Deletions of the derivative chromosome 9 occur at the time of the Philadelphia translocation and provide a powerful and independent prognostic indicator in chronic myeloid leukemia

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Chronic myeloid leukemia (CML) is characterized by formation of the BCR-ABL fusion gene, usually as a consequence of the Philadelphia (Ph) translocation between chromosomes 9 and 22. Large deletions on the derivative chromosome 9 have recently been reported, but it was unclear whether deletions arose during disease progression or at the time of the Ph translocation. Fluorescence in situ hybridization (FISH) analysis was used to assess the deletion status of 253 patients with CML. The strength of deletion status as a prognostic indicator was then compared to the Sokal and Hasford scoring systems. The frequency of deletions was similar at diagnosis and after disease progression but was significantly increased in patients with variant Ph translocations. In patients with a deletion, all Ph+ metaphases carried the deletion. The median survival of patients with and without deletions was 38 months and 88 months, respectively (P = .0001). By contrast the survival difference between Sokal or Hasford high-risk and non–high-risk patients was of only borderline significance (P = .057 and P = .034). The results indicate that deletions occur at the time of the Ph translocation. An apparently simple reciprocal translocation may therefore result in considerable genetic heterogeneity ab initio, a concept that is likely to apply to other malignancies associated with translocations. Deletion status is also a powerful and independent prognostic factor for patients with CML. The prognostic significance of deletion status should now be studied prospectively and, if confirmed, should be incorporated into management decisions and the analysis of clinical trials. (Blood. 2001; 98:1732-1738) © 2001 by The American Society of Hematology

Introduction

Chronic myeloid leukemia (CML) is a clonal hematologic malignancy that results from transformation of a multipotent hematopoietic stem cell.1-3 The molecular hallmark of CML is the formation of a BCR-ABL fusion gene, usually formed as a consequence of the Philadelphia (Ph) translocation involving chromosomes 9 and 22.4-6 BCR-ABL plays a pivotal role in the pathogenesis of CML and its formation is likely to represent the initiating event. In support of this concept transgenic and retroviral transduction studies have demonstrated that expression of BCR-ABL in murine bone marrow cells resulted in leukemia, with some cases closely resembling CML.7-13 In one recent transgenic model the leukemia could be reversed by down-regulating BCR-ABL14.

Chronic myeloid leukemia is a biphasic disease with an initial chronic phase that is readily controlled. However, this is followed by an ill-defined accelerated phase, and then a terminal blastic phase that resembles an acute leukemia, which is usually refractory to therapy. Transformation to blast crisis is accompanied by secondary cytogenetic changes in about 85% of cases, but the molecular basis for this transformation is poorly understood. A number of molecular changes have been identified in a minority of cases of blast crisis, including mutations or deletions of p53, p16INK4a, and the retinoblastoma protein, and mutation or overexpression of Ras and EVI-1.1,2 However, none provide a method for prospectively distinguishing those patients who will progress rapidly to blast crisis from those patients whose disease pursues an indolent course.

Treatment options for patients with chronic phase CML currently include hydroxyurea, interferon α (IFN-α; with or without cytosine arabinoside), and allogeneic or autologous stem cell transplantation. Recently there has been considerable interest in STI 571, a novel agent specifically designed to inhibit the tyrosine kinase activity of BCR-ABL, and which has shown excellent early results in all phases of the disease.16,17 Other inhibitors of BCR-ABL signal transduction such as farnesyltransferase inhibitors are also showing promise.1,18,19 Allogeneic transplantation is potentially curative but may be associated with considerable morbidity and mortality, whereas other forms of current treatment are less dangerous but merely delay the onset of blast crisis. One important goal therefore remains the identification at diagnosis of those patients who have a worse prognosis and in whom more intensive...
treatment modalities are justified. Patients with CML show considerable clinical heterogeneity in chronic phase and a number of prognostic scoring systems have been reported, each based on multiple clinical and laboratory parameters at diagnosis. However, these are not sufficiently robust to routinely form the basis of individual management decisions.

