

**IN SEARCH OF THE ORIGINAL LEUKEMIC CLONE IN CHRONIC MYELOID LEUKEMIA
PATIENTS IN COMPLETE MOLECULAR REMISSION AFTER STEM CELL
TRANSPLANTATION OR IMATINIB**

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Artigo 48º, § 3º: *A Faculdade não responde pelas doutrinas expendidas na dissertação.*

(Regulamento da Faculdade de Medicina da Universidade do Porto – Decreto-Lei n.º 19337 de 29 de Janeiro de 1931)

Ao abrigo do Art.º 8º do Decreto-Lei n.º388/70, fazem parte integrante desta tese a Introdução, Métodos, Resultados e Discussão do artigo publicado na revista Blood em Agosto de 2010 de que sou primeiro autor:

In search of the original leukemic clone in chronic myeloid leukemia patients in complete molecular remission after stem cell transplantation or imatinib. Sobrinho-Simões M, Wilczek V, Score J, Cross NC, Apperley JF, Melo JV. Blood. 2010 Aug 26;116(8):1329-35.

Na tese são também apresentados elementos extraídos do artigo publicado na revista Leukemia em Outubro de 2010 de que sou co-autor:

Analysis of genomic breakpoints in p190 and p210 BCR-ABL indicate distinct mechanisms of formation. Score J, Calasanz MJ, Ottman O, Pane F, Yeh RF, Sobrinho-Simões MA, Kreil S, Ward D, Hidalgo-Curtis C, Melo JV, Wiemels J, Nadel B, Cross NC, Grand FH. Leukemia. 2010 Oct;24(10):1742-50.

Finalmente, são também utilizados elementos da minha dissertação de Mestrado, intitulada “**Strategies for detection of rare *BCR-ABL* transcripts**”, apresentada em 2004 na Faculdade de Medicina da Universidade do Porto. As secções de Materiais e Métodos e Resultados dessa dissertação fazem parte dos documentos anexos a este documento.

Contribuição pessoal

No artigo de que sou primeiro autor e na dissertação de Mestrado fui responsável pelo desenho e execução do trabalho experimental, obtenção e análise de resultados, e escrita dos manuscritos, com a colaboração dos restantes autores e a supervisão da Professora Junia Melo e do Professor José Eduardo Guimarães (supervisores da dissertação de Mestrado). No artigo de que sou co-autor colaborei na execução do trabalho experimental e na revisão do manuscrito.

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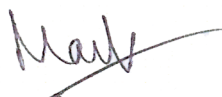
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Abbreviations

ALL - acute lymphoblastic leukemia
AP - accelerated phase
BC - blast crisis
CCytR - complete cytogenetic response
cDNA - complementary DNA
CHR - complete hematologic response
CML - chronic myeloid leukemia
CMR - complete molecular response
CP - chronic phase
DLI - donor lymphocyte infusion
DNA - deoxyribonucleic acid
EFS - event-free survival
FU - follow-up
gBCR-ABL - genomic *BCR-ABL* fusion
gDNA - genomic DNA
GTC - guanidinium thiocyanate
HSCT - hematopoietic stem cell transplantation
IFN α - interferon α
IL - interleukin
IRIS - international randomized study of interferon versus STI571
IS - international scale
LTR - long-term remission
MCytR - major cytogenetic response
MMR - major molecular response
MNC - mononuclear cell
MRD - minimal residual disease
mRNA - messenger RNA
OS - overall survival
PCR - polymerase chain reaction
PCytR - partial cytogenetic response
Ph - Philadelphia chromosome

RNA - ribonucleic acid

RQ-PCR - real-time quantitative PCR

RT-PCR - reverse-transcriptase PCR

SCF - stem cell factor

TKI - tyrosine kinase inhibitor

VUD - voluntary unrelated donor

WBC - white blood cell

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Resumo

Existe uma forte associação causal entre o gene de fusão *BCR-ABL* e a leucemia mielóide crónica (LMC). Daí o interesse na compreensão da origem do rearranjo *BCR-ABL*, assim como na utilização do *BCR-ABL* para medir doença residual em doentes com LMC. Uma proporção relevante dos indivíduos saudáveis tem ARNm de *BCR-ABL* no sangue periférico. Este facto levanta questões relativas 1) ao desenvolvimento da LMC; e 2) ao significado da ocorrência de níveis baixos de transcritos de *BCR-ABL* no sangue periférico de doentes em remissão prolongada (RP) após transplante de células estaminais hematopoiéticas (TCEH), a maioria dos quais não sofre recidiva da doença e está funcionalmente curada. Em alguns doentes tratados com imatinib, os transcritos de *BCR-ABL* tornam-se indetectáveis, atingindo aquilo a que se convencionou chamar resposta molecular completa (RMC). Não é claro se este grau de resposta deve ser considerado alcançável por todos os doentes dado que transcritos de *BCR-ABL* são detectáveis em indivíduos saudáveis e em doentes em RP pós-TCEH. Na maioria dos doentes tratados com imatinib que atingem RMC a interrupção da terapêutica leva a recidiva da doença em poucos meses. Nos doentes que permanecem livres de recidiva sem terapêutica, não se sabe se a RMC corresponde à erradicação do clone leucémico. Começámos por tentar esclarecer se a positividade para transcritos que se observa em indivíduos saudáveis tem origem em células precursoras hematopoiéticas imaturas (CD34+), uma tarefa dificultada pela escassez destas células. O sistema experimental que utilizámos resultou numa proliferação substancial das células CD34+. Infelizmente para o nosso objectivo, esta proliferação acompanhou-se de diferenciação mielóide e de diminuição da expressão do *BCR-ABL*. Abandonámos esta abordagem dado que não conseguimos demonstrar que resultasse num aumento da sensibilidade para a detecção de hipotéticas raras células CD34+ que fossem *BCR-ABL*-positivas.

Em contraste com os transcritos de *BCR-ABL*, cada fusão genómica de *BCR-ABL* (*gBCR-ABL*) é, na prática, característica do caso de LMC a que se associa.

Sequenciámos as fusões *gBCR-ABL* de doentes com LMC com o intuito de procurar o clone leucémico original em doentes em RP pós-TCEH e em RMC em tratamento com imatinib. Para isso desenhamos reacções em cadeia da polimerase (RCP) quantitativas, baseadas em ADN, específicas para cada caso, e altamente sensíveis.

As sequências de *gBCR-ABL* foram também utilizadas para tentar entender a gênese do rearranjo. O estudo das fusões *gBCR-ABL* (provenientes de casos de LMC p210 e de leucemia linfoblástica aguda p190 e p210) não encontrou evidência de “clustering” relevante, nem associação significativa com sequências específicas. A única exceção diz respeito à menor frequência em regiões repetitivas dos pontos-de-quebra no *BCR* em fusões p210. Este estudo sugere que as fusões p210 e p190 resultam de mecanismos diferentes.

Quanto ao nosso objectivo principal, procurámos perceber melhor a qualidade da resposta molecular em doentes tratados com imatinib em RMC e em doentes em RP pós-TCEH, usando uma RCP para *gBCR-ABL*, específica de cada doente. Detectámos a fusão *gBCR-ABL* em 5 de 5 doentes em RMC com imatinib, em amostras negativas para transcritos; 4 destes 5 doentes tornaram-se negativos para *gBCR-ABL* com a continuação da terapêutica. Pelo contrário, só detectámos *gBCR-ABL* em 1 de 9 doentes em RP (13 a 27 anos) pós-TCEH, apesar de 8 destes 9 doentes terem tido transcritos detectáveis.

Em conclusão, em doentes tratados com imatinib, a ausência de transcritos não deve ser interpretada como sinónimo de desaparecimento do clone leucémico. Demonstrámos que a continuação da terapêutica com imatinib após alcançar RMC pode resultar em diminuição da doença residual. Pós-TCEH, encontramos escassa evidência em favor da noção de que os transcritos ocasionalmente detectados nestes doentes resultam do clone leucémico original.

Abstract

The *BCR-ABL* fusion gene has a strong causal association with chronic myeloid leukemia (CML). This justifies the interest in understanding the origin of the *BCR-ABL* rearrangement, and in using *BCR-ABL* to measure residual disease in CML patients. *BCR-ABL* mRNA is present in the peripheral blood of a significant proportion of healthy individuals, which raises questions relating to 1) the development of CML; and 2) the interpretation of the low-level positivity for *BCR-ABL* mRNA that is occasionally seen in patients in long-term remission (LTR) post-hematopoietic stem cell transplantation (HSCT), most of which will not experience disease relapse and are functionally cured. With imatinib, some patient become negative for *BCR-ABL* transcripts, achieving what has been termed a complete molecular response (CMR). It is not clear whether this is an attainable ultimate goal of therapy for the majority of patients, given that transcripts are detectable in healthy individuals and in patients in LTR post-HSCT. Most imatinib-treated patients in CMR relapse within months if therapy is stopped; it is not known whether CMR equates with eradication of the CML clone in those patients that remain relapse-free without imatinib.

We initially wanted to investigate whether the transcript-positivity of healthy individuals originates from immature hematopoietic progenitor cells (CD34+), a task that is complicated by their paucity. The experimental system we used to expand CD34+ cells *in vitro* produced significant proliferation, which was associated with myeloid differentiation and decreased *BCR-ABL* expression. We did not pursue this approach further since we could not demonstrate that it would increase the sensitivity of detection of putative rare CD34+, *BCR-ABL*-positive cells.

Unlike *BCR-ABL* transcripts, the genomic *BCR-ABL* fusion (*gBCR-ABL*) is virtually unique to each individual CML case. We sequenced *gBCR-ABL* from CML patients in order to design patient-specific, highly sensitive, DNA quantitative polymerase chain reactions (PCR) to test follow-up samples from patients in LTR post-HSCT and in CMR on imatinib for the presence of the original leukemic clone.

The *gBCR-ABL* sequences were also used to try to understand the genesis of the rearrangement. Analysis of breakpoints from p210 CML and p190 and p210 acute lymphoblastic leukemia cases found no evidence for significant clustering and no association with sequence motifs, except for a breakpoint deficit in repeat regions

within *BCR* for p210 cases; it suggests that p190 and p210 rearrangements are formed by different mechanisms.

Returning to our main objective, we sought to elucidate the quality of the molecular response in imatinib-treated patients in CMR and in patients in LTR post-HSCT using a genomic DNA based PCR. In 5 imatinib-treated patients in CMR, *gBCR-ABL* was detected in transcript-negative samples; 4 patients became *gBCR-ABL* negative with continuing imatinib therapy. In contrast, of 9 patients in LTR (13-27 years) post-HSCT, *gBCR-ABL* was detected in only 1, despite occasional transcript-positive samples in 8 of them.

We concluded that, in imatinib-treated patients, absence of transcripts should not be interpreted as absence of the leukemic clone, although continuing imatinib after achievement of CMR may lead to further reduction of residual disease. Post-HSCT, we found little evidence that the transcripts occasionally detected originate from the leukemic clone.

Foreword

Back in 2002, I and my supervisors chose as the theme of my graduate studies “O significado da presença de leucócitos *BCR-ABL*-positivos em indivíduos saudáveis e em doentes com leucemia mielóide crónica em remissão completa molecular”. Translating this (admittedly cumbersome) phrase into English, we set out to investigate “The significance of *BCR-ABL*-positive leukocytes in healthy individuals and chronic myeloid leukemia (CML) patients in complete molecular remission”.

Some healthy individuals have a small amount of detectable *BCR-ABL* transcripts in their peripheral blood. This has significant implications, both in understanding the genesis of CML and in evaluating minimal residual disease after allogeneic hematopoietic stem cell transplantation (HSCT) or treatment with tyrosine kinase inhibitors (TKIs).

We initially approached the question of the cellular origin of *BCR-ABL* transcripts in healthy individuals. This is a crucial point, since *BCR-ABL* translocations would only have leukemogenic potential if they occurred in hematopoietic precursor cells capable of self-renewal and clonal expansion, as opposed to more mature, differentiated leukocytes. Specifically, we attempted to establish an experimental design that would increase the sensitivity of detection of rare *BCR-ABL*-positive cells among the immature hematopoietic progenitor (CD34+) fraction of leukocytes by their *in vitro* expansion. These experiments were reported in the Master thesis presented to the Faculty of Medicine of the University of Porto in 2004, entitled “Strategies for Detection of Rare *BCR-ABL* Transcripts”.

To summarize the results, we found that CD34+, *BCR-ABL*-positive cells from chronic phase CML patients could indeed be significantly expanded *in vitro*. However, this expansion was accompanied by differentiation (demonstrated by a progressive loss of CD34 surface expression in CD33+ cells) and a decrease in *BCR-ABL* expression (demonstrated by a reduction in *BCR-ABL* transcripts quantified by real-time PCR in cells before and after culture). The combined effect of the cellular expansion and the reduction in *BCR-ABL* expression resulted in a marginal net gain of *BCR-ABL*

transcripts after culture, which we considered insufficient to justify the artificiality of the experimental system.

We then decided to change direction - not to pursue further the *BCR-ABL* positivity of healthy individuals and to instead focus on molecular residual disease assessment post-HSCT or imatinib. We were particularly interested in 2 observations:

- a) low level positivity for *BCR-ABL* transcripts is occasionally found in patients in long-term remission post-HSCT, that remain free of disease relapse and are considered “functionally cured”;
- b) a proportion of patients that achieve a stable complete molecular response (defined as undetectable *BCR-ABL* transcripts using sensitive molecular assays) on imatinib, relapse if therapy is stopped.

These phenomena, somewhat paradoxical, demonstrate that it is still unclear whether “cure” of CML requires eradication of the original *BCR-ABL*-positive leukemic clone. On the one hand, the significance of transcript negativity is critically dependent on the sensitivity of the assay that is used; on the other hand, occasional low level transcripts may not be a manifestation of the original leukemic clone, but instead result from the kind of “benign” *BCR-ABL* rearrangements seen in healthy individuals.

Given that each patient’s genomic *BCR-ABL* fusion sequence is virtually unique, we developed a highly sensitive, patient-specific real-time quantitative PCR for genomic *BCR-ABL*. We used it to test whether 1) the original leukemic clone is present late after HSCT and is the cause of the occasional low-level transcript positivity seen in patients who are relapse-free long-term HSCT survivors, and 2) whether the original leukemic clone is detectable in patients that are transcript-negative on imatinib therapy.

Introduction

Few, if any, human malignancies have been more extensively studied than CML. Both in the past and in the present, knowledge garnered from investigations into this model disease has had profound implications in our understanding of neoplastic hematologic disorders and in the wider field of oncology.

Background and historical landmarks

CML was the first form of leukemia to be recognized. Two case reports originated from Edinburgh in 1845 describe CML in terms that are recognizable today - “Case of hypertrophy of the spleen and liver, in which death took place from suppuration of the blood”¹ and “Case of disease of the spleen, in which death took place in consequence of the presence of purulent matter in the blood”.² The authors of these reports favoured an infectious cause of the disease; Rudolph Virchow, who published an account of a very similar case a few weeks afterwards,³ was the first to suggest the non-suppurative, neoplastic nature of the process. Virchow is also credited with first using the term “leukämie”, from the Greek for white (*leukos*) blood (*haima*).⁴

In 1960, Nowell and Hungerford found an abnormal small chromosome in bone marrow cultures of CML patients.⁵ This, later named the Philadelphia (Ph) chromosome, was the first consistent chromosomal abnormality associated with a neoplasm. The Ph chromosome was identified as a shortened chromosome 22 (22q-) in 1970;⁶ three years later, the cytogenetic abnormality was shown to result from a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11).⁷

During the late 1960s and 1970s, it was demonstrated that CML was clonal and affected a pluripotent hematopoietic stem cell.^{8;9}

The development of hematopoietic stem cell transplantation (HSCT) had a major impact in the treatment of CML. The first report of successful HSCT in CML dates from 1979, using syngeneic twin donors.¹⁰ The Ph-positive clone disappeared in the HSCT recipients. It was soon realized that HLA-matched siblings and unrelated volunteers

could be used as donors; and, more importantly, that HSCT could be “curative” in CML.¹¹

During the 1980s, it was demonstrated that the translocation involved the *ABL* proto-oncogene (9q34) and the *BCR* gene (22q11),¹²⁻¹⁴ leading to the formation of a *BCR-ABL* fusion gene. This is transcribed into a chimeric mRNA,¹⁵ which in turn encodes a Bcr-Abl fusion protein. Abl is a non-receptor tyrosine kinase; in Bcr-Abl, the tyrosine kinase activity of the Abl moiety is increased and deregulated.

In 1990, the tyrosine kinase activity of Bcr-Abl was shown to be central to its transformation ability.¹⁶ The hybrid protein is believed to be the principal cause of chronic phase CML. Finally, the development of imatinib, a highly effective and specific inhibitor of Bcr-Abl tyrosine kinase,¹⁷ radically changed the approach to the treatment of CML in the late 1990s.¹⁸ Imatinib is the recommended first-line therapy for most newly-diagnosed CML patients; it was the first, prototypical drug in the class of Bcr-Abl tyrosine kinase inhibitors (TKIs), two of which – dasatinib and nilotinib – have already been approved for imatinib-intolerant and resistant disease.¹⁹

The mechanism first described in CML – a chromosome translocation leading to the formation of a fusion gene, which generates a fusion protein with oncogenic properties – has proven to be operative in a number of leukemias. The success of imatinib in the treatment of CML illustrates the possibilities of molecularly targeted cancer therapies.

The disease

CML is a clonal myeloproliferative disorder of the pluripotent hemopoietic stem cell. In the United States of America the annual incidence is 1-2 cases per 100,000 individuals, accounting for approximately 15% of all leukemias and 7-20% of adult leukemias. Males are more often affected than females, with a 1.3-2.2 to 1 ratio. There has been no change in incidence in the last 50 years. The incidence increases with age, with a median age at presentation of 45-55 years.²⁰

The natural history of CML typically involves gradual progression through three phases: chronic phase (CP), accelerated phase (AP) and blast crisis (BC).

Approximately 85% of patients present in CP.²⁰ The dominating feature in newly diagnosed patients is a massive myeloid expansion, causing leukocytosis with a predominance of granulocytes – 50-70% of patients present with leukocyte counts above $100 \times 10^9/L$, including cells in all stages of granulocytic differentiation, from blasts (usually less than 5%) to mature granulocytes. Thrombocytosis, anemia, basophilia and monocytosis may also be present at diagnosis. The marrow is characteristically hypercellular, with a greatly increased myeloid/erythroid ratio; in over 90% of patients bone marrow blasts are $\leq 5\%$. The most frequent clinical sign at diagnosis is splenomegaly, present in 40-50% of cases; hepatomegaly and lymphadenopathy are less common. The increasing frequency of routine blood tests and physical examinations has led to a change in the presentation features of the disease, with a tendency to earlier diagnosis, often in patients that are still asymptomatic.

Characteristically, in CP the differentiation capacity of the leukemic cells is largely preserved. The subtle morphological, biochemical and functional defects that have been described in cells from CML-CP (reviewed in Clarkson *et al.*²¹) do not compromise their ability to carry out their physiological functions.

The indolent CP is followed after 2-6 years by progression to AP/BC. This progression is characterised by progressive maturation arrest with increasing blasts, increase in tumour burden, poor response to therapy and cytogenetic clonal evolution.

The AP of CML is usually short-lived and somewhat ill-defined. In BC, the clinical picture is that of an aggressive acute leukemia with a phenotype that is myeloid in about two thirds of patients and lymphoid in one third. BC results in the patient's death, usually within 3-6 months.

Cytogenetics of CML

The cytogenetic hallmark of CML is the Ph chromosome (Figure 1) – a shortened chromosome 22 (22q-) that results from a reciprocal translocation between the long arms of chromosomes 9 and 22.⁵⁻⁷ Over 90% of patients with clinical and morphological features of CML have a Ph chromosome. Of the 5-10% that have a normal karyotype, about one-third to half can be shown to harbour a *BCR-ABL* fusion

gene, molecularly equivalent to the one present in the Ph-positive patients. Ph-positive CML and Ph-negative, *BCR-ABL*-positive CML have similar clinical features and response to treatment.^{22;23}

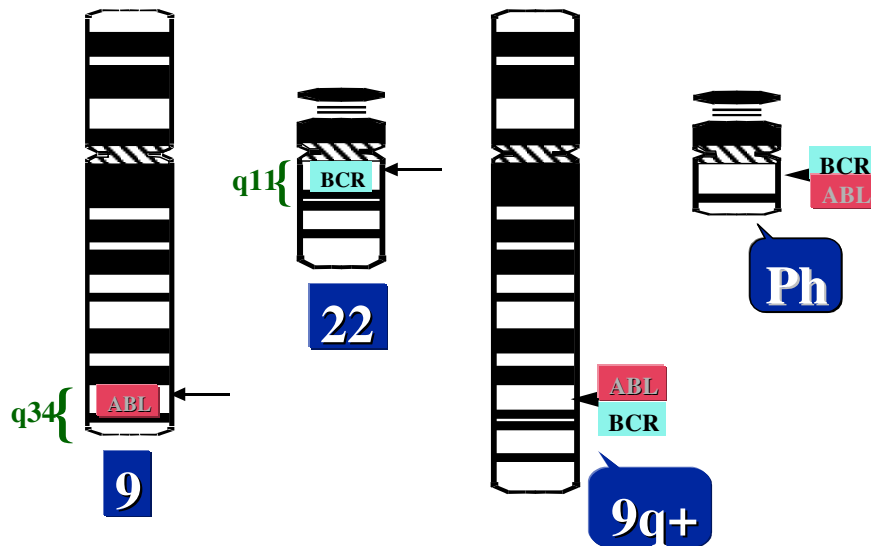


Figure 1. The normal chromosomes 9 and 22 and the t(9;22) reciprocal translocation. The Ph chromosome – a shortened chromosome 22 (22q-) – results from an exchange of chromosome material between the long arms (q) of chromosomes 9 and 22. The *ABL* gene is located at 9q24 and the *BCR* gene at 22q11. As a result of the translocation, the Ph chromosome bears the oncogene *BCR-ABL* and a reciprocal fusion gene, *ABL-BCR*, is formed in 9q+. Figure reproduced by permission of Prof. Junia Melo.

The acquisition of cytogenetic changes in addition to the Ph chromosome is associated with progression from CP to BC. Around 60 to 80% of patients in blastic transformation have additional non-random karyotypic changes, most frequently +8, +Ph, +19 and i(17)q.^{24;25}

The development of fluorescence *in situ* hybridisation (FISH) techniques has revealed the presence of deletions in the derivative chromosome 9q+ in 10 to 15% of patients with CML.²⁶ These were not detectable by conventional cytogenetic analysis. The deletions are variable in size and can in some cases span several megabases. They are adjacent to and most often span the translocation breakpoint, involving both chromosome 9 and 22 sequences. They appear to originate simultaneously with the Ph

translocation. The presence of these deletions has been associated with a shorter duration of chronic phase, earlier disease progression and decreased survival in patients treated with hydroxyurea or interferon α (IFN α)-based regimens.²⁷⁻²⁹ There are also data suggesting an increased rate of relapse in patients with deletions after allogeneic HSCT.²⁹

Initial reports looking at the effect of 9q+ deletions on prognosis in imatinib-treated patients gave conflicting results, with some authors reporting lower response rates and shorter time to progression while others did not confirm it.³⁰⁻³² It is worth noting that these studies included few patients in early CP-CML. A more recent and comprehensive study in early CP-CML found no prognostic impact of 9q+ deletions in imatinib-treated patients.³³

The Ph chromosome and the *BCR-ABL* fusion are not exclusive to CML. They are present in a group of acute lymphoblastic leukemias (ALL) with poor prognosis – up to 30% of adult and 5% of childhood ALL.³⁴

***BCR-ABL* diversity at the genomic and mRNA levels**

BCR-ABL fusions are heterogeneous (Figure 2). This diversity is mostly due to the *BCR* component of the fusion.³⁵

The breakpoint in *ABL* can occur anywhere along a region more than 300kb long in the 5' end of the gene. The breakpoint is usually intronic and may be located upstream of exon 1b, downstream of exon 1a or, more commonly, between the two alternative first exons^{36;37} (Figure 2). *BCR-ABL* can therefore contain both or none of the alternative *ABL* first exons or exon 1a alone. Even if present in the genomic fusion, *ABL* first exons are spliced out during RNA processing. As such, at the mRNA level, *BCR* exons are fused to *ABL* exon a2.

The breakpoints in *BCR* are usually located in one of three breakpoint cluster regions (*bcr*, hence the name of the gene) (Figure 2). In 95% of CML patients and approximately one third of *BCR-ABL*-positive ALL patients the breakpoint is within a 5.8kb central segment of the gene known as the major breakpoint cluster region (M-

bcr).¹⁴ This includes exons 12-16 (e12-e16, formerly named b1-b5).³⁸ Most of the breakpoints occur in the introns downstream of e13 or e14, leading to e13a2 or e14a2 chimeric mRNAs (formerly b2a2 and b3a2, respectively). A minority of about 5% of patients co-express e13a2 and e14a2 transcripts, probably due to alternative splicing of e14.^{39;40} Both types of mRNAs are 8.5kb long and are translated into a 210kD protein, p210^{Bcr-Abl}. The type of junction does not appear to carry prognostic significance.³⁵

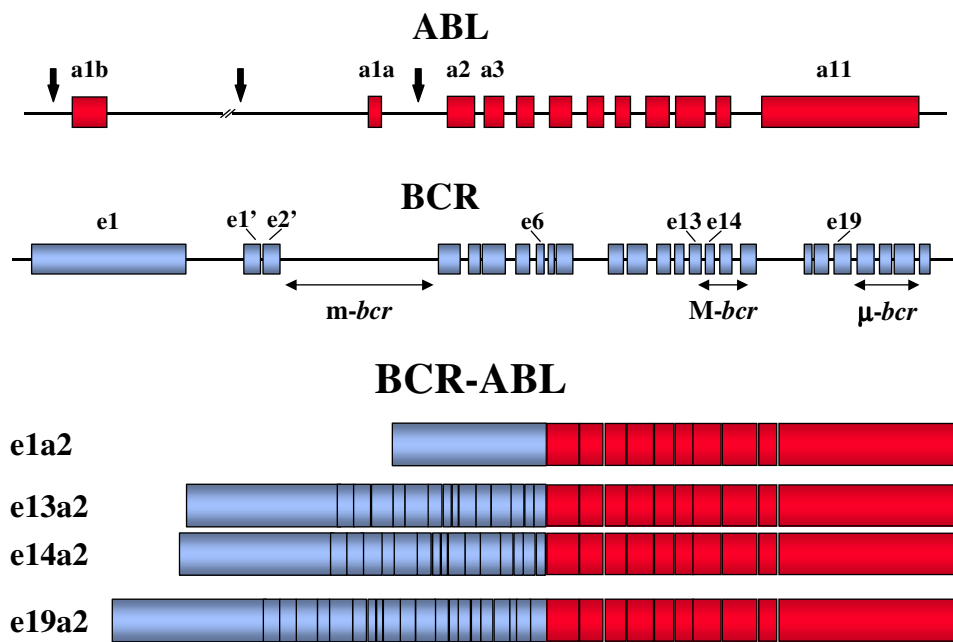


Figure 2. Locations of breakpoints in *ABL* (solid arrows) and on the three breakpoint cluster regions in *BCR*. Structure of the resulting chimeric mRNAs. Figure reproduced by permission of Prof. Junia Melo.

In two thirds of *BCR-ABL*-positive ALL patients and in rare CML cases, the *BCR* breakpoint occurs in a 54.4kb intronic sequence between the two alternative second exons, e2' and e2. This is located 5' of the M-*bcr* and is termed minor breakpoint cluster region (m-*bcr*) (Figure 2). The resulting fusion gene contains both *BCR* alternative first exons (e1 and e1') and e2'. During RNA processing, e1' and e2' are spliced out to generate a 7kb *BCR-ABL* mRNA in which *BCR* e1 is fused to *ABL* a2. The shorter segment of *BCR* present in the fusion leads to a smaller, 190kD, protein - p190^{Bcr-Abl} (also referred to as 185^{Bcr-Abl}).

More than 90% of p210^{Bcr-Abl}-positive CML and ALL patients have detectable p190^{Bcr-Abl}-type mRNA transcripts.⁴¹ These e1a2 transcripts are expressed at a low level when compared with the “dominant” e13a2 and e14a2 messages and probably result from alternative splicing or missplicing of primary RNA transcripts. They do not appear to have pathogenic significance.

A third breakpoint cluster region, 3' of the M-*bcr*, is named “micro” (μ -*bcr*) (Figure 2). This is between *BCR* e19 and e20 and leads to an e19a2 *BCR-ABL* mRNA. The resulting 230kD protein (p230^{Bcr-Abl}) is associated with chronic neutrophilic leukemia, characterised by an expansion of mature neutrophils, thrombocytosis and indolent clinical course, with less propensity to transform than p210^{Bcr-Abl}-positive CML.⁴² The less aggressive course of this disease may be due to a lower level of expression of the e19a2 mRNA and p230^{Bcr-Abl} when compared with “classical” CML.⁴³

In addition to the e1a2, e13a2, e14a2 and e19a2 transcripts, a number of other *BCR-ABL* fusions with “atypical” breakpoints have been described in sporadic cases of CML and ALL.^{35;44}

Aetiology of the *BCR-ABL* rearrangement

The cause of the *BCR-ABL* rearrangement is unknown. Exposure to ionising radiation is an established risk factor for CML⁴⁵⁻⁴⁷ and *in vitro* irradiation of myeloid cell lines generates *BCR-ABL* fusions indistinguishable from those found in CML.⁴⁸ The physical proximity of normal *BCR* and *ABL* in hematopoietic cells during certain phases of the cell cycle may favour aberrant recombination, if breakage should occur.⁴⁹ The existence of a 76kb duplicon (a duplicated genomic sequence) in close proximity to *BCR* and *ABL* may facilitate the rearrangement.⁵⁰

Despite the above, the mechanism behind the formation of the *BCR-ABL* fusion remains elusive. An important reason for this is that few genomic fusions had been characterized prior to our work. In CML, the few studies that have addressed this issue found *BCR-ABL* breakpoints to be often associated with repeat regions, particularly *Alu* repeats, in both *ABL* and *BCR*.^{51;52} This has not been confirmed by the study we co-authored (see **Concluding Remarks**).⁵³

Normal *ABL* and *BCR*

ABL and *BCR* are ubiquitously expressed in mammalian tissues. The precise functions of their protein products are not fully understood.

ABL is the human homologue of v-*abl*, the oncogene of the Abelson murine leukemia virus (A-MuLV).⁵⁴ It contains 12 exons located at 9q34. It encodes a 145kD protein, Abl, with two isoforms derived from alternative splicing of its first exon.

Functionally, Abl is a non-receptor tyrosine kinase that interacts with a variety of cellular proteins and is involved in a number of cellular processes, namely regulation of cell cycle, DNA damage response, transduction of integrin-mediated signalling, cytoskeletal organization and cell migration.^{55;56}

The two alternative isoforms of Abl differ in that one of them has in its amino terminal a myristoylation site that allows it to attach to the plasma membrane. Abl contains three domains (SH1-SH3) that are highly homologous to domains found in Src, the prototypical non-receptor tyrosine kinase. SH1 is the tyrosine kinase domain; SH2 and SH3 are non-catalytic domains that allow interaction with other proteins. Additionally, the molecule includes proline-rich sequences that provide binding sites for the SH3 domains of other proteins,⁵⁷ actin⁵⁸ and DNA-binding motifs,⁵⁹ nuclear localisation⁶⁰ and export⁶¹ signals.

ABL knockout mice are runted, have very high neonatal mortality and are lymphopenic.^{62;63}

The *BCR* gene comprises 23 exons (including alternative exons 1' and 2') located at 22q11. Its main product is the ubiquitously expressed 160kD Bcr protein which includes several functional motifs.^{44;64}

The first exon encodes a coiled-coil oligomerisation domain⁶⁵ and a serine-threonine kinase domain⁶⁶ whose only known substrates are Bcr itself (autophosphorylation) and Bap-1 (Bcr associated protein-1).⁶⁷ It also encodes several SH2-binding domains, which enable it to associate with the SH2 domain of Abl⁶⁸ and other proteins.^{67;69}

Bcr appears to play a dual role in guanine nucleotide-binding protein (G protein) signal transduction. The central region of the molecule contains a guanine nucleotide exchange factor (GEF) domain⁷⁰ and the C-terminal region a domain that acts as a GTPase-activating protein (GAP) for Rac,⁷¹ a member of the superfamily of G proteins of which Ras is the best known element. GEFs activate G proteins by promoting the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP). GAPs “turn off” G proteins by accelerating the rate of GTP hydrolysis.

BCR knockout mice are viable and their only phenotypic abnormality is an increased oxidative burst in neutrophils.⁷²

The p210^{Bcr-Abl} Oncoprotein

In p210^{Bcr-Abl} an N-terminal segment of Bcr is joined to a C-terminal segment of Abl (Figure 3).

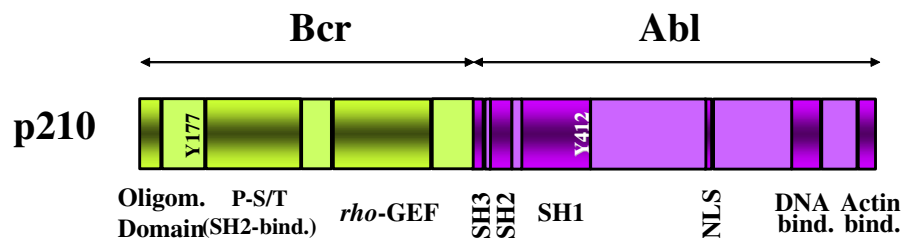


Figure 3. Structure of the p210^{Bcr-Abl} oncoprotein. An N-terminal Bcr segment is fused to a C-terminal Abl segment. The Bcr moiety contains an oligomerisation domain, a serine-threonine kinase domain (P-S/T), Src-homology 2 (SH2)-binding domains and a guanine nucleotide exchange factor (GEF) domain. Tyrosine 177 (Y177) is essential for binding of adapter proteins. The normal Bcr also includes a C-terminal GTPase-activating domain which is not part of p210^{Bcr-Abl}. Present in the Abl component are the Src-homology domains (SH1, SH2 and SH3), nuclear localization signal (NLS) and DNA and actin-binding motifs. Tyrosine 412 (Y412) is a site of autophosphorylation. Not depicted, but included in the Abl portion, are additional NLS, DNA and actin-binding motifs, proline-rich SH3-binding sequences and a nuclear export signal. Figure reproduced by permission of Prof. Junia Melo.

The functional domains encoded by the first exon of *BCR* are the only ones present in all 3 forms of the Bcr-Abl oncoprotein (p210, p190 and p230). These include the oligomerisation, serine-threonine kinase and SH2-binding domains. Both the oligomerisation domain⁶⁵ and a SH2-binding domain located between amino acids 192 and 242⁶⁸ are essential for Bcr-Abl-mediated transformation. Tyrosine 177 is essential for binding the adapter protein Grb-2. This interaction is required for Bcr-Abl-induced Ras activation.⁶⁹ Additionally, p210^{Bcr-Abl} includes the Bcr GEF domain.

The Abl component of the fusion protein includes its SH1, SH2 and SH3 domains; the proline-rich protein-interaction domains; the DNA and actin-binding motifs; and the nuclear localisation and export signals.

The central determinant of the oncogenicity of p210^{Bcr-Abl} is the tyrosine kinase activity of the Abl-derived SH1 domain. The tyrosine kinase activity of normal Abl is tightly regulated under physiological conditions. In Bcr-Abl, the truncation of the N-terminal portion of Abl and its fusion with Bcr sequences leads to an increase and deregulation of the tyrosine kinase activity of the Abl component.⁷³ This uncontrolled tyrosine kinase activity results in activation of mitogenic signalling pathways, altered cellular adhesion to bone marrow stromal cells and extracellular matrix and a reduced response to apoptotic stimuli.^{44;74} The relative contribution of these multiple effects to the phenotype of CML-CP is still poorly understood.

