Abnormal Methylation of the Calcitonin Gene Marks Progression of Chronic Myelogenous Leukemia

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The clinical aspects of disease progression in chronic myelogenous leukemia (CML) are well established, but the nature of the molecular events responsible is not known. We have previously reported a consistent pattern of novel sites of methylation in the 5' region of the calcitonin (CT) gene and other chromosome 11p loci in acute myelogenous and and lymphoid leukemias. In the present study, CT gene methylation patterns were investigated in peripheral blood from 51 patients with CML. Abnormal patterns were found in only 2 of 31 patients in chronic phase, but in 5 of 8 patients in accelerated phase, and in 11 of 12 patients in blast crisis \((P < .005)\). For one patient studied in blast crisis, abnormal CT gene methylation was found in the peripheral blast cells but not in the granulocytes. In two of three patients studied with CML and having normal peripheral cell patterns, abnormal patterns were found in marrow blast cells. In one patient, only partial normalization of the CT gene methylation pattern was seen after chemotherapy induction of a second chronic phase and the patient relapsed 5 months later. Our findings indicate that abnormal methylation of the 5' region of the CT gene is regularly a marker of disease progression in CML which may prove clinically useful. This abnormal methylation site is part of an imbalance in DNA methylation that may play a role in the progressive genetic instability which characterizes the advancing stages of CML.

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IN THIS REPORT, we characterize a new molecular marker, involving DNA methylation, for the progression of stages in chronic myelogenous leukemia (CML). The clinical course of patients with this disorder is defined by stages corresponding to increasing aggressiveness of disease.\(^1\) The initial chronic phase is marked by a benign proliferation of differentiating blood cells. In contrast, the terminal phase, blast crisis, resembles acute leukemia. A less well-defined phase, often preceding blast crisis, is termed “accelerated” disease, and involves patients who manifest one or more characteristics of disease progression without having the full-blown manifestations of blast crisis.

The molecular determinants of progression of CML are not well defined or understood. The only genetic abnormality consistently found in CML is a translocation between the BCR region on chromosome 22 and the ABL proto-oncogene on chromosome 9, which gives rise to a chimeric gene encoding a protein with tyrosine kinase activity.\(^2\) This translocation is present in the chronic phase, has been shown to directly activate the CML phenotype in mice,\(^4\) and is believed to be an early etiologic event in the development of human CML. In terms of progression of CML, the results of some studies suggest that there is a correlation between the exact site of the breakpoint in the BCR and the disease phase or duration of the chronic phase.\(^5\) However, other studies have been unable to confirm these findings.\(^6\)

Evidence exists that different breakpoint sites in the BCR and/or alternative splicing of the BCR/ABL fusion gene transcript leads to two species of mRNA, but there was no correlation between the type of mRNA found and the phase of disease.\(^6,11\) A search for activating point mutations or alternative splicing of the BCR/ABL fusion gene may provide not only a useful marker for monitoring patients with this disorder, but also clues to the mechanisms that cause increasing genetic instability during the progression of CML.

MATERIALS AND METHODS

Patient specimens. All patients had CML as determined by the appropriate clinical criteria and a Philadelphia chromosome on cytogenetic analysis or a rearrangement in the major BCR by Southern blot hybridization. The phases of disease, chronic versus accelerated versus blast crisis, were determined by the currently accepted criteria.\(^7\) All samples were obtained according to the guidelines of the institutional review boards at each institution.

Peripheral blood or marrow mononuclear cells were separated by density gradient centrifugation\(^16,20\) before extraction of DNA. In some cases, a modification was used to isolate granulocytes.\(^17\) These fractionation procedures yielded greater than 85% blast cells, greater than 95% granulocytes, and 97% mononuclear cells in their respective fractions.

Southern blot analysis of CT methylation. The probe is a previously detailed 1.7-kb fragment derived from a genomic cosmid clone of the 5' region of the human CT gene. The preparation of DNA, complete digestion with the isoschizomers Msp I (methyla-

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RESULTS

The frequency of abnormal CT gene methylation increases in stages of CML progression. Peripheral blood from 51 patients with various phases of CML were assessed for CT gene DNA methylation in the present study (Table 1). In all normal tissues studied, including early and late cells in hematopoietic cell maturation and normal granulocytes and earlier myeloid lineage progenitor cells, the 5' CT gene probe detects dominant Hpa II fragments of 0.5, 0.6, and 2.0 kb and a minor 1.0-kb fragment (Fig 1, lane 2), while Msp I digests show 0.5-, 0.6-, and 1.0-kb bands (Fig 1, lane 1). We have established that the 2.0-kb fragments in Hpa II digests is derived at the expense of the 1.0-kb fragment because of 50% to 90% methylation of a CCGG site in the first intron of the CT gene in all normal tissues. For the patient samples in this study, as in our previous work, the loss of one or more of the normal bands in Hpa II digests with concomitant gain of new bands (hypermethylation), or reduction of the ratio of the 2.0-kb to the 1.0-kb band below 0.6 (hypomethylation), were scored as abnormal methylation events.

In agreement with our previous report, abnormal CT gene methylation was seldom seen in peripheral blood DNA from patients with chronic phase CML (Fig 1, lanes 3 through 5, and Table 1). In contrast, abnormal DNA methylation was much more commonly seen in patients with accelerated disease (Fig 2 and Table 1). In patients in the blast crisis phase of CML, this abnormal DNA methylation change was almost invariably present (Fig 1, lanes 6 through 11, and Table 1), as we had previously observed for patients with acute leukemias.

