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DNA Methylation: Its Role in Cancer Development and Therapy

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Inflammation and Methylation Changes

Changes in the genome which result in cancer promotion remain a complex interplay of aberrant methylation in the setting of a multitude of epigenetic changes. The events leading to these disruptions in methylation have been postulated; though remain to be fully elucidated. A causal relationship between inflammation and cancer has long been accepted in multiple tumor types, most prominently colon cancer; an association supported by the evidence that non-steroidal anti-inflammatory medications can potentially stop the development of colon cancer. Investigations in patients with ulcerative colitis who have a predisposition to the development of colon cancer have established a potential link between inflammation and cancer by the demonstration of hypermethylated gene promoters in early dysplastic lesions.[1] The mechanisms responsible for the contribution of inflammation to cancer remain to be fully understood. Mutagenesis resulting from the production of halogenated nucleotide precursors in activated neutrophils and eosinophils with subsequent incorporation into DNA has been demonstrated *in vitro*.[2,3] The link between inflammation-induced halogenated nucleotides and aberrant methylation stems from the demonstration that methyl-binding proteins cannot distinguish between methylated cytosine residues and chlorinated or brominated cytosine nucleotides. Furthermore, DNMT1 also could not distinguish between 5-methylcytosine and some 5-halocytosine products which could potentially result in mistaken methylation at sites of inflammation.[4] These halogenated cytosine residues were not recognized by DNA repair enzymes suggesting the possibility of accumulation of heritable changes within the genome.

Infection with *Helicobacter pylori* has been implicated in the development of gastric cancer with a relative risk increase of 2.2- to 6 fold when infection is detected.[5–8] The mechanism of cancer development in this setting has been attributed to inflammation leading to cell proliferation resulting in a propensity for gene mutation. Aberrant methylation due to *H. pylori* infection has been suggested as an additional mechanism bridging the gap between inflammation and cancer. In a series of 154 healthy volunteers and 72 patients with gastric cancer, methylation of 8 regions of 7 CpG islands was assessed.[14] Among the healthy volunteers, the extent of DNA methylation was 5.4- to 303-times higher in the *H. pylori* positive cases compared to the *H. pylori* negative cases ($p<0.0001$) suggesting an increase in methylation levels due to the presence of *H. pylori*. However, the comparison of methylation levels between healthy volunteers and patients with gastric cancer stratified by *H. pylori* status

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revealed an increase in methylation in *H. pylori* positive gastric cancer patients in one of the gene promoters studied when compared to *H. pylori* positive individuals without gastric cancer. However, in patients with *H. pylori* negative status, those with gastric cancer had significantly higher methylation levels in CpG islands of all genes chosen for study than in healthy volunteers. That *H. pylori* positive individuals have higher methylation levels than *H. pylori* negative gastric cancer patients could result from *H. pylori* infection conferring aberrant methylation status on all gastric cells including stem cells such that cancer-inducing stem cells persist while the gastric cells die. After resolution of the infection, it could appear that the methylation changes have resolved despite the continued presence of methylated, gastric stem cells capable of promoting cancer. The increased methylation in *H. pylori* positive individuals, the independence of methylation status from age and gender, and the association between prior *H. pylori* exposure and subsequent gastric cancer risk suggests that aberrant methylation is a potential biomarker for cancer prediction and surveillance among patients with *H. pylori* infection.[9] With the proposed association between *H. pylori* infection, aberrant methylation and subsequent gastric cancer evolves the question of whether eradication of *H. pylori* infection can mitigate increased methylation with a potential to halt carcinogenesis. Fuccio, et al. investigated this idea.[10] In previous work by them and others, it has been demonstrated that *E-cadherin*, a tumor suppressor gene, is aberrantly methylated in *H. pylori* infected gastric specimens as well as in gastric tumor specimens. A decrease in methylation of *E-cadherin* has been demonstrated after treatment of *H. pylori* infection, suggesting the possibility that methylation changes induced by infection can be mitigated by treatment directed against the bacteria.[10–12]

Alcohol mediated methylation changes

Chronic alcohol consumption has a number of health-related consequences and is causally related to the development of cancers of the oral cavity, pharynx, larynx, esophagus, liver, colon, rectum and breast. Since the beginning of the 20th century, it has been hypothesized that alcohol and/or its metabolites act as carcinogens, through a variety of mechanisms including the activation of other pro-carcinogens, the generation of reactive oxygen species as well as metabolism to carcinogens such as acetaldehyde.[13] Methylation changes due to a variety of alcohol related factors have been proposed as an additional mechanism of carcinogenesis. S-adenosyl-L-methionine (SAMe), a coenzyme which acts as a methyl donor, is principally produced in the liver from L-methionine and ATP. The isoenzymes of methionine adenosyltransferase, which catalyze this reaction, are encoded by the *MAT1A* (MAT I and III isoenzymes) and *MAT2A* (MATII) genes. MATI and MATIII are predominantly active in adult liver while MATII is found in fetal and regenerating liver. Because the enzymes encoded by *MAT1A* are most able to maintain adequate levels of SAMe, the gene is active in normal liver. However, in the presence of liver injury such as from alcohol use, the production of nitric oxide and reactive oxygen species are thought to result in decreased levels of MATI and III with resultant decrease in SAMe production. Subsequent diminished levels of *MAT1A* products are due to an as yet unknown mechanism of promoter hypermethylation. The decrease in SAMe, a methyl donor, is thought to result in hypomethylation of *MAT2A* such that MATII production is increased resulting in an increase in hepatocyte growth and promotion of cell division cycle. *MAT2A* is hypomethylated in hepatocellular cancer and hypermethylated in normal liver supporting the above as a mechanism of methylation changes resulting in cancer promotion.[13,14]

The reduced intake amongst alcoholics of key nutrients as well as the perturbation of enzymatic processes such as the production of SAMe by alcohol ingestion leads to further disturbance of important methylation reactions. One such example is the folate and methionine deficiency found in alcoholics which is associated with higher rates of colorectal cancer, an association that is not seen in occasional drinkers with replete levels of these nutritional factors.[15] The

hypomethylation detected in colorectal cancer, thought to be in part a result of folate depletion, remains an association despite the findings that folate supplementation resulted in higher rates of carcinoma in a randomized controlled trial. Folate depletion appears to play an important role in the very early stages of carcinogenesis with changes in methylation levels impacting the development of tumors later on. Folate supplementation in the setting of pre-existing cancer cells, however, appears to be a tumor promoter due to the increased folate requirements of cancer cells and the associated increase in folate receptors on malignant cells.[16]

DNA methylation changes and aging

The association between aging and increased incidence of cancer has long been noted and has been largely attributed to the accumulation of genetic mutations over time. Of late, the alterations in the epigenome as a result of aging have gained increased attention. As seen in patients already diagnosed with cancer, aging brings about a global hypomethylation with hypermethylation of specific gene promoters. A pertinent example is found in sporadic colorectal tumors with microsatellite instability. Hypermethylation of the *MLH1* gene in these patients led to an interest in the possibility that this finding was a precursor to the development of cancer. Normal colonic mucosa in patients with colorectal cancer was obtained and the 700-bp region upstream of *MLH1* was analyzed.[17] Partially methylated alleles were noted in 44% of samples from patients under the age of 60 years, while 83% of patients above the age of 80 demonstrated increased methylation levels from the 51 CpG islands studied. Fully methylated alleles were detected in the majority of microsatellite unstable specimens and in only 20% of those tumors without microsatellite instability. The location of the hypermethylated regions was detected in cryptal cells close to the lumen. The results from this series suggest that molecular changes accumulate with age with a contribution from environmental influences resulting in methylation changes over the entire promoter region of *MLH1* and subsequent development of microsatellite unstable colon cancers. An important caveat to these findings is the fact that the samples were derived from patients with documented colon cancer such that definitive conclusions about the molecular sequence of events resulting in carcinoma cannot as yet be made.

Disease-Associated Methylation Changes

Myelodsyplastic Syndromes

The myelodysplastic syndromes encompass a group of stem cell disorders characterized by ineffective erythropoiesis with varying probability of leukemic transformation. The disease is characterized by the presence of cytopenias and is often diagnosed on routine laboratory examination.[18] Patients are sometimes asymptomatic, but may suffer from symptoms secondary to low blood cell counts, infection or autoimmune manifestations. Approximately 10,000 cases are diagnosed annually and most commonly in persons over the age of 60. Based on the most recent SEER data, 3 year survival rates remain a dismal 35 percent.[19]

The genetic map of MDS has not yet been fully elucidated; however, the methylation of several gene promoters has been demonstrated and is thought to contribute to its pathogenesis. In particular, interstitial deletions of chromosome 5 are associated with a distinct sub-type of MDS known as 5q syndrome as well as therapy-related MDS and some types of leukemia.[20,21] Investigation into a possible tumor suppressor gene in this region of chromosome 5 led to the identification of the *reversion induced LIM (RIL)* gene located in this region as a candidate tumor suppressor gene.[22] Methylation of *RIL* was identified in only 6% of patients classified as having low-risk MDS while over 50% of patients with intermediate- to high-risk MDS demonstrated aberrant methylation of the gene. Furthermore, among the higher risk patients, those with *RIL* methylation had a median survival of only 55 weeks compared with 119 weeks in the samples without methylation suggesting a role of the gene in the pathogenesis

of MDS. Methylation of the *p15*^{INK4b} gene, which inhibits cyclin dependent kinases 4 and 6, has been associated with MDS cases and seems to correlate with percentage of blasts. As methylation of the gene was uniformly found in cases of AML arising from MDS, its presence may signal disease evolution.[23] Similar results were obtained by Tien, et al. in which an increasing proportion of *p15*^{INK4b} methylated cells were found in patients as their disease evolved into AML suggesting a relationship with leukemic transformation.[24] While a detailed understanding of the methylation changes driving the pathogenesis of MDS has not yet been reached, the effort to clarify the pertinent changes to be used for prognostication and targeted therapy is well under way.

