Predicting response to epigenetic therapy

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Drugs targeting the epigenome are new promising cancer treatment modalities; however, not all patients receive the same benefit from these drugs. In contrast to conventional chemotherapy, responses may take several months after the initiation of treatment to occur. Accordingly, identification of good pretreatment predictors of response is of great value. Many clinical parameters and molecular targets have been tested in preclinical and clinical studies with varying results, leaving room for optimization. Here we provide an overview of markers that may predict the efficacy of FDA- and EMA-approved epigenetic drugs.

Introduction

Traditionally, cancer patients have been offered the type of chemotherapy that has shown efficacy in the largest proportion of individuals suffering from that particular type of cancer. However, given the rapidly increasing therapeutic options, we are beginning to envision a paradigm shift in cancer treatment. Today, an increasing number of cancer patients are tested for one or more biomarkers in clinical practice, there is a constant pursuit to identify better markers to predict response to existing and upcoming drugs.

Drugs that target the epigenome are promising novel treatment modalities, but not all patients achieve the same benefit from epigenetic therapy and responses are often not evident until after several months of treatment. Identification of good predictive biomarkers for epigenetic therapy would be of great value because patients with minimal chances of response could be spared long-term treatment with an inefficient drug with unpleasant side effects, and could be offered alternative treatment strategies.

This Review focuses on predictors of response to the two classes of epigenetic drugs currently approved by the European Medicines Agency (EMA) and/or the US FDA for cancer treatment: DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis). These drugs may be used individually, in combination with each other, or even in combination with conventional chemotherapy.

What characterizes a good biomarker?

A biomarker is generally defined as a substance that can be measured objectively and is an indicator of either a clinically important aspect of a pathogenic process or of a pharmacologic response to a therapeutic intervention. In cancer, most biomarker assays are based on the detection of aberrantly expressed proteins, mRNAs, microRNAs (miRs), or genetic or epigenetic alterations that are specific to the cancer cells. Irrespective of its nature, a biomarker should have high diagnostic sensitivity and specificity as well as a high positive predictive value (PPV) and negative predictive value (NPV) (Table 1). PPV and NPV are highly dependent on the prevalence of the disease. Therefore, PPV and NPV can only be estimated from cross-sectional studies. Conversely, the diagnostic sensitivity and specificity are intrinsic to the test and may therefore also be derived from case-control studies.

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It is important to realize that the performance of a biomarker is only as good as the assay employed for its measurement and will be compromised if the assay does not have a sufficiently high analytical sensitivity and specificity. Also, if the substance is found at low levels in unaffected or non-responding individuals, the biomarker assay should preferably be quantitative to define a cut-off that provides optimal diagnostic sensitivity and specificity.

Apart from diagnostic sensitivity and specificity, it is important that the biomarker can be detected in readily accessible tissues or body fluids in order to save the patients from a potentially harmful invasive procedure. Finally, the biomarker assay should be based on a methodology that is user friendly and cost efficient (2).

When conducting and reporting biomarker studies, it is important to realize that several aspects of study design, selection of biomarker assay, and statistical analyses may affect the overall outcome of the study. Specific guidelines have been developed that may be helpful when designing, conducting, and reporting biomarker studies (3). In particular, it is recommended that predictive biomarker studies generally should be conducted within randomized trials and that assays should be used at a more advanced stage of development (4).

Predicting response to DNMTis

Recent multicenter studies demonstrated that DNMTis have significant efficacy in the treatment of hematological malignancies (5–9) and have led to the approval of two DNMTis, azacytidine and decitabine, by the FDA and EMA. However, the FDA and EMA have not approved the drugs for similar indications (Table 2). Still, only about 50% of patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) achieve a clinical response to treatment with DNMTis (10, 11). The value of DNMTis in patients that obtain stable disease is still unclear; however, a survival benefit can be observed in patients that obtain hematological improvement. Accordingly, conventional complete remission (CR) and complete remission with incomplete blood count recovery (CRi), as measured by standard parameters (bone marrow blast and peripheral blood cell counts), are not necessarily good markers for predicting outcome (12–14).

The varying efficacy of the drugs may relate to different mechanisms of action in individual patients. In vitro studies indicate that DNMTis can reprogram somatic cells by DNA demethylation of aberrantly silenced genes (Figure 1 and ref. 15). However, the exact mechanisms of action of DNMTis in patients are currently unknown; reactivation of epigenetically silenced tumor suppressor genes and genes involved in normal...
Pharmacologic factors with potential impact on DNMTi resistance

*Human nucleoside transporters.* Cellular uptake is crucial for the efficacy of azanucleosides. It has been shown in vitro that azacytidine and decitabine use different human nucleoside transporters (hNTs), and that cytotoxicity is dependent on hNT presence (27, 28). These observations suggest that hNTs may be useful biomarkers for the efficacy of DNMTis, but clinical data are still not available.

