10% clonal bone marrow plasma cells, and the absence of end-organ damage, CRAB. In contrast to MGUS, patients with SMM will progress to symptomatic myeloma at a higher rate of about 10% per year [5]. Symptomatic myeloma is diagnosed by the presence of ≥10% clonal bone marrow plasma cells, serum and/or urine M-protein and evidence of end-organ damage, CRAB. In the milieu of the bone marrow microenvironment, interactions between myeloma plasma cells and bone marrow stromal cells will stimulate an abundant secretion of paracrine factors, including IL-6 and IGF1, which will confer survival and proliferation benefits to myeloma plasma cells [6]. At the terminal stage, myeloma plasma cells may become independent of the bone marrow microenvironment and progress into extramedullary myeloma, such as plasma cell leukemia (PCL) or extramedullary plasmacytoma.

Genetically, despite universal upregulation of D-type cyclins (D1, D2 or D3), myeloma is a highly heterogeneous disease, characterized by specific gains or losses of chromosomes and/or reciprocal translocations [7,8]. Based on the gains or losses of chromosomes, myeloma can be categorized into hyperdiploid or non-

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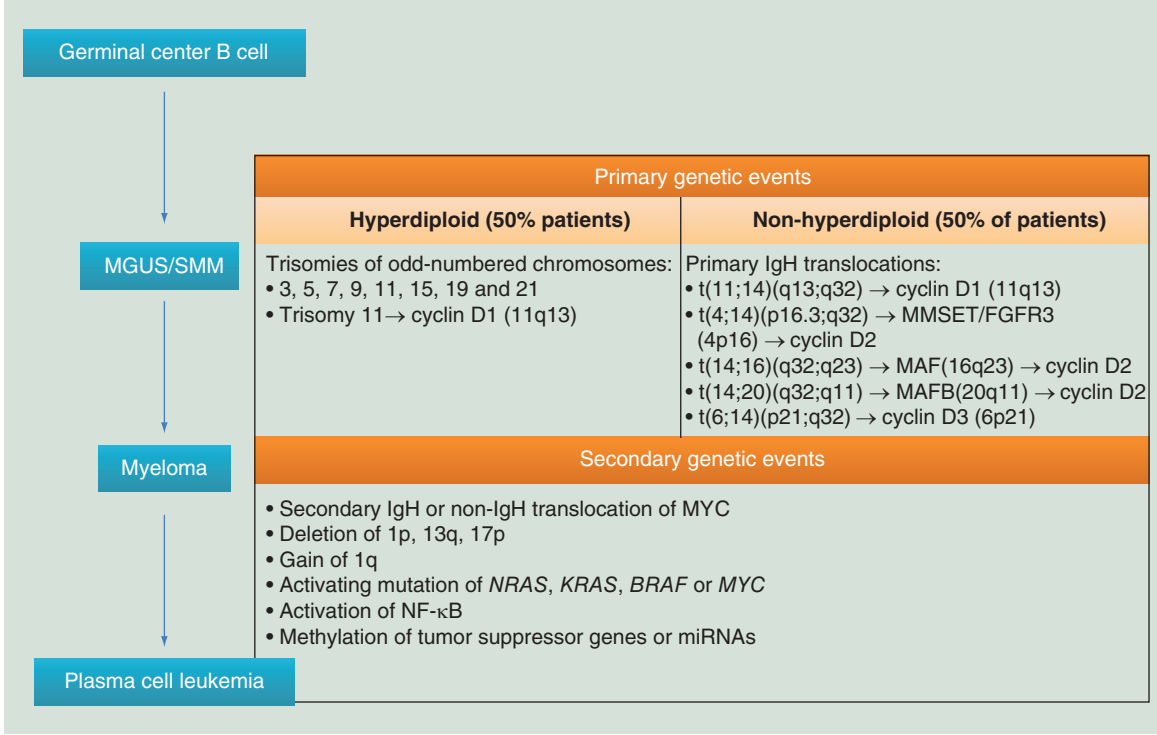

Figure 1. Clinical stages and pathogenetics of multiple myeloma. Multiple myeloma is initiated from immortalization of a postgerminal center B cell, which will present as MGUS, in which primary genetic events have been well-established. Occasionally, MGUS is followed by a transition stage of SMM. Multiple myeloma is associated with accumulation of secondary genetic events, which will enable myeloma plasma cells become independent of the bone marrow microenvironment, resulting in PLC.
MGUS: Monoclonal gammopathy of undetermined significance; PCL: Plasma cell leukemia; SMM: Smoldering multiple myeloma.

hyperdiploid subtypes [9]. About half of myeloma patients carry a hyperdiploid karyotype (48–74 chromosomes), which is characterized by trisomies of odd-numbered chromosomes, including 3, 5, 7, 9, 11, 15, 19 and 21; but not 13 [10–12]. Among trisomies, trisomy 11 is associated with direct upregulation of cyclin D1 expression. On the other hand, another half of myeloma patients carry a non-hyperdiploid karyotype (<48 or >74 chromosomes), which can be further subdivided into hypodiploid (<44/45 chromosomes), pseudodiploid (46 chromosomes with numerical and/or structural aberration [13]) or near-tetraploid (≥75 chromosomes) [10–12]. Non-hyperdiploid myeloma is typically associated with a primary translocation involving the *IgH* chain gene located at 14q32 and a partner oncogene [14,15]. With strong enhancer activity of the *IgH* locus, the partner oncogene is therefore associated with increased expression, contributing to myelomagenesis. Most common recurrent primary translocations include t(11;14)(q13;q32), t(4;14)(p16.3;q32), t(14;16)(q32;q23), t(14;20)(q32;q11) and t(6;14)(p21;q32). Among these translocations, t(11;14)(q13;q32) and t(6;14)(p21;q32) result in direct upregulation of cyclin D1 (11q13) and D3 (6p21), respectively. On the other hand, cyclin D2 is indirectly induced through the increased expression of FGFR3 (4p16.3), WHSC1 (alias: MMSET; 4p16.3), MAF (also known as c-MAF; 16q23) or MAFB (20q11.2-q13.1). Importantly, while the mechanism underlying the development of hyperdiploid or non-hyperdiploid myeloma remains unknown, a hyperdiploid karyotype indicates better prognosis, treatment outcome and longer survival than a non-hyperdiploid karyotype [12,16–18].