***BCR-ABL* in healthy individuals**

Two landmark studies, using highly sensitive nested reverse-transcriptase polymerase chain reaction (RT-PCR) protocols, found that *BCR-ABL* mRNA transcripts could be detected at a very low level in leukocytes from healthy individuals.

Biernaux *et al.*⁷⁵ detected *BCR-ABL* transcripts in leukocytes from the peripheral blood of 22 out of 73 (30%) healthy adults and 1 out of 22 (4%) children. They found no *BCR-ABL* transcripts in 22 samples of umbilical cord blood. In 18 of the 23 positive cases, the amplification product corresponded to an e14a2 junction; 3 of the cases produced e13a2 transcripts; 4 cases expressed both types of p210^{Bcr-Abl} transcripts.

These findings were confirmed and expanded in the work by Bose *et al.*⁷⁶ They tested the total amount of cDNA generated from 10^8 peripheral blood leukocytes of normal individuals or from 10^8 cells from Ph-negative cell lines for the presence of p210- or p190-type transcripts. They found p210-type transcripts (e13a2 and/or e14a2) in leukocytes from 4 out of 15 (27%) healthy adults and 3 out of 7 (43%) Ph-negative cell lines. Of the 4 positive individuals, 2 co-expressed additional hybrid mRNAs with unusual junctions, such as e15a2, e16a2 and e17a2. When they used a PCR protocol with primers designed to amplify hybrid mRNAs with an e1a2 junction (p190-type fusion), *BCR-ABL* transcripts were found in 11 out of 16 (69%) healthy adults and 7 out of 7 (100%) Ph-negative cell lines. The majority of the transcripts (74%) had the expected e1a2 junction, but in 7 samples larger fragments were also observed. These were shown by sequencing to represent *BCR-ABL* transcripts with atypical junctions such as e2a2, e2a3, e1a3, e4a2 and e5a3, and fusions containing *BCR* and/or *ABL* intronic sequences in various rearrangements between e1 and a2. Of the 4 individuals positive for the p210-type transcripts, 3 co-expressed p190-type transcripts.

It is noteworthy that several of the p210- and p190-type transcripts that were detected have aberrant junctions and encode truncated Bcr-Abl proteins that would presumably not have oncogenic properties.

Based on the sensitivity of the RT-PCR protocols used in these studies, the number of *BCR-ABL*-positive leukocytes in the blood of healthy individuals is estimated to be extremely low, probably around 1-10 per 10^8 leukocytes.⁷⁶

To produce a leukemic phenotype, these fusion genes would have to fulfil at least two criteria:

1. the structure of the gene fusion must allow for the production of a functional protein with oncogenic properties;
2. the gene fusion must occur in an immature hematopoietic progenitor cell, with the capacity for self-renewal and clonal expansion.

In terms of the structure of the gene fusions, Bose *et al.*⁷⁶ demonstrated the presence of both leukemogenic and non-leukemogenic transcripts in normal individuals. However,

they did not address the question of which cell type originated these transcripts - whether mature, differentiated cells, destined to die after a limited number of cell divisions, or immature progenitors. This is a very relevant point, since *BCR-ABL* has been shown to transform hematopoietic stem cells, but to be insufficient, by itself, to transform committed myeloid progenitors lacking inherent self-renewal capacity (unlike other leukemia-associated fusion genes, such as *MLL-ENL* and *MOZ-TIF2*).^{77;78}

The Master thesis was an attempt at dealing with the second point. We wanted to test immature hematopoietic progenitor cells (CD34+) from normal individuals for the presence of these transcripts, a task complicated by the rarity of these cells in peripheral blood. The *in vitro* expansion of CD34+ cells would increase the amount of cells available for testing by RT-PCR, thereby increasing its sensitivity in detecting rare *BCR-ABL*-positive cells that might be present.⁷⁹ This approach would have yielded optimal results if two conditions were met:

1. if the rare *BCR-ABL*-positive cells that might be present among the CD34+ cells would proliferate at least as much as the majority of the *BCR-ABL*-negative cells;
2. if the expansion had no deleterious impact on *BCR-ABL* expression.

This was not the case, since we concluded, from our experiments, that cellular expansion was accompanied by a decrease in *BCR-ABL* expression.

The detailed description of the Material and Methods and of the Results can be found in **Annex I**. In summary, we confirmed the feasibility of expanding CD34+ cells *in vitro* in medium enriched with IL-1 β , IL-3, IL-6 and SCF.⁸⁰ After 12 days in culture, the number of viable CML-CP cells increased, on average, over 21-fold, from an initial number of 400,000 to 7,500,000 cells or more. The single sample of CD34+ cells from the peripheral blood of a normal individual that we tested showed even greater expansion - 36-fold on day 12. Although Garin *et al.*⁸⁰ found a similar degree of proliferation of CML and normal CD34+ cells in these culture conditions, their work also demonstrates that there is significant inter-individual variability, which may account for the larger expansion of the normal CD34+ cells we tested when compared with the CML-CP samples.

As occurs in most *in vitro* expansion systems, the proliferation of CD34+ cells was accompanied by differentiation. The CD34+ cells differentiated in a myeloid direction, as indicated by a progressive loss of CD34 surface expression while maintaining CD33 positivity. Corroborating previous data in this experimental setting,⁸⁰ the decline in CD34 positivity was faster in the CML-CP than in the normal cells, a known characteristic of CML primitive cells.⁸¹ The amount of normal cells expressing CD33 on day 0 was of the same order of magnitude as that found in the CML samples (over 85%). This is in contrast with previous reports which indicate that, when compared with their normal counterparts, a higher proportion of CML CD34+ cells express CD33.^{80;82}

To test the effect of *in vitro* expansion on *BCR-ABL* expression, we used real-time PCR to quantify *BCR-ABL* transcripts in CML-CP CD34+ cells cultured for 7 and 12 days. The results indicate that, after 12 days in culture, the amount of *BCR-ABL* transcripts decreased, on average, to about one-fifth of its day-0 values.

The association of myeloid differentiation of CD34+ cells with a decrease in *BCR-ABL* transcript levels that occurred in this experimental setting is consistent with the observation that protein levels of p210^{Bcr-Abl} in CML cells, and Bcr and Abl in normal myeloid cells, are inversely related to maturation.⁸³

If we transpose the data obtained from CML-CP cells to the rare *BCR-ABL*-positive cells that might exist among the CD34+ cells of normal individuals, the combination of a 21-fold increase in the number of *BCR-ABL*-positive cells with a 5-fold decrease in their *BCR-ABL* mRNA expression indicates that culturing these cells for 12 days under the conditions described would provide a net gain of *BCR-ABL* transcripts of about 4-fold.

We concluded that the experimental approach we devised resulted in only a minor gain in *BCR-ABL* transcripts. We considered this gain insufficient to justify the increased artificiality of the approach.

Measuring residual disease

The response to treatment in CML is measured at three different levels, progressively more sensitive in the detection of residual disease: hematologic, cytogenetic and molecular.¹⁹

Complete hematologic response (CHR) is defined as a white blood cell (WBC) count inferior to $10 \times 10^9/L$; no myeloblasts, promyelocytes or myelocytes in a WBC differential with less than 5% basophils; a platelet count inferior to $450 \times 10^9/L$; and a non-palpable spleen. At least 20 bone marrow metaphases are required to define cytogenetic response: a patient with 1% to 35% Ph-positive cells is said to have achieved a partial cytogenetic response (PCyR), whereas the absence of Ph-positive cells defines a complete cytogenetic response (CCyR). Major cytogenetic response (MCyR) usually encompasses both PCyR and CCyR.¹⁹

Minimal residual disease (MRD) at the molecular level is usually determined by measuring *BCR-ABL* mRNA using a real-time quantitative polymerase chain reaction (RQ-PCR).^{84;85} There have been substantial efforts to ensure that there is harmonization of methodologies used to measure *BCR-ABL* transcripts, and to define an internationally agreed scale to report results. This is known as the international scale (IS).⁸⁵ A major molecular response (MMR) is defined as a 3-log_{10} (1000-fold) reduction in the level of *BCR-ABL* transcripts compared with a standardized baseline obtained from a group of patients at diagnosis,⁸⁴ and corresponds to $\leq 0.1\%$ in the IS.⁸⁵ Expression of the results of *BCR-ABL* mRNA quantification in the IS requires the definition of a laboratory-specific conversion factor.⁸⁵

A deeper level of molecular response, in which *BCR-ABL* transcripts become undetectable using strict sensitivity criteria, is referred to as a complete molecular response (CMR). The interpretation of results at the threshold of sensitivity of the assay is difficult, and the concept of CMR is fraught with biologic⁸⁶ and technical^{85;87} limitations (see **Discussion** and **Concluding Remarks**).

Treatment: hematopoietic stem cell transplantation (HSCT)

Until the development of HSCT, CML progression was considered virtually inevitable, and the disease was almost invariably lethal.^{4;11}

Myeloablative conditioning produces a profound reduction in leukemic burden; however, this by itself is not enough to achieve long-term control of the disease. The importance of an immune-mediated “graft-versus-leukemia” effect is demonstrated by an increased relapse rate in recipients of syngeneic transplants when compared with HLA-identical siblings,⁸⁸ and in recipients of T cell-depleted grafts;⁸⁹ and in the remarkable efficacy of donor lymphocyte infusions (DLI) in restoring remission after relapse post-HSCT.^{90;91}

Post-HSCT, it has been observed that low level positivity for *BCR-ABL* mRNA in the peripheral blood is not a harbinger of inevitable relapse, particularly in patients that are in long-term remission (LTR), and continues to be observed more than 10 years after HSCT.⁹²⁻⁹⁶ Although the majority of relapses post-HSCT occur early after the procedure, and the relapse risk decreases with increasing time post-HSCT, relapse as late as 14 years after HSCT has been reported.⁹⁷ It remains unknown if these rare transcripts are a manifestation of the original leukemic clone or a result of the kind of “benign” *BCR-ABL* rearrangements seen in healthy individuals.

Treatment: imatinib

The development of imatinib mesylate (formerly known as STI571) dramatically changed the treatment approach to CML. Imatinib is the recommended first-line therapy for newly-diagnosed CML-CP.¹⁹ The landmark IRIS (International Randomized Study of Interferon versus STI571) trial compared IFN α plus low-dose cytarabine with imatinib 400mg per day in the treatment of newly-diagnosed CML-CP patients. The trial demonstrated the superiority of imatinib in terms of hematologic, cytogenetic and molecular responses, and in freedom from progression to AP/BC.^{84;98} The high rate of early crossover between the two arms of the study precluded an analysis of overall survival; an overall survival advantage of imatinib could only be shown by retrospective, historical comparisons.^{99;100}

Subsequent reports of IRIS have shown that the response to imatinib is durable, with 8-year event-free survival (EFS), progression-free survival and overall survival (OS) of, respectively, 81%, 92% and 85% (93% OS when only CML-related deaths were considered).¹⁰¹⁻¹⁰³ CML-CP patients responding to imatinib had a low risk of progression to AP/BC, which decreased with time on imatinib. Most events of progression to AP/BC occurred early, with minimal risk after 3 years on therapy, and no evidence for an increase over time with 8 years follow-up. The annual rates of progression to AP/BC in years 4 to 8 after initiation of imatinib were 0.9%, 0.5%, 0%, 0% and 0.4%, respectively. Only 15 (3%) of the patients who achieved CCytr progressed to AP/BC, 14 of them within 2 years of achieving CCytr.^{102;103}

An observational, single institution, intention-to-treat analysis reported on 204 consecutive adult patients with newly-diagnosed CML-CP treated with imatinib at the Hammersmith Hospital.¹⁰⁴ At 5 years, estimated probabilities of OS and EFS – events defined as in the IRIS trial (i.e., death from any cause during treatment, progression to AP/BC, loss of a CHR, loss of a MCytr, or an increasing white-cell count) were, respectively, 83% and 81%,¹⁰⁴ comparable with the IRIS 5-year follow data (OS 89% and EFS 83%).¹⁰¹

It is worth noting that the IRIS trial did not consider as an “event” discontinuation of imatinib due to failure to achieve a MCytr, or due to intolerance. Taking these situations into consideration, the Hammersmith study found that the 5-year probability of being in cytogenetic remission while still receiving imatinib was 63%.¹⁰⁴

The frequency of CMR in imatinib-treated patients was initially reported as 4% after a median of 19 months.⁸⁴ There is evidence that this number increases with continuing therapy, with 13 of 29 first-line imatinib-treated patients achieving CMR after a median follow-up of 81 months.¹⁰⁵ The maintenance of this level of response appears to be largely dependent on continued suppression of the *BCR-ABL* clone by imatinib, as illustrated by the finding of early relapse in 6 of 12 patients that discontinued imatinib after being in continuous CMR for more than 2 years.¹⁰⁶

In vitro treatment of CML cells with imatinib results in the accumulation of quiescent CML precursors.^{107;108} As a phenotypically similar population of cells isolated from newly diagnosed patients can serially engraft immunodeficient mice, it is possible that these cells are imatinib-resistant CML stem cells; however, this has not been experimentally demonstrated. It is unresolved whether or not the survival of these cells results from a failure of Bcr-Abl inhibition by imatinib. Recent evidence suggests that imatinib does inhibit Bcr-Abl activity in primitive CML cells, and that their survival is Bcr-Abl-independent.¹⁰⁹

Although imatinib has shown remarkable efficacy in the treatment of CML-CP, HSCT remains the only proven curative treatment for CML.

Objectives and Aims

When finishing the Master thesis, we identified the following as one of the questions to be addressed in future work: Does the presence of *BCR-ABL*-positive leukocytes in healthy individuals have an impact in the evaluation of MRD in treated CML patients?

Since low-level positivity for *BCR-ABL* transcripts late after HSCT is not an indication of impending relapse, and some patients with CMR on imatinib relapse after imatinib cessation, we sought to elucidate the quality of the molecular response in patients in LTR post-HSCT and in imatinib-treated patients who achieved CMR, using a genomic DNA (gDNA)-based approach as opposed to the usual mRNA-based approach.

Given that the *ABL* breakpoint is distributed randomly over a very large - over 150kb - genomic area, the likelihood that two CML patients share the same breakpoint in both genes, and therefore have identical *BCR-ABL* fusion sequences, is close to zero. Hence, the genomic *BCR-ABL* (*gBCR-ABL*) fusion is a “molecular signature” of each individual CML case. Based on this characteristic, we designed a patient-specific RQ-PCR for *gBCR-ABL* in order to elucidate the nature and quality of CMR upon imatinib therapy, and to establish whether the original leukemic clone is present late after HSCT and is the cause of the occasional low-level positivity for *BCR-ABL* transcripts seen in patients who are relapse-free long-term HSCT survivors.

Materials and Methods

Patients, specimens and treatment regimens

We studied 12 patients submitted to myeloablative HSCT with high-dose cyclophosphamide and total body irradiation conditioning, and 5 imatinib-treated patients who achieved CMR, as previously defined¹⁰⁵ (Table 1). In the latter group, at least 4 consecutive samples negative for *BCR-ABL* mRNA, collected over a minimum of 8 months, were analyzed. We also collected WBC from 24 healthy adult volunteers as controls for RQ-PCR for *BCR-ABL* mRNA, tested exactly as the patients and as previously published^{96;110} (Table 2). Informed consent for the use of cells for research was obtained in accordance with the Declaration of Helsinki and with approval from the Hammersmith and Queen Charlotte's and Chelsea Research Ethics Committee Institutional Review Board.

Table 1. Clinical features of the patients analyzed in this study.

Characteristic	Category			
	LTR post-HSCT/DLI	Early post-HSCT	Imatinib-treated	Total
No. of patients	9	3	5	17
Median age at diagnosis, y (range)	34 (24-43)	32 (22-32)	41 (38-56)	34 (22-56)
Patient gender				
Female	3	0	4	7
Male	6	3	1	10
Median duration imatinib therapy,* mo (range)	NA	NA	76 (63-79)	NA
Donor				
Sibling	4	1	NA	5
VUD	3	2	NA	5
Syngeneic	2	0	NA	2
DLI	2	3	NA	5

LTR indicates long-term remission; HSCT, hematopoietic stem cell transplantation; DLI, donor lymphocyte infusion; VUD, voluntary unrelated donor; and NA, not applicable.

Table 1.*Additional therapy in imatinib-treated patients: all 5 imatinib-treated patients received hydroxyurea. Only 1 of the 5 imatinib-treated patients received Interferon- α (IFN α) prior to imatinib; this patient (represented in Figure 4B, the sole patient who did not achieve g*BCR-ABL* negativity) was on IFN α for 32 months, reaching a minor cytogenetic response. IFN α was stopped 7 months before start of imatinib. This same patient also received homoharringtonine for 7 months, during months 33 to 39 of imatinib therapy. Of the 4 additional patients, 1 received IFN α during months 11 to 13 of imatinib therapy.

Table 2. Results of RQ-PCR for *BCR-ABL*, expressed as the % ratio of *BCR-ABL/ABL* transcripts, on healthy adult volunteers.

Quantification			
Person number	BCR-ABL transcripts*	ABL transcripts*	BCR-ABL/ABL ratio (%)
1 ♥	13	1.40×10^4	0.096
2 ♥	18	2.81×10^4	0.064
3	0	3.35×10^4	0.000
4	0	5.26×10^4	0.000
5	0	7.35×10^4	0.000
6 ♥	0	3.24×10^4	0.000
7 ♥	0	1.22×10^4	0.000
8 ♥	0	2.04×10^4	0.000
9 ♥	5	3.48×10^4	0.013
10	0	1.48×10^4	0.000
11	0	2.70×10^4	0.000
12	0	2.57×10^4	0.000
13 ♥	0	2.10×10^4	0.000
14	0	3.75×10^4	0.000
15	0	3.45×10^4	0.000
16	0	3.82×10^4	0.000
17	0	5.37×10^4	0.000
18	0	9.38×10^4	0.000
19 ♥	0	1.07×10^5	0.000
20	0	2.52×10^4	0.000
21	0	4.28×10^4	0.000
22	0	3.04×10^4	0.000
23	0	3.05×10^4	0.000
24	0	4.65×10^4	0.000

* Average of 3 identical replicate PCR reactions.

Table 2. Peripheral blood red cells were lysed to isolate the WBC, which were then lysed in guanidinium thiocyanate (GTC), and subjected to RNA extraction, cDNA synthesis (reverse transcription) and real-time PCR amplification on an ABI 7500 Fast thermocycler (ABI), according to the same protocol used for testing CML patients' samples [Foroni *et al*, 2009 - Manuscript reference no.110]. All steps of the procedure were carried out in a laboratory entirely separate from that routinely used for MRD testing, under strict conditions to prevent molecular contamination, and in parallel to a series of relevant negative controls. Stored GTC lysate aliquots from 8 individuals (3 positive and 5 negative on the first test), marked with a ♥ symbol on the table, were subjected to a second RNA extraction and cDNA synthesis for repeat of the same test at

a different date, yielding essentially the same results, with the exception of individual no. 9 who, on a repeat PCR, scored negative (zero *BCR-ABL* transcripts). The latter case illustrates the inaccuracy of the results at the very low level of sensitivity of the assay (5 *BCR-ABL* transcripts on the first test), probably reflecting a well documented PCR ‘sampling effect’ [Melo *et al*, 1996 - Manuscript ref. no. 79].

For the 12 HSCT patients, the stem cell donors were HLA-identical siblings (Sib) in 5 cases, HLA-matched unrelated volunteers (VUD) in 5 cases, and syngeneic siblings (Syn) in 2 cases. In 5 cases, the patients met criteria for molecular relapse post-HSCT (as defined in Mughal *et al.*⁹⁴) and were treated with DLI - 2 with a single bulk dose and 3 with an escalating dose schedule.^{111;112} Of the 12 patients, 3 (2 VUD, 1 Sib) were studied in CMR within 6 to 12 months post-HSCT, at molecular relapse and after re-induction of CMR with DLI. Nine patients (4 Sib, 3 VUD, 2 Syn), 2 of whom received DLI for relapse, were studied in LTR post-HSCT/DLI – defined as being relapse-free for a median of 193 months (range: 161 to 333) after last treatment.

Sequencing of the *gBCR-ABL* fusions was done on gDNA from WBC collected before HSCT or imatinib therapy and stored at -80°C as dry pellets or as guanidinium thiocyanate (GTC) lysates. In 1 case, due to the lack of a pre-imatinib sample, WBC collected early in the course of imatinib therapy were used. The QIAamp DNA blood minikit (QIAGEN) was used to extract gDNAs. The quality of the gDNAs was tested with a PCR for an 871bp fragment of *ABL* that included an intronic sequence, as described in Melo *et al.*¹¹³

Follow-up (FU) gDNA samples were collected from peripheral blood WBC after red blood cell lysis using the QIAamp DNA blood minikit (QIAGEN). In some cases, the same kit was used to extract gDNA from WBC GTC lysates stored at -80°C. The FU gDNA samples were quantified using a spectrophotometer and diluted in PCR-grade water to 50ng/μL. The quality and concentration of the gDNAs were checked with a TaqMan RQ-PCR for a genomic fragment of albumin, with a probe (Applied Biosystems) labelled with FAM reporter and TAMRA quencher.

For all the FU gDNAs, the amount of *BCR-ABL* transcripts was measured in cDNAs synthesized from the same WBC samples and quantified as a *BCR-ABL* to *ABL* mRNA

ratio as described in Kaeda *et al.*⁹⁶ and Foroni *et al.*¹¹⁰ Per standard MRD PCR protocol,^{96;110} a minimum of 1×10^4 *ABL* transcripts was required to ensure good cDNA quality, with more than 90% of the samples analyzed in the study containing $>3 \times 10^4$ *ABL* transcripts (average 6×10^4).

Sequencing of g*BCR-ABL* fusions

The g*BCR-ABL* fusions were sequenced using either an inverse PCR (7 cases) or a long-range PCR (10 cases) approach. For the former, 5µg of the gDNA were digested with *RsaI*, a blunt-end cutter that digests the region between exon 13 (e13) and e15 of the major breakpoint cluster region of *BCR* into two fragments: a 776bp fragment including most of intron 13 and e14, and a 1703bp fragment that comprises most of intron 14. The digested gDNA was extracted with phenol/chloroform, completion of digestion checked by agarose gel electrophoresis, and quantified using a spectrophotometer. A ligation reaction containing 500ng of the digested gDNA, 5 units of high concentration T4 DNA ligase and 5X ligase buffer (Invitrogen) in a final volume of 500µL was incubated at 14°C for 16 to 24 h. The digested gDNA was diluted in the ligation reaction in order to favour circularization of the restriction fragments over intermolecular ligation. The ligated gDNA was purified using the QIAquick Gel Extraction kit (QIAGEN) and amplified using 2 pairs of inverse PCR primers, located in the 5' ends of intron 13 or intron 14, and the *Pfu*-Turbo polymerase (Stratagene). The PCR products were then visualized under ultraviolet light in ethidium bromide-stained agarose gels. The inverse PCR was successful when 2 bands could be seen: a wild-type *BCR* band of a known size (767 and 1693bp for intron 13 and intron 14 inverse PCR primers, respectively) and a shifted band corresponding to the rearranged *BCR-ABL* allele. The shifted band was cut out from the gel, purified using the QIAquick Gel Extraction kit (QIAGEN) and cloned using the TOPO-TA cloning kit (Invitrogen). Bacterial colonies were selected according to the size of the insert using a PCR with forward and reverse vector primers. Constructs of the desired size were sequenced in both forward and reverse orientation using the same primers as for the PCR.

When the inverse PCR method was not successful (6 of 13 cases) and in 4 additional cases, a long-range genomic PCR for *BCR-ABL* was used.⁵³ The reaction used the

Expand Long Template PCR System (Roche Applied Science) polymerase and Buffer 2 from the kit. The reaction conditions and thermocycling parameters were optimized to amplify templates of up to 12kb. The PCRs were multiplex reactions with 1 of 2 forward primers, located in *BCR* e13 or e14, and 1 of 4 mixes of 5 reverse primers each, located in *ABL* intronic sequences – a total of 20 reverse primers spanning more than 150kb of the *ABL* breakpoint area, from 5' of exon 1b to 3' of exon a3. The patient-specific PCR products were gel purified and sequenced in a forward orientation using consecutive *BCR* primers, from 5' to 3', until the *BCR-ABL* junction was reached.

Patient-specific RQ-PCR for g*BCR-ABL*

The TaqMan technology (Applied Biosystems [ABI]) was used to detect g*BCR-ABL*. Individual g*BCR-ABL* sequences – all intronic – were submitted to ABI for design of RQ-PCR primers and probe using the Custom TaqMan Genomic Assays Service. The design was “directed” so as to avoid repetitive sequences (namely *Alu* elements) and so that the forward primer was located in *BCR*, the reverse primer in *ABL* and the probe would anneal to the junction. (See Table 3 for primers/probe sequences.) The RQ-PCR reactions were set up in 96-well Fast Optical Reaction plates to a final volume of 30μL using 20X primers/probe mix, 2X TaqMan Universal PCR Master Mix (plates and reagents from ABI), 6μL of gDNA at 50ng/μL and PCR-grade water. The reactions were amplified in the 7500 Fast thermal cycler (ABI) under the following conditions: 2 min at 50°C, 10 min at 95°C and 50 cycles of 15 sec at 95°C followed by 1 min at 60°C. The results were analyzed using the 7500 Fast System SDS Software Version 1.3. The specificity of the RQ-PCR reactions was tested using at least 2 different *BCR-ABL*-positive gDNAs from CP-CML patients or cell lines. The sensitivity and quantitative nature of the RQ-PCR was tested using serial 10-fold dilutions of pre-imatinib/HSCT gDNAs in gDNA from HL60 (*BCR-ABL*-negative cell line). The series covered a range of 6 logs of dilution of pre-imatinib/HSCT gDNA, from 10ng/μL (10^1) to 0.1pg/μL (10^{-4}), diluted in HL60 gDNA at 50ng/μL. The 10^1 , 10^0 and 10^{-1} dilutions were tested in triplicate and the 10^{-2} , 10^{-3} and 10^{-4} in 6 replicate reactions. A standard curve was obtained by plotting the logarithmic value of the dilution against the average cycle threshold (C_t) of the positive reactions at each dilution of the pre-imatinib/HSCT gDNA.

Follow-up gDNA samples were tested in 36 replicate reactions, each loaded with 300ng gDNA (6µL of gDNA at 50ng/µL) – a total of 10.8µg of gDNA per FU sample.

Table 3. Patient-specific RQ-PCR primers and probes for *gBCR-ABL* amplification.

Pt N°	Forward Primer	Probe	Reverse Primer
11	GTCGAGCTGGATGGATACTACTTT	CCTTTCCCTATCTTTAATATCC	GCAGAGAGACTGAGGAAGTGTTC
12	GTCAGTGGTTTGCCTGTATTGTG	CTGGATCCTACCCCTTAACC	TCGGTAATTCACCCAAGGGACTA
16	GCAGGTGGATCGAGTAATTGCA	CCAGTCTCTTGCCAAACC	AGAAAATTGCATAATTGGACCTCTCACT
17	CCACAGCAGAGCAGATTGG	CTGCTCTGCACATTAAA	GCAATACCTGGCACCACATACA
18	CCCTAGCCTGTCTCAGATCCT	CATTATGGGCAGCTCACC	AACCTTTAGGAAGTGTCTCTGAAAGAG
19	CCCGGGACAACAGAAGCT	CAAGACTGCAAGAGATCA	CTCTGCAAAGAGGTGTGTGTGA
20	TGGTAACACATGAGTTGCACTGT	TTCAGTATCATTAGTCAACTGCC	GTCACCAAAGATGAAATACAAATGGCTAA
24	CTCCTCTCCTCCAGCTACCT	CCAGCAGTAGTGAGCATC	CAACTGTGGCAGCTGACACT
26	CATGAGGTGCTGGTGTTCAC	CAGACCAACAATTAGGAATAC	GGTGCATCAGTGCCGTAACCT
30	GCCTGTATTGTGAAACCAACTGGAT	ATGGCCCCGGCTGTG	AGTACCTCATTCTACCAAGTTCTGA
33	GGCTCTATGGGTTTCTGAATGTCAT	CCACTCGAAGAAATC	ACTTCTTTGTGACCTTAGGGTAGACA
36	CATGAGGTGCTGGTGTTCAC	CCACAATTAGGTGTTAAGACCA	GTGGAAGCAATAGAACTCAAATGCA
37	CCTTAACCTTTGCCCCATAGTACA	CACTACAGGCAGCCATC	GGAGTGCAATGCTGCTGATAGTT
41	GGAGTGTTTGTGCTGGTTGATG	CTGGGTGTGGACAGACT	TCGTGGTTTACATCCCTTTGTTCT
45	ACAGAAAGCTTACCAGGGATTGTG	ACTAATACACACACCTTTTC	TGGTAAACCTCAGCAAACTAACT
46	GCTTGTAGGGCCTCTGTCT	CCCGACACATTCCTATGTCC	GCCATTTTAATCTTTTACCCCTAGTCA
47	GCCTGTATTGTGAAACCAACTGGAT	CCTGAGATCCAAGGAAATT	CCCCTGTGCAATGTTCTGATT

Table 3. Each patient is identified by a unique patient number. All sequences are 5' to 3'. The probes were FAM-labelled, with an MGB non-fluorescent quencher.

Patient-specific RQ-PCR versus nested RQ-PCR for *gBCR-ABL*

We compared the sensitivity of the patient-specific RQ-PCR for *gBCR-ABL* with a nested approach in which the RQ-PCR was done on conventional PCR products obtained with primers external to the RQ-PCR primers.

The gDNA from 4 CP-CML patients was serially 10-fold diluted in HL60 gDNA at 50ng/µL, as described in “**Patient-specific RQ-PCR for *gBCR-ABL***”. The lowest concentration that yielded positive results by RQ-PCR (10^{-3} dilution) was tested in 36 replicate RQ-PCR reactions (5µL of gDNA as template in 25µL of final volume; reagents and thermal cycling conditions as described in “**Patient-specific RQ-PCR for *gBCR-ABL***”).

After determining the optimal PCR conditions and combinations of *BCR* forward with *ABL* reverse primers that yielded specific amplification from the CP-CML gDNA of

each patient, 36 replicate conventional PCR reactions were seeded with 5 μ L of template at the 10⁻³ dilution in a final reaction volume of 20 μ L. These were subjected to 30 cycles of amplification, with an annealing temperature ranging from 58 to 66°C. From each of the replicate PCR reactions, 2.5 μ L were used to seed a 25 μ L RQ-PCR.

Results

Specificity and sensitivity of patient-specific RQ-PCR for *gBCR-ABL*

All patient-specific RQ-PCRs for *gBCR-ABL* were specific, that is, there was no amplification from *BCR-ABL*-positive gDNAs obtained from a source other than the individual patient in question (a minimum of 3 different *BCR-ABL*-positive gDNAs – 2 CML samples and 1 cell line – were tested to establish specificity).

The threshold of sensitivity of the assays was at the 10^{-3} dilution of the pre-imatinib/HSCT gDNA, corresponding to a 1pg/ μ L concentration of the pre-imatinib/HSCT gDNA. Because each reaction was loaded with 6 μ L of gDNA at this concentration, each individual reaction contained on average 6pg of pre-imatinib/HSCT gDNA – the estimated gDNA content of 1 human diploid cell¹¹⁴ and, therefore, the theoretical maximal sensitivity achievable if one postulates the presence of 1 *BCR-ABL* copy per cell of the leukemic clone.

At the limit of sensitivity, the RQ-PCRs were not reproducibly positive, with 1 to 4 of 6 replicate reactions positive and a larger variation of C_t values (ΔC_t) between replicates than with less diluted samples, as has been observed with other leukemia-associated fusion genes.^{79;115;116} Less diluted pre-imatinib/HSCT gDNA samples gave reproducible results, with all replicates positive and lower ΔC_t .

All patient-specific RQ-PCRs generated standard curves over 5 logs of dilution (10^1 to 10^{-3}) of acceptable quality as defined in¹¹⁷, that is, a slope between -3.0 and -3.9 and a correlation coefficient of more than 0.95; the majority of them were close to the optimal theoretical slope of -3.3 and correlation coefficient of 1.

Comparison of the sensitivity of RQ-PCR with nested RQ-PCR for *gBCR-ABL*

The numbers of positive reactions of the 36 replicate single-step RQ-PCRs and nested RQ-PCRs with the 10^{-3} diluted template were compared by the Wilcoxon signed rank test. The median difference between the 2 tests was 12.5%, which was not statistically

significant ($p=0.71$). The average number of positive reactions was 13.25 (range: 8 to 20) with single-step RQ-PCR and 11.5 (range: 5 to 15) with nested RQ-PCR; in 3 of the 4 CP-CML samples tested, there was, in fact, a higher number of positive reactions with single-step RQ-PCR than with nested RQ-PCR. Because we could not show an advantage of the 2-step (nested) RQ-PCR assay, it was not adopted for this study.

Residual disease assessment by patient-specific RQ-PCR for *gBCR-ABL*

From each of the 5 patients in CMR on imatinib, between 3 and 5 FU samples negative for *BCR-ABL* transcripts were tested by patient-specific RQ-PCR for the presence of *gBCR-ABL*. In all 5 patients, *gBCR-ABL* was detected in 1 to 5 samples negative for *BCR-ABL* mRNA (Figures 4 and 5). In 4 of the 5 patients, *gBCR-ABL* became undetectable with continuing imatinib therapy - 2 consecutive *gBCR-ABL*-negative samples in 3 patients, and 1 *gBCR-ABL*-negative sample in 1 patient. In all cases, the *gBCR-ABL*-positive sample(s) preceded the *gBCR-ABL*-negative sample(s) (Figures 4A and 5). Negativity for *gBCR-ABL* was found after a median time on imatinib of 70.5 months (range: 60 to 75). In 1 patient, all 5 FU samples tested were *gBCR-ABL*-positive (Figure 4B).

Altogether, from the 5 patients in CMR on imatinib, of 19 samples negative for *BCR-ABL* transcripts, 12 were *gBCR-ABL*-positive, all at a very low level – 1 to 6 positive reactions of 36 replicates, with C_t values at the limit of detection of the standard curve.

Three HSCT-treated patients were tested in CMR early after HSCT (between 6 and 12 months), at relapse, and within 6 to 28 months after reinduction of molecular remission by DLI. In the 3 cases, *gBCR-ABL* was detected in the pre-relapse CMR samples at a level comparable with the limit of sensitivity of the standard curve. At relapse, *gBCR-ABL* was detected at a much higher level (1 to 2 logs above the limit of sensitivity of the standard curve); in the 3 cases it became undetectable after DLI (2 FU samples from each of the 3 patients were *gBCR-ABL*-negative; Figures 6 and 7).

Nine patients were studied in LTR post-HSCT/DLI (Figures 8 and 9). These 9 patients had *BCR-ABL* mRNA levels measured and quantified on a median of 8 occasions (range: 7 to 20). One of the 9 patients was negative for *BCR-ABL* transcripts in all 7 FU

samples. Eight patients, while not meeting the criteria for molecular relapse,⁹⁴ were intermittently low-level positive for *BCR-ABL* mRNA. The proportion of samples positive for *BCR-ABL* mRNA ranged from 22% (2 of 9) to 67% (8 of 12); 3 patients had 4 consecutive samples positive for *BCR-ABL* mRNA collected over 18 to 26 months. In 7 of the 9 patients, 10 or more *BCR-ABL* transcripts were detected on at least one occasion.

Between 1 and 5 FU samples (median time from HSCT/DLI to sample collection: 159 months; range: 80 to 320) from each patient were tested by patient-specific RQ-PCR for *gBCR-ABL*. In 8 of the patients all FU samples were *gBCR-ABL*-negative. In only 1 patient the original *gBCR-ABL* fusion was detected in 1 of 5 samples tested, all of which were positive for *BCR-ABL* transcripts (Figure 8B). The *gBCR-ABL*-positive sample – collected 157 months post-HSCT - was positive in 1 of 36 replicate reactions, with a C_t at the limit of sensitivity of the standard curve (Figure 10).

