PART- II

Chapter- 4

Promoter methylation status in acute myeloid leukemia
4.1. Introduction

Acute myeloid leukemia (AML), a clonal hematological malignancy is a heterogeneous disease characterized by many different genetic defects. Chromosomal translocations involving oncogenes and transcription factors, activation of signal transduction pathways, and alterations of growth factor receptors have been documented in AML [Caligiuri MA et al. 1997, Lowenberg B et al. 1999]. Disruptions of tumor suppressor networks are also associated with most types of tumorigenesis. This might be achieved through deletions, mutations or epigenetic processes and the latter play a major role in human carcinogenesis [Jones PA and Laird PW 1999].

Hypermethylation of the CpG islands near the promoter regions is followed by binding of complexes of methyl-CpG binding proteins, transcriptional corepressors, chromatin remodelling proteins and histone deacetylases (HDAC) to the hypermethylated DNA regions, resulting in a transcriptionally repressive chromatin state [Rountree MR et al. 2001, Esteller M 2002, Herman JG and Baylin SB 2003, Egger G et al. 2004]. In this context abnormal methylation of CpG islands may exert the same effects as a mutation or deletion in coding region in one copy of the gene and thus represent an alternative mechanism to contribute to the loss of function of one or both alleles [Rountree MR et al. 2001, Herman JG and Baylin SB 2003]. Several genes of basic cellular pathways have been shown to be affected by aberrant CpG island methylation in human cancers [Esteller M et al. 2001, Herman JG and Baylin SB 2003].

Attempts are being made to characterise the methylome associated with various types of cancer, and relate them to the clinical and other parameters to evaluate their significance. Despite a broad tumor specific pattern of global methylation, there is significant inter-individual variability. For example, methylation of p15 has been reported in 30-90% and E-cadherin (CDH1) in 30-80% of AML cases [Melki JR et al. 1999, Toyota M et al. 2001, Leone G et al. 2002]. These differences may indicate differences in the subtypes of AML included in the studies, etiology of the disease, or differences in the cohort of patients.

In the present work, I examined the methylation status of nine well characterized cancer related genes in 63 de novo AML patient samples at diagnosis and explored possible correlation between methylation patterns and clinical parameters. Methylation was analyzed using the methyl-specific polymerase chain reaction (MSP) of bisulfite treated DNA [Herman JG et al. 1996]. The list of candidate genes comprises the cell cycle regulators p15, p16, p14, p73 and p21, the E-cadherin (CDH1) (tumor-cell invasion or tumor architecture), hMLH1 (repair of DNA damage), retinoic acid receptor β2 (RARβ2), and the cytokine regulator suppressor of cytokine signaling-1 (SOCS1). It has been shown previously that the expression of each of these genes may be affected by aberrant CpG island methylation in association with transcriptional silencing in various human cancers [Cameron EE et
Chapter 4  Promoter methylation status in acute myeloid leukemia


The results indicate higher frequency of p16 gene methylation over that reported in other studies and a statistically significant reduction in overall survival of patients with increase in number of methylated genes.

4.2. Materials and Methods

4.2.1. Patients

Sixty-three de novo Acute Myeloid Leukemia (AML) patients without prior treatment were subjects of this study. Patients' characteristics can be seen in Table 3.1.1, chapter 3.1. Presence of the AML1-ETO translocation was detected through RT-PCR. FLT3 alteration was scored through standard techniques, details in next chapter. Peripheral blood of healthy volunteers was used as control.

4.2.2. Genomic DNA isolation

Genomic DNA was isolated from whole blood/bone marrow samples as described in chapter 2.

4.2.3 Bisulfite Modification

Genomic DNA was modified with sodium bisulfite as previously described [Herman JG et al. 1996]. Briefly 2 μg genomic DNA in a volume of 50 μl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. Thirty μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite at pH 5.0, both freshly prepared, were added and mixed and samples were incubated under mineral oil at 50°C for 16 hours. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer's protocol (Promega, USA) and eluted into 50 μl of hot (60-70°C) triple distilled water. Modification was completed by NaOH (final conc. 0.3 M) treatment for 5 min at 37°C, followed by salt ethanol precipitation in presence of PEG. DNA was resuspended in 30 μl triple distilled water and used immediately or stored at -20°C.

4.2.4. Methylation specific polymerase chain reaction

DNA methylation patterns in the CpG islands of the nine genes were determined by methylation specific PCR (MSP). MSP distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated (but not methylated) cytosine to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA. The PCR mixture contained 1X PCR buffer, 1.5 mM MgCl2 (5.5 mM for p15M), 0.2 mM dNTPs (0.8 mM for p15M), 5% DMSO, 20 pmol to 50 pmol in each reaction.
primers that specifically recognize either methylated or unmethylated gene sequences (primer sequences for p15, p16, p14, p73, hMLH1, RARβ2, CDH1, SOCS1 and p21 are listed in chapter 2 primer table) and bisulfite-modified DNA in final volume of 50 µl. Reactions were hot-started at 95°C for 5 min and held at 80°C before addition of 1.5 U Taq polymerase (Bangalore Genei, India). Thermal cycle was 30-35 cycles (30 sec at 95°C, 30 sec at 54°C to 68°C and 30 sec at 72°C) followed by a final 4 min extension at 72°C. Genomic DNA from normal lymphocytes treated in vitro with SssI methyltransferase (New England BioLabs, USA) in presence of S-adenosylmethionine (for p14, hMLH1, RARβ2, SOCS1) or genomic DNA of Raji B cell line (for p16, p15, p73, p21, CDH1) was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated alleles. Primers specific for untreated DNA were used to check for completion of modification. PCR products were electrophoresed on 3% agarose gel, stained with ethidium bromide, and visualized under UV transillumination (Figure 4.1).