During disease progression, additional translocations and other genetic alterations occur [7,19]. For instance, dysregulation of *MYC* oncogene [20,21], amplification of 1q21 [22], and deletion of 1p, 13q and 17p [23] have been detected in newly diagnosed or relapsed myeloma patients. Importantly, deletion of 17p and gain of 1q21 are independently associated with poor prognosis, therefore, they are now widely accepted as criteria for defining high-risk patients for risk-stratified clinical trials [24,25]. On the other hand, activating mutation of oncogenic RAS signaling pathway at *NRAS*, *KRAS* and/or *BRAF* occurs in about 50% of myeloma patients [26,27], implicating ample opportunity toward personalized treatment by the availability of novel drugs targeting RAS signaling pathway [28].

DNA methylation in cancer

DNA methylation is a form of heritable epigenetic alteration which involves the catalytic addition of a methyl (-CH_3) group to the carbon 5 position on a cytosine ring in a CpG dinucleotide, resulting in the formation of 5-methylcytosine [29,30]. During cell division, DNA methylation pattern of the parental DNA strand is maintained on the newly synthesized daughter strand by DNMT1, attributed by the higher enzyme specificity for hemimethylated DNA [31,32]. On the other hand, DNMT3A and 3B are mainly responsible for introducing *de novo* methylation pattern on DNA [33,34]. Under physiological conditions, DNA methylation predominantly occurs at a majority of CpG dinucleotide sites interspersing across the human genome, leading to suppression of intergenic retroviral elements, regulation of tissue-specific gene expression, genetic imprinting and X-chromosome inactivation [35,36].

During evolution, CpG dinucleotides have been progressively depleted from the mammalian genome due to deamination of 5-methylcytosine into thymine, and hence occur at a frequency lower than expected [37,38]. Nonetheless, CpG-rich regions are concentrated in gene promoters as promoter-associated CpG islands or in repetitive DNA regions such as *LINE1* elements or *Alu* repeats [39]. A CpG island refers to any stretch of genomic DNA >200 bp in length with a high GC content >50% and a ratio of observed/expected CpG >0.60 [40]. Over 50% of CpG islands are embedded on or located in the proximity of a transcription start site [38,41]. Moreover, at least 70% of annotated gene promoters are associated with one or more CpG islands [38,42]. CpG islands in normal cells, in particular promoter-associated CpG islands of tumor suppressor genes or miRNAs, are mostly unmethylated and associated with open chromatin configurations, such as trimethyl H3K4 and/or acetylated H3K9, which will allow recruitment and binding of transcription factors, leading to expression of genes or miRNAs. In contrast, during carcinogenesis, the promoter-associated CpG islands of tumor suppressor genes or miRNAs are usually methylated and resulted in recruitment of histone methyltransferase, methyl-CpG-binding domain protein and histone deacetylase, leading to formation of close chromatin configurations, such as dimethyl H3K9 and/or trimethyl H3K27, which will preclude binding of transcription factor, and eventually silencing of genes or miRNAs (Figure 2) [43–45].

In carcinogenesis, in contrast to an oncogene, which can be activated by gain-of-function mutation of a single allele, the Knudson's double-hit hypothesis stipulates that a cancer phenotype will only occur when both alleles of a tumor suppressor gene are abolished. For example, biallelic inactivation of a tumor suppressor gene may occur when a first hit is rendered by chromosomal/interstitial deletion or loss-of-function mutation of one allele, and a second hit by methylation-mediated silencing. Therefore, methylation-mediated silencing of tumor suppressor gene or miRNA may serve as the second hit to fulfill the Knudson's hypothesis [46,47].

Methylation of tumor suppressor protein-coding genes

In myeloma, DNA methylation has been shown to result in the suppression of specific tumor suppressor genes [48–52]. To date, most methylated tumor suppressor genes reported in myeloma target either one of the following pathways, including the cell cycle, death-associated protein kinase (DAPK)/p14ARF/MDM2/TP53 intrinsic TP53-dependent apoptosis pathway, IL-6/JAK/STAT signaling pathway, WNT signaling pathway and HIF-1 regulatory pathway [51–53].

In the regulation of cell cycle, G1/S progression is promoted by D-type cyclins and CDK4 and 6. In contrast, it is counteracted by cyclin-dependent kinase inhibitors, including the INK4 family and the CIP/KIP family, resulting in cell cycle arrest [54,55]. Indeed, frequent methylation-mediated loss of *CDKN2A* and *CDKN2B*, members of the INK4 family, has been demonstrated in myeloma patients at diagnosis [49–50,56–63]. Furthermore, methylation of *CDKN2A* was shown to be associated with disease progression and inferior survival for myeloma patients [49,57,59,61].

TP53 is pivotal to a network of TP53-dependent tumor suppression. However, deletion or loss-of-function mutation of *TP53* is infrequently detected in myeloma at diagnosis [64–67]. Hence, it has been hypothesized that the TP53-dependent tumor suppression in myeloma might be impaired by inactivation of other tumor suppressor genes, such as *DAPK1*, involved in the TP53 network. DAPK1 may indirectly activate TP53 through p14ARF-dependent inhibition of a negative regulator of TP53, MDM2, and hence acts as a tumor suppressor in the TP53 network [68,69]. Indeed, frequent methylation of *DAPK1* has been reported in myeloma patients at diagnosis and shown to be associated with disease progression and shortened overall survival [57–60,70–72]. Furthermore, Chim *et al.* demonstrated that, in myeloma cell line completely methylated for *DAPK1*, hypomethylation treatment resulted in demethylation and re-expression of *DAPK1*, indicating the methylation-mediated silencing of *DAPK1* was reversible [71]. Moreover, methylation of *DAPK1* appeared to be mutually exclusive with other genes along the same pathway, such as p14ARF and apoptotic protease activating factor 1 [71]. Later,