*Cytidine and deoxycytidine kinase.* The next crucial step in DNMTi processing is the initial mono-phosphorylation of azacytidine and decitabine by cytidine kinase and deoxycytidine kinase (DCK), respectively. Accordingly, disruption of DCK may confer decitabine resistance, as demonstrated by a DCK point mutation in the HL60 cell line (29). DCK mutations are rare in patients (30), but a borderline significant lower expression of DCK was observed in non-responders (31). CDA/DCK ratio was negatively correlated with clinical response to decitabine (31).

**Clinical predictors**

*The French prognostic score for MDS patients.* Itzykson et al. evaluated 282 higher-risk MDS patients (International Prognostic Scoring System [IPSS] intermediate-2 [INT-2] and high-risk group; ref. 37) treated with azacytidine, and found that bone marrow blasts > 15%, abnormal karyotype, and previous treatment with low-dose cytarabine independently predicted poor response to azacytidine (Table 3). In addition, performance status ≥ 2, presence of circulating blasts, red blood cell transfusion dependency ≥ 4 units/8 weeks, and intermediate- or high-risk cytogenetics independently predicted poorer overall survival. Based on these factors, Itzykson et al. developed the French prognostic score for overall survival (Table 4 and refs. 12, 13). This prognostic score was validated in 161 higher-risk MDS patients treated in the...
AZA001 trial (6), who represented an independent but highly selected patient cohort. The prognostic score has recently been further validated in two independent patient cohorts of 60 (38) and 90 (39) patients, respectively. In addition, this score identified patients who obtained CR; all CRs were observed in the low- or intermediate-risk group (38).

**Clinical predictors in patients with CMML.** The impact of different clinical factors was evaluated in 76 patients with chronic myelomonocytic leukemia (CMML) treated with azacitidine (8). No predictive factors for clinical response were identified, while increased bone marrow blasts, splenomegaly, and high white blood cell counts were associated with significantly shorter survival. However, by multivariate analysis only bone marrow blast count and splenomegaly retained impact on overall survival.

**Platelet doubling time.** In a cohort of 90 patients with MDS, CMML, and AML treated with azacitidine, an increase in platelet counts of at least two-fold at the initiation of the second treatment cycle, as compared with the pretreatment values, was associated with significantly better overall survival (39).

**Cytogenetic and molecular predictors**

**Cytogenetic abnormalities.** Poor-risk cytogenetics in MDS and AML has been associated with shorter response duration and shorter overall survival (refs. 12, 38, 39, and Table 5). However, among patients with poor-risk cytogenetics, better clinical response rates and a relatively favorable outcome in patients with deletions or loss of chromosome 7 were observed (6, 7, 12, 35, 40–42). The explanation for this is currently unclear; interestingly, however, chromosome 7 harbors EZH2, which encodes the catalytic component of the polycomb repressive complex 2 histone methyltransferase complex. One study showed that EZH2 may directly recruit DNMTs to promoters (43), which theoretically may lead to global hypomethylation. However, a direct interaction between EZH2 and DNMT has not been consistently substantiated, and at this point no association has been shown between EZH2 mutational status and outcome of azacitidine treatment.

**Point mutations.** Mutations in epigenetic regulators are identified in most cancers, and mutations in enzymes that are involved in the regulation of DNA methylation are particularly frequent in hematological malignancies. It seems logical that mutations in these enzymes would influence the response to DNMT inhibitors and thus

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**Table 3**

Clinical markers for response to DNMT inhibitors

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Patients types included</th>
<th>Treatment</th>
<th>Number of patients</th>
<th>Predict overall survival</th>
<th>Predict therapy response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>French prognostic score</td>
<td>MDS (INT-2, high risk)</td>
<td>Azacitidine</td>
<td>282; 161</td>
<td>C</td>
<td>C</td>
<td>12, 13</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-2, high risk), CMML</td>
<td>Azacitidine</td>
<td>60</td>
<td>C</td>
<td>C</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-1, INT-2, high risk), CMML, AML</td>
<td>Azacitidine</td>
<td>90</td>
<td>C</td>
<td>NE</td>
<td>39</td>
</tr>
<tr>
<td>Splenomegaly; bone marrow blast count</td>
<td>MDS (INT-1, INT-2, high risk), CMML, AML</td>
<td>Azacitidine</td>
<td>76</td>
<td>C</td>
<td>—</td>
<td>8</td>
</tr>
<tr>
<td>Platelet doubling time</td>
<td>MDS (INT-1, INT-2, high risk), CMML, AML</td>
<td>Azacitidine</td>
<td>90</td>
<td>C</td>
<td>NE</td>
<td>39</td>
</tr>
</tbody>
</table>

Dash indicates no correlation; C, correlation; NE, correlation not examined.