Altogether, from the 9 patients in LTR post-HSCT/DLI, a total of 24 FU samples were tested for patient-specific *gBCR-ABL*. Only 1 of these samples was *gBCR-ABL*-positive. Fourteen of the 24 samples were positive for *BCR-ABL* transcripts (5/14, from 4 patients, with 10 or more transcripts), with *BCR-ABL* to *ABL* mRNA ratios ranging from 0.001 to 0.019% (or 0.0022 and 0.04218% on the IS⁸⁵). This low level of *BCR-ABL* transcripts is similar to that also found sporadically in normal adults tested by the same cDNA RQ-PCR assay (Table 2).¹¹⁰

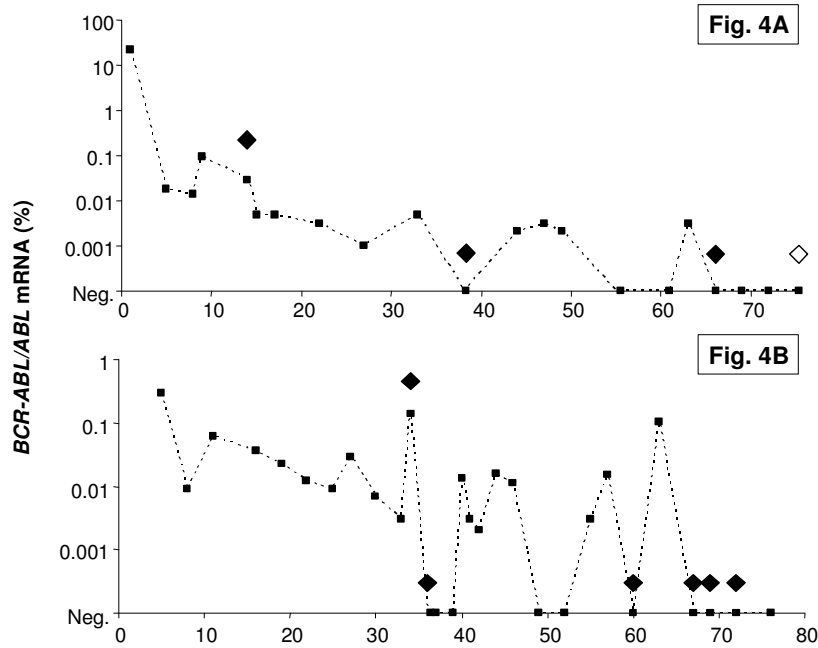


Figure 4. Representative patterns of residual disease found in imatinib-treated patients achieving CMR. Panels A and B show 2 individual patients. On the x-axis are months on imatinib. The results of the quantitation of *BCR-ABL* transcripts (dashed line with ■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the real-time quantitative polymerase chain reaction for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. (A) *gBCR-ABL* was initially detected in transcript-negative samples but became undetectable with continuing imatinib therapy. A similar pattern was seen in 3 other patients tested (Figure 5). (B) The single patient, of 5 tested, in which all transcript-negative samples tested were *gBCR-ABL*-positive.

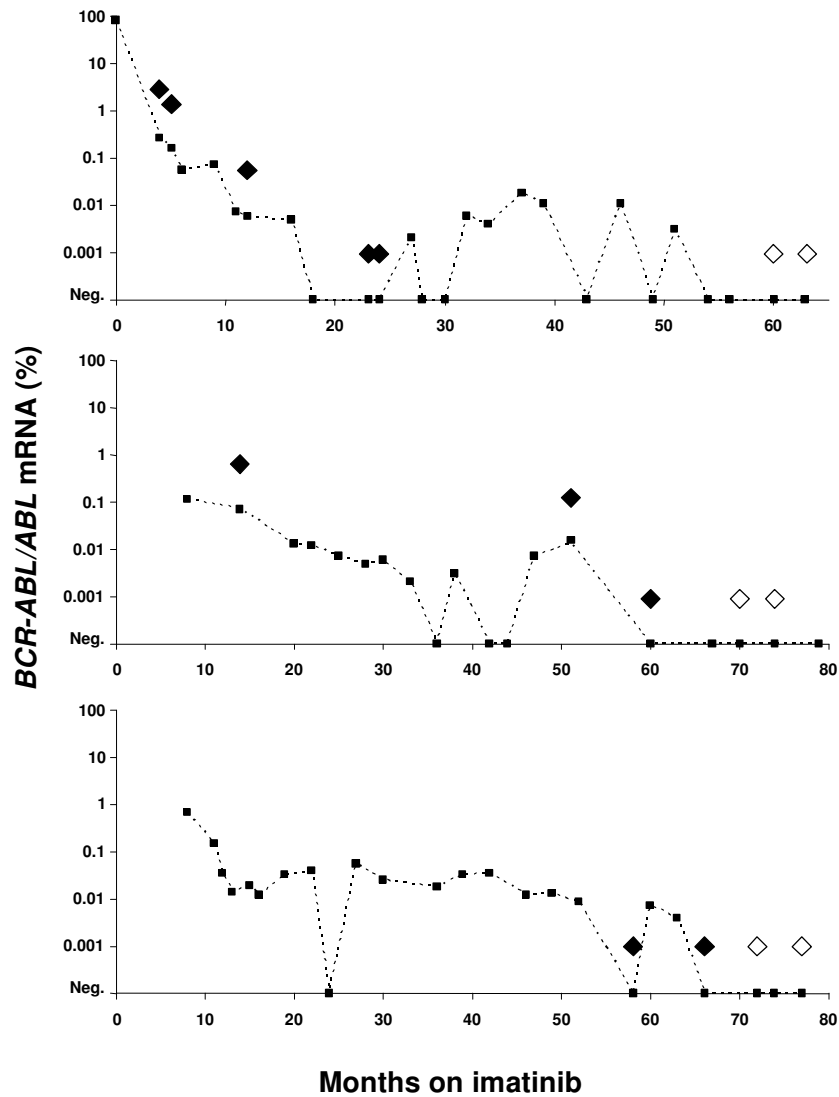


Figure 5. Patterns of residual disease found in 3 additional imatinib-treated patients achieving CMR. On the x-axis are months on imatinib. The results of the quantitation of *BCR-ABL* transcripts (dashed line with ■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. In the 3 patients depicted, *gBCR-ABL* was initially detected in transcript-negative samples but became undetectable with continuing imatinib therapy.

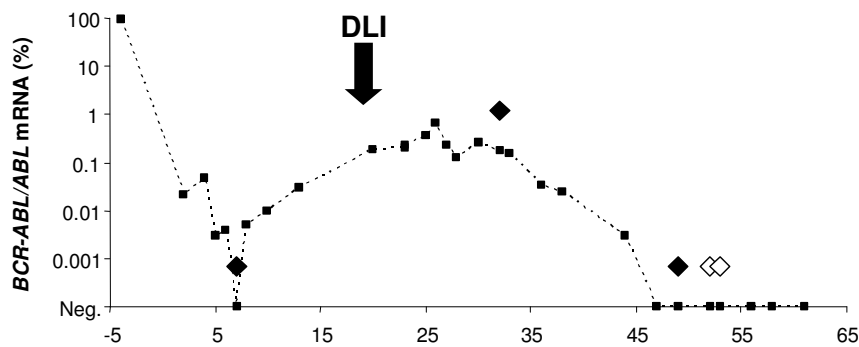


Figure 6. Representative pattern of residual disease found in patients tested early after HSCT, at relapse and post-DLI. One of 3 patients tested, all with similar patterns, is shown. On the x-axis are months post-HSCT. The results of the quantitation of *BCR-ABL* transcripts (dashed line with ■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. *gBCR-ABL* was detected in a transcript-negative sample pre-relapse and at relapse; after DLI, a transcript-negative sample was *gBCR-ABL*-positive; later samples were *gBCR-ABL*-negative. A similar pattern was seen in 2 other comparable patients tested (Figure 7).

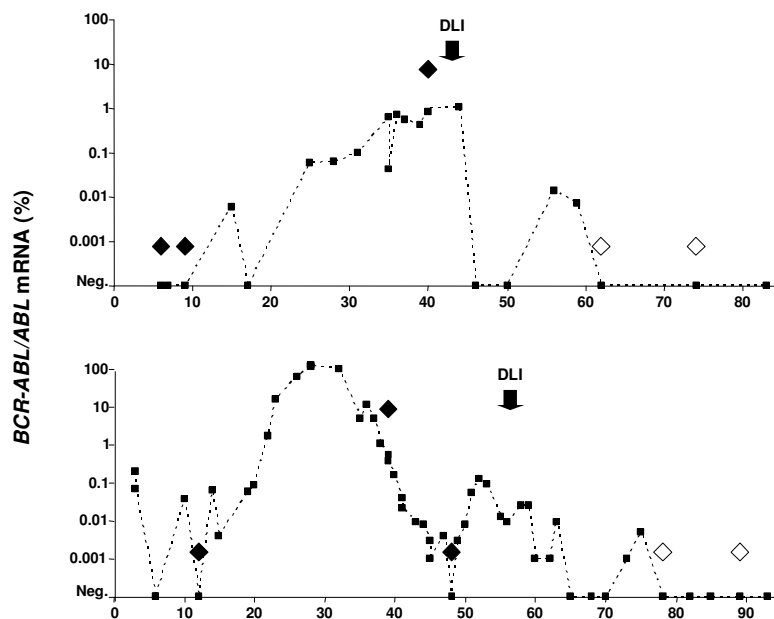


Figure 7. Patterns of residual disease found in 2 additional patients tested early after HSCT, at relapse and post-DLI. On the x-axis are months post-HSCT. The results of the quantitation of *BCR-ABL* transcripts (dashed line with ■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. *gBCR-ABL* was detected in transcript-negative samples pre-relapse and at relapse; both patients achieved *gBCR-ABL*-negativity post-DLI.

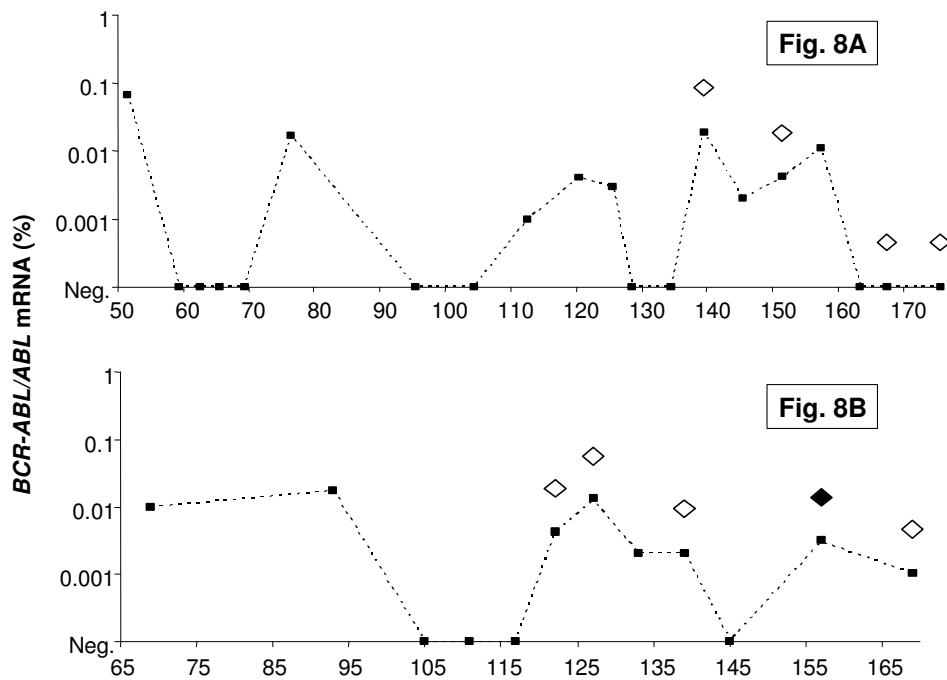


Figure 8. Representative patterns of residual disease found in patients in LTR post-HSCT/DLI. Panels A and B show 2 individual patients who, despite not meeting criteria for molecular relapse, were persistently positive for *BCR-ABL* transcripts at a low level. On the x-axis are months post-HSCT/DLI. The results of the quantitation of *BCR-ABL* transcripts (dashed line with ■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. (A) All 4 samples tested, 2 of which were transcript-positive, were *gBCR-ABL*-negative. (B) Of 5 transcript-positive samples tested, 1 was *gBCR-ABL*-positive, at the very limit of sensitivity of the assay. Of 24 samples tested from 9 patients in LTR post-HSCT/DLI, this was the single instance of *gBCR-ABL* positivity, despite 14 of 24 samples being transcript-positive. The patterns found in the 7 additional patients tested in LTR post-HSCT/DLI are shown in Figure 9.

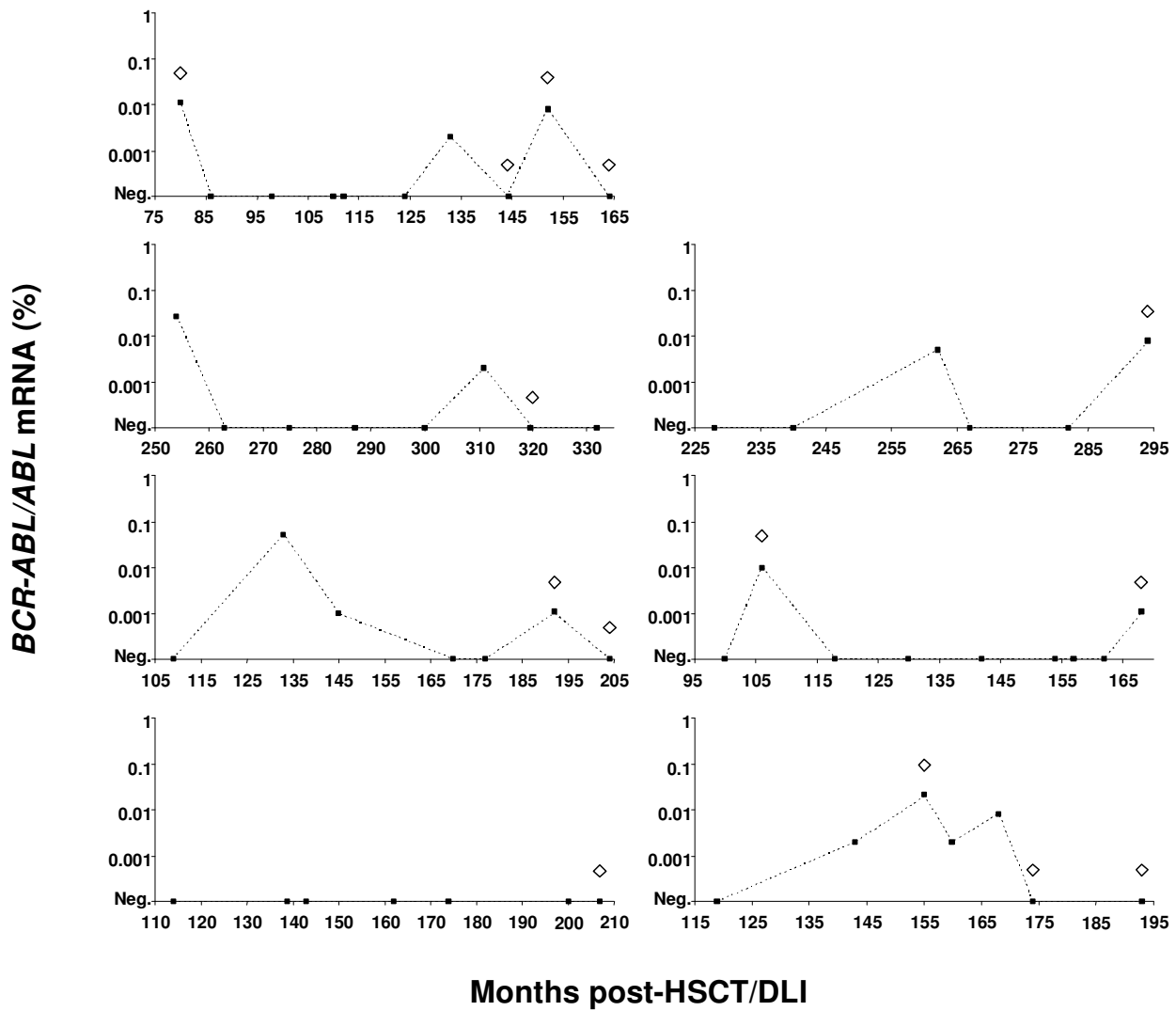


Figure 9. Patterns of residual disease found in 7 additional patients in LTR post-HSCT/DLI. On the x-axis are months post-HSCT/DLI. The results of the quantitation of *BCR-ABL* transcripts (dashed line with ■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. All 14 samples tested, 7 of which were transcript-positive, were *gBCR-ABL*-negative.

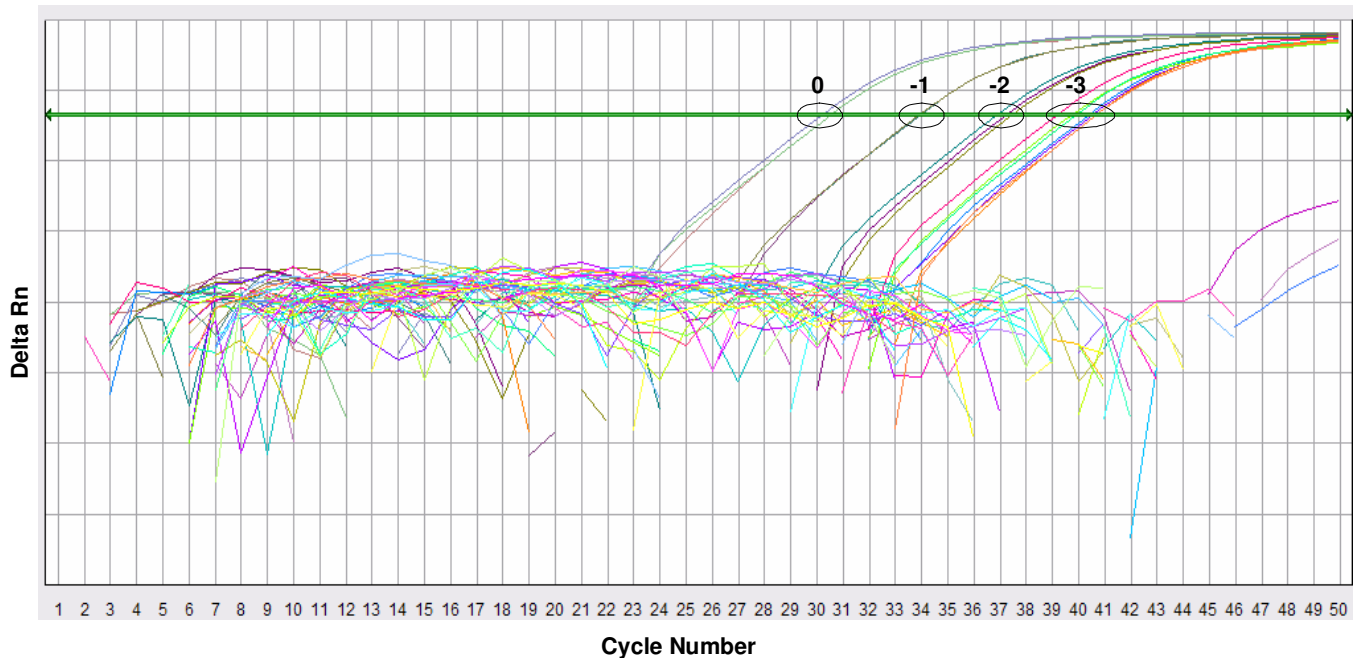


Figure 10. Amplification plot of the serial dilutions of pre-HSCT gDNA and of the only *gBCR-ABL*-positive sample from a patient in LTR post-HSCT. Circles 0, -1, -2 and -3 enclose the cycle threshold (Ct) of the serial 10-fold dilutions of pre-HSCT gDNA – from 10^0 (1ng/ μ L) to 10^{-3} (1pg/ μ L). The 10^{-3} dilution was tested in 6 replicate reactions; previous dilutions were tested in triplicate. The average Ct of each dilution was used to calculate a standard curve. Included in circle -3 is also the amplification plot of the single sample from a patient in LTR post-HSCT that was positive – 1 of 36 replicates, Ct – 40.7. Thus, circle -3 highlights the close parallel of the *gBCR-ABL*-positive sample with the limit of sensitivity of the standard curve; and the wider variation in Ct seen at the limiting dilution of pre-HSCT gDNA. We did not report as positive samples with a $Ct \geq 45$, nor samples with a $Ct \geq 3$ cycles above the highest Ct of the 10^{-3} dilution of the standard curve.

Discussion

It remains unknown whether the curative effect of HSCT in CML depends on eradication of the *BCR-ABL*-positive clone. This question is increasingly relevant in the era of effective tyrosine kinase inhibitors (TKIs), able to induce CMR in a proportion of patients.¹¹⁸ It is a matter of concern that “surviving” *BCR-ABL*-positive cells post-HSCT or imatinib might not only lead to relapse, but also acquire the genetic lesions that are associated with progression to BC or the development of resistance to TKIs.¹¹⁹⁻¹²¹

The concept of CMR should be used with caution. The RQ-PCR for *BCR-ABL* transcripts that is typically used to measure molecular MRD has technical pitfalls and significant inter-laboratory variability,⁸⁵ and it has been estimated that a residual leukemic burden of up to 10^6 *BCR-ABL*-positive cells may lie below its threshold of sensitivity.⁸⁶ On the other hand, attempts to increase even further its sensitivity may run into the opposite pitfall, that is, the difficulty in interpreting a low level, intermittent *BCR-ABL* transcript positivity,⁷⁹ which may be seen inconsequentially in healthy individuals^{75;76} (Table 2), in CML patients in LTR post-HSCT,⁹²⁻⁹⁵ or maybe even in very rare patients who remain in prolonged cytogenetic remission after IFN α discontinuation.¹²²⁻¹²⁴

The method used for RQ-PCR amplification of g*BCR-ABL* proved to be patient-specific and highly sensitive, because it was able to detect g*BCR-ABL* on gDNA diluted to 6pg per reaction – the DNA content of a single human diploid cell.¹¹⁴ Its sensitivity was further demonstrated by the g*BCR-ABL* positivity of samples that were negative for *BCR-ABL* transcripts from both patients in CMR on imatinib and those post-HSCT in CMR before relapse using a highly sensitive assay conducted in a reference MRD laboratory.^{84;85} Further evidence that our assay was thoroughly optimised for sensitivity was the fact that an additional nesting step of RQ-PCR amplification of first-round PCR products did not lead to any increase in “reproducible sensitivity”. Because nested amplification is not only more labor-intensive, technically demanding, and costly but, above all, more prone to false-positivity due to the increased risk of PCR contamination, we felt confident that it was unwarranted for this study.

In all 5 patients studied in CMR on imatinib, *gBCR-ABL* was detected in transcript-negative samples. In 1 of the 5, *gBCR-ABL* was detected in all (n=5) FU samples tested; 4 of the 5 patients became *gBCR-ABL*-negative with continuing imatinib therapy. These results expand on the findings from a recent publication¹²⁵ and indicate that, in imatinib-treated patients, absence of transcripts should not be interpreted as absence of the leukemic clone. In addition, our results fit with the reported continuing reduction in amount of residual disease (measured by mRNA) in long-term treatment with imatinib,¹⁰⁵ and demonstrate such a reduction in a proportion of the patients previously classified as CMR by *BCR-ABL* mRNA quantification.

Contrasting with the detection of *gBCR-ABL* in transcript-negative samples from every patient in CMR on imatinib, we found very little evidence that the leukemic clone is present in LTR post-HSCT, despite occasional transcript-positivity in 8 of the 9 patients. In fact, the original leukemic clone was detected in only 1 of the 9 patients. The positivity of the *gBCR-ABL* test from this patient was at the very limit of sensitivity of the assay – 1 positive reaction of 36 replicates, with a C_t corresponding to the threshold of detection of the standard curve (Figure 10). Four other samples from this patient, all positive for *BCR-ABL* transcripts, were *gBCR-ABL*-negative. This patient (Figure 8B) received a syngeneic graft and was free of relapse at last FU, more than 14 years after HSCT, with no further therapy. While syngeneic graft recipients are known to have an increased risk of relapse compared with recipients of allografts,⁸⁸ relapse would be uncommon more than 14 years post-HSCT. Barring the possibility of PCR contamination (which can never be totally ruled out, but for which we have no evidence in very extensive negative controls over the course of the experiments), this *gBCR-ABL*-positivity is the sole evidence we have of survival of the original CML clone late after HSCT. This is consistent with the rarity of CML relapse after very prolonged remissions post-HSCT. The other syngeneic graft recipient in this study was 333 months post-HSCT and free of relapse at last FU; he had 2 FU samples positive for *BCR-ABL* transcripts of 8 tested. The only sample from this patient that was tested by both methods was negative for both *gBCR-ABL* and for transcripts.

An earlier attempt to compare gDNA with cDNA in MRD assessment in the post-HSCT setting found largely concordant results between the two approaches.¹²⁶ This was done in the pre-RQ-PCR era and it should be noted that, compared with the present study, the

FU period post-HSCT was shorter and there was very little evidence of transcript positivity more than 2 years after HSCT.

The fact that we were able to consistently detect the original leukemic clone in samples negative for *BCR-ABL* transcripts from imatinib-treated patients (12/19 samples from 5/5 patients) and from HSCT-treated patients pre-relapse (5/5 samples from 3/3 patients) demonstrates the high sensitivity of the gDNA RQ-PCR approach we used. Yet, 13 of 14 transcript-positive samples from 9 patients late after HSCT were conclusively g*BCR-ABL*-negative, which raises an intriguing question as to the origin of such *BCR-ABL* transcripts in this group of patients. Occasional low-level positivity for *BCR-ABL* mRNA might be explainable by PCR contamination, an event that is probably inevitable, at however a low level, in laboratories that handle large numbers of *BCR-ABL*-positive samples. It is, however, very unlikely that PCR contamination can explain the 4 consecutive samples positive for *BCR-ABL* transcripts seen in 3 patients.

Two alternative scenarios for this type of pattern (Figure 8) can be envisaged. The low-level transcript positivity observed in some post-HSCT patients may be a manifestation not of the original leukemic clone, but of different, “benign” *BCR-ABL*-positive cells, as have been detected in healthy subjects^{75,76} (Table 2). There is no indication of whether some subjects may be particularly prone to generate such cells, but that could be the case of these patients (or their donors, because the patients received hematopoietic stem cell grafts). Furthermore, this sustained positivity could be a manifestation of different, short-lived, *BCR-ABL*-positive cells, or of a single, longer-lived but not oncogenic (“fully transformed”), *BCR-ABL* clone. It is also tempting to speculate that, if such inconsequential *BCR-ABL* recombinations were indeed to occur and produce a functional protein, their detection would be less likely in imatinib-treated than in post-HSCT patients, because tyrosine kinase inhibition would abrogate any marginal proliferative advantage afforded by Bcr-Abl.

Alternatively, the *BCR-ABL* transcripts may be a manifestation of an extremely low number of cells from the original leukemic clone, in which *BCR-ABL* is being very actively transcribed. This would generate a much larger amount of mRNA than gDNA targets for PCR amplification, thus making the residual disease less likely to be detected by genomic-based methods than cDNA-based methods. There are no data to corroborate

this hypothesis. In fact, it could be speculatively argued against it that, should putative surviving cells be expressing unusually large amounts of *BCR-ABL*, this could make them particularly good targets for the graft-versus-leukemia effect that is known to be crucial to the therapeutic efficacy of HSCT and DLI. Thus, such high *BCR-ABL* expressing clones should die out and not be repeatedly detectable at the transcript level.

There are several advantages to using a gDNA-based PCR as opposed to mRNA-based PCR for analysis of residual disease. Genomic DNA is much more stable than mRNA, which is very susceptible to degradation; extraction of gDNA is technically easier than the extraction of mRNA followed by cDNA synthesis; genomic DNA PCR of a target gene results in amplification whether or not that gene is actually being transcribed. Furthermore, a gDNA-based PCR does not need to rely on a control gene for normalisation of results – arguably, the biggest source of variation in RT-PCR. In the case of CML, the use of clone-specific primers makes the assay less susceptible to contamination and excludes the possible amplification of the “benign” clones seen in healthy subjects. The major disadvantage of the method we describe is the need to sequence the *BCR-ABL* fusion from each individual at diagnosis, the need to demonstrate linearity and sensitivity for each patient-specific primers/probe combination, and the need for multiple reactions to test large amounts of gDNA and thus maximize sensitivity. However, as for many other originally complex and technically demanding techniques, both processes may be further optimized and automated for future use in a routine setting. In the meantime, the use of gDNA PCR for *BCR-ABL* cannot be advocated for immediate implementation as a routine MRD test, until its reliability and predictive values are soundly established and compared in different laboratories.

Our gDNA-based method can be quantitative over a wide range of disease burdens, as seen by the good quality standard curves. However, similarly to what occurs with mRNA quantification, the very low levels of residual disease seen in patients negative for transcripts are below the range of reproducible and accurate quantification.

In conclusion, using a sensitive, patient-specific gDNA-based RQ-PCR for detection of *BCR-ABL*, we could demonstrate the presence of residual amounts of the original leukemic clone in 5 of 5 CML patients treated with imatinib who were negative for

BCR-ABL transcripts. In 4 of these patients, the leukemic clone became undetectable with continuing imatinib therapy. In contrast, in only 1 of 9 patients in LTR post-HSCT there was evidence for the survival of the original leukemic clone, which is consistent with the low risk of disease relapse observed many years post-HSCT. The low-level positivity for *BCR-ABL* transcripts that is seen in some of these patients (sometimes persistently) remains puzzling, as we found no evidence that it is caused by residual amounts of the original leukemic clone. Clarification of this issue will require prospective collection of larger blood samples for sub-cellular fractionation to ascertain the origin of these rare transcripts.

Because the period of observation is not strictly comparable for the 2 groups of patients, it is possible that the apparent difference in the degree of “elimination” of the leukemic clone between patients treated with HSCT or imatinib will disappear with longer exposure to the drug. Likewise, a larger cohort of patients with longer follow-up will help ascertain whether the type of “deeper CMR” we demonstrated upon continuing imatinib therapy correlates with response (ie, relapse or sustained remission) to interruption of the kinase inhibitor.

Recently published results suggest that the presence of detectable *gBCR-ABL* in CMR patients is compatible with sustaining CMR after stopping imatinib (see **Concluding Remarks**).¹²⁷

Concluding Remarks

This thesis describes a limited attempt at addressing Oncology's two "Big Cs" - "Cause" and "Cure" - in the CML field. Admittedly, these concepts are ill-defined, crude, and, to some extent, even unscientific ("Causes" and "Cures" is preferable, emphasizing that they are very likely heterogeneous in nature), but they are operational.

Regarding CML causation, we initially focused on the presence of rare *BCR-ABL* transcripts in healthy individuals (a finding we confirmed using the same protocol used for testing CML patients' samples, as seen in Table 2). We were specifically interested in the cellular origin of these transcripts, and whether they might originate from the immature hematopoietic progenitor (CD34+) fraction of leukocytes. As previously described, the experimental approach we designed did not result in a sizable increase in sensitivity of detection of *BCR-ABL* transcripts within this cellular population.

Still within causation, the *gBCR-ABL* fusions (of the p210 type) we sequenced were used to investigate the genesis of *BCR-ABL* rearrangements in CML (p210 *BCR-ABL*) and in Ph-positive ALL (p210 and p190 *BCR-ABL*), using a genomics approach.⁵³ Breakpoint sequence analysis found no evidence for significant clustering and no association with specific motifs, with the exception of a breakpoint deficit in repeat regions of *BCR* in the p210 cases. This is at variance with previous publications that had found an association with *Alu* elements in both *BCR* and *ABL* in CML.^{51;52} It is worth noting that these prior studies were based on a smaller number of cases, and could have been biased by analyzing only cases whose sequencing of *gBCR-ABL* fusion was dependent on the presence of specific restriction enzyme recognition sites. The patterns of deletions and insertions at the breakpoint junctions were different in p190 and p210 cases, suggesting that the rearrangements originate through different mechanisms.⁵³

Extra-chromosomal recombination assays identified functional cryptic recombination signal sequences (cRSSs) in a minority of ALL cases tested (3 of 13), suggesting that aberrant RAG (Recombinase-Activating Gene enzyme complexes) activity might create double-stranded breaks in a subset of ALL cases. No functional cRSSs were identified in the two CML cases tested.⁵³

Recognizing that our results were inconclusive in terms of causative mechanisms in CML, we decided to pursue the second C – “Cure(s)”. We turned to the question of whether the low level positivity for *BCR-ABL* transcripts that is occasionally seen in patients in LTR post-HSCT, without disease relapse, is a manifestation of the original leukemic clone, as has been hypothesized.¹¹⁸ Using a patient-specific, highly-sensitive RQ-PCR for *gBCR-ABL*, we found very little evidence to support this notion. In fact, *gBCR-ABL* was detected in only 1 of 9 patients in LTR post-HSCT, despite 8 of them being positive for transcripts.¹²⁸

Although we cannot definitely rule it out, our results provide the most compelling evidence to date against the hypothesis that the original leukemic clone survives in patients in LTR post-HSCT. This is the case even in patients that are transcript-positive (sometimes consistently); and it suggests that, in most instances, this transcript-positivity may result from the same type of inconsequential, “benign” translocations that are well documented, by us and others, in healthy individuals.^{75;76;127;128}

In imatinib-treated patients, we focused on a question that, in a way, is the “mirror image” of the one we addressed in transplanted patients. We used the same patient-specific, highly-sensitive RQ-PCR for *gBCR-ABL* to test patients in sustained CMR for the presence of the original leukemic clone. In 5 of 5 patients tested, we were able to detect the *gBCR-ABL* in transcript-negative samples; in 4 of these 5 patients, *gBCR-ABL* became undetectable with continuing imatinib therapy.¹²⁸

We concluded that, in imatinib-treated patients, non-detectable transcripts should not be interpreted as eradication of the leukemic clone; and continuing imatinib therapy after achievement of a CMR leads, at least in some patients, to further reduction in residual disease.¹²⁸

The verification that CMR does not correspond with disease eradication is consistent with previous estimates that up to 10^6 leukemic cells may lie below the sensitivity of PCR-based methods used for routine MRD assessment.⁸⁶ It is also consistent with the observation that some patients in sustained CMR relapse shortly after imatinib interruption.¹⁰⁶

Recent reports^{127;129} have furthered our understanding of how patients in CMR respond to imatinib interruption. The Stop Imatinib (STIM) trial is a prospective, multicenter trial that enrolled 100 imatinib-treated CML patients in sustained (>2 years duration) CMR and assessed the occurrence of molecular relapse after imatinib interruption.¹²⁹ Molecular relapse was detected by quantification of *BCR-ABL* transcripts in peripheral blood through RQ-PCR (monthly during the first year, every 2 months during year 2 and every 3 months after year 2). Of 69 patients (34 of which had received IFN α prior to imatinib) with a minimum FU of 12 months, 42 (61%) relapsed. Most relapses occurred early after imatinib interruption: 35 of 42 within the first 3 months, 40 of 42 within 6 months; one patient relapsed at month 7 and one at month 19. For these 69 patients, the estimated molecular relapse-free survival was 41% at 12 months and 38% at 24 months.¹²⁹

The relapse rate was similar in patients receiving imatinib after IFN α therapy and those who received imatinib first-line. Using multivariate Cox regression model analysis, a lower Sokal risk score, longer duration of imatinib therapy (≥ 50 months) and male gender were found to be independently associated with a smaller probability of molecular relapse.¹²⁹

All 42 relapsed patients responded to re-introduction of imatinib, 16 of them showing a decrease in *BCR-ABL* transcripts and 26 re-entering CMR after 1-5 months of imatinib.¹²⁹

A similar trial is being conducted in Australia, with comparable results.^{127;130} As in the STIM trial, imatinib was stopped in patients in sustained (>2 years) CMR, which were then frequently tested for molecular relapse (defined as detectable *BCR-ABL* transcripts). A total of 35 patients were enrolled, both with and without IFN α therapy prior to imatinib. With a median FU of 21 months, the estimated molecular relapse rate was 60%; a total of 12 patients remained in continuous CMR, without any therapy, for at least 12 months. As in the STIM trial, the vast majority of relapses occurred in the first 6 months after stopping imatinib. In the Australian study, the latest relapse has been observed 24 months after imatinib cessation.¹³⁰

Again corroborating the results from STIM, in the Australian trial all relapsed patients responded to imatinib resumption, with most re-entering CMR within 6 months.¹³⁰

In both studies, there are no reports of cytogenetic or hematologic relapse, nor of disease progression to AP/BC.