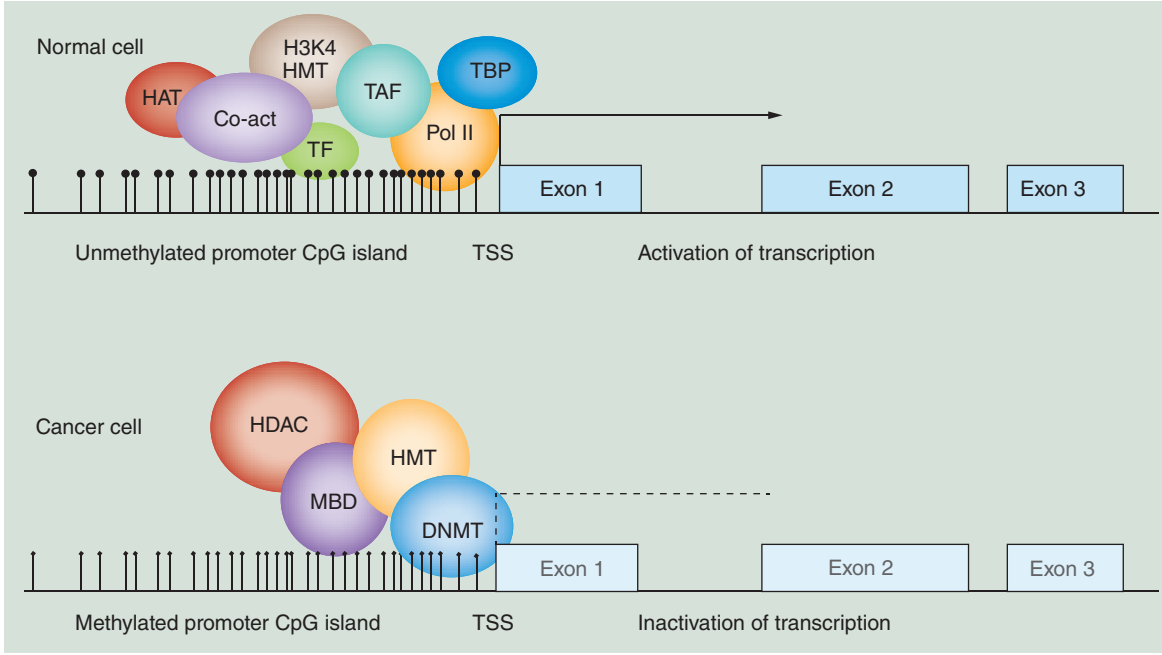

Figure 2. Methylation-mediated inactivation of tumor suppressor protein-coding gene or noncoding miRNA.
In normal cells, the promoter-associated CpG island of a tumor suppressor gene or miRNA is typically unmethylated and associated with an open chromatin configuration, and therefore allows the access of TF, HAT, HMT and RNA polymerase complexes, resulting in activation of gene or miRNA transcription. In cancer cell, the promoter-associated CpG island of a tumor suppressor gene or miRNA is usually methylated by DNMT and associated with a close chromatin configuration by the recruitment of HDAC and HMT, and hence inactivation of gene or miRNA transcription.
DNMT: DNA methyltransferase; HAT: Histone acetyltransferase; HDAC: Histone deacetylase; HMT: Histone methyltransferase; TF: Transcription factor.

Chim *et al.* further validated the prognostic impact of *DAPK1* methylation for predicting inferior survival in a uniformly treated cohort of myeloma patients [72].

On the other hand, within the bone marrow microenvironment, IL-6 is crucial in mediating survival and proliferation of myeloma plasma cells [6,19]. Binding of IL-6 to IL-6 receptors on the surface of plasma cells triggers receptor dimerization, and subsequent activation of JAK/STAT signaling pathway, which will activate downstream MAPKs and PI3K/Akt signaling pathways for cell growth and survival [73,74]. Meanwhile, the JAK/STAT signaling pathway is controlled by three major classes of negative regulators, including SOCS, PIAS, and PTP (also known as SHP). Among them, methylation of *SHP1* was frequently detected in myeloma plasma cells at diagnosis [56–57,75–76]. Importantly, Chim *et al.* showed that restoration of SHP1 was associated with reduced level of phosphorylated STAT3 and therefore inactivation of the JAK/STAT signaling pathway, thereby illustrating the tumor suppressor role of SHP1 in myeloma [75]. On the other hand, the frequency of methylation of *SOCS1* in myeloma remained controversial [59,75–78]. By selecting a CpG island in the promoter instead of the second exon, Chim *et al.* showed that methylation of *SOCS1* was absent in primary myeloma samples, and hence unimportant for the constitutive activation of JAK/STAT signaling in myeloma [75].

In addition, both canonical and noncanonical WNT signaling pathways have been implicated in myelomagenesis [79,80]. Upon binding of WNT ligands to the cell surface frizzled (Fz) receptors, cytoplasmic β-catenin will be stabilized and translocated into the nucleus, and form complex with LEF/TCF DNA-binding factors for the transcription of genes, such as *cyclin D1*, *MYC* and *STAT3*, leading to enhanced cell growth and proliferation [81,82]. By contrast, the WNT signaling pathway is antagonized by specific soluble negative regulators, namely WIF1, SFRPs and DKK family of secreted proteins. As each of these soluble WNT antagonists, except SFRP3, is associated with a CpG island at the promoter region, Chim *et al.* studied methylation of *WIF1*, *DKK-3*, *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5*, and *APC* gene in primary samples of myeloma at diagnosis [83]. Intriguingly, in addition to the fact that frequent methylation of each of them was observed, it was found that 42% of patients had methylation of at least one of these seven negative regulators, indicating a possibility of stepwise augmentation of WNT signaling pathway during disease progression of myeloma. Later, Jost *et al.* reaf-

firmed frequent methylation of SFRPs in myeloma [84]. Functionally, the tumor suppressor role of SFRP1 was verified by a dose- and time-dependent inhibition of cellular proliferation upon coculture of myeloma cells completely methylated for *SFRP1* with recombinant SFRP1 [83]. Moreover, restoration of SFRP1, by either coculture with recombinant SFRP1 or re-expression upon demethylation treatment, led to downregulation of WNT signaling, thus testifying an important role of WNT signaling in the pathogenesis of myeloma [83]. Therefore, *SFRPs* are tumor suppressors frequently hypermethylated in myeloma.

Furthermore, as the myeloma bone marrow microenvironment is considered a hypoxic niche, HIF-1 pathway has been implicated in myeloma-associated angiogenesis [53]. In the presence of oxygen at physiological level, HIF-1α is hydroxylated by EGLN3, leading to VHL-mediated HIF-1 degradation through ubiquitination. Conversely, in hypoxic condition, HIF-1 was found elevated and correlated with expression of angiogenic factors, including IL-8 and VEGF, and hence angiogenesis in the bone marrow of myeloma. [53,85]. Both *EGLN3* and *VHL* were found methylated in myeloma at diagnosis, hence possibly accounting for the upregulation of HIF-1α [86,87].

Recently, genome-wide studies of DNA methylation in myeloma have been reported [88–91].