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Figure 1
Mechanism of action DNMT inhibitors. (A) Under normal circumstances, the DNMTs copy the methylation pattern of the parental DNA strand after replication, ensuring that methylation patterns are maintained during cell division. (B) During treatment, DNMT inhibitors are incorporated into DNA and RNA, where they covalently bind and thus inactivate DNMTs. After successive cell divisions, the original DNA methylation pattern is lost.
be of prognostic importance, but the results are contradictory. Itzykson et al. observed a correlation between clinical response and mutations in the DNA dioxygenase TET2 in 86 patients with MDS and AML treated with azacitidine (44). Significantly better response rates, but no difference in overall survival, were observed among patients with TET2 mutations. Meanwhile, correlation between TET2 mutational status and clinical response or overall survival was not observed in 38 patients with higher-risk MDS treated with azacitidine and valproic acid (45), or in 39 patients with CMML treated with decitabine (46), respectively.

A positive correlation between mutations in the DNA methytransferase DNMT3A and clinical response was observed in 46 patients with AML treated with decitabine; this response, however, did not translate into an overall survival benefit (47).

In a recent study, the impact of several point mutations on the clinical response and mutations in the DNA dioxygenase TET2 in 86 patients with MDS and AML treated with azacitidine (44). Significantly better response rates, but no difference in overall survival, were observed among patients with TET2 mutations. Meanwhile, correlation between TET2 mutational status and clinical response or overall survival was not observed in 38 patients with higher-risk MDS treated with azacitidine and valproic acid (45), or in 39 patients with CMML treated with decitabine (46), respectively.

A positive correlation between mutations in the DNA methyltransferase DNMT3A and clinical response was observed in 46 patients with AML treated with decitabine; this response, however, did not translate into an overall survival benefit (47).

In a recent study, the impact of several point mutations on the response to treatment was examined in 92 MDS, MDS/MPN, and secondary AML (sAML) patients treated with either azacitidine, azacitidine plus lenalidomide, decitabine, or decitabine plus azacitidine (48). TET2 and/or DNMT3A mutations were associated with a better overall response rate and progression-free survival, but not overall survival. Mutations of the putative polycomb-associated protein ASXL1 were correlated with poor overall survival, while mutations of the splice factor 3B (SF3B1) were associated with better overall survival. However, these data need confirmation because this patient cohort was heterogeneous with regard to both diagnosis and choice of treatment modalities, and only about 50% of the examined samples were collected before the initiation of DNMTi treatment.

**DNA methylation**

Several groups investigated whether responses to DNMTis are predicted by pretreatment methylation levels at individual gene promoters, at combinations of genes, or by global screening.

**CDKN2B**. The relationship between clinical response to DNMTi and methylation status of the tumor suppressor gene CDKN2B, which encodes the cell cycle inhibitor p15, has been examined in several studies in patients with MDS (17, 35, 49–52). Some reported a positive correlation between low-level pretreatment CDKN2B methylation and clinical response, while others observed a correlation between CDKN2B demethylation/expression during decitabine treatment and clinical response (16, 49, 52). Yet other groups did not detect any correlation at all (17, 34, 53). The varying results are likely due to variation in patient groups, combinations of epigenetic therapies, and methodologies for monitoring DNA methylation; in particular, not all groups performed quantitative analyses.

**BCL2L10**. Methylation of the anti-apoptotic Bcl-2 family member BCL2L10 has been negatively correlated to response to azacitidine and associated with a significantly poorer overall survival in patients with more than 50% BCL2L10 methylation. These results were based on an initial analysis of 38 — and validation in 27 — azacitidine-treated patients with higher-risk MDS (45). By contrast, others showed that patients with azacitidine-resistant MDS/AML have an increased fraction of BCL2L10-positive cells in the bone marrow, and that patients with low BCL2L10 expression had significantly better overall survival (54).