4.2.5. Statistical analysis
For statistical analysis, AML patients were classified into two different groups according to the number of methylated genes observed in each individual sample: Group A (0 to 2 methylated genes) and Group B (more than 2 methylated genes). For survival analyses, patients were followed up to death or last contact. Kaplan-Meier plots were generated to compare the survival distribution of patients in these two groups (Group A and B). Finally, the log-rank test was used to compare the survival distributions of these groups. All P value indicates two sided comparisons and a P<0.05 was considered significant. Statistical data analyses were performed using the statistical software R [R Development Core Team (2006). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org].

4.3. Results
4.3.1. Profile of promoter CpG island methylation pattern in AML
At least one of the nine promoter regions studied was found hypermethylated in 80% (50/63) of the primary AML patient samples, while 47.6% (30/63) harbored two or more hypermethylated genes and 25.4% (16/63) harbored more than two hypermethylated genes (Figure 4.2A). The frequency of hypermethylated genes among the AML patient samples was 39.7% (25/63) for RARβ2, 27% (17/63) for E-cadherin and hMLH1, 23.8% (15/63) for p15, 22.2% (14/63) for p16, 15.8% (10/63) for p14, 14.3% (9/63) for p73, 20% (8/40) for SOCS1 and 0% (0/63) for p21 (Figure 4.2B). An overview of profile of the hypermethylation status of the AML samples is given in Figure 4.3.
Figure 4.1. Representative MSP analysis of AML patient samples. Lane U: amplified products with primers recognizing the unmethylated gene sequence. Lane M: amplified products with primers recognizing the methylated gene sequence. N: healthy volunteers served as negative control for methylated specific primers and positive control for unmethylated specific primers. B, water served as no template controls; Mr: Molecular weight marker. RajiB cell line or in vitro methylated DNA (IVM) served as positive control for methylated specific primers.

78
Figure 4.2. (A) Distribution of the number of hypermethylated genes among the analyzed AML patient samples. (B) Frequency of hypermethylation for each gene among the AML patients.

Figure 4.3. Methylation profile of 9 cancer related genes in 63 primary AML patient samples. Black boxes indicate methylated gene, white boxes indicate unmethylated gene, boxes with ND indicate not done due to DNA insufficiency. FAB, French-American-British subtype; UC, unclassified, a AML1-ETO positive patients, b Patients with FLT3 alteration.
4.4. Discussion

The addition of methyl group to the cytosine of CpG islands of gene promoter regions is associated with gene silencing and is observed in X-linked or imprinted genes under normal condition. This epigenetic mechanism produces a form of cell memory and provides heritable states of differential gene expression [Riggs AD and Pfeifer GP 1992]. However, aberrant use of the phenomenon with increase of age or during malignancy is well established and an attempt to use it in cancer as a prognostic marker or in design of therapy is underway [Issa JP et al. 2004].

A large volume of data investigating the epigenetic inactivation of genes associated with cell cycle arrest, DNA repair and tumor metastasis has been generated in a variety of tumors [Cameron EE et al. 1995, Corn PG et al. 1999, Graff JR et al. 1995,

The different studies on promoter hypermethylation in various hematological malignancies including AML indicated a high incidence of promoter hypermethylation, 70-100% of the samples carrying hypermethylation of at least one gene, many samples registering multiple gene promoter hypermethylation. Higher incidence of multiple gene hypermethylation in younger patients has been reported by some groups [Toyota M et al. 2001] while others including this study failed to find any correlation of methylation index (ratio of number of genes methylated to the number of genes analyzed) with age [Galm O et al. 2005, Ekmekci CG et al. 2004]. Some studies also linked methylation with specific FAB subtypes but others have discounted it [Galm O et al. 2005, Ekmekci CG et al. 2004].

Various studies indicate that the patterns of epigenetic inactivation of genes among different malignancies differ and the profile of promoter hypermethylation is tumor type and gene specific. For example, p14 and APC hypermethylation occurs frequently in gastrointestinal cancers and GST P1 is affected in steroid related malignancies [Esteller M et al. 2001]. The p16 gene is frequently hypermethylated in a wide variety of solid tumors, underlining that disruption of the cyclin D-Rb cell cycle control pathway contributes significantly towards development of such neoplasms [Esteller M et al. 2001]. However, in hematopoietic malignancies, and AML in particular, epigenetic inactivation of p16 is rare; instead, the 25 kb upstream p15 gene, inducible by TGFβ, and with similar CDK inhibitor activity as p16 is affected [Esteller M et al. 2001, Herman JG et al. 1997].