In the first study, Walker *et al.* investigated the global DNA methylation patterns of different stages of myeloma using a methylation array (Infinium HumanMethylation27 BeadChip, Illumina, CA, USA), which interrogated methylation status of 27,578 CpG sites encompassing 14,495 genes [88]. The global DNA methylation patterns of normal B cells (n = 6), normal plasma cells (n = 3), MGUS (n = 4), myeloma at diagnosis (n = 161), PCL (n = 7) and myeloma cell lines (n = 9) were identified and analyzed. First, of these 27,578 CpG sites interrogated, 16,268 sites were differentially methylated, and most of them were located on promoters, transcription start site or first exon of genes. Among them, global hypomethylation (as indicated by hypomethylation of 3407 probes, majority localized outside CpG islands) and specific hypermethylation of 77 genes – that is, methylation within CpG islands, occurred during progression from MGUS to symptomatic myeloma, mainly affecting genes involved in the pathways of development, cell cycle and transcriptional regulation. These included *CALCA*, *ONECUT2*, *GATA4* and *CDKN2B* but not *CDKN2A*. Moreover, during progression from myeloma to PCL, additional DNA methylation occurred. In particular, CpG hypermethylation occurred not only within promoter-associated CpG islands but also in non-promoter-associated CpG sites.

Therefore, progression of MGUS to myeloma is marked by global hypomethylation and gene-specific hypermethylation, and evolution of myeloma to PCL is characterized by additional gene methylation. Moreover, methylation of genes involved in adhesion to the bone marrow microenvironment contributes to bone marrow-independent growth and proliferation in the circulation as plasma cell leukemia. Furthermore, among different cytogenetic subtypes according to the translocation/cyclin D (TC) classification system, patients with t(4;14) were associated with significantly higher degree of DNA methylation by the number of methylated genes. As histone methylation and DNA methylation are linked [92], Walker *et al.* postulated that the differentially abundant DNA methylation associated with t(4;14) was due to the histone methyltransferase activity inherent with the upregulation of MMSET. On the other hand, two distinct methylation profiles with survival significance were shown within patients with hyperdiploidy, consistent with similar findings that four subclasses of hyperdiploid myeloma with survival difference in response to bortezomib were characterized by gene expression profiling [11].

Therefore, these data were consistent with DNA methylation changes in cancer, which is global hypomethylation but gene-specific hypermethylation [44,93]. However, the mechanisms accounting for the differential methylation intensity amongst different cytogenetic subtypes remain to be elucidated.

Subsequently, Kaiser *et al.* identified methylation-repressed genes with prognostic significance among myeloma patients at diagnosis [89]. They studied global methylation profile of CD138-sorted plasma cells from 159 patients enrolled in the MRC Myeloma IX trial, in which 1970 patients were enrolled with a median follow-up of 5.9 years. When correlated with overall survivals, 207 methylation probes, mapping to 195 genes, were shown to be prognostically significant, and referred as 'p-DMR'. Interestingly, a significant portion of these p-DMRs were sites targeted by EZH2 polycomb repressive complex.

Moreover, in 115 patients with both methylation array and gene expression profiling data, correlation of methylation and gene expression were studied. In contrast to high methylation associated with gene silencing, low methylation was found associated with a transcription-ready state only, which may or may not result in gene expression. Among methylated genes with known function in cancer, seven hypermethylated genes were shortlisted for multivariate analysis together with clinical (International Staging System stage, serum lactate dehydrogenase, β2-microglobulin, calcium, creatinine levels) and cytogenetic alterations

including t(4;14), t(14;16), del(17p) and amp(1q21). Four genes, including *GPX3* (5q33.1), *RBP1* (3q23), *SPARC* (osteonectin; 5q33.1) and *TGFB1* (19q13.2), were shown to be independent adverse prognostic factors. In particular, 35% (55) patients had at least one of these four genes methylated, among which 55%, 25%, 11% and 9% had 1, 2, 3 or all four genes hypermethylated. Furthermore, methylation of these four genes increased progressively from MGUS to myeloma and PCL, and hence associated with disease progression. Indeed, other studies have demonstrated that these four genes were associated with growth suppression properties. Moreover, methylation-mediated silencing of these genes has been shown to be associated with clinical impacts in other cancers [94–97].

Furthermore, Heuck *et al.* also reported a similar study, which mapped and compared global DNA methylation patterns of normal plasma cells (n = 8), MGUS (n = 11), SMM (n = 4), myeloma at diagnosis (n = 13) and myeloma at relapse (n = 2) [90]. By studying DNA libraries of HpaII fragments (only unmethylated but not methylated CCGG is digested) and MspI fragments (both unmethylated and methylated CCGG are digested) from each sample, using a custom array interrogating 14,214 gene promoters, several key findings, consistent with those of Walker *et al.* [88], were observed. First, it was shown that unsupervised clustering of DNA methylation profiles could distinguish between normal plasma cells, MGUS, myeloma at diagnosis and myeloma at relapse, indicating a stage-specific methylation profile of myeloma. Second, as compared with normal plasma cells, MGUS and myeloma at diagnosis showed global DNA hypomethylation whereas myeloma at relapse showed global DNA hypermethylation. Moreover, genes hypermethylated in myeloma at diagnosis and at relapse were found to be commonly involved in the regulation of cell cycle, DNA replication, recombination and repair, etc. Furthermore, during disease progression, DNA methylation was found preferentially in chromosomes 1, 9, 11, 15, 17, 19, 20 and 21 [90].

More recently, based on the high-resolution Infinium HumanMethylation450 BeadChip (Illumina), which provides a more comprehensive methylation array encompassing >485,000 CpG sites including CpG sites in promoter, 5'-UTR, gene body, intron and 3'-UTR, Agirre *et al.* investigated the methylomes of normal plasma cells (n = 3), non-tumoral tonsil plasma cells (n = 8), MGUS (n = 16) and myeloma at diagnosis (n = 104) [91]. Moreover, by whole-genome bisulfite sequencing (WGBS), which is able to determine methylation of almost all CpG sites in the genome, the global DNA methylation pattern of one normal plasma cell sample, one hypermethylated myeloma case and one hypomethylated myeloma case had been further characterized.