**Multiple genes**. In 317 patients with MDS, a methylation signature consisting of 10 hypermethylated genes (CDH1, CDH13, ERα, NOR, NPM2, OLIG2, CDKN2B, PGRA, PDZ, and RIL) was identified among 24 genes previously shown to be methylated in MDS/AML (including several known tumor suppressors). The pretreatment methylation level of these genes was not correlated with clinical response to decitabine, but reduction of methylation after more than four months of treatment (across all 10 genes) was positively correlated to clinical response in a cohort of 34 patients (34).

Promoter methylation of four genes (APC, RASSF1A, CDH13, and CDKN2A) has been shown to correlate negatively to survival in non–small-cell lung cancer (NSCLC). Analysis of methylation levels of four genes (CDKN2B, APC, RASSF1A, and CDH13) was associated with better clinical response and overall survival (55). Moreover, a positive correlation between low-level pre-treatment CDKN2B methylation and clinical response in a cohort of 34 patients was observed.

**Table 4**

<table>
<thead>
<tr>
<th>French prognostic score</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>0</td>
</tr>
<tr>
<td>Performance status</td>
<td>2</td>
</tr>
<tr>
<td>Presence of circulating blasts</td>
<td>No</td>
</tr>
<tr>
<td>RBC TDA ≥ 4 units/8 weeks</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Cytogenic risk group</td>
<td>Low</td>
</tr>
</tbody>
</table>

*RBC TDA, red blood cell transfusion dependency.* In low-risk groups (score 0), median survival is 32.1 months. In intermediate-risk groups (score 1–3), median survival is 15.0 months. In high-risk groups (score 4–5), median survival is 6.1 months.
### Table 5