Other Hematologic Malignancies

Acute Leukemias—Hypermethylation of the 5′ CpG island within the *p15*^{INK4b} gene is found in the overwhelming majority of acute myelogenous leukemia (AML) series studied, thus confirming its role in AML, particularly in light of the fact that this aberrant methylation is not detected in normal bone marrow progenitor cells.[25–27] While *p15*^{INK4b} methylation is the most frequently associated with AML, several other gene promoters have demonstrated aberrant methylation including *ER*, *PR*, *RIL*, *BRCA1*, and *Apaf-1*, among others.[22,28–31]

Several genes have been identified as being aberrantly mutated in acute lymphocytic leukemia (ALL) and are thought to contribute to the cellular proliferation and apoptosis resistance found in leukemic cells. Some of these candidate genes include those that target the cell cycle, such as *p21*, *p15* and *p16*, and those that influence apoptosis, including *DAPK* and *TMS-1*. In addition, *E-cadherin* and *H-cadherin* both methylated in ALL are thought to inhibit apoptotic signaling in addition to their cell adhesion effects.[32–35] The number of methylated genes in a series of 251 patients with ALL was analyzed and found to correlate with survival such that the survival rate at 11 years in those patients with only 1 methylated gene was 45.5% vs 7.8% in the group with 4 or more methylated genes ($p = 0.0004$).[36] As techniques in identifying aberrant methylation have improved, the ability to detect wide-scale methylation changes has evolved. Several more candidate genes have been identified in lymphoblasts from ALL patients and have even allowed differentiation between B- and T-ALL. *In vitro*, modulation of the methylation status of these genes by exposure to demethylating agents as well as demonstration of cell line growth inhibition has been demonstrated providing a basis for future targeted therapies in this disease.[37] While the ability to restore gene expression after exposure to demethylating agents has been proven in a variety of cell lines, whether this restoration of gene expression results in cell growth inhibition has not been demonstrated to our knowledge.

Chronic Leukemias—In contrast with AML, chronic myelogenous leukemia (CML) samples have not demonstrated a consistent inactivation of *p15*^{INK4b}.[26,38] Interestingly, methylation of *p15*^{INK4b} was not detected even in patients with blast crisis. Methylation of the *C-ABL* promoter has been studied in patients with CML and appears to correlate with disease progression.[39] Though of questionable prognostic significance, the changes in methylation of the oncogene promoter are apparent in a proportion of patients with chronic phase CML and appear to increase in patients with blast crisis. *In vitro* treatment with IFN-alpha demonstrated reversion of the promoter to an unmethylated state and correlated with response to the agent.[39–41] The reported lack of methylated *p15*^{INK4b} in CML samples has been attributed to less sensitive methods of detection. In an attempt to identify aberrant methylation through more sensitive methods, Nguyen, et al. utilized a quantitative bisulfite PCR assay and detected *p15*^{INK4b} methylation in 24% of CML samples from all phases of disease.[42] Furthermore, *C-ABL* promoter methylation did not appear to have prognostic significance.

In patients with chronic lymphocytic leukemia (CLL), the predominant finding has been global hypomethylation, though hypermethylation of some gene promoters have been noted such as

E-cadherin[43] and *DXS255*.[44] Though analysis for hypermethylation has principally been undertaken one gene at a time, the utility of genome wide methylation detection has become more apparent as the methodology has evolved. Also termed restriction landmark genomic scanning (RLGS), the process involves two-dimensional electrophoresis and utilization of radiolabeled restriction endonucleases which are methylation sensitive resulting in a map of radiolabeled unmethylated sites in the DNA being studied.[45] A total of 193 methylated sequences were identified in a series of 10 CLL samples and were deemed to be nonrandom changes. One gene with a hypermethylated promoter, *G-protein coupled metabotropic glutamate receptor 7* (*GRM7*), was evaluated further because of its 5′ CpG spanning the regulatory region as well as its effects on signaling in apoptosis.[46] Methylation of *GRM7* was confirmed by bisulfite PCR sequencing. *In vitro*, treatment with decitabine resulted in upregulation of expression of *GRM7*, even at low doses. In a similar study also utilizing RLGS, secreted frizzled-related protein 4 (*SFRP4*), a Wnt pathway anatagonist, was found to be methylated in all of the CLL samples studied. Treatment of cells with decitabine restored gene expression. However, whether this was associated with inhibition of cell growth was not reported.[47]

Solid Tumors—A detailed description of the methylation patterns of every solid tumor is beyond the scope of this article. However, analysis has revealed several key aberrantly methylated genes common to several tumor types.

Perhaps one of the most extensively studied of such genes is *human MutL homolog 1*, *hMLH1*, a mismatch repair (MMR) gene. Deficiency in MMR has been a hallmark of colorectal cancer arising from hereditary nonpolyposis colorectal cancer (HNPCC) and is detected in an estimated 13% of sporadic colon tumors.[48,49] Sporadic cases of colorectal cancer are associated with hypermethylation of *hMLH1*, resulting in a deficiency in MMR, and are associated with those tumors exhibiting microsatellite instability (MSI).[50] *In vitro* utilization of decitabine results in reexpression of *hMLH1* as well as restoration of the cells' mismatch repair ability. Quantitative anaylsis of *hMLH1* methylation has revealed differences in sporadic MSI colorectal tumors from those arising out of HNPCC, a distinction that has been often difficult to make.[51] A significant difference in methylation of *hMLH1* has been detected in MSI tumors based on the site of origin such that proximal tumors are associated with a significantly greater frequency of *hMLH1* than distal colon cancers further supporting the heterogeneous nature of these cancers.[52] Promoter hypermethylation of *hMLH1* with associated decreased mRNA protein expression has been detected in over half of non-small cell lung cancer specimens in one series. Furthermore, matched sputum samples from this cohort demonstrated these methylation changes as well.[53] Amongst samples from patients with adenocarcinoma of the lung, the decrease in *hMLH1* expression differed by sex with over 80% of samples from women and about half of samples from men demonstrating reduced protein expression.[54] The rates of lung cancer amongst women without a history of smoking are much higher in Taiwan than in other places including the U.S. In a series of non-smoking female patients from Taiwan, the methylation of *hMLH1* was detected in almost 70% of samples.[55] Whether these epigenetic changes can be found in women without a smoking history in other parts of the world or whether they will impact prognosis and treatment response remain to be determined.

Approximately, 13% of ovarian cancer samples display hypermethylation of the *hMLH1* promoter, with concomitant decrease in *hMLH1* protein expression in tumors. *In vitro* studies of ovarian cancer cell lines display increasing methylation of *hMLH1* with increasing cisplatin resistance. Pre-treatment with 5-azacitidine restored platinum susceptibility in these cell lines.[56] In a patient series, the methylation status of hMLH1 in patients treated with carboplatin and a taxane revealed that 25% of patients developed hypermethylation of the promoter at relapse when they did not demonstrate increase in methylation prior to exposure to chemotherapy.[57]

Furthermore, hypermethylation at relapse correlated with poorer survival independent of other factors.

Though inactivation of the BRCA1 gene has been most commonly associated with inherited forms of breast cancer, its hypermethylation is thought to play a role in some sporadic tumors as well. Up to 30% of sporadic breast tumors are thought to have a *BRCA1* hypermethylation. In a study confirming this percentage, Wei, et al. demonstrated that the methylation was more common in tumor samples from young patients with hormone receptor negative, high grade tumors, an observation similar to that seen in patients with hereditary *BRCA1* deletion.[58] Approximately 15% of sporadic ovarian tumors demonstrate *BRCA1* hypermethylation.[59, 60] In a series comparing the methylation status of *BRCA1* amongst tumor samples from patients with benign ovarian tumors, borderline tumors as well as carcinomas, promoter methylation was detected in 31% of carcinomas but in none of the benign or borderline tumors.[61] Interestingly, decreased *BRCA1* expression was noted in 16% of benign tumors, 38% of borderline tumors and 72% of carcinomas suggesting the possibility that pathways upstream to *BRCA1* are altered resulting in diminished expression without evidence of loss of heterozygosity or hypermethylation of *BRCA1* in the low grade tumors. In the ovarian cancer samples, loss of heterozygosity and hypermethylation were thought to account for the decreased *BRCA1* expression. A significant correlation between methylation of *BRCA*1 and reduced protein expression was demonstrated.

A number of genes have been associated with hypermethylation in non-small cell lung cancer patients. In fact, hypermethylation of several genes has been associated with a decrease in overall survival.[62,63] However, few of these studies have been replicated with the exception of those involving the tumor suppressor gene *p16*, the product of which inhibits cyclin-dependent kinase 4. In the largest series to date by Gu, et al., tumor samples from 150 patients with various stages of non-small cell lung cancer of either squamous or adenocarcinoma histology were analyzed.[64] Those with methylated *p16* had a statistically significant decreased median survival compared to those who did not (21.7 months vs. 62.5 months, $p = 0.0001$). Methylation of the *p16* promoter is thought to be induced by tobacco smoke exposure as its direct correlation with number of pack-years smoked, duration of smoking history and indirect correlation with time elapsed since smoking cessation have been statistically significant in a large series of patients with NSCLC.[65] When studied in combination with other genes in a series of samples from patients with NSCLC, healthy volunteers as well as smokers without evidence of lung cancer, the hypermethylation of *p16* appeared to be a later event during lung cancer progression. None of the 4 genes studied was hypermethylated in the normal volunteers.[66] The above are a few examples of genes hypermethylated in various solid tumors, which may contribute to the establishment and/or progression of cancer. A more detailed list is included in Table 1.