First, both HumanMethylation450 array and WGBS showed that global DNA hypomethylation was associated with the progression from MGUS to symptomatic myeloma, concurring with the data of HumanMethylation27 array [88]. On the other hand, in contrast Walker *et al.* reported that MGUS were closely clustered with normal plasma cells, Agirre *et al.* demonstrated that MGUS could be distinguished from normal plasma cells by the global DNA methylation pattern generated using HumanMethylation450, in which, accounted by the enhanced coverage of CpG sites outside of both CpG islands and coding regions, 6874 and 2514 CpG sites were respectively hypomethylated and hypermethylated during the progression from normal to MGUS. However, unlike Walker *et al.* discovered that DNA hypermethylation was significantly associated in 15 cases with t(4;14), Agirre *et al.* reported that there was no association between DNA methylation and clinically relevant cytogenetic subtypes, including ploidy, deletion of *TP53* and *IgH* translocation, which only contained three cases with t(4;14).

Second, based on functional chromatin states defined by chromatin immunoprecipitation (ChIP)-seq in immortalized mature B cells, which is similar to that of normal plasma cells, and RNA-seq data from myeloma cases (n = 11) and normal plasma cells (n = 4), Agirre *et al.* discovered that a majority (>75%) of hypomethylated CpG sites in myeloma were located in heterochromatic regions with low impact on gene expression. On the other hand, majority (about 60%) of hypermethylated CpG sites in myeloma were located in intronic enhancer regions and significantly associated with reversible silencing of the associated genes, which contrasted with only about 15% hypermethylated CpG sites localized to the promoter regions.

Third, interestingly, by 794 hypermethylated CpG sites located in the intronic enhancer regions of the myeloma cases, Agirre *et al.* showed these CpGs sites were associated 631 genes, which were significantly associated with B-cell differentiation by gene ontology analysis. Moreover, these hypermethylated enhancer regions were found overlapped with binding sites of B-cell-specific transcription factors. Furthermore, by comparing methylation profiles across different differentiation stages, including embryonic stem cells, common lymphoid progenitors, precursor B cells, immature B cells, naive B cells, germinal centre B cells and normal plasma cells, hypermethylation of these intronic enhancer-associated CpG sites in myeloma was similar to those in embryonic stem cells and common lymphoid progenitors, suggesting that DNA

methylation in myeloma might stem from epigenetic reprogramming back into the undifferentiated stage or acquisition of progenitior cell properties, hence implicating possibility of myeloma stem cells. Collectively, this high-resolution methylome study of myeloma suggested that DNA methylation of intronic enhancer-associated CpG islands, majority being involved in B-cell differentiation. Moreover, methylation-silencing of these B-cell differentiation genes, recapitulates methylation profile of stem cell, and hence a potential myeloma stem cell methylation signature.

Methylation of tumor suppressor miRNAs in myeloma

miRNAs are a class of short, single-stranded, non-coding RNA molecules manifesting multifaceted functions in the regulation of development, differentiation, immunity and carcinogenesis [98–100]. Specific seed region sequence (i.e., the second–seventh/eight nucleotides from the 5'-end) of the mature miRNA may recognize and bind to the 3'-UTR of target protein-coding genes, leading to inhibition of translation and hence gene silencing [101,102]. In carcinogenesis, dysregulation of miRNAs has been identified [103,104]. Overexpressed miRNAs targeting tumor suppressor genes are known as oncomiRs, whereas downregulated miRNAs targeting oncogenes are known as tumor suppressor miRNAs [105,106]. Moreover, DNA methylation has also been shown to be an alternative mechanism leading to inactivation of tumor suppressor miRNAs with promoter-associated CpG islands [107,108]. Nevertheless, despite that dysregulation of miRNAs has been reported in myeloma, studies on the role of DNA methylation in the regulation of tumor suppressor miRNAs remains scanty [109].

miR-34 family miRNAs: *miR-34a*

Among the first tumor suppressor miRNAs discovered in human cancers, *miR-34* family miRNAs (*miR-34a*, *miR-34b* and *miR-34c*) have been identified as the direct downstream targets and tumor suppressor effectors of the TP53 [110–115]. In cells with *TP53* mutation or deletion, the expression of *miR-34* family miRNAs was downregulated, resulting in loss of repression on their oncogenic target genes, including *BCL2*, *CDK6*, *MET* and *SIRT1* [116–121]. Moreover, both *miR-34a* (located on 1p36) and *miR-34b/c* cluster (located on 11q23) were found associated with a promoter-associated CpG island overlapping a TP53 binding site. Furthermore, methylation-mediated loss of *miR-34* family miRNAs and consequent upregulation of their oncogenic target genes has been reported in different kinds of solid cancers, including colorectal, gastric, lung and ovarian cancer [122–126]. In myeloma, despite that deletion of 17p, which harbors a copy of *TP53*, is an adverse prognostic factor indicating shorter event-free survival and overall survival for myeloma patients [64,67], biallelic loss or loss-of-function mutation of the *TP53* infrequently occurs in myeloma patients at diagnosis [65,66]. Hence, it has been postulated that perturbation of TP53-mediated tumor suppressor network might be associated with methylation-mediated suppression of *miR-34* family miRNAs. In a study of methylation of *miR-34a* in multiple hematological cancers, Chim *et al.* have examined DNA methylation of *miR-34a* in myeloma bone marrow samples at diagnosis or relapse/progression, in addition to normal controls and myeloma cell lines [127]. It was found that 50% of the myeloma cell lines but not normal controls showed methylation of *miR-34a*. By contrast, methylation of *miR-34a* was only detected in 5.5% of primary myeloma samples at diagnosis. Moreover, in patients with paired marrow samples at both diagnosis and relapse/progression, *miR-34a* methylation was not acquired during relapse/progression. Collectively, unlike the frequent occurrence in epithelial cancers, methylation of *miR-34a* appears not to be important in the pathogenesis or progression of myeloma.

miR-34 family miRNAs: *miR-34b* & *miR-34c* cluster

In contrast to *miR-34a*, Wong *et al.* later revealed that methylation of *miR-34b/c* was important in the progression of myeloma [128]. First, methylation of *miR-34b/c* was shown to be negative in normal controls, but positive in 75% of myeloma cell lines, showing a tumor-specific pattern of methylation. Second, completely *miR-34b/c* methylated myeloma cell lines had lower expression of *miR-34b* than those completely unmethylated cells. Moreover, hypomethylation treatment of myeloma cell lines completely methylated for *miR-34b/c* led to *miR-34b/c* demethylation with concomitant re-expression of mature *miR-34b*. Therefore, these data demonstrated that methylation resulted in reversible silencing of miRNA expression. Third, in myeloma cell lines with complete methylation of *miR-34b/c*, restoration of *miR-34b* reduced cell proliferation and enhanced apoptosis, testifying the tumor suppressor function of *miR-34b* in myeloma cells. Most importantly, in primary samples, methylation of *miR-34b/c* was detected in only 5.3% of myeloma at diagnosis but 52.2% of myeloma at relapse/progression ($p < 0.001$), indicating a much more frequent methylation of *miR-34b/c* at relapse/progression than diagnosis. Moreover, in 12 patients with paired bone marrow samples collected both at diagnosis and relapse/progression, 54.5% of patients showed gain of *miR-34b/c* methylation at the time of relapse, suggesting methylation of *miR-34b/c* is acquired dur-

ing relapse/progression, suggestive of a pathogenetically important role of *miR-34b/c* methylation in myeloma relapse/progression.