**Molecular markers for response to DNMTi**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Patients types included</th>
<th>Treatment</th>
<th>Number of patients</th>
<th>Predict overall survival</th>
<th>Predict therapy response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDA</strong></td>
<td>MDS (not otherwise specified)</td>
<td>Azacytidine or decitabine</td>
<td>90</td>
<td>C</td>
<td>NE</td>
<td>32</td>
</tr>
<tr>
<td><strong>CDA/DCK ratio</strong></td>
<td>MDS (all IPSS groups)</td>
<td>Decitabine</td>
<td>32</td>
<td>NE</td>
<td>C</td>
<td>31</td>
</tr>
<tr>
<td><strong>Poor-risk cytogenetics</strong></td>
<td>MDS (INT-2, high risk), CMML</td>
<td>Azacytidine</td>
<td>60</td>
<td>C</td>
<td>C</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-1, INT-2, high risk), CMML, AML</td>
<td>Azacytidine</td>
<td>90</td>
<td>C</td>
<td>NE</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-2, high risk)</td>
<td>Azacytidine</td>
<td>282; 161</td>
<td>C</td>
<td>C</td>
<td>12, 13</td>
</tr>
<tr>
<td><strong>Isolated chromosome 7 abnormalities</strong></td>
<td>MDS (INT-2, high risk), CMML, AML, AML &lt; 30% blasts</td>
<td>Azacytidine</td>
<td>358</td>
<td>C</td>
<td>NE</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>MDS (all IPSS groups), AML &lt; 30% blasts</td>
<td>Azacytidine</td>
<td>34</td>
<td>NE</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-2, high risk), CMML, AML &lt; 30% blasts</td>
<td>Decitabine</td>
<td>124</td>
<td>NE</td>
<td>C</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-1, INT-2, high risk), CMML, AML &lt; 30% blasts</td>
<td>Decitabine</td>
<td>170</td>
<td>NE</td>
<td>C</td>
<td>7</td>
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<tr>
<td></td>
<td>AML</td>
<td>Decitabine</td>
<td>23</td>
<td>NE</td>
<td>C</td>
<td>42</td>
</tr>
<tr>
<td><strong>TET2 mutation</strong></td>
<td>MDS (INT-1, INT-2, high risk), AML</td>
<td>Azacytidine</td>
<td>86</td>
<td>—</td>
<td>C</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-2, high risk), CMML</td>
<td>Azacytidine</td>
<td>38, 27</td>
<td>—</td>
<td>—</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>CMML</td>
<td>Decitabine</td>
<td>39</td>
<td>—</td>
<td>—</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>MDS (all IPSS groups), MDS/MPN, sAML</td>
<td>Azacytidine, azacytidine plus lenalidomide, decitabine or azacytidine plus decitabine</td>
<td>92</td>
<td>—</td>
<td>C</td>
<td>48</td>
</tr>
<tr>
<td><strong>DNMT3A mutation</strong></td>
<td>AML</td>
<td>Decitabine</td>
<td>46</td>
<td>—</td>
<td>C</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>MDS (all IPSS groups), MDS/MPN, sAML</td>
<td>Azacytidine, azacytidine plus lenalidomide, decitabine or azacytidine plus decitabine</td>
<td>92</td>
<td>—</td>
<td>C</td>
<td>48</td>
</tr>
<tr>
<td><strong>ASXL1 mutation</strong></td>
<td>MDS (all IPSS groups), MDS/MPN, sAML</td>
<td>Azacytidine, azacytidine plus lenalidomide, decitabine or azacytidine plus decitabine</td>
<td>92</td>
<td>C</td>
<td>—</td>
<td>48</td>
</tr>
<tr>
<td><strong>SF3B1 mutation</strong></td>
<td>MDS (all IPSS groups), MDS/MPN, sAML</td>
<td>Azacytidine, azacytidine plus lenalidomide, decitabine or azacytidine plus decitabine</td>
<td>92</td>
<td>C</td>
<td>—</td>
<td>48</td>
</tr>
<tr>
<td><strong>CDKN2B methylation</strong></td>
<td>MDS (all IPSS groups), AML &lt; 30% blasts</td>
<td>Azacytidine</td>
<td>34</td>
<td>NE</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-2, high risk), CMML, AML</td>
<td>Azacytidine plus entinostat</td>
<td>30</td>
<td>NE</td>
<td>—</td>
<td>17</td>
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<tr>
<td></td>
<td>MDS (INT-1, INT-2, high risk), CMML, AML</td>
<td>Decitabine</td>
<td>23</td>
<td>NE</td>
<td>C</td>
<td>49</td>
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<tr>
<td></td>
<td>AML, CML</td>
<td>Decitabine</td>
<td>41</td>
<td>NE</td>
<td>C</td>
<td>51</td>
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<tr>
<td></td>
<td>MDS, AML</td>
<td>Decitabine plus valproic acid</td>
<td>54</td>
<td>NE</td>
<td>C</td>
<td>50</td>
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<tr>
<td></td>
<td>MDS, CMML</td>
<td>Decitabine</td>
<td>95</td>
<td>NE</td>
<td>—</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>AML, MDS, CML, ALL</td>
<td>Decitabine</td>
<td>50</td>
<td>NE</td>
<td>—</td>
<td>53</td>
</tr>
<tr>
<td><strong>BCL2L10 methylation/expression</strong></td>
<td>MDS (INT-2, high risk), CMML</td>
<td>Azacytidine</td>
<td>38, 27</td>
<td>C</td>
<td>C</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-2, high risk), AML &lt; 30% blasts</td>
<td>Azacytidine</td>
<td>77</td>
<td>C</td>
<td>C</td>
<td>54</td>
</tr>
<tr>
<td><strong>10-Gene methylation signature</strong></td>
<td>MDS (all IPSS), CMML</td>
<td>Decitabine</td>
<td>34</td>
<td>—</td>
<td>C</td>
<td>34</td>
</tr>
<tr>
<td><strong>4-Gene methylation signature</strong></td>
<td>NSCLC</td>
<td>Azacytidine plus entinostat</td>
<td>26</td>
<td>NE</td>
<td>C</td>
<td>55</td>
</tr>
<tr>
<td>≥ 2 hypermethylated TSG</td>
<td>MDS (all IPSS), AML</td>
<td>Azacytidine</td>
<td>63</td>
<td>C</td>
<td>—</td>
<td>56</td>
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<tr>
<td><strong>Global methylation</strong></td>
<td>AML</td>
<td>Decitabine</td>
<td>16</td>
<td>NE</td>
<td>C</td>
<td>57</td>
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<tr>
<td><strong>CJun; CMYB</strong></td>
<td>CMML</td>
<td>Decitabine</td>
<td>36</td>
<td>C</td>
<td>C</td>
<td>46</td>
</tr>
<tr>
<td><strong>Fas</strong></td>
<td>MDS (all IPSS groups), AML</td>
<td>Azacytidine</td>
<td>38</td>
<td>—</td>
<td>C</td>
<td>61</td>
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<tr>
<td><strong>PI-PLCζ1</strong></td>
<td>MDS (INT-2, high risk), AML</td>
<td>Azacytidine</td>
<td>18</td>
<td>NE</td>
<td>C</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-1, low risk)</td>
<td>Azacytidine</td>
<td>26</td>
<td>NE</td>
<td>C</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>MDS (INT-1, low risk)</td>
<td>Azacytidine</td>
<td>32</td>
<td>NE</td>
<td>C</td>
<td>64</td>
</tr>
<tr>
<td><strong>miR-29b</strong></td>
<td>AML</td>
<td>Decitabine</td>
<td>23</td>
<td>NE</td>
<td>C</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>AML</td>
<td>Azacytidine, valproic acid plus ATRA</td>
<td>45</td>
<td>—</td>
<td>—</td>
<td>68</td>
</tr>
</tbody>
</table>