Methylation Changes as a Biomarker

The research associating methylation changes with the development of cancer have identified these epigenetic modifications as early events thought to often precede the appearance of tumor. Furthermore, investigations into whether therapeutic intervention of these epigenetic changes can alter the course of disease are being undertaken. A corollary to these observations is an interest in utilizing methylation changes as biomarkers to aid in the detection and monitoring of cancer. Though current screening methods for breast cancer have made significant strides in the detection of early stage breast cancer, the combination of mammography and MRI have a combined specificity of 77% and a sensitivity of 94% with increased cost.[67] Whether the incorporation of MRI into the screening of high-risk women improves survival remains to be determined. The quest for more accurate detection of breast cancer in women has led to the analysis of nipple duct fluid as a means of direct assessment. However, the process has been

hampered by difficulties in sampling of nipple duct fluid as well as the lack of predictive capability by cytologic analysis.[68] In one series, the methylation status of 11 tumor suppressor genes from ductal epithelial cells predicted sporadic and hereditary cancer with an accuracy approaching 90%.[69] Importantly, it appears that methylation changes are apparent in the ductal epithelial cells obtained from nipple duct fluid regardless of the location of the primary breast tumor.

In patients with non-small cell lung cancer (NSCLC), the heterogeneity in clinical behavior amongst patients with early stage disease has prompted the investigation of methods to improve prognostication. In a nested case-control study of patients with stage I NSCLC, promoter methylation of the cyclin-dependent kinase inhibitor 2A gene *p16* and the H-cadherin gene *CDH13,* was associated with an odds ratio of recurrent cancer of 25 when primary tumor and resected lymph nodes were evaluated by methylation specific PCR.[63] This association was independent of age, race, sex, tumor histology, stage or smoking history. While efforts to predict the risk of recurrence in patients with an existing diagnosis of NSCLC will hopefully result in the ability to determine which patients need adjuvant therapy, research into methods to screen patients at risk for NSCLC may have even more far-reaching effects. In the first prospective study of its kind, Belinksy, et al. studied the methylation pattern of 14 genes in exfoliated cells from sputum.[70] Study participants were recruited beginning in 1993 as part of the University of Colorado Cancer Center Sputum Screening Cohort Study and had a history of at least 30 pack-years of smoking with no prior diagnosis of cancer. Participants were followed periodically and sputum samples obtained routinely. The methylation patterns of sputum cells from patients who developed NSCLC were compared with those patients who did not. Of the 14 genes studied, 6 were identified as being associated with a greater than 50% likelihood of developing cancer. When 3 or more of the 6 genes were methylated, a 6.5 fold increased risk of cancer was noted. The sensitivity and specificity of this association was 64%. In a similar study, the sputum samples obtained from patients with and without squamous cell lung cancer participating in a lung cancer surveillance study were analyzed.[71] The presence of methylation of the *p16* and *O^6-methylguanine-DNA methyltransferase* (MGMT) promoters were determined by PCR. Aberrant methylation was detected in 100% of the patients with squamous cell carcinoma and was identified in samples from up to 3 years prior to diagnosis. The finding of p16 and MGMT methylation in high risk patients without cancer approximated their lifetime risk of lung cancer. At the time the study was published, two of these patients did in fact develop lung cancer. The search for a screening approach in patients at risk for NSCLC continues, however, results from prospective studies such as this certainly provide a basis for future investigations. The determination of additional methylated genes can perhaps increase the accuracy of sputum analysis and may provide a determination of which patients would benefit from frequent radiologic assessment.[70]

Hepatocellular carcinoma (HCC) poses challenges in early detection both in the U.S. and globally, and improvements in screening in high risk patients could have a far-reaching impact in this often incurable disease. Using blood samples from a community screening program in Taiwan, the DNA of patients with and without a diagnosis of HCC was extracted and methylation specific PCR was performed to detect the pre-diagnosis methylation status of three genes, *p15, p16* and *ras association domain family 1a (RASSF1A).*[72] Amongst the 50 cases of HCC, *RASSF1A* was the most frequently methylated at a rate of 70%. Six HCC patients did not demonstrate methylation of any of the three genes. When combined with other risk factors such as hepatitis B surface antigen status, anti-hepatitis C antibody status, smoking and alcohol use, the hypermethylation biomarkers had a predictive accuracy of 89%, a sensitivity of 84% and a specificity of 94%. Methylation changes were detected in the samples up to 9 years prior to the diagnosis of HCC. The current method of screening patients at risk for HCC includes alpha-fetoprotein testing in combination with ultrasound. The effectiveness of such an approach remains controversial and the establishment of a molecular signature predicting for

the development of HCC could prove useful, particularly in the setting of chemopreventive techniques. The ease of blood sampling may also increase compliance and potentially be more cost-effective and sensitive than routine radiologic screening.

As research into the treatment of various cancers has progressed over the last few decades, the notion of customizing therapy to a particular tumor's characteristics has gained increased interest. No example better illustrates this concept than the use of temozolomide in the treatment of glioma. The use of this alkylator in combination with radiation therapy became standard of care in the adjuvant treatment of glioblastoma with the demonstration of a clinically meaningful survival advantage of 2.5 months in the group that received temozolomide with radiation therapy as opposed to those who received radiation alone.[73] Given that *MGMT* repairs the damage caused by such alkyating agents as temozolomide, an investigation into whether *MGMT* methylation affected response to the drug was initiated.[74] The *MGMT* promoter methylation status was evaluated in the tumors of patients enrolled in the adjuvant trial comparing radiation therapy with and without temozolomide described above. *MGMT* promoter methylation was detected in 45% of the assessable cases and was associated with a more favorable prognosis regardless of the therapy administered. Among those patients who received temozolomide with radiation therapy, the presence of *MGMT* promoter methylation was associated with a survival advantage with a median survival of 21.7 months compared to 15.3 months in the radiation alone cohort ($p = 0.007$). An association has also been described between *MGMT* promoter methylation and the incidence of pseudoprogression on MRI (defined as the radiologic appearance of tumor progression due to increased contrast enhancement). This radiographic change is thought to be caused by blood-brain barrier disruption from radiation therapy resulting in capillary disruption, fluid transudation and finally brain edema. In a series of patients with glioblastoma treated with radiation therapy and temozolomide, MRI pseudoprogression was noted in 91% of patients with *MGMT* promoter methylation and in 41% of those with unmethylated promoter ($p = 0.0002$).[75] Overall survival was significantly longer in patients with *MGMT* promoter methylation and in those patients who developed radiologic pseudoprogression ($p = 0.001$ and $p = 0.045$, respectively). Thus, methylation status may potentially be used to offer treatment to those patients with glioma for whom it has the most chance of efficacy while also providing a means to ensure that treatment is not prematurely discontinued.

One disease in which genetic alterations have had a significant impact on use of noninvasive screening methods is colorectal cancer. With the publishing of the most recent joint guidelines of the screening and surveillance for the early detection of cancer and adenomatous polyps by the American Cancer Society, US Multi-Society Task Force on Colorectal Cancer and the American College of Radiology, the first time incorporation of a fecal DNA test has the potential to significantly impact providers' approach to colorectal cancer screening.[76] Though the test does not include a search for methylation changes, such DNA alterations in fecal DNA are found in patients with colorectal cancer and could impact the further development of screening tests in the future. In a series studying the fecal DNA of patients with colorectal cancer and adenomas as well as samples from normal individuals revealed a higher rate of methylation of the target genes studied in the patients with cancer.[77] In fact, of the three genes studied, over 96% of patients with cancer and 81% of patients with precancerous lesions had at least one methylated gene. In the normal individuals, only 1 patient of the 24 studied had a methylated gene. This analysis, which was performed using methylation-specific PCR had a sensitivity of 93.7% and a specificity of 77.1%. The presence of free-circulating tumor DNA in patients with colorectal cancer has provided an even easier option for testing in patients and a foundation on which to build further genetic testing.[78,79] Hypermethylated *p16* in circulating DNA of patients with colorectal cancer has been associated with advanced stage and has been found in patients with recurrent disease.[80,81] Methylation changes in the serum yielding a diagnosis of colorectal cancer could further build on existing testing and provide an even less

invasive means of diagnosis than fecal DNA testing. Epigenetic changes in the serum of patients already diagnosed with colorectal cancer provide a potential means of prognostication before and during therapy.

The disease most notably associated with hypermethylation due to the use of demethylating agents in its treatment is the myelodysplastic syndromes (MDS). In an assessment of methylation patterns of several key genes including $p15^{INK4B}$, E-cadherin (*CDH1*), *hypermethylated in cancer 1*(*HIC1*), and estrogen receptor (*ER*), hypermethylation was associated with leukemic transformation and poor prognosis in low-risk MDS.[82] In particular, patients with early MDS who demonstrated hypermethylation of at least one gene had a significantly shorter survival (20 months vs. 102 months, p=0.002). Analysis of the methylation patterns of $p15^{INK4B}$, *CDH1* and *HIC1* in elderly patients with high risk MDS or acute myeloid leukemia (AML) following MDS who underwent standard induction therapy revealed absence of complete responses (CR) in those patients with hypermethylation of all three genes (p = 0.03).[83] CR was achieved in over 50% of the patients with no hypermethylation which is the same or marginally better than CR rates in other studies of similar patients.[884,85] This was the first study to demonstrate the effect of methylation on the response to treatment amongst patients with high risk MDS and AML arising from MDS. Whether incorporation of agents that modulate hypermethylation can improve outcomes in these patients remains to be determined.

