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Genomic Instability in Chronic Myelogenous Leukemia

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A thesis submitted to the faculty of Graduate Studies and Research

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of

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Abstract

Chronic myelogenous leukemia (CML) is a disease arising from a reciprocal translocation between chromosome 9 and 22. Chromosome 22 encodes the *Bcr* gene, while chromosome 9 gives rise to the proto-oncogene, *c-abl*. In CML, these two genes form a fusion protein known as Bcr/Abl, which contains a constitutively activated protein tyrosine kinase. Different molecular weights of this protein manifest from this initial translocation, including; a p210kDa weight, which manifests into a CML type of leukemia; a p190kDa weight, which gives rise to an acute leukemia, known as acute lymphocytic leukemia (ALL); and a p230kDa weight, which manifest into a rare form of leukemia, known as chronic neutrophilic leukemia (CNL) CML has an inevitable progression from the chronic to acute phase, in which many secondary abnormalities accumulate. These secondary chromosomal abnormalities suggest that additional genetic events may be critical for progression to the blast crisis. However, it is still unclear as to whether Bcr/Abl is the only genetic event required or if there are other major genetic events required for the transition of this disease. Therefore, the focus of my research has been to address genomic instability as it relates to CML.

Initially, I addressed genomic instability by utilizing Inter Simple Sequence Repeat Polymerase Chain Reaction (Inter-SSR PCR), in combination with primers which consist of a set of eight CA repeats. Using Inter-SSR PCR, I compared $P190^{Bcr/Abl}$ pre-leukemic mice (about 100 days, before onset of leukemia) and $P190^{Bcr/Abl}$ leukemic mice to control mice (BL6/CBA), and found an increased number of insertions and deletions in the pre-leukemic and leukemic mice compared to control. I also showed that the

disruption of a DNA repair pathway. These experiments demonstrate that Bcr/Abl is inducing a mutator phenotype, seen as an increase in altered bands and point mutations, and that this can be reversed with the inhibitor STI571. The results from the $P210^{Bcr/Abl}$ inducible mice confirm previous results seen in the $P190^{Bcr/Abl}$ transgenic mice and reconfirm that Bcr/Abl is required for the induction and maintenance of disease. Finally, these results suggest that Bcr/Abl causes genomic instability seen upon disease progression and that Bcr/Abl induces genomic instability potentially through defects in DNA repair pathways.

Résumé

La leucémie myélogénique chronique (LMC) est une maladie découlant d'une translocation réciproque entre les chromosomes 9 et 22. Alors que le chromosome 22 code pour le gène *Bcr*, le chromosome 9 code pour le proto-oncogène *c-abl*. Dans les cas de LMC, ces deux gènes donnent naissance à une protéine de fusion, connue sous le nom de Bcr/Abl, qui est une protéine kinase constitutivement activée. Différents isoformes de la protéine résultent de la translocation initiale et donnent lieu à différents types de leucémie. L'isoforme de 210kDa de Bcr/Abl génère une LMC, un isoforme de 190kDa entraîne une leucémie aigüe connue sous le nom de leucémie lymphocytique aigüe (LLA) alors qu'un isoforme de 230kDa donne lieu à une leucémie rare nommée leucémie neutrophilique chronique (LNC). La LCM présente une progression inévitable de la phase chronique à aigüe, progression durant laquelle plusieurs anomalies chromosomiques s'accumulent. Ces anomalies chromosomiques suggèrent que l'instabilité génomique est critique pour la progression de la maladie vers la phase blastique terminale. Il est toujours incertain, cependant, si Bcr/Abl est le seul évenement génétique nécéssaire ou s'il y a un autre évenement requispour la transition de la maladie. L'essentiel de mon étude est d'adresser l'instabilité génomique, comme cela survient dans les cas de leucémie myélogénique chronique.

J'ai tout d'abord abordé l'instabilité génomique en utilisant l'Inter Simple Sequence Repeat Polymerase Chain Reaction ou Inter-SSR PCR, réaction amplifiant des séquences bordées de répétitions de CA. À l'aide de l' Inter-SSR PCR, j'ai comparé des souris $P190^{Bcr/Abl}$ pré-leucémiques (environ 100 jours avant le début de la leucémie) et

leucémiques à des souris contrôles (BL6/CBA). J'ai trouvé un nombre accru de bandes altérées (insertions et deletions) à la fois chez les souris pré-leucémiques et leucémiques, comparé au contrôle. J'ai aussi prouvé que la fréquence des bandes altérées pouvait être diminuées par l'administration d'un inhibiteur spécifique de la protéine kinase c-Abl, STI571 (officiellement CGP57148, Novartis). Ensuite j'ai adressé l'instabilité génomique en utilisant les souris transgéniques $P190^{Bcr/Abl}$ (line 623) et le système in vivo de test de mutagenèse "Big Blue" (Stratagene). Mes résultats démontrent une augmentation de 2 à 3 fois du nombre de mutatons ponctuelles chez les $P190^{Bcr/Abl}$ X Big Blue en phase pré-leucémiques, comparées au contrôle (C57/BL6). Afin de voir si la fréquence de ces mutations pouvait être reversée, ces souris furent injectées avec l'inhibiteur STI571, pendant 10 jours consecutifs. J'ai observé une diminution de la fréquence des mutations suivant injection, à la fois au rein et au foie. Final lement, j'ai adressé l'instabilité génomiques à l'aide de souris transgéniques $P210^{Bcr/Abl}$ possédant un système de répression à la tétracycline, toujours en utilisant le système de Inter-SSR PCR. Des souris doublement transgéniques (*BCR/ABL1*-transactivateur de la tétracycline, *tTA*) ont été générées en croisant des femelles trans-répondeur avec des mâles portant le transcctivateur mouse mammary tumor virus (*MMTV*)*tTA*, sous administration continue de tétracycline (0,5g/L), débutant 5 jours avant l'accouplement. L'arrêt de l'administration de tétracycline aux animaux doublement transgéniques permet l'expression de *BCR-ABL1* et résulte en le développement d'une leucémie léthale chez 100% des souris, dans un laps de temps constant pour chaque ligne. Mes résultats démontrent une augmentation des insertions et déletions lors de l'arrêt de l'administration de tétracycline, chez les souris $P210^{Bcr/Abl}$. Ceci a été démontré être réversible, dépendemment du nombre de fois où les animaux ont étés remis au traitement. Final

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Preface

The Guidelines Concerning Thesis Preparation Issued by the Faculty of Graduate Studies and Research at McGill University reads as follows:

> "The candidate has the option, subject to the approval of their department, of including as part of the thesis, copies of the text of a paper(s) submitted for publication, or clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.
>
> If the option is chosen, connecting texts, providing logical bridges between different pages, are mandatory. The thesis must conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in French and English (3) an introduction which clearly states the rationale and objectives of the study, (4) comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final conclusion and/or summary.
>
> Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g. in the appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

I have chosen to write my thesis according to these guidelines, with all four papers submitted for publication. The thesis is organized into 7 chapters: (I) a general introduction and literature review with references, (II-V) manuscripts, each with its own abstract, introduction, methods, results and references, (V) a general discussion of all results with references, and (VI) claims to original research.

Publications Arising from the Work of this Thesis

First-Author Publications

1. **Brain, J.**, Goodyer, N., and Laneuville, P. Measurement of Genomic Instability in Pre-Leukemic P190$^{Bcr/Abl}$ Transgenic Mice Using Inter-SSR PCR. 2001 *Cancer Research* (submitted)

2. **Brain, J.**, Saksena, A., and Laneuville, P. The Kinase Inhibitor STI571 Reverses Bcr/Abl induced Increased Point Mutation Frequencies in Pre-Leukemic P190$^{Bcr/Abl}$ Transgenic Mice. 2001 *Leukemia Research* (submitted)

3. **Brain, J.**, Huettner, C.S., Goodyer, N., Tenen, D.G., and Laneuville, P. Genomic Instabililty in an Induced P210$^{Bcr/Abl}$ Transgenic Mouse Using Inter-SSR PCR Genomic Scanning. 2001 *Blood* (submitted)

4. **Brain, J.**, Yu, Q., Saksena, A., Leyland-Jones, B., and Laneuville, P. Over Expression of DNA Repair Genes in P190$^{Bcr/Abl}$ Pre-Leukemic Mice 2001 *Oncogene* (submitted)

The specific contributions of various authors and others to these manuscripts are as follows:

In manuscript number one, N.G. helped to run some of the gels as well as helping with the data analysis.

In manuscript number two, A.S. helped to make media and prepare new solutions and media plates.

In manuscript number three, C.S.H. gave the kind gift of the P210$^{Bcr/Abl}$ mice as well as the DNA extracted from the blood of these mice. N.G. helped to run some of the gels and also helped with the data analysis.

In manuscript number four, Q.Y, helped by doing all the computer analysis of the array membranes. A.S. helped by preparing some of the probes and hybridization/washes for the membranes.

Other Publications:

5. Yu, Q., **Brain, J.**, Laneuville, P., and Osmond, D.G. Suppressed apoptosis of pre-B cells in bone marrow of pre-leukemic $P190^{Bcr/Abl}$ transgenic mice. 2001 *Leukemia* (15)5 819-827.

In manuscript five, J.B. was responsible for the genotyping and organ preparation of the $P190^{Bcr/Abl}$ transgenic mice.

List of Figures

Chapter I Literature Review

Chapter II Measurement of Genomic Instability in Pre-Leukemic $P190^{Bcr/Abl}$ Transgenic Mice Using Inter-SSR PCR (*submitted*)

Chapter III The Inhibitor STI571 Reverses Bcr/Abl Induced Point Mutation Frequencies in Pre-Leukemic $P190^{Bcr/Abl}$ Transgenic Mice (*submitted*)

Chapter IV Genomic Instability in an Induced $P210^{Bcr/Abl}$ Transgenic Mouse Using Inter-SSR PCR Genomic Scanning (*submitted*)

60. CML accounts for 20% of all leukemic deaths and is the leukemia with the poorest prognosis, it is therefore one of the most highly studied of all the leukemias.

2. Molecular Biology and Philadelphia Positive Leukemias

The cytogenetic hallmark of CML is one of the products of an unequal reciprocal translocation between chromosomes 9 and 22, known as the Philadelphia Chromosome. This reciprocal translocation, t(9;22)(q34;q11), is found in almost 95% of CML patients. The 5% of patients which do not carry this translocation, still manifest a CML like disease on a histological level. Chromosome 22, gives rise to a protein known as Bcr or "break cluster region," while chromosome 9 gives rise to a proto-oncogene, known as *c-Abl*. The 9 and 22 fusion therefore, gives rise to a fusion protein known as Bcr/Abl.[7; 8] The translocation gives rise to a shortened 22q- or Ph chromosome containing the *Bcr/Abl* fusion gene, while also gaining a 9q+ *Abl/Bcr* gene. The majority of the breakpoints in *bcr* are scattered, but all lie within the middle of the gene in one of two introns separating exons b2 and b3 or exons b3 and b4 (figure 1). A number of different breakpoints will manifest into different length fusion proteins, namely p210kDa, p190kDa and p230kDa. The p210kDa length, is the most common and associated with CML. In CML, the break occurs within a 5.8kb area spanning *bcr* exons 12-16 (b1-b5), defined as the major breakpoint cluster region (M-*bcr*). The p190kDa length, which is commonly associated with acute lymphocytic leukemia (ALL), is characterized by a break point further upstream in the 54.4kb region between the alternative *bcr* exons e2′ and e2, known as the minor breakpoint cluster region (m-*bcr*). Finally, the p230kDa length is more commonly

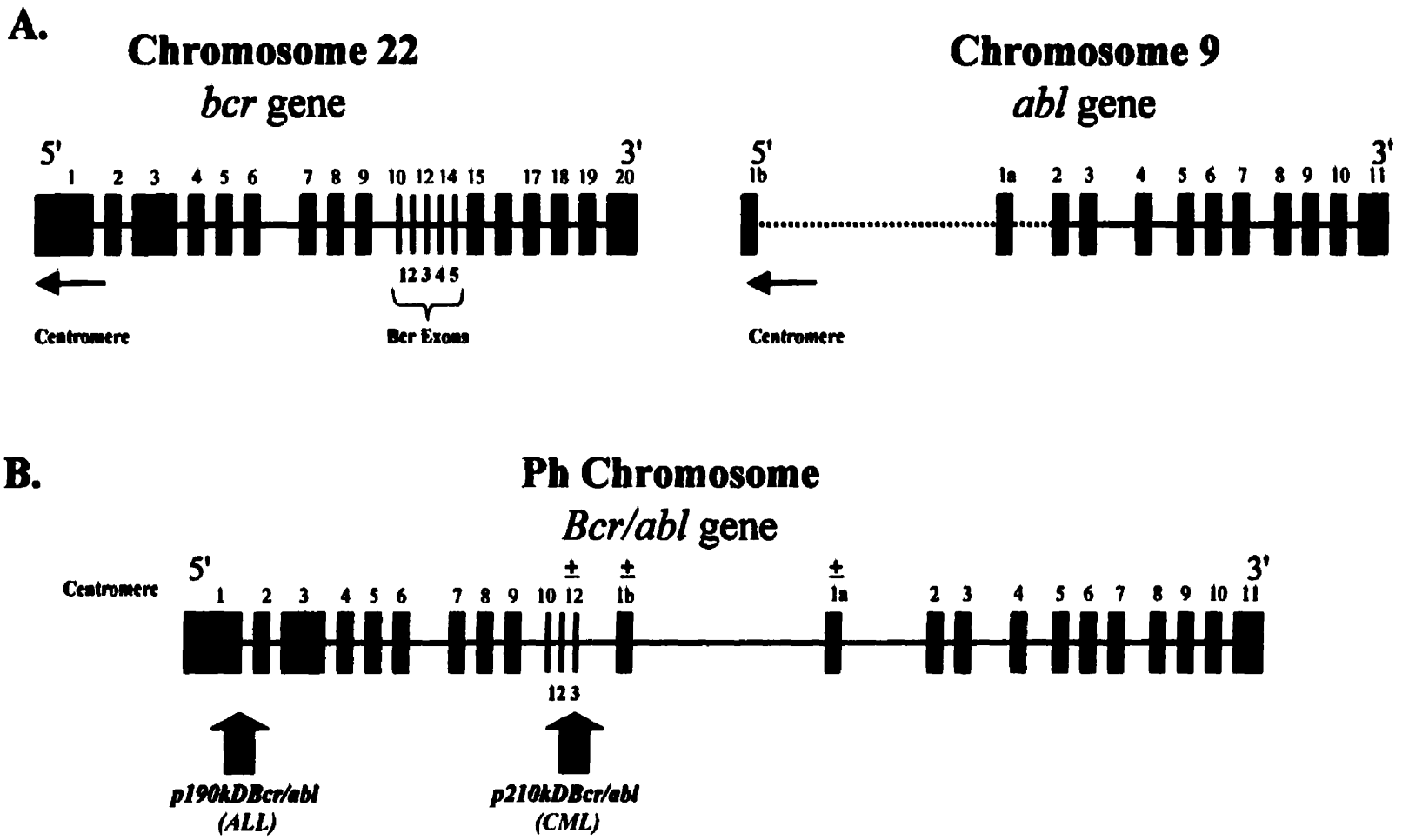

Figure 1: The Cytogenetic Hallmark of CML- The Philadelphia Chromosome. Exons appear as numbered boxes and vertical lines. Introns appear within the horizontal lines. A. Chromosome 22 transcribes the *bcr* gene, while chromosome 9 transcribes the *abl* gene. The long dashed line within the *abl* gene represents the extended region in which breakpoints occur. B. The *bcr/abl* fusion gene. Arrows indicate breakpoints within the *bcr* gene which give rise to either a CML or ALL type of leukemia. (Adapted from Kurzrock R.)

associated with chronic neutrophilic leukemia (CNL), this break is associated with the break cluster region (μ-*bcr*) downstream of exon 19.[9; 10]

The Ph+ type of ALL accounts for 20-30% of all ALL cases in adults but only 5% of childhood cases. However, it is still the most common karyotypic anomaly associated with this disease. Ph+ ALL, is associated with unusually high white counts, resistance to chemotherapy and a poor prognosis in both children and adults. Furthermore, the target cell in ALL is within the lymphoid lineage, whereas in CML it is within the myeloid and lymphoid lineages. The rarest form of Ph+ leukemia is CNL, in which the neutrophillis in particular are effected.[11]

Thus far, researchers have clearly identified the translocation of this disease along with the fusion protein it manifests, as well as several signal transduction pathways associated with its neoplastic transformation. One major question that still needs to be addressed in CML is the cause of the initial translocation. The only known risk factor that is associated with CML is previous exposure to high-dose ionizing radiation.[12; 13] Although many investigators have tried to address this question directly, this appears to be a multifaceted question to address. It may be that certain genes or cell types are more susceptible to this type of translocation or rearrangement. Even more specifically, cells within the immune system that require Ig rearrangement, such as B and T cells. It is also possible that the cause is environmental. If this is the case, biologists will need to collaborate in the future to address this multifaceted question and begin to answer the question as to the precise etiology of this disease.

3. Clinical Features of Chronic Phase CML

From a clinical perspective, the chronic phase of CML does not pose a great risk to the majority of patients as the elevated levels of white blood cells can be controlled with cytotoxic agents and the neutrophil and platelet functions are largely normal. As the granulocyte count rises to 50,000 cells/μl the spleen becomes palpably enlarged and other symptoms may manifest such as fatigue, night sweats and weight loss. Other symptoms may include bone pain, a fullness in the upper abdomen and splenomegaly. Splenomegaly is the most common physical finding upon initial examinations, and is found in about 95% of CML patients.[14]

Hematological features at diagnosis include a leukocyte count which is greater than 100,000 cells/μl. Typically, CML patients have an average white cell count at diagnosis of about 200,000 cells/μl. All stages of myeloid development are represented accordingly, as though bone marrow had leaked directly into the circulation. The percentage of immature cells is directly proportional to the magnitude of the white count. A prognosis of CML is made by looking at blood cell differential counts which usually reveals absolute basophilia and an increase in myelocytes.[15] Most patients are anemic at diagnosis but it is only mild and therefore they do not require blood transfusions. Platelet counts vary between patients however, nucleated red cells are found in the blood of almost all CML patients.[16] About 80% of CML patients show myelodysplastic changes including various unclassifiable leukocytes and plentiful eosinophil myelocytes, upon diagnosis. CML patients are also lacking or show low levels of leukocyte alkaline phosphatase (LAP) enzyme. Although the physiological function of this enzyme remains unclear, decreased levels of LAP are used as a prognostic factor for entry into remission.[17]

Marrow biopsy shows a decrease in marrow fat space due to crowding of the marrow cavity by proliferation of all stages and classes of myeloid cells, which eventually effect the erythroid differentiation. There is an abnormal number of immature to mature neutrophils in the patients marrow as compared to blood, meaning there is a problem with the release of immature cells into the peripheral blood.[18; 19]

4. Clinical Features of Blast Crisis

CML usually progresses to a blast crisis within 5 years. During the first years after onset, the leukocyte and platelet counts rise, red cell counts fall and immature myeloid forms spill into the bloodstream. However, in the Japanese survivors of the atomic bombing in Hiroshima, the first Ph+ cell appeared 6 years before the white blood cell count reached 100,000 cells/μl. These studies revealed that the original Ph+ clone may take on average, 11 years to reach the full blast crisis.[20]

With an average of about 3.5 years after diagnosis the chronic phase gives rise to the acute blast crisis phase. It is unclear what the cause of this transition is but it may involve the cooperation of several mutations to induce the phenotype of blast crisis. The symptoms of entry into blast crisis are not unlike the symptoms at diagnosis but become much more severe. Patients in the acute blast crisis stage also experience pain from osteolytic bone lesions, myalgias and arthralgias.[21] Usually, myeloblasts make up most of the cells during blast crisis, however in some cases myeloid blast occurs from the transformation of promyelocytes or eosinophil progenitors. In about a third of cases the blast is of B cell lineage, which is more typical in acute lymphoblastic leukemia. Lymphoid T cell blast crisis does occur in CML, but is very rare.[22]

Around 85%-90% of patients die from complications arising during blast crisis, in which the most common are cytopenic bleeding, infection, and marrow aplasia, which is aggravated by progressive myelofibrosis.[23; 24] Some patients die from leukostatic lesions, while others succumb to therapy related thrombocytopenia, myocardial failure, pulmonary fibrosis, uremia or a combination of other adverse side effects.[25]

5. Treatment of CML

Treatment of chronic phase CML is aimed at reducing the proliferating myeloid mass and relieving problems created by hyperleukocytosis, thrombocytosis, and splenomegaly. With treatment the quality of life for a CML patient is greatly improved, however survival time is not extended as CML is not a chemocurable disease.[26]

For over forty years busulfan (Myleran; 1,4-dimethanesulfonyloxybutane) has been one of the most popular agents in treating chronic phase CML. Busulfan is an alkylating agent which is active at the stem cell level and can induce remission within sixteen weeks for continuous low-dose administration.[27] The patients white count falls and the spleen shrinks, blood counts return to normal and the patient gains weight. Leukocyte doubling time is an indicator as to whether treatment should be continued, with each relapse the leukocyte doubling time becomes shorter and shorter and therefore the effect of busulfan becomes less and less.[28; 29] Busulfan is not used for treatment in blast crisis as the doubling time of the leukocytes is far greater than the capacity of the drug. There are several noted side effects for this drug including severe marrow hypoplasia and a reduction in platelet formation which is disproportionate to its other myelosuppressive actions.[30-32]

Hydroxyurea, another drug used to combat CML, is a ribonucleotide reductase inhibitor and is a cell cycle-specific antagonist of DNA synthesis in S phase and is more effective than busulfan in sustaining remission during the chronic phase.[33] Hydroxyurea combats the accelerated phase of blast crisis as it has been shown to be more toxic to CML cells than to normal cells in a clonogenic assay *in vitro*. Hydroxyurea, as with busulfan is not capable of preventing blast crisis or inducing a cytogenetic remission.[34]

Interferon-α (IFN-α) has become one of the most popular treatments for CML, as it induces some cytogenetic responses and prolongs duration of chronic phase and survival of patients. Although IFN-α is one of the most popular treatments, its mechanism of action remains unclear. Several investigators have shown using *in vitro* and *in vivo* studies that the effect IFN-α has on CML cells may involve adhesion. CML cells have an alteration in β–1 integrin function making these cells defective in stromal adherence and IFN-α has been shown to reverse this effect.[35-37] IFN-α is effective in that 70% of patients undergo hematologic remission and in 25% of patients complete cytogenetic remission is seen (loss of the Ph chromosome).[38; 39] However, nearly all of these complete cytogenetic remission patients were shown to be Bcr/Abl positive upon PCR amplification.[40; 41] Therefore, IFN-α is a good treatment for CML but again not a cure for the disease.