We and others have recently reported previously unrecognized deletions adjacent to the t(9;22) breakpoint on the derivative chromosome 9. The deletions were large, spanning up to several megabases, displayed variable breakpoints, and usually resulted in genomic loss of sequences from both the chromosome 9 and chromosome 22 sides of the translocation breakpoint. However, it was not clear whether the deletions arose during disease progression, as a consequence of genomic instability, or alternatively whether they occurred at the time of the original Ph translocation. Moreover, although it was reported that the deletions may be associated with a more rapid onset of blast crisis, the number of patients studied was small and the survival difference was explained by the fact that few patients with deletions had received IFN-α. Here we present several lines of evidence to show that the deletions occur at the time of the Ph translocation and thus result in previously unsuspected genetic heterogeneity from the onset of the disease. Furthermore, our results suggest that deletion status at diagnosis is a powerful and independent prognostic indicator for patients with CML.

### Patients, materials, and methods

#### Patient samples and clinical and laboratory data

Fixed cytogenetic preparations from cultured bone marrow samples were obtained from 253 patients with CML, diagnosed between January 1989 and March 2000, in the Department of Hematology at Addenbrooke’s Hospital, Cambridge, United Kingdom; the Oncology Cytogenetics Service, Christie Hospital, Manchester, United Kingdom; from patients entered into the United Kingdom Medical Research Council (MRC) CML III trial; and patients diagnosed in the Victorian Cancer Cytogenetics Service, St Vincent’s Hospital, Melbourne, Australia. The patients were unselected for type of therapy and were treated at various centers in the United Kingdom and Victoria, Australia. Patients in the CML III trial were previously untreated and were randomized to receive either hydroxyurea or IFN. These patients were unselected for stage of disease. A diagnostic sample was available for analysis in 123 patients, a subsequent chronic phase sample was analyzed in 66 patients, and an accelerated phase blast crisis sample was available for analysis in 27 patients. Paired samples, where both a diagnostic sample and an accelerated phase/blast crisis sample were available, were analyzed in a further 37 patients. Risk categories were determined as previously described. Data for 55 of these patients have previously been reported.

#### Fluorescent in situ probes and detection systems

**Triple-probe/3-color system.** This system (Figure 1) was used as described. Briefly, it uses 3 probes, each labeled with a separately colored fluorochrome. These are: (1) ASS probe, a 350-kb cosmids contig that contains the ASS and 8604 Met genes, both mapping proximal to the first exon of ABL and labeled with Spectrum Aqua; (2) ABL probe (Vysis, Downers Grove, IL), a 300-kb cosmids contig that contains the 3′ region of the ABL gene (exons 3-11), labeled with Spectrum Orange; and (3) BCR probe (Vysis), an approximately 300-kb contig that begins between exons 13 and 14 of BCR and extends well beyond the M-bcr region, labeled with Spectrum Green.

**Dual–fluorescent in situ hybridization BCR-ABL detection system.** This system (Figure 1) was used according to the manufacturer’s instructions (Qbiogene, Middlesex, United Kingdom). It uses 2 probes: (1) ABL probe, a 600-kb contig spanning the breakpoint region on ABL labeled with fluorescein isothiocyanate (FITC) and (2) BCR, a 500-kb contig containing the major and minor breakpoint regions of BCR labeled with Texas red.

#### Digital imaging and analysis

All fluorescent in situ hybridization (FISH) images were captured and analyzed with a Smart Capture 2 imaging station, (Digital Scientific, Cambridge, United Kingdom). For each detection system up to 50 images were captured automatically for each patient. A minimum of 30 Ph+ metaphase cells were analyzed in 247 patients in which the normal chromosome 9 and 22 displayed appropriate signal patterns. In 6 patients, fewer than 30 Ph+ metaphase cells were available and results were confirmed by additional analysis of 200 interphase nuclei. The derivative chromosome 9 was identified in patients with deletions by the presence of a heterochromatin block on an appropriately sized chromosome by reverse DAPI (4,6-diamidino-2-phenylindole) banding.