The epigenetic changes well-established in MDS and AML arising from MDS have also been investigated in other types of AML. While the attainment of a complete response with standard induction therapy is feasible in most patients, the relapse rate is greater than 50%. Prognostication of individuals at greater risk of relapse could prove useful in the effort to develop risk-adapted therapy. In an evaluation of over 180 patients with and without AML, methylation of *ERα* and $p15^{INK4B}$ occurred specifically in patients with AML and in none of the controls.[86] Increased methylation levels in one or both genes in patients in complete remission were associated with decreased relapse free survival. Importantly, all patients who demonstrated hypermethylation of *ERα* and $p15^{INK4B}$ relapsed whereas 17% of patients with methylation of neither gene had recurrence. The most obvious utility of studying methylation changes in cancer cells is the creation of novel agents; however, a very important potential outcome of this increasing knowledge is also in the arena of diagnostics. While the ultimate goal is the eradication of cancer through the development of preventive tactics, the fact remains that a significant proportion of the population will likely be affected by cancer and the development of biomarkers to use as diagnostic and monitoring tools can be an important part of our goals for the very near future.

Therapeutic Modulation of Gene Hypermethylation

Azacitidine

Azacitidine in MDS—Azacitidine (5-azacytidine) differs from its analogue cytidine by the substitution of a nitrogen atom for a carbon atom in the 5 position of the heterocyclic ring.[87] The mechanism of action of azacitidine as a hypomethylating agent is through its inhibition of DNA methyltransferase 1 in replicating cells. It is also cytotoxic through the incorporation into RNA of 5-aza-deoxycytidine triphosphate, a byproduct of a series of reduction and phosphorylation reactions of the parent compound 5-azacytidine.[88] *In vitro* studies suggest the predominant activity of azacitidine is DNA demethylation with lower dose administration.[89] In 2004, the Food and Drug Administration (FDA) approved azacitidine as an injectable suspension for the treatment of the following subtypes of MDS as designated in the FAB classification: refractory anemia (RA), RA with ringed sideroblasts (RARS) (if accompanied by neutropenia, thrombocytopenia or anemia requiring transfusion), refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEB-T) or chronic myelomonocytic

leukemia. The three trials which supported the FDA approval of the agent in the treatment of MDS were Cancer and Leukemia Group B (CALGB) 9221, 8921 and 8421, all sponsored by the National Cancer Institute. CALGB 9221 was a randomized controlled trial of subcutaneously administered azacitidine compared with best supportive care in patients with MDS. This randomized trial was undertaken as a result of the activity of azacitidine in two phase II studies.[90] Both studies, CALGB 8421 and 8921, investigated a 7 day continuous schedule of azacitidine at 75 mg/m^2/day, however, it was administered intravenously in the former and subcutaneously in the latter. Of the 43 evaluable patients in CALGB 8421, 21 had a response (49%) with a median survival of 13.3 months for all patients. The mean number of cycles required for best response was 3.8 with a range of 2 to 11 cycles suggesting the need for prolonged administration. CALGB 8921 produced similar results with a response rate of approximately 50% with a 27% complete response (CR) rate compared with a 12% CR rate in CALGB 8421.[91] Based on the activity of azacitidine in these phase II studies, CALGB 9221 was undertaken. In the trial, 191 patients were randomized to either azacitidine 75 mg/m^2/day administered subcutaneously for 7 days or best supportive care. Responses, defined as complete and partial responses as well as hematologic improvement (see Table 2 for response criteria), were detected in 60% of patients receiving the drug compared with 5% in the patients who received best supportive care ($p < 0.001$). Cross-over was allowed in the study and approximately half of the patients receiving supportive care eventually received azacitidine. To correct for this confounding effect, the survival analysis was performed on three different groups. In one group were the patients who either received supportive care throughout or changed to azacitidine after 6 months of supportive care. In a second group were the patients who were randomized to supportive care but who received azacitidine within the first 6 months of randomization. The final group included those patients who were initially randomized to azacitidine therapy. The analysis revealed a median survival after the landmark 6 month date of 11, 14, and 18 months, respectively. The difference in survival was significantly different between the group who received azacitidine when compared with the supportive care group regardless of whether the latter crossed over late or did not cross over at all ($p = 0.03$). Amongst the supportive care patients, those who crossed over early had a longer survival than those who crossed over late or never, however, this difference was not statistically significant ($p = 0.11$).[92] In addition to improved efficacy, quality of life assessments revealed significant improvements in multiple facets, particularly fatigue and psychological well-being in the azacitidine arm than in the supportive care arm.[93] At the time these important trials were conducted, the WHO classification of MDS, the International Working Group (IWG) response criteria and the International Prognostic Scoring System (IPSS) were not yet in use. To determine if the documented response rates in the three CALGB trials would change with the use of the WHO classification and the IWG response criteria, an additional comprehensive analysis including pathology review was undertaken.[94] The response rates across the studies were 40% to 47% validating the prior results. The baseline diagnoses of patients did not change significantly with the exception of the patients subsequently classified as having AML utilizing the WHO criteria. The response rates in the AML patients across the three studies ranged from 35% to 48%. Of those patients with AML, the median duration of response was 7.3 months and ranged from 2.2 to 25.9 months. Though upon reanalysis only 7% of the patients in CALGB 9221 were determined to have AML, the overall survival was 19.3 months in the patients assigned to the treatment arm compared with 12.9 months in the patients who received supportive care.

The use of azacitidine in these trials was at a standard dose of 75 mg/m^2/day though route of admininistration differed. While the response rates approximated each other across the studies regardless of whether the agent was given subcutaneously or intravenously, the lack of extensive pharmacokinetic data on this drug prompted a randomized trial amongst patients with MDS to determine the bioavailability and compare the single-dose pharmacokinetics of the two routes.[95] Patients received 75 mg/m^2 as a single dose in a cross-over design with a

minimum of 7 days and a maximum of 28 days between drug administrations. The area under the curve (AUC) values for the subcutaneous administration was 89% of those for the intravenous administration and ranged of 70% to 112% with a significantly longer half-life, 0.69 hours ± 0.14 hours compared with 0.36 hours ± 0.02 in the intravenous administration. This pharmacokinetic study helped to validate the more easily administered subcutaneous dosing. To further ease the treatment of MDS patients with azacitidine, a multi-center, phase II study of alternative dosing schedules was initiated.[96] Over 150 patients with both low and high risk MDS received azacitidine in one of three ways: A) 75 mg/m^2/day for 5 consecutive days, then 2 days of rest, followed by 2 additional days at 75 mg/m^2/day, B) 50 mg/m^2/day for 5 consecutive days, followed by 2 days of rest, and then an additional 5 days at 50 mg/m^2/day, and C) 75 mg/m^2/day for 5 days. Analysis of the initial 6-month phase of the trial demonstrates consistent rates of hematologic improvement and transfusion dependence among all three cohorts. The dosing schedules were all well tolerated though grade 3–4 hematologic adverse events were greatest in group A at 44% versus 18% in group C. The 12 month phase of the trial is ongoing, so whether the 7 day schedule can be curtailed remains to be determined, though preliminary results appear promising.

Since the results of the phase III trial demonstrating an overall survival advantage in patients with MDS who receive azacitidine compared to those who receive best supportive care only, another phase III trial (AZA-001) was reported which compared azacitidine to conventional care regimens (CCR).[97] CCR was defined as best supportive care, low-dose cytarabine or standard induction/consolidation regimens. Patients with higher risk MDS with an IPSS of Int-2 or High were eligible. A total of 358 patients were randomized at 79 international sites. Overall survival in the azacitidine group was statistically significantly higher than in the CCR cohort (24.4 months vs 15 months, p = 0.001). When azacitidine was compared with each of the three groups within the CCR cohort, the differences in the median overall survival times were as follows: 12.9 months in the best supportive care group (p = 0.0003), 9.1 months in the low-dose cytarabine patients (p = 0.016) and 8.7 months in the standard induction/consolidation group (p = 0.19). As in prior trials, azacitidine was tolerated well with no unexpected events. While the establishment of a CR has translated to a survival benefit in patients with AML, the same has not been established in patients with MDS, although the assumption has been that better responses result in better survival.[98] Analysis of the AZA-001 for survival benefit according to response has helped to partially dispel this notion. Patients who achieved a CR by azacitidine treatment benefited with a prolonged survival when compared to patients who achieved CR in the CCR group though this did not reach statistical significance (26.3 mo vs. 21.9 mo, p = 0.078). Similarly, patients who achieved hematologic improvement or a partial response also benefited suggesting that obtaining a CR is not a requirement to extend survival. Furthermore, these results indicate that the extension of azacitidine treatment in the absence of disease progression is a reasonable approach in an effort to better the chances of survival prolongation.[99] The initial studies of azacitidine in the treatment of MDS began over twenty years ago and its proven efficacy in multiple trials has placed it at the forefront of MDS therapy.