The most revolutionary advance in the treatment of CML involves a new class of drugs known as the signal transduction inhibitors (STI), which have the ability to block or prevent a protein from exerting its oncogenic role. These molecules were designed to compete with the adenosine triphosphate (ATP) binding site or the catalytic region of the protein.[42] The first of these drugs is known as STI571 (formally known as CGP57148B,

Novartis Pharmaceuticals), and is one of the first signal transduction inhibitors to be used in clinical trials. This drug is an example of what is yet to come, not only in this evolution in molecular biology but also among these new concepts in drug design, which is to target a specific protein. Inhibition of Bcr/Abl by STI571 results in transcriptional modulation of genes involved in control of cell cycle, cell adhesion and cytoskeleton organization.[43] This leads the Ph+ cell to undergo an apoptotic death, which is the first CML drug besides IFN-α to do so. Previous treatments, may decrease the number of Ph+ cells but the patient would still appear Ph+ cytogenetically. Whereas STI571 has the ability to render the Ph+ patient Ph negative both on a cell level and a cytogenetic level, through it's ability to render apoptosis in the cell. This drug, while in phase II clinical trials, showed little toxicity but proved highly effective, whereas patients given 300mg/d or more entered a complete cytogenetic remission as well as some patients showing a cytogenetic response. It also appears to be effective in many patients with acute Ph+ leukemias, mainly of the lymphoid lineage.[45] Remission is not sustained however, in many of these acute patients, therefore it is still to be determined as to whether it will have an effect on the acute patients. This drug has just recently been approved by the FDA, and is now known as Gleevec, it will be interesting to see what the long term effects of this drug are. The development of this drug is a huge achievement in drug design and for the treatment of CML and hopefully it is a glimpse of what is yet to come for future treatment of leukemias and furthermore of solid tumors.

The results of chemotherapeutic treatment for CML not only destroys the Ph+ clones but also severely disrupts the normal hematopoietic system. One way to circumvent this problem is by destroying all marrow parenchymal elements, leukemic and normal with ablative doses of irradiation and chemotherapy, followed by transplantation of normal

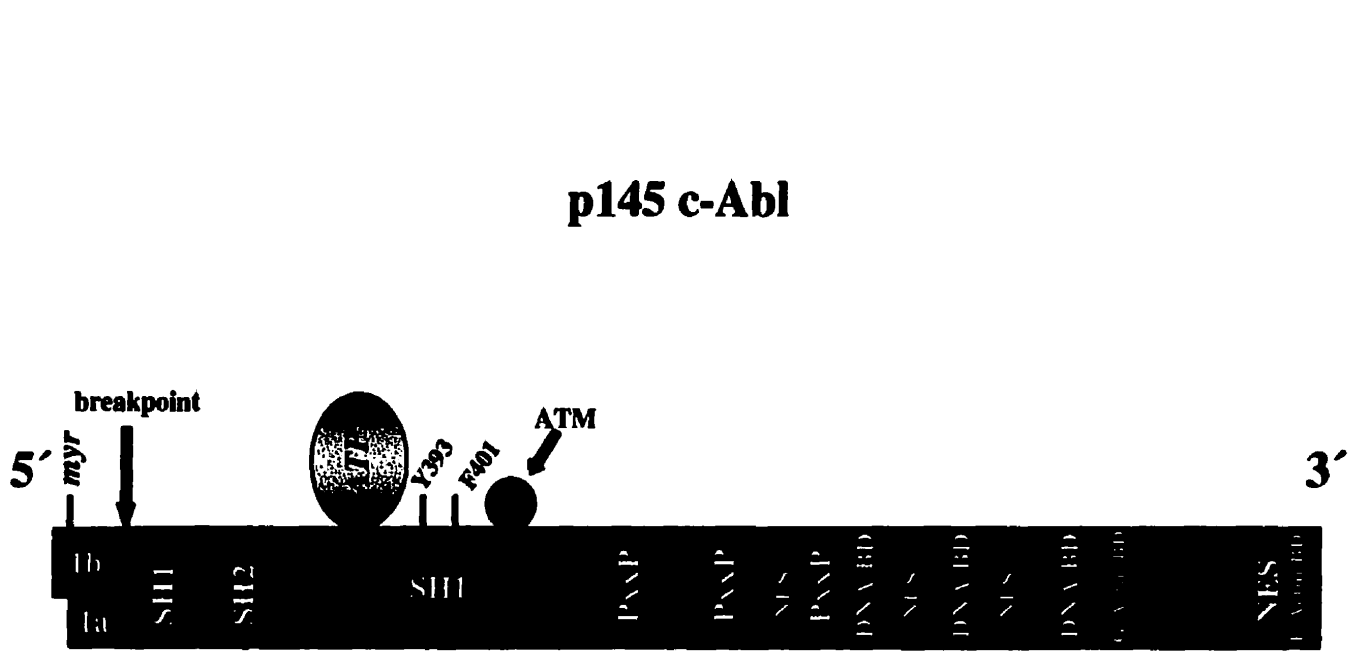

Figure 2: Structure of c-Abl protein. At the 5′, N terminal region is a myristolation (myr) site for attachment to the plasma membrane. Isoform type 1a is slightly smaller than isoform type 1b. There are three SRC-homology (SH) domains situated near the N terminal region. Y393 is the major autophosphorylation site within the SH1, kinase domain. Phenylalanine 401 (F401) is highly conserved in protein tyrosine kinases containing SH3 domains. The middle region of the protein contains proline-rich domains (PxxP), which are capable of binding SH3 domains as well as nuclear localization signals (NLS). The 3′, carboxy terminal region contains DNA binding domains and a F and G actin binding domain. An ATM Phosphorylation site is shown and the arrow at the 5 region is the position of the breakpoint in the Bcr/Abl fusion protein. (Adapted from Deininger,M.)

the center of the molecule which interact with SH3 domains of other proteins. The large C-terminal end includes three nuclear localization signals, a DNA binding domain and actin-binding motifs.[53-55]

It appears that c-Abl may have many important roles both in the nucleus and in the cytoplasm, although several are yet to be delineated. It remains unclear what role c-Abl plays in the cytoplasm. It is known however, that it co-localizes with F-actin. When c-Abl is found in the cytoplasm, it is found almost exclusively bound to F-actin, suggesting that the cytoplasmic pool of c-Abl may contribute to normal signal transduction pathways.[54; 56] It is unclear how important these cytoplasmic roles are as c-Abl is localized more frequently in the nucleus than in the cytoplasm. Some known substrates for c-Abl include the product of *enabled*, which interacts with the SH3 domain and Crk which interacts with the C terminal region of c-Abl.[57-59]

It is unclear as to how or why c-Abl shuttles between the nucleus and the cytoplasm, since c-Abl has three nuclear localization signals. It may have several important roles in the nucleus. Thus far, nuclear c-Abl has been implicated in the regulation of cell cycle and in response to genotoxic stress. In the nucleus, c-Abl appears to inhibit cell growth through a direct interaction with the retinoblastoma protein (Rb). It appears that c-Abl may be regulated in a cell cycle dependent manner as it binds to DNA during interphase and is inhibited in G_1 by binding of the C terminal domain of Rb.[56] Rb is phosphorylated in late G_1 by cyclin-associated kinases and this causes a dissociation of Rb and a repression of the c-Abl kinase. This allows the DNA-bound active c-Abl kinase to phosphorylate adjacent proteins that are involved in transcription at the onset of S-phase. One substrate for c-Abl is the carboxy-terminal repeat domain of mammalian RNA polymerase II, mediated by the SH2 domain of c-Abl.[60]

One reason why c-Abl appears to be more nuclear localized than cytoplasmic is due to its response to DNA damage. Some of the most recent discoveries have been in the role c-Abl plays in response to ionizing radiation (figure 3). It appears that c-Abl is activated by ataxia telanglectasia mutant (*ATM*) protein in response to radiation.[61] Ataxia telangiectasia (AT) is a rare human autosomal recessive disorder, which has several phenotypes including neuronal degeneration, immune dysfunction, premature ageing and increased cancer risk.[62] The gene mutated in AT, *ATM*, encodes a protein kinase. It appears that c-Abl is a downstream target of phosphorylation by the ATM kinase in the cellular response to ionizing radiation.[63] Meaning, that ATM is involved in the activation of c-Abl by DNA damage and further studies show that this may occur during G_1 arrest.[61] Further studies also suggest that c-Abl interacts with DNA dependent protein kinase (DNA-PK) in response to DNA damage.[64] DNA-PK is involved in double-strand break repair and V(D)J recombination.[65] Other studies suggest c-Abl binds to p53 and enhances its transcriptional activity by blocking cell cycle progression in response to DNA damage. p53 is a major transcription factor and is known as one of the main "gate keeper" proteins.[66] Finally, nuclear c-Abl has been shown to be activated due to the stress response of DNA damaging agents. Cells deficient in c-Abl fail to activate Jun kinase (JNK/SAP kinase) after ionizing radiation or alkylating agent exposure.[59] Whereas, the stress response to tumor-necrosis factor is stimulated by a c-Abl independent mechanism. More recent studies show a relationship between p73 and c-Abl. p73 is a structural and functional homologue of the p53 tumor suppressor protein, and is regulated by a c-Abl dependent mechanism.[67] p73 participates in the apoptotic response to DNA damage.[68]

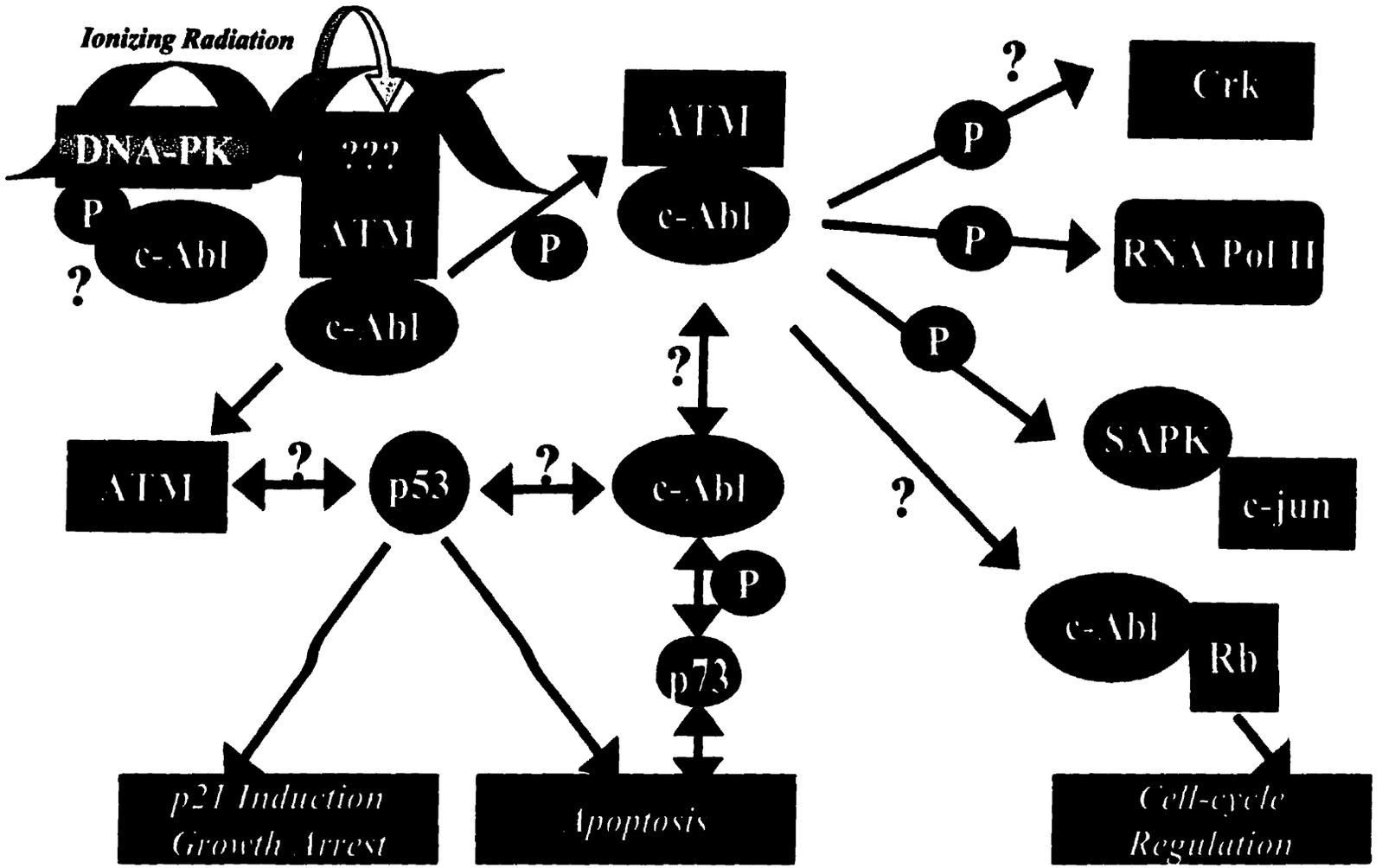

Figure 3: Role of c-Abl in DNA repair. ATM is constitutively bound to c-Abl following radiation induced DNA damage. ATM phosphorylates c-Abl, which activates Crk, RNA polymerase II for transcriptional regulation, and SAPK. ATM may also associate with p53, Rb, and p73 influencing cell-cycle regulation and apoptosis. C-Abl can also associate with DNA protein kinase (DNAPK) on DNA itself. All the "p" points marked stand for phosphorylation sites. (Adapted from Brown, L.)

It seems to be clear from these experiments that c-Abl has several important roles but the majority of these results were obtained using *in vitro* models and so the actual relevance *in vivo* is still controversial. In an attempt to answer some of these questions *in vivo* a *c-abl* knockout mouse was generated but failed to resolve many of these questions. However, these knockouts did reveal some information concerning the role of c-abl *in vivo*. The *c-abl* knockout mice have a high rate of neonatal death, with the survivors showing defects in B and T cell development, as well as developmental abnormalities in the spleen, cranium, and eye[69; 70] These mice also showed an increased susceptibility to infection and to carcinogens, which may have something to do with the relationship of c-Abl and the response to genotoxic stress.[71] The mutant mice homozygous for a deletion in the C-terminal region of *c-abl* have similar phenotypes to the complete *c-abl* knockout mice, suggesting that the C-terminal region of *c-abl* is essential for proper function.[69] These knockout studies clearly show the importance of *c-abl* in development and even more specifically in the lymphoid lineage. Future studies on *c-abl* should therefore attempt to address the issues of the relationship between c-Abl, genotoxic stress and DNA repair *in vivo* in order to understand the importance of c-Abl in these events.

7. Functions of *BCR*

BCR transcribes a 160kDa protein which is ubiquitously expressed, although the exact function is still largely unknown. Much is known about the structure of *BCR* (figure 4), as the first N-terminal exon encodes a serine-threonine kinase. The known substrates of this kinase domain are Bap-1, which is a member of the 14-3-3 family of proteins involved in apoptosis.[72] Another target for this domain is *BCR* itself, as it

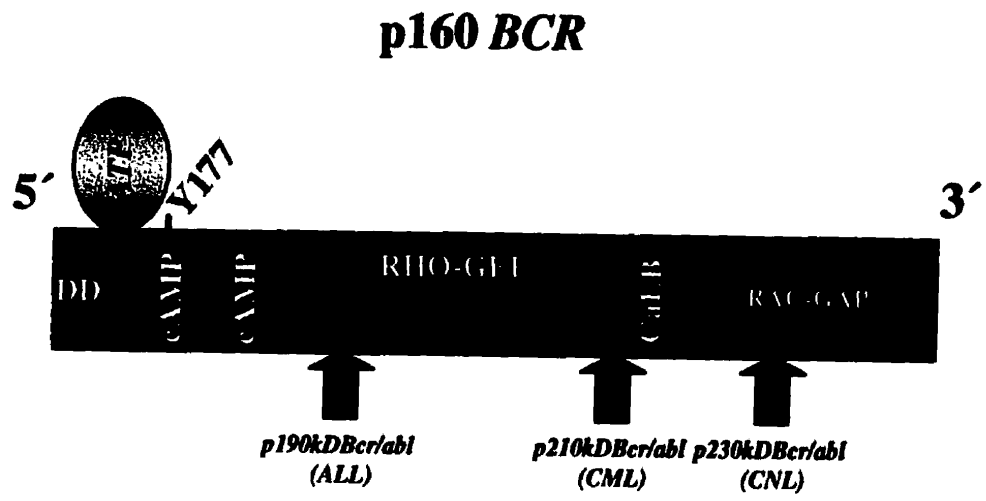

Figure 4: Structure of BCR protein. At the 5 ´, N terminus end of the protein, lies a dimerization domain (DD) and two cyclic adenosine monophosphate kinase homologous domains (cAMP). Also at this terminus is the tyrosine 177 (Y177) binding site which is essential for Grb-2 binding. In the center of the protein lies a region homologous to Rho guanidine nucleotide exchange factors (Rho-GEF) as well as dbl-like and pleckstrin homology (PH) domains. Within the C´ terminal domain lies a putative site for calcium-dependent lipid binding (CaLB) and a domain with functional activation for Rac-GTPase (Rac-GAP). The arrows indicate the position of breakpoints within Bcr which give rise to different Ph+ leukemias. (Adapted from Deininger,M.)

contains a coil-coil domain at the N-terminal region which allows dimer formation *in vivo*.[73] The central region of the *BCR* gene contains a region with dbl-like and pleckstrin-homology (PH) domains that stimulate the exchange of guanidine triphosphate (GTP) to guanidine diphosphate (GDP) on Rho guanidine exchange factors, this in turn may activate several transcription factors such as NF-κB.[74; 75] The C-terminus region has GTPase activity for Rac, which is part of a superfamily that regulates actin polymerization and is also known to regulate the activity of NADPH oxidase in phagocytic cells.[76] Finally, Bcr can be phosphorylated on several tyrosine residues, with the most important being tyrosine 177 (Y177). This tyrosine site has been shown to bind the adaptor protein Grb-2, which is involved in activation of the Ras pathway.[77] Also, very interestingly, c-Abl has been shown to phosphorylate Bcr in a COS1 cell line, resulting in a reduction in the Bcr kinase activity.[76-78]

BCR knockout mice show normal lymphoid function and hematopoietic development. These mice do however, form a connection between *BCR in vivo* and regulation of Rac-mediated superoxide production by the NADPH-oxidase system of leukocytes.[76; 78; 79] The results conclude that *BCR* functions as a regulator of the respiratory burst in some hematopoietic cells including, phagocytes (neutrophils, macrophages) and B cells, which are all specialized cells with an active NADPH-associated oxidative burst complex.[76; 78] These knockout studies are most interesting because all these cells which are affected by respiratory burst, are involved in Ph+ leukemias. Further studies are still needed to address the normal role of *BCR* and more importantly the physiological effects of how it functions as a serine/threonine kinase.