miR-194-2-192 cluster miRNAs

In another study of TP53-regulated tumor suppressor miRNAs in myeloma, Pichiorri *et al.* reported that a TP53-inducible miRNA cluster, *miR-194-2-192* (located on 11p13), was also silenced by DNA methylation in myeloma cell lines [129]. In brief, among myeloma cell lines of different *TP53* statuses, a higher expression of *miR-194-2-192* was associated with intact *TP53*. Moreover, in myeloma cell lines with intact *TP53*, treatment with TP53 activator, Nutlin-3a, which is a small molecule inhibitor of MDM2, further augmented the expression of *miR-194-2-192*. Furthermore, by ChIP and luciferase assay, TP53 was shown to bind and activate the promoter of *miR-194-2-192*, confirming that these are TP53-inducible miRNAs. Moreover, restoration of mature *miR-194* or *miR-192* resulted in inhibited cellular proliferation and cell migration, but enhanced apoptosis and cell cycle arrest, indicating their tumor suppressor function in myeloma cells. However, in some primary myeloma samples, *miR-194-2-192* was not upregulated upon treatment with Nutlin-3a even in the presence of intact *TP53*. Subsequently, it was proven in myeloma cell lines that, in addition to *TP53* mutation or deletion, methylation of promoter-associated CpG island served as an alternative silencing mechanism of *miR-194-2-192*. By inversely correlated expression in myeloma cell lines, *miR-194-2-192* was hypothesized to target *MDM2*, *IGF1* and *IGF1R*. Indeed, by luciferase assays, *MDM2*, *IGF1* and *IGF1R* were all verified to be direct targets of *miR-194-2-192*. Together, methylation and hence silencing of TP53-inducible *miR-194-2-192* led to overexpression of MDM2, IGF1 and IGF1R, with consequent downregulation of TP53-mediated tumor suppression, but upregulation of IGF1 oncogenic signaling, conferring survival benefit to myeloma cells. Later, Corthals *et al.* reported that lower expression of *miR-194* was associated with inferior survival of myeloma patients, thereby supporting the tumor suppressor role of *miR-194* in myeloma [130].

miR-203

Approximately 50% of myeloma patients are associated with translocation of the *IgH* chain gene enhancer located on 14q32, leading to high expression of its partner oncogene [131,132]. On the other hand, as translocation involves double-strand DNA break, the chromosomal breakpoint region is prone to loss of DNA, which may harbor tumor suppressor gene or miRNA [133]. Indeed, methylation-mediated silencing of *miR-203*, which is a tumor suppressor miRNA located on 14q32, has been reported in hepatocellular carcinoma, chronic myeloid leukemia and other hematological cancers [134–137]. Therefore, Wong *et al.* postulated that methylation of *miR-203*, which is also prone to be deleted by double-strand DNA break, may play a role in myeloma [138]. In this study, methylation of *miR-203* was shown to be absent in normal controls, but detected in 25% of myeloma cell lines. Upon hypomethylation treatment, the promoter-associated CpG island of *miR-203* in completely methylated myeloma cell lines became demethylated, which was associated with re-expression of mature *miR-203*. Moreover, by bioinformatics prediction and luciferase assay, *CREB1* was identified as a novel direct target of *miR-203* in myeloma cells. In myeloma cell lines completely methylated for *miR-203*, restoration of *miR-203* led to lowered expression of the CREB1 and reduced cellular proliferation. In primary samples, methylation of *miR-203* was detected in 25% of MGUS, 24% of myeloma at diagnosis and 21% of myeloma at relapse/progression. As frequent methylation of *miR-203* occurs at MGUS, which is a precursor of symptomatic myeloma, *miR-203* methylation could be an early event during myelomagenesis.

miR-129-2

Another frequently methylated tumor suppressor miRNA in a variety of human cancer is *miR-129-2*, which belongs to the *miR-129* family. Despite mature *miR-129-2* (located on 11p11) share an identical sequence with mature *miR-129-1* (located on 7q32), a CpG island is associated with *miR-129-2* but not *miR-129-1*. Therefore, *miR-129-2*, but not *miR-129-1*, may be regulated by DNA methylation. Indeed, methylation and hence inactivation of *miR-129-2* has been demonstrated in a panel of solid cancers, including colorectal, endometrial, gastric cancer, esophageal squamous cell carcinoma and hepatocellular carcinoma [139–144]. Moreover, it has been illustrated that restoration of *miR-129* led to inhibition of cell proliferation, in association with suppression of oncogenic *CDK6* and *SOX4* mRNAs, thereby exemplifying the tumor suppressor function of *miR-129* [140–142,145–146]. In a study of methylation of *miR-129-2* in different hematological cancers, Wong *et al.* reported the role of DNA methylation of *miR-129-2* in primary myeloma samples ranging from asymptomatic MGUS, symptomatic myeloma at diagnosis and myeloma at relapse/progression [147]. In this study, methylation of *miR-129-2* was shown

absent in a compartment-specific control of CD138-sorted normal plasma cells in addition to normal buffy coat controls, but aberrantly methylated in seven out eight (87.5%) myeloma cell lines. In primary samples, methylation of *miR-129-2* was detected in 27.5% of MGUS, 49.5% of myeloma at diagnosis and 41.4% of myeloma at relapse, implicating a role in progression from MGUS to symptomatic myeloma ($p = 0.023$). Furthermore, frequent methylation of *miR-129-2* in MGUS suggests that it is an early event in myelomagenesis. Finally, as frequent methylation of *miR-129-2* occurs in myeloma at diagnosis, study of the prognostic potential of *miR-129-2* methylation is warranted in uniformly treated myeloma patients.