Azacitidine in Leukemia—One of the first published reports of the use of azacitidine in leukemia patients was published in 1973 as a phase I trial in pediatric AML resistant to cytarabine.[100] Of the 14 patients with AML, 5 achieved a complete response lasting at least 3 months. Of the 22 patients with ALL, one achieved a complete response for 3 months. The maximally tolerated dose in this trial was 150 to 200 mg/m^2 over 15 minutes for 5 days every 14 days with dose-limiting toxicities of nausea/vomiting and diarrhea at doses above 150 mg/m2. Myelosuppression accompanied nearly all of the responses. When investigated in combination with etoposide in children with AML who suffered from induction failures, the two drug combination resulted in a 60% complete response rate.[101]

Similarly, when investigated in combination with amsacrine and etoposide in children with AML in a randomized phase II study, the addition of azacitidine yielded significantly higher complete response rate in those patients who had failed primary induction than when the two drugs were used without azacitidine (53% vs. 18% p = 0.03).[102] Azacitidine appears to have some activity in pediatric patients with AML particularly those with primary induction failure, but remains to be studied in a randomized phase III trial to confirm these initial findings.

The activity of azacitidine in the MDS patients with high blast counts, later classified as having AML has prompted its study in adults with acute leukemia. In a retrospective analysis of patients with a diagnosis of MDS but were qualified as having AML by WHO criteria and had received azacitidine, an overall response rate of 60% was detected. Complete responders comprised 20% of the cases analyzed.[103] As previously discussed, in the combined analysis of CALGB 8241, 8921, 9221 after the WHO criteria were developed, the 27 patients classified as having AML demonstrated a median overall survival of 19.3 months compared with 21.9 months in the 25 patients who received best supportive care alone.[94] In a series of 17 patients with AML, azacitidine was administered at a dose of 75 mg/m^2 subcutaneously for 5 days every 4 weeks. The patients were nearly evenly represented with regard to those who were receiving azacitidine as first line therapy and those who had relapse or were refractory to standard induction. The overall response rate was 65%, the majority of which were complete responses. Response was observed after a median of 3 cycles per patient. Patients who underwent first line therapy did better than relapse/refractory patients. Median duration of response was 18 weeks. The five day regimen was well-tolerated and further supports the activity of single agent azacitidine in AML.[104] In a series of 33 patients over the age 60 with AML, patients were offered azacitidine, standard induction therapy or supportive are alone. When compared with standard induction therapy, those patients who received azacitidine had similar median survival rates (276 days vs. 397 days, p = 0.7). The patients who received azacitidine had significantly less transfusional support, bacteremia as well as days in the hospital.[105] In a phase II study in elderly patients with AML, patients were given standard 7 day dosing of azacitidine followed by gemtuzumab on day 8.[106] Of the 13 patients treated thus far, 11 achieved a CR (76%) with no treatment related toxicities. Nine patients remain alive and in CR at a median of 7 months of therapy. All treatment was administered as an outpatient. Completion of this study may provide an option for elderly patients with AML in whom treatment related mortality from standard induction therapy remains approximately 30%.

Azacitidine in Solid Tumors—Initial studies investigating the efficacy of azacitidine in patients with solid tumors date back over 30 years ago, though with disappointing results. In one series of over 150 patients, five partial regressions were seen which lasted 28 to 77 days. At a starting dose of 225 mg/m^2 for 5 days every 3 weeks, toxicity resulted in a dose reduction to 150 mg/m^2.[107] Responses were few and were associated with significant toxicity resulting in 13 deaths. Published in the same year, in a series of 177 patients, a lower dose with a more prolonged schedule resulted in a 17% response rate in patients with breast cancer and 21% in patients with lymphoma. Though this schedule was less toxic, the responses were transient and resistance appeared to develop quickly.[108] Since these early trials, the investigation of azacitidine in patients with non-hematological cancers had nearly halted until recently when a more in depth understanding of the epigenetic modulation of cancer evolved. Based on their preclinical work demonstrating the ability of azacitidine to reverse *in vitro* platinum resistance in ovarian cancer cells, Bast and colleagues administered azacitidine at 75 mg/m^2 for 5 days followed by carboplatin at an AUC of 5 on day 2 repeated every 28 days after its tolerability was demonstrated in a phase I trial.[109] Thirty patients with confirmed ovarian cancer who had either progressed within six months of platinum therapy or who progressed while receiving a platinum were eligible for participation. When including stable disease, the response rate was

47% with a one-year survival rate of 53%. Platinum resistant patients had even better responses when compared to the patients with platinum refractory disease.

In patients with prostate cancer, loss of androgen receptor expression is associated with resistance to androgen blockade and is thought to result from hypermethylation of the androgen promoter. *In vitro* data suggests that exposure to a demethylating agent can reverse this loss of androgen receptor expression and possibly restore responsiveness to androgen blockade.[110] Interim results of a phase II trial in patients with castration resistant prostate cancer who were treated with azacitidine at 75 mg/m^2 for 5 days of every 28 days with continued complete androgen blockade suggests this reversal may be possible.[111] The primary endpoint was modulation of prostate specific antigen (PSA) doubling time. Of the 20 evaluable patients thus far, the PSA doubling time was significantly prolonged when compared to baseline ($p = 0.01$) with a median PFS of 13.1 weeks. Biologic evaluation included plasma DNA methylation which was noted to be significantly decreased over time Studies of azacitidine in patients with solid tumors at the doses and schedules initially investigated did not demonstrate significant anti-tumor activity. As the knowledge of the role of methylation in epithelial cancers has evolved, trials investigating azacitidine, particularly in combination with other agents, permit the scientific community to revisit the possibility of its use in solid tumors.

Decitabine

Decitabine in MDS—Decitabine, first synthesized over 40 years ago, is also known as 5-aza-2′-deoxycitabine. Its mechanisms of action are attributed to the ability of its triphosphate metabolite to incorporate into DNA resulting adduct formation with DNMT leading to cellular differentiation or apoptosis. Because decitabine contains a deoxyribose group, it is only incorporated into DNA, unlike azacytidine which is able to incorporate into RNA. At relatively low doses, the depletion of DNMT by decitabine causes DNA demethyation and gene re-expression.[112,113] Phase II studies of decitabine in patients with high-risk MDS demonstrated its initial efficacy. One of a 72-hour continuous infusion to 29 patients resulted in a response rate of 54%.[114] The major toxicity was myelosuppression which resulted in 5 toxicity associated deaths. The results of this small study prompted a larger, multi-institutional phase II study also in elderly patients with high-risk MDS.[120] Decitabine was administered to 66 patients at a dose of 15 mg/m^2 over 4 hours, every 8 hours for 3 days. Dosing was repeated every 6 weeks. The prior results were confirmed with an overall response rate of 49%, for patients with IPSS high-risk score, the response rate increased to 64%. Response was defined as complete and partial responses as well as hematologic improvement. Myelosuppression was the most common toxicity and resulted in a 7% treatment related death rate. The pivotal phase III, which supported the FDA approval of decitabine, was a randomized trial initiated on the basis of decitabine's efficacy in phase II trials included 170 patients with a diagnosis of MDS.[83] Patients received either 15 mg/m^2 every 8 hours for 3 days repeated every 6 weeks or best supportive care. Patients who received decitabine had a significantly higher overall response rate (defined as CR + PR) (17% vs. 0%, $p < 0.001$), as well as a longer median time to AML transformation or death (12.1 months vs. 7.8 months, $p = 0.16$). Median survival did not differ significantly between the two groups though transfusion requirements were significantly less in the treatment arm. The discrepancy in response rates between this phase III trial and the prior phase II trials has been postulated to be secondary to the higher median number of cycles administered in the latter.[83]

The FDA approved schedule of 15 mg/m^2 administered as a continuous three hour infusion every 8 hours for 3 days can be cumbersome due to the schedule and instability of the agent making it not very amenable to outpatient treatment. Preclinical data suggest a more protracted administration may be more efficacious.[87] Based on these observations, a phase I trial of varying doses of decitabine was undertaken in patients with MDS and acute leukemia at doses

ranging from 5 to 20 mg/m2/day intravenously over 1 hour for 10 doses over 2 weeks.[116] Some patients received a more prolonged administration at 15 mg/m^2 for 15 to 20 days. Though the therapy was well-tolerated and responses were seen at all dose levels, the patients who were treated at 15 mg/m^2 for 10 days had the highest response rate at 65%. Dose escalation or prolonged administration resulted in a response rate of 11%. Based on these findings as well as the notion that maintenance of higher dose intensity maximizes hypomethylation, a randomized study of three dosing schedules in patients with MDS and CMML was initiated.[117] Ninety-five patients were treated with one of three dosing schedules: (1) 20 mg/m^2 intravenously each day for 5 days, (2) 20 mg/m^2 subcutaneously daily for 5 days, (3) 10 mg/m^2 intravenously each day for 10 days. Courses were repeated every 4 weeks instead of every 6–8 weeks and treatment was administered regardless of myelosuppression, unless complications due to low blood counts were encountered. The highest CR rate was in the 5 day intravenous schedule (39%) compared with 21% in the 5-day subcutaneous group and 24% in the 10 day intravenous group. At the time of publication, 65% of patients on the 5 day intravenous schedule were still on therapy compared with 21% and 35% in the subcutaneous and 10 day intravenous schedules, respectively. Pharmacodynamic analysis of LINE-1 methylation as a marker of global methylation as well as the methylation status of *p15^{INK4B}* was performed. Results of these analyses demonstrated the greatest hypomethylation and induction of *p15^{INK4B}* in the cohort with the highest complete response rate. That the median number of cycles administered in this study was 6+ compared with a median of 3 cycles in other decitabine and azacitidine studies implies the utility of administration at a higher dose intensity. Though the CR rate of 39% in the 5 day intravenous dosing was superior to the other dose schedules in this study, the 21% CR rate in the more easily administered subcutaneous schedule merits consideration of a randomized comparison of the two.