Ross *et al.*¹²⁷ used a semi-quantitative gDNA-based PCR approach, similar to our own, to look for evidence of *gBCR-ABL* in a subset of 18 patients (9 treated with imatinib *de novo* and 9 treated with imatinib after IFN α) enrolled in the Australian trial.¹²⁷ Of the 18 patients, 8 remained in CMR after a median FU of 2 years (range: 12 to 41 months), while 10 (6 imatinib *de novo* and 4 imatinib after IFN α) experienced molecular relapse after a median 4 months (range: 2 to 8 months) off imatinib. Seventeen of the 18 patients had detectable *gBCR-ABL* on at least one occasion during the study period – only 1 patient was *gBCR-ABL* negative on all samples tested. At study entry (i.e., at the time of imatinib cessation), *gBCR-ABL* was detectable in 3 of the 8 patients that remained in CMR and in 7 of 9 patients that relapsed (1 patient who relapsed did not have a sample available for *gBCR-ABL* testing at study entry). At variance with patients who remained in CMR (in which *gBCR-ABL* remained quantitatively stable or became undetectable), an increase in the amount of *gBCR-ABL* could be demonstrated in most relapsed patients.¹²⁷

The results of Ross *et al.*¹²⁷ indicate that the presence of residual amounts of the original leukemic clone is compatible with a sustained CMR off imatinib. The presence of detectable *gBCR-ABL* at the time of imatinib cessation did not discriminate between relapsing and non-relapsing patients, suggesting that, below a still undefined threshold of residual disease, factors other than the size of the CML clone may contribute to the probability of remaining relapse-free without TKI therapy.¹²⁷

The prognostic implications of the degree of cytogenetic response to imatinib^{101;102;104} (as indeed to IFN α ¹³¹) are well established, and the achievement of a CCyTR is nowadays considered the minimum satisfactory goal in the treatment of CML-CP.¹⁹ Due to the good prognosis associated with CCyTR, the exact value of deeper molecular responses has been more difficult to ascertain. This is reflected in the CML management recommendations from the European LeukemiaNet which define imatinib failure in the

first-line therapy of CML-CP on the basis of hematologic and cytogenetic levels of response.¹⁹ For patients in CCyR, achieving MMR at 18 months defines an optimal response; not achieving MMR defines a response that is considered suboptimal. In other words, patients that achieve CCyR within 18 months of starting imatinib, and remain in CCyR, are never deemed therapy failures, irrespective of their level of molecular response.¹⁹

A recent analysis of IRIS patients randomized to first-line imatinib provides convincing evidence that deeper levels of response (MMR) at certain landmark points are associated with long-term prognosis (7 years FU).¹³² Among patients in CCyR, achievement of MMR at the 18-month landmark was associated with durability of the CCyR – an estimated 97% of patients with MMR at 18 months remained in CCyR at 84 months, whereas only 74% of the patients not achieving MMR remained in CCyR at that time.¹³² In IRIS, none of the patients that achieved MMR at 12 or 18 months progressed to AP/BC under imatinib at 8 years FU.^{103;132}

This study¹³² corroborates previous findings of a progressive decrease in *BCR-ABL* transcripts with continuing imatinib therapy, up to 7 years FU.^{84;104;105} It remains to be verified whether this trend continues with longer FU; and, if so, whether currently standard methods of transcript quantification will still be informative in an enlarging population of patients with very low levels of residual disease.

The reliability of MRD quantification decreases with diminishing levels of residual disease, despite the efforts to standardize methods of transcript quantification. This is highlighted in a recent review of 2466 patients treated with imatinib frontline in 8 different studies, which found a much larger variation in the rate of MMR at 12 months than in the rate of CCyR at the same time point.¹³³ The problem becomes even more acute in the definition of CMR. The sensitivity of a PCR assay is always critically dependent on the amount of patient material that is sampled,⁷⁹ and there is significant inter-laboratory variability.⁸⁵ Importantly, it is the practice of some laboratories (in accordance with published recommendations) to define a cut-off value for positivity of 5-10 *BCR-ABL* transcripts.⁸⁵ This is based on the lowest *BCR-ABL* plasmid dilution that can be amplified reliably; and on the low level of *BCR-ABL* transcripts that is found in healthy individuals.^{75;76} It is likely that establishing such a cut-off results in under

reporting of very small amounts of transcripts that can be biologically relevant, i.e., manifestations of the leukemic clone.

While we do not dispute that transcript quantification has proven robust enough for clinical decision-making, the gDNA-based method we have used provided additional information. In patients in LTR post-HSCT, our results argue against the notion that the low-level positivity for transcripts that is occasionally seen is derived from the original leukemic clone, at least in most patients. However, we should stress that our results do not prove eradication of the *BCR-ABL* clone.¹²⁸

In patients who achieve profound molecular responses on imatinib, we conclusively demonstrate that the leukemic clone is detectable in patients in CMR, and that continuing imatinib leads to further reduction in the amount of residual disease.¹²⁸ Using a similar approach to the one we describe, the results reported by Ross *et al.*¹²⁷ in patients that interrupted imatinib therapy after achieving a sustained CMR demonstrate that the detection of *gBCR-ABL* at the time of imatinib cessation is not predictive of molecular relapse; and that patients can remain in CMR for longer than 1 year without imatinib despite being persistently *gBCR-ABL*-positive. A longer FU of these patients is required to ascertain whether late relapses will become more frequent with longer periods without imatinib. At present, we do not know whether a proportion of patients will remain in “true” LTR without TKI therapy (perhaps indefinitely?), despite the presence of a detectable pool of leukemic cells.

Considerations on the nature of the mechanisms that might be keeping such cells in check, without causing relapse, are largely of a speculative nature, and are beyond the scope of this thesis. These mechanisms could be intrinsic to the leukemic clone and result in a limited proliferative capacity; and/or could be immunologic in nature, leading to a continued suppression of the clone (for a recent review see ¹³⁰).

A report from Burchert *et al.*¹³⁴ offers support to the idea that immunologic mechanisms are important and that they may be enhanced by IFN α . The authors describe 20 patients treated with a combination of imatinib and IFN α , in whom imatinib was stopped and IFN α continued as maintenance therapy. At the time of imatinib cessation, 19 of the 20 patients were in CCyR, 15 had achieved MMR and 2 CMR. Imatinib discontinuation

led to molecular relapse (defined in this instance as an increase in *BCR-ABL* transcript load to >1% in the IS) in 5 patients, with kinetics that are comparable with those seen in the STIM and Australian imatinib discontinuation trials described above. Of the 15 patients who did not suffer molecular relapse, 10 improved the depth of their molecular remission on IFN α alone. The results suggest that IFN α -induced cytotoxic T lymphocytes reactive against a leukemia-associated antigen may contribute to this effect.¹³⁴

An alternative scenario to the persistence of the CML clone would be its extinction under prolonged imatinib therapy. This possibility is suggested in 2 mathematical modelling studies, based on different assumptions about CML stem cell biology^{135;136} (for a recent review see ¹³⁰).

The value of the information provided by the gDNA-based method we describe increases in relevance with decreasing amounts of residual disease. The longest available molecular FU data indicate that the median level of residual disease continues to decline at 7 years on imatinib.¹³² The so-called second generation TKIs, dasatinib and nilotinib, are more potent inhibitors of Bcr-Abl than imatinib; this has translated into higher rates, and faster achievement of, CCyR and MMR, when compared with imatinib, in the treatment of newly-diagnosed CML-CP.^{137;138} Transcript-based methods are informative enough to establish that residual disease is still decreasing, both in imatinib-treated patients and in those receiving second generation TKIs. It is likely that a gDNA-based method in the mould of the one we describe will have more widespread, practical relevance when the curve of *BCR-ABL* transcripts in TKI-treated patients bottoms out.

It has not been possible to conclusively demonstrate whether the “last CML cell” has been eradicated post-HSCT, or can be eradicated with TKI therapy.

References

1. Bennett JH. Case of hypertrophy of the spleen and liver, in which death took place from suppuration of the blood. The Edinburgh Medical and Surgical Journal 1845;64:413-423.
2. Craigie D. Case of disease of the spleen, in which death took place in consequence of the presence of purulent matter in the blood. The Edinburgh Medical and Surgical Journal 1845;64:400-413.
3. Virchow R. Weisses Blut. Frorieps Notizen 1845;36:151-156 (as cited in ref.4).
4. Geary CG. The story of chronic myeloid leukaemia. Br.J.Haematol. 2000;110:2-11.
5. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science 1960;132:1497.
6. Caspersson T, Gahrton G, Lindsten J, Zech L. Identification of the Philadelphia chromosome as a number 22 by quinacrine mustard fluorescence analysis. Exp.Cell Res. 1970;63:238-240.
7. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973;243:290-293.
8. Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. Proc.Natl.Acad.Sci.U.S.A. 1967;58:1468-1471.
9. Martin PJ, Najfeld V, Hansen JA et al. Involvement of the B-lymphoid system in chronic myelogenous leukaemia. Nature 1980;287:49-50.
10. Fefer A, Cheever MA, Thomas ED et al. Disappearance of Ph1-positive cells in four patients with chronic granulocytic leukemia after chemotherapy, irradiation and marrow transplantation from an identical twin. N.Engl.J.Med. 1979;300:333-337.
11. Pavlu J, Szydlo RM, Goldman JM, Apperley JF. Three decades of transplantation for chronic myeloid leukemia: what have we learnt? Blood 2011;117:755-763.
12. Bartram CR, de Klein A, Hagemeijer A et al. Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 1983;306:277-280.

13. Heisterkamp N, Stephenson JR, Groffen J et al. Localization of the c-abl oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. *Nature* 1983;306:239-242.
14. Groffen J, Stephenson JR, Heisterkamp N et al. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* 1984;36:93-99.
15. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. *Nature* 1985;315:550-554.
16. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* 1990;247:1079-1082.
17. Druker BJ, Tamura S, Buchdunger E et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat.Med.* 1996;2:561-566.
18. Druker BJ, Talpaz M, Resta DJ et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N.Engl.J.Med.* 2001;344:1031-1037.
19. Baccarani M, Cortes J, Pane F et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *J.Clin.Oncol.* 2009;27:6041-6051.
20. Garcia-Manero G, Faderl S, O'Brien S et al. Chronic myelogenous leukemia: a review and update of therapeutic strategies. *Cancer* 2003;98:437-457.
21. Clarkson B, Strife A, Wisniewski D, Lambek CL, Liu C. Chronic myelogenous leukemia as a paradigm of early cancer and possible curative strategies. *Leukemia* 2003;17:1211-1262.
22. Cortes JE, Talpaz M, Beran M et al. Philadelphia chromosome-negative chronic myelogenous leukemia with rearrangement of the breakpoint cluster region. Long-term follow-up results. *Cancer* 1995;75:464-470.
23. Kurzrock R, Blick MB, Talpaz M et al. Rearrangement in the breakpoint cluster region and the clinical course in Philadelphia-negative chronic myelogenous leukemia. *Ann.Intern.Med.* 1986;105:673-679.
24. Johansson B, Fioretos T, Mitelman F. Cytogenetic and molecular genetic evolution of chronic myeloid leukemia. *Acta Haematol.* 2002;107:76-94.
25. Bernstein R. Cytogenetics of chronic myelogenous leukemia. *Semin.Hematol.* 1988;25:20-34.

26. Huntly BJ, Bench A, Green AR. Double jeopardy from a single translocation: deletions of the derivative chromosome 9 in chronic myeloid leukemia. *Blood* 2003;102:1160-1168.
27. Sinclair PB, Nacheva EP, Leversha M et al. Large deletions at the t(9;22) breakpoint are common and may identify a poor-prognosis subgroup of patients with chronic myeloid leukemia. *Blood* 2000;95:738-743.
28. Huntly BJ, Reid AG, Bench AJ et al. 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. *Blood* 2001;98:1732-1738.
29. Kolomietz E, Al Maghrabi J, Brennan S et al. Primary chromosomal rearrangements of leukemia are frequently accompanied by extensive submicroscopic deletions and may lead to altered prognosis. *Blood* 2001;97:3581-3588.
30. Huntly BJ, Guilhot F, Reid AG et al. Imatinib improves but may not fully reverse the poor prognosis of patients with CML with derivative chromosome 9 deletions. *Blood* 2003;102:2205-2212.
31. Quintas-Cardama A, Kantarjian H, Talpaz M et al. Imatinib mesylate therapy may overcome the poor prognostic significance of deletions of derivative chromosome 9 in patients with chronic myelogenous leukemia. *Blood* 2005;105:2281-2286.
32. Kim DH, Popradi G, Sriharsha L et al. No significance of derivative chromosome 9 deletion on the clearance kinetics of BCR/ABL fusion transcripts, cytogenetic or molecular response, loss of response, or treatment failure to imatinib mesylate therapy for chronic myeloid leukemia. *Cancer* 2008;113:772-781.
33. Castagnetti F, Testoni N, Luatti S et al. Deletions of the derivative chromosome 9 do not influence the response and the outcome of chronic myeloid leukemia in early chronic phase treated with imatinib mesylate: GIMEMA CML Working Party analysis. *J.Clin.Oncol.* 2010;28:2748-2754.
34. Kurzrock R, Kantarjian HM, Druker BJ, Talpaz M. Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics. *Ann.Intern.Med.* 2003;138:819-830.
35. Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* 1996;88:2375-2384.
36. Melo JV, Gordon DE, Cross NC, Goldman JM. The ABL-BCR fusion gene is expressed in chronic myeloid leukemia. *Blood* 1993;81:158-165.

37. Melo JV, Gordon DE, Tuszynski A et al. Expression of the ABL-BCR fusion gene in Philadelphia-positive acute lymphoblastic leukemia. *Blood* 1993;81:2488-2491.
38. Chisoe SL, Bodenteich A, Wang YF et al. Sequence and analysis of the human ABL gene, the BCR gene, and regions involved in the Philadelphia chromosomal translocation. *Genomics* 1995;27:67-82.
39. Shepherd P, Suffolk R, Halsey J, Allan N. Analysis of molecular breakpoint and m-RNA transcripts in a prospective randomized trial of interferon in chronic myeloid leukaemia: no correlation with clinical features, cytogenetic response, duration of chronic phase, or survival. *Br.J.Haematol.* 1995;89:546-554.
40. Melo JV, Hochhaus A, Yan XH, Goldman JM. Lack of correlation between ABL-BCR expression and response to interferon-alpha in chronic myeloid leukaemia. *Br.J.Haematol.* 1996;92:684-686.
41. van Rhee F, Hochhaus A, Lin F et al. p190 BCR-ABL mRNA is expressed at low levels in p210-positive chronic myeloid and acute lymphoblastic leukemias. *Blood* 1996;87:5213-5217.
42. Pane F, Frigeri F, Sindona M et al. Neutrophilic-chronic myeloid leukemia (CML-N): a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction) Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction). *Blood* 1996;88:2410-2414.
43. Verstovsek S, Lin H, Kantarjian H et al. Neutrophilic-chronic myeloid leukemia: low levels of p230 BCR/ABL mRNA and undetectable BCR/ABL protein may predict an indolent course. *Cancer* 2002;94:2416-2425.
44. Barnes DJ, Melo JV. Cytogenetic and molecular genetic aspects of chronic myeloid leukaemia. *Acta Haematol.* 2002;108:180-202.
45. Tanaka K, Takechi M, Hong J et al. 9;22 translocation and bcr rearrangements in chronic myelocytic leukemia patients among atomic bomb survivors. *J.Radiat.Res.(Tokyo)* 1989;30:352-358.
46. Corso A, Lazzarino M, Morra E et al. Chronic myelogenous leukemia and exposure to ionizing radiation--a retrospective study of 443 patients. *Ann.Hematol.* 1995;70:79-82.
47. Preston DL, Kusumi S, Tomonaga M et al. Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987 [published erratum appears in *Radiat Res* 1994 Jul;139(1):129]. *Radiat.Res.* 1994;137:S68-97.

48. Deininger MW, Bose S, Gora TJ et al. Selective induction of leukemia-associated fusion genes by high-dose ionizing radiation. *Cancer Res.* 1998;58:421-425.
49. Neves H, Ramos C, da Silva MG, Parreira A, Parreira L. The nuclear topography of ABL, BCR, PML, and RARalpha genes: evidence for gene proximity in specific phases of the cell cycle and stages of hematopoietic differentiation. *Blood* 1999;93:1197-1207.
50. Saglio G, Storlazzi CT, Giugliano E et al. A 76-kb duplicon maps close to the BCR gene on chromosome 22 and the ABL gene on chromosome 9: possible involvement in the genesis of the Philadelphia chromosome translocation. *Proc.Natl.Acad.Sci.U.S.A* 2002;99:9882-9887.
51. Jeffs AR, Benjes SM, Smith TL, Sowerby SJ, Morris CM. The BCR gene recombines preferentially with Alu elements in complex BCR- ABL translocations of chronic myeloid leukaemia. *Hum.Mol.Genet.* 1998;7:767-776.
52. Mattarucchi E, Guerini V, Rambaldi A et al. Microhomologies and interspersed repeat elements at genomic breakpoints in chronic myeloid leukemia. *Genes Chromosomes.Cancer* 2008;47:625-632.
53. Score J, Calasanz MJ, Ottman O et al. Analysis of genomic breakpoints in p190 and p210 BCR-ABL indicate distinct mechanisms of formation. *Leukemia* 2010;24:1742-1750.
54. Abelson HT, Rabstein LS. Lymphosarcoma: virus-induced thymic-independent disease in mice. *Cancer Res.* 1970;30:2213-2222.
55. Van Etten RA. Cycling, stressed-out and nervous: cellular functions of c-Abl. *Trends Cell Biol.* 1999;9:179-186.
56. Pendergast AM. The Abl family kinases: mechanisms of regulation and signaling. *Adv.Cancer Res.* 2002;85:51-100.
57. Sattler M, Salgia R, Okuda K et al. The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway. *Oncogene* 1996;12:839-846.
58. McWhirter JR, Wang JY. An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias. *EMBO J.* 1993;12:1533-1546.
59. Kipreos ET, Wang JY. Cell cycle-regulated binding of c-Abl tyrosine kinase to DNA. *Science* 1992;256:382-385.

60. Wen ST, Jackson PK, Van Etten RA. The cytostatic function of c-Abl is controlled by multiple nuclear localization signals and requires the p53 and Rb tumor suppressor gene products. *EMBO J.* 1996;15:1583-1595.
61. Taagepera S, McDonald D, Loeb JE et al. Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. *Proc.Natl.Acad.Sci.U.S.A* 1998;95:7457-7462.
62. Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* 1991;65:1153-1163.
63. Schwartzberg PL, Stall AM, Hardin JD et al. Mice homozygous for the ablm1 mutation show poor viability and depletion of selected B and T cell populations. *Cell* 1991;65:1165-1175.
64. Laurent E, Talpaz M, Kantarjian H, Kurzrock R. The BCR gene and Philadelphia chromosome-positive leukemogenesis. *Cancer Res.* 2001;61:2343-2355.
65. McWhirter JR, Galasso DL, Wang JY. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. *Mol.Cell Biol.* 1993;13:7587-7595.
66. Maru Y, Witte ON. The BCR gene encodes a novel serine/threonine kinase activity within a single exon. *Cell* 1991;67:459-468.
67. Reuther GW, Fu H, Cripe LD, Collier RJ, Pendergast AM. Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science* 1994;266:129-133.
68. Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON. BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non- phosphotyrosine-dependent manner. *Cell* 1991;66:161-171.
69. Pendergast AM, Quilliam LA, Cripe LD et al. BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. *Cell* 1993;75:175-185.
70. Cen H, Papageorge AG, Zippel R, Lowy DR, Zhang K. Isolation of multiple mouse cDNAs with coding homology to *Saccharomyces cerevisiae* CDC25: identification of a region related to Bcr, Vav, Dbl and CDC24. *EMBO J.* 1992;11:4007-4015.
71. Diekmann D, Brill S, Garrett MD et al. Bcr encodes a GTPase-activating protein for p21rac. *Nature* 1991;351:400-402.

72. Voncken JW, van Schaick H, Kaartinen V et al. Increased neutrophil respiratory burst in bcr-null mutants. *Cell* 1995;80:719-728.
73. Hantschel O, Superti-Furga G. Regulation of the c-Abl and Bcr-Abl Tyrosine Kinases. *Nat.Rev.Mol.Cell Biol.* 2004;5:33-44.
74. Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood* 2000;96:3343-3356.
75. Biernaux C, Loos M, Sels A, Huez G, Stryckmans P. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. *Blood* 1995;86:3118-3122.
76. Bose S, Deininger M, Gora TJ, Goldman JM, Melo JV. The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. *Blood* 1998;92:3362-3367.
77. Cozzio A, Passegue E, Ayton PM et al. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes Dev.* 2003;17:3029-3035.
78. Huntly BJ, Shigematsu H, Deguchi K et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell* 2004;6:587-596.
79. Melo JV, Yan XH, Diamond J et al. Reverse transcription/polymerase chain reaction (RT/PCR) amplification of very small numbers of transcripts: the risk in misinterpreting negative results. *Leukemia* 1996;10:1217-1221.
80. Garin MI, Apperley JF, Melo JV. *Ex vivo* expansion and characterisation of CD34+ cells derived from chronic myeloid leukaemia bone marrow and peripheral blood, and from normal bone marrow and mobilised peripheral blood. *Eur.J.Haematol.* 2000;64:85-92.
81. Strife A, Perez A, Lambek C et al. Characterization of lineage-negative blast subpopulations derived from normal and chronic myelogenous leukemia bone marrows and determination of their responsiveness to human c-kit ligand. *Cancer Res.* 1993;53:401-409.
82. Strife A, Perez A, Lambek C et al. Characterization of lineage-negative blast subpopulations derived from normal and chronic myelogenous leukemia bone marrows and determination of their responsiveness to human c-kit ligand. *Cancer Res.* 1993;53:401-409.

83. Wetzler M, Talpaz M, Van Etten RA et al. Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. *J.Clin.Invest.* 1993;92:1925-1939.
84. Hughes TP, Kaeda J, Branford S et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N.Engl.J.Med.* 2003;349:1423-1432.
85. Hughes T, Deininger M, Hochhaus A et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood* 2006;108:28-37.
86. Kaeda J, Chase A, Goldman JM. Cytogenetic and molecular monitoring of residual disease in chronic myeloid leukaemia. *Acta Haematol.* 2002;107:64-75.
87. Gabert J, Beillard E, Van DV, V et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. *Leukemia* 2003;17:2318-2357.
88. Gale RP, Horowitz MM, Ash RC et al. Identical-twin bone marrow transplants for leukemia. *Ann.Intern.Med.* 1994;120:646-652.
89. Apperley JF, Jones L, Hale G et al. Bone marrow transplantation for patients with chronic myeloid leukaemia: T-cell depletion with Campath-1 reduces the incidence of graft-versus-host disease but may increase the risk of leukaemic relapse. *Bone Marrow Transplant.* 1986;1:53-66.
90. Kolb HJ, Mittermuller J, Clemm C et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 1990;76:2462-2465.
91. Chalandon Y, Passweg JR, Schmid C et al. Outcome of patients developing GVHD after DLI given to treat CML relapse: a study by the Chronic Leukemia Working Party of the EBMT. *Bone Marrow Transplant.* 2010;45:558-564.
92. van Rhee F, Lin F, Cross NC et al. Detection of residual leukaemia more than 10 years after allogeneic bone marrow transplantation for chronic myelogenous leukaemia. *Bone Marrow Transplant.* 1994;14:609-612.
93. Pignon JM, Henni T, Amselem S et al. Frequent detection of minimal residual disease by use of the polymerase chain reaction in long-term survivors after bone marrow transplantation for chronic myeloid leukemia. *Leukemia* 1990;4:83-86.

94. Mughal TI, Yong A, Szydlo RM et al. Molecular studies in patients with chronic myeloid leukaemia in remission 5 years after allogeneic stem cell transplant define the risk of subsequent relapse. *Br.J.Haematol.* 2001;115:569-574.
95. Radich JP, Gooley T, Bryant E et al. The significance of bcr-abl molecular detection in chronic myeloid leukemia patients "late," 18 months or more after transplantation. *Blood* 2001;98:1701-1707.
96. Kaeda J, O'Shea D, Szydlo RM et al. Serial measurement of BCR-ABL transcripts in the peripheral blood after allogeneic stem cell transplantation for chronic myeloid leukemia: an attempt to define patients who may not require further therapy. *Blood* 2006;107:4171-4176.
97. Yong AS, Goldman JM. Relapse of chronic myeloid leukaemia 14 years after allogeneic bone marrow transplantation. *Bone Marrow Transplant.* 1999;23:827-828.
98. O'Brien SG, Guilhot F, Larson RA et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N.Engl.J.Med.* 2003;348:994-1004.
99. Roy L, Guilhot J, Krahnke T et al. Survival advantage from imatinib compared with the combination interferon-alpha plus cytarabine in chronic-phase chronic myelogenous leukemia: historical comparison between two phase 3 trials. *Blood* 2006;108:1478-1484.
100. Kantarjian HM, Talpaz M, O'Brien S et al. Survival benefit with imatinib mesylate versus interferon-alpha-based regimens in newly diagnosed chronic-phase chronic myelogenous leukemia. *Blood* 2006;108:1835-1840.
101. Druker BJ, Guilhot F, O'Brien SG et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N.Engl.J.Med.* 2006;355:2408-2417.
102. Hochhaus A, O'Brien SG, Guilhot F et al. Six-year follow-up of patients receiving imatinib for the first-line treatment of chronic myeloid leukemia. *Leukemia* 2009;23:1054-1061.
103. Deininger M, O'Brien SG, Guilhot F et al. International Randomized Study of Interferon Vs STI571 (IRIS) 8-year follow up: sustained survival and low risk for progression or events in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib [abstract]. *Blood* 2009;114: Abstract 1126.
104. de Lavallade H., Apperley JF, Khorashad JS et al. Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. *J.Clin.Oncol.* 2008;26:3358-3363.

105. Branford S, Seymour JF, Grigg A et al. BCR-ABL messenger RNA levels continue to decline in patients with chronic phase chronic myeloid leukemia treated with imatinib for more than 5 years and approximately half of all first-line treated patients have stable undetectable BCR-ABL using strict sensitivity criteria. *Clin.Cancer Res.* 2007;13:7080-7085.
106. Rousselot P, Huguet F, Rea D et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood* 2007;109:58-60.
107. Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. *Blood* 1999;94:2056-2064.
108. Graham SM, Jorgensen HG, Allan E et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* 2002;99:319-325.
109. Corbin AS, Agarwal A, Loriaux M et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J.Clin.Invest* 2011;121:396-409.
110. Foroni L, Gerrard G, Nna E et al. Technical aspects and clinical applications of measuring BCR-ABL1 transcripts number in chronic myeloid leukemia. *Am.J.Hematol.* 2009;84:517-522.
111. Mackinnon S, Papadopoulos EB, Carabasi MH et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood* 1995;86:1261-1268.
112. Dazzi F, Szydlo RM, Craddock C et al. Comparison of single-dose and escalating-dose regimens of donor lymphocyte infusion for relapse after allografting for chronic myeloid leukemia. *Blood* 2000;95:67-71.
113. Melo JV, Kent NS, Yan XH, Goldman JM. Controls for reverse transcriptase-polymerase chain reaction amplification of BCR-ABL transcripts. *Blood* 1994;84:3984-3986.
114. Jeffreys AJ, Wilson V, Neumann R, Keyte J. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. *Nucleic Acids Res.* 1988;16:10953-10971.
115. Burmeister T, Marschalek R, Schneider B et al. Monitoring minimal residual disease by quantification of genomic chromosomal breakpoint sequences in acute leukemias with MLL aberrations. *Leukemia* 2006;20:451-457.

116. van der Velden VH, Cazzaniga G, Schrauder A et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. *Leukemia* 2007;21:604-611.
117. van der Velden VH, Hochhaus A, Cazzaniga G et al. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003;17:1013-1034.
118. Goldman J, Gordon M. Why do chronic myelogenous leukemia stem cells survive allogeneic stem cell transplantation or imatinib: does it really matter? *Leuk.Lymphoma* 2006;47:1-7.
119. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nat.Rev.Cancer* 2007;7:441-453.
120. Chu S, Xu H, Shah NP et al. Detection of BCR-ABL kinase mutations in CD34+ cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment. *Blood* 2005;105:2093-2098.
121. Barnes DJ, Melo JV. Primitive, quiescent and difficult to kill: the role of non-proliferating stem cells in chronic myeloid leukemia. *Cell Cycle* 2006;5:2862-2866.
122. Bonifazi F, De VA, Rosti G et al. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. *Blood* 2001;98:3074-3081.
123. Mahon FX, Delbrel X, Cony-Makhoul P et al. Follow-up of complete cytogenetic remission in patients with chronic myeloid leukemia after cessation of interferon alfa. *J.Clin.Oncol.* 2002;20:214-220.
124. Verbeek W, Konig H, Boehm J et al. Continuous complete hematological and cytogenetic remission with molecular minimal residual disease 9 years after discontinuation of interferon-alpha in a patient with Philadelphia chromosome-positive chronic myeloid leukemia. *Acta Haematol.* 2006;115:109-112.
125. Mattarucchi E, Spinelli O, Rambaldi A et al. Molecular monitoring of residual disease in chronic myeloid leukemia by genomic DNA compared with conventional mRNA analysis. *J.Mol.Diagn.* 2009;11:482-487.
126. Zhang JG, Lin F, Chase A, Goldman JM, Cross NC. Comparison of genomic DNA and cDNA for detection of residual disease after treatment of chronic myeloid leukemia with allogeneic bone marrow transplantation. *Blood* 1996;87:2588-2593.
127. Ross DM, Branford S, Seymour JF et al. Patients with chronic myeloid leukemia who maintain a complete molecular response after stopping imatinib treatment

have evidence of persistent leukemia by DNA PCR. *Leukemia* 2010;24:1719-1724.

128. Sobrinho-Simões M, Wilczek V, Score J et al. In search of the original leukemic clone in chronic myeloid leukemia patients in complete molecular remission after stem cell transplantation or imatinib. *Blood* 2010;116:1329-1335.
129. Mahon FX, Rea D, Guilhot J et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol.* 2010;11:1029-1035.
130. Ross DM, Hughes TP, Melo JV. Do we have to kill the last CML cell? *Leukemia* 2010 [Epub ahead of print].
131. Guilhot F, Chastang C, Michallet M et al. Interferon alfa-2b combined with cytarabine versus interferon alone in chronic myelogenous leukemia. French Chronic Myeloid Leukemia Study Group. *N.Engl.J.Med.* 1997;337:223-229.
132. Hughes TP, Hochhaus A, Branford S et al. Long-term prognostic significance of early molecular response to imatinib in newly diagnosed chronic myeloid leukemia: an analysis from the International Randomized Study of Interferon and STI571 (IRIS). *Blood* 2010;116:3758-3765.
133. Baccarani M, Rosti G, Martinelli G et al. Evaluating the response to imatinib in Philadelphia-positive chronic myeloid leukemia (Ph+ CML): the value of major molecular response (MMoR) at 12 months [abstract]. *Blood* 2010;116: Abstract 668.
134. Burchert A, Muller MC, Kostrewa P et al. Sustained molecular response with interferon alfa maintenance after induction therapy with imatinib plus interferon alfa in patients with chronic myeloid leukemia. *J.Clin.Oncol.* 2010;28:1429-1435.
135. Roeder I, Horn M, Glauche I et al. Dynamic modeling of imatinib-treated chronic myeloid leukemia: functional insights and clinical implications. *Nat.Med.* 2006;12:1181-1184.
136. Lenaerts T, Pacheco JM, Traulsen A, Dingli D. Tyrosine kinase inhibitor therapy can cure chronic myeloid leukemia without hitting leukemic stem cells. *Haematologica* 2010;95:900-907.
137. Saglio G, Kim DW, Issaragrisil S et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N.Engl.J.Med.* 2010;362:2251-2259.

138. Kantarjian H, Shah NP, Hochhaus A et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *N.Engl.J.Med.* 2010;362:2260-2270.

ANNEX I

Strategies for detection of rare BCR-ABL transcripts

Materials and Methods; Results

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Master thesis, Faculdade de Medicina da Universidade do Porto | 2004

Materials and Methods

Blood Samples

Peripheral blood and leukapheresis samples from newly diagnosed CML-CP patients and normal individuals were collected at the Hammersmith Hospital after informed consent was obtained.

Processing of Blood Samples

A - Mononuclear cell (MNC) isolation

Blood samples were diluted in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate buffered saline solution (PBS). MNC were isolated within 18 h of collection by centrifugation over Lymphoprep (Nycomed, Oslo, Norway) for 2,200 rpm, 30 min, 20°C. The MNC were washed in PBS and cryopreserved in liquid nitrogen or utilised fresh for CD34+ separation.

B - CD34+ cell selection

CD34+ cells were positively selected from MNC using an indirect immunomagnetic separation system (MACS CD34 Progenitor Cell Isolation Kit, MS+ and LS Separation Columns, MiniMACS and MidiMACS Separators, Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions.

Cell Culture Medium and Conditions

Cells were cultured in RPMI 1640 medium without HEPES supplemented with 88 IU/mL penicillin, 88 µg/mL streptomycin, 3.5 mM L-glutamine (Gibco, Invitrogen Ltd, Paisley, UK) and 8.8% fetal calf serum (FCS) (Harlan Sera-Lab, UK), herein referred to as 'RF-10'. The cell cultures were incubated in 5% CO₂ at 37°C and 95% humidity.

Cryopreservation of Cells

The cells in suspension were spun down (1500 rpm, 5 min) and re-suspended in RF-10 at the desired concentration (usually ranging from $2 \times 10^7/\text{mL}$ to $6 \times 10^7/\text{mL}$).

Separately, a cryopreserving solution was prepared with the following composition (total volume equal to the total volume of concentrated cell suspension): RF-10 - 20%; FCS - 60%; dimethyl-sulfoxide (Koch Light Ltd., Haverhill, England) - 20%. Both the cell suspension and the cryopreserving solution were left in the refrigerator for approximately 30 min. During this interval, 2 mL screw-top plastic ampoules (cryo-vials, Sterilin Ltd., Teddington, England), for liquid nitrogen storage, were cooled inside a -20°C freezer. The cell suspension and cryopreserving solution were taken out from the refrigerator and put in an ice bath. The cryopreserving solution was added dropwise to the cell suspension while this was under constant mild agitation. The mixture of cell suspension and cryopreserving solution was then aliquoted into the pre-cooled cryo-vials. The cryo-vials were put inside a Nalgene Cryo 1°C container (Nalgene), which was at room temperature. The Nalgene container was then placed in a -80°C freezer for a minimum of 24 hours, after which the cryo-vials were transferred to liquid nitrogen storage.

Thawing of Cells

The cryo-vials containing the cryopreserved cell suspensions were brought from the liquid nitrogen to a 37°C water-bath. As soon as the suspensions were thawed, they were transferred to 50 mL tubes and 8 mL of RF-10 were added drop wise, under constant mild agitation. The cells were centrifuged (1,500 rpm; 5 min), washed in RF-10 and re-suspended in RF-10.