miR-124-1

In human cancers, *miR-124-1* is among the first methylated tumor suppressor miRNAs discovered in colon cancer [107]. Moreover, methylation-mediated silencing and hence impaired tumor suppressor function of *miR-124-1* was verified in acute lymphoblastic leukemia, glioblastoma, hepatocellular carcinoma, cervical and pancreatic cancers [148–152]. Recently, methylation of *miR-124-1* was studied in a variety of hematological cancers, including myeloma [135,153]. In myeloma, methylation of *miR-124-1* was detected in 75% of myeloma cell lines but not in normal controls. Moreover, upon hypomethylation treatment, myeloma cell lines with completely methylated for *miR-124-1* showed demethylation of the *miR-124-1* locus, and consequent re-expression of mature *miR-124*, demonstrating reversible methylation-mediated silencing of *miR-124-1*. Furthermore, by ChIP assay, demethylation of *miR-124-1* promoter was associated with augmentation of euchromatic trimethyl H3K4, implicating the interaction between methylation and histone modification in the regulation of miRNA expression. Furthermore, the re-expression of *miR-124-1* was shown to correlate with downregulation of its direct target oncogene, CDK6, thereby illustrating the tumor suppressor role of *miR-124-1* in myeloma. However, in contrast to myeloma cell lines, methylation of *miR-124-1* was detected rarely in 2% of myeloma both at diagnosis or relapse/progression. Therefore, methylation of tumor suppressive *miR-124-1* appeared unimportant in the pathogenesis or relapse/progression of myeloma, and the frequent methylation of *miR-124-1* might be acquired *in vitro* during continual culture of these cell lines.

miR-152 & miR-10b-5p

Based on a genome-wide approach, Zhang *et al.* have identified *miR-152* and *miR-10b-5p* as tumor suppressor miRNAs hypermethylated in relapsed/refractory myeloma [154]. In brief, by ChIP-chip, which involved capture of methylated DNA using anti-5-methylcytosine (5-mC) antibody and subsequent quantification using a DNA chip (Affymetrix), it was observed that primary relapsed/refractory myeloma (n = 8) had a differentially increased global DNA methylation pattern than normal plasma cells (n = 2; pooled from 6). In particular, in relapsed/refractory myeloma, the noncoding regions became much more extensively methylated than normal plasma cells. Moreover, these hypermethylated non-coding regions were associated with 127 miRNAs, which might be regulated by DNA methylation. Among them, by integrative analysis with miRNA expression profiling of myeloma cell lines (n = 6) treated with or without 5-azadC, candidate miRNAs likely to be silenced by DNA methylation were shortlisted. As a result, demethylated and hence re-expressed miRNAs, including *miR-152* and *miR-10b-5p*, were further validated. Upon 5-azadC treatment of myeloma cell lines completely methylated for *miR-152* or *miR-10b-5p*, promoter demethylation and concomitant re-expression of the *miR-152* or *miR-10b-5p* was observed, thereby testifying methylation-mediated silencing of both miRNAs. Moreover, restoration of *miR-152* or *miR-10b-5p* in completely methylated myeloma cell lines led to reduced cellular proliferation and enhanced apoptosis in the presence or absence of bone marrow stromal cells, which mimicked the bone marrow microenvironment, thereby demonstrating the inherent tumor suppressor function. Furthermore, by bioinformatics search, putative target genes of *miR-152* (*DNMT1* and *E2F3*) and *miR-10b-5p* (*E2F3*, *BTRC* and *MYCBP*) were identified. Finally, downregulation of *miR-152* and *miR-10b-5p* in myeloma, with concomitant high expression of these predicted putative target genes were confirmed in publicly available gene expression datasets of myeloma patients and normal plasma cells. Indeed, overexpression of each of *miR-152* and *miR-10b-5p* was associated with downregulation of their respective predicted target genes. In summary, methylation-mediated silencing of tumor suppressive *miR-152* and *miR-10b-5p* was identified in relapsed/refractory myeloma.

Conclusion & future perspective

Collectively, these data have demonstrated the role of DNA methylation in the repression of tumor suppressor protein-coding genes or non-coding miRNAs in myeloma (Table 1). Further studies to elucidate the mechanisms underlying aberrant DNA methylation in myeloma are warranted. In this connec-

tion, given that the histone methyltransferase activity of MMSET might recruit DNMTs, it has been proposed that t(4;14)-mediated upregulation of MMSET might account for the increased number of genes hypermethylated in cases with t(4;14) as compared with other cytogenetic subtypes [88,155]. On the other hand, despite the distinct methylation profile of t(4;14) is mainly governed by histone methyltransferase activity of MMSET, the mechanisms leading to the differential methylation profiles of other cytogenetic subtypes remain enigmatic.

Recently, *DNMTs* have been shown to be direct targets of miRNAs. For instance, *DNMT3A* and *3B* have been shown to be the direct targets of *miR-29* family miRNAs, restoration of which was associated with DNA hypomethylation and re-expression of tumor suppressor genes in cancer cell lines, thereby implicating a potential of *miR-29* family miRNAs in the development of aberrant DNA methylation in cancer [156,157]. Similarly, *DNMT1* has been shown to be direct target of *miR-152* [158,159]. Therefore, the interplay or regulatory loop between DNA methylation and miRNAs warrants further studies.

Current data also indicated that DNA methylation of tumor suppressor genes or miRNAs may be associated with disease progression and/or prognostic significance. Therefore, DNA methylation may pose as novel molecular biomarkers for identifying high-

Table 1. Summary of methylated tumor suppressor protein-coding genes and non-coding miRNAs in multiple myeloma†.