8. Functions of *Abl/Bcr*

transformation.[82] These oncogenic forms of c-Abl have the ability to transform cells *in vitro* based on their ability to promote a growth factor independent system, as well as *in vivo* using transgenic mice.[83] A cancer cell itself becomes transformed by activating mitogenic signaling, inhibiting apoptosis and sometimes by inhibiting or showing a disruption of adhesion molecules.[82] There are several oncogenic forms of c-Abl including v-Abl, Bcr/Abl and Tel/Abl. Oncogenic c-Abl uses similar pathways to induce transformation through several signal transduction pathways, including pathways which specify altered adhesion to stroma cells and the extracellular matrix.[84] Furthermore, the activation of various signaling cascades. such as Ras/Raf/Erk, Jak-STAT, PI3-K and *myc*, generates a mitogenic signal (figure 5). Finally, the activated Bcr/Abl oncogene inhibits apoptosis through the activation of survival pathways.[85; 86]

9. Bcr/Abl Signal Transduction-Altered Adhesion

A significant area that is affected by expression of Bcr/Abl are the signaling proteins involved in cellular adhesion. It was first shown that CML progenitor cells exhibit decreased adhesion to bone marrow stroma cells and also to the extracellular matrix within the bone marrow.[84] Adhesion to stroma negatively regulates cell proliferation in the bone marrow.[87] INF-α is one of the most popular therapeutics used to treat CML and appears to reverse this adhesive defect.[36] Although, it is still unclear as to the exact mechanism, recent data suggests a role for β–integrins in the interaction between stroma and progenitor cells. CML cells express an adhesion

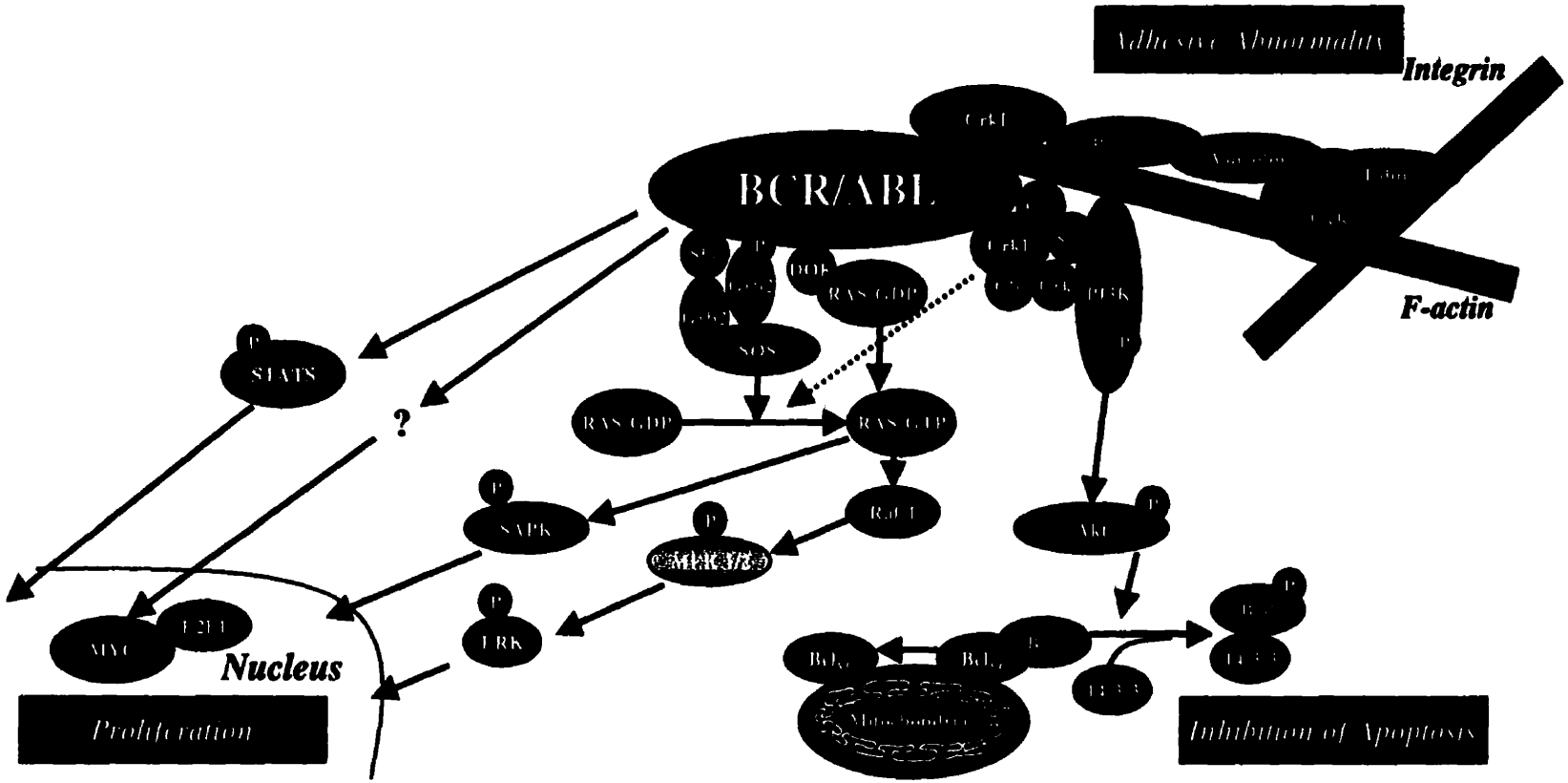

Figure 5: Pathways Involved in Bcr/Abl Signal Transduction. Constitutive activation of c-Abl protein tyrosine kinase induces tyrosine phosphorylation of many substrates including Bcr/Abl itself, as well as several adaptor proteins. Bcr/Abl exhibits adhesive abnormalities through integrin proteins, CrkL and P13K. Bcr/Abl increases proliferation through the Ras/Raf-1 pathway as well as possibly through MYC and STAT activation. Finally, Bcr/Abl shows inhibition of apoptosis through association with several apoptotic proteins such as Akt, Bad and again P13-K. It is important to note that this diagram may not include all signaling proteins known to associate with Bcr/Abl. (Adapted from Gotoh, A., and Deininger, M.)

inhibitory variant of β1 integrin which is not present in normal cells.[37] This variant may have adverse effects on the normal integrin signaling. In addition, CrkL, which is one of the most abundantly tyrosine-phosphorylated proteins in Bcr/Abl transformed cells, is involved in integrin-mediated cell adhesion regulation of cellular motility.[88] This is through an association with focal adhesion proteins such as paxillin, which then in turn activates other focal adhesion molecules, such as focal adhesion kinase (FAK), vinculin and talin.[89; 90] Recently it has also been demonstrated that Bcr/Abl upregulates another protein involved in integrin regulated signaling, the α6 integrin, which may potentially contribute to transformation.[91] It is clear that Bcr/Abl affects integrin function and adhesion, both through activation of proteins such as FAK and through expression of a β1 integrin variant. Even more convincing is that INF-α may reverse some of these adhesive effects, as loss of adhesion may help to eliminate the cancer burden.

10. Bcr/Abl Signal Transduction-Activation of Mitogenic Signaling

Several proteins have been identified which can become activated by Bcr/Abl and therefore activate the Ras and MAP kinase pathways, one such protein is the adaptor protein Grb-2.[92] The autophosphorylation of tyrosine 177 within Bcr provides a docking site for this adaptor protein.[92; 93] Recruitment of Grb2/SOS complexes to Bcr/Abl together with the proper localization at the membrane, stabilizes Ras in its active GTP-bound state.[94] Two other adaptor proteins can bind Bcr/Abl substrates, Shc, via its SH2 domain and CrkL, via its SH3 domain.[95; 96] However, new studies have also shown that CrkL may not be as significant for Ras activation in Bcr/Abl transformation cells as

previously thought, as it appears to be restricted to myeloid cells and is not required for transformation in this line.[97] Ras appears to be constitutively activated throughout the transition of this disease and no additional Ras mutations appear even in at the blast phase of the disease.[98] It is still unclear which mitogen-activated protein kinase (MAPK) pathway is downstream of Ras in Ph+ cells. Activation of Erk 1/2 through the Mek1/Mek2 pathways occurs downstream by the stimulation of cytokine receptors, such as IL-3.[99] The activation of the JNK/SAPK pathway has been shown to be required for malignant transformation, signaling through Ras may be through the exchange factor Rac.[100; 101] Bcr/Abl enhances JNK function as measured by transcription of *Jun* responsive promoters. Finally, there is some evidence that the mitogen-activated protein kinase p38, is also activated in Bcr/Abl transformed cells.[102]

Within the CML chronic phase, the progenitor cells are still dependent upon external growth factors for survival and proliferation. There is evidence for an autocrine loop dependent upon the Bcr/Abl induced secretion of growth factors, as it has been reported that Bcr/Abl induces an IL-3 and G-CSF autocrine loop in early progenitor cells.[111] Furthermore, Bcr/Abl has been shown to activate growth factors as well as cytokines, including the oncostatin Mβ receptor.[112]

Cross-talk between Bcr/Abl and cytokine receptors may provide an additional means to activate mitogenic signaling pathways. It has been observed that Bcr/Abl associates with the βc subunit of the IL-3 receptor and through the Kit receptor.[103; 104] Even more evidence for this association is observed when normal progenitor cells are stimulated with the Kit ligand and the proteins activated are similar to those in CML progenitor cells. Although, most receptors/tyrosine kinases activate similar pathways. One protein that is highly prominent in this context is Dok-1 ($p62^{DOK}$), which can form

complexes with CrkL, RasGAP, and Bcr/Abl.[105] The importance of Dok-1 for cellular transformation remains unclear, as it has been proven non-essential for transformation of growth-factor independent myeloid cells.[105]

Constitutive tyrosine phosphorylation of several Stat transcription factors have been reported in Bcr/Abl positive cell lines and in CML primary cells. STATs 1, 5 and 6 have all appeared activated, with STAT5 contributing to malignant transformation.[106-108] The effects of STAT5 is through transcriptional activation of Bcl-x_L which has an anti-apoptotic role.[109] Even more interesting, is the fact that Bcr/Abl appears to directly activate STAT1 and STAT5 independent of any JAK proteins.[110] Studies also show that activation of STAT6 occurs in P190Bcr/Abl transformed cells but not in P210Bcr/Abl transformed cells.[110] These results are the first to show differences between the signaling mediated by the two different forms of Bcr/Abl. STAT6 may be the signaling protein responsible for the lineage specificity of these proteins, future studies will help to elucidate the relevance of this.

PI3 kinase (P13-K) activity has been shown to be required for the inhbittion of apoptosis in Bcr/Abl positive cells.[113] When the p110 catalytic subunit of P13 kinase is inhibited with a pharmacological inhibitor, there was an inhibition of the proliferation of Ph+ cell lines and colony formation by CML primary cells.[113] This data suggests that the P13 kinase pathway is required for Bcr/Abl transformation. There are several proteins activated in the PI3 kinase pathway, which either bind the p85 regulatory subunit, including Cbl as well as adaptor proteins like Crk and CrkL and other proteins which are activated downstream of P13-K, most importantly the serine-threonine kinase Akt.[114; 115] Akt is a protein implicated in anti-apoptotic signaling. Several reports on Akt identified a protein known as Bad, which is a key substrate of Akt and a product of the IL-3 signaling

cascade.[116; 117] Bad cannot bind to anti-apoptotic proteins, such as Bcl-x_L when it is phosphorylated as it becomes trapped by the cytoplasmic 14-3-3 proteins.[116] This therefore allows for an anti-apoptotic signal and cell survival.

SHIP-1 and SHIP-2 are two inositol phosphatases which become activated in response to growth factor signals by Bcr/Abl, thus possibly giving Bcr/Abl a role in phosphoinositol metabolism.[118] SHIP-2 is tyrosine phosphorylated and therefore can associate with Shc. Since recent evidence has implicated Bcr/Abl in growth factor mediated signaling, the finding that both PI(3,4,5,)P3 and PI(3,4)P2 are constitutively tyrosine phosphorylated in CML primary hematopoietic progenitor cells may have important implications in Bcr/Abl myeloid expansion.[119]

The last cascade that is implicated in Bcr/Abl expression involves the *myc* pathway. Consistent with the transcription factor, over-expression of *myc* has been observed in many human malignancies.[120; 121] Bcr/Abl induces the expression of *myc*, dependent on its SH2 domain, although the pathway linking these two are unknown.[122] Reports using cell lines which express v-Abl, show that this induction is mediated through Ras/Raf, cyclin-dependent kinases (cdks) and *E2F* transcription factors that activate the *MYC* promoter.[123] Similar findings have been reported in Bcr/Abl transformed cell lines but the significance in Ph+ cells is unknown.[124] It is likely that *myc* is important in Ph+ cells and if this is the case *myc* may play a role in proliferation or as an apoptotic signal.

11. Bcr/Abl Signal Transduction-Inhibition of apoptosis

other proteins involved in the proteasome pathway, to see if Bcr/Abl plays a role in their degradation.

Although several proteins have been found to be either phosphorylated or activated in response to Bcr/Abl, it still remains unclear which are necessary for transformation. Many of the studies were initially done in cell lines and therefore it is not clear what their importance to *in vivo* transformation is. The other theme is of cross talk between pathways, which is another fundamental issue that must also be addressed. Future focus on these questions will help to elucidate which signal transduction pathways are important for Bcr/Abl transformation.

12. Experimental Models Used to Study CML-Cell Lines

There are several experimental models used to study CML including cell lines and transgenic mouse models. Each of these models have specific advantages and disadvantages. Fibroblast cell lines, are a popular tool because they are easy to use and well understood as their anchorage-independent growth in soft agar is a standard *in vitro* test of tumorgenicity.[133] Bcr/Abl has different effects on different fibroblast cell lines, for example, $P210^{Bcr/Abl}$ transforms Rat-1 fibroblasts but has no effect on NIH3T3 cells.[85; 134; 135] Another limitation in this system is that there are differences in the signal transduction proteins activated by hematopoietic cell lines and fibroblastic cell lines. This makes fibroblastic cell lines good for basic tumorgenicity questions and signal transduction questions, but may not be a good indication of what is actually occurring in a hematopoietic cell.

There are several hematopoietic cell lines used to study CML, including K562 for myeloid differentiation and BV173 for lymphoid differentiation.[136] Although, these lines are specific to a hematopoietic lineage, they are derived from the blast crisis, meaning they may have other genetic abnormalities besides Bcr/Abl. This makes it difficult to differentiate what events Bcr/Abl is responsible for as opposed to other genetic lesions. The ideal cell line would be from chronic phase CML, so one could observe the development of these genetic lesions or events, but this cell line does not yet exist.

Another set of cell lines used in CML experimental studies are cell lines which switch from being growth factor-dependent to being growth factor independent when the oncoprotein is activated. These cell lines include murine cell lines such as Baf/3 or 32D and human cell lines, such as MO7. It is not clear how these cell lines apply to clinical CML as these cells are still factor-dependent. A recent study showed that the growth factor, IL-3 is not required for transformation in CML retrovirally induced mice.[137] The very popular 32D cell line is growth factor dependent on IL-3 and IL-3 independent upon Bcr/Abl transformation. These new results may imply that the studies done on 32D cell lines have no *in vivo* relevance.

Several promising studies have shown progress in using a cell line which has a multilineage hematopoietic differentiation, as none of the previously described cell lines have this capability. One report utilized a murine FDCPmix cell line transduced with a temperature sensitive mutant of Bcr/Abl in which it become partially factor-independent at a permissive temperature.[138] The results using these cell lines exhibited characteristics which are much more similar to what is observed in chronic phase CML. Another promising approach is to transfect embryonic stem (ES) cells with Bcr/Abl. In these experiments an expansion of the myeloid lineage at the expense of the erythroid

compartment, a major characteristic of the disease was observed. Interestingly, these experiments also demonstrated increased proliferation but had little effect on apoptosis, which is also characteristic of the chronic phase of the disease.[139] In another study, Bcr/Abl was transfected into ES cells then transplanted into irradiated mice, and the results show that these mice manifest a leukemic syndrome, with many features similar to CML.[139] This may be an important tool in the future to look at transformed Bcr/Abl cells in an *in vitro* model system.

One of the best cell line models to study CML is to use primary cells from patient material. In this model, not only can chronic phase cells be used but also cellular properties of CML cells versus normal cells can be addressed. However, the time allocated for experiments on these primary cell lines is very short because these cells mature when placed in culture. Other setbacks to using primary cells is that there is considerable variation between patients and the system is unreliable unless the cell subsets are clearly defined, such as CD34+. The variation between the patients may have something to do with the duration at which the original clone was introduced into the patient, this may have an influence on secondary genetic events. The advent of the inhibitor STI571 has helped to make all of these cell lines more useful, as the activity of Bcr/Abl can be turned off by introduction of this inhibitor into tissue culture. Currently, treating cultured cells with STI571 as a control has become standard. Clearly, as important as these cell lines are for experimental purposes, no question is truly addressed until these experiments are addressed *in vivo*.

13. Experimental Models Used to Study CML-Animal Models

There are several animal models used to address CML *in vivo*. The first animal model consists of the engraftment of Bcr/Abl transformed cell lines in syngeneic mice. This is done by transforming factor independent cell lines with Bcr/Abl, such as 32D, and transplanting them into syngeneic recipients.[140] This system gives rise to an aggressive leukemia and is a good system to test drugs and the leukemogenicity when mutants of Bcr/Abl are made and transducted into 32D cells before transplantation. The main disadvantages to this sytem is that the leukemia is an aggressive, acute form, as there is no chronic phase in these mice.

Another mouse model system has been designed by the engraftment of immunodeficient mice with human Bcr/Abl positive cells. Cells taken from human CML blast crisis patients are easily propagated in severe combined immunodeficiency (SCID) mice.[141] The distribution of cells is very similar to the acute phase of the disease. Recent reports show that if the cell count is large enough (1×10^8) then chronic phase CML cells can be used for this same system. When these chronic phase cells were used, up to 10% human cells were detectable in the recipient bone marrow, although there were several colonies which were Bcr/Abl negative, indicating the patients normal cells were also transplanted.[142]

The next set of animal models consist of transgenic mice in which both P190 and P210 models have been developed. Several different promoters have been used to drive expression of Bcr/Abl in transgenic mice, although one major difficulty is that Bcr/Abl has a toxic effect on embryogenesis. Recently, a P210 mouse model with a tetracycline-repressible promoter overcame this problem. These mice are able to obtain a complete reversal of the leukemia upon administration of tetracycline.[143] Another problem with the transgenic mice is that the P210 form of the protein, relative to CML, is less efficient in

inducing leukemia than the P190 form.[144] The most interesting yet, is that all the mice develop a B or T-lymphoid phenotype, characteristic of ALL, not chronic phase CML, no matter which form of the protein is expressed, P210 or P190.[145; 146] In fact, of the transgenic models developed thus far, myeloid leukemia is very rare. One major problem with all the transgenic mouse models, is the promoters targeting to one type of cell or cell lineage. One example of this is in a recent study using the Tec promoter, which is a cytoplasmic tyrosine kinase mostly expressed in the hematopoietic lineage. In this study, Bcr/Abl was expressed from the Tec promoter and the animals developed a CML like disease within ten months.[147] This may be due to the targeting of the hematopoietic lineage specifically. New advances in transgenic models, to develop new promoters and inducible systems, will help to develop better transgenic animal models in the future, of which mimic the human disease more closely.

The last animal model system is perhaps one of the most useful, this is by the transduction of murine bone marrow cells with the Bcr/Abl retroviruses.[148; 149] This is similar to the engraftment system except retroviruses are used as a vector instead of a murine cell line. When the bone marrow is transplanted with the retrovirus containing $P210^{Bcr/Abl}$, a myeloproliferative syndrome is observed. This was not the case in all the mice, with about a quarter developing a myeloproliferative syndrome, while others showed macrophage tumors, B-ALL, T-ALL and erythroleukemia.[148; 149] An explanation for this could be that the initial infection was of different committed progenitor cells. Improvements on these initial experiments include high-titer Bcr/Abl retroviral stocks, improved culture conditions and the introduction of the murine stem cell virus LTR for improved target cell efficiency. All these improvements together helped in two recent studies which showed the induction of a transplantable CML-like disease in 100% of

apoptosis, but in the Bcr/Abl expressing cells, this arrest allows for mismatch repair and no apoptosis occurs. Over several cell cycles, this may lead to an accumulation of mutations in Bcr/Abl positive cells, which finally leads to blastic formation. It is indisputable that high cell turnover and proliferation will eventually lead to genetic mutations over time, whether genomic instability is the primary cause for the transition of this disease is disputable.