Culture of CD34+ Primary Cells

Cryopreserved CD34+ primary cells from two newly diagnosed CML-CP patients and a normal individual were thawed and cultured in RF-10 supplemented with 1ng/mL recombinant human interleukin- 1β (rhIL- 1β) and 50ng/mL each of rhIL-3, rhIL-6 and stem cell factor (rhSCF) (First Link Ltd., West Midlands, UK and StemCell

Technologies Inc., British Columbia, Canada). Control cells were cultured in RF-10 without cytokine supplementation. Cultures were seeded at a concentration of 2×10^5 cells/mL in 24-well plates (24WP) (2 mL initial volume) or 25 cm² flasks (TCF) (3 mL initial volume). Cultures initiated in 24-well plates were transferred to 25 cm² flasks when their volume exceeded the capacity of the well. Cultures were incubated in 5% CO₂ at 37°C and 95% humidity for up to 22 days. Cell cultures were expanded on days 3, 5, 7 and 14 by adding an equal volume of fresh medium with cytokines. Cells were collected at the start and at several time points during the culture for assessing proliferation, differentiation and *BCR-ABL* expression.

Cell Count and Viability

Cell concentration was measured using an automated cell counter (SYSMEX corporation, Kobe, Japan). Cell viability was assessed by mixing an aliquot of cell suspension with an equal volume of a 0.5% aqueous solution of Trypan-blue (Sigma-Aldrich Company Ltd., Dorset, UK) and counting the cells on a Neubauer chamber under a light microscope. The absolute number of viable cells in culture was estimated by combining the two measurements.

Immunophenotyping

Immunophenotyping was performed by flow cytometry. Aliquots of 5×10^5 cells were stained with 1 µL CD34-FITC, CD33-PE or both antibodies (Becton Dickinson, San Jose, CA, USA), incubated on ice for 30 min in the dark and washed twice in PBS. Negative controls using isotype-matched antibodies were used to establish the background fluorescence level for each sample. Stained cells were analysed in a FACScan flow cytometer (Becton- Dickinson). FACS data were analysed using the WinMDI, Version 2.8 software (Freeware from <http://facs.scripps.edu/>).

RNA Extraction

Total RNA was extracted using the QIAGEN RNeasy Mini kit (QIAGEN Ltd., West Sussex, UK), according to the manufacturer's instructions (RNeasy mini protocol for isolation of total RNA from animal cells, RNeasy mini handbook, pp31-35). At the end

of the protocol, the RNA was eluted from the RNeasy column with 30 μL of DEPC-treated water. The RNA solutions were used immediately for reverse transcription (cDNA synthesis) or stored at -80°C .

Reverse Transcription

A total of 25 μL RNA were reverse-transcribed in a 50 μL reaction. Reverse-transcription was done using random hexamer primers, 5x RT buffer, 0.1 M DTT and M-MLV reverse transcriptase (RT) (Invitrogen Ltd, Paisley, UK), and RNasin[®] RNase inhibitor (Promega, Madison, WI, USA). The composition of the reaction mix is shown on Table 1. A 10 mM dNTP pool contains 10 mM of each dATP, dCTP, dGTP and dTTP. A mix including all reagents was prepared for “n + 1” samples. The mix was then aliquoted into “n” 0.5mL microcentrifuge tubes. The RNA was denatured by heating to 65°C for 5 min, then added to the mix and incubated at 37°C for 2 h. The reaction was stopped by 10 min incubation at 65°C and the cDNA was stored at 4°C (short term) or -20°C .

Table 1

Reagents	50 μL reaction
DEPC- H_2O (μL)	7.0
5x RT buffer (μL)	10.0
0.1 M DTT (μL)	0.5
10 mM dNTPs (μL)	5.0
5 mg/mL pd(N) ₆ (μL)	1.0
40 U/ μL RNAsin (μL)	1.0
200 U/ μL RT (μL)	2.0

Real-Time PCR

Real-time PCR (TaqMan[™]) was used to quantitate *BCR-ABL* mRNA expression. Titrations of the *BCR-ABL* probe and primers and optimisation of the PCR mixes and conditions had been previously performed in our laboratory. During these optimisations,

it was ensured that no amplification of genomic DNA occurred under the established protocol. Each reaction was seeded with 2 μ L cDNA in a final reaction volume of 25 μ L. Table 2 lists the *BCR-ABL* primers and probe used and table 3 the reaction composition. HotGoldStar *Taq* polymerase, 10x real-time PCR buffer without $MgCl_2$, 50 mM $MgCl_2$ and 5mM dNTPs mix were purchased from Eurogentec Ltd. (Hampshire, UK). The *BCR-ABL* probe (OSWEL, Southampton, UK) was labelled at the 5' end with the reporter dye molecule FAM and at the 3' end with the quencher dye molecule TAMRA.

Table 2

Primer/Probe(*)	Location	Sequence (5'-3')
BCT1+	<i>BCR</i> exon 13	TCCGCTGACCATCAATAAGGA
ABT3-	<i>ABL</i> exon 2	TTGAGCCTCAGGGTCTGAGTG
ABL-P2 (probe)	<i>ABL</i> exon 2	CCCTTCAGCGGCCAGTAGCATCTGA

(*) The suffixes + and – indicate forward and reverse primers, respectively.

Table 3

Reagents	Real time BCR-ABL PCR
10x real-time PCR buffer (μ L)	2.5
$MgCl_2$ (mM)	3.5
dNTPs (mM)	0.2
Forward primer (μ M)	0.3
Reverse primer (μ M)	0.3
Probe (μ M)	0.2
<i>Taq</i> polymerase (U/ μ L)	0.025
cDNA (μ L)	2
H ₂ O up to final volume of (μ L)	25

Amplification of mRNA from the ubiquitously expressed *GAPDH* gene was used as a control for cDNA quality and for normalisation of the *BCR-ABL* amplification values. A commercially available 20x concentrated *GAPDH* primer/probe mix was used according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Table 4 describes the *GAPDH* real-time PCR reaction composition using 2

μL cDNA template in 25 μL final volume. The *GAPDH* probe was labelled at the 5' end with the reporter dye VIC and at the 3' end with the quencher dye TAMRA.

Table 4

Reagents	Real time GAPDH PCR
10x real-time PCR buffer (μL)	2.5
MgCl ₂ (mM)	5
dNTPs (mM)	0.4
Primers/probe mix (μL)	1.25
<i>Taq</i> polymerase (U/μL)	0.025
cDNA (μL)	2
H ₂ O up to final volume of (μL)	25

The reactions were conducted in 96-well optical reaction plates closed with optical caps (Applied Biosystems). Amplification was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems). The cycling conditions were: 50°C/2 min; 95°C/10 min; 95°C/15 sec; 60°C/1 min – 50 cycles of the last two steps. A logarithmic dilution of 10⁶ KYO1 (a CML myeloid blast crisis cell line that expresses the e13a2 type of BCR-ABL transcript) cDNA was included in each plate for use as a standard for quantification. All PCR amplifications were run at least in duplicate and all values are expressed as *BCR-ABL/GAPDH* ratios.

Results

Expansion of CD34+ Cells

CD34+ cells from a newly diagnosed CML-CP patient were cultured in RF-10 supplemented with rhIL-1 β , rhIL-3, rhIL-6 and rhSCF for up to 22 days. The maximum proliferation was observed on day 14, when the number of viable cells in culture had increased, on average, about 27-fold. Control cells cultured in RF-10 without added cytokines did not expand (Fig. 4). On day 14, cultures with cytokines had a mean

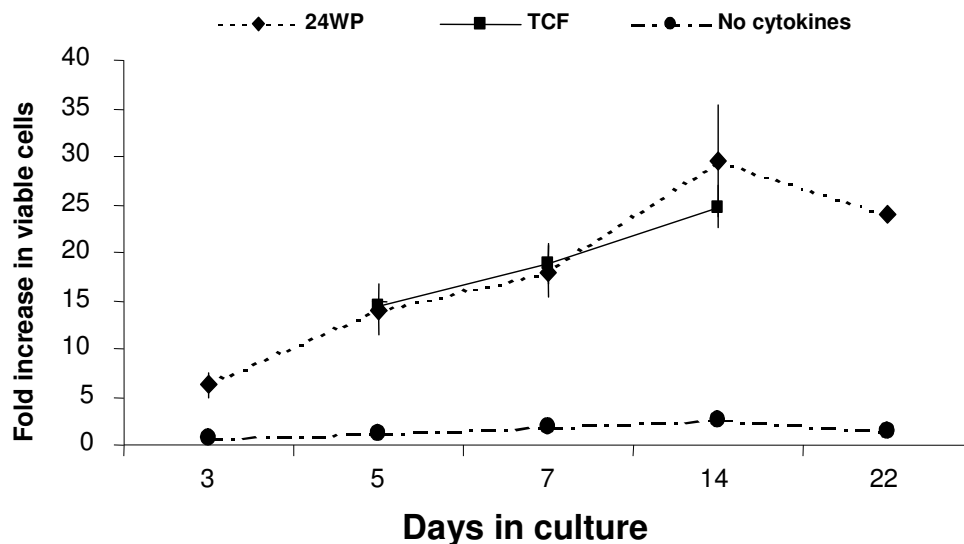


Figure 4. Expansion of CD34+ cells from a newly diagnosed CML-CP patient when cultured in RF-10 supplemented with rhIL-1 β (1 ng/mL), rhIL-3, rhIL-6 and rhSCF (50 ng/mL each). Cultures were seeded at 2×10^5 cells/mL in 24-well plates (24WP) or 25 cm² tissue culture flasks (TCF) (initial volume of 2 or 3 mL, respectively). Culture volume was doubled at each data point by adding fresh medium. On day 3, 24WP cultures were transferred to TCF. As a control, cells were cultured in RF-10 without added cytokines. Results are expressed as the mean and standard deviation of the fold increase in viable cells.

viability of approximately 75% (Fig. 5).

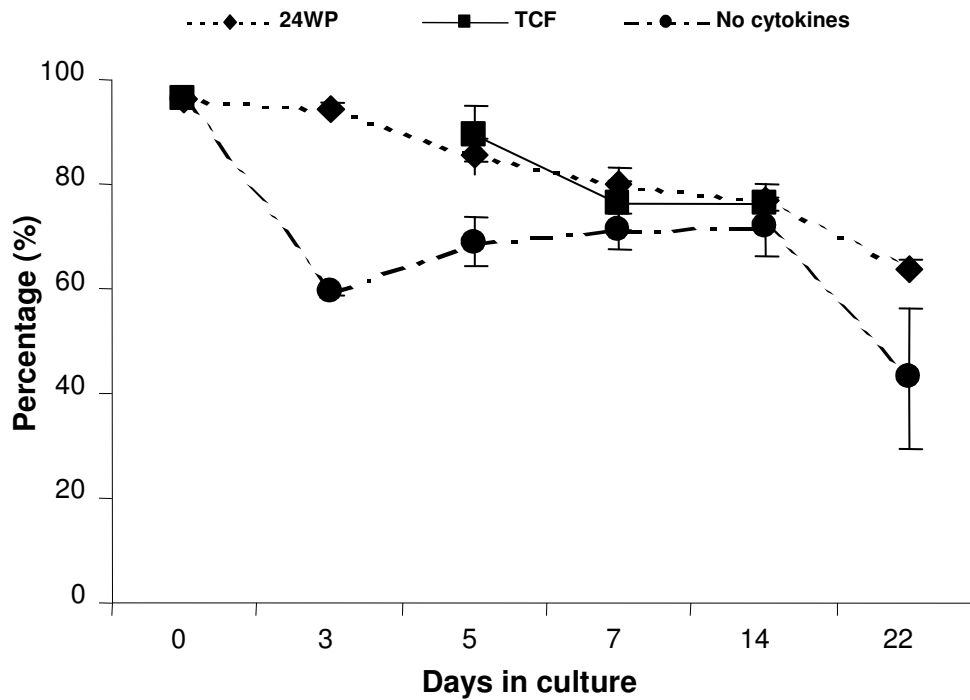


Figure 5. Viability of CML-CP CD34+ cells in culture. Culture conditions are as described in figure 4. Results are expressed as mean and standard deviation of the percentage of viable cells.

In subsequent experiments, all cultures of CD34+ cells were initiated with 2 mL of a 2×10^5 cells/mL suspension in 24WP and cultured for 12 days, with culture volume doubled on days 3, 5 and 7. The proliferation of CD34+ cells from two newly diagnosed CML-CP patients was compared with that of CD34+ cells from a normal individual (Fig. 6). The normal cells proliferated more than the CML cells – in mean values, the number of normal cells had increased by over 17-fold on day 7 and 36-fold on day 12, whereas CML-CP cells increased 13.3 and 21.7-fold, respectively. In absolute cell numbers, from an initial number of 4×10^5 cells per culture, the normal cell culture contained 14.5×10^6 cells on day 12 and the CML-CP cell cultures contained between 7.5×10^6 and 9.9×10^6 ($n=3$, mean= 8.7×10^6). Viabilities of these cultures are presented in figure 7. At day 12, the mean viability of CML-CP cells was 73%, whereas normal cells were 85% viable.

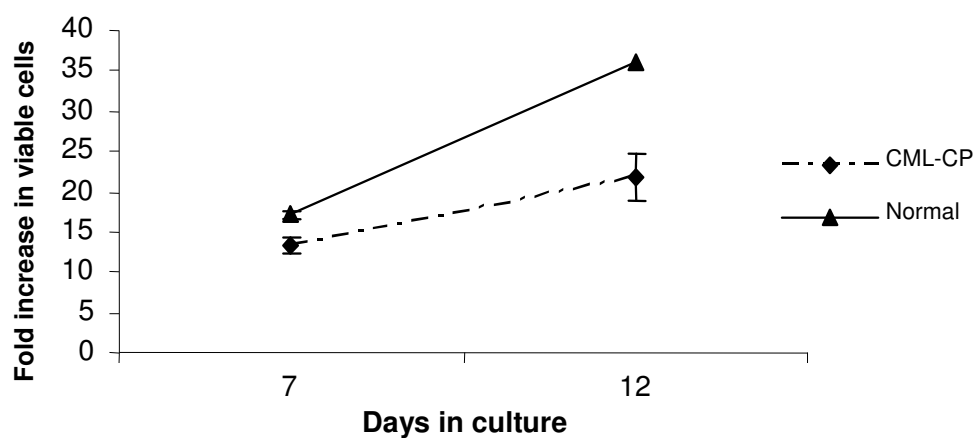


Figure 6. Expansion of CD34+ cells from 2 CML-CP patients and 1 normal individual. Cells were grown in medium supplemented with cytokines as described. Results are expressed as mean and standard deviation of the fold increase in viable cells.

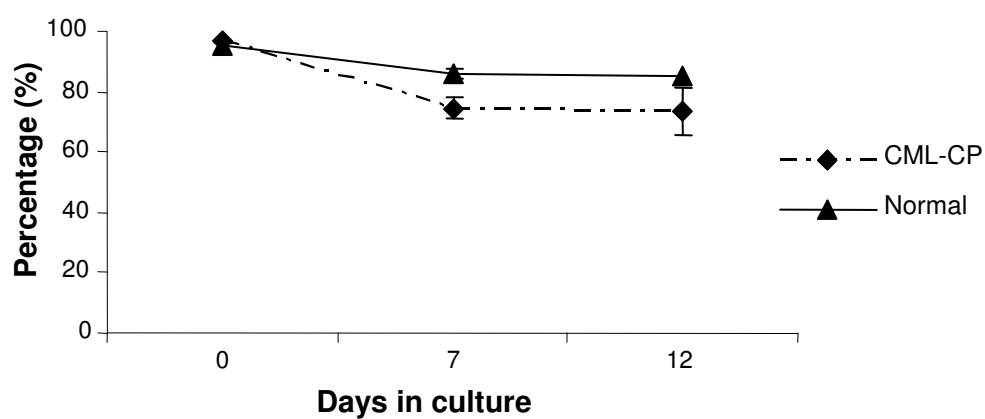


Figure 7. Viabilities of CD34+ cells from 2 CML-CP patients and 1 normal individual. Results are expressed as mean and standard deviation of the percentage of viable cells.

Immunophenotype of cultured CD34+ Cells

Cell surface expression of CD34, CD33 and co-expression of the two antigens was assessed by flow cytometry on days 0, 7 and 12 of culture of CD34+ cells from 2 CML-CP patients and 1 normal individual. There were no significant differences in the immunophenotype of the CML-CP and normal cells on day 0. The cells had been selected for CD34 positivity using an immunomagnetic separation system – on day 0, all three samples were more than 95% CD34+. The proportion of CD33+ cells was 94% and 86% in the CML-CP samples and 87% in the normal sample. CD34 and CD33 were co-expressed in 91% and 82% of the CML-CP cells and 82% of the normal cells.

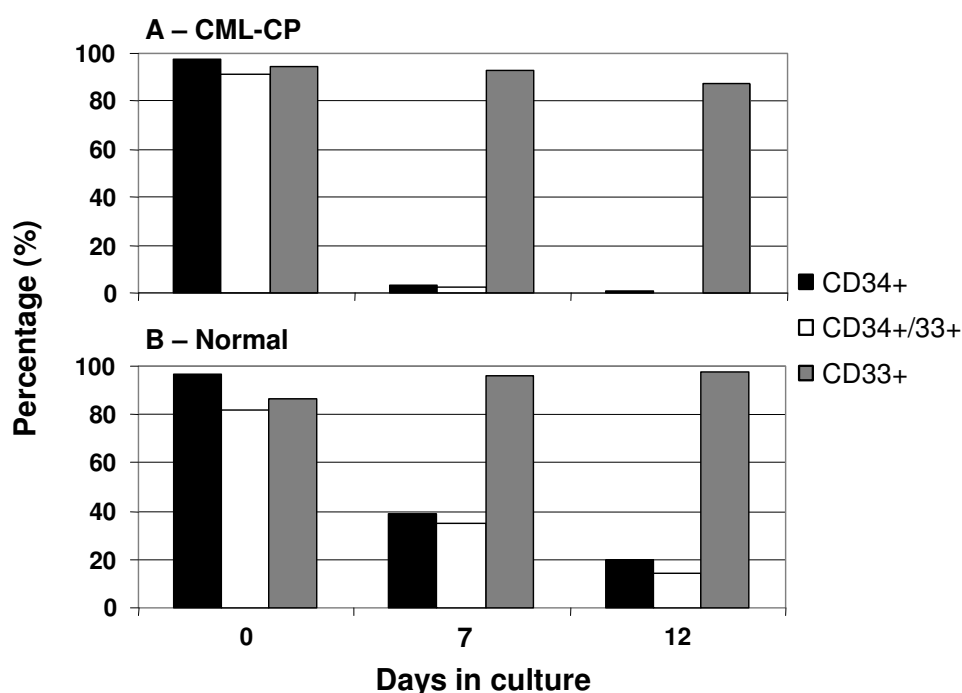


Figure 8. Immunophenotype of CD34+ cells from 2 CML-CP patients (**A** is representative of 2 experiments) and 1 normal individual (**B**) grown in medium supplemented with cytokines as described in “Materials and Methods”. Results are expressed as the percentage of CD34+, CD33+ and double positive (CD34+/CD33+) cells within the live cells gate.

In the CML-CP cells, CD34 positivity declined to 7% and 3% on day 7 and to less than 1% on day 12. In the normal sample the rate of decline of CD34 positivity was much less pronounced, with 39% and 20% of cells CD34+ on days 7 and 12, respectively. Both CML-CP and normal cells maintained high levels (minimum 86% positive cells) of CD33 expression during culture (Figs. 8 and 9).

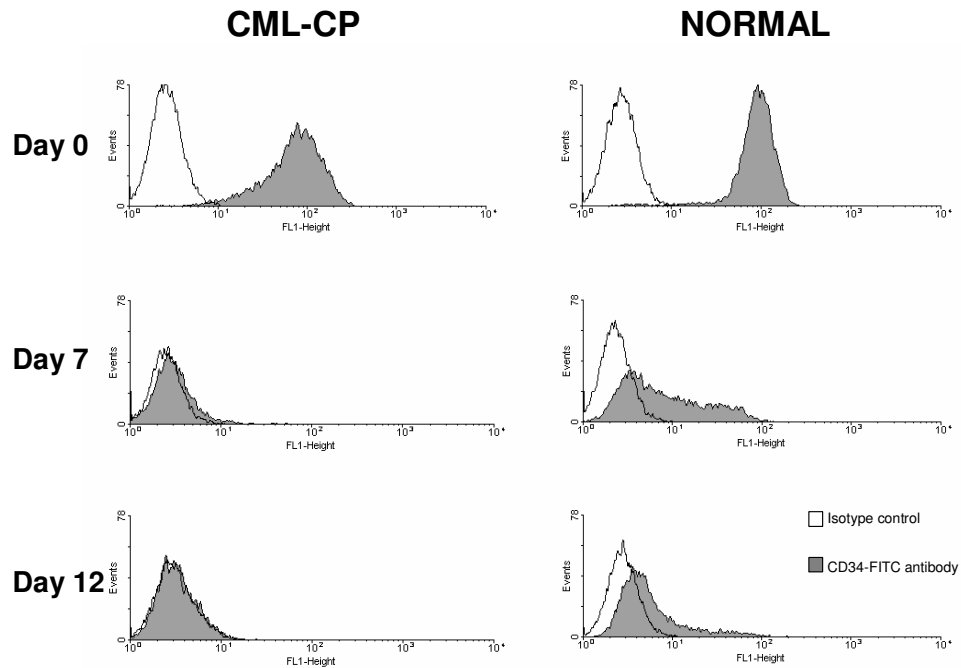


Figure 9. Surface CD34 expression in cells from a CML-CP patient and a normal individual cultured in the medium with cytokines described in “Materials and Methods”. The x-axis indicates FITC fluorescence and the y-axis cell number.

***BCR-ABL* Expression in Cultured Cells**

CML-CP CD34+ cells showed a reduction in *BCR-ABL* mRNA levels during *in vitro* culture (Fig. 10). The results were consistent in the two patient samples that were analysed. *BCR-ABL* expression decreased more rapidly during the first 7 days in culture, to about 30% of its initial value. By day 12, one sample expressed 18.5% and

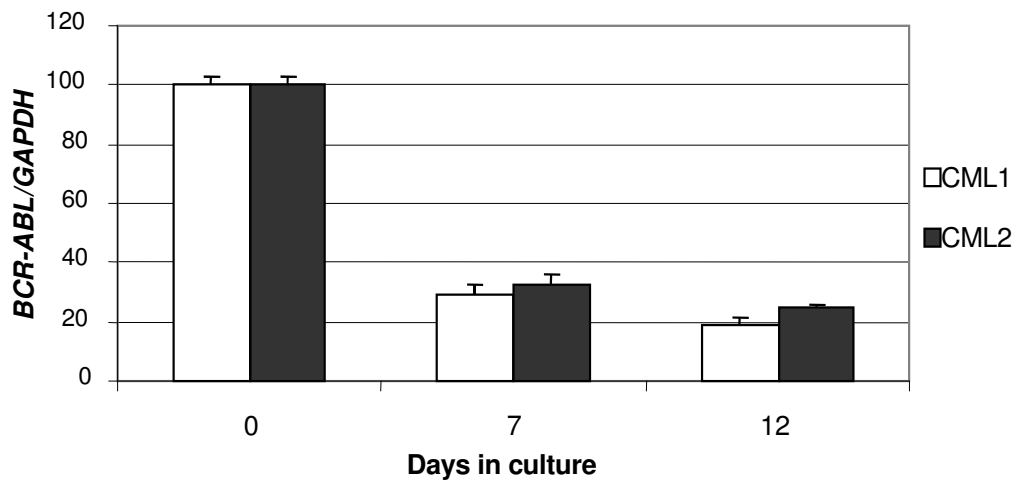


Figure 10. Changes in *BCR-ABL* mRNA levels in cultured CD34+ cells from two newly diagnosed CML-CP patients (CML1 and 2). Cells were cultured in RF-10 supplemented with cytokines, as described in “Material and Methods”. Cells were collected on days 0, 7 and 12, RNA extracted using the QIAGEN kit and reverse transcribed into cDNA. *BCR-ABL* and *GAPDH* mRNA transcripts were amplified by real-time PCR and *BCR-ABL* transcripts quantitated by determining the *BCR-ABL/GAPDH* ratio. CML1 cDNAs were derived from 1.7×10^6 or 3.7×10^6 cells; all CML2 cDNAs were derived from 1.5×10^6 cells. For each data point, 2 or 7 cDNA aliquots were tested, each in replicate reactions. Results are expressed as mean and standard deviation of the *BCR-ABL/GAPDH* ratios, normalized to an arbitrary value of 100 on day 0.

the other 24.8% of the day 0 amount of *BCR-ABL* transcripts.

Although quantifying *BCR-ABL* expression as the *BCR-ABL/GAPDH* transcript ratio should theoretically compensate for variations in the amount of cells that are used for RNA extraction and cDNA synthesis, we tested this hypothesis by measuring *BCR-ABL/GAPDH* ratios in cDNAs derived from 10^6 , 1.5×10^6 and 2×10^6 CML-CP CD34+ cells. The *BCR-ABL/GAPDH* ratios were lower on the 10^6 and 2×10^6 cells aliquots than

on the 1.5×10^6 cells aliquots (Fig. 11). The average *BCR-ABL/GAPDH* ratios in the 10^6 and 2×10^6 cells aliquots were respectively 82.9 and 90.8% of those measured in the aliquots containing 1.5×10^6 cells.

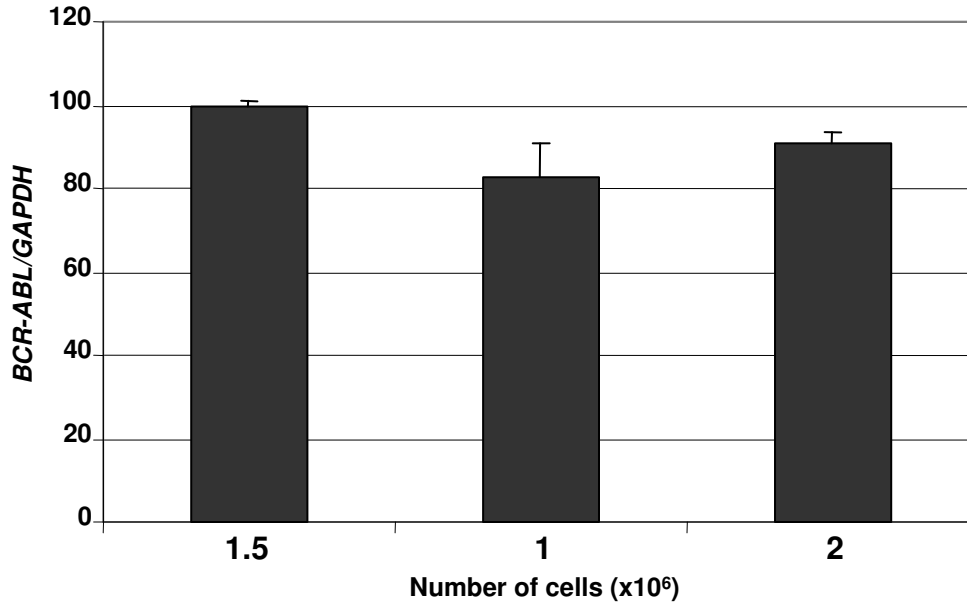


Figure 11. Variations in *BCR-ABL/GAPDH* ratios caused by differences in the number of cells used for cDNA synthesis. Cryopreserved CD34+ cells from a CML-CP patient were thawed and divided into aliquots containing 10^6 , 1.5×10^6 and 2×10^6 cells (2 aliquots each). These were processed in parallel through RNA extraction (QIAGEN kit), cDNA synthesis and real-time PCR amplification of *BCR-ABL* and *GAPDH* transcripts. Results are expressed as mean and standard deviation of the *BCR-ABL/GAPDH* ratios, normalized to an arbitrary value of 100 on the aliquots containing 1.5×10^6 cells.

BCR-ABL expression was also measured in CML-CP CD34+ cells that were cultured for 24h in medium without added cytokines. The *BCR-ABL/GAPDH* ratio of day 1 cells was 45% of the day 0 value (Fig. 12).

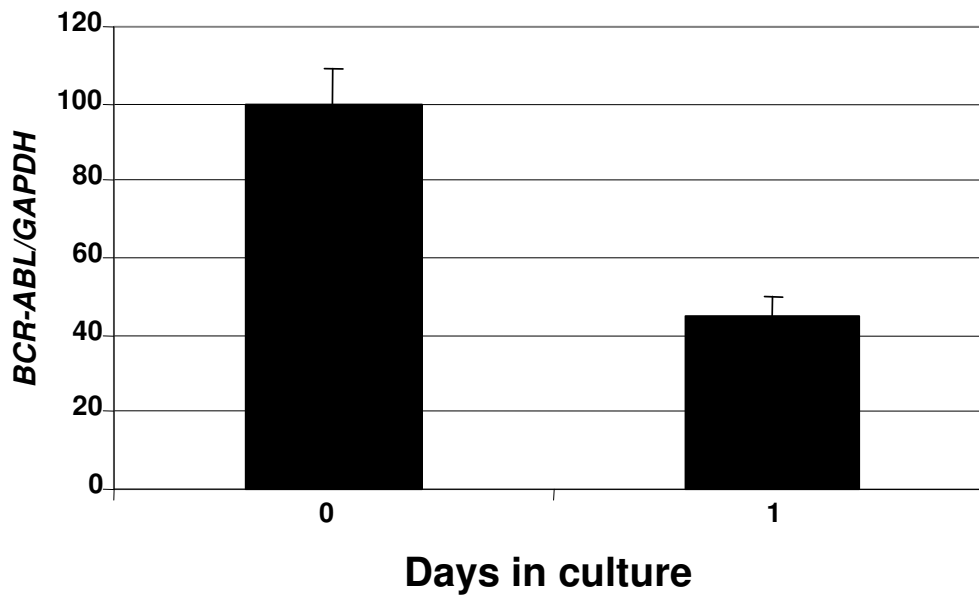


Figure 12. Decrease in *BCR-ABL* expression with 24h culture of CML-CP cells in medium without cytokines. CD34+ cells from a CML-CP patient were cultured in RF-10 for 24h. RNA was extracted (QIAGEN kit) from 2 aliquots of 10^6 cells each, reverse transcribed to cDNA and *BCR-ABL* and *GAPDH* transcripts amplified by real-time PCR. The same procedure was followed for 2 aliquots of 10^6 cells straight after thawing. Each cDNA was tested in replicate. Results are expressed as means and standard deviations of the *BCR-ABL/GAPDH* ratios, normalized to an arbitrary value of 100 on day 0.

ANNEX II

In search of the original leukemic in chronic myeloid leukemia patients in complete molecular remission after stem cell transplantation or imatinib.

Sobrinho-Simões M, Wilczek V, Score J, Cross NC, Apperley JF, Melo JV. Blood. 2010 Aug 26;116(8):1329-35.

Analysis of genomic breakpoints in p190 and p210 BCR-ABL indicate distinct mechanisms of formation.

Score J, Calasanz MJ, Ottman O, Pane F, Yeh RF, Sobrinho-Simões MA, Kreil S, Ward D, Hidalgo-Curtis C, Melo JV, Wiemels J, Nadel B, Cross NC, Grand FH. Leukemia. 2010 Oct;24(10):1742-50.

In search of the original leukemic clone in chronic myeloid leukemia patients in complete molecular remission after stem cell transplantation or imatinib

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It is not clear if absence of *BCR-ABL* transcripts—complete molecular response (CMR)—is synonymous with, or required for, cure of chronic myeloid leukemia (CML). Some patients achieve CMR with imatinib (IM), but most relapse shortly after treatment discontinuation. Furthermore, most patients in long-term remission (LTR) post-stem cell transplantation (SCT) are considered functionally cured, although some remain occasionally positive for low-level *BCR-ABL* mRNA. Interpretation of the latter is complicated be-

cause it has been observed in healthy subjects. We designed a patient-specific, highly sensitive, DNA quantitative polymerase chain reaction to test follow-up samples for the original leukemic clone, identified by its unique genomic *BCR-ABL* fusion (*gBCR-ABL*). In 5 IM-treated patients in CMR, *gBCR-ABL* was detected in transcript-negative samples; 4 patients became *gBCR-ABL*-negative with continuing IM therapy. In contrast, of 9 patients in LTR (13-27 years) post-SCT, *gBCR-ABL* was detected in only

1, despite occasional transcript-positive samples in 8 of them. In conclusion, in IM-treated patients, absence of transcripts should not be interpreted as absence of the leukemic clone, although continuing IM after achievement of CMR may lead to further reduction of residual disease. Post-SCT, we found little evidence that the transcripts occasionally detected originate from the leukemic clone. (*Blood*. 2010;116(8):1329-1335)

Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder consequent to a t(9;22)(q34;q11) reciprocal translocation that generates the Philadelphia (Ph) chromosome harbouring the *BCR-ABL* hybrid gene. The encoded Bcr-Abl fusion protein has a constitutively active tyrosine kinase that is essential (and, arguably, under certain circumstances, sufficient¹) for leukemic transformation in CML.²

The natural course of the disease is progression from an indolent chronic phase (CP) to a very aggressive blast crisis (BC), throughout which the *BCR-ABL*-positive clone acquires additional genetic abnormalities and progressively loses its differentiation ability.³ CML progression was considered virtually inevitable, and the disease was almost invariably lethal, until the development of allogeneic stem cell transplantation (SCT). In the last decade, the management of CML has been revolutionized by the development of Bcr-Abl tyrosine kinase inhibitors (TKIs). Imatinib mesylate (IM), the prototype of these drugs, is now the recommended first line therapy for most newly diagnosed CML patients in CP.⁴

Both SCT and IM are able to induce substantial reductions in disease burden, but the question of whether “cure” of CML—defined as long-term freedom from clinical relapse—requires eradication of the original *BCR-ABL* clone is unresolved. Minimal residual disease (MRD) at the molecular level is usually determined by measuring *BCR-ABL* mRNA using a real-time quantitative polymerase chain reaction (RQ-PCR).^{5,6} The interpretation of results at the threshold of sensitivity of the assay is difficult. The concept of complete molecular response (CMR)—defined as

absence of detectable *BCR-ABL* transcripts using strict sensitivity criteria—is fraught with biologic⁷ and technical^{6,8} limitations. In addition, low-level positivity for *BCR-ABL* transcripts has been found in the peripheral blood of a significant proportion of healthy subjects.^{9,10}

Post-SCT, it has been observed that low level positivity for *BCR-ABL* mRNA in the peripheral blood is not a harbinger of inevitable relapse, particularly in patients who are in long-term remission (LTR), and continues to be observed more than 10 years after SCT.¹¹⁻¹⁵ Although the majority of relapses post-SCT occur early after the procedure, and the relapse risk decreases with increasing time post-SCT, relapse as late as 14 years after SCT has been reported.¹⁶ It remains unknown if these rare transcripts are a manifestation of the original leukemic clone or a result of the kind of “benign” *BCR-ABL* rearrangements seen in healthy subjects.

Although SCT remains the only proven curative treatment for CML, IM has shown remarkable efficacy. The frequency of CMR in IM-treated patients was initially reported as 4% after a median of 19 months.⁵ However, there is evidence that this number increases with continuing therapy, with 13 of 29 first-line IM-treated patients achieving CMR after a median follow-up of 81 months.¹⁷ The maintenance of this level of response appears to be largely dependent on continued suppression of the *BCR-ABL* clone by IM, as illustrated by the finding of early relapse in 6 of 12 patients who discontinued IM after being in continuous CMR for more than 2 years.¹⁸

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An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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Table 1. Clinical features of the patients analyzed in this study

Characteristic	Category			
	LTR post-SCT/DLI	Early post-SCT	IM-treated	Total
No. of patients	9	3	5	17
Median age at diagnosis, y (range)	34 (24-43)	32 (22-32)	41 (38-56)	34 (22-56)
Patient sex				
Female	3	0	4	7
Male	6	3	1	10
Median duration of IM therapy,* mo (range)	NA	NA	76 (63-79)	NA
Donor				
Sibling	4	1	NA	5
VUD	3	2	NA	5
Syngeneic	2	0	NA	2
DLI	2	3	NA	5

LTR indicates long-term remission; SCT, stem cell transplantation; DLI, donor lymphocyte infusion; IM, imatinib; VUD, voluntary unrelated donor; and NA, not applicable; .