Gene/miRNA	Genomic location	Function	Ref.
Protein-coding gene			
CDKN2A	9p21.3	Inhibitor of cell cycle progression	[49,50,56–63]
CDKN2B	9p21.3	Inhibitor of cell cycle progression	[49,50,56–63]
DAPK1	9q21.33	Apoptosis	[57–60,70–72]
SOCS1	16p13.13	Inhibitor of JAK/STAT signaling pathway	[59,75–78]
SHP1	12p13.31	Inhibitor of JAK/STAT signaling pathway	[56,57,75–76]
WIF1	12q14.3	Inhibitor of WNT signaling pathway	[83,84]
DKK3	11p15.3	Inhibitor of WNT signaling pathway	[83,84]
SFRP1	8p11.21	Inhibitor of WNT signaling pathway	[83,84]
SFRP2	4q31.3	Inhibitor of WNT signaling pathway	[83,84]
SFRP4	7p14.1	Inhibitor of WNT signaling pathway	[83,84]
SFRP5	10q24.2	Inhibitor of WNT signaling pathway	[83,84]
APC	5q22.2	Inhibitor of WNT signaling pathway	[83,84]
GPX3	5q33.1	Scavenger of reactive oxygen species	[89]
TGFB1	19q13.2	Chemosensitivity; inhibition of metastasis	[89]
RBP1	3q23	Intracellular storage and transfer of retinoic acid	[89]
SPARC	5q33.1	Chemosensitivity; inhibition of cellular proliferation	[89]
VHL	3p25.3	Proteasomal degradation of HIF	[86]
EGLN3	14q13.1	Proteasomal degradation of HIF	[87]
Noncoding miRNA			
miR-34a	1p36	Apoptosis; targeting *BCL2* and *CDK4/6*	[127]
miR-34b/c	11q23	Apoptosis; targeting *CREB* and *MET*	[128]
miR-124-1	8p23	Inhibitor of cell cycle progression; targeting *CDK6*	[153]
miR-203	14q32	Antiproliferation; targeting *CREB*	[138]
miR-129-2	11p11	Antiproliferation; targeting *SOX4*	[147]
miR-194-2-192	11q13	Apoptosis; targeting *MDM2*, *IGF* and *IGF1R*	[129,130]
miR-10b-5p	2q31	Apoptosis; targeting *E2F3*, *BTRC* and *MYCBP*	[154]
miR-152	17q21	Apoptosis; targeting *DNMT1* and *E2F3*	[154]

†Include those discussed in this review.

risk patients who may benefit from more intensive treatments, such as allogeneic stem cell transplantation [160,161]. Furthermore, as epigenetic drugs, such as azacitidine and decitabine, have been used with success in cancer therapy, understanding of methylation-silenced tumor suppressor genes or miRNAs may serve as the scientific rationale for incorporating epigenetic drugs in myeloma therapy [162,163].

Based on the aforementioned genome-wide methylation studies, which mainly focused on annotated gene promoters of protein-coding genes, methylation of non-coding region remains largely unexplored. Moreover, as most primary miRNA promoters have not been well identified, current data on methylation of tumor suppressor miRNAs may only represent the tip of the iceberg. On the other hand, long non-coding RNAs,

Executive summary

Multiple myeloma

- Multiple myeloma is a form of mature B-cell neoplasm characterized by clonal proliferation of plasma cells in the bone marrow.
- Clinically, this disease can be classified into asymptomatic stages of monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma, and symptomatic stages of myeloma at diagnosis, at relapse/progression, and extramedullary plasma cell leukemia.
- Genetically, based on primary genetic alternations, this disease is mainly classified into hyperdiploid or non-hyperdiploid.
- Hyperdiploid myeloma is characterized by trisomies of odd-numbered chromosomes, including 3, 5, 7, 9, 11, 15, 19 and 21; but not 13.
- Non-hyperdiploid myeloma is characterized by a primary translocation involving the *IgH* locus at 14q32 and a partner oncogene, for example, most common primary translocations include t(11;14)(q13;q32), t(4;14)(p16.3;q32), t(14;16)(q32;q23), t(14;20)(q32;q11) and t(6;14)(p21;q32).
- Secondary genetic alternations include secondary translocation of *MYC* gene, amplification of 1q21, deletion of 1p, 13q and 17p, and activating mutation of *RAS* and *NF-κB*, among others.

DNA methylation in cancer

- DNA methylation refers to the catalytic addition of a methyl (-CH_3) group to the carbon 5 position on a cytosine ring in a CpG dinucleotide, leading to the formation of 5-methylcytosine.
- DNA methylation of promoter-associated CpG island, which is a cluster of CpG sites, serves as an alternative mechanism of transcriptional silencing of the associated tumor suppressor gene.

Methylation of tumor suppressor protein-coding genes

- By a candidate gene approach or a cancer pathway approach, DNA methylation of tumor suppressor genes in myeloma mainly converges to one of the following signaling pathways, including the cell cycle, DAPK/p14ARF/MDM2/TP53 intrinsic TP53-dependent apoptosis pathway, IL6/JAK/STAT signaling pathway, WNT signaling pathway and HIF-1 regulatory pathway.
- By a genome-wide approach, myeloma is characterized by a global DNA hypomethylation but a gene-specific hypermethylation.
- Genome-wide analysis of the DNA methylomes reveals distinct methylation profiles across different clinical stages of myeloma, including normal plasma cells, MGUS, myeloma at diagnosis and myeloma at relapse.
- Genome-wide analysis identifies methylation of intronic enhancers in myeloma samples, which resembles that of undifferentiated stem cells, implicating an epigenetic reprogramming back to the undifferentiated state or an acquisition of progenitor cell properties involved during the pathogenesis of myeloma.
- Global DNA hypermethylation is significantly associated with translocation t(4;14), whereas gene-specific hypermethylation of each of *GPX3*, *RBP1*, *SPARC* and *TGFB1* are independently associated with adverse prognostic impact.

Methylation of tumor suppressor miRNAs in myeloma

- DNA methylation is involved in the transcriptional silencing of tumor suppressor non-coding miRNAs, including *miR-34a*, *miR-34b/c*, *miR-124-1*, *miR-129-2*, *miR-194-2-192*, *miR-203*, *miR-152* and *miR-10b-5p*, in myeloma.
- Methylation of *miR-34a*, *miR-34b/c* and *miR-194-2-192*, which are transcriptional targets of TP53, implicated a role of the TP53 pathway in the pathogenesis of myeloma. Of which, methylation of *miR-34b/c* is associated with relapse/progression of myeloma.
- Methylation of *miR-203*, *miR-129-2* and *miR-152*/*miR-10b-5p* occurs at MGUS, at diagnosis, and at relapsed/refractory, respectively.

Conclusion & future perspective

- Future investigation of DNA methylation in myeloma is warranted.

which belong to a novel class of functional RNA molecules of >200 nucleotides with little or no protein-coding capacity, emerge to play role in carcinogenesis by acting as oncogene or tumor suppressor gene [164,165]. Indeed, hypermethylation of a tumor suppressor long non-coding RNA *MEG3* has been demonstrated in myeloma [166]. Hence, the role of DNA methylation of non-coding RNAs in myeloma warrants further studies.

In conclusion, DNA methylation of tumor suppressor protein-coding genes and non-coding miRNAs plays a crucial role in the myeloma pathogenesis, diagnosis, prognosis and treatment. Therefore, future investigation of the role of DNA methylation in the regulation of non-coding RNAs is warranted.

Author contributions

CS Chim, KY Wong helped to draft the manuscript. All authors read and approved the final manuscript.

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