#### Statistical analysis

All calculations were performed with the SPSS statistical package (SPSS, Chicago IL.). Medians and interquartile ranges were calculated for age and clinical and laboratory findings at diagnosis for patients with and without

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**Figure 1.** Analysis of Ph+ metaphase cells with or without a deletion of the derivative chromosome 9 using the triple-probe or D-FISH systems. Each panel shows a cartoon of the expected hybridization signals together with a partial metaphase. Using the triple-probe system (A,B), a blue signal is absent from the derivative chromosome 9 (der 9) in a cell carrying a deletion of that chromosome (B). Using the D-FISH system (C,D), note the absence of the colocalized red/green signal on the derivative chromosome 9 in a cell carrying a deletion of that chromosome (D).
deletions (Table 1) and tested for any significant differences with the Mann-Whitney U test (for continuous variables) or χ² analysis and Fisher exact tests (for categorical variables). Survival time was calculated from month of presentation to month of death, with a median follow-up of 34 months (range, 1-117 months). Patients who died in chronic phase for reasons unrelated to CML and bone marrow transplant recipients were censored at the time of death and transplantation, respectively. Survival data were calculated with the Kaplan-Meier estimator and significance was assessed with the log-rank test. The analysis was performed both by time censoring at the time of transplantation or death due to causes unrelated to CML and also by excluding these patients from analysis. Patient age, sex, platelet count, percentage of peripheral blood blasts, classical or variant translocation, and initial Hasford and Sokal scores were considered potentially confounding prognostic factors. The respective P values for the univariate analyses were age (P = .002), sex (P = .44), percentage peripheral blood blasts (P = .001), platelets (P = .1), classical/variant translocation (P = .14), initial Sokal group (P = .04), and initial Hasford group (P = .08). Univariate analysis was performed for these variables and those significant at the P < .2 level were included in a multivariate analysis from which a forward stepping procedure was used to derive the most significant model. The hazard ratio of deletion status as a univariate analysis was then compared to the hazard ratios following adjustment for initial Hasford and Sokal scores and to the hazard ratio for the most significant model.

### Results

Bone marrow samples from 253 patients with CML were studied using the triple-probe and dual-FISH (D-FISH) systems. Figure 1 shows the hybridization patterns expected in “normal” Ph+ cells and in Ph+ cells carrying a deletion of the derivative chromosome 9. Deletions were detected in 39 of 253 patients (15%) of whom 30 demonstrated deletion of both chromosome 9 and 22 sequences. Deletions of only chromosome 9 sequences were detected in 7 patients, with deletion of only chromosome 22 sequences detected in 2 patients. There was complete concordance between the triple-probe and D-FISH analyses.

#### Deletions occur at the time of the Ph translocation

Deletions of the derivative chromosome 9 may reflect genomic instability during disease progression or may arise at the time of the initial Ph translocation. We have investigated this issue in several ways. First, patients analyzed in different phases of the disease were found to exhibit virtually identical frequencies of deletions. In the whole cohort, 15% (39 of 253) of samples carried a deletion. In samples taken at diagnosis, 14% (22 of 160) carried a deletion compared to 16% (10 of 64) of samples taken following progression to accelerated phase or blast crisis (χ², P = .72). Sequential paired samples were also analyzed, with the second sample taken following disease progression. In 34 of 37 patients the initial chronic phase sample lacked a deletion. In these 34 patients none of the 1306 Ph+ metaphases obtained after disease progression (30 blast crisis, 4 accelerated phase) had acquired a deletion.

Second, deletions were observed in 16 of 41 (39%) patients with a variant translocation compared to 25 of 212 (11%) patients with a classical Ph translocation (χ², P < .001). Because variant translocations are thought to result from a complex series of recombination events, this difference is consistent with a model in which each recombination event has a finite probability of resulting in a deletion adjacent to the breakpoint.

Third, if deletions occurred during disease progression it should be possible to identify cells carrying the Ph translocation but no deletion. We therefore analyzed a total of 1524 metaphases from the 39 patients shown to carry a deletion. In every metaphase both a Ph translocation and a deletion were demonstrated.

Taken together these data demonstrate that the recombination event producing an apparently reciprocal translocation also results in large genomic deletions. This process gives rise to previously unsuspected genetic heterogeneity and is likely to be widely applicable to other malignancies associated with translocations.