Though the evidence suggests that at least 3 cycles of treatment may be required to yield the best response when treating MDS patients with decitabine or azacitidine, the optimal treatment duration is unknown as well as the optimal treatment approach upon disease recurrence. This question was posed as part of a trial by Ruter, et al.[118] Patients with MDS were enrolled based on their prior participation in phase II trials in which the use of decitabine was continued for 2 additional cycles beyond best response. In the 22 patients studied, decitabine retreatment at the standard dose of 15 mg/m^2 over 1 hour, three times per day for 3 days resulted in a response rate of 45%. Despite the ability to again achieve response with decitabine retreatment, the quality and duration of response were not equal to that achieved initially suggesting that extension of therapy and possibly maintenance dosing may become a component of MDS treatment approaches. As more and more patients previously treated with azacitidine or refractory to azacitidine were encountered, a trial to investigate the response rate to decitabine in patients with prior azacitidine exposure was undertaken.[119] Fourteen patients were treated with dectiabine at 20 mg/m2 intravenously over 1 hour for 5 days, and repeated every 4 weeks. An interim analysis demonstrated a CR and PR rate of 28% with decreases in global methylation detected. While decitabine appears to demonstrate activity in patients with MDS whose disease progresses on or is refractory azacitidine, these results are preliminary and are not derived from a randomized trial.

Decitabine in Leukemias—In one of the first published reports of the use of decitabine in patients with previously treated ALL and AML, the response rate, which included complete and partial responses, was 37% utilizing a continuous infusion dosing schedule ranging from 36 to 60 hours.[120] The survival after response was 5 months ± 3 (mean, ± SD) and responses were short-lived. Since initial observations of activity in acute leukemia, the study of decitabine has also been performed in patients undergoing allogeneic transplantation. In a series of patients with AML or transformed CML who had relapsed after prior transplant, decitabine was administered prior to a repeat infusion of stem cells from the original donor.[121] Fourteen patients were treated with doses of decitabine ranging from 100 to 150 mg/m^2 every 12 hours

for 5 days. Therapy was very well-tolerated with no grade 3 or 4 toxicities attributed to the agent. Eight of the 14 patients had a complete or partial hematologic remission, the latter defined as no evidence of peripheral or bone marrow blasts but with platelet count below 100/μL. Median survival was 190 days (11 to 1215 days). When high-dose decitabine was incorporated into a preparative regimen with busulfan and cyclophosphamide in patients with hematologic malignancies eligible for allogeneic stem cell transplant, 21 of 23 patients were engrafted and achieved disease remission.[122] The median survival was 17.2 months with a disease free survival of 8.9 months. With a long term follow-up of over 3 years, 26% remained alive. Amongst the patients with AML, 40% were alive at the end of 3 years. While decitabine appears to have clinical activity in patients with acute leukemia, its precise role remains to be determined by the multiple ongoing trials in the disease.

The anti-leukemic effects of dectiabine were also investigated in patients with CML whose disease was in either blastic or accelerated phase. Patients were given doses of either 75 mg/m2 over 6 hours, every 12 hours for 10 doses (750 mg/m^2/cycle) or 100 mg/m^2 over 6 hours, every 12 hours for 10 doses (1000 mg/m^2 per cycle).[123] Overall response rates were 25% in the patients in blastic phase and 53% in accelerated phase, with myelosuppression being the most common serious side effect. Based on these promising results, a larger trial was initiated in 130 patients with CML in various phases.[124] Patients initially accrued to the trial were treated with 1000 mg/m^2 per course (100 mg/m^2 over 6 hours, repeated every 12 hours for 5 days) and this dose was eventually reduced to 500 mg/m^2 per course (50 mg/m^2 over 6 hours, repeated every 12 hours for 5 days) based on the occurrence of prolonged and delayed myelosuppression. Objective response rates were 28% in the blastic phase, 55% in the accelerated phase and 63% in the chronic phase with a 3 year survival rate of 27% in the accelerated phase. Similar to prior experiences in MDS, the time to best response was after a median of 3 courses supporting the notion of improved drug activity with lower dose and more prolonged drug administration, possibly by increased hypomethylation. The results of this study preceded the advent of imatinib, which completely changed the treatment approach to CML patients. As the development of intolerance and resistance to the drug was increasingly encountered, the search for effective agents in this arena was actively pursued. In that vein, a phase II study in CML patients of all phases who were either intolerant or refractory to imatinib therapy was initiated.[125] Thirty-five patients were treated with decitabine at a dose of 15 mg/m^2 intravenously over one hour daily for 5 days followed by a 2 day rest and then repeated for another 5 days. Each cycle would be repeated every 6 weeks. After the first 10 patients were treated, the dose was lowered to 10 mg/m2 with the same schedule because of prolonged myelosuppression. Because of prior data supporting a longer duration of treatment, it was recommended that patients be treated with at least 2 cycles whether or not a response was seen. The total hematologic response rates defined as the rates of complete and partial responses were 83% among the patients with chronic phase disease, 41% in those with accelerated phase and 34% in the patients with blastic phase. The highest rate of major cytogenetic responses was detected in patients with chronic phase at a rate of 25%. Duration of responses was generally short-lived, but the similarity of response rates to decitabine in this setting and in the trial of imatinib naïve patients support a differing mechanism of action between the two agents. Imatinib resistance does not appear to imply decitabine resistance. The activity of the agent in this setting merits its study in combination with novel therapies and could provide an option in patients whose disease loses imatinib sensitivity.

Given the differing mechanisms of action of imatinib and decitabine, the subsequent logical pursuit was the combination of the two agents in patients with CML. *In vitro* synergy of the two agents further supported the endeavor, although continued imatinib sensitivity appeared to be necessary for this synergy.[126] Twenty-eight patients with CML in either accelerated or blastic phase were given 15 mg/m^2 of decitabine intravenously for 10 days over a 2 week period with 600 mg of imatinib daily as part of a phase II trial.[127] About 90% of the patients had

previously been treated with imatinib. The rate of combined complete and partial responses were 45% in the patients in accelerated phase and 20% in those in blastic phase such that combination therapy did not impart a greater advantage than decitabine alone in this population of imatinib exposed patients. These results were consistent with the in vitro studies of combination imatinib treatment which suggested the requirement for residual imatinib sensitivity for the combination therapy to be any more effective. Thus, decitabine therapy does not appear to reverse imatinib resistance.

Decitabine in Solid Tumors—Like azacitidine, early evidence demonstrating any activity of decitabine in solid tumors have been scant. In one of the earliest trials of the agent in solid tumors, a phase I trial with pharmacokinetics identified 3 infusions at 75 mg/m^2 over 1 hour every 7 hours as the recommended phase II dose.[128] Myelosuppression was the dose limiting toxicity with a nadir at day 22. One patient with an undifferentiated tumor of the ethmoid sinus responded to therapy at a dose of 100 mg/m^2 and was in a complete response 15 months after therapy. In a followup trial by the EORTC, decitabine was administered to approximately 100 patients with colorectal cancer, squamous cell carcinoma of the head and neck, melanoma or renal cell carcinoma at a dose of 75 mg/m^2 intravenously for one hour, every 7 hours for 3 doses and repeated every 5 weeks.[129] Only one partial response was noted in a patient with melanoma. Multiple clinical trials in other types of epithelial cancers yielded similar results with little to no activity in prostate, non-seminomatous testicular and ovarian cancers.[130–132] The existence of hypermethylated genes in non-small cell lung cancer as well as the activity of decitabine in lung cancer cell lines.[133] prompted multiple clinical trials of the agent in patients with advanced disease. In a phase I/II trial, decitabine was administered as a single 8 hour infusion ranging from 200–660 mg/m^2 in pretreated patients with lung cancer.[133] Of the 15 patients treated, 9 were assessable and had a median survival of 6.7 months. Three of these patients survived longer than 15 months.

The investigations of decitabine as a single agent in solid tumors yielded less than dramatic results, which prompted the pursuit of combination studies. *In vitro*, decitabine has demonstrated the ability to reverse cisplatin resistance in ovarian cancer cells as well as act synergistically with the platinum in multiple human tumor cell lines.[134,135] This combined with the fact that cisplatin is an accepted therapy in patients with non-small cell lung cancer resulted in a phase I/II trial of the combination in patients with advanced tumors.[136] In the phase I portion of the trial, a combination of 100 mg/m^2 of cisplatin was combined with escalating doses of decitabine. The recommended phase II decitabine dose based on the initial phase of this trial was 67 mg/m^2 as a one hour infusion daily for three days prior to cisplatin administration on the third day. In the phase II portion of the trial, 14 patients with previously untreated stage IIIB or IV non-small cell lung cancer were treated with the combination. There were no objective responses, a disappointing result particularly in light of the 15% response rate to single agent cisplatin in prior studies.