*Additional therapy in IM-treated patients: all 5 IM-treated patients received hydroxyurea. Only 1 of the 5 IM-treated patients received Interferon- α (IFN α) prior to IM; this patient (represented in Figure 1B, the sole patient who did not achieve *gBCR-ABL* negativity) was on IFN α for 32 months, reaching a minor cytogenetic response. IFN α was stopped 7 months before start of IM. This same patient also received homoharringtonine for 7 months, during months 33 to 39 of IM therapy. Of the 4 additional patients, 1 received IFN α during months 11 to 13 of IM therapy.

We sought to elucidate the quality of the molecular response in IM-treated patients in CMR and in patients in LTR post-SCT using a genomic DNA (gDNA)-based PCR. Given that the *ABL* breakpoint is distributed randomly over a very large—more than 150-kb—genomic area, the likelihood that 2 CML patients share the same breakpoint in both genes, and therefore have identical *BCR-ABL* fusion sequences, is close to zero. Hence, the genomic *BCR-ABL* (*gBCR-ABL*) fusion is a “molecular signature” of each individual CML case. Based on this characteristic, we designed a patient-specific RQ-PCR for *gBCR-ABL* to establish whether the original leukemic clone is present late after SCT and is the cause of the occasional low-level positivity for *BCR-ABL* transcripts seen in patients who are relapse-free long-term SCT survivors.

Methods

Patients, specimens, and treatment regimens

We studied 12 patients submitted to myeloablative SCT with high-dose cyclophosphamide and total body irradiation conditioning, and 5 IM-treated patients who achieved sustained CMR, as previously defined¹⁷ (Table 1). In the latter group, at least 4 consecutive samples negative for *BCR-ABL* mRNA, collected over a minimum of 8 months, were analyzed. We also collected white blood cells (WBCs) from 24 healthy adult volunteers as controls for RQ-PCR for *BCR-ABL* mRNA, tested exactly as the patients and as previously published^{15,19} (supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Informed consent for the use of cells for research was obtained in accordance with the Declaration of Helsinki and with approval from the Hammersmith and Queen Charlotte's and Chelsea Research Ethics Committee Institutional Review Board.

For the 12 SCT patients, the stem cell donors were HLA-identical siblings (Sib) in 5 cases, HLA-matched unrelated volunteers (VUD) in 5 cases, and syngeneic siblings (Syn) in 2 cases. In 5 cases, the patients met criteria for molecular relapse post-SCT (as defined in Mughal et al¹³) and were treated with donor lymphocyte infusions (DLI)—2 with a single bulk dose and 3 with an escalating dose schedule.^{20,21} Of the 12 patients, 3 (2 VUD, 1 Sib) were studied in CMR within 6 to 12 months post-SCT, at molecular relapse and after reinduction of CMR with DLI. Nine patients (4 Sib, 3 VUD, 2 Syn), 2 of whom received DLI for relapse, were studied in LTR post-SCT/DLI, defined as being relapse-free for a median of 193 months (range: 161 to 333) after last treatment.

Sequencing of the *gBCR-ABL* fusions was done on gDNA from WBCs collected before SCT or IM therapy and stored at -80°C as dry pellets or as guanidinium thiocyanate (GTC) lysates. In 1 case, due to the lack of a

pre-IM sample, WBCs collected early in the course of IM therapy were used. The QIAamp DNA blood minikit (QIAGEN) was used to extract gDNAs. The quality of the gDNAs was tested with a PCR for an 871-bp fragment of *ABL* that included an intronic sequence, as described in Melo et al.²²

Follow-up (FU) gDNA samples were collected from peripheral blood WBCs after red blood cell lysis using the QIAamp DNA blood minikit (QIAGEN). In some cases, the same kit was used to extract gDNA from WBC GTC lysates stored at -80°C . The FU gDNA samples were quantified using a spectrophotometer and diluted in PCR-grade water to 50 ng/ μL . The quality and concentration of the gDNAs were checked with a TaqMan RQ-PCR for a genomic fragment of albumin, with a probe (Applied Biosystems) labeled with FAM reporter and TAMRA quencher.

For all the FU gDNAs, the amount of *BCR-ABL* transcripts was measured in cDNAs synthesized from the same WBC samples and quantified as a *BCR-ABL* to *ABL* mRNA ratio as described in Kaeda et al¹⁵ and Foroni.¹⁹ Per standard MRD PCR protocol,^{15,19} a minimum of 1×10^4 *Abl* transcripts was required to ensure good cDNA quality, with more than 90% of the samples analyzed in the study containing $> 3 \times 10^4$ *ABL* transcripts (average 6×10^4).

Sequencing of *gBCR-ABL* fusions

The *gBCR-ABL* fusions were sequenced using either an inverse PCR (7 cases) or a long-range PCR (10 cases) approach. For the former, 5 μg of the gDNA were digested with *RsaI*, a blunt-end cutter that digests the region between exon 13 (e13) and e15 of the major breakpoint cluster region of *BCR* into 2 fragments: a 776-bp fragment including most of intron 13 and e14, and a 1703-bp fragment that comprises most of intron 14. The digested gDNA was extracted with phenol/chloroform, completion of digestion checked by agarose gel electrophoresis, and quantified using a spectrophotometer. A ligation reaction containing 500 ng of the digested gDNA, 5 units of high concentration T4 DNA ligase and 5X ligase buffer (Invitrogen) in a final volume of 500 μL was incubated at 14°C for 16 to 24 hours. The ligated gDNA was purified using the QIAquick Gel Extraction kit (QIAGEN) and amplified using 2 pairs of inverse PCR primers, located in the 5' ends of intron 13 or intron 14, and the *Pfu*-Turbo polymerase (Stratagene). The PCR products were then visualized under ultraviolet light in ethidium bromide-stained agarose gels. The inverse PCR was successful when

2 bands could be seen: a wild-type *BCR* band of a known size (767 and 1693 bp for intron 13 and intron 14 inverse PCR primers, respectively) and a shifted band corresponding to the rearranged *BCR-ABL* allele. The shifted band was cut out from the gel, purified using the QIAquick Gel Extraction kit (QIAGEN) and cloned using the TOPO-TA cloning kit (Invitrogen). Bacterial colonies were selected according to the size of the insert using a PCR with forward and reverse vector primers. Constructs of the desired size

were sequenced in both forward and reverse orientation using the same primers as for the PCR.

When the inverse PCR method was not successful (6 of 13 cases) and in 4 additional cases, a long-range genomic PCR for *BCR-ABL* was used.²³ The reaction used the Expand Long Template PCR System (Roche Applied Science) polymerase and Buffer 2 from the kit. The reaction conditions and thermocycling parameters were optimized to amplify templates of up to 12 kb. The PCRs were multiplex reactions with 1 of 2 forward primers, located in *BCR* e13 or e14, and 1 of 4 mixes of 5 reverse primers each, located in *ABL* intronic sequences—a total of 20 reverse primers spanning more than 150 kb of the *ABL* breakpoint area, from 5' of exon 1b to 3' of exon a3. The patient-specific PCR products were gel purified and sequenced in a forward orientation using consecutive *BCR* primers, from 5' to 3', until the *BCR-ABL* junction was reached.

Patient-specific RQ-PCR for gBCR-ABL

The TaqMan technology (Applied Biosystems [ABI]) was used to detect g*BCR-ABL*. Individual g*BCR-ABL* sequences—all intronic—were submitted to ABI for design of RQ-PCR primers and probe using the Custom TaqMan Genomic Assays Service. The design was “directed” so as to avoid repetitive sequences (namely *Alu* elements) and so that the forward primer was located in *BCR*, the reverse primer in *ABL* and the probe would anneal to the junction. (See supplemental Table 2 for primers/probe sequences and size of products.) The RQ-PCR reactions were set up in 96-well Fast Optical Reaction plates to a final volume of 30 μ L using 20 $\times$ primers/probe mix, 2 $\times$ TaqMan Universal PCR Master Mix (plates and reagents from ABI), 6 μ L of gDNA at 50 ng/ μ L, and PCR-grade water. The reactions were amplified in the 7500 Fast thermal cycler (ABI) under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, and 50 cycles of 15 seconds at 95°C followed by 1 minute at 60°C. The results were analyzed using the 7500 Fast System SDS Software Version 1.3.

The specificity of the RQ-PCR reactions was tested using at least 2 different *BCR-ABL*-positive gDNAs from CP-CML patients or cell lines. The sensitivity and quantitative nature of the RQ-PCR was tested using serial 10-fold dilutions of pre-IM/SCT gDNAs in gDNA from HL60 (*BCR-ABL*-negative cell line). The series covered a range of 6 logs of dilution of pre-IM/SCT gDNA, from 10 ng/ μ L (10^1) to 0.1 pg/ μ L (10^{-4}), diluted in HL60 gDNA at 50 ng/ μ L. The 10^1 , 10^0 , and 10^{-1} dilutions were tested in triplicate and the 10^{-2} , 10^{-3} , and 10^{-4} in 6 replicate reactions. A standard curve was obtained by plotting the logarithmic value of the dilution against the average cycle threshold (C_t) of the positive reactions at each dilution of the pre-IM/SCT gDNA. Follow-up gDNA samples were tested in 36 replicate reactions, each loaded with 300 ng of gDNA (6 μ L of gDNA at 50 ng/ μ L)—a total of 10.8 μ g of gDNA per FU sample.

Patient-specific RQ-PCR versus nested RQ-PCR for gBCR-ABL

We compared the sensitivity of the patient-specific RQ-PCR for g*BCR-ABL* with a nested approach in which the RQ-PCR was done on conventional PCR products obtained with primers external to the RQ-PCR primers.

The gDNA from 4 CP-CML patients was serially 10-fold diluted in HL60 gDNA at 50 ng/ μ L as described in “Patient-specific RQ-PCR for gBCR-ABL.” The lowest concentration that yielded positive results by RQ-PCR (10^{-3} dilution) was tested in 36 replicate RQ-PCR reactions (5 μ L of gDNA as template in 25 μ L of final volume; reagents, and thermal cycling conditions as described in “Patient-specific RQ-PCR for gBCR-ABL”).

After determining the optimal PCR conditions and combinations of *BCR* forward with *ABL* reverse primers that yielded specific amplification from the CP-CML gDNA of each patient, 36 replicate conventional PCR reactions were seeded with 5 μ L of template at the 10^{-3} dilution in a final reaction volume of 20 μ L. These were subjected to 30 cycles of amplification, with an annealing temperature ranging from 58 to 66°C. From each of the replicate PCR reactions, 2.5 μ L were used to seed a 25- μ L RQ-PCR.

Results

Specificity and sensitivity of patient-specific RQ-PCR for gBCR-ABL

All patient-specific RQ-PCRs for g*BCR-ABL* were specific, that is, there was no amplification from *BCR-ABL*-positive gDNAs obtained from a source other than the individual patient in question (a minimum of 3 different *BCR-ABL*-positive gDNAs—2 CML samples and 1 cell line—were tested to establish specificity).

The threshold of sensitivity of the assays was at the 10^{-3} dilution of the pre-IM/SCT gDNA, corresponding to a 1 pg/ μ L concentration of the pre-IM/SCT gDNA. Because each reaction was loaded with 6 μ L of gDNA at this concentration, each individual reaction contained on average 6 pg of pre-IM/SCT gDNA—the estimated gDNA content of 1 human diploid cell²⁴ and, therefore, the theoretical maximal sensitivity achievable if one postulates the presence of 1 *BCR-ABL* copy per cell of the leukemic clone.

At the limit of sensitivity, the RQ-PCRs were not reproducibly positive, with 1 to 4 of 6 replicate reactions positive and a larger variation of C_t values (ΔC_t) between replicates than with less diluted samples, as has been observed with other leukemia-associated fusion genes.²⁵⁻²⁷ Less diluted pre-IM/SCT gDNA samples gave reproducible results, with all replicates positive and lower ΔC_t .

All patient-specific RQ-PCRs generated standard curves over 5 logs of dilution (10^1 to 10^{-3}) of acceptable quality as defined in,²⁸ that is, a slope between -3.0 and -3.9 and a correlation coefficient of more than 0.95; the majority of them were close to the optimal theoretical slope of -3.3 and correlation coefficient of 1.

Comparison of the sensitivity of RQ-PCR with nested RQ-PCR for gBCR-ABL

The numbers of positive reactions of the 36 replicate single-step RQ-PCRs and nested RQ-PCRs with the 10^{-3} diluted template were compared by the Wilcoxon signed rank test. The median difference between the 2 tests was 12.5%, which was not statistically significant ($P = .71$). The average number of positive reactions was 13.25 (range: 8 to 20) with single-step RQ-PCR and 11.5 (range: 5 to 15) with nested RQ-PCR; in 3 of the 4 CP-CML samples tested, there was, in fact, a higher number of positive reactions with single-step RQ-PCR than with nested RQ-PCR. Because we could not show an advantage of the 2-step (nested) RQ-PCR assay, it was not adopted for this study.

Residual disease assessment by patient-specific RQ-PCR for gBCR-ABL

From each of the 5 patients in CMR on IM, between 3 and 5 FU samples negative for *BCR-ABL* transcripts were tested by patient-specific RQ-PCR for the presence of g*BCR-ABL*. In all 5 patients, g*BCR-ABL* was detected in 1 to 5 samples negative for *BCR-ABL* mRNA (Figure 1 and supplemental Figure 1). In 4 of the 5 patients, g*BCR-ABL* became undetectable with continuing IM therapy—2 consecutive g*BCR-ABL*-negative samples in 3 patients, and 1 g*BCR-ABL*-negative sample in 1 patient. In all cases, the g*BCR-ABL*-positive sample(s) preceded the g*BCR-ABL*-negative sample(s) (Figure 1A). Negativity for g*BCR-ABL* was found after a median time on IM of 70.5 months (range: 60 to 75). In 1 patient, all 5 FU samples tested were g*BCR-ABL*-positive (Figure 1B).

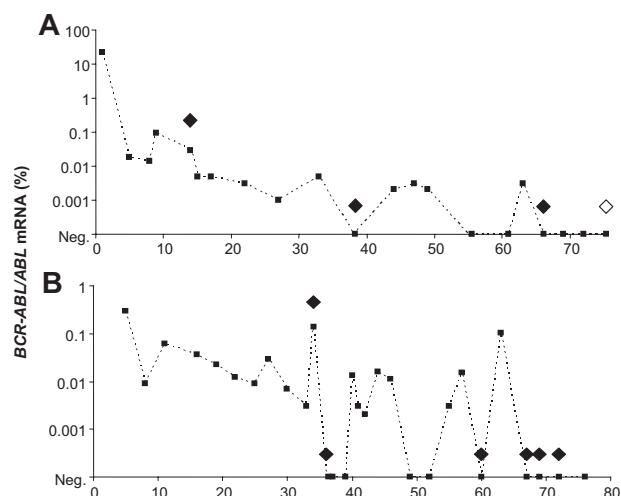


Figure 1. Representative patterns of residual disease found in IM-treated patients achieving CMR. Panels A and B show 2 individual patients. On the x-axis are months on imatinib (IM). The results of the quantitation of *BCR-ABL* transcripts (■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the real-time quantitative polymerase chain reaction for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. (A) *gBCR-ABL* was initially detected in transcript-negative samples but became undetectable with continuing IM therapy. A similar pattern was seen in 3 other patients tested. (B) The single patient, of 5 tested, in which all transcript-negative samples tested were *gBCR-ABL*-positive.

Altogether, from the 5 patients in CMR on IM, of 19 samples negative for *BCR-ABL* transcripts, 12 were *gBCR-ABL*-positive, all at a very low level—1 to 6 positive reactions of 36 replicates, with C_t values at the limit of detection of the standard curve.

Three SCT-treated patients were tested in CMR early after SCT (between 6 and 12 months), at relapse, and within 6 to 28 months after reinduction of molecular remission by DLI. In the 3 cases, *gBCR-ABL* was detected in the pre-relapse CMR samples at a level comparable with the limit of sensitivity of the standard curve. At relapse, *gBCR-ABL* was detected at a much higher level (1 to 2 logs above the limit of sensitivity of the standard curve); in the 3 cases it became undetectable after DLI (2 FU samples from each of the 3 patients were *gBCR-ABL*-negative; Figure 2 and supplemental Figure 2).

Nine patients were studied in LTR post-SCT/DLI (Figure 3 and supplemental Figure 3). These 9 patients had *BCR-ABL* mRNA levels measured and quantified on a median of 8 occasions (range: 7 to 20). One of the 9 patients was negative for *BCR-ABL*

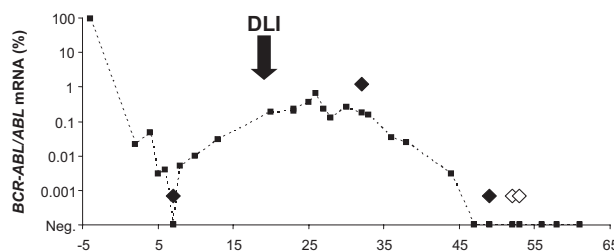


Figure 2. Representative pattern of residual disease found in patients tested early after SCT, at relapse and post-DLI. One of 3 patients tested, all with similar patterns, is shown. On the x-axis are months post-stem cell transplantation (SCT). The results of the quantitation of *BCR-ABL* transcripts (■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. *gBCR-ABL* was detected in a transcript-negative sample prerelapse and at relapse; after donor lymphocyte infusion (DLI), a transcript-negative sample was *gBCR-ABL*-positive; later samples were *gBCR-ABL*-negative.

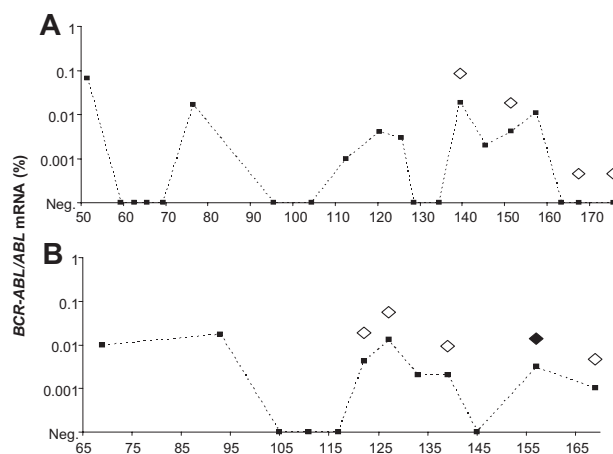


Figure 3. Representative patterns of residual disease found in patients in LTR post-SCT/DLI. Panels A and B show 2 individual patients who, despite not meeting criteria for molecular relapse, were persistently positive for *BCR-ABL* transcripts at a low level. On the x-axis are months post-SCT/DLI. The results of the quantitation of *BCR-ABL* transcripts (■) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (y-axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: ◆, *gBCR-ABL*-positive; ◇, *gBCR-ABL*-negative. (A) All 4 samples tested, 2 of which were transcript-positive, were *gBCR-ABL*-negative. (B) Of 5 transcript-positive samples tested, 1 was *gBCR-ABL*-positive, at the very limit of sensitivity of the assay. Of 24 samples tested from 9 patients in long-term remission (LTR) post-SCT/DLI, this was the single instance of *gBCR-ABL* positivity, despite 14 of 24 samples being transcript-positive.

transcripts in all 7 FU samples. Eight patients, while not meeting the criteria for molecular relapse,¹³ were intermittently low-level positive for *BCR-ABL* mRNA. The proportion of samples positive for *BCR-ABL* mRNA ranged from 22% (2 of 9) to 67% (8 of 12); 3 patients had 4 consecutive samples positive for *BCR-ABL* mRNA collected over 18 to 26 months. In 7 of the 9 patients, 10 or more *BCR-ABL* transcripts were detected on at least 1 occasion.

Between 1 and 5 FU samples (median time from SCT/DLI to sample collection: 159 months; range: 80 to 320) from each patient were tested by patient-specific RQ-PCR for *gBCR-ABL*. In 8 of the patients all follow-up samples were *gBCR-ABL*-negative. In only 1 patient the original *gBCR-ABL* fusion was detected in 1 of 5 samples tested, all of which were positive for *BCR-ABL* transcripts (Figure 3). The *gBCR-ABL*-positive sample—collected 157 months post-SCT—was positive in 1 of 36 replicate reactions, with a C_t at the limit of sensitivity of the standard curve (supplemental Figure 4).

Altogether, from the 9 patients in LTR post-SCT/DLI, a total of 24 FU samples were tested for patient-specific *gBCR-ABL*. Only 1 of these samples was *gBCR-ABL*-positive. Fourteen of the 24 samples were positive for *BCR-ABL* transcripts (5/14, from 4 patients, with 10 or more transcripts), with *BCR-ABL* to *ABL* mRNA ratios ranging from 0.001 to 0.019% (or 0.0022 and 0.04218% on the International Scale⁶). This low level of *BCR-ABL* transcripts is similar to that also found sporadically in normal adults tested by the same cDNA RQ-PCR assay (supplemental Table 1).¹⁹

Discussion

It remains unknown whether the curative effect of SCT in CML depends on eradication of the *BCR-ABL*-positive clone. This question is increasingly relevant in the era of effective TKIs, able to induce CMR in a proportion of patients.²⁹ It is a matter of concern

that “surviving” *BCR-ABL*-positive cells post-SCT or IM might not only lead to relapse, but also acquire the genetic lesions that are associated with progression to BC or the development of resistance to TKIs.^{3,30,31}

The concept of CMR should be used with caution. The RQ-PCR for *BCR-ABL* transcripts that is typically used to measure molecular MRD has technical pitfalls and significant inter-laboratory variability,⁶ and it has been estimated that a residual leukemic burden of up to 10^6 *BCR-ABL*-positive cells may lie below its threshold of sensitivity.⁷ On the other hand, attempts to increase even further its sensitivity may run into the opposite pitfall, that is, the difficulty in interpreting a low level, intermittent *BCR-ABL* transcript positivity,²⁵ which may be seen inconsequentially in healthy subjects^{9,10} (supplemental Table 1), in CML patients in LTR post-SCT,¹¹⁻¹⁴ or maybe even in very rare patients who remain in prolonged cytogenetic remission after interferon- α discontinuation.³²⁻³⁴

The method used for RQ-PCR amplification of *gBCR-ABL* proved to be patient-specific and highly sensitive, because it was able to detect *BCR-ABL* on DNA diluted to 6 pg per reaction—the DNA content of a single human diploid cell.²⁴ Its sensitivity was further demonstrated by the *gBCR-ABL* positivity of samples that were negative for *BCR-ABL* transcripts from both patients in CMR on IM and those post-SCT in CMR before relapse using a highly sensitive assay conducted in a reference MRD laboratory.^{5,6} Further evidence that our assay was thoroughly optimised for sensitivity was the fact that an additional nesting step of RQ-PCR amplification of first-round PCR products did not lead to any increase in “reproducible sensitivity.” Because nested amplification is not only more labor-intensive, technically demanding, and costly but, above all, more prone to false-positivity due to the increased risk of PCR contamination, we felt confident that it was unwarranted for this study.

In all 5 patients studied in CMR on IM, *gBCR-ABL* was detected in transcript-negative samples. In 1 of the 5, *gBCR-ABL* was detected in all ($n = 5$) FU samples tested; 4 of the 5 patients became *gBCR-ABL*-negative with continuing IM therapy. These results expand on the findings from a recent publication³⁵ and indicate that, in IM-treated patients, absence of transcripts should not be interpreted as absence of the leukemic clone. In addition, our results fit with the reported continuing reduction in amount of residual disease (measured by mRNA) in long-term treatment with IM,¹⁷ and demonstrate such a reduction in a proportion of the patients previously classified as CMR by *BCR-ABL* mRNA quantification.

Contrasting with the detection of *gBCR-ABL* in transcript-negative samples from every patient in CMR on IM, we found very little evidence that the leukemic clone is present in LTR post-SCT, despite occasional transcript-positivity in 8 of the 9 patients. In fact, the original leukemic clone was detected in only 1 of the 9 patients. The positivity of the *gBCR-ABL* test from this patient was at the very limit of sensitivity of the assay—1 positive reaction of 36 replicates, with a C_t corresponding to the threshold of detection of the standard curve (supplemental Figure 4). Four other samples from this patient, all positive for *BCR-ABL* transcripts, were *gBCR-ABL*-negative. This patient (Figure 3B) received a syngeneic graft and was free of relapse at last follow-up, more than 14 years after SCT, with no further therapy. While syngeneic graft recipients are known to have an increased risk of relapse compared with recipients of allografts,³⁶ relapse would be uncommon more than 14 years post-SCT. Barring the possibility of PCR contamination (which can never be totally ruled out, but for which we have no

evidence in very extensive negative controls over the course of the experiments), this *gBCR-ABL* positivity is the sole evidence we have of survival of the original CML clone late after SCT. This is consistent with the rarity of CML relapse after very prolonged remissions post-SCT. The other syngeneic graft recipient in this study was 333 months post-SCT and free of relapse at last follow-up; he had 2 FU samples positive for *BCR-ABL* transcripts of 8 tested. The only sample from this patient who was tested by both methods was negative for both *gBCR-ABL* and for transcripts.

An earlier attempt to compare gDNA with cDNA in MRD assessment in the post-SCT setting found largely concordant results between the 2 approaches.³⁷ This was done in the pre-RQ-PCR era and it should be noted that, compared with the present study, the FU period post-SCT was shorter and there was very little evidence of transcript positivity more than 2 years after SCT.

The fact that we were able to consistently detect the original leukemic clone in samples negative for *BCR-ABL* transcripts from IM-treated patients (12/19 samples from 5/5 patients) and from SCT-treated patients pre-relapse (5/5 samples from 3/3 patients) demonstrates the high sensitivity of the gDNA RQ-PCR approach we used. Yet, 13 of 14 transcript-positive samples from 9 patients late after SCT were conclusively *gBCR-ABL*-negative, which raises an intriguing question as to the origin of such *BCR-ABL* transcripts in this group of patients. Occasional low-level positivity for *BCR-ABL* mRNA might be explainable by PCR contamination, an event that is probably inevitable, at however low a level, in laboratories that handle large numbers of *BCR-ABL*-positive samples. It is, however, very unlikely that PCR contamination can explain the 4 consecutive samples positive for *BCR-ABL* transcripts seen in 3 patients.

Two alternative scenarios for this type of pattern (Figure 3) can be envisaged. The low-level transcript positivity observed in some post-SCT patients may be a manifestation not of the original leukemic clone, but of different, “benign” *BCR-ABL*-positive cells, as have been detected in healthy subjects^{9,10} (supplemental Table 1). There is no indication of whether some subjects may be particularly prone to generate such cells, but that could be the case of these patients (or their donors, because the patients received hematopoietic stem cell grafts). Furthermore, this sustained positivity could be a manifestation of different, short-lived, *BCR-ABL*-positive cells, or of a single, longer-lived but not oncogenic (“fully transformed”), *BCR-ABL* clone. It is also tempting to speculate that, if such inconsequential *BCR-ABL* recombinations were indeed to occur and produce a functional protein, their detection would be less likely in IM-treated than in post-SCT patients, because tyrosine kinase inhibition would abrogate any marginal proliferative advantage afforded by Bcr-Abl.

Alternatively, the *BCR-ABL* transcripts may be a manifestation of an extremely low number of cells from the original leukemic clone, in which *BCR-ABL* is being very actively transcribed. This would generate a much larger amount of mRNA than gDNA targets for PCR amplification, thus making the residual disease less likely to be detected by genomic-based methods than cDNA-based methods. There are no data to corroborate this hypothesis. In fact, it could be speculatively argued against it that, should putative surviving cells be expressing unusually large amounts of *BCR-ABL*, this could make them particularly good targets for the graft-versus-leukemia effect that is known to be crucial to the therapeutic efficacy of SCT and DLI. Thus, such high *BCR-ABL* expressing clones should die out and not be repeatedly detectable at the transcript level.

There are several advantages to using a gDNA-based PCR as opposed to mRNA-based PCR for analysis of residual disease. Genomic DNA is much more stable than mRNA, which is very susceptible to degradation; extraction of gDNA is technically easier than the extraction of mRNA followed by cDNA synthesis; genomic DNA PCR of a target gene results in amplification whether or not that gene is actually being transcribed. Furthermore, a gDNA-based PCR does not need to rely on a control gene for normalization of results—arguably, the biggest source of variation in RT-PCR. In the case of CML, the use of clone-specific primers makes the assay less susceptible to contamination and excludes the possible amplification of the “benign” clones seen in healthy subjects. The major disadvantage of the method we describe is the need to sequence the *BCR-ABL* fusion from each individual at diagnosis, the need to demonstrate linearity and sensitivity for each patient-specific primers/probe combination, and the need for multiple reactions to test large amounts of gDNA and thus maximize sensitivity. However, as for many other originally complex and technically demanding techniques, both processes may be further optimized and automated for future use in a routine setting. In the meantime, the use of gDNA PCR for *BCR-ABL* cannot be advocated for immediate implementation as a routine MRD test, until its reliability and predictive values are soundly established and compared in different laboratories.

Our gDNA-based method can be quantitative over a wide range of disease burdens, as seen by the good quality standard curves. However, similar to what occurs with mRNA quantification, the very low levels of residual disease seen in patients negative for transcripts are below the range of reproducible and accurate quantification.

In conclusion, using a sensitive, patient-specific gDNA-based RQ-PCR for detection of *BCR-ABL*, we could demonstrate the presence of residual amounts of the original leukemic clone in 5 of 5 CML patients treated with IM who were negative for *BCR-ABL* transcripts. In 4 of these patients, the leukemic clone became undetectable with continuing IM therapy. In contrast, in only 1 of 9 patients in LTR post-SCT there was evidence for the survival of the original leukemic clone, which is consistent with the low risk of disease relapse observed many years post-SCT. The low-level positivity for *BCR-ABL* transcripts that is seen in some of these patients (sometimes persistently) remains puzzling, as we found no evidence that it is caused by residual amounts of the original

leukemic clone. Clarification of this issue will require prospective collection of larger blood samples for subcellular fractionation to ascertain the origin of these rare transcripts.

Because the period of observation is not strictly comparable for the 2 groups of patients, it is possible that the apparent difference in the degree of “elimination” of the leukemic clone between patients treated with SCT or IM will disappear with longer exposure to the drug. Likewise, a larger cohort of patients with longer follow-up will help ascertain whether the type of “deeper CMR” we demonstrated upon continuing IM therapy correlates with response (ie, relapse or sustained remission) to interruption of the kinase inhibitor.

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Authorship

Contribution: M.S.-S. designed the study, collected patient data, processed samples, performed experiments, analyzed data, and wrote the report; V.W. processed samples and performed experiments; J.S. designed vital experimental assays; N.C.P.C. helped design the study, analyze the data, and write the report; J.F.A. provided clinical care and recorded clinical data; and J.V.M. conceived and designed the study, supervised its execution, helped write the report, and had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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References

- Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science*. 1990;247(4944):824-830.
- Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood*. 2000;96(10):3343-3356.
- Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nat Rev Cancer*. 2007;7(6):441-453.
- Baccarani M, Cortes J, Pane F, et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *J Clin Oncol*. 2009;27(35):6041-6051.
- Hughes TP, Kaeda J, Branford S, et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N Engl J Med*. 2003;349(15):1423-1432.
- Hughes T, Deininger M, Hochhaus A, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood*. 2006;108(1):28-37.
- Kaeda J, Chase A, Goldman JM. Cytogenetic and molecular monitoring of residual disease in chronic myeloid leukaemia. *Acta Haematol*. 2002;107(2):64-75.
- Gabert J, Beillard E, van Der Velden V, et al. Standardization and quality control studies of ‘real-time’ quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia—a Europe Against Cancer program. *Leukemia*. 2003;17(12):2318-2357.
- Bose S, Deininger M, Gora-Tybor J, Goldman JM, Melo JV. The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. *Blood*. 1998;92(9):3362-3367.
- Biernaux C, Loos M, Sels A, Huez G, Stryckmans P. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. *Blood*. 1995;86(8):3118-3122.
- van Rhee F, Lin F, Cross NC, et al. Detection of residual leukaemia more than 10 years after allogeneic bone marrow transplantation for chronic myelogenous leukaemia. *Bone Marrow Transplant*. 1994;14(4):609-612.
- Pignon JM, Henni T, Amselem S, et al. Frequent detection of minimal residual disease by use of the polymerase chain reaction in long-term survivors after bone marrow transplantation for chronic myeloid leukemia. *Leukemia*. 1990;4(2):83-86.
- Mughal TI, Yong A, Szydlo RM, et al. Molecular studies in patients with chronic myeloid leukaemia in remission 5 years after allogeneic stem cell transplant define the risk of subsequent relapse. *Br J Haematol*. 2001;115(3):569-574.
- Radich JP, Gooley T, Bryant E, et al. The significance of bcr-abl molecular detection in chronic myeloid leukemia patients “late,” 18 months or more after transplantation. *Blood*. 2001;98(6):1701-1707.

15. Kaeda J, O'Shea D, Szydlo RM, et al. Serial measurement of BCR-ABL transcripts in the peripheral blood after allogeneic stem cell transplantation for chronic myeloid leukemia: an attempt to define patients who may not require further therapy. *Blood*. 2006;107(10):4171-4176.
16. Yong AS, Goldman JM. Relapse of chronic myeloid leukaemia 14 years after allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 1999;23(8):827-828.
17. Branford S, Seymour JF, Grigg A, et al. BCR-ABL messenger RNA levels continue to decline in patients with chronic phase chronic myeloid leukemia treated with imatinib for more than 5 years and approximately half of all first-line treated patients have stable undetectable BCR-ABL using strict sensitivity criteria. *Clin Cancer Res*. 2007;13(23):7080-7085.
18. Rousselot P, Huguet F, Rea D, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood*. 2007;109(1):58-60.
19. Foroni L, Gerrard G, Nna E, et al. Technical aspects and clinical applications of measuring BCR-ABL1 transcripts number in chronic myeloid leukemia. *Am J Hematol*. 2009;84(8):517-522.
20. Mackinnon S, Papadopoulos EB, Carabasi MH, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood*. 1995;86(4):1261-1268.
21. Dazzi F, Szydlo RM, Craddock C, et al. Comparison of single-dose and escalating-dose regimens of donor lymphocyte infusion for relapse after allografting for chronic myeloid leukemia. *Blood*. 2000;95(1):67-71.
22. Melo JV, Kent NS, Yan XH, Goldman JM. Controls for reverse transcriptase-polymerase chain reaction amplification of BCR-ABL transcripts. *Blood*. 1994;84(11):3984-3986.
23. Score J, Calasanz MJ, Ottmann O, et al. Analysis of genomic breakpoints in p190 and p210 BCR-ABL indicate distinct mechanisms of formation. [Submitted for publication]
24. Jeffreys AJ, Wilson V, Neumann R, Keyte J. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. *Nucleic Acids Res*. 1988;16(23):10953-10971.
25. Melo JV, Yan XH, Diamond J, et al. Reverse transcription/polymerase chain reaction (RT/PCR) amplification of very small numbers of transcripts: the risk in misinterpreting negative results. *Leukemia*. 1996;10(7):1217-1221.
26. Burmeister T, Marschalek R, Schneider B, et al. Monitoring minimal residual disease by quantification of genomic chromosomal breakpoint sequences in acute leukemias with MLL aberrations. *Leukemia*. 2006;20(3):451-457.
27. van Der Velden V, Cazzaniga G, Schrauder A, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. *Leukemia*. 2007;21(4):604-611.
28. van Der Velden V, Hochhaus A, Cazzaniga G, et al. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia*. 2003;17(6):1013-1034.
29. Goldman J, Gordon M. Why do chronic myelogenous leukemia stem cells survive allogeneic stem cell transplantation or imatinib: does it really matter? *Leuk Lymphoma*. 2006;47(1):1-7.
30. Chu S, Xu H, Shah NP, et al. Detection of BCR-ABL kinase mutations in CD34+ cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment. *Blood*. 2005;105(5):2093-2098.
31. Barnes DJ, Melo JV. Primitive, quiescent and difficult to kill: the role of non-proliferating stem cells in chronic myeloid leukemia. *Cell Cycle*. 2006;5(24):2862-2866.
32. Bonifazi F, de Vivo A, Rosti G, et al. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. *Blood*. 2001;98(10):3074-3081.
33. Mahon FX, Delbrel X, Cony-Makhoul P, et al. Follow-up of complete cytogenetic remission in patients with chronic myeloid leukemia after cessation of interferon alfa. *J Clin Oncol*. 2002;20(1):214-220.
34. Verbeek W, Konig H, Boehm J, et al. Continuous complete hematological and cytogenetic remission with molecular minimal residual disease 9 years after discontinuation of interferon-alpha in a patient with Philadelphia chromosome-positive chronic myeloid leukemia. *Acta Haematol*. 2006;115(1-2):109-112.
35. Mattarucci E, Spinelli O, Rambaldi A, et al. Molecular monitoring of residual disease in chronic myeloid leukemia by genomic DNA compared with conventional mRNA analysis. *J Mol Diagn*. 2009;11(5):482-487.
36. Gale RP, Horowitz MM, Ash RC, et al. Identical-twin bone marrow transplants for leukemia. *Ann Intern Med*. 1994;120(8):646-652.
37. Zhang JG, Lin F, Chase A, Goldman JM, Cross NC. Comparison of genomic DNA and cDNA for detection of residual disease after treatment of chronic myeloid leukemia with allogeneic bone marrow transplantation. *Blood*. 1996;87(6):2588-2593.