#### Deletion status is a powerful prognostic indicator in CML

Survival data were available from 241 patients (39 with and 202 without a deletion). The clinical and laboratory characteristics of patients with and without deletions are shown in Table 1 and are similar for both. However, Kaplan-Meier analysis revealed a striking difference in survival (Figure 2). The estimated median survival time for patients with deletions was 38 months (95% CI [confidence interval], 36.5-39.4) compared to 88 months (95% CI,
after correction for initial Hasford and Sokal scores, and for survival 81 months for patients without deletions versus 53 months for patients with deletions, with a similar median survival 76 months versus 60 months, log-rank

The prognostic strength of deletion status, Sokal score, and Hasford score were then compared in 210 patients for whom all the necessary clinical information was available. As shown in Table 3 and Figure 2, Sokal and Hasford high-risk groups had estimated median survivals of 56 and 55 months, respectively, compared to 37 months for patients with deletions. Moreover, in contrast to the survival difference reported here because there was no significant survival difference between patients with classical or variant Ph translocations (median survival 76 months versus 60 months, log-rank

### Table 2. Comparison of hazard ratios for deletion status following adjustment for other potential prognostic factors

<table>
<thead>
<tr>
<th>Adjustment</th>
<th>Hazard ratio (95% CI)</th>
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<tbody>
<tr>
<td>Deletion status alone</td>
<td>3.2 (1.7-5.0)</td>
</tr>
<tr>
<td>All significant variables*</td>
<td>3.0 (1.7-5.5)</td>
</tr>
<tr>
<td>Initial Sokal score</td>
<td>3.0 (1.6-4.9)</td>
</tr>
<tr>
<td>Initial Hasford score</td>
<td>3.0 (1.4-4.8)</td>
</tr>
</tbody>
</table>

*Hazard ratio was derived from the forward stepping procedure following correction for the initial age and percentage peripheral blood blasts (the only two variables to provide additional prognostic value in the multivariate model constructed).
of 78 (14%), 9 of 67 (13%), and 12 of 65 (18%) patients, respectively, in the Sokal low-, intermediate-, and high-risk groups and 10 of 80 (13%), 15 of 84 (18%), and 7 of 46 (15%) patients in the 3 respective Hasford risk groups. These results agree well with our findings that the hazard ratios associated with deletion status remain virtually unchanged following adjustment for Sokal or Hasford score, implying independence of deletion status as a prognostic factor (Table 2 and above). Consistent with this concept, the Sokal and Hasford scoring systems retain prognostic significance if analysis is restricted to patients without a deletion (Sokal high-risk versus non–high-risk \( P = .03 \); Hasford high-risk versus non–high-risk \( P = .04 \)). The striking prognostic power of deletion status relative to the Sokal and Hasford systems is likely to reflect the fact that deletion status directly detects a molecular event with a critical role in the progression of CML.

**Discussion**

Patients with CML display considerable clinical heterogeneity during the chronic phase of the disease, with some individuals progressing rapidly to blast crisis and death, whereas others remain well controlled for many years. The molecular basis for this variability remains obscure. Here we demonstrate that the Ph translocation event itself can give rise to considerable genetic variability in the form of large deletions of sequences on the derivative chromosome 9. These results show that the pathogenetic consequences of an apparently “simple” translocation may frequently be more complex than previously realized, a concept that is likely to apply to other malignancies with chromosomal translocations.

It has previously been suggested that deletions of the derivative chromosome 9 may be associated with a worse survival. However, the number of patients studied was small (55 in total and only 16 of whom had deletions). There was also a much higher proportion of variant Ph translocations (21 of 55, 38%) within this cohort than occur randomly. Moreover, the significance of the survival difference was complicated by the fact that few patients with deletions had received IFN-\( \alpha \) treatment. Here we provide definitive evidence in a large cohort of patients that deletion status is a powerful prognostic indicator for patients with CML. Our results suggest that the prognostic significance of deletion status is much more potent than the widely used Sokal and Hasford scoring systems. This observation has several important clinical consequences. Allogeneic stem cell transplantation is potentially curative, but the procedure is associated with significant morbidity and mortality, especially in older patients or those with unrelated donors. As contrast IFN-\( \alpha \), with or without cytosine arabinoside, is much less dangerous and is effective at controlling chronic phase, but most patients still progress to blast crisis. As a result it has been suggested that Sokal or Hasford low-risk patients aged more than 30 years should receive a trial of IFN-\( \alpha \) for up to 2 years prior to consideration of allogeneic transplantation. However, our data demonstrate that approximately one third of patients carrying a deletion fall into the Sokal or Hasford low-risk categories. It will therefore be essential to consider deletion status when weighing the risks associated with different treatment modalities. Because the presence of a deletion is likely to influence the outcome of treatment with new agents such as STI 571 or farnesyl transferase inhibitors, it will also be necessary to consider deletion status when analyzing future clinical trials of both conventional and novel therapies.