There are several ways to address genomic instability as it exists in cancer. One way to address mutator phenotype is by using the replication error phenomenon (RER) to look at defects in DNA mismatch repair genes.[158] Two ways to scan all chromosomal arms with DNA probes is by restriction fragment length polymorphisms and the newly developed spectral karyotyping analysis (SKY).[159; 160] The use of these methods helped to develop a diagnostic factor for loss of heterozygosity in colorectal cancers.[161] Other techniques, such as flow cytometry, fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization also help to find widespread rearrangements in cancers such as amplifications, deletions, insertions and translocations.[162-164] Other methods include PCR to look at methylation sites, or to observe regions of methylation.[165] While other PCR techniques include microsatellite analysis to identify specific genes and Inter-SSR PCR to span the genome using $(CA)_n$ repeats to detect amplifications, deletions and insertions randomly.[165-167]

There are also mouse models used to address genomic instability, most of which contain a reporter gene for mutation frequencies. One such mouse which measures the basal point mutation rate is the Big Blue/LIZ transgenic mouse. The chromosomally-integrated λ bacteriophage shuttle vector (LIZ) contains a bacterial *lacI* gene as a target for mutation and *alacZ* as a reporter gene (ie. Big Blue® mutation detection system

[Stratagene]).[168] Although there are several methods which have been used to address genomic instability in CML, it is still unclear what events are necessary for the transition of this disease. The most recent studies lead to DNA repair being one major event, but these results are still unclear.

15. Main Question/Experimental Rational

This disease has an inevitable progression from the chronic to acute phase. During the transition from chronic to blast crisis, a high frequency of secondary chromosomal abnormalities has been reported, which include the loss of tumor suppressor genes, including the p53 or retinoblastoma (Rb) genes.[152; 154] These chromosomal abnormalities suggest that these additional genetic events may be critical for the progression to blast crisis. Both the expression of the dominant oncogene *Bcr/Abl* and the concurrent loss of an allele of the *c-abl* gene could potentially directly initiate genomic instability in Ph+ leukemias. It is still unclear however, as to whether Bcr/Abl is the only genetic event required or if there is another major genetic event required for the transition of this disease. A major interest therefore, is to examine the role of Bcr/Abl in genomic instability as it occurs during the transition of this disease.

Our laboratory has previously addressed genomic instability by using 32D cell lines which express the $P210^{Bcr/Abl}$, in which we have shown that Bcr/Abl does indeed alter the stability of the genome. These cells exhibit cytogenetic abnormalities, show cell cycle abnormalities and inhibition of apoptosis.[169] We have also addressed genomic instability by utilizing the $P190^{Bcr/Abl}$ transgenic mice (line 623) and the Big Blue assay System (Stratagene). Our results showed a two to three fold increase in point mutation

rate observed in the $P190^{Bcr/Abl}$ x Big Blue pre-leukemic mice (about 100 days, before onset of leukemia) compared to control mice (C57/BL6).[170] These mice were then injected with 50mg/kg of the c-Abl specific kinase inhibitor, STI571 (formally known as CGP57148B, Novartis) for ten consecutive days, to see if these mutation frequencies could be reduced. We observed a decrease in mutation frequencies upon injection with the inhibitor, in both the kidney and the spleen. We also addressed the question of genomic instability by utilizing Inter Simple Sequence Repeat Polymerase Chain Reaction (Inter-SSR PCR), in combination with primers which consist of a set of eight CA repeats. These CA repeats appear in all species tested and are the most frequent repeats in the human genome, with an estimated copy number of 50,000 to 100,000 per haploid genome. Using Inter-SSR PCR we compared the $P190^{Bcr/Abl}$ pre-leukemic mice and $P190^{Bcr/Abl}$ leukemic mice to control mice (BL6/CBA), and found an increased number of altered bands (insertions and deletions) in the pre-leukemic and leukemic mice compared to control. We demonstrated that the frequency of altered bands can be decreased using the c-Abl specific kinase inhibitor, STI571. Lastly, we again utilized this system to address genomic instability in a $P210^{Bcr/Abl}$ transgenic mouse with a tetracycline repressible system.[171] Double transgenic mice (*BCR-ABL1*-tetracycline transactivator (*tTA*)) were generated by breeding female transresponder mice with male mouse mammary tumor virus (*MMTV*)-*tTA* transactivator mice under continuous administration of tetracycline (0.5g/l) in the drinking water, starting five days before mating. Withdrawal of tetracycline administration in double transgenic animals allowed expression of *BCR-ABL1* and resulted in the development of lethal leukemia in 100% of the mice within a time frame that was consistent with each line.[171]

Our results showed an increase in insertions and deletions when the tetracycline is withdrawn from the $P210^{Bcr/Abl}$ transgenic mice and therefore Bcr/Abl is expressed. These results confirm the previous results seen in the $P190^{Bcr/Abl}$ transgenic mice and reconfirm that Bcr/Abl is required for induction and maintenance of disease and that Bcr/Abl itself is responsible for this instability seen upon disease progression.

Lastly, our laboratory addressed genomic instability by utilizing cDNA expression arrays to compare the $P190^{Bcr/Abl}$ pre-leukemic mice to the control mice (BL6/CBA). Using this system we note an increase in several genes in the pre-leukemic mice including several genes involved in DNA repair. This defect in the DNA repair genes may be what is driving this alleged genomic instability during the transition of this disease.

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Chapter II Measurement of Genomic Instability in Pre-Leukemic P190$^{Bcr/Abl}$ Transgenic Mice Using Inter-SSR PCR

Preface

The expression of Bcr/Abl is associated with a high degree of genomic instability, including the loss of the tumor suppressor genes p53 and Rb. CML disease has an inevitable progression from the chronic to acute phase but it is unclear as to whether additional genetic events are required for this transition. A major interest is therefore to examine the role of Bcr/Abl in this alleged genomic instability. In this chapter, we utilized the P190$^{Bcr/Abl}$ transgenic mice to observe changes in the genome as they occur *in vivo*. We examined genomic instability in these mice by using the Inter-SSR PCR assay system to observe gross changes within the genome such as insertions and deletions. We observed an increase in altered bands (insertions and deletions) in these mice compared to control mice, both at the pre-leukemic stage (100 days before the onset of leukemia) and at the leukemic stage. We further demonstrate, that these altered bands can be decreased using the c-Abl specific kinase inhibitor, STI571. This chapter therefore concludes that Bcr/Abl alone can confer genomic instability before leukemic onset.

Abstract

Bcr/Abl associated leukemias are characterized by a high degree of chromosomal and genomic instability. The genomic instability is usually associated with disease progression as in chronic myelogenous leukemia (CML) or a poor prognosis as observed in Ph-positive acute lymphoblastic leukemia (ALL). It is unclear whether the phenotype of genomic instability is a primary consequence of Bcr/Abl expression or if it is secondarily acquired in the multistep process of tumor evolution. To address this issue, we measured the frequency of insertions and deletions in $P190^{Bcr/Abl}$ transgenic mice. These mice ubiquitously express Bcr/Abl for an average of three months before developing B-cell type lymphoma/leukemia. Genome scanning for insertions and deletions in samples of DNA extracted from kidney and spleen tissues taken from pre-leukemic animals was performed using the Inter Simple Sequence Repeat Polymerase Chain Reaction (Inter-SSR PCR). We observed an increased frequency of insertions and deletions in the tissues of pre-leukemic animals, which could be partially reversed with the Abl-specific inhibitor STI571. These results suggest that the expression of Bcr/Abl can directly induce a mutator phenotype that antedates overt neoplastic transformation, and that STI571 appears to be capable of reversing this effect.

Introduction

Bcr/Abl associated leukemias in man are characterized by the presence of the translocation t(9;22)(q34;q11) and the cytogenetic hallmark Philadelphia chromosome (Ph).[1] This rearrangement inserts the *c-abl* gene, which encodes a protein tyrosine kinase, from chromosome 9 into the break cluster region (*bcr*) gene on chromosome 22 to create a fusion *Bcr/Abl* gene. Depending upon the breakpoint position within the *bcr* gene on chromosome 22, Bcr/Abl fusion proteins with constitutively activated protein tyrosine kinase activity of 190, 210 or 230kD are characteristically expressed in acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML) and chronic neutrophilic leukemia (CNL) respectively.[2] The expression of Bcr/Abl is associated with a high degree of chromosomal and genomic instability. This effect is best observed in the acquisition of secondary cytogenetic abnormalities and gene mutations associated with the progression of chronic phase CML to CML blast crisis, and the adverse prognosis of Bcr/Abl positive ALL compared to that of Bcr/Abl negative ALL.[3] The mechanism of this observed genomic instability is unknown.

In addition to the expression of *Bcr/Abl*, the reciprocal translocation t(9;22) results in the variable expression of *abl/bcr* from the derivative chromosome, and loss of one normal allele of the *bcr* and *c-abl* genes.[4] The natural history of CML patients expressing *abl/bcr* does not appear to differ from that observed in patients who do not express *abl/bcr*, suggesting that its expression is unlikely to contribute to genomic instability.[4] The loss of one normal *bcr* allele is also unlikely to play a role. Normal *bcr* is ubiquitously expressed and negatively regulates oxidative burst in B cells and neutrophils.

Whereas homozygous *bcr* knock-out mice are susceptible to endotoxin mediated shock, heterozygous *bcr* knock-out mice appear normal.[5] The *c-abl* proto-oncogene however has been implicated in cell cycle regulation and response to DNA damage following genotoxic stress. Over-expression of c-Abl induces G_1 cell-cycle arrest while deficiency of c-Abl confers a susceptibility to enhanced cellular transformation by dominant oncogenes.[6,7,2] Thus, both the expression of the dominant oncogene *Bcr/Abl* and the concurrent loss of an allele of the tumor suppressor gene *c-abl* could potentially directly initiate genomic instability in Ph-positive leukemias. Conversely, genomic instability might result from secondary genetic or epigenetic events.[8]

In previous studies, we have shown that retroviral transduction and expression of *Bcr/Abl* encoding P210 into 32D cl3(G) cells results in inhibition of apoptosis altered cell cycle regulation, and induces rapid numerical and structural chromosomal abnormalities.[9,10] More recently, we measured the basal point mutation rate in heterozygote $P190^{Bcr/Abl}$/LIZ transgenic mice. The chromosomally-integrated λ bacteriophage shuttle vector (LIZ) contains a bacterial *lacI* gene as a target for mutation and *αlacZ* as a reporter gene (ie. Big Blue® mutation detection system [Stratagene]). The line of $P190^{Bcr/Abl}$ mice used express *Bcr/Abl* ubiquitously before developing B-cell lymphoma/leukemia with a latency of approximately 100 days.[11] In this pre-leukemic period, when there is no sign of cellular transformation, we observed a 2-3 fold steady-state increase in the frequency of point mutations in $P190^{Bcr/Abl}$/LIZ mice compared to control.[12] Although these studies demonstrated that the expression of *Bcr/Abl* can directly induce a mutator phenotype antedating overt leukemic transformation, the

Methods

Transgenic Mice/DNA Isolation:

Transgenic $P190^{Bcr/Abl}$ (line 623) were kindly donated by Dr. John Groffen (Children's Hospital Los Angeles). The derivation and phenotype of this mouse line has been extensively described elsewhere.[15] These mice are characterized by the ubiquitous expression of *Bcr/Abl* that is driven by a truncated mouse metallothionein promoter, and which precedes the onset of a B-cell lymphoma/leukemia with a latency of approximately 100 days after birth. BL6/CBA mice were used as controls (Jackson Lab, New York, USA). The mice were genotyped by using tail DNA and Southern Blot Analysis. Pre-leukemic mice were sacrificed and the kidneys and spleen removed for whole genomic DNA isolation using the Wizard DNA Isolation Kit (Promega, Madison, WI, USA). Tumor tissue isolated from mice that had undergone leukemic transformation was similarly processed.

STI571 Kinase Inhibitor:

The c-Abl specific kinase inhibitor, STI571 (formally known as CGP57148B), was provided by Novartis Pharmaceuticals (Basel, Switzerland). A stock solution of STI571 was prepared by dissolving 35.7mg of STI571 in 1 mL of 100% DMSO. The injection solution was prepared by making a final concentration of 10% DMSO and STI571 of the initial stock in sterile PBS. Mice were weighed and injected intraperitoneally with 50 mg/kg of STI571 daily for 10 consecutive days. This dose of STI571 has been previously shown to completely inhibit Bcr/Abl in a mouse tumorigenic assay.[16]

PCR:

PCR amplification was carried out using 1 μM of the RG primer ($(CA)_8RG$); 50ng genomic DNA; 0.3 units Taq polymerase (Gibco, Rockville, MD, USA) and 1μCi $^{32}P\alpha dCTP$ (250 μCi)(Amersham, Arlington Heights, IL, USA), in a 20μL total mix of PCR buffer (10mM tris-HCL, pH 9.0; 2% formamide; 50mM KCL; 0.2mM dNTPs; 1.5mM $MgCL_2$; 0.01% gelatin; 0.01% triton X-100). The primer RG, consists of eight CA repeats anchored by two nucleotides, where G is guanine and R is a 50:50 mix of the purines adenine (A) and guanine (G) (Fig. 1A).

Amplification was performed using a Perkin Elmer Cycler (Cetus), with an initial denaturation for 3 minutes at 94°C; followed by 30 PCR cycles at 94°C for 30 seconds, at 52°C for 45 seconds, and at 72°C for 2 minutes. A final extension at 72°C was performed for 7 minutes.

Gel Analysis:

The PCR product was loaded on an 8% nondenaturing polyacrylamide gel, run at 1500 constant volts, dried and exposed to film (Biomax, Kodak, Amersham, Arlington Heights, IL, USA) at room temperature for two days. The gels were analyzed using one normal control as a standard for each tissue (Fig. 1B). Using this control, an average of 20 bands were counted and compared to each sample run to count insertions and deletions (Fig 1C). Repeat analysis of all samples was performed (average of five PCR reactions per tissue sample) to minimize the effect of experimental variability. Data analysis was completed and the statistical calculations were carried out using Microsoft Excel and SigmaPlot.

Results

Demonstration of Genomic Instability in Pre-leukemic P190$^{Bcr/Abl}$ Transgenic Mice Spleen: In order to determine whether the P190$^{Bcr/Abl}$ transgenic mice accumulate mutations in lymphocytes before developing B-lymphocyte leukemia/lymphoma, we examined DNA isolated from the spleen of pre-leukemic P190$^{Bcr/Abl}$ transgenic mice for the presence of insertions and deletions using Inter-SSR PCR. The results obtained are shown and compared to that observed in normal control spleen and B-cell tumors and spleens from P190$^{Bcr/Abl}$ mice that had undergone leukemic transformation (Fig. 2A). Whereas no insertions or deletions were observed in control mice spleen DNA, spleens from P190$^{Bcr/Abl}$ pre-leukemic mice contained an average of three such events. The observed rate of insertions and deletions in P190$^{Bcr/Abl}$ pre-leukemic mice was approximately half that observed in tumors and leukemia infiltrated spleens. These results suggest that Bcr/Abl expression can directly induce genomic instability characterized by DNA insertions and deletions before leukemic transformation. To examine whether this instability could be decreased using a c-Abl specific inhibitor, mice were injected with the inhibitor STI571. We observed an increased frequency of insertions and deletions in control mice injected with STI571 that was approximately of the same magnitude as that seen in pre-leukemic P190$^{Bcr/Abl}$ mice with or without treatment with STI571. This was an unexpected result that raises the possibility that the inhibition of normal c-Abl may be mutagenic in normal spleen lymphocytes (ie. STI571 is equally effective at inhibiting normal Abl and Bcr/Abl proteins). This observation

could be in keeping with the tumor suppressor role some investigators have assigned to c-Abl.

Demonstration of Genomic Instability in Pre-leukemic P190$^{Bcr/Abl}$ Transgenic Mice Kidney: In order to determine whether the P190$^{Bcr/Abl}$ transgenic mice similarly accumulated mutations in non-lymphoid, non-transforming Bcr/Abl expressing tissues, we examined DNA isolated from pre-leukemic P190$^{Bcr/Abl}$ transgenic mouse kidneys using inter-SSR PCR (Fig. 2B). Previous studies in our laboratory have shown high expression of Bcr/Abl in the kidney by Western Blot analysis and demonstrated that this is associated with an increased frequency of point mutations even though oncogenic transformation is restricted to B-lymphocytes. We observed an increased frequency of altered bands in the pre-leukemic P190$^{Bcr/Abl}$ mice compared to control. A basal rate of insertions and deletions could be detected in normal control kidney DNA that was not observed in normal spleen tissue.

In contrast to what we observed in normal spleen tissue, the basal mutation rate was not increased in the kidneys of mice treated with STI571. This difference might be explained by the masking of a mutagenic STI571 effect by the basal mutation rate observed in control kidney, insensitivity of kidney tissue to the loss of normal c-Abl function, or by some other mechanism. The mutation frequency in preleukemic kidney tissue was higher than that observed in spleen and it was possible to show that this could be partially reduced with STI571 treatment.

Combined, these results confirm the ability of Bcr/Abl to directly increase the frequency of insertions and deletions. Although our results indicate the potential beneficial effect of STI571 to decrease this by inhibiting Bcr/Abl, the observed increase

in mutation frequency in normal spleen tissue of mice treated with STI571 raises some concern that inhibition of normal c-Abl may be mutagenic in susceptible cells.

genotoxic stress at these levels of Bcr/Abl in preleukemic mice.[20] Yet the level of Bcr/Abl expression is sufficient to induce leukemia. The only abnormality that we have observed is a subtle abnormality of bone marrow B-lymphocyte development.[21] We have shown in a previous study using this animal model system a 2-3 fold increase in the point mutation frequency in the spleen and kidney.[12] In the present study, using Inter-SSR PCR, we were able to show that the increased frequency of insertions and deletions occur in both spleen and kidney tissues before neoplastic transformation, providing strong evidence that Bcr/Abl can directly induce genomic instability. The mutation frequency in kidney was greater than that observed in spleen tissue and was consistent with the higher expression of Bcr/Abl observed.

The mechanism(s) responsible for the Bcr/Abl associated mutator phenotype that we describe is unknown but could involve functional disruption of a DNA repair protein or pathway. Recent studies have shown altered regulation of replication factor C (RFCp140) in CML clinical samples and Bcr/Abl expressing cell lines.[22] RFCp140 plays a significant role in DNA recombination and repair as part of a larger protein complex by recruiting PCNA and attaching it to single stranded DNA.[23,24] Recent studies have shown that RFC is important for efficient binding of PCNA for catalyzed elongation of the singly primed DNA template but RFC is not required for repair to occur. When mutants of PCNA were constructed for the binding regions of RFC, elongation still took place but was not as efficient.[25] Other studies indicate that c-Abl may play an important role in the regulation of the cell cycle and cell response to genotoxic stress. DNA damage causes activation of ataxia-telangiectasia mutated (ATM) kinase which phosphorylates both c-Abl and p53 and results in their activation.[17,18,26] Activated c-Abl in turn phosphorylates the recombination repair associated protein Rad51, and the p53 homologous protein

observation and the risk of inducing secondary cancers from the pharmacological inhibition of c-Abl in normal tissues deserves further investigation.

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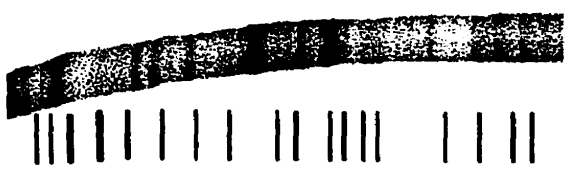
Figure Legends

Figure 1 A: Inter-Simple Sequence Repeat (SSR) PCR is a highly effective method for detecting differences within $(CA)_n$ repeats. The two primers consist of CA repeats of different lengths and amplify the region of DNA within these repeats. **B:** Gel banding patterns observed for normal mouse spleen (number 72) and kidney (number 95). The eighteen bands shown were used to score for the presence of insertions and deletions in test samples. **C:** Typical RG primer banding pattern from kidney samples, of control and a P190 transgenic mouse. A deletion is present in the $P190^{Bcr/Abl}$ mouse sample.