The lack of activity in these solid tumor trials led to a search for alternate dosing schedules in hopes of finding a more efficacious treatment approach. With the few responses seen in the trial of non-small cell lung cancer patients treated with an 8 hour infusion[133] Aparicio, et al. found that 20 to 40 mg/m^2 over a 72 hour infusion was well-tolerated with the expected dose limiting toxicity of myelosuppression. There were no objective tumor responses and methylation changes in tumor biopsies were not consistent.[137] Samlowski, et al. tested a 7 day continuous infusion in an effort to identify a maximally hypomethylating dose with ultimate goal of using decitabine in future combination trials. Dose-limiting myelosuppression was encountered at the lowest dose of 2 mg/m^2/day for 7 days. The methylation changes induced by decitabine were transient and reverted to normal soon after treatment cessation.[138] Building on these findings, a phase I trial investigating a 72 hour infusion of decitabine in patients with primary thoracic malignancies or extrathroacic primary cancers with thoracic metastases was

undertaken with the intent to also identify molecular changes induced by the agent.[139] Thirty-five patients were enrolled and the maximum tolerated dose was 60 to 75 mg/m^2 (which equaled total dose administered over 72 hour infusion). Those patients with fewer prior cytotoxic regimens were able to tolerate the higher dose. Tumor biopsies were performed on a proportion of patients to determine molecular response after treatment. Although there were no objective radiologic responses, approximately 30% exhibited a molecular response after treatment as evidenced by target gene induction.[139] The genes of interest in this study were *NY-ESO-1*, *MAGE-3* and *p16*. A tumor suppressor gene, *p16*, has been shown to be aberrantly methylated in lung cancer as previously discussed.[140] *NY-ESO-1* and *MAGE-3* encode cancer-testis antigens and are thought to be expressed in patients with lung cancer and can elicit an immune response by cytotoxic T lypmphocytes, though antigen expression in tumors is not thought to be robust enough to result in any significant cancer immunotoxicity.[141,142] In the phase I trial described above, 8 of 22 patients who underwent tumor biopsies demonstrated induction of one of the three genes studied. In the 3 patients' tumors where *NY-ESO-1* induction, serologic activity was noted suggesting that decitabine treatment could in fact elicit an immunogenic response. Clinical trials investigating decitabine at a lower dose and in a more chronic administration are ongoing.

Other Demethylating Agents

As described in the previous sections, the demethylating capabilities of the 5-azacytosine analogs prevail at lower doses with cytoxicity the primary mechanism of action at higher doses. In those studies in which methylation changes in target genes were noted after treatment with a demethylating agent, the hypomethylation effects were noted to be lost within days after therapy cessation. An orally bioavailable alternative to the currently approved agents would permit a more chronic dosing schedule with the potential for more durable methylation changes. Zebularine, a cytidine analog, which was first developed with the intent to use as combination therapy with nucleoside analogs because of its ability to inhibit cytidine deaminase, the enzyme responsible for inactivation of nucleoside analogs[143,144] Many years later, its ability to inhibit DNA methylation was characterized.[145] This newly discovered demethylating activity as well as the fact that zebularine was known to be more stable in aqueous solution than azacitidine and decitabine made it an attractive agent for development as a potential oral DNMT inhibitor. When administered chronically in a mouse intestinal cancer model, the average number of polyps decreased from 58 to 1 in female mice with decreased DNA methylation.[146] The therapy was well-tolerated in the chronic dosing schedule. However, when zebularine was administered orally to primates, bioavailability was less than 1%, a disappointing finding considering its *in vivo* activity when administered orally to murine tumor models.[147]

The activity of 5-fluorodeoxycytidine (FdCyd), a fluorinated pyrimidine, was initially ascribed to its deamination to 5-fluorodeoxyuridine (FdUrd), another fluorinated pyrimidine. After deamination, conversion of FdUrd to FdUMP resulted in an irreversible binding to thymidylate synthase and subsequent DNA synthesis inhibition.[148] However, the increased activity against tumor models of FdCyd when compared with other fluorinated pyrimidines yielded a search for other potential mechanisms of action. Furthermore, because the deamination of FdCyd was thought to be a necessity for the production of an active compound, then a logical conclusion may be that inhibition of cytidine and deoxycytidylate deaminase would result in less cyotoxicity. However, it was found that FdCyd was incorporated into the DNA of MCF-7 human breast cancer cells and that the addition of tetrahydrouridine (THU), a deaminase inhibitor, resulted in an increase in the extent of FdCyd incorporation into DNA and resultant cytotoxicity, which challenged this concept. FdCyd was additionally found to inhibit DNMT and decrease methylation of MCF-7 DNA.[149] Given the demethylating capability of FdCyd when used in combination with THU, a phase I trial of the combination was initiated and is ongoing.[150]

The addition of a 2-(*p*-nitrophenyl)ethoxycarbonyl (NPEOC) group to the N4 carbon of the azacytosine ring of decitabine created N4-NPEOC-DAC, another nucleoside analog.[151] *In vitro* studies have demonstrated the ability to cleave this NPEOC group by carboxylesterase enzymes resulting in the production of decitabine. The compound has demonstrated molecular changes in human cancer cell lines as evidenced by decreased global and gene specific methylation, however, this effect was limited to cells expressing carboxylesterase 1. The agent is still early in its development and i*n vivo* efficacy is being tested. Whether a bioavailable oral formulation of N4-NPEOC-DAC can be produced also remains to be determined.

All the agents discussed thus far are nucleoside analogs and require incorporation into RNA or DNA in order to inhibit DNMT. Non-nucleoside analogs which have demethylation capabilities appear to act by directly impairing the action of DNMT. RG108, which was designed to block the active site of DNMT1, is a small molecule with a significantly longer half-life in aqueous solution than 5-azacitidine (~20 days vs. 17 hours).[152] It has exhibited a significant concentration dependent demethylating capability in vitro without cytoxicity. In a comparison study with other non-nucleoside and nucleoside analogs, RG108 was the only agent which directly inhibited purified recombinant DNMT.[153] Other non-nucleoside analogs include hydralazine and procainamide, drugs that have been traditionally utilized for their anti-hypertensive and anti-arrythmic properties, respectively. Their DNA demethylating capabilities were first noted in 1988.[154] A more recent discovery has been the ability of (–)-epigallocatechin-3-gallate (EGCG), the principal polyphenol in green tea, to inhibit DNMT.[155] The biological effects of these agents were studied and compared with azacitidine by Chuang, et al and found that azacitidine had a much more profound effect on DNA methylation and gene reactivation in various cell lines than any of these agents.[156] However, the compounds did exhibit some degree of demethylation such that their tolerability and mild activity may be considered as part of a longer term therapy. The advent of novel agents and the identification of novel activities for known agents have provided the basis for further study in an evolving arena of cancer therapeutics and will hopefully yield agents that can be safely administered orally with improved efficacy.

Histone Acetylation and Methylation

The focus of this article has been on the role of DNA methylation in silencing the transcription of key tumor suppressor genes and thus promoting the development and progression of cancer. However, other epigenetic changes working in concert with DNA methylation affect either the establishment or promulgation of cancer and have the potential to be exploited in the development of novel therapeutic approaches. The configuration of the chromatin structure can vary and dictate whether certain genes will be transcribed. The nucleosome is comprised of 146 base pairs of DNA wrapped around a complex of eight histones. The configuration of nucleosomes is partly dependent on the methylation status of the genes such that its shape differs when genes are methylated and silent or unmethylated and transcriptionally active.[88,157] The configuration of the nucleosome is also affected by the acetylation of the lysine residues on the histones. Removal of acetylases by histone deactylase (HDAC) results in a positive charge thereby attracting the histones more tightly to the DNA causing transcriptional inactivation. Conversely, histone acetylase activity produces a negative charge on the histones such that the chromatin structure accessible for transcription. Aberrant activity of histone deacetylases is thought to play a role in carcinogenesis through the suppression of genes involved in cell differentiation and apoptosis.[158,159] Furthermore, methylation of lysine residues on particular histones by histone methylases also regulates their activity such that methylation of lysine-4 in histone-3 is associated with transcriptional activation whereas methylation of lysine-9 in histone-4 is associated with the converse[160,161] Methylation and histone acetylation work in concert to regulate gene transcription as evidenced by the key observation that the binding of 5-methyl-cytosine binding protein causes the recruitment of

HDAC.[162] *In vitro*, this interaction has been corroborated by the synergistic activation of several genes including, *p16CDKN2A*, with the concomitant exposure to an HDAC inhibitor with either azacitidine or decitabine.[163,164,165]