ATRA, all-trans retinoic acid; MPN, myeloproliferative neoplasms.
ylation status of these genes in plasma samples from 26 patients with NSCLC before treatment with azacitidine and entinostat (HDACi), showed higher clinical response rates in patients with methylation of two or more genes (55). Among 63 patients with MDS and AML treated with azacitidine, those with methylation of at least two genes from a panel of 24 tumor suppressor genes had a shorter overall survival (56). However, the number of methylated genes did not correlate with the treatment response to azacitidine.

Global methylation. Another approach has been to examine the methylation status of repetitive elements during treatment, which is independent of the presence of tumor cells after therapy. Several studies have shown significant demethylation of LINE1 and Alu elements during treatment by both azacitidine and decitabine (17, 51, 52). However, prognostic effect of neither pretreatment methylation levels nor methylation changes during treatment has been documented.

A recent study analyzed the global DNA methylation level using MethylCap-seq in 16 patients with AML treated with decitabine. A trend toward a higher baseline methylation level and more pronounced methylation decrease during treatment was observed among responding patients (57).

Gene expression

DNMT3B amplification. Overexpression of DNMT3B mRNA and protein due to gene amplification is frequently observed in human cancers (58). Interestingly, cell lines harboring the DNMT3B amplification were less sensitive to azacitidine, decitabine, and SGI-110, but clinical data are still not available.

CJUN and CMYB. The gene expression levels of CJUN and CMYB have been identified as potential biomarkers in a cohort of 36 decitabine-treated patients with CMML (46). CJUN has previously been shown to promote aberrant monocyte transformation (59). CJUN expression was significantly lower in monocytes from responding patients, and higher CJUN expression was correlated to shorter survival (46). Deregression of CMYB has been implicated in leukemia (60), and higher CMYB expression was also associated with shorter survival (46).

Fas expression. Expression of the pro-apoptotic protein Fas in CD45lo/CD34+ bone marrow cells from patients with MDS (all IPSS groups) or sAML has been positively correlated with response to azacitidine. A correlation between promoter methylation and Fas expression was also observed. Among 63 patients, low Fas expression at diagnosis (presumably due to hypermethylation) was correlated to clinical response, while no association between Fas expression and overall survival was observed (61). In 38 patients Fas expression was examined before and after at least 3 cycles of azacitidine, and responding patients (23 of 38) had a significant increase in Fas expression.

Phosphoantistide-phospholipase C β1. Phosphoantistide-phospholipase C β1 (PLCβ1) is a key enzyme in lipid-signaling pathways that acts on cell proliferation and differentiation. PLCβ1 is highly expressed in the early stages of hematopoietic differentiation (62), is hypermethylated in higher-risk MDS patients, and may be a specific target for azacitidine (63). Among 18 patients an increase in PLCβ1 expression and a decrease in PLCβ1 methylation were observed in 9 of 10 patients with hematological response. The same group observed a similar association in two cohorts of 32 and 26 patients with low-risk MDS treated with azacitidine (64, 65). In the latter cohort, the PLCβ1 target cyclin D3 was induced in responding patients, supporting the notion that the PLCβ1 pathway is activated during azacitidine treatment (65). Due to the involvement of PLCβ1 in early hematopoietic differentiation, it is hypothesized that PLCβ1 upregulation by demethylation leads to differentiation.

miR-29b. miR-29b is involved in the regulation of DNA methylation by targeting the DNA methyltransferases DNMT3A/3B and DNMT1 (41, 66). In a phase II clinical trial in older AML patients treated with decitabine, a positive correlation between the clinical response and high pre-treatment levels of miR-29b was observed (42). In vitro studies from the same group have recently shown that priming of AML cell lines and primary AML blasts with a new HDACi (AR-42) leads to upregulation of miR-29b expression and enhanced anti-leukemic effect of subsequently administered decitabine (67). Yang et al. (68) reported, however, a lack of association between pretreatment miR-29b expression levels and clinical responses to azacitidine in patients with AML. The results obtained by these studies may be explained by the different sources used for miR analysis (peripheral blood vs. bone marrow) and the use of decitabine (42), which may more efficiently downregulate DNMTs.