Our data also provide an explanation for conflicting previous reports of the prognostic significance of variant Ph translocations. Patients with a variant Ph translocation have a more than 3-fold increase in the frequency of deletions, although patients with a deletion still represent a minority (39%) of patients with a variant Ph translocation. Differences in the proportion of patients carrying a deletion are therefore likely to account for previous controversy concerning the prognostic significance of variant compared to classical Ph translocations.

What might be the molecular mechanism whereby deletions confer such a poor prognosis? Several models can be envisaged. First, deletions will result in loss of the reciprocal fusion gene, ABL-BCR. However, current evidence suggests that this is unlikely to be the critical event because no ABL-BCR protein has been detected and ABL-BCR expression does not correlate with prognosis as assessed by cytogenetic response. Second, a deletion on the derivative chromosome 9 may act as a surrogate marker for smaller intronic deletions on the Ph chromosome, which may influence the level of BCR-ABL expression. Blast crisis is sometimes associated with the acquisition of an additional Ph chromosome suggesting that BCR-ABL dosage may be important.

A third potential mechanism would involve the loss of one or more genes within the deleted region. Such loss may be sufficient to produce a neoplastic effect (haploinsufficiency) or may require subsequent inactivation of the corresponding normal allele(s). The deletions are large, extending up to 5.5 Mb on the chromosome 9 side of the translocation breakpoint and up to 17 Mb on the chromosome 22 side of the breakpoint (Sinclair et al25 and B.J.P., unpublished data, April, 2001). Both of these regions are gene rich. The chromosome 9 region contains 44 known genes and another 40 predicted genes and the chromosome 22 region contains 138 known genes and a further 91 predicted genes based on genome sequence analysis.44 The chromosome 22 region contains 3 known tumor suppressor genes, the chromatin remodeling gene hSNF5/ INI1, the neurofibromatosis type 2 gene NF2, and MNI1/MGCRI-PEN, a putative tumor suppressor gene associated with sporadic meningioma and rearranged in a myeloproliferative disorder with t(12;22). Both the chromosome 9 and chromosome 22 regions contain a number of other genes encoding transcription factors/cofactors (PBX3, LMX1B), components of signal transduction

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**Table 3. Comparison of prognostic significance of deletion status, Sokal score, and Hasford score**

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<thead>
<tr>
<th>Prognostic factor</th>
<th>Non–high-risk</th>
<th>High-risk</th>
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<tr>
<td>Percent of patients</td>
<td>Median survival (95% CI)</td>
<td>Percent of patients</td>
</tr>
<tr>
<td>Deletionstatus</td>
<td>85</td>
<td>81 (51-110)</td>
</tr>
<tr>
<td>Sokal</td>
<td>63</td>
<td>72 (51-93)</td>
</tr>
<tr>
<td>Hasford</td>
<td>78</td>
<td>76 (49-103)</td>
</tr>
</tbody>
</table>

Survival differences were assessed by log-rank analysis. Non–high-risk refers to patients lacking a deletion or, in the Sokal and Hasford systems, the combined low- and intermediate-risk groups. High-risk refers to patients with a deletion or to the Sokal and Hasford high-risk groups.
pathways (the serine/threonine phosphatase PP2A,\textsuperscript{90} Ras inhibitor \textit{INP},\textsuperscript{31} lim domain protein kinase \textit{LM-K2},\textsuperscript{52} GM-CSF/IL-3/IL-5 receptor common chain \textit{B} \textsuperscript{31}), or cell cycle control proteins (\textit{CDK9}).\textsuperscript{55} Subset analysis of a large series of deletions of varying sizes will be necessary to identify a critical deleted region and thus prioritize candidate genes for further analysis.

Finally, deletions may represent a consequence of genetic instability within the target cell at the time of the Ph translocation. In this case the poor prognosis would reflect a predisposition to subsequent additional genetic alterations within the malignant clone. Patients with chronic phase CML do not exhibit genomic instability as assessed by microsatellite analysis,\textsuperscript{55,56} but these data do not exclude more subtle levels of genetic instability. It is also worth emphasizing that further layers of complexity may exist because the 4 mechanisms discussed above are not mutually exclusive.

**Acknowledgments**

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**References**