These findings subsequently provided the rationale for clinical trials investigating the combination of a demethylating agent with and HDAC inhibitor. The first trial published with this combination utilized azacitidine for 5, 10 or 15 days followed by a 7 day continuous infusion of sodium phenylbutyrate in patients with MDS or AML.[166] Responses were more frequent in those patients who received the most prolonged duration of azacitidine exposure, but treatment was well-tolerated in all groups. Correlative studies were a major focus of this trial, and interestingly, histone acetylation was detected prior to any phenlbutyrate exposure suggesting additional epigenetic effects of azacitidine. Administration of both agents was associated with acetylation of histones H3 and H4. In patients who demonstrated pretreatment methylation of *p15*, all patients who responded demonstrated demethylation after therapy. Conversely, none of the patients without a response exhibited promoter demethylation post-treatment. Valproic acid, which has an established role as an anti-convulsant and as a mood stabilizer, has been shown to inhibit histone deacetylase.[167,168] In a phase 1/2 study of the combination of azacitidine with escalating doses of valproic acid in patients with AML showed an overall complete response rate of 22%.[169] Median survival amongst patients who responded was 15.3 months (range, 4.6–20.2+ months). Induction of DNA hypomethylation and histone acetylation was evident in the patient samples, although effects were transient. In another trial, this time combining decitabine with valproic acid, an optimal biologic dose (OBD) of decitabine alone in patients with relapsed AML or elderly patients with de novo disease was first identified.[170] This OBD was defined as the dose at which at least 5 of 6 patients enrolled demonstrated a ≥100% increase in the mRNA expression of *p15* and/or *ER*. Decitabine was administered intravenously days 1 through 10 and repeated every 28 days. After the OBD was identified, valproic acid was added in increasing doses from day 5 until day 21. The OBD of decitabine was 20 mg/m2/day for 10 consecutive days. The maximum tolerated dose of valproic acid in combination with decitabine was 20 mg/kg/d with encephalopathy being the dose limiting toxicity. The overall response rate was 44% and re-expression of *ER* was significantly associated with leukemia response ($p < 0.05$). However, gene re-expression did not appear to be greater with the combination of the two agents when compared to the decitabine alone phase of the trial. Though responses were found in this trial, it is unclear what valproic acid contributed. This combined with its encephalopathy limit its development particularly in the elderly population with AML. The use of other HDAC inhibitors such as vorinostat have been initiated in combination with DNA demethylating agents based on single agent activity of vorinostat in AML and MDS.[171] Based on *in vitro* data demonstrating greater apoptosis with sequential therapy,[172] a phase I/II trial in patients with MDS and leukemia was conducted evaluation azacitidine administered for 3 days prior to the addition of vorinostat.[173] A total of 23 patients were treated and 18 were evaluable. The overall complete response rate was 61% with a probability of survival at 1 year of 86.4%. The median number of cycles to response was 2. The most significant adverse events were grade 3 fatigue which appeared to be related to the duration of vorinostat with those patients who received the drug for 14 days rather than 7 days more likely to experience the side effect. Based on supportive in vitro data, a phase I trial in AML patients is addressing the question of whether there is an improvement in clinical activity with sequential administration of a demethylating agent and an HDAC inhibitors.[174] In this ongoing trial, patients received vorinostat and decitabine either sequentially or concurrently. The maximum tolerated dose has not yet been reached in the three dose levels planned and no grade 3 or 4 toxicities have been encountered. Several clinical responses have been encountered thus far suggesting activity for the combination. A number of clinical trials with other HDAC inhibitors are also ongoing. The utility of the combination of an HDAC inhibitor with a demethylating agent illustrates another example frequently encountered in cancer therapy research; as the mechanisms of cell and

cancer growth are definitively understood more opportunities to exploit those integral survival mechanisms arise.

Conclusion

Though the knowledge that methylation of tumor suppressor genes dates back for almost 30 years, it is only recently that we have gained enough insight into this DNA modification to use it to our advantage. While the most obvious outcome of our increasing understanding of cancer epigenetics is the development of new therapies, these discoveries have also afforded us the possibility to better understand the development of cancer. Associations between cancer and inflammation, aging and environmental toxins have long been identified, but the sequence of events leading to carcinogenesis are now coming to light in the context of epigenetic changes. That the treatment of *H. pylori* infection could possibly halt the development of gastric cancer has far-reaching possibilities the world over. The discovery of azacitidine and decitabine decades ago initially thought only to be cytotoxic agents have now been more completely understood, and, as a result changed the treatment of myelodysplastic disease, for which we previously had no survival prolonging therapies. Future gains in pharmaceutical research will likely offer patients more efficacious, safer and orally bioavailable agents and further revolutionize treatment approaches. With every new discovery in the epigenetic landscape of tumors comes a new opportunity for targeted agents. As our understanding of the intricate machinery of the cancer cell continues to evolve, we are hopeful that the days of therapies targeting only the rapidly dividing nature of cancer cells begin to fade and be replaced by more sophisticated and effective means of cancer growth inhibition and ultimately, cancer prevention.

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Table 1

Methylated Genes by Disease Type

Disease	Methylated Genes	References
MDS	*P15^{INK4B}, CDH1, HIC1, FHIT, RIL, SOCS, RASSF1A, calcitonin*	Aggerholm, et al.,[82] Lin, et al.,[175] Boumber, et al, [22] Wu, et al.,[176] Johan, et al.,[177] Ihalainen, et al. [178]
Acute Myelogenous Leukemia	RIL, SFRP, NOR1, CDH13, p15, NPM2, OLIG2, PGR, HIN1, SLC26A4, ESR1, IGSF4, p73, SHP1	Boumber, et al.,[22] Jost, et al., [179] Kroeger, et al.,[180] Hess, et al.,[181] Schmelz, et al.,[182] Oka, et al.[183]
Acute Lymphocytic Leukemia	Hck, PARK2, PACRG, SHP1	Hoshino, et al.,[184] Agirre, et al.,[185] Oka, et al.[183]
Chronic Myelogenous Leukemia	PARK2, PACRG, SOCS1, p16, p14, CDH-13, JunB, SHP1, ABL1, p15, HOXA	Agirre, et al.,[185] Liu, et al.,[186] Nagy, et al.,[187] Roman-Gomez, et al.,[188] Yang, et al.,[189] Oka, et al.,[190] Shteper, et al.,[191] Nguyen, et al.,[42] Strathdee, et al.[192]
Chronic Lymphocytic Leukemia	p15, PTPRO, DAPK1, ARLTS1, HoxA4, SFRP, TWIST2, GRM7, hTERT	Papagergiou, et al.,[193] Motiwala, et al.,[194] Raval, et al.,[195] Petrocca, et al.,[196] Strathdee, et al.,[197] Liu, et al.,[47] Raval, et al.,[198] Rush, et al.,[46] Bechter, et al.[199]
Ovarian Cancer	BRCA1, ARLTS1, HOXA9, HOXB5, SCGB3A1, CRABP1, hMLH1, ER, RASSF1A, RAR beta, E-cadherin, H-cadherin, APC, GSTP1, MGMT	Esteller, et al.,[200] Esteller, et al.,[201] Petrocca, et al.,[196] Wu, et al.,[176] Watanabe, et al.,[202] Wiley, et al.,[203] Choi, et al.,[204], Makarla, et al.[205]
Non-Small Cell Lung Cancer	ARLTS1, RASSF2, p16, H-cadherin, RASSF1A, APC, MGMT, ASC, DAPK	Petrocca, et al.,[206], Cooper, et al., [207], Belinsky, et al.,[70] Toyooka, et al.,[208] Toyooka, et al.,[209] Machida, et al.,[210] Brock, et al.[211]
Colorectal Cancer	p16, RIL, RASSF2, p53, RAR, cyclin A1, CDH-13, DNMT, hMLH1, p14, APC, MGMT	Herman, et al.,[212] Boumber, et al.,[22] Cooper, et al.,[212] Vousden, et al.,[213] Rhee,[214] Gazzoli, et al.,[215] Shen, et al.,[216] Whitehall, et al.[217]
Breast Cancer	BRCA1, ID4, p16, RASSF2, ER, PR, E-Cadherin, MGMT, HOXA5, DAPK, Twist, cyclinD2, GSTP1, RAR-beta	Esteller, et al.,[200] Esteller, et al.,[201] Noetzel, et al.,[217] Herman, et al.,[219] Cooper, et al.,[207] Droufakou, et al.,[220] Sarrio, et al.,[221] Munot, et al.,[222] Xu, et al.,[223] Stearns, et al.[224]
Prostate Cancer	GSTpi, APC, MGMT, RASSF1A, p16, PTGS2, ARF	Lee, et al.,[225] Hu, et al.,[226] Yegnasubarmian, et al.,[227] Fu, et al.[228]
Renal Cancer	VHL, UCHL1, FHIT, DAPK, SFRP-1, CDH-1, PTGS2, RASSF1A, APAF-1, DLC1, HOXB13	Herman, et al.,[229] Kagara, et al.,[230] Kvasha, et al.,[231] Christoph, et al.,[232] Gumz, et al.,[233] Costa, et al.,[234] Christoph, et al.[235], Zhang, et al.,[236] Okuda, et al.[237]
Pancreatic Cancer	p16, RASSF1A, Cyclin D2, SOCS-1, RAR beta, APC, E-cadherin, DAPK	Fukushima, et al.,[238] Dammann, et al.,[239] Matsubayashi, et al.,[240] Fukushima, et al.,[241] Ueki, et al.,[242] Esteller, et al.,[243] Winter, et al.,[244] Dansranjavin, et al.[245]
Gastric Cancer	GSTP1, RASSF1A, COX-2, hMLH1, p16, DAPK, p14, p15	Kang, et al.,[246] To, et al.[247]
Sarcoma	RASSF1A, MGMT, MST1, MST2	Seidel, et al.,[248] Kawaguchi, et al.,[249] Seidel, et al.[250]
Melanoma	RASSF1A, RARbeta, MGMT, DAPK, APC	Spugnardi, et al.,[251] Hoon, et al.,[252] Worm, et al.[253]
Mesothelioma	RASSF1A, DAPK, RARbeta, PGR1, ESR1, CDH1, p16	Fischer, et al.,[254] Tsou, et al.,[255] Hirao, et al.[256]

Table 2

Response Criteria in CALGB 9221 Trial[92] (Silverman JCO 2002)

Complete Response	Normal bone marrow appearance or with < 5% blasts. No transfusion requirement, normal peripheral blood counts.
Partial Response	Bone marrow blast count is ≤ 50% of pre-treatment count. ≥ 50% increase in the difference between baseline blood count and normal value in all three peripheral blood cell lines. No transfusion requirement.
Hematologic Improvement	. ≥ 50% increase in the difference between baseline blood count and normal value in all one or two peripheral blood cell lines. Transfusion requirement is less than 50% of pre-treatment requirement.