Table S1. Results of RQ-RT/PCR for *BCR-ABL*, expressed as the % ratio of *BCR-ABL*/*ABL* transcripts, on healthy adult volunteers. Peripheral blood red cells were lysed to isolate the WBC, which were then lysed in guanidinium thiocyanate (GTC), and subjected to RNA extraction, cDNA synthesis (reverse transcription) and real-time PCR amplification on an ABI 7500 Fast thermocycler (Applied Biosystems, Foster City, CA), according to the same protocol used for testing CML patients' samples [Foroni et al, 2009 - Manuscript reference no.19]. All steps of the procedure were carried out in a laboratory entirely separate from that routinely used for MRD testing, under strict conditions to prevent molecular contamination, and in parallel to a series of relevant negative controls. Stored GTC lysate aliquots from 8 individuals (3 positive and 5 negative on the first test), marked with a ♥ symbol on the table, were subjected to a second RNA extraction and cDNA synthesis for repeat of the same test at a different date, yielding essentially the same results, with the exception of individual no. 9 who, on a repeat PCR, scored negative (zero *BCR-ABL* transcripts). The latter case illustrates the inaccuracy of the results at the very low level of sensitivity of the assay (5 *BCR-ABL* transcripts on the first test), probably reflecting a well documented PCR 'sampling effect' [see e.g., Manuscript ref. no. 25].

Person number	BCR-ABL transcripts*	Quantification	
		ABL transcripts*	BCR-ABL/ABL ratio (%)
1 ♥	13	1.40×10^4	0.096
2 ♥	18	2.81×10^4	0.064
3	0	3.35×10^4	0.000
4	0	5.26×10^4	0.000
5	0	7.35×10^4	0.000
6 ♥	0	3.24×10^4	0.000
7 ♥	0	1.22×10^4	0.000
8 ♥	0	2.04×10^4	0.000
9 ♥	5	3.48×10^4	0.013
10	0	1.48×10^4	0.000
11	0	2.70×10^4	0.000
12	0	2.57×10^4	0.000
13 ♥	0	2.10×10^4	0.000
14	0	3.75×10^4	0.000
15	0	3.45×10^4	0.000
16	0	3.82×10^4	0.000
17	0	5.37×10^4	0.000
18	0	9.38×10^4	0.000
19 ♥	0	1.07×10^5	0.000
20	0	2.52×10^4	0.000
21	0	4.28×10^4	0.000
22	0	3.04×10^4	0.000
23	0	3.05×10^4	0.000
24	0	4.65×10^4	0.000

* Average of 3 identical replicate PCR reactions.

Table S2. Patient-specific RQ-PCR primers and probes for *gBCR-ABL* amplification. Each patient is identified by a unique patient number. All sequences are 5' to 3'. The probes were FAM-labelled, with an MGB non-fluorescent quencher.

Pt N°	Forward Primer	Probe	Reverse Primer
11	GTCGAGCTGGATGGATACTACTTTT	CCTTTCCTATCTTTAATATCC	GCAGAGAGACTGAGGAACTGTTC
12	GTCACTGGTTTGCCTGTATTGTG	CTGGATCCTACCCCTTAACC	TCGGTAATTCACCCAAGGGACTA
16	GCAGGTGGATCGAGTAATTGCA	CCAGTCTCTTGCCAAACC	AGAAAATTGCATAATTTGGACCTCTCACT
17	CCACAGCAGAGCAGATTTGG	CTGCTCTGCACATTAAA	GCAATACCTGGCACCACATACA
18	CCCTAGCCTGTCTCAGATCCT	CATTATGGGCAGCTCACC	AACCTTTAGGAACTGTTTCTCTGAAAGAG
19	CCCGGGACAACAGAAGCT	CAAGACTGCAAGAGATCA	CTCTGCAAAGAGGTGTGTGTGA
20	TGGTAACACATGAGTTGCACTGT	TTCAGTATCATTAGTCAACTGCC	GTCACCAAAGATGAAATACAAATGGCTAA
24	CTCCTCTCCTCCAGCTACCT	CCAGCAGTAGTGAGCATC	CAACTGTGGCACCTGACACT
26	CATGAGGTGCTGGTGTTTAC	CAGACCACAATTAGGAATAC	GGTGCATCAGTGCCGTAAC
30	GCCTGTATTGTGAAACCAACTGGAT	ATGGCCCCGGCTGTG	AGTACCTCATTCTCACCAAGTTCTGA
33	GGCTCTATGGGTTTCTGAATGTCAT	CCACTCGAAGAAATC	ACTTCTTTGTGACCTTAGGGTAGACA
36	CATGAGGTGCTGGTGTTTAC	CCACAATTAGGTGTTAAGACCA	GTGGAAGCAATAGAACTCAAATGCA
37	CCTTAACTCTTTGCCCCATAGTACA	CACTACAGGCAGCCATC	GGAGTGCAATGCTGCTGATAGTT
41	GGAGTGTTTGTGCTGGTTGATG	CTGGGTGTGGACAGACT	TCGTGGTTTACATCCCTTTGTTTCT
45	ACAGAAAGCTTACCAGGGATTGTTG	ACTAACTACACACACCTTTTC	TTGGTAAACCCTCAGCAAACTAACT
46	GCTTGTTAGGGCCTCTTGTCT	CCCGACACATTCCTATGTCC	GCCATTTTAATCTTTTACCCCCTAGTCA
47	GCCTGTATTGTGAAACCAACTGGAT	CCTGAGATCCAAGGAAATT	CCCCTTGTGCAATGTTCTGATT

Figure S1. Patterns of residual disease found in 3 additional IM-treated

patients achieving CMR. On the X axis are months on IM. The results of the quantitation of *BCR-ABL* transcripts (dashed line with black squares) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (Y axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: black – *gBCR-ABL*-positive; white – *gBCR-ABL*-negative. In the 3 patients depicted, *gBCR-ABL* was initially detected in transcript-negative samples but became undetectable with continuing IM therapy.

Figure S2. Patterns of residual disease found in 2 additional patients tested

early after SCT, at relapse and post-DLI. On the X axis are months post-SCT. The results of the quantitation of *BCR-ABL* transcripts (dashed line with black squares) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (Y axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: black – *gBCR-ABL*-positive; white – *gBCR-ABL*-negative. *gBCR-ABL* was detected in a transcript-negative samples pre-relapse and at relapse; both patients achieved *gBCR-ABL*-negativity post-DLI.

Figure S3. Patterns of residual disease found in 7 additional patients in

LTR post-SCT/DLI. On the X axis are months post-SCT/DLI. The results of the quantitation of *BCR-ABL* transcripts (dashed line with black squares) are expressed as the ratio of *BCR-ABL* to *ABL* mRNA (Y axis, in a logarithmic scale). The results of the RQ-PCR for *gBCR-ABL* are represented as diamonds: black – *gBCR-ABL*-positive; white – *gBCR-ABL*-negative. All 14 samples tested, 7 of which were transcript-positive, were *gBCR-ABL*-negative.

Figure S4. Amplification plot of the serial dilutions of pre-SCT gDNA and

of the only *gBCR-ABL*-positive sample from a patient in long term remission (LTR) post-SCT. Circles 0, -1, -2 and -3 enclose the cycle threshold (Ct) of the serial 10-fold dilutions of pre-SCT gDNA – from 100 (1ng/μL) to 10⁻³ (1pg/μL). The 10⁻³

dilution was tested in 6 replicate reactions; previous dilutions were tested in triplicate. The average Ct of each dilution was used to calculate a standard curve. Included in circle -3 is also the amplification plot of the single sample from a patient in LTR post-SCT that was positive – 1 of 36 replicates, Ct – 40.7. Thus, circle -3 highlights the close parallel of the gBCR-ABL positive sample with the limit of sensitivity of the standard curve; and the wider variation in Ct seen at the limiting dilution of pre-SCT gDNA. We did not report as positive samples with a $Ct \geq 45$, nor samples with a $Ct \geq 3$ cycles above the highest Ct of the 10^{-3} dilution of the standard curve.

Figure S1

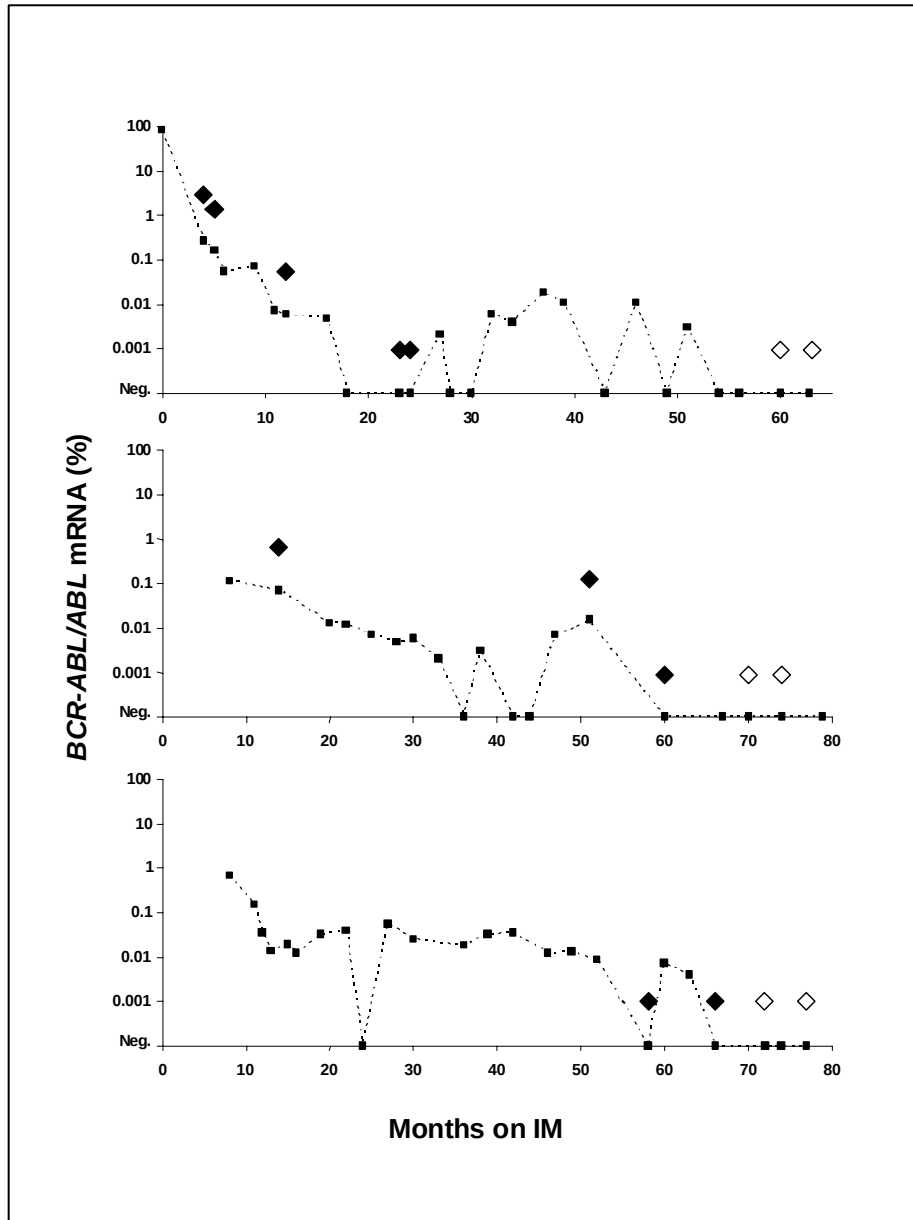


Figure S2

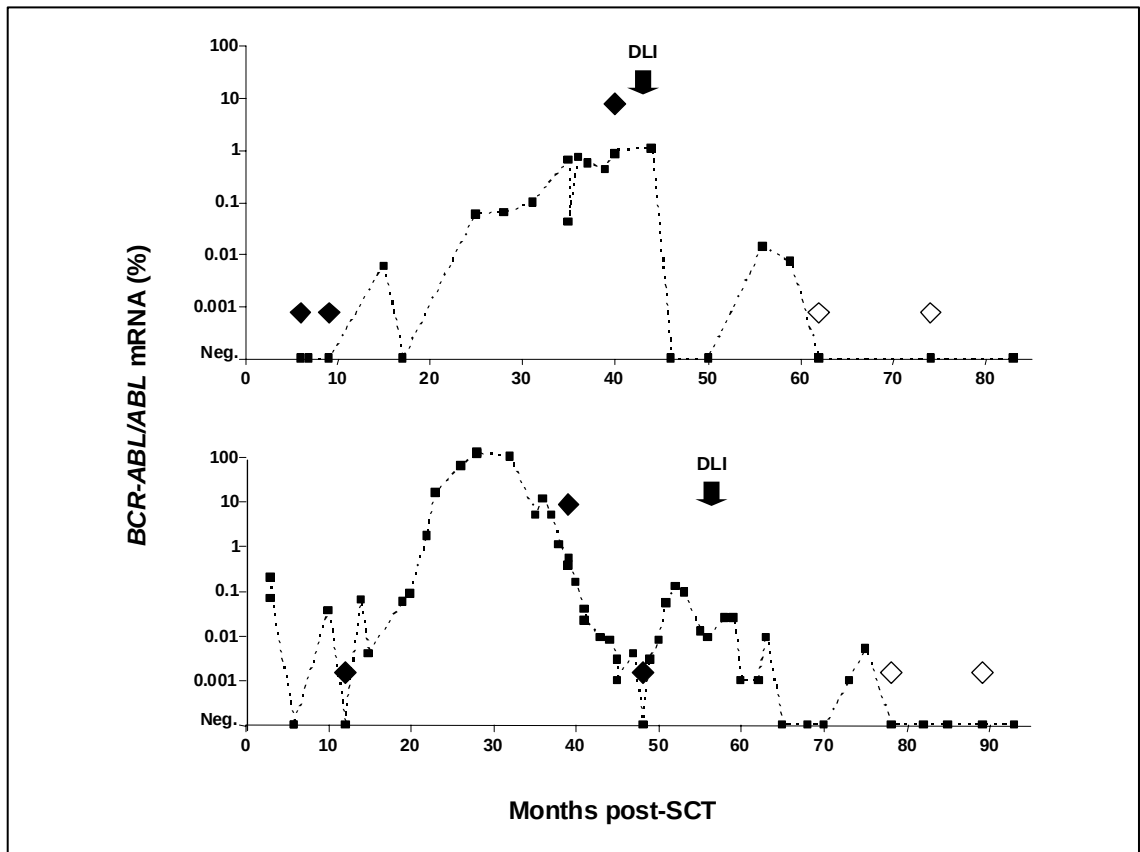


Figure S3

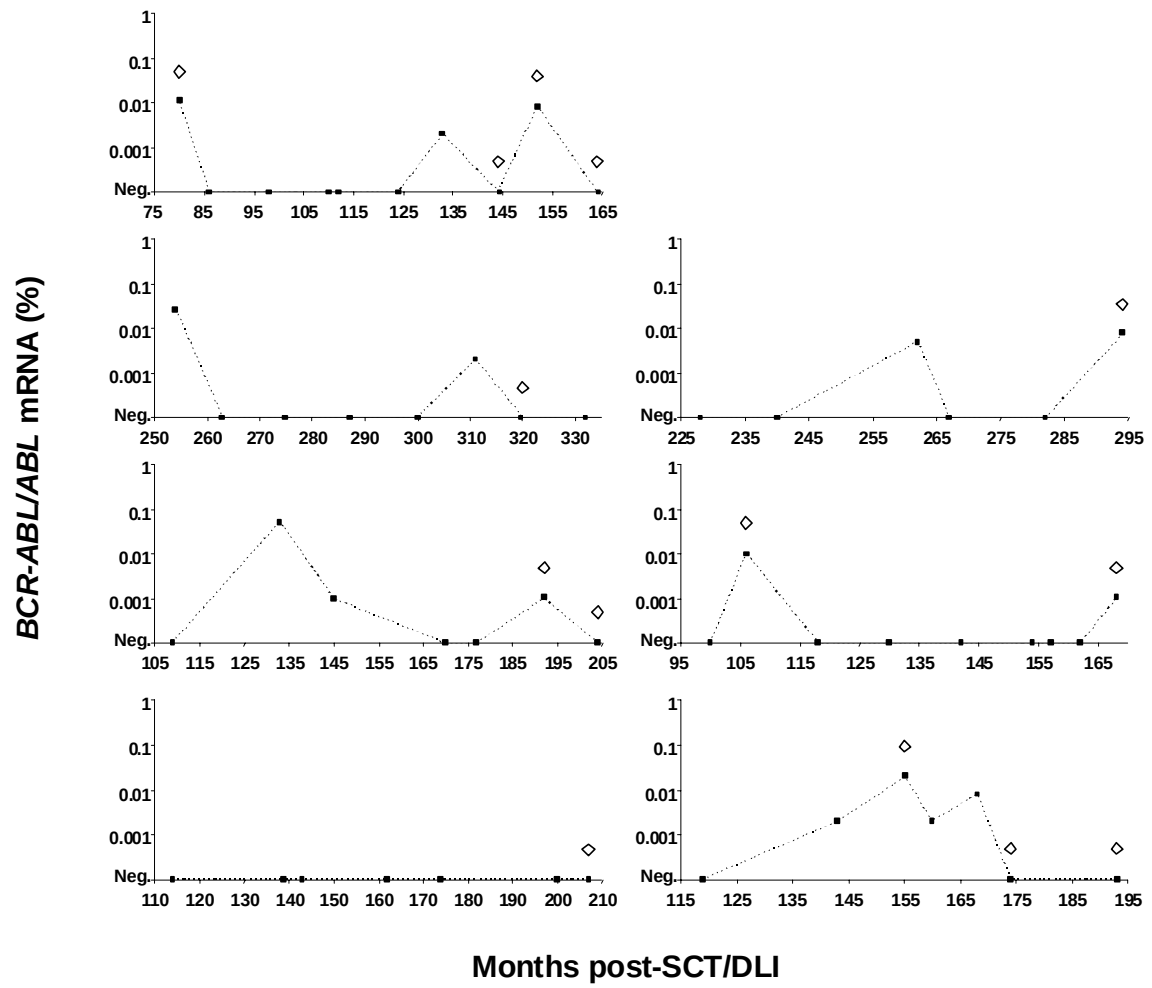
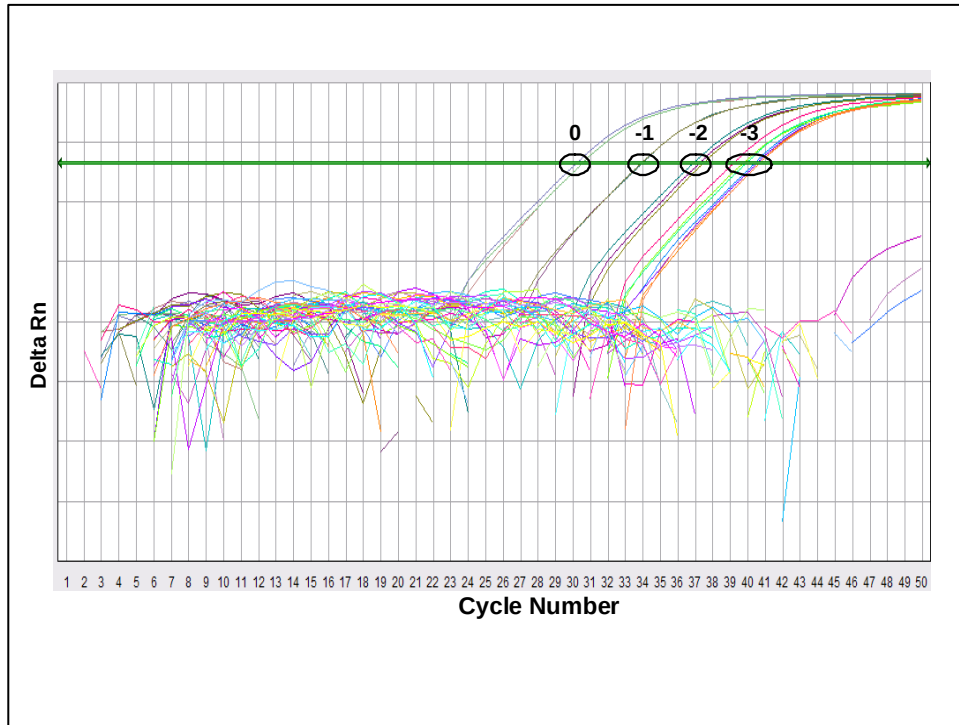


Figure S4



ORIGINAL ARTICLE

Analysis of genomic breakpoints in p190 and p210 BCR–ABL indicate distinct mechanisms of formation

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We sought to understand the genesis of the t(9;22) by characterizing genomic breakpoints in chronic myeloid leukemia (CML) and BCR–ABL-positive acute lymphoblastic leukemia (ALL). BCR–ABL breakpoints were identified in p190 ALL (n=25), p210 ALL (n=25) and p210 CML (n=32); reciprocal breakpoints were identified in 54 cases. No evidence for significant clustering and no association with sequence motifs was found except for a breakpoint deficit in repeat regions within BCR for p210 cases. Comparison of reciprocal breakpoints, however, showed differences in the patterns of deletion/insertions between p190 and p210. To explore the possibility that recombinase-activating gene (RAG) activity might be involved in ALL, we performed extra-chromosomal recombination assays for cases with breakpoints close to potential cryptic recombination signal sequence (cRSS) sites. Of 13 ALL cases tested, 1/10 with p190 and 1/3 with p210 precisely recapitulated the forward BCR–ABL breakpoint and 1/10 with p190 precisely recapitulated the reciprocal breakpoint. In contrast, neither of the p210 CMLs tested showed functional cRSSs. Thus, although the t(9;22) does not arise from aberrant variable (V), joining (J) and diversity (D) (V(D)J) recombination, our data suggest that in a subset of ALL cases RAG might create one of the initiating double-strand breaks.

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Keywords: BCR–ABL; breakpoints; RAG

Introduction

Chromosomal translocations that produce oncogenic fusion genes are common in hematological malignancies, but the mechanism by which they are formed is incompletely understood. Broadly there are three non-mutually exclusive factors that are believed to be relevant to this process: (i) biological selection for specific gene fusions or deregulated gene expression,¹ (ii) susceptibility of particular chromosomal regions to breakage² and (iii) the opportunity to recombine due to physical proximity.³ Analysis to date has indicated that translocations in leukemia result from non-homologous end joining (NHEJ) following two double stranded DNA breaks; homologous recombination does not have a significant role.^{4,5}

The paradigm for translocations in leukemia is the Philadelphia (Ph) chromosome, the smaller derivative of the t(9;22).⁶ The resultant BCR–ABL fusion gene is the defining marker of chronic myeloid leukemia (CML).⁶ In CML, the breakpoints in the majority of patients occur within the 5.8 kb major breakpoint cluster region (M-BCR) in the middle of BCR, resulting in a p210 BCR–ABL protein. The Ph chromosome is also found in 30% of adult acute lymphoblastic leukemia (ALL), 3–5% of childhood ALL and 1% of acute myeloid leukemia.⁶ In contrast to CML, only about 30–50% of patients with Ph-positive ALL harbor the p210 fusion protein, with the remaining 50–70% being characterized by the smaller p190 fusion protein resulting from breakpoints in the BCR minor breakpoint cluster region (m-BCR) within the 72 kb BCR intron 1. Within ABL, most breaks fall within the 140 kb region between exon 1b and exon 2, however, in CML breaks upstream of exon 1b and between exons 2 and 3 also occur in some cases (Figure 1). Despite this knowledge, the precise mechanism for the formation of the BCR–ABL fusion gene remains largely unknown. One of the reasons for this is that only a small number of genomic fusion junctions have been fully characterized, particularly for p190 for which only 2 breakpoints have been fully sequenced.^{7,8} Nevertheless, studies in CML have noted that BCR–ABL breakpoints are often associated with repeat regions.^{5,6,9,10} Also, it has been observed that BCR and ABL tend to be in close proximity compared with control genes during cell division in hematopoietic stem cells, potentially providing the opportunity for aberrant recombination should breakage occur.³

The mechanisms behind many chromosomal translocations in lymphoid malignancies are clearer and are believed to be the result of the aberrant activity of the machinery for V(D)J or class switch recombination.^{11,12} In V(D)J recombination, which takes place early in B-cell differentiation, the recombinase-activating gene (RAG) enzyme complex recognises specific recombination signal sequences (RSSs) flanking the V(D)J segments. RAG makes specific double stranded breaks that are subsequently ligated by NHEJ into a coding joint and a signal joint. The RAG complex can occasionally mistarget recombination to cryptic RSSs and juxtapose proto-oncogenes, such as LMO2 and HOX2, to loci that encode antigen receptors.^{13–15} It is also possible for two non-antigen receptor loci to be joined erroneously by illegitimate V(D)J recombination, for example the SIL and SCL genes in some patients with T-ALL.^{12,16} In addition, in some B-cell lymphomas associated with Immunoglobulin H translocations, breakpoint regions

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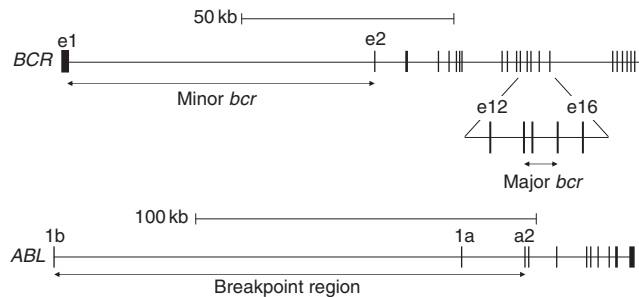


Figure 1 Translocation breakpoint clusters in the *ABL* and *BCR* genes (to scale).

adopting a non-B form might be mistargeted by RAG even in the absence of RSSs.¹⁷

In CML it is thought that the transforming event takes place in a multipotent stem cell, inferred from the range of cell lineages that have been shown to be Ph or *BCR-ABL* positive.¹⁸ In Ph-positive ALL, it is debated whether the acquisition of the Ph chromosome occurs in a multipotent stem cell,^{19–21} a committed lymphoid progenitor^{22–24} or whether both are possible.²⁵ It has been suggested that p190 *BCR-ABL* might specifically direct lymphoid development, however, this fusion is seen occasionally in CML,²⁶ and *in vivo* experiments have shown that both p210 and p190 can give rise to CML or B-ALL depending on whether stem cells or committed progenitor cells are transduced.²⁷ It is possible, therefore, that the rarity of p190 in human CML is not a direct consequence of the activity of this fusion, but rather that *BCR* intron 1 breaks may be much more frequent in ALL because they are formed by a lymphoid-specific mechanism.²⁷ Such a mechanism could be the action of RAG, which is only present in lymphoid-restricted cells.

In this study we sought to characterize and compare breakpoint sequences in p210 CML, p210 ALL and p190 ALL to help clarify the mechanism giving rise to the t(9;22) in ALL. We aimed specifically to test the hypothesis that RAG-mediated recombination may have a role in the genesis of p190 *BCR-ABL*.

Materials and methods

Patient samples

Patients were randomly selected for analysis on the basis of sample availability (CML, $n=32$; p210 ALL, $n=32$; p190 ALL, $n=43$) from centers in the UK, Spain, Germany and Italy. The presence of e13a2 and/or e14a2 (p210) or e1a2 (p190) *BCR-ABL* fusion mRNA was determined by reverse transcriptase-PCR.²⁸ For samples with limited DNA, multiple displacement amplification (GenomiPhi, GE Healthcare, Amersham, UK) was undertaken according to the manufacturer's instructions. The study was approved by the Internal Review Boards from participating institutions and informed consent was provided according to the Declaration of Helsinki.

Detection of forward and reciprocal breakpoints

To sublocalise p190 *BCR-ABL* genomic breakpoints, fluorescence *in situ* hybridization was performed in some cases using fosmid clones selected from the Ensembl database (<http://www.ensembl.org>) and obtained from the Sanger Institute (Hinxton, UK). The clones spanned *BCR* intron 1 and *ABL* exon 1b to a3 (clones E11, E8, G4, F1, D5; Supplementary Figure S1).

Fosmid clones were labelled and hybridized as previously described²⁹ after validation on normal control samples.

For amplification of p190 and p210 breakpoints by long template PCR (LT-PCR) all primers were designed using Primer3 (<http://frodo.wi.mit.edu/primer3/>), checked to be free of single-nucleotide polymorphisms (<http://genome.ucsc.edu/>) and tested on normal control DNA with a suitable reverse primer. To amplify breakpoint bands up to 12 kb in size, the Expand Long-Template LT-PCR system 2 (Roche, Burgess Hill, UK) was used with an annealing temperature of 64 °C and an elongation time of 8 min. Initially, a simplex (standard two primer) PCR protocol was used to detect both p190 and p210 breakpoints, however, a multiplex PCR strategy was later implemented, whereby every *BCR* forward primer was mixed with 5 *ABL* reverse primers (Supplementary Table S1). All breakpoint junctions amplified by LT-PCR were confirmed by reamplification from genomic DNA and sequencing across the breakpoint using an Applied Biosystems 3100 (Foster City, CA, USA). The reverse *ABL* primers were the same for the p210 and p190 screening, but the forward *BCR* primers were different. We attempted to identify reciprocal (*ABL-BCR*) breakpoints in all cases with characterized forward (*BCR-ABL*) breakpoints using *ABL* forward and *BCR* reverse primers approximately 2–4 kb either side of where the break was estimated to be. If no product was amplified after multiple attempts with multiple primer combinations, multiplex ligation-dependent probe amplification (MLPA) was used to detect large deletions, as previously described.³⁰ All nucleotide positions refer to NM_004327 and NM_007313 for *BCR* and *ABL*, respectively, with the A from the ATG start codon considered as position 1.

Statistical analysis

The Kolmogorov–Smirnov test was carried out to test for differences between the distributions of breakpoints in the different sub-types of *BCR-ABL*-positive leukemia. Scan statistics were used to detect any clustering of breakpoints and the Silverman's smoothed bootstrap test was then used to provide evidence for the numbers of clusters. The proximity of breakpoints to either known or unknown sequence motifs within 50 bp either side of the breaks were tested for by comparing the observed breakpoints to 200 randomly generated breakpoints.³¹

In silico analysis of *BCR-ABL* forward breakpoints for cryptic RSSs

Before functional analysis, the EMBOSS Fuzznuc tool (<http://bioweb.pasteur.fr/seqanal/interfaces/fuzznuc.html>) was used to analyze all CML and ALL breakpoints to select the most likely candidates for illegitimate RAG activity. The search was configured using the consensus heptamer (5'-CACAGTG-3') and nonamer (5'-ACAAAAACC-3') sequences separated by 12 or 23 nucleotides. The CAC in the heptamer had to be present and up to nine mismatches were allowed in the remaining heptamer and nonamer sequences.^{32,33}

Functional analysis of breakpoints to determine illegitimate RAG activity

The recombination plasmids used for the extra-chromosomal recombination assays have been described previously.³⁴ Briefly, the two fragments to be tested for V(D)J recombination are separated by a transcription stop sequence (oop). The P_{tac} promoter will transcribe the chloramphenicol acetyltransferase gene when transformed into *E.coli* only when the stop signal has

Initially we pursued a fluorescence *in situ* hybridization strategy to sublocalize the breakpoints. Five fosmids were isolated (two within *BCR* intron 1 and three within *ABL* intron 1) that were used in pairwise combinations to identify the approximate breakpoint positions in five cases (Supplementary Figure S2). This information was then used to direct LT-PCR screening, resulting in the successful identification of forward breakpoints for all five cases (#67, #68, #69, #70, #71).

For many cases suitable cytogenetic material was not available and so we designed PCR screens on the basis of the strategy that had been successfully used for p210 cases. Initially a simplex LT-PCR screen was performed by combining 10 *BCR* forward spanning intron 1 and 10 *ABL* reverse primers (all *ABL* primers used were the same as for p210 screening). This screen consisted of 100 individual reactions per case and successfully amplified the breakpoints in 4 of 13 cases tested. An additional 10 *ABL* primers were designed and a multiplex LT-PCR strategy similar to p210 screening was implemented. The multiplex amplified one of nine cases that tested negative with the simplex screen and a further 9 of 25 cases, yielding a total of 19 breakpoints from 43 cases. All 19 p190 breakpoints within the *BCR* fell within the second half of intron 1 despite the fact that primers were equally spaced along the entire intron. To focus more specifically on this region, a further 11 *BCR* primers were designed and, in addition, 5 *ABL* primers upstream of exon 1b were also included. Inclusion of these primers in the multiplex assay resulted in the identification of six new breakpoints. In total, therefore, 25/43 (58%) of the p190 breakpoints were successfully amplified (Supplementary Table S4). A summary of the breakpoint analysis for all cases is shown on Table 1.

Statistical analysis of forward breakpoints

In addition to the 82 *BCR*-*ABL* breakpoints found in this study, an additional 18 p210 CML forward breakpoints (Supplementary Table S5) that had been identified by the same LT-PCR multiplex technique at a second center were used for the statistical analysis. No significant differences in the distribution of forward breakpoints between the different subtypes of leukemia or mRNA fusion were seen (Supplementary Table S6). Similarly no breakpoint clusters were observed, although some breakpoint preferences were apparent that approached statistical significance. For p210 ALL, two distinct broad clusters were seen, whereas for p210 CML these were less distinct (Figure 3a). There was no clustering of p190 *BCR* breakpoints although, as noted above, it is striking that they all fell in the second half of *BCR* intron 1 (Figure 3b). Within *ABL* there was a marginal excess of breakpoints towards the 3' end of the breakpoint cluster region for p190 ALL, whereas for p210 cases there were two broad clusters that were more pronounced when all cases were considered together (Figure 3c).