Figure 2 A: Observed Insertions and Deletions from samples amplified with the RG primer. Samples include, control spleen (Control,n=3) from normal mouse strain (BL6/CBA) and pre-leukemic (PL, n=5) or leukemic spleens (L Spl, n=3) from $P190^{Bcr/Abl}$ transgenic mice with (Inj, n=3) or without injection (n=3) with the kinase inhibitor, STI571. Also included are tumor samples from lymphatic tissue (Tumor, n=9). The error bars indicate the standard deviation from the mean. The difference between the control and pre-leukemic spleen is significant (p=0.0006). **B:** Observed Insertions and Deletions from samples amplified with the RG primer. Samples include, control kidney (Control, n=3) from normal mouse strain (BL6/CBA) and pre-leukemic (PL, n=5) kidney (Kid) from $P190^{Bcr/Abl}$ transgenic mice with injection (Inj) (n=3) or without injection (n=3) with the kinase inhibitor STI571. The error bars indicate the standard deviation from the mean. The difference between the control and pre-leukemic kidney is significant (p=0.007). The difference between the control injected kidney and the pre-leukemic kidney is significant (p=0.045).

Figure 1B

Normal 72 Spleen



Normal 95 Kidney

Figure 1C

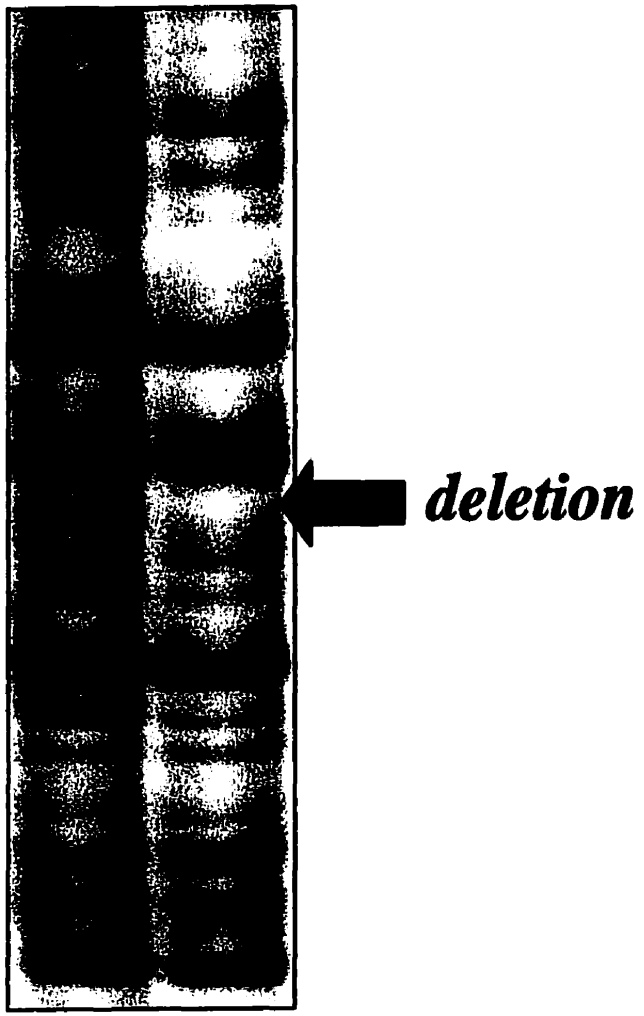


Figure 2A

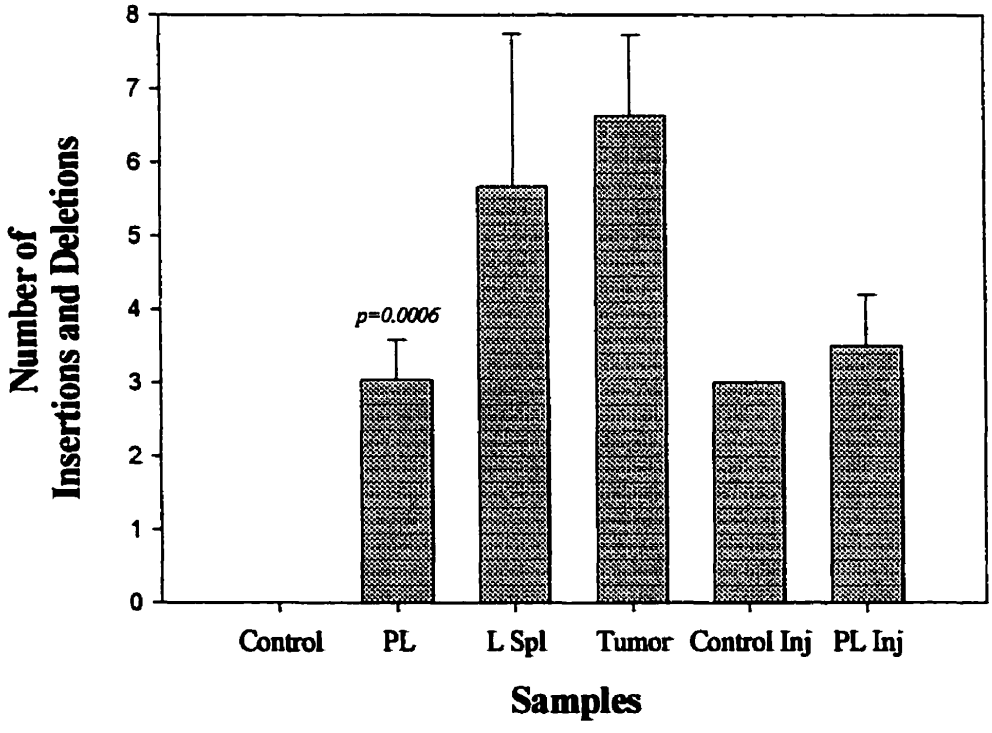
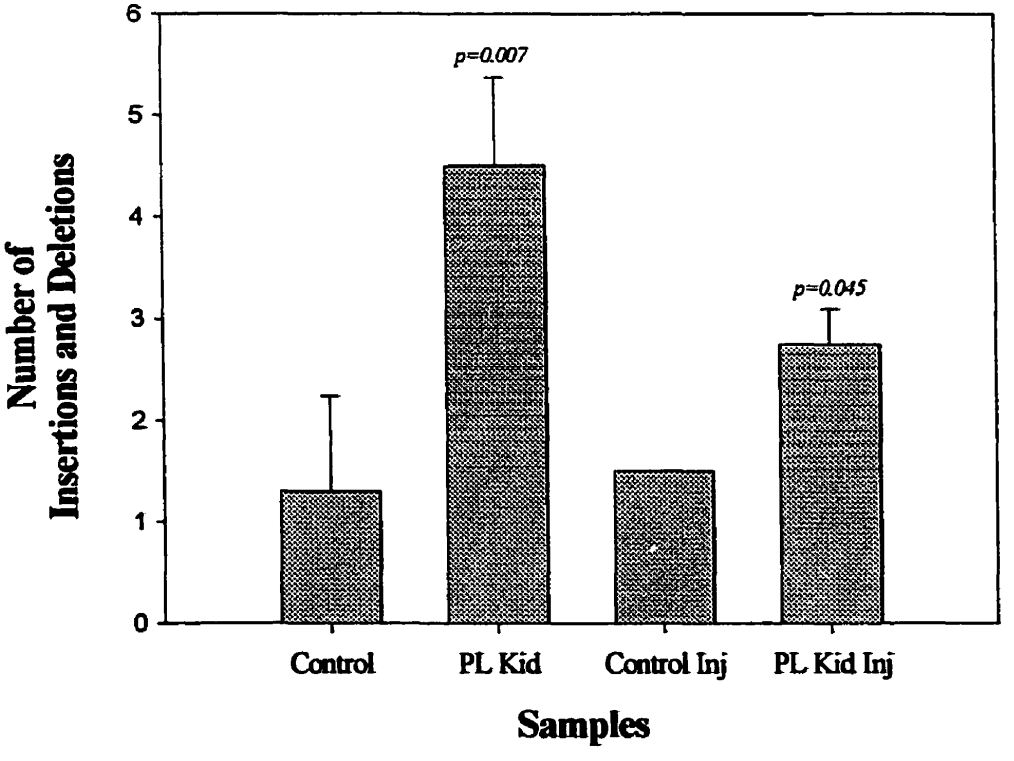

Number of Insertions and Deletions
8
7
6
5
4
3
2
1
0
p=0.0006
Control
PL
L Spl
Tumor
Control Inj
PL Inj
Samples

Figure 2B


Number of Insertions and Deletions
6
5
4
3
2
1
0
p=0.007
p=0.045
Control
PL Kid
Control Inj
PL Kid Inj
Samples

Chapter III The Inhibitor STI571 Reverses Bcr/Abl-Induced Point Mutation Frequencies in Pre-Leukemic $P190^{Bc/Abl}$ Transgenic Mice

Preface

In the previous chapter, altered bands (insertions and deletions) were observed in the pre-leukemic and leukemic $P190^{Bcr/Abl}$ transgenic mice. These bands were also found to decrease upon treatment with STI571. In this chapter, we further address this genomic instability associated with Bcr/Abl to observe point mutations as they occur in the $P190^{Bcr/Abl}$ transgenic mice. We take this one step further to see if these point mutations or mutation frequencies could be reversed using the inhibitor STI571. The measurement of endogenous point mutation rates in these animals was performed during the pre-leukemic period when Bcr/Abl was widely expressed before there were any signs of leukemic transformation. A two to three fold increase in point mutation frequency was observed in mice expressing $P190^{Bcr/Abl}$ compared to control mice. We further these studies to see if these observed mutation frequencies could be decreased with the inhibitor STI571. The point mutation frequency in spleen and kidney tissues of treated animals was significantly decreased compared to control. These results, along with the results in chapter II, illustrate that Bcr/Abl is responsible for this observed genomic instability and that this can be decreased using the c-Abl specific kinase inhibitor STI571.

Abstract

Chronic myelogenous leukemia (CML) and 25% of adult onset acute lymphoblastic leukemia (ALL) are associated with the expression of Bcr/Abl, a constitutively activated protein tyrosine kinase. Bcr/Abl associated leukemias are characterised by a high degree of chromosomal and genomic instability. It is unclear if the phenotype of genomic instability is a primary consequence of Bcr/Abl expression or if it is acquired secondarily. We have attempted to answer this question in previous studies by measuring the frequency of point mutations in double heterozygote transgenic mice derived from mating heterozygous $P190^{Bcr/Abl}$ transgenic mice (line 623) and the Big Blue Mice® (Stratagene). Our results showed a two to three fold increase in the point mutation frequency in pre-leukemic (ie. about 100 days before the onset of leukemia) P190 mice, compared to control mice (C57/BL6). In the present report, we extended these prior studies to ascertain if Bcr/Abl induced point mutations is a reversible phenotype. Pre-leukemic $P190^{Bcr/Abl}$/Big Blue double heterozygous and C57/BL6 control mice were injected with the c-Abl specific kinase inhibitor STI571 for ten consecutive days. We observed a decrease in the Bcr/Abl induced mutation frequencies in spleen and kidney tissue from mice treated with STI571. These results confirm that Bcr/Abl can directly and reversibly induce an increase in point mutation frequencies that could contribute to the genomic instability observed in Bcr/Abl positive leukemias.

Introduction

The cytogenetic hallmark of Bcr/Abl associated leukemias is the presence of the Philadelphia chromosome (Ph), representing the reciprocal translocation t(9;22).[1] At the molecular level, the proto-oncogene *c-ABL* normally residing on chromosome 9 is rearranged in a head to tail orientation in the *BCR* (break cluster region) gene on chromosome 22. The resulting chimeric *BCR/ABL* gene expresses a fusion Bcr/Abl protein, with a constitutively activated protein tyrosine kinase. Depending on the location of the breakpoint in the *BCR* gene, Bcr/Abl proteins of 190kDa, 210kDa, or 230kDa may be created which are most commonly associated with acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), and chronic neutrophilic leukemia (CNL) respectively.[2] In some instances, *Abl/Bcr* gene products may also be co-expressed from the derivative chromosomal breakpoint.[3] This translocation event results in the loss of one copy each of the normal *c-ABL* and *BCR* genes. The functions of Bcr and c-Abl are not well characterized. The ubiquitously expressed Bcr protein is known to act as a negative regulator of oxidative burst in neutrophils and a homozygous deletion of its function in transgenic knock-out mice renders the animals susceptible to gram negative endotoxin mediated septic shock.[4] In contrast, the c-Abl proto-oncogene has been implicated in the cell cycle response to genotoxic stress, DNA repair, and exhibits characteristics of a tumor suppressor gene.[5;6] Some of the major activities attributed to Bcr/Abl include the activation of mitotic signal transduction pathways, inhibition of apoptosis, decreased adhesion of bone marrow stromal elements and the induction of

increased cellular motility.[2] Though it is clear that expression of Bcr/Abl is the main leukemogenic event in Bcr/Abl associated leukemias, the potential contribution of loss of function of *BCR* and *c-ABL* to the pathogenesis of these leukemias remains to be determined.

Genomic instability is a key clinical feature of Bcr/Abl associated leukemias. Patients with Ph-positive ALL have a significantly worse prognosis than patients who do not express Bcr/Abl.[7] Patients with CML, however best exhibit this genomic instability. As the disease progresses from a chronic to an acute terminal blast phase, cells frequently acquire secondary cytogenetic abnormalities. These include, alteration of DNA methylation, and develop mutations of a wide variety of genes including the loss of tumor suppressors genes such as p53 and Rb.[8] The natural history of CML illustrates the general progression of neoplastic tumors, namely that a succession of genetic events are required for disease progression. In some instances it is clear that an initial early event can increase the rate at which secondary genetic events will occur (ie. hereditary mismatched DNA repair defects in colon cancer).[9] Studies conducted in our laboratory suggest that the expression of Bcr/Abl may be directly mutagenic and can induce chromosomal abnormalities.[10-12] In one set of experiments we generated double heterozygote transgenic mice that expressed the 190kDa Bcr/Abl protein which contain a λLIZ shuttle vector permitting measurement of the frequency of mutations of a *LacI* gene insert *in vivo* using the Big Blue Mutagenesis Assay System (Stratagene). The measurement of endogenous point mutation rates in these animals was performed during the pre-leukemic period when Bcr/Abl was widely expressed before there were any signs of leukemic transformation. A two to three fold increase in the point mutation frequency

was observed in mice expressing P190 Bcr/Abl compared to control mice, thus providing strong evidence that the expression of Bcr/Abl may be directly mutagenic.[13]

In the present report, we utilize our pre-leukemic $P190^{Bcr/Abl}$ transgenic animal model to assess whether Bcr/Abl associated mutagenesis could be reversed with the c-Abl specific protein tyrosine kinase inhibitor STI571 (Novartis). STI571 has recently been approved for clinical use after demonstrating high efficacy in the treatment of CML and Ph positive ALL.[14;15] The successful reversal of Bcr/Abl associated mutagenesis by STI571 would raise expectations of the drugs ability to halt or decrease the rate of tumor progression in Bcr/Abl associated leukemias in the longer term. Pre-leukemic mice derived by mating of homozygous $P190^{Bcr/Abl}$ and Big Blue transgenic mice were injected with 50 mg/kg of STI571 for ten consecutive days. The point mutation frequency in spleen and kidney tissues of treated animals was significantly decreased compared to control. These results confirm that Bcr/Abl induced point mutagenesis is a direct and at least partially reversible phenotype that should translate in reduced risk of leukemic progression in Bcr/Abl associated leukemias.

Materials and Methods

STI571 Kinase Inhibitor:

The kinase inhibitor, STI571 (formally known as CGP57148B), was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). A stock solution of STI571 was prepared by dissolving 35.7mg of STI571 in 1mL of 100% DMSO. The injection was prepared by making a final concentration of 10% DMSO and STI571 of the initial stock in sterile PBS. Mice were weighed and injected intraperitoneally with 50mg/kg of STI571 daily for 10 consecutive days. This dose of STI571 has been previously shown to completely inhibit Bcr/Abl in a mouse tumorgenic assay.[16]

Transgenic Mice:

We utilized a leukemic mouse model, known as the transgenic line 623 $P190^{Bcr/Abl}$.[17] Genotyping of the mice was performed using the standard Southern Blot method. In brief, a one inch piece of tail was cut from each mouse and placed in tail buffer, including proteinase K (Stratagene, San Diego, USA). DNA was extracted using the phenol/chloroform method, followed by an ethanol wash. The pure DNA was overnight digested with two restriction enzymes, *XhoI* and *HindIII* (Gibco, Life Technologies, Grand Island, NY). After a brief re-precipitation with ethanol, the samples were run on a 1% agarose gel at 30V overnight. The gel was transferred to a Hybond-N+ (Amersham, Illinois, USA) Nylon Transfer Membrane using 0.25N HCL and 0.40M NaOH. The probe consisted of a 600bp fragment (*XhoI* (X)/*BglII* (Bg)) of *bcr* exon (E)1. The probe was labeled using the $^{32}P\alpha dCTP$ (250μCi) (Amersham, Illinois, USA) and the Stratagene Prime-It RMT Random Primer Labeling Kit (Stratagene, San Diego,

CA). The membrane was probed overnight at 42°C and was washed and exposed to film at -80°C for 2-3 days.

The $p190^{bcr\text{-}abl}$ mice were crossed with the Big Blue transgenic mouse (Stratagene) to create Big Blue x $p190^{bcr\text{-}abl}$ double heterozygotes. As a control, we also crossed a Big Blue x C57/BL6 mouse. The Big Blue Transgenic mouse is a reporter mouse, which contains a λLIZ shuttle vector. The λLIZ shuttle vector contains the *Lac*I gene and the α–portion of the *LacZ* gene. The *Lac*I gene in the shuttle vector is the target for mutagenesis. The α*LacZ* gene in the shuttle vector, which encodes the α–portion of β–galactosidase, is the reporter gene. The *Lac*I gene encodes a region of the lac repressor protein, which represses transcription of the *LacZ* gene. Mutations in the *Lac*I gene that inactivate the lac repressor protein allow transcription of the *LacZ* gene, resulting in production of β–galactosidase, the reporter protein. After injection of the double heterozygote mice with 50mg/kg of STI571 for 10 days, the mice were then sacrificed and the kidneys and spleens removed.

Big Blue Assay System-DNA extraction:

Extraction of whole genomic DNA was done using the RecoverEase™ DNA Isolation Kit (Stratagene). Kidney and spleen were homogenized using a Wheaton Dounce tissue grinder and 7ml of lysis buffer (Stratagene). The tissue was put through a cell strainer and into a conical tube. The samples were then centrifuged at 11,000 x g for 12 minutes at 4°C. The supernatant was discarded and the cell nuclei pellet was allowed to dry. The pellet was incubated at 50°C for 45 minutes with proteinase K (Stratagene) and 20μl RNAce-It™ ribonuclease cocktail/ml of digestion buffer (Stratagene). The samples were then transferred to a dialysis cup (Stratagene) and dialyzed in 500ml of TE

buffer (10mM tris-HCL (pH 7.5)/ 1mM EDTA) per sample, while stirring the buffer gently with a magnetic stir bar. The samples were then stored in a sterile microcentrifuge tube at 4°C.

Big Blue Assay System -Packaging of Shuttle Vector:

LB-tetracycline agar plates were streaked with the bacteria from the glycerol stock, E.coli line SCS-8 (Stratagene). The plate was incubated overnight in a stationary 37°C air incubator. In a 50ml conical tube, a bacterial colony was added to 20ml of NZY broth (NaCl, $MgSO_4 \cdot 7H_20$, yeast extract, casein peptone) and 250μL of a 20% (w/v) maltose and 1 M $MgSO_4$ solution. This was incubated at 37°C for 4-6 hours, while shaking at 200rpm. The culture was then centrifuged at 1000 x g for 10 minutes to pellet the bacterial cells. The supernatant is discarded and the pellet is resuspended in 10ml of sterile 10mM $MgSO_4$. The bacterial cells were resuspended in 10mM $MgSO_4$ to a density of A_{600}=0.50.

The λLIZ shuttle vector was rescued using the Transpack® Packaging Extract Kit (Stratagene). This kit is a patent from Stratagene, and contains two 90 minute incubation periods at 30°C, with two pre-weighted tubes. The samples are resuspended to make a final volume of 1ml with SM buffer (NaCl, $MgSO_4 \cdot 7H_20$, 1M tris-HCl, 2%(w/v) gelatin) and 50μL chloroform.

Plate for Lytic Growth:

The shuttle vector containing the packaged DNA was then plated for lytic growth on an E.Coli (SCS-8 line, Stratagene) and 5-Bromo-4-Chloro-3-Indolyl-ß-D-galactopyranoside (X-gal) (Fisher) containing agar (Big Blue Media, Stratagene) lawn.