Different patterns of CT gene methylation accompany myeloid versus lymphoid blast crisis. The most common abnormal pattern of CT gene methylation in blast crisis (8 of 11 abnormal samples) was the appearance of bands greater than 2.0 kb in the Hpa II digests (Fig 1, lanes 6 through 9). This finding represents, in some cells in the tumor clone, abnormal methylation of virtually all of the CCGG sites in the 5' half of the gene and is the characteristic pattern seen in patients with acute myelogenous leukemia. Of five CML patients with this pattern who were classified as to histologic type of blast crisis, all had a myeloid pattern. In three patients (examples in Fig 1, lanes 10 and 11), a smaller number of CCGG sites in the CT gene are abnormally methylated, or loss of all normal methylation (hypomethylation) was seen. This pattern is typical of ALL and two of the three patients with these patterns had a lymphoid leukemia pattern during blast crisis.

Abnormal CT gene methylation marks subclonal evolution of disease progression in CML. In four patients, we separated blast cells from more mature mononuclear cells or granulocytes and studied patterns of CT gene methylation. Our previous studies of AML had shown that both mature cells and blasts within the tumor clone manifest the same abnormal pattern of CT gene methylation. In CML, we now find that this is not always the case. In one patient in blast crisis (Fig 1, lanes 12 and 13) the peripheral blast cells had an abnormal pattern, but the granulocytes, while still exhibiting a normal pattern, were closer to normal. In marrow cells from one patient in blast crisis (Fig 1, lane 6), both blast cells and more mature mononuclear cells (data not shown) had the same abnormal CT gene methylation pattern. We have also examined separated blasts and mononuclear cells from marrow of two patients in the chronic phase of CML. In one of these patients, the blasts exhibited an abnormal CT gene methylation pattern, while the mononuclear cells were normal (Fig 1, lanes 14 and 15). In the other patient, both blasts and mononuclear cells were normal (data not shown). Because, in chronic-phase CML, all immature and mature cells are from the tumor clone, these findings suggest that (1) separated blasts may show abnormalities in CML before evidence of such abnormalities in peripheral blood, and (2) subclonal progression toward blast crisis may not obliterate earlier clones still in chronic phase and from which normal-appearing cells, with normal CT gene methylation, continue to be produced.

A final case (Fig 1, lanes 16 and 17) also suggested coexistence of subclones in blast crisis. In this patient, who had progressed from CML to blast crisis, a second chronic phase was induced with chemotherapy. Comparison of lanes 16 (blast crisis) and 17 (induced chronic phase) show that, while the normal 0.6- and 0.5-kb bands reappeared with return of the patient to the chronic phase, the normal 2.0-kb band remained distinctively underrepresented with respect to the 1.0-kb band in the chronic phase DNA sample. This, chronic phase was presumably derived from a subclone separate from that which was dominant in blast crisis. Alternatively, the therapy may have induced these DNA methylation changes in the blast crisis subclone. In either case, the CT gene region in the chronic phase remained abnormally methylated, suggesting that the cells remained in a different phase of disease progression, relative to primary chronic phase. This patient relapsed to blast crisis within 5 months after the induction of the second chronic phase.

DISCUSSION

We show in the present study of patients with CML, that abnormal methylation of the CT gene is a consistent marker
for the phase of blast crisis. Different patterns of abnormalities occur in myeloid versus lymphoid blast crisis and represent the same differences previously seen between patients with AML and ALL. The abnormal pattern is not simply a measure of the blast content, because it can be seen in separated, normal-appearing granulocytes in both CML (this study) and AML. The abnormal methylation of the CT gene also can be seen in some patients with the accelerated phase before entry into blast crisis, suggesting that the change in methylation may be a true marker of tumor progression. In keeping with this hypothesis, in an accompanying report, Malinen et al25 not only report the same high incidence of abnormal CT gene methylation in patients with the accelerated and blast crisis phases of CML, but also show that the changes evolve with advancing disease in individual patients followed in longitudinal studies.

The above findings must be put in context with our previous studies. We have shown that abnormal CT gene methylation is a marker of malignancy in other systems as well.17,22,23 This change is part of a more widespread process on the short arm of chromosome 11,18 a region known to contain tumor suppressor genes.26 Furthermore, an overall imbalance of DNA methylation involving widespread hypomethylation and regional hypermethylation is a consistent molecular change in human neoplastic cells.18,27,28 In colonic epithelium, these changes occur in benign lesions preceding cancer formation.23,28 suggesting that alterations in methylation may mark precipitating events in malignant transformation. These methylation changes, in turn, have the potential to alter chromatin conformation in such a way as to silence the transcription of tumor suppressor genes and/or create predisposition to genetic instability.29,30 Finally, we have found most recently that these previously mentioned DNA methylation abnormalities are accompanied by abnormally high expression of the DNA methyltransferase gene, which encodes for the enzyme that catalyzes
DNA methylation. This latter change occurs in colon tumor progression before histologic changes in colonic mucosa.

In summary, we have found that abnormal DNA methylation patterns of the CT gene consistently mark stages of progression in CML. This change probably reflects a more widespread process that may involve chromosome 11p and other regions and which could mark and/or contribute to the genetic instability events mediating progression of this leukemia. This methylation change may also serve as a useful clinical marker for monitoring patients with CML. Further studies of this process, including studies of DNA methyltransferase gene expression, may provide an even more sensitive index for this purpose.

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