Predicting response to HDACis

HDACis have considerable antiproliferative and apoptotic activities, making them potential anticancer agents. The HDAC family contains 18 enzymes, grouped into 4 classes that regulate the acetylation level of histones, and several non-histone substrates, including a variety of proteins involved in, for example, cell cycle control, apoptosis, and angiogenesis (69). However, it is still not clear by which key pathways HDACis modify tumor growth in patients (Figure 2). Like the DNMTis, the most promising results are observed in hematological malignancies, with only limited effects in solid tumors. Currently, two HDACis, vorinostat and romidepsin, are FDA approved for treatment of refractory cutaneous T cell lymphoma (CTCL) in patients who have received at least two prior regimens. Romidepsin is also approved for peripheral T cell lymphoma.

Molecular predictors

Acetylation. At this point, only molecular predictors have been identified as biomarkers for HDACi therapy (Table 6). Thus far, the most extensively studied biomarker for HDACi activity is acetylation levels of the target proteins before and after treatment in peripheral blood or tumor tissue, but no correlation to clinical response has been found (70–74). Indeed, hyperacetylation was generally observed in all patients irrespective of response to HDACi (72–74).

Gene expression signature. Gene expression profiling of HDACi-treated cell lines indicated that HDACis are only involved in the regulation of 2%–5% of all human genes (75). In 10 patients mRNA expression in CTCL biopsies taken 4, 8, and 24 hours after administration of the pan-HDACi panobinostat showed altered expression (mainly downregulation) in less than 10% of all genes at the four-hour time point, at which peak changes were observed (73). No correlation was observed between gene expression and response, which could obviously be due to the low number of patients. Similar studies have been done in vitro, leading to identification of a nine-gene signature predictive for response in lung cancer cell lines (70), but this signature has not been validated in vivo.

Several studies have demonstrated that many HDACis increase
the level of the cell cycle inhibitor p21 both in vitro (76, 77) and in vivo (72, 78), but no correlation between p21 induction and clinical response has been observed. Interestingly, p21 is upregulated independent of p53, and stratification according to disruption of p21 regulatory pathways (e.g., p53 mutation) may identify patients that benefit from HDACi. Due to the variable functions of the HDACs in multiple pathways, gene signatures are likely to vary with the tumor type, the HDACi being applied, and the concentration of the HDACi.

**HDAC expression level.** The expression levels of the HDACs themselves have been suggested as a predictive biomarker. Most studies have quantified HDAC expression by immunohistochemistry (IHC), and many HDACs are overexpressed in human cancers (70, 79). Marquard et al. (80) examined the expression levels of HDAC1,-2, and -6 and acetylated histone H4 in 73 CTCL biopsies. Overexpression of HDAC2 and histone H4 acetylation were correlated with more aggressive forms of CTCL. In two clinical trials, a correlation was observed between pretreatment HDAC2 expression and histone acetylation in the tumor tissue (81), and it was suggested that HDAC2 expression potentially can identify patients who will benefit from HDACi treatment (81, 82).

**HR23B.** A genome-wide loss-of-function screen indicated that RAD23 homolog B (HR23B) sensitizes tumor cells to HDACis (83). Under normal conditions HDACs inhibit the expression of HR23B. HDACi-mediated HR23B overexpression leads to proteasome overload, aberrant protein degradation, and apoptosis. Accordingly, cells depleted of HR23B are less sensitive to vorinostat-induced apoptosis (84). High HR23B expression by IHC was positively correlated to clinical response (PPV = 71.7%) in a phase II clinical trial with 65 vorinostat-treated CTCL patients (84). Sequential samples from a fraction of these patients showed that HR23B expression remained high throughout the time of response. However, a recent study in malignant pleural mesothelioma cell lines shows that vorinostat induced apoptosis is independent of HR23B (85), indicating that the role of HR23B may be cell type dependent.

**STAT signaling.** In a functional screen of 40 human B and T cell lymphoma cell lines, high baseline levels of activated STAT1, STAT3, and STAT5 correlated with resistance to vorinostat (86). STATs are transcription factors that participate in chromatin remodeling and enable transcription of several anti-apoptotic proteins. These factors were evaluated in 48 pretreatment CTCL biopsies from patients enrolled in a vorinostat phase IIb clinical trial, and it was shown that nuclear accumulation of STAT1, and high levels of phosphorylated STAT3, in the malignant T cells correlated with lack of clinical response.