Mutation Frequencies:

Results

Effects of STI571 on the Kidney of P190$^{Bcr/Abl}$ Pre-Leukemic Mice

In order to determine whether the P190$^{Bcr/Abl}$ transgenic mice accumulate mutations in non-lymphoid, non-transforming Bcr/Abl expressing tissues, and to see if these mutations could be reversed, we examined mutation frequencies of DNA isolated from pre-leukemic P190$^{Bcr/Abl}$ x Big Blue transgenic mice (Table 1). A total of 77,400 to 190,585 plaques were counted for each mouse sample. The baseline mutation frequency for the normal kidneys was 2.19×10^{-5} (SD$\pm$1.16). In the pre-leukemic kidneys there was an increase in the mutation frequency, as previously described, at 6.70×10^{-5} (SD$\pm$1.10). After injection with the c-Abl inhibitor STI571, there was a slight increase in the normal kidneys, 2.46×10^{-5} (SD$\pm$0.52), with a decrease in the pre-leukemic kidneys, 5.08×10^{-5} (SD$\pm$1.38).

Effects of STI571 on the Spleen of P190$^{Bcr/Abl}$ Pre-Leukemic Mice

In order to determine whether the P190$^{Bcr/Abl}$ transgenic mice accumulate mutations in lymphocytes before developing B-lymphocyte leukemia/lymphoma, and to see if these mutations could be reversed, we examined mutation frequencies from spleen DNA isolated from pre-leukemic P190$^{Bcr/Abl}$ x Big Blue transgenic mice (Table 2). A total of 72,000 to 225,723 plaques were counted for each mouse sample. The baseline mutation frequency for the normal spleens was 2.70×10^{-5} (SD$\pm$0.80). In the pre-leukemic spleen there was an increase in the mutation frequency, as previously described, at 6.53×10^{-5} (SD$\pm$3.87). After injection with STI571, there was a slight increase in the normal spleen,

2.90×10^{-5} (SD±0.57), with a decrease in the pre-leukemic spleens, 2.90×10^{-5} (SD±0.64).

Effects of STI571 on the Kidney and Spleen of $P190^{Bcr/Abl}$ Pre-Leukemic Mice

In order to determine the overall effect of the inhibitor on both tissues combined, we compared injected tissue with the inhibitor versus non-injected tissue (Fig. 1). The difference between the baseline (normal mice) mutation frequencies and the pre-leukemic mice are highly significant ($p=0.010$), meaning that the pre-leukemic $P190^{Bcr/Abl}$ mice are accumulating mutations before leukemic onset. In order to determine whether this increase in the mutation frequency could be reversed or decreased upon injection, we compared the injected mice to the non-injected mice, showing no effect in the normal mice upon injection with the inhibitor. However, there is a decrease in the mutation frequency in the pre-leukemic injected mice compared to the non-injected pre-leukemic mice and this is highly significant ($p=0.042$).

Discussion

Whether Bcr/Abl is a direct inducer of genomic instability or is secondarily acquired within the evolution of this disease has been the subject of a recurring debate.[7] In the present study, we addressed this question by taking advantage of the $P190^{Bcr/Abl}$ line 623 transgenic mice, which express Bcr/Abl before developing leukemia with an average latency of 100 days after birth. This system makes it possible to investigate the effect of Bcr/Abl expression in different tissues without confounding a direct effect with the secondary abnormalities that might arise following neoplastic transformation. We have shown in a previous study using this animal model system a 2-3 fold increase in the point mutation frequency in the spleen and kidney.[13] We extended these experiments in the current report to determine if the increase in Bcr/Abl associated mutation frequency could be decreased with the c-Abl specific kinase inhibitor STI571. Significant inhibition was observed confirming that the mutator phenotype associated with Bcr/Abl is a direct consequence of its activity.

The mechanism(s) responsible for the Bcr/Abl associated mutator phenotype that we describe is unknown but could involve functional disruption of a DNA repair protein or pathway. Recent studies have shown altered regulation of replication factor C (RFCp140) in CML clinical samples and Bcr/Abl expressing cell lines.[18] RFCp140 plays a significant role in DNA recombination and repair as part of a larger protein complex by recruiting PCNA and attaching it to single stranded DNA.[19;20] Additional studies have shown that RFC is important for efficient binding of PCNA for catalyzed

there has been some concern about the potential for STI571 to be mutagenic or increase the risk of secondary malignancy.[24;31] We did not observe an increased rate of point mutagenesis in our control mice treated with STI571. This does not exclude the possibility that a mutagenic effect could be observed under different experimental conditions or using a different mutation detection system, therefore this issue merits further study.

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Figure Legends

Table 1: Mutation Frequencies in the kidneys of $P190^{Bcr/Abl}$ Pre-leukemic mice and Normal control mice (C57/BL6). The injected mice were administered STI571 at 50mg/kg mouse per day for ten consecutive days.

Table 2: Mutation Frequencies in the spleen of $P190^{Bcr/Abl}$ Pre-leukemic mice and normal control mice (C57/BL6). The injected mice were administered STI571 at 50mg/kg mouse per day for ten consecutive days.

Figure 1: Combined mutation frequencies in both the kidney and spleen combined for normal control (Normal) and $P190^{Bcr/Abl}$ Pre-leukemic mice (P190) with (Inj) and without injection with the inhibitor STI571.

Table 1:

Injected	Mouse	Total pfu	Mutant plaques	Mutation Frequency x 10^{-5}	Mean x 10-5 $\pm$ s.d.
No	P190-99	110,336	6	5.44	6.70 $\pm$ 1.10
No	P190-103	190,585	9	7.43	
No	P190-230	123,063	9	7.31	
No	Normal-48	139,886	6	3.40	2.19 $\pm$ 1.16
No	Normal-40	164,590	6	2.10	
No	Normal-62	93,216	1	1.07	
Yes	P190-113	77,400	5	6.46	5.08 $\pm$ 1.38
Yes	P190-116	99,584	4	4.00	
Yes	P190-182	150,252	7	4.60	
Yes	Normal-73	141,558	4	2.82	2.46 $\pm$ 0.52
Yes	Normal-72	106,998	2	1.86	
Yes	Normal-118	111,030	3	2.70	

Table 2:

Injected	Mouse	Total pfu	Mutant plaques	Mutation Frequency x 10^{-5}	Mean x 10-5 $\pm$ s.d.
No	P190-99	117,094	13	11.0	6.53 $\pm$ 3.87
No	P190-85	72,000	3	4.16	
No	P190-230	225,723	10	4.43	
No	Normal-40	99,532	2	2.00	2.70 $\pm$ 0.80
No	Normal-48	108,844	4	3.60	
No	Normal-62	112,327	3	2.67	
Yes	P190-113	149,399	4	2.60	2.40 $\pm$ 0.57
Yes	P190-116	160,274	3	1.80	
Yes	P190-182	136,766	4	2.92	
Yes	Normal-72	141,860	5	3.50	2.90 $\pm$ 0.64
Yes	Normal-118	124,227	4	3.22	
Yes	Normal-73	88,187	2	2.26	

Chapter IV Genomic Instability in an Induced $P210^{Bcr/Abl}$ Transgenic Mouse Using Inter-SSR PCR Genomic Scanning

Preface

In the two previous chapters, altered bands (insertions and deletions) and an increase in mutation frequencies were observed in the pre-leukemic and leukemic $P190^{Bcr/Abl}$ transgenic mice. These bands and mutation frequencies were also found to decrease upon treatment with STI571. In this chapter, we further address this genomic instability associated with Bcr/Abl to observe altered bands as they occur in inducible $P210^{Bcr/Abl}$ transgenic mice. Blood samples were taken at several timepoints from the $P210^{Bcr/Abl}$ transgenic mice to observe altered bands (insertions and deletions) as these mice express $P210^{Bcr/Abl}$. A sharp increase in altered bands was observed in these mice upon expression of Bcr/Abl. The results, of chapters II and III, illustrate that Bcr/Abl is responsible for this observed genomic instability and that this can be decreased by using the c-Abl specific kinase inhibitor STI571. The results in this chapter, take these studies one step further to include $P210^{Bcr/Abl}$ inducible mice. These mice exhibited an increase in altered bands upon expression of $P210^{Bcr/Abl}$. This further confirms that Bcr/Abl is solely responsible for this observed genomic instability, and confirms that these observations occur in both the P210kDa and the P190kDa weights of the Bcr/Abl protein.

Abstract

The hallmark of chronic myelogenous leukemia (CML) is a translocation between chromosome 9 and chromosome 22. Chromosome 22 gives rise to a protein known as Bcr, while chromosome 9 gives rise to a proto-oncogene, known as c-Abl. These two proteins combine to form a Bcr/Abl fusion protein which contains a constitutively activated protein tyrosine kinase. Depending upon the breakpoint within the *bcr* gene, several length fusion proteins will manifest, the most common are the P190, P210 or P230kDa lengths, these give rise to different leukemic diseases, acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML) and chronic neutrophilic leukemia (CNL), respectively. Bcr/Abl leukemias are characterized by a high incidence of genomic instability. In order to determine whether *Bcr/Abl* is the initiator of this instability, we utilize a system known as Inter-SSR PCR to address genomic instability in a $P210^{Bcr/Abl}$ transgenic mouse with a tetracycline repressible system. Inter-SSR PCR is a method for genomic scanning, which utilizes primers which consist of CA repeats. After multiple cycles of PCR amplification, it becomes feasible to detect instability of the genome by observing insertions and deletions within the DNA. Here, we took blood samples at several timepoints from the $P210^{Bcr/Abl}$ repressible mice, to look for insertions and deletions within the genome upon repression and expression of Bcr/Abl. We observe a sharp increase in altered bands, insertions and deletions, in mice after expression of Bcr/Abl in the $P210^{Bcr/Abl}$ mice compared to control mice. These results clearly demonstrate that this disease is Bcr/Abl dependent and that *Bcr/Abl* is the initial cause of genomic instability seen in the Ph+ leukemias.

Introduction

Chronic myelogenous leukemia (CML) is a disease characterized by a translocation involving chromosome 9 and chromosome 22, known as the Philadelphia Chromosome. Chromosome 22, transcribes a protein known as Bcr, while chromosome 9 gives rise to a proto-oncogene, known as *c-abl*.[1] The independent roles of both Bcr and c-Abl still remain unclear, however, one role attributed to Bcr is as a negative regulator of oxidative burst in B cells and in neutrophils.[2] The *c-abl* proto-oncogene, may have several important roles in DNA repair and may function as a tumor suppressor, as it has been associated with G1/S arrest.[3; 4] The product of these two genes forms a fusion protein, known as Bcr/Abl. This fusion protein has several molecular weights, the most common being the P210kDa length, which is associated with CML and the P190kDa length, which is more commonly associated with acute lymphocytic leukemia (ALL).[5] In each case, the fusion protein gives rise to the protein tyrosine kinase, c-Abl, which becomes constitutively activated. The effects this fusion protein, and therefore this activated protein tyrosine kinase, have on the hematopoietic system specifically, is yet to be elucidated. It is clear however, through several signal transduction studies both *in vitro* and *in vivo*, that Bcr/Abl enhances proliferation, prolongs viability and alters cell adhesion and mobility to induce transformation.[5]

This disease has an inevitable progression from the chronic to acute phase. During the transition from chronic to blast crisis, a high frequency of secondary chromosomal abnormalities have been reported, which include the loss of tumor suppressor genes, including the p53 or retinoblastoma (Rb) genes.[6-9] These chromosomal

abnormalities suggest that these additional genetic events may be responsible for the progression to blast crisis. A major interest therefore, is to examine the role of Bcr/Abl in genomic instability as it occurs during the transition of this disease.

Our laboratory has previously addressed genomic instability by using 32D cell lines which express $P210^{Bcr/Abl}$, by which we show that Bcr/Abl does indeed alter the stability of the genome. These cells exhibit cytogenetic abnormalities, show cell cycle abnormalities and inhibition of apoptosis.[10] We also addressed genomic instability by utilizing the $P190^{Bcr/Abl}$ transgenic mouse (line 623) and the Big Blue Assay System (Stratagene)[11]. Our results show a two to three fold increase in point mutation rate observed in $P190^{Bcr/Abl}$ x Big Blue pre-leukemic mice (about 100 days, before onset of leukemia) compared to control mice (C57/BL6).[12]

We also addressed this question of genomic instability by utilizing Inter Simple sequence Repeat Polymerase Chain Reaction (Inter SSR-PCR), in combination with primers which consist of a set of eight CA repeats (Fig. 1A).[13] These CA repeats appear in all species tested and are the most frequent repeats in the human genome, with an estimated copy number of 50,000 to 100,000 per haploid genome.[14] Using Inter-SSR PCR we compared the $P190^{Bcr/Abl}$ pre-leukemic and leukemic mice to the control mice (BL6/CBA), and found an increase in altered bands (insertions and deletions) in the pre-leukemic and leukemic mice compared to control. We also showed that these altered bands can be decreased using the c-Abl specific kinase inhibitor, STI571 (Novartis) *(Brain et al., submitted data).*

In this study, we again utilize the Inter-SSR PCR system to address genomic instability (Fig. 1B). In these studies, we use a $P210^{Bcr/Abl}$ transgenic mouse with a tetracycline repressible system. Double transgenic mice (*BCR-ABL1*-tetracycline

transactivator (*tTA*)) were generated by breeding female transresponder mice with male mouse mammary tumor virus (*MMTV*)-*tTA* transactivator mice under continuous administration of tetracycline (0.5g/l) in the drinking water, starting five days before mating. Withdrawal of tetracycline administration in double transgenic animals allowed expression of *BCR-ABL1* and resulted in the development of lethal leukemia in 100% of the mice within a time frame that was consistent with each line.[15]

Our results show an increase in insertions and deletions when the tetracycline is withdrawn from the $P210^{Bcr/Abl}$ transgenic mice and therefore Bcr/Abl is expressed. These results confirm the previous results observed in the $P190^{Bcr/Abl}$ transgenic mice and reconfirm that Bcr/Abl is required for induction and maintenance of disease and that Bcr/Abl itself is responsible for this instability seen upon disease progression.

Methods

Transgenic Mice/DNA Isolation:

Two founder mouse lines (F.2 and F.27) were used with several controls for each line (table 1). The derivation and phenotype of these mouse lines has been extensively described elsewhere.[15] Mice were bled at thirty day intervals with or without tetracycline administration, to express or suppress Bcr/Abl expression. Whole genomic DNA was isolated using the Wizard DNA Isolation Kit (Promega, Madison, WI, USA).

PCR:

PCR amplification was carried out using 1 μM of the primer $(CA)_8RG$; 50ng genomic DNA; 0.3 units Taq polymerase (Gibco, Rockville, MD, USA) and 1μCi $^{32}P\alpha dCTP$ (Amersham, Arlington Heights, IL, USA), in a 20μL total mix of PCR buffer (10mM tris-HCL, pH 9.0; 2% formamide; 50mM KCL; 0.2mM dNTPs; 1.5mM $MgCl_2$; 0.01% gelatin; 0.01% triton X-100). The primer RG, consists of eight CA repeats anchored by two sets of nucleotides, where R is a 50:50 mix of the purines adenine and guanine and where G is guanine.

The reaction was amplified using a Perkin Elmer Cycler (Cetus), with an initial denaturation for 3 minutes at 94°C; followed by 30 PCR cycles at 94°C for 30 seconds, at 52°C for 45 seconds, and at 72°C for 2 minutes. A final extension at 72°C was performed for 7 minutes.

The PCR product was loaded on an 8% nondenaturing polyacrylamide gel, run at 1500 constant volts, dried and exposed to film (Biomax, Kodak, Amersham, Arlington Heights, IL, USA) at room temperature for two days.

Analysis:

The gels were analyzed using one normal control as a standard for each timepoint and for each mouse line (fig. 1C). Using this control, an average of 25-26 bands were counted and compared to each sample run to count insertions and deletions. Repeat analysis of all samples was performed (average of five PCR reactions per tissue sample) to minimize the effect of experimental variability. Data analysis was completed and the statistical calculations were carried out using Microsoft Excel and SigmaPlot.

Results

Tetracycline repressible $P210^{Bcr/Abl}$ transgenic mice line F.27 exhibit genomic instability when expressing $P210^{Bcr/Abl}$. In order to determine whether the $P210^{Bcr/Abl}$ transgenic mice are accumulating mutations once Bcr/Abl is expressed, we examined whole genomic DNA isolated from blood samples, using various timepoints. The first two timepoints taken were controls, where the mice were administered tetracycline in their drinking water, and therefore Bcr/Abl expression is suppressed. The mice were then taken off of the tetracycline for the remainder of the study (to express Bcr/Abl).

When comparing a single $P210^{Bcr/Abl}$ (+/+) mouse to a control (-/-) mouse (Fig. 2), we observe a sharp increase in insertions and deletions after administration of tetracycline, at timepoint three. When comparing another set of mice, we observe more of a gradual increase in insertions and deletions in the $P210^{Bcr/Abl}$ mouse upon expression of Bcr/Abl (Fig. 3). To look at all the mice from line F.27 combined, when there are three controls and three $P210^{Bcr/Abl}$ mice for each timepoint (Fig. 4), we observe an overall increase in insertions and deletions in the $P210^{Bcr/Abl}$ mice compared to control mice. The difference between the control mice and the $P210^{Bcr/Abl}$ mice is significant ($p=0.010$), upon expression of Bcr/Abl.

After induction of Bcr/Abl in the $P210^{Bcr/Abl}$ transgenic mice, there was a significant difference found in the number of insertions and deletions compared to control mice. Consequently, in the first two timepoints, when Bcr/Abl is not expressed, the difference between control and $P210^{Bcr/Abl}$ transgenic mice was not significant. These $P210^{Bcr/Abl}$ mice, therefore show an increase in mutations (insertions and deletions) as

soon as Bcr/Abl is expressed. This confirms that this accumulation of mutations is strictly Bcr/Abl dependent.

Tetracycline repressible $P210^{Bcr/Abl}$ transgenic mouse line F.2 is not capable of reversing genomic instability. In order to determine whether these mutations (insertions and deletions) seen in the $P210^{Bcr/Abl}$ transgenic mice, could be reversed, we stopped the expression of Bcr/Abl at certain time intervals to see if the mutations would decrease. The first two timepoints taken were controls, where the mice were administered tetracycline in their drinking water, and therefore suppress Bcr/Abl expression. The mice were then taken off the tetracycline at timepoint three and five (to express Bcr/Abl). The mice were then re-administered tetracycline at timepoint four (to suppress Bcr/Abl).

When we observed all the mice for line F.2 (Fig. 5), it is clear that this genomic instability observed as insertions and deletions, is not reversible. The difference in the control mice and the $P210^{Bcr/Abl}$ mice at the first induced timepoint was significant ($p=0.012$), however this significance stayed the same upon suppression of Bcr/Abl or expression of Bcr/Abl. If these mice were reversible, there would not be a significant difference between Bcr/Abl expressing and control at the timepoints where Bcr/Abl is suppressed. This is most likely because the initial thirty-day expression of Bcr/Abl in this mouse line already rendered secondary effects.

Discussion

Previously, our laboratory had addressed the question of genomic instability by utilizing 32D cell lines which express $P210^{Bcr/Abl}$. These lines exhibited secondary cytogenetic abnormalities, showed inhibition of apoptosis and exhibited cell cycle abnormalities.[10] We next addressed genomic instability *in vivo* by using $P190^{Bcr/Abl}$ transgenic mice, which express Bcr/Abl ubiquitously. The $P190^{Bcr/Abl}$ transgenic mice were crossed with the Big Blue transgenic mice (Stratagene) to observe point mutations at a pre-leukemic stage. We observed a two to three fold increase in point mutation rate in the pre-leukemic mice compared to control.[12] We therefore decided to take these *in vivo* studies one step further using Inter-Simple Sequence Repeat PCR. This system, uses CA repeats as primers to compare insertions or deletions of the entire genome.[13]

Here, we address genomic instability by utilizing a $P210^{Bcr/Abl}$ tetracycline repressible system whereby we can express or repress Bcr/Abl. The two key questions to address here is whether Bcr/Abl induces genomic instability directly, through observed altered bands (insertions and deletions) and whether this instability can be reversed using this repressible mouse model system.

We observe a sharp increase in altered bands directly after tetracycline withdrawal, and Bcr/Abl is expressed in mouse line F.2. This is highly significant at the third timepoint ($p=0.010$). This is consistent with previous data in our laboratory, where we noted an increase in insertions and deletions in the $P190^{Bcr/Abl}$ pre-leukemic mice compared to controls. We also observe a sharp increase in mouse line F.27, after one induction of Bcr/Abl ($p=0.012$), however, this observed genomic instability was shown to not be reversible in this mouse line. This is perhaps due to Bcr/Abl causing secondary

mutations in these mice upon the first thirty-day induction of Bcr/Abl. Previous studies on this mouse line indicate that these mice had the longest latency of disease, surviving up to eleven weeks after the expression of Bcr/Abl.[15] It appears then, that Bcr/Abl is slowly exerting these secondary effects on this mouse line to finally induce leukemia. This may also be due to the existence of a single Bcr/Abl positive clone lingering even after the mice are suppressing Bcr/Abl. This has been apparent in patients with CML, as even after bone marrow transplant, there is sometimes still an appearance of Bcr/Abl positive clones[16].