Studies showed p15 methylation varying between 40-80% in AML cases. One Australian study reported some methylation of p16 in 20 AML cases studied [Melki JR et al. 1999]. I observed that the methylation of p15 is lesser and that of p16 is more frequent in our patient cohort than that reported. Together the frequency adds up to ~45%. Both p15 and p16 genes were methylated in 4 patients and all of them had methylation of multiple genes, 4 in 2 and 5 in the other 2. A study from Japan with 29 childhood AML cases also registered 11 cases of p16 and 10 cases of p15 promoter hypermethylation [Guo SX et al. 2000].

Studies of experimental lung tumors in rodent system indicate that malignancies induced by different carcinogens differ in their methylation profile [Issa JP et al. 1996]. Differential methylation profile of our data may suggest etiological variation of the disease in this area compared to the other studies.

P14 gene hypermethylation has not been detected in therapy related AML and this study is the only one on de novo AML, where methylation has been detected in only a few cases (Figure 4.2B). A study from Hong Kong and another from Germany reported absence of p21 promoter methylation in AML patients [Chim CS et al. 2005,
My results also failed to detect promoter methylation of p21 gene in the 63 AML patients studied. The leukemia specific PML-RAR\(\alpha\) and AML1-ETO translocations are rarely associated with functional methylation of the RAR\(\beta2\) promoter [Tabe Y et al. 2006]. In this cohort of patients 7 are AML-ETO positive and 7 are of AML-M3 subtype; among them only one M3 patient is methylated at RAR\(\beta2\) promoter. RAR\(\beta2\) promoter methylation is detected in 40% of the samples in this study, against 18% and 20% in others [Galm O et al. 2005, Ekmekci CG et al. 2004]. Frequency of CDH1 gene methylation varied from 10-70% in different studies [Melki JR et al. 1999, Galm O et al. 2005, Ekmekci CG et al. 2004, Corn PG et al. 2000]. SOCS1, a member of suppressor of cytokine signaling genes with tumor suppressor activity, was methylated in 60% of newly diagnosed AML samples from Taiwan [Chen CY et al. 2003], 39% in Turkey [Ekmekci CG et al. 2004] and 45% in the study from Germany [Galm O et al. 2005]. I have observed such methylation in 20% (8/40) cases in this cohort of AML samples. The hMLH1 gene is involved in the mismatch repair pathway and its absence is associated with microsatellite instability in solid tumors. Aberrant methylation of hMLH1 detectable in 0-20% of AML cases and reduced mRNA expression of hMLH1 in leukemia samples have also been reported [Lenz G et al. 2004]. The p53 gene family member p73 shows significant sequence homology and functional overlap with p53 and activates p53 responsive genes like p21, MDM2 and others. Its possible inactivation through methylation has been studied by a few groups including one from India who did not detect any case of hypermethylation [Sahu GR et al. 2005]. Others detected low frequencies all below 20% [Pluta A et al. 2006] which supports the trend of my data.

In vitro experiments have shown that, in contrast to the genetic aberrations, the promoter hypermethylation associated with silencing of tumor suppressor genes is a reversible phenomenon [Bachman KE et al. 1999, Cameron EE et al. 1999, Paz MF et al. 2003]. So, there is a scope of using demethylating agents for therapy to reverse the silencing of genes repressed by hypermethylation. The therapeutical efficacy of the demethylating agents 5-aza-2'-deoxycytidine and 5-azacytidine has been demonstrated in clinical trials in patients with MDS, AML and chronic myeloid leukaemia [Willemze R et al. 1997, Wijermans P et al. 2000, Silverman LR et al. 2002, Kantarjian HM et al. 2003, Issa JP et al. 2004].

Attempts to relate methylation profile of de novo AML with overall survival or disease free survival (DFS) have not produced positive results when the genes are considered separately. However, when the patients were stratified by the number of genes methylated those with more methylated genes showed shorter duration of disease free survival and overall survival in a study with acute lymphoblastic leukemia [Roman-Gomez J et al. 2004].
In this study, patients with methylation of 3 or more genes have a poorer overall survival (OS) than patients with 1 or 2 methylated genes or patients lacking promoter methylation. Log rank test analysis confirmed that higher methylation was associated with a shorter OS ($P = 0.015$). This indicates that therapeutic efficacy of demethylating agents is a distinct possibility in patients of this region.

A recent study on methylation level of p15 and estrogen receptor α (ERα) in AML patients at remission showed that higher methylation is associated with lower OS and DFS [Agrawal S et al. 2007].

In summary, these results indicate that aberrant promoter methylation affecting simultaneously several fundamental molecular pathways is a common phenomenon in de novo AML irrespective of geographic/ethnic background, though the extent of involvement of individual genes differ. The methylation profile may be an important factor in predicting the clinical outcome of the disease. This is the first report on methylation of multiple genes in AML patients from India.