**Oxidative stress.** Vorinostat resistance has been linked to increased tolerance of oxidative stress (74). The expression levels of 17 genes involved in antioxidation, selected from preclinical studies, were examined in 21 patients with AML treated with vorinostat in a phase I clinical trial (74). Nonresponders had higher baseline expression levels of these 17 genes compared with patients with hematological improvement, partial response, or CR. The same group showed that a decrease in the cellular glutathione levels increased the sensitivity to vorinostat in cell lines and in primary leukemic cells (87).

**Conclusion**

Ideally, the identification of good predictive biomarkers allows selection of personalized therapy and thereby maximizes the benefit of treatment. However, despite comprehensive knowledge of the biology and function of epigenetic therapy, the search for specific biomarkers for response and survival is not straightforward. Disappointingly, the novel high-throughput epigenetic screening methodologies have not yet been useful for this purpose.

There may be several reasons why the identification of biomarkers for epigenetic therapy has been less successful. First, most studies have been performed in relatively small and miscellaneous patient cohorts, and the findings need confirmation in larger, independent studies. Second, it is likely that a particular biomarker will only be useful for a specific agent, since each individual epigenetic drug has a different pharmacological profile. Among the DNMTis, decitabine is incorporated into DNA, while azacytidine is mainly incorporated into RNA; the in vitro effects of the two agents differ (18, 88), and it is possible that they have different effects in vivo (10). Similarly, some HDACis inhibit all classes of HDACs, while others target only one or two; e.g., vorinostat is a pan-inhibitor, while romidepsin is a class I inhibitor. Thus, it is

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Patients types included</th>
<th>Treatment</th>
<th>Number of patients</th>
<th>Predict overall survival</th>
<th>Predict therapy response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone acetylation</td>
<td>Head and neck cancer, CTCL, AML, ALL, CLL, CML, MDS</td>
<td>Vorinostat, Romidepsin, Panobinostat</td>
<td>14, 10, 41</td>
<td>NE, NE, NE</td>
<td>—, —, 74</td>
<td>72, 73, 74</td>
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<td>Gene expression signature</td>
<td>CTCL</td>
<td>Panobinostat</td>
<td>10</td>
<td>NE</td>
<td>—</td>
<td>73</td>
</tr>
<tr>
<td>P21 induction</td>
<td>Head and neck cancer, Glioblastoma</td>
<td>Romidepsin, Vorinostat, and doxorubicin</td>
<td>14, 66</td>
<td>NE, NE</td>
<td>—, 78</td>
<td>81, 82</td>
</tr>
<tr>
<td>HDAC2 expression level</td>
<td>Solid tumors, Glioblastoma</td>
<td>Vorinostat and doxorubicin, Valproic acid and epirubicin</td>
<td>32, 44</td>
<td>NE, NE</td>
<td>NE, NE</td>
<td>81, 82</td>
</tr>
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<td>HR23B</td>
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<td>65</td>
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<td>C</td>
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<tr>
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<td>NE</td>
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<td>86</td>
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<tr>
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<td>AML</td>
<td>Vorinostat</td>
<td>21</td>
<td>NE</td>
<td>C</td>
<td>74</td>
</tr>
</tbody>
</table>

**Table 6**

Molecular markers for response to HDACis
likely that each individual drug will require a specific biomarker. Third, epigenetic drugs are being used in combination, which may further complicate the identification of relevant biomarkers. Fourth, each individual patient might respond for different reasons, such as reactivation of tumor suppressor genes, restoration of sensitivity to conventional chemotherapy, induction of immunogenicity, induction of terminal differentiation, or combinations thereof. Finally, great variation in drug sensitivity may exist for each cancer type. The recent next-generation sequencing studies have taught us that each tumor harbors a wealth of mutations, and at this point it is unclear whether some of these will be pertinent biomarkers for the efficacy of epigenetic therapy.

Currently, only the clinical markers have been verified by independent research groups, which is a requirement for implementation in clinical practice. The most promising biomarkers are likely to be measurements of the biological effects during treatment; this, however, may be hampered by the elimination of malignant cells. One solution might be to investigate changes in the constitutive methylation patterns as, for example, LINE1 elements ($\beta$), but although significant demethylation is observed during treatment with azanucleosides, there is no evidence of its prognostic value.

In conclusion, there is a lack of proof of a relation between molecular mechanisms of action and biomarkers. Thus, for the time being, there is still much to uncover before the responses to epigenetic therapy can be consistently predicted, but hopefully many large clinical trials in combination with novel high-throughput screening methods will enable us to identify good biomarkers in the near future.

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