In addition to clustering, the distributions of the breakpoints in *BCR* were directly compared with those in *ABL* for all p210 and p190 cases, however, no significant difference was found

(see Supplementary Figure S3). To check whether the subsets of breakpoints were associated with known sequence motifs, including functional RSSs, the frequencies of specific sequence motifs (Supplementary Table S7) within 50bp of sequence

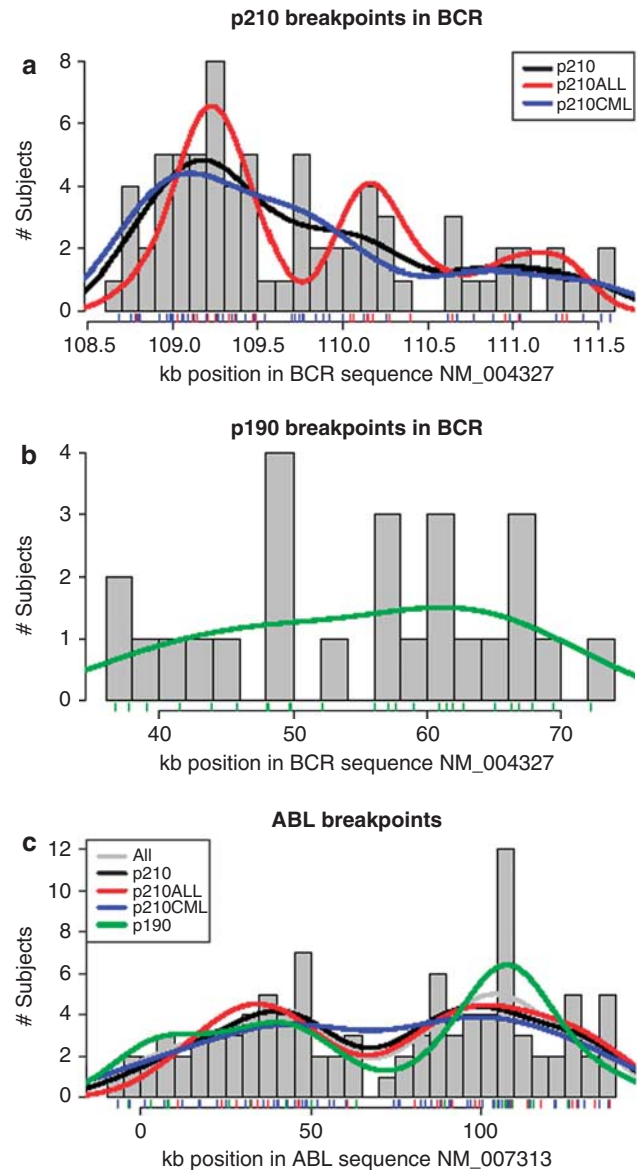


Figure 3 Evidence for weak clusters in *BCR* and *ABL*. Breakpoint positions are shown grouped into 30 intervals (vertical bars) and density curves were determined by using the Sheather-Jones solve-the-equation bandwidth method with the peaks indicating clustering. (a) p210 *BCR* breakpoints ($n = 75$). (b) p190 *BCR* breakpoints ($n = 25$). (c) *ABL* breakpoints ($n = 100$).

Table 1 Summary of breakpoint analysis

	No. of cases analyzed	Forward breakpoints identified number (%)	Reciprocal breakpoints identified number (%)	Large deletions detected by MLPA No. found/no. tested
CML	32	32 (100)	21 (66)	5/9
p210 ALL	32	25 (78)	12 (48)	5/7
P190 ALL	43	25 (58)	21 (84)	1/2

Abbreviations: ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; MLPA, multiplex ligation-dependent probe amplification.

flanking the breakpoints were compared with those observed in the 50 bp flanking random breakpoints sampled from the entire BCR or ABL sequences. Only one significant association was found: a deficit of BCR breakpoints falling in repeat regions for p210 CML and all p210 cases considered as a single group (Supplementary Figure S4). Undirected searches were also performed to see whether any unknown motifs were enriched 50 bp either side of the breakpoints. The only consistent result generated by this method was an association of CTGNNTTG with the BCR breakpoints in p210 ALL, however this motif has no known function and so the significance of this finding is unclear.

Structure of forward and reciprocal junction sequences for p210 and p190 cases

Short stretches of microhomology (2–4 bp) at the forward breakpoints were found in 7/25 (28%) p190, 5/25 (20%) p210 ALL and 10/32 (31%) p210 CML. In addition, 2 p210 CMLs (#8 and #10) cases had microhomologies of 11 and 17 bp, respectively. To identify reciprocal breakpoints, LT-PCR was used to screen all 82 cases in which forward breakpoints had been found. This yielded 54 (66%) positive bands that were sequenced and confirmed. Of these, 21 were from cases with p210 CML (21/32; 66%), 12 with p210 ALL (12/25; 48%) and 21 with p190 ALL (21/25; 84%). Table 2 shows examples of

different types of reciprocal breakpoints and Supplementary Table S8 shows all the results. No products were obtained for the remaining 28 cases despite repeated attempts with multiple primer combinations.

Just seven cases had perfectly balanced translocations: three with p190 (#73, #74, #78), two with p210 ALL (#49, #53) and two with p210 CML (#2, #10). All other cases showed deletions, duplications or insertions at the breakpoints. Of the remaining 47 cases, 8 showed duplication of sequence derived from either or both genes (indicating staggered breaks incompatible with RAG-mediated activity), 28 had deletions and 11 had both duplications and deletions. The median number of duplicated nucleotides was 4 bp for the p190s ($n=7$, range 3–7 bp), 3 bp for the p210 ALLs ($n=5$, range 2–1000 bp) and 173 bp for the p210 CMLs ($n=7$, range 1–534 bp). The median size of deletions was 4 bp for p190s ($n=15$, range 1–27 bp), 24 bp for the p210 ALLs ($n=8$, range 1–7883 bp) and 29 bp for p210 CMLs ($n=16$, range 1–985 bp). All additional sequences at the reciprocal breakpoints could have been derived by duplication of neighboring sequences. In addition, two cases had insertions of unrelated sequence in their forward fusions. Case #65 (p190) had a complex rearrangement with an insertion of approximately 3 kb from chromosome 11. This was consistent with the karyotype that revealed a three-way translocation t(9;22;11)(q34;q11;p11), as well as several other abnormalities. The second case (#48; p210 ALL) had an

Table 2 Forward and reciprocal breakpoints for six p190 ALL cases. Short regions of homology are shown in bold, BCR-derived sequences are underlined and deleted sequences are shown as dots

#58	Duplication within one gene
BCR	<u>GGGACTCGTGTGGGGACAAGCTGAACAGTGTGCTGATCTCTGATCACC</u> <u>ACTCAAGCCACAAGTG</u>
BCR-ABL	<u>GGGACTCGTGTGGGGACAAGCTGAACAGTGTG</u> TTAG CAGCCTAAAATGGTGGCTTTACCAGTTT
ABL	AAAAGTAAGCTGTTGTTACTTTAAGCAAATAT TTAG CAGCCTAAAATGGTGGCTTTACCAGTTT
ABL-BCR	AAAAGTAAGCTGTTGTTACTTTAAGCAAATAT TTAG ATCTCTGATCACCCTCAAGCCACAAGT
#62	Duplication within one gene, deletion within the other
BCR	<u>GTTCTGCCACCTGCCCTTTGAGGCTGTG</u> TGGG CACGGTTCCTCTAGAGCTCCATGTCTAGCAG
BCR-ABL	<u>GTTCTGCCACCTGCCCTTTGAGGCTGTG</u> TGGG AGTGAGATCTTACCTCTACAGAAATTTAAAAA
ABL	AGGTCAGGAGTTTCAACCCAGCTGTGCAATCTAGTGAGATCTTACCTCTACAGAAATTTAAAAA
ABL-BCR	AGGTCAGGAGTTTCAACCCA TGGG CACGGTTCCTCTAGAGCTCCATGTCTAGCAG
#64	Deletion within both genes
BCR	<u>CTTTGCAGGAAGGATGTGGCTGATGAGACCATCAACCTCAGTCCTGCCAAGGGTGAGGCGGCCC</u>
BCR-ABL	<u>CTTTGCAGGAAGGATGTGGCTGATGAGACCATGCCCAGTTAGTGATTTTTAAATTGTAGTTCCCT</u>
ABL	TCTAGGGTTACAGGCATGAGCTACTGTGCCTGGCCAGTTAGTGATTTTTAAATTGTAGTTCCCT
ABL-BCR	TCTAGGGTTAC CAGTCCTGCCAAGGGTGAGGCGGCCC
#70	Identical short stretches of homology, deletion of 1bp in one gene
BCR	<u>TCGTCTTCAGATAAACAGCAAAACCGTTGG</u> TTAA AGCTGGTCTAACCCACCTTATTTTGTGTGT
BCR-ABL	<u>TCGTCTTCAGATAAACAGCAAAACCGTTGG</u> TTT TACAAGACTATTTTTAGCCTTTTTTACAAGA
ABL	GAAAGGGATTGCTGTTTTTTATTACAGAT TTT TACAAGACTATTTTTAGCCTTTTTTACAAGA
ABL-BCR	GAAAGGGATTGCTGTTTTTTATTACAGA . TTAA AGCTGGTCTAACCCACCTTATTTTGTGTGT
#72	Deletion within one gene
BCR	<u>TCCTTTTGAAGGTCATTGTTTTCTTAAACAGATGGTTATTTGGACTCTTACAAGTTCTCTGTA</u>
BCR-ABL	<u>TCCTTTTGAAGGTCATTGTTTTCTTAAACAGAGCAATCCAGGGCTTTAATATCCTTTTTGTA</u>
ABL	GTCTCAAAAAAAAAAGAAAAGAAAATTAGAACTCAATCCAGGGCTTTAATATCCTTTTTGTA
ABL-BCR	GTCTCAAAAAAAAAAGAAAAGAAAATTAGAACT . . <u>TTATTTGGACTCTTACAAGTTCTCTGTA</u>
#74	No deletion, duplication or insertion
BCR	<u>GGGGAAGTGGTTCCCGAGGAAGGAGGCAGAGGAGTGATGATTTTGTAGAGAAGGCTAAGAGAGG</u>
BCR ABL	<u>GGGGAAGTGGTTCCCGAGGAAGGAGGCAGAGGATCTTCAGATATCTAAAAGAACTTTGTAAACA</u>
ABL	GGGGATGTTTCTGTTGTTGCTCCTTGCTGAGTATCTTCAGATATCTAAAAGAACTT . GTAACA
ABL-BCR	GGGGATGTTTCTGTTGTTGCTCCTTGCTGAGTAGTGATGATTTTGTAGAGAAGGCTAAGAGAGG

Abbreviation: ALL, acute lymphoblastic leukemia.

insertion of 57 bp of intronic sequence from the phosphatidylserine decarboxylase gene, downstream of *BCR*. Neither of the reciprocal breakpoints was successfully amplified from these cases.

Deletions of the 9q+ chromosome at the reciprocal breakpoint have been described in about 15% of CML cases.³⁰ To determine whether the failure to amplify reciprocal breakpoints was owing to the presence of large deletions, we performed MLPA analysis for 18 of the 28 of the reciprocal breakpoint-negative cases for whom adequate DNA was available. Eleven cases (CML, $n = 5$; p210 ALL, $n = 5$ and p190 ALL, $n = 1$) showed deletion of at least two probes, indicating large deletions (Table 1; Supplementary Table S8). Seven cases showed no deletion or deletion of just one probe (CML, $n = 4$; p210 ALL, $n = 2$ and p190 ALL, $n = 1$). In addition, distances of the forward breakpoints from CpG and CAC/GTG sequences were analyzed (Supplementary Table S9), but no obvious differences were found between the different subtypes of leukemia.

Functional analysis of breakpoints

To determine whether RAG might have a role in the genesis of the t(9;22) we performed Fuzznuc analysis to identify candidate cRSS sequences close to the breakpoints. In total, 11/25 (44%) p190 ALL, 11/25 (40%) p210 ALL and 12/32 (38%) p210 CML forward breakpoints had candidate cRSSs, in which the start of the forward or reverse cryptic heptamer fell within 10 bp of the breakpoint (Supplementary Table S10). Cases with p190 ALL ($n = 10$), p210 ALL ($n = 3$) and p210 CML ($n = 2$) were selected on the basis of proximity of the cRSS site to the breakpoint, as well as ensuring a mixture of both *ABL* and *BCR* sequences and a mixture of 12 and 23 cRSSs (Supplementary Table S11).

To test whether these candidate cRSS sequences could indeed act as RAG substrates we performed extra-chromosomal recombination assays using all relevant permutations. PCR and sequencing of the resulting colonies revealed that the high level of recombination events seen in the control vectors containing two pairs of authentic RSSs (RS32 and RS39) were indeed mediated by RAG (Supplementary Table S12). In contrast, the majority of colonies derived from *BCR-ABL* plasmids showed evidence of break repair (63% of all breakpoint-derived colonies) rather than RAG-mediated recombination. Of the colonies with potential RAG-mediated events, the great majority were non-specific with 44/49 *BCR-ABL* plasmids (24% of all colonies) using fortuitous RSS sites within the plasmid core DNA. There were a smaller number of non-breakpoint specific V(D)J recombination events (11% of all colonies; 33/49 of *BCR-ABL* plasmids) that arose from other fortuitous sites in either *BCR* or *ABL*. Altogether, this demonstrates the great sensitivity of our recombination substrate assay. Moreover, this indicates that a number of functional cRSS are present at random in a given sequence, including *BCR* and *ABL* breakpoint regions. The fact that such cryptic sites are not usually seen at *BCR-ABL* junctions indicates that standard V(D)J-mediated translocation is not a common event leading to the t(9;22).

Identification of 'specific' cRSSs at the breakpoints in four cases

Two cases, p210 ALL (#34) and p190 ALL (#64) recapitulated the exact forward breakpoint in a minority of colonies in at least two separate transfections. Case #34/RS2 yielded seven colonies (of 37 sequenced; 19%) that used the predicted 12 cRSS to make a signal joint (Figure 4a) with six of these seven having the joint exactly at the breakpoint in *ABL* (the other colony had a signal

joint with a 4 bp deletion in *ABL*). Case #64/RS3 yielded six colonies (of 43 sequenced; 14%) that used the predicted reverse 12 cRSS to make a coding joint. Of the six colonies, three recapitulated the exact forward breakpoint despite the breakpoint being 4 bp upstream of the putative cRSS (Figure 4b and Supplementary Figure S6). Although there is larger than usual resection back into the signal end than might be expected by conventional V(D)J recombination, experiments in cells that lack some components of the NHEJ pathway show differences in the structures of breakpoints, such as larger deletions at the breakpoint.^{35,36} Both of these cases yielded multiple sequence variants in both transfections, usually with the addition of non-templated nucleotides or small deletions around the breakpoint. In addition, case #62/RS8 yielded a signal joint 4 bp upstream of the *BCR* forward breakpoint, but co-incident with the reciprocal breakpoint (Supplementary Figure S5).

Discussion

Although the t(9;22) was the first chromosomal abnormality to be associated with a hematological malignancy,³⁷ the mechanisms behind its formation and indeed those of many primary myeloid malignancies remains elusive. Sequence analysis of myeloid translocation breakpoints has revealed that the majority probably result from NHEJ following DNA breakage,^{4,33,38,39} however, the reason why some fusions are seen very frequently compared with other known or potential fusions is still unclear. By contrast, the mechanisms giving rise to many chromosomal translocations in lymphoid malignancies are believed to be the result of illegitimate lymphoid-specific recombination mechanisms, such as V(D)J^{16,34,40,41} or class switch recombination.^{11,42}

In this study, we aimed to determine whether there were any consistent differences between t(9;22) breakpoints in CML and ALL, and to understand why the p190 fusion is nearly always associated with lymphoid disease. We characterized 82 forward *BCR-ABL* genomic breakpoints and 54 reverse *ABL-BCR* genomic breakpoints using LT-PCR and identified 11 cases with large 9q+ deletions by MLPA (Table 1). The reasons why fewer breakpoints were successfully identified in ALL compared with CML are probably two-fold; (i) the quality and amount of DNA available for some ALL cases was less than that for CML and (ii) for p210 cases we identified several breakpoints by a simplex screen following the failure to amplify fusion junctions by multiplex PCR; for p190 the follow-up simplex screen was not performed because of the very large number of amplifications required for each case.

Statistical analysis did not reveal any differences in breakpoint distribution between the subtypes of leukemia, and only very weak evidence for clustering was observed. For p190, breaks in *BCR* were confined to the 3' end of intron 1, confirming Southern blot studies on a small number of Ph-positive, *BCR-ABL*-positive acute leukemias.⁴³ However, we found no evidence for any clustering within the m-*BCR*, contrary to previous reports that suggested subclustering of these breakpoints in *Alu* repeat regions.^{10,44} Analysis of sequence motifs found one significant association: a deficit of repeat regions in p210 *BCR* breakpoints. This contrasts with other publications that have indicated an association of repeats, particularly *Alu* elements, in both *ABL* and *BCR*.^{5,9} These previous observations, however, were made on small numbers of cases and may have been biased owing to the fact that the methodology used to amplify breakpoints required the presence of specific restriction enzyme recognition sites.

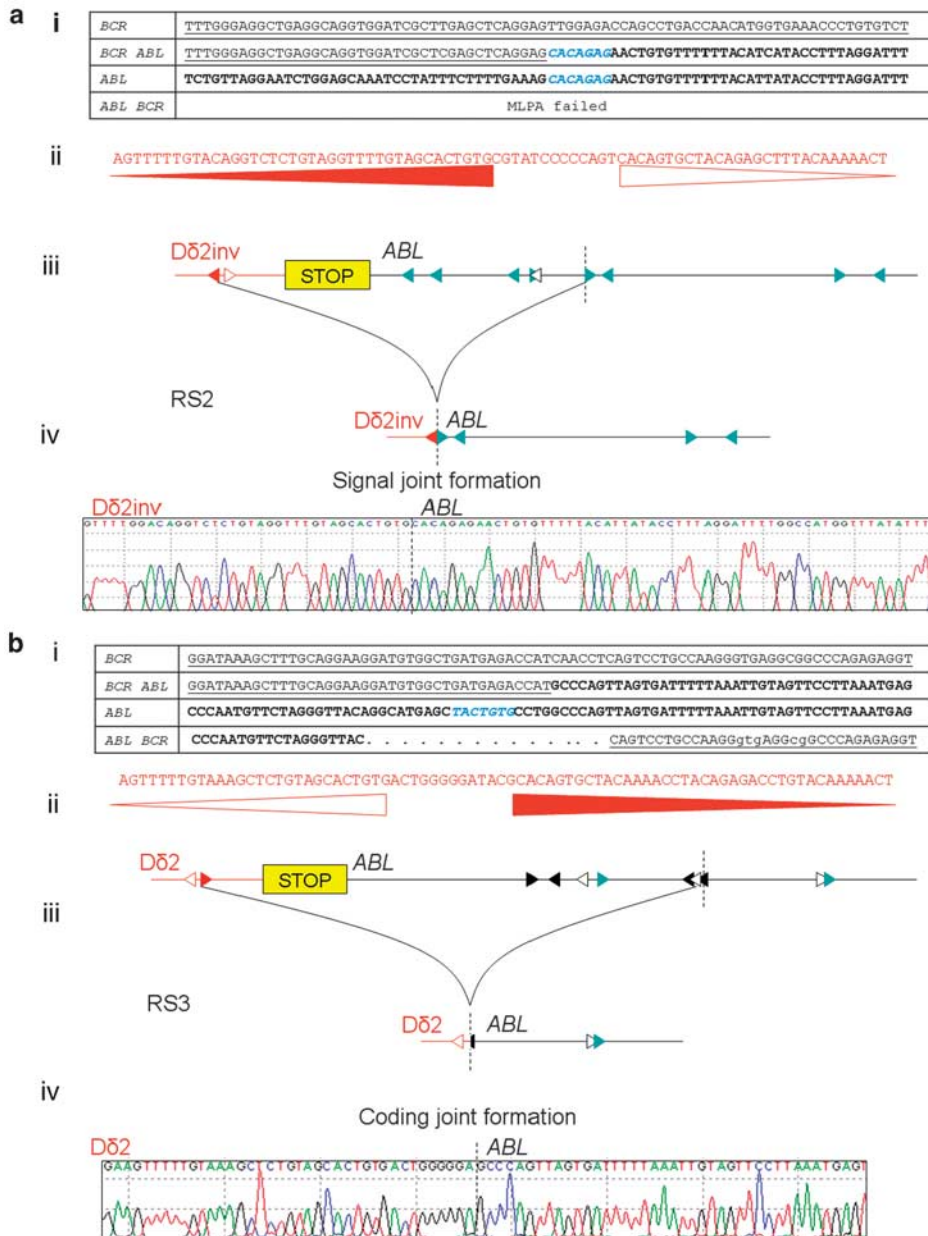


Figure 4 Potential RAG mediated breaks in #34 and #64. **(a)** Case #34 RS2 'specific' V(D)J recombination found in seven colonies from the cell culture assay. **(i)** #34 forward breakpoint sequence, with the putative heptamer in blue italics. **(ii)** Dδ2inv (inverted) authentic RSS sequence from the T-cell receptor alpha gene on chromosome 14q11.2, showing the reverse 23 RSS and the forward 12 RSS. **(iii)** Schematic representation of the signal joint formation, #34 ABL cassette is to scale with all the cryptic RSSs represented by triangles (12 RSSs are white and 12/23 RSSs are green). The authentic RSSs in Dδ2inv are represented by red empty or solid triangles (12 and 23 RSSs, respectively). **(iv)** Electropherogram from colony sequencing with the breakpoint marked by the vertical black line. **(b)** #64 RS3 'specific' V(D)J recombination found in six colonies from the cell culture assay. **(i)** #64 forward and reciprocal breakpoint sequence, with the putative heptamer in blue italics. **(ii)** Dδ2 authentic RSS sequence, showing the reverse 12 RSS and the forward 23 RSS. **(iii)** Schematic representation of the coding joint formation, #64 ABL cassette is to scale with all the cryptic RSSs represented by triangles (12 RSSs are white, 23 RSSs are black and 12/23 RSSs are green). The authentic RSSs in Dδ2 are represented by red empty or solid triangles (12 or 23 RSSs, respectively). **(iv)** Electropherogram from colony sequencing with the breakpoint marked by the vertical black line.

Amplification of the reciprocal breakpoints was more successful in p190 compared with p210 cases (21/25 versus 33/57; $P=0.02$, Fisher's exact test). This is consistent with previously published data generated by fluorescence *in situ* hybridization and reverse transcriptase-PCR showing that p190 cases had a much lower frequency of large 9q+ deletions,^{45,46} compared with the established figure of around 15% in CML.⁴⁷ Fine breakpoint mapping revealed that the sizes of deletions and

duplications at the breakpoint junctions were smaller in p190 compared with p210 cases. Assuming that any LT-PCR-negative/MLPA-negative cases had deletions that were too large to be detected by LT-PCR, but too small for MLPA, the median size of deletions was 6 bp for p190 and 7883 bp for p210. These data suggest that p190 and p210 are formed by different mechanisms. We did not observe any consistent difference between CML and p210 ALL breakpoints, but this is perhaps not

unexpected as an unknown proportion of p210 ALLs may in fact be derived from early transformation of a CML clone. As previously described, we did not find large tracts of homology between ABL and BCR at the breakpoint junctions, but small deletions and duplications suggestive of NHEJ were frequent. Short stretches of microhomology (2–4 bp) at the forward breakpoints were found in 7/25 (28%) p190, 5/25 (20%) p210 ALL and 10/32 (31%) p210 CML. In addition, 2 p210 CMLs (#8 and #10) cases had microhomologies of 11 and 17 bp, respectively.

As p190 is largely restricted to lymphoid disease we considered the possibility that RAG may be involved in generating this fusion. The two hallmarks of V(D)J recombination are the addition of non-templated nucleotides and palindromic sequences, neither of which were observed in our cases. This does not, however, preclude the possibility that one or both breaks in ALL may be accomplished by aberrant RAG activity. We, therefore, searched for potential cRSS signals using previously defined criteria.^{32,33} Because of high degeneracy, these sequences are expected to occur on average every 5000 bp and, therefore, we identified several candidate ALL and CML cases. As it is not possible to predict the functionality of cRSSs on the basis of sequence alone, we selected a representative subset for analysis by the extra-chromosomal recombination assay.³⁴ Although no strong or recurrent RAG recognition sites were observed, it is striking that we found precise recapitulation of the forward breakpoint in two ALL cases multiple times in multiple transfections. Furthermore, one p190 ALL case was identified, with a cut site at the reciprocal breakpoint. By contrast specific cRSSs were not identified in the two CML cases tested.

Taken together, our data support the hypothesis that p190 and p210 BCR-ABL are formed by different mechanisms. In particular the presence of functional cryptic RSS sequences close to some ALL breakpoints suggests the involvement of RAG, which in turn indicates that these fusions must have arisen in a committed lymphoid progenitor cell; however, these were exceptional cases rather than the rule.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

The study was designed by JS, NCPC and FHG. Laboratory analysis was performed by JS, MAS-S, SK, CH-C, DW. Data analysis was performed by JS, RFY, JW, BN. Samples and data were provided by MJC, OO, FP, MAS-S, JVM. The initial paper was written by JS, NCPC and FHG; all authors contributed to the final version.

References

- Deininger MW, Bose S, Gora-Tybor J, Yan XH, Goldman JM, Melo JV. Selective induction of leukemia-associated fusion genes by high-dose ionizing radiation. *Cancer Res* 1998; **58**: 421–425.
- Stanulla M, Wang J, Chervinsky DS, Thandla S, Aplan PD. DNA cleavage within the MLL breakpoint cluster region is a specific event which occurs as part of higher-order chromatin fragmentation during the initial stages of apoptosis. *Mol Cell Biol* 1997; **17**: 4070–4079.
- Neves H, Ramos C, da Silva MG, Parreira A, Parreira L. The nuclear topography of ABL, BCR, PML, and RARalpha genes: evidence for gene proximity in specific phases of the cell cycle and stages of hematopoietic differentiation. *Blood* 1999; **93**: 1197–1207.
- Reiter A, Saussele S, Grimwade D, Wiemels JL, Segal MR, Lafage-Pochitaloff M et al. Genomic anatomy of the specific reciprocal translocation t(15;17) in acute promyelocytic leukemia. *Genes Chromosomes Cancer* 2003; **36**: 175–188.
- Mattarucchi E, Guerini V, Rambaldi A, Campiotti L, Venco A, Pasquali F et al. Microhomologies and interspersed repeat elements at genomic breakpoints in chronic myeloid leukemia. *Genes Chromosomes Cancer* 2008; **47**: 625–632.
- Melo JV. BCR-ABL gene variants. *Baillieres Clin Haematol* 1997; **10**: 203–222.
- van der Feltz MJ, Shivji MK, Allen PB, Heisterkamp N, Groffen J, Wiedemann LM. Nucleotide sequence of both reciprocal translocation junction regions in a patient with Ph positive acute lymphoblastic leukaemia, with a breakpoint within the first intron of the BCR gene. *Nucleic Acids Res* 1989; **17**: 1–10.
- Chen SJ, Chen Z, Font MP, d'Auriol L, Larsen CJ, Berger R. Structural alterations of the BCR and ABL genes in Ph1 positive acute leukemias with rearrangements in the BCR gene first intron: further evidence implicating Alu sequences in the chromosome translocation. *Nucleic Acids Res* 1989; **17**: 7631–7642.
- Jeffs AR, Benjes SM, Smith TL, Sowerby SJ, Morris CM. The BCR gene recombines preferentially with Alu elements in complex BCR-ABL translocations of chronic myeloid leukaemia. *Hum Mol Genet* 1998; **7**: 767–776.
- Chen SJ, Chen Z, d'Auriol L, Le Coniat M, Grausz D, Berger R. Ph1+bcr- acute leukemias: implication of Alu sequences in a chromosomal translocation occurring in the new cluster region within the BCR gene. *Oncogene* 1989; **4**: 195–202.
- Robbiani DF, Bothmer A, Callen E, Reina-San-Martin B, Dorsett Y, Difilippantonio S et al. AID is required for the chromosomal breaks in c-myc that lead to c-myc/IgH translocations. *Cell* 2008; **135**: 1028–1038.
- Tsai AG, Lu H, Raghavan SC, Muschen M, Hsieh CL, Lieber MR. Human chromosomal translocations at CpG sites and a theoretical basis for their lineage and stage specificity. *Cell* 2008; **135**: 1130–1142.
- Marculescu R, Vanura K, Montpellier B, Roulland S, Le T, Navarro JM et al. Recombinase, chromosomal translocations and lymphoid neoplasia: targeting mistakes and repair failures. *DNA Repair (Amst)* 2006; **5**: 1246–1258.
- Roth DB. Restraining the V(D)J recombinase. *Nat Rev Immunol* 2003; **3**: 656–666.
- Brandt VL, Roth DB. Recent insights into the formation of RAG-induced chromosomal translocations. *Adv Exp Med Biol* 2009; **650**: 32–45.
- Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by 'illegitimate' V-(D)-J recombinase activity. *Science* 1990; **250**: 1426–1429.
- Raghavan SC, Swanson PC, Ma Y, Lieber MR. Double-strand break formation by the RAG complex at the bcl-2 major breakpoint region and at other non-B DNA structures *in vitro*. *Mol Cell Biol* 2005; **25**: 5904–5919.
- Fialkow PJ, Denman AM, Jacobson RJ, Lowenthal MN. Chronic myelocytic leukemia. Origin of some lymphocytes from leukemic stem cells. *J Clin Invest* 1978; **62**: 815–823.
- Cobaleda C, Gutierrez-Cianca N, Perez-Losada J, Flores T, Garcia-Sanz R, Gonzalez M et al. A primitive hematopoietic cell is the target for the leukemic transformation in human Philadelphia-positive acute lymphoblastic leukemia. *Blood* 2000; **95**: 1007–1013.
- Schenk TM, Keyhani A, Bottcher S, Kliche KO, Goodacre A, Guo JQ et al. Multilineage involvement of Philadelphia chromosome positive acute lymphoblastic leukemia. *Leukemia* 1998; **12**: 666–674.
- Zaliova M, Fronkova E, Krejickova K, Muzikova K, Mejstrikova E, Sary J et al. Quantification of fusion transcript reveals a subgroup with distinct biological properties and predicts relapse in BCR/ABL-positive ALL: implications for residual disease monitoring. *Leukemia* 2009; **23**: 944–951.

- 22 Bacher U, Haferlach T, Hiddemann W, Schnittger S, Kern W, Schoch C. Additional clonal abnormalities in Philadelphia-positive ALL and CML demonstrate a different cytogenetic pattern at diagnosis and follow different pathways at progression. *Cancer Genet Cytogenet* 2005; **157**: 53–61.
- 23 Turhan AG, Eaves CJ, Kalousek DK, Eaves AC, Humphries RK. Molecular analysis of clonality and bcr rearrangements in Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* 1988; **71**: 1495–1498.
- 24 Hotfilder M, Rottgers S, Rosemann A, Schrauder A, Schrappe M, Pieters R et al. Leukemic stem cells in childhood high-risk ALL/t(9;22) and t(4;11) are present in primitive lymphoid-restricted CD34+. *Cancer Res* 2005; **65**: 1442–1449.
- 25 Castor A, Nilsson L, Astrand-Grundstrom I, Buitenhuis M, Ramirez C, Anderson K et al. Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. *Nat Med* 2005; **11**: 630–637.
- 26 Melo JV, Myint H, Galton DA, Goldman JM. P190BCR-ABL chronic myeloid leukaemia: the missing link with chronic myelomonocytic leukaemia? *Leukemia* 1994; **8**: 208–211.
- 27 Li S, Ilaria Jr RL, Million RP, Daley GQ, Van Etten RA. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J Exp Med* 1999; **189**: 1399–1412.
- 28 Cross NCP, Melo JV, Feng L, Goldman JM. An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. *Leukemia* 1994; **8**: 186–189.
- 29 Baxter EJ, Kulkarni S, Vizmanos JL, Jaju R, Martinelli G, Testoni N et al. Novel translocations that disrupt the platelet-derived growth factor receptor beta (PDGFRB) gene in BCR-ABL-negative chronic myeloproliferative disorders. *Br J Haematol* 2003; **120**: 251–256.
- 30 Kreil S, Pfirrmann M, Haferlach C, Waghorn K, Chase A, Hehlmann R et al. Heterogeneous prognostic impact of derivative chromosome 9 deletions in chronic myelogenous leukemia. *Blood* 2007; **110**: 1283–1290.
- 31 Segal MR, Wiemels JL. Clustering of translocation breakpoints. *Journal of the American Statistical Association* 2002; **97**: 66–76.
- 32 Gellert M. V(D)J recombination: RAG proteins, repair factors, and regulation. *Annu Rev Biochem* 2002; **71**: 101–132.
- 33 Wiemels JL, Leonard BC, Wang Y, Segal MR, Hunger SP, Smith MT et al. Site-specific translocation and evidence of postnatal origin of the t(1;19) E2A-PBX1 fusion in childhood acute lymphoblastic leukemia. *Proc Natl Acad Sci USA* 2002; **99**: 15101–15106.
- 34 Marculescu R, Le T, Simon P, Jaeger U, Nadel B. V(D)J-mediated translocations in lymphoid neoplasms: a functional assessment of genomic instability by cryptic sites. *J Exp Med* 2002; **195**: 85–98.
- 35 Weinstock DM, Jasin M. Alternative pathways for the repair of RAG-induced DNA breaks. *Mol Cell Biol* 2006; **26**: 131–139.
- 36 Weinstock DM, Brunet E, Jasin M. Formation of NHEJ-derived reciprocal chromosomal translocations does not require Ku70. *Nat Cell Biol* 2007; **9**: 978–981.
- 37 Nowell PC, Hungerford DA. Chromosome studies on normal and leukemic human leukocytes. *J Natl Cancer Inst* 1960; **25**: 85–109.
- 38 Reichel M, Gillert E, Nilson I, Siegler G, Greil J, Fey GH et al. Fine structure of translocation breakpoints in leukemic blasts with chromosomal translocation t(4;11): the DNA damage-repair model of translocation. *Oncogene* 1998; **17**: 3035–3044.
- 39 Zhang JG, Goldman JM, Cross NCP. Characterization of genomic BCR-ABL breakpoints in chronic myeloid leukaemia by PCR. *Br J Haematol* 1995; **90**: 138–146.
- 40 Tycko B, Sklar J. Chromosomal translocations in lymphoid neoplasia: a reappraisal of the recombinase model. *Cancer Cells* 1990; **2**: 1–8.
- 41 Raghavan SC, Swanson PC, Wu X, Hsieh CL, Lieber MR. A non-B-DNA structure at the Bcl-2 major breakpoint region is cleaved by the RAG complex. *Nature* 2004; **428**: 88–93.
- 42 Pasqualucci L, Bhagat G, Jankovic M, Compagno M, Smith P, Muramatsu M et al. AID is required for germinal center-derived lymphomagenesis. *Nat Genet* 2008; **40**: 108–112.
- 43 Chen SJ, Chen Z, Hillion J, Gausz D, Loiseau P, Flandrin G et al. Ph1-positive, bcr-negative acute leukemias: clustering of breakpoints on chromosome 22 in the 3' end of the BCR gene first intron. *Blood* 1989; **73**: 1312–1315.
- 44 Chen SJ, Chen Z, Font MP, d'Auriol L, Larsen CJ, Berger R. Structural alterations of the BCR and ABL genes in Ph1 positive acute leukemias with rearrangements in the BCR gene first intron: further evidence implicating Alu sequences in the chromosome translocation. *Nucleic Acids Res* 1989; **17**: 7631–7642.
- 45 Melo JV, Gordon DE, Tuszynski A, Dhut S, Young BD, Goldman JM. Expression of the ABL-BCR fusion gene in Philadelphia-positive acute lymphoblastic leukemia. *Blood* 1993; **81**: 2488–2491.
- 46 Specchia G, Albano F, Anelli L, Storlazzi CT, Zagaria A, Mancini M et al. Deletions on der(9) chromosome in adult Ph-positive acute lymphoblastic leukemia occur with a frequency similar to that observed in chronic myeloid leukemia. *Leukemia* 2003; **17**: 528–531.
- 47 Huntly BJ, Reid AG, Bench AJ, Campbell LJ, Telford N, Shepherd P et al. 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. *Blood* 2001; **98**: 1732–1738.

Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)