The next item to contemplate is what causes these mutations within the DNA to begin with. The first obvious mutation might be occurring in a DNA repair protein. One DNA repair protein that has been shown recently to be altered in CML patients and Bcr/Abl expressing cell lines is replication factor C (RFCp140).[17] This protein has a significant role in DNA recombination and repair.[18,19] It may be possible that the mutations observed in these experiments are in conjunction with mutations in a DNA repair pathway and this theme is a subject for future studies.

These experiments, taken together, clearly demonstrate that Bcr/Abl is the initial cause of genomic instability seen in CML. This observed previously in the $P190^{Bcr/Abl}$ mice, and here in the $P210^{Bcr/Abl}$ mice. Future experiments should focus upon what mutations are occurring to cause these genomic alterations and which ones are the most significant for the progression of this disease.

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Figure Legends

Figure 1 A: Inter-Simple Sequence Repeat (SSR) PCR is a highly effective method for detecting differences within $(CA)_n$ repeats. The two primers consist of CA repeats of different lengths and the region of DNA within the repeats is amplified. **B:** Typical RG primer gel scan from Inter-SSR PCR samples taken from three different $P210^{Bcr/Abl}$ transgenic mice. Note here, the insertion and deletions in the third lane compared to samples in the first two lanes. **C:** Control banding patterns for mouse line F.27 and F.2 using primer RG. Scan of samples used 26 bands for line F.27 and 25 bands for line F.2, where bands used for controls are marked to the right of the gel scan. Sample J189(-/-) was used for mouse line F.27, while C609(-/-) was used for mouse line F.2.

Table 1: Table showing mice from founder lines F.2 and F.27. $P210^{Bcr/Abl}$ (+/+) mice were used and either of ((-/-), or (+/-), or (-/+)) mice were used as controls.

Figure 2: Observed insertions and deletions from two line F.27 mice, J189(-/-) control mouse and C703(+/+) induced $P210^{Bcr/Abl}$ mouse. The first two timepoints are controls, where $P210^{Bcr/Abl}$ is not induced (tetracycline administration). The mice are induced (withdrawal of tetracycline) after the second timepoint and therefore the (+/+) mouse expresses Bcr/Abl (+Bcr/Abl).

Figure 3: Observed insertions and deletions from two line F.27 mice, J194(-/-) control mouse and C606(+/+) induced $P210^{Bcr/Abl}$ mouse. The first two timepoints are controls,

where $P210^{Bcr/Abl}$ is not induced (tetracycline administration). The mice are induced (withdrawal of tetracycline) after the second timepoint and therefore the (+/+) mouse expresses Bcr/Abl (+Bcr/Abl).

Figure 4: Insertions and deletions from all line F.27 mice combined, which is a total of six mice, three (+/+) mice and three control mice. The first two timepoints are controls, where $P210^{Bcr/Abl}$ is not induced (tetracycline administration). The mice are induced (withdrawal of tetracycline) after the second timepoint and therefore the (+/+) mice expresses Bcr/Abl (+bcr/abl). The p value is shown after the induction of Bcr/Abl, at the third timepoint, which is p=0.010.

Figure 5: Insertions and deletions from all line F.2 mice combined, which is a total of six mice, three (+/+) mice and three control mice. The first two timepoints are controls, where $P210^{Bcr/Abl}$ is not induced (tetracycline administration). The mice are induced (withdrawal of tetracycline) after the second timepoint and therefore the (+/+) mice expresses Bcr/Abl (+bcr/abl). In order to see if these observations are reversible, the mice were reverted back (timepoint four), where the (+/+) mice are not expressing Bcr/Abl (tetracycline administration). The p value is shown after the first induction of Bcr/Abl, at the third timepoint, which is p=0.012.

Figure 1A:

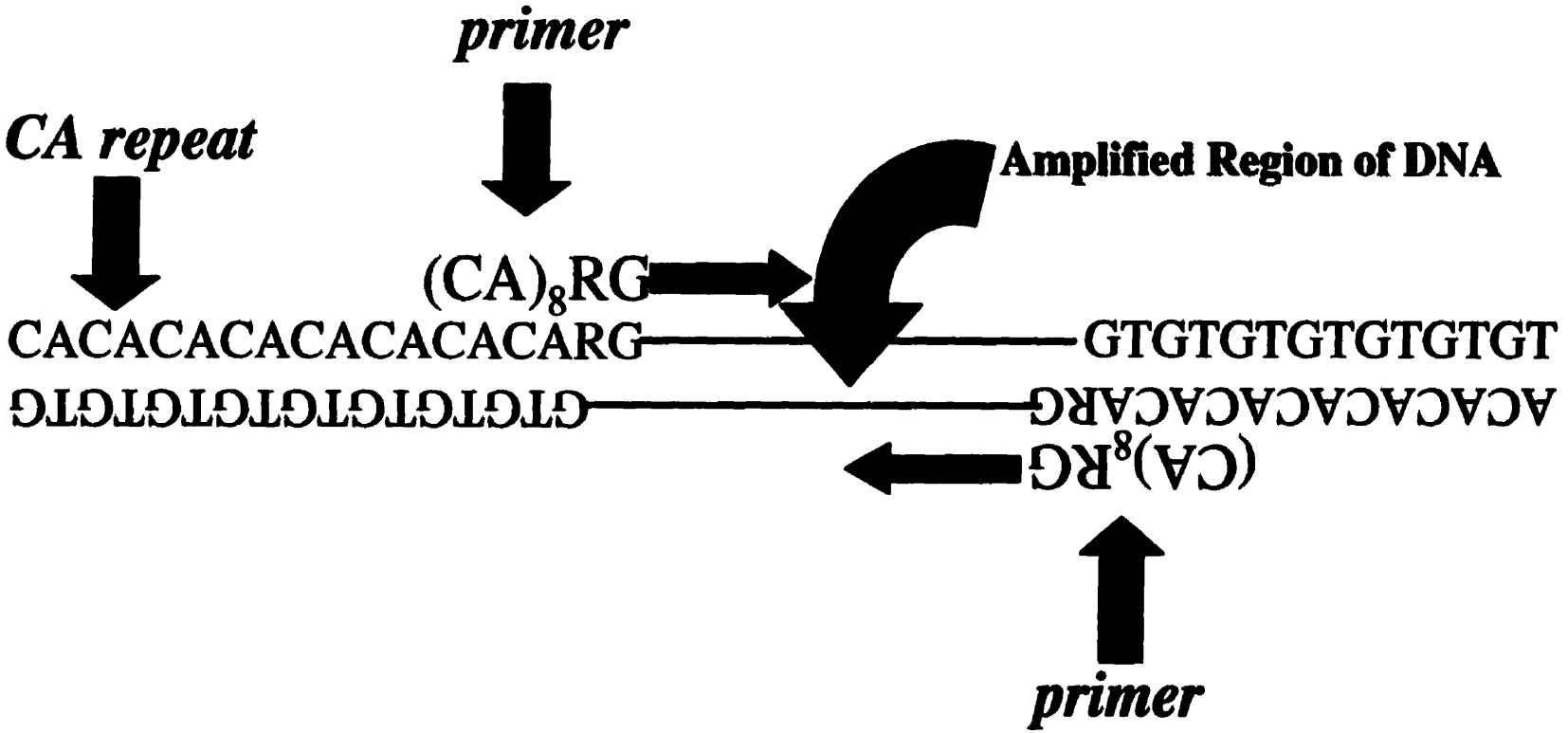

primer
CA repeat
Amplified Region of DNA
(CA)8RG
CACACACACACACACARG
GTGTGTGTGTGTGTGT
GTGTGTGTGTGTGTGTG
ACACACACACACARG
(CA)8RG
primer

Figure 1C:

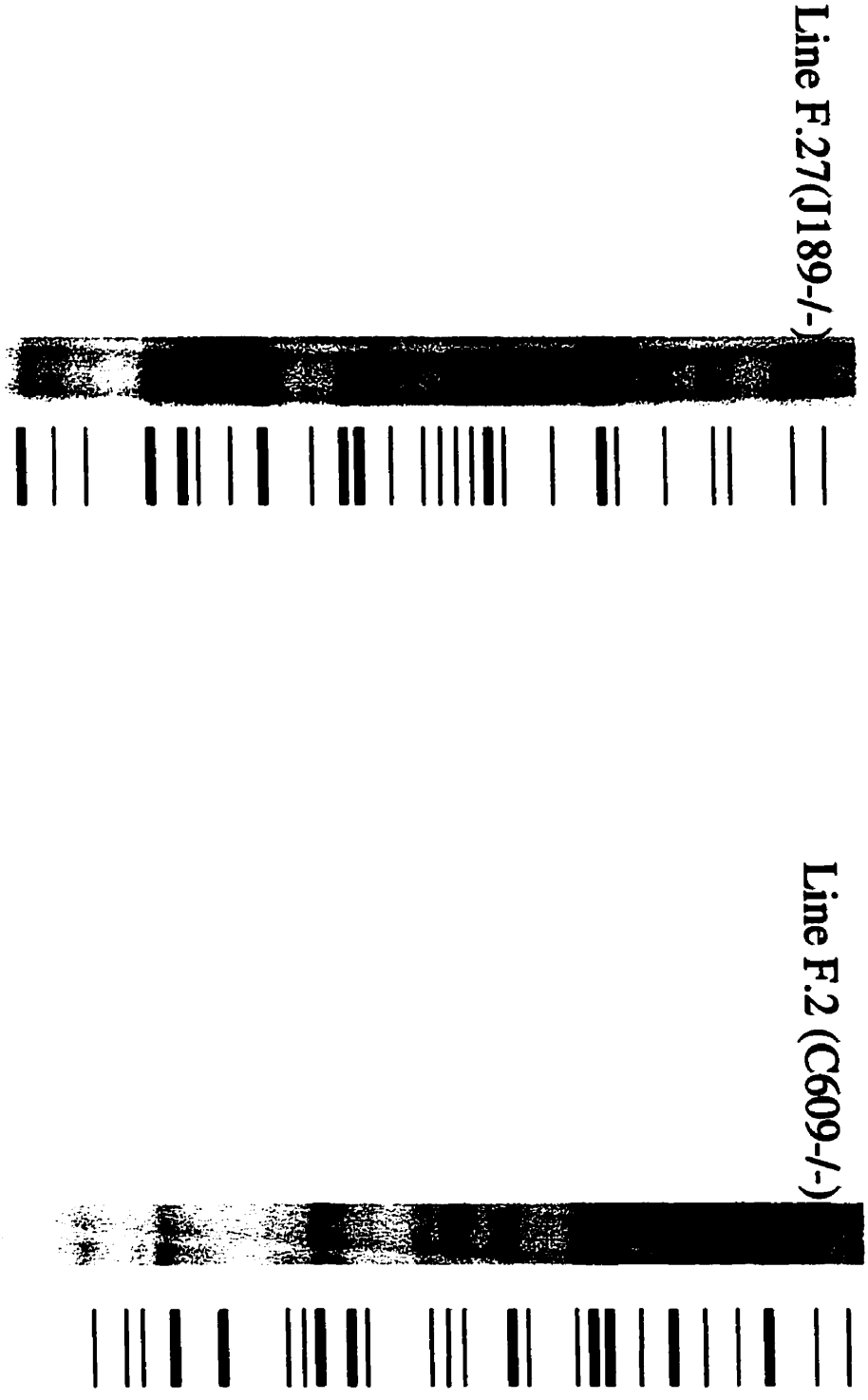


Table 1:

Genotype	Gene Expression	Develop Leukemia?
(+/+)	BCR/ABL-tTA	yes
(+/-)	BCR/ABL without tTA transgene	no-control
(-/+)	no BCR/ABL transgene, only tTA	no-control
(-/-)	wildtype mice	no-control

Figure 2:

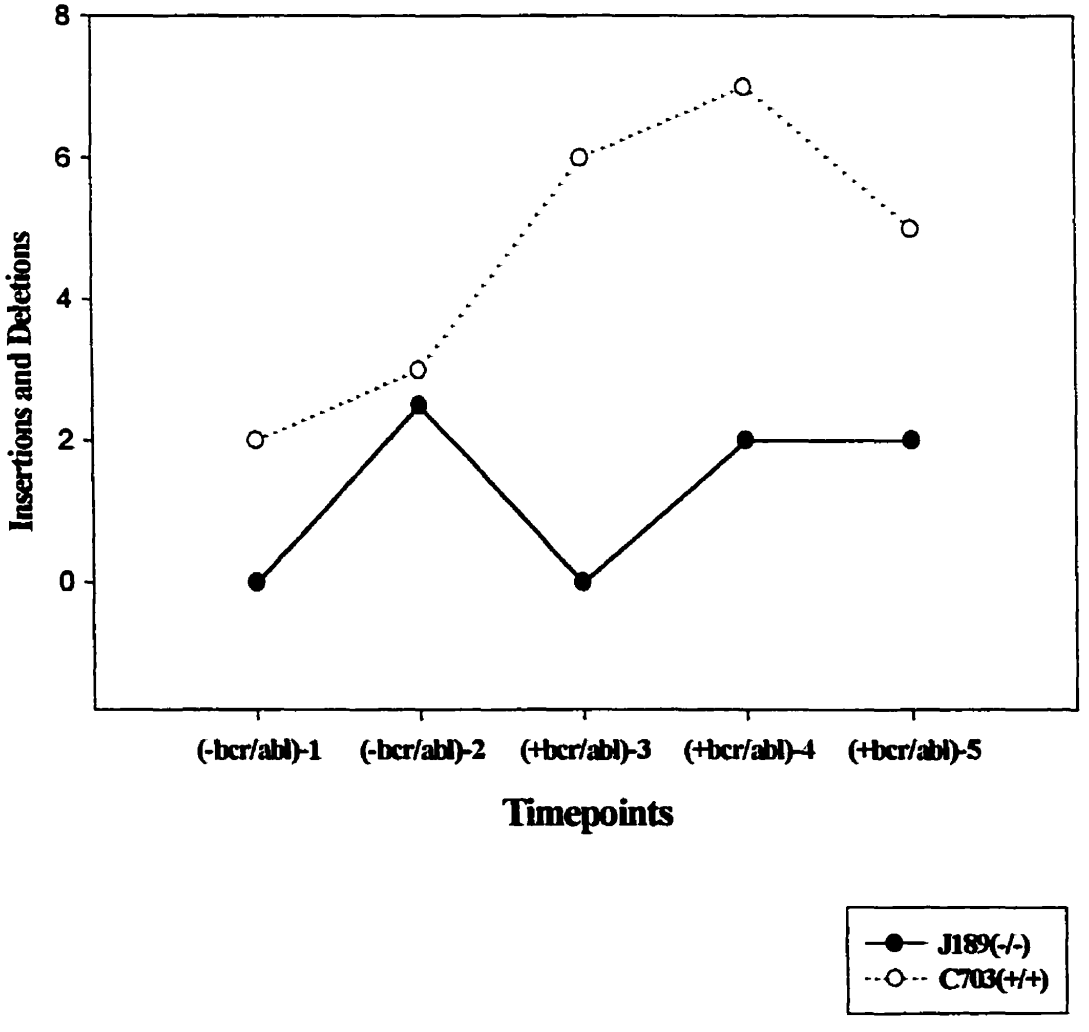

8
6
4
2
0
Insertions and Deletions
(-bcr/abl)-1
(-bcr/abl)-2
(+bcr/abl)-3
(+bcr/abl)-4
(+bcr/abl)-5
Timepoints
J189(-/-)
C703(+/+)

Figure 3:

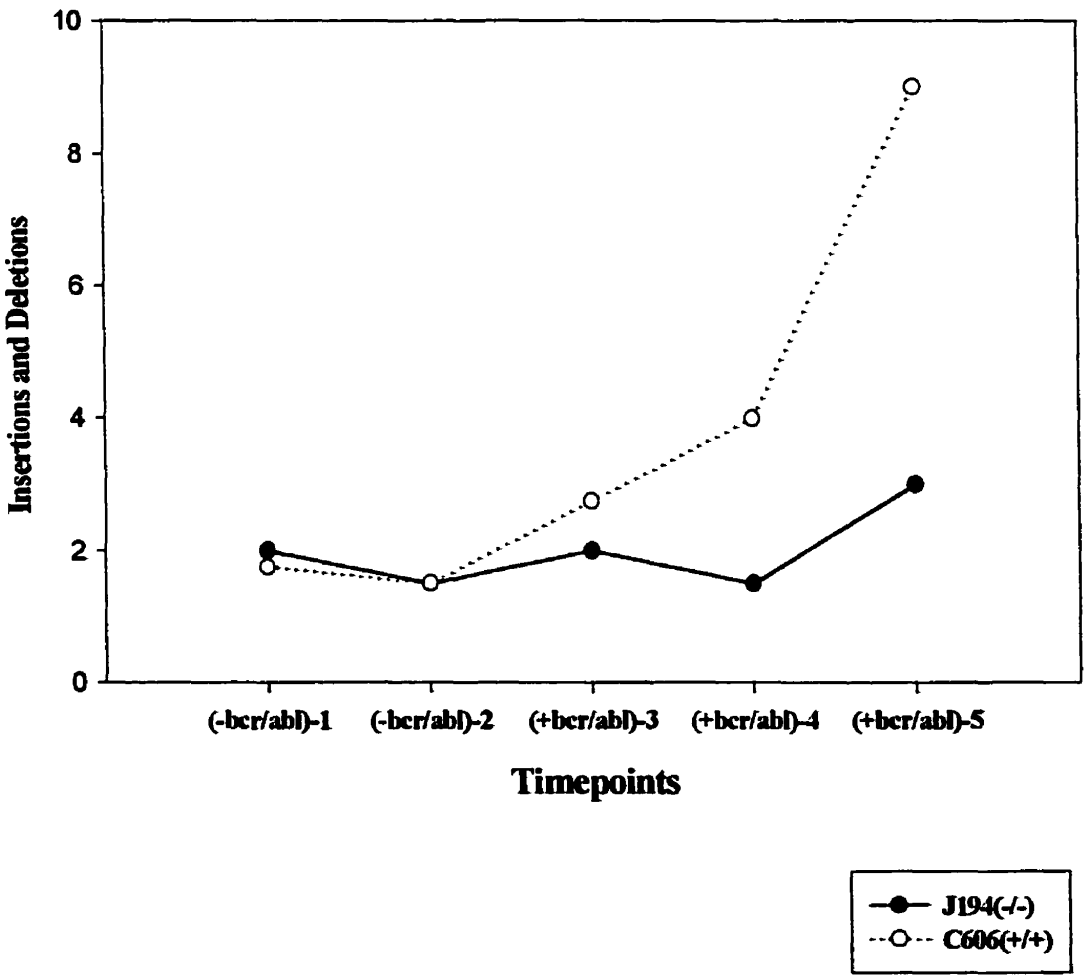

10
8
6
4
2
0
Insertions and Deletions
(-bcr/abl)-1
(-bcr/abl)-2
(+bcr/abl)-3
(+bcr/abl)-4
(+bcr/abl)-5
Timepoints
J194(-/-)
C606(+/+)

Figure 4:

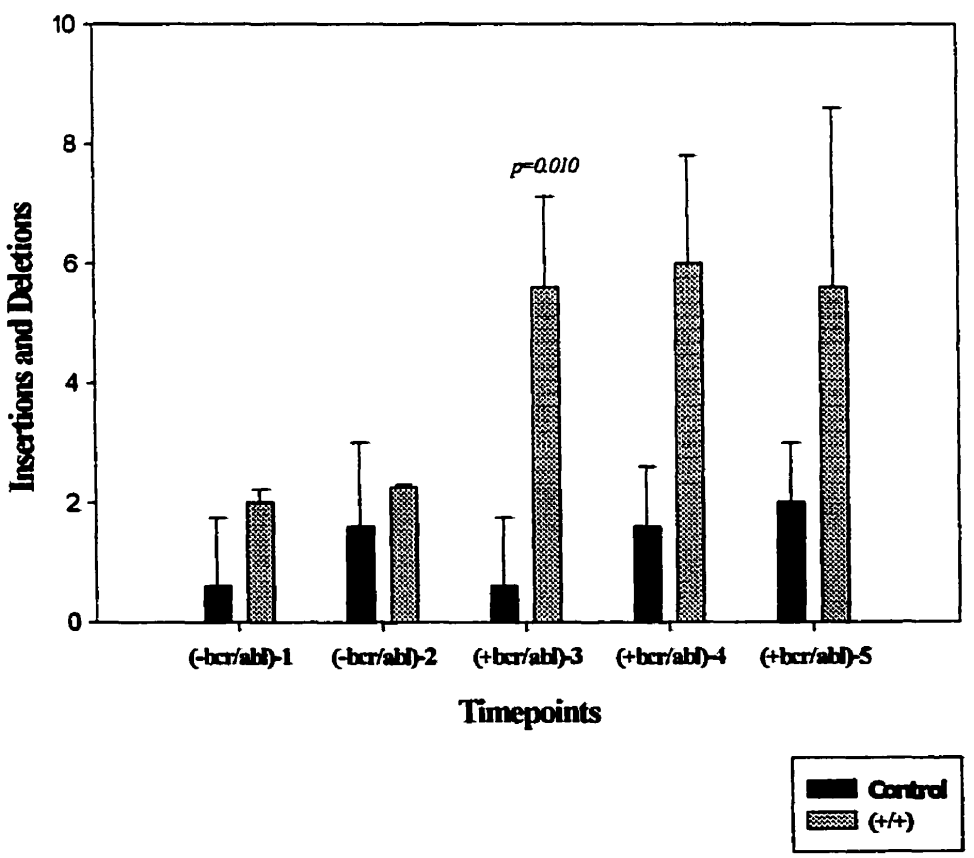
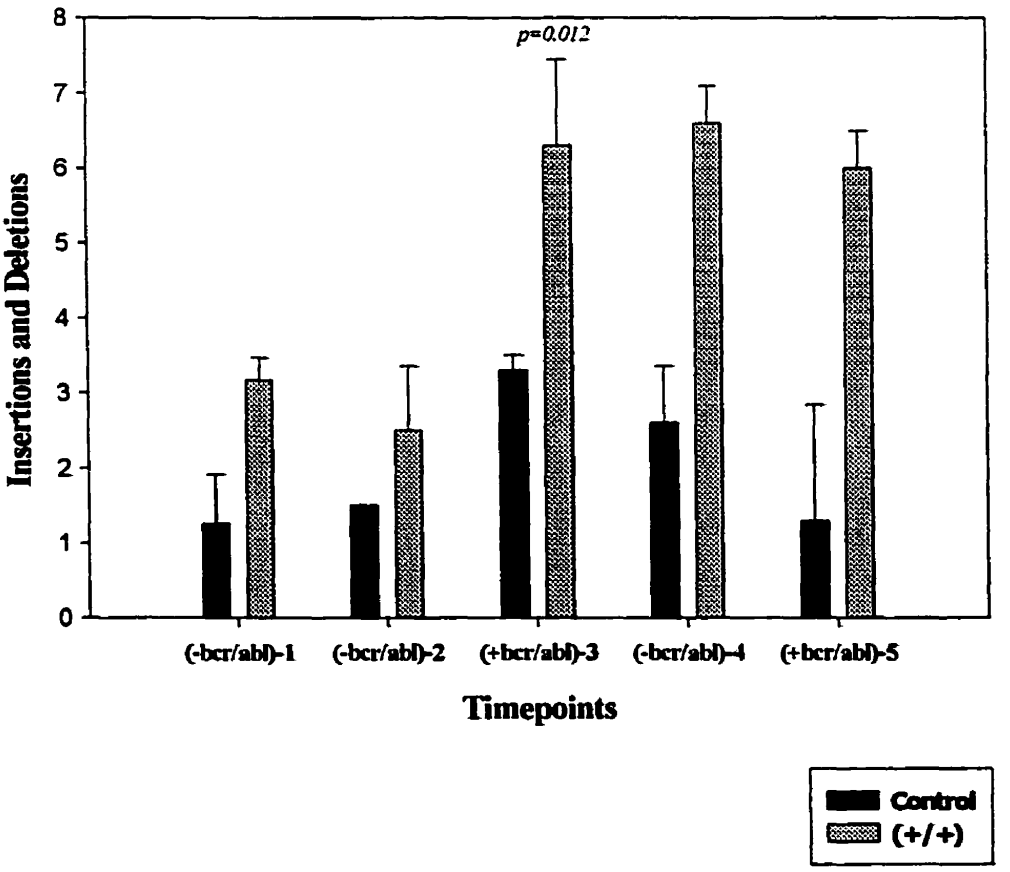

10
8
6
4
2
0
Insertions and Deletions
p=0.010
(-bcr/abl)-1
(-bcr/abl)-2
(+bcr/abl)-3
(+bcr/abl)-4
(+bcr/abl)-5
Timepoints
Control
(+/+)

Figure 5:


p=0.012
Insertions and Deletions
0
1
2
3
4
5
6
7
8
(-bcr/abl)-1
(-bcr/abl)-2
(+bcr/abl)-3
(-bcr/abl)-4
(+bcr/abl)-5
Timepoints
Control
(+/+)

Chapter V Over expression of DNA Repair Genes in P190$^{Bcr/Abl}$ Pre-Leukemic Mice

Preface

In the three previous chapters, altered bands (insertions and deletions) and an increase in mutation frequencies were observed in the pre-leukemic and leukemic P190$^{Bcr/Abl}$ transgenic mice. These bands and mutation frequencies were also found to decrease upon treatment with STI571. These studies were further addressed to include a P210$^{Bcr/Abl}$ inducible mouse system, in which an increase in altered bands was also observed. In this chapter, we further these genomic instability questions associated with Bcr/Abl to observe genetic changes in the P190$^{Bcr/Abl}$ transgenic mice using the MicroArray system. These mice have several genes both under and over expressed in the pre-leukemic phase compared to the normal, control mice. Some of these include genes involved in mitogenic signaling and DNA repair. These studies demonstrate that Bcr/Abl not only alters gene expression but also provides direct evidence for which Bcr/Abl induces this genomic instability. The results in this chapter elucidate some of the observations in previous chapters, as specific genes may be involved in rendering instability during the course of this disease. Bcr/Abl therefore, is not only responsible for this alleged genomic instability, by the induction of point mutations, and an increase in altered bands, but it is also directly responsible for altered gene expression.

Abstract

The Philadelphia chromosome is the cytogenetic hallmark of chronic myelogenous leukemia. This reciprocal translocation involves chromosome 9 and 22, and manifests into the fusion protein Bcr/Abl. This disease has an inevitable progression from the chronic phase to the acute phase and is associated with genomic instability. It is unclear as to whether this genomic instability is solely driven by Bcr/Abl or requires another major secondary event. Our focus is therefore to address this genomic instability as it occurs during the course of this disease. In the current study, we utilize a cDNA expression array to identify genes whose expression is altered in pre-leukemic $P190^{Bcr/Abl}$ mice compared to control BL6/CBA mice. The upregulation of several genes was associated with the expression of Bcr/Abl in the pre-leukemic $P190^{Bcr/Abl}$ mice. These include Rad23 excision repair protein homologue B (*RAD23B*) and Xeroderma pigmentosum group C repair complementing 58kDa protein (*XP-C repair*). Reduced expression of a number of genes was also associated with Bcr/Abl expression. Some of these genes included MAP kinase p38, heat shock protein p86 (*HSP86*), glutathione S-transferase 5, prothymosin alpha, cytoplasmic beta-actin and glutathione reductase. The changes within these genes show that Bcr/Abl confers a mutator phenotype through altered regulation of several genes. Also, these changes indicate that Bcr/Abl expression can induce significant changes associated with cellular stress and DNA repair pathways before recognizable cellular transformation occurs.

Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder characterized in the chronic phase by an overproduction of mature and immature granulocytes. CML is the result of an unequal reciprocal translocation between chromosome 9 and 22 (t9;22 at q34;q11), known as the Philadelphia chromosome (Ph+).[1] Chromosome 22 transcribes a protein known as Bcr, while chromosome 9 gives rise to a proto-oncoprotein known as c-Abl. The product of these two genes form a fusion protein known as Bcr/Abl. Bcr/Abl has several molecular weights, the most common forms are the 190kDa and 210kDa weights. P210 is associated with CML, while P190 is more commonly associated with acute lymphocytic leukemia (ALL).[2] In each case, the formation of these fusion proteins results in the constitutive activation of the protein tyrosine kinase c-Abl.

CML has an inevitable progression from the chronic to acute phase. During the transition of this disease, several secondary chromosomal abnormalities have been reported, including the loss of the tumor suppressor genes p53 and retinoblastoma (Rb).[3-6] These chromosomal abnormalities suggest that additional genetic events may be responsible for the progression to blast crisis. A major question is whether there is another major secondary event required for the transition of this disease, or if this is solely driven by Bcr/Abl.

Previously, our laboratory had addressed the question of genomic instability in CML, by utilizing 32D cell lines which express $P210^{Bcr/Abl}$.[7,8] These lines exhibited secondary cytogenetic abnormalities, show inhibition of apoptosis and exhibit cell cycle

abnormalities. We next addressed genomic instability *in vivo* by using $P190^{Bcr/Abl}$ transgenic mice, which express Bcr/Abl ubiquitously. The $P190^{Bcr/Abl}$ transgenic mice were crossed with the Big Blue reporter mice (Stratagene) to observe point mutations at a pre-leukemic stage. We observed a two to three fold increase in point mutation rate in the pre-leukemic mice compared to the control mice.[9] We then demonstrated that the observed point mutation rate can be partially reversed by a c-Abl specific inhibitor, STI571 (*Brain, et. al., submitted data*). We therefore decided to take the *in vivo* studies one step further using Inter-Simple Sequence Repeat PCR (Inter-SSR PCR). This system uses CA repeats as primers to compare insertions or deletions of the entire genome.[25] We demonstrate using this Inter-SSR PCR system, that Bcr/Abl is directly responsible for the observed insertions and deletions in the $P190^{Bcr/Abl}$ and $P210^{Bcr/Abl}$ mouse models (*Brain, et. al., submitted data*).

Most studies on Bcr/Abl are in cell lines in which Bcr/Abl has already transformed the cell. In this study, we utilized a $P190^{Bcr/Abl}$ transgenic mouse to study the early molecular events associated with the expression of Bcr/Abl that antedates leukemic transformation. In an effort to determine what roles Bcr/Abl may play before transformation, we used the microarray or cDNA expression arrays to identify specific genes involved in Bcr/Abl transformation *in vivo*. This microarray system has the ability to identify genes both under and over expressed in a mouse model system. The array membranes used in this study can identify up to 588 mouse genes. This covers a wide spectra of genes and includes genes involved in mitogenic signaling, cell adhesion, cellular stress response and DNA repair.

When we compared the pre-leukemic $P190^{Bcr/Abl}$ mice to the control mice (BL6/CBA), we note an upregulation in several genes, including *RAD23*, XPC repair

gene, the lung Kruppel-like Factor and the Cek5 ligand. Several genes were also shown to be down regulated, including p38 MAP kinase, heat shock protein p86 (*HSP86*), glutathione S-transferase 5, prothymosin alpha, cytoplasmic beta-actin and glutathione reductase. These results suggest that Bcr/Abl alone can induce genomic alterations before the onset of leukemia. These results also suggest that Bcr/Abl can confer this by affecting specific genes, including genes involved in mitogenic signaling and DNA replication and repair.

Methods

Isolation of Total RNA from Tissue:

The $P190^{Bcr/Abl}$ transgenic mice were genotyped by Southern Blot analysis. Mice were sacrificed and the kidneys and spleens were removed. Total RNA was extracted using Trizol (GibcoBRL,Rockville, MD, USA).

Integrity of RNA:

RNA concentration was determined by spectrophotometric measurements at A_{260} and a ratio of A_{260}/A_{280}. The integrity of the RNA itself was tested by running 5μg of total RNA on a formaldehyde gel.

Probe Synthesis from Total RNA:

The probes were synthesized using the Atlas™ cDNA expression array (Clontech, Palo Alto, CA, USA). In brief, 4-10μg of total RNA was added to a mix including 5x reaction buffer (Clontech, Palo Alto, CA, USA), 10 x dNTP mix (Clontech, Palo Alto, CA, USA), DTT (Clontech, Palo Alto, CA, USA), CDS primer mix (Clontech, Palo Alto, CA, USA) and ^{32}PαdATP (Amersham, Arlington Heights, IL, USA). This was incubated at 70°C and 48°C for 2 minutes each. MMLV reverse transcriptase (Clontech, Palo Alto, CA, USA) was added and again incubated at 48°C for 25 minutes. The reaction was stopped by adding a 10x termination mix (Clontech, Palo Alto, CA, USA).

Column Chromatography:

To purify the labeled cDNA from unincorporated ^{32}P labeled nucleotides and small cDNA fragments, column chromatography was used for each probe. This was done

by using several buffers and the NucleoSpin Extraction Kit (Clontech, Palo Alto, CA, USA).

Hybridizing cDNA Probes to the Array:

Pre-hybridization was conducted using ExpressHyb (Clontech, Palo Alto, CA, USA) and sheared salmon testes (Sigma, Oakville, ON, Canada). The Atlas membranes, (Clontech) which have the ability to identify 588 mouse genes, were placed at 68°C with continuous agitation for 30 minutes. The labeled probe was incubated at 68°C for 20 minutes with 1/10 total volume of 10X denaturing solution (1M NaOH, 10mM EDTA). A final incubation of the probe was done at 68°C for 10 minutes with C_0t-1 Mouse DNA (Clontech, Palo Alto, CA, USA) and 2X neutralizing solution (1 M NaH_2PO_4, pH 7.0. The probe was then added to the membrane and the prehybridization solution, hybridization was conducted overnight at 68°C with continuous agitation.

Wash:

The membranes were washed, four times for one half hour at 68°C with wash solution 1 (2XSSC, 1%SDS). Followed by a second wash for a half an hour at 68°C with wash solution 2 (0.1XSSC, 0.5%SDS). A final wash was conducted at room temperature with 2XSSC for 5 minutes. The membranes were exposed to a phosphoimaging screen at room temperature over night.

Membrane Analysis:

The membranes were analyzed by using the Clontech Atlas Image Software package (Clontech, Palo Alto, CA, USA). A gene was defined as over expressed when the intensity was at least twice the reference (ratio=2). A gene was defined as under expressed when the intensity was at most half the reference (ratio=0.5 or less). A gene

was defined as turned on when the gene is not expressed at all in the reference and at least twice the intensity of the background. All genes were tested at least twice in order to determine expression.

Results

Gene Expression in the Kidneys of Pre-leukemic P190$^{Bcr/Abl}$ Transgenic Mice: We examined total RNA isolated from the kidney of pre-leukemic P190$^{Bcr/Abl}$ transgenic mice using microarray analysis to look for the presence of genes expressed in the pre-leukemic kidney compared to control. The results obtained are shown (table 1) and compared to that observed in normal control kidney. The upregulation of several genes was associated with the expression of Bcr/Abl. These include but are not limited to, lung Kruppel-like factor (*LKLF*), a zinc finger transcription factor which plays an important role in embryonic development and inhibits apoptosis and promotes T-lymphocyte cell survival.[10-13] Another gene identified to be upregulated was the Eph receptor tyrosine kinase Cek5 ligand. Activation of the EphB2 receptor has been shown to control integrin activity via R-Ras and reduce cell adhesion.[14,15] The role that upregulation of LKLF and Cek5 ligand plays in inhibiting apoptosis and cell adhesion, two mechanisms implicated in the pathogenesis of Bcr/Abl induced leukemia, merits further attention. The over expression of two DNA repair genes was also observed, including *RAD23* and *XP-C*. Both *RAD23* and *XPC* are involved in nucleotide excision repair.[16-21] Reduced expression of a number of genes was also associated with Bcr/Abl expression. In order of decreasing difference that included tumor cell growth inhibiting factor-1 (*TIF-1*), related filament/plasma membrane associated protein (*merlin, NF-2*), ornithine decarboxylase (*ODC*), prothymosin alpha (*PTMA*), MAP kinase p38, heat shock protein p86 (*HSP86*), glutathione S-transferase 5, prothymosin alpha, cytoplasmic beta-actin and glutathione reductase. These changes indicate that Bcr/Abl expression can induce significant changes

associated with cellular stress, redox pathways and DNA repair, before recognizable cellular transformation occurs.

Gene Expression in the Spleens of Pre-leukemic P190$^{Bcr/Abl}$ Transgenic Mice: We examined total RNA isolated from the spleen of pre-leukemic P190$^{Bcr/Abl}$ transgenic mice using microarray analysis to look for the presence of genes expressed in the pre-leukemic spleen compared to control. The results obtained are shown (table 2) and compared to that observed in normal control spleen. The upregulation of several genes were associated with the expression of Bcr/Abl in the spleen. These include, paired box protein (*PAX5*), B-cell transcription factor (*BSAP*), 7S nerve growth factor alpha subunit (*alpha-NGF*) and *KLK4*. *PAX5* is a transcription repressor and activator and is required for B cells to maintain their B-lymphoid identity throughout B cell development.[22,23] The B cell transcription factor, activator protein (*BSAP*) is encoded by the *PAX5* gene and is essential for B-cell differentiation. *BSAP* may also be responsible for the growth arrest of pre-B cells.[24] The changes in the expression of these genes indicate that Bcr/Abl expression can induce significant changes in the spleen, before recognizable cellular transformation occurs.

Discussion

CML has an inevitable progression from the chronic to acute phase. A major question is whether there is another major secondary event required for the transition of this disease, or if this is solely driven by Bcr/Abl. In this study, we utilized a $P190^{Bcr/Abl}$ transgenic mouse to study the early molecular events associated with the expression of Bcr/Abl. These mice express Bcr/Abl before developing a B-cell leukemia/lymphoma with a median latency of approximately 100 days. In order to determine what effects Bcr/Abl has before leukemic transformation, we utilized the $P190^{Bcr/Abl}$ pre-leukemic mice and cDNA expression arrays to study the events that take place before leukemic transformation.

When we compared the pre-leukemic $P190^{Bcr/Abl}$ mice to the control mice (BL6/CBA), we noted an upregulation in several genes, including *RAD23*, *XPC* repair gene, lung Kruppel-like Factor, Cek5 ligand, *PAX5* and *BSAP*. We also observed several genes which were down regulated. In order of decreasing difference these included, tumor cell growth inhibiting factor-1(*TIF-1*), related filament/plasma membrane associated protein (*merlin, NF-2*), ornithine decarboxylase (*ODC*), prothymosin alpha (*PTMA*), MAP kinase p38, heat shock protein p86 (*HSP86*), glutathione S-transferase 5, prothymosin alpha, cytoplasmic beta-actin and glutathione reductase. We also observed a gene which was turned on in the pre-leukemic kidney, the prolactin receptor. Prolactin receptor is involved in mammary development and lactation, it is not known if this receptor plays a role in Bcr/Abl oncogenesis, however this should be a subject for future studies.[26]

We observed more significant differences in gene expression in the kidney than in the spleen. This is similar to previous results in our laboratory and might have something to do with the high expression of Bcr/Abl in the kidney. We also observed no differences in gene expression when comparing the sick spleen to the pre-leukemic spleen (n=3). This may be again due to the low expression of Bcr/Abl in the spleen and due to an accumulation of erythroblasts in the spleen. It is important to note here as well, that these experimental findings are limited by the genes which appear on the membrane and therefore this membrane in particular may have more kidney specific genes than genes specific to the spleen.

Of the genes which were shown to be over expressed, several are of particular interest to CML and to cancer biology. Cathepsin D, for example is a proteolytic enzyme which is used as a prognostic factor in squamous cell carcinoma of the skin.[27] While LKLF has been implicated in blood vessel stabilization during embryogenesis and may be involved in angiogenesis.[28] The protease kallikrein 4 (*KLK4*), may also be involved in cellular proliferation and is upregulated in human endometrial cancer cell lines.[29] Non-muscle myosin light chain 3 may also contribute to the neoplastic transformation as it is involved with cellular movement and cell shape changes.[30] Of the genes which were shown to be under expressed, several also had interesting implications to cancer biology. Tumor cell growth inhibiting factor-1 (*TIF-1*) inhibits growth of human lung cell carcinoma in soft agar, and therefore is thought to inhibit tumor growth.[31] Merlin (*NF-2*), is a tumor suppressor whose absence results in the occurrence of multiple tumors of the nervous system.[32] Ornithine decarboxylase (*ODC*) is an enzyme involved in the synthesis of polyamines and has been shown to have altered expression in transformed cell lines.[33] Prothymosin alpha (*PTMA*) is a nuclear protein which is thought to play a role in cellular

proliferation.[34] MAP kinase p38, is involved in mitogenic signaling and has been shown to be involved in Bcr/Abl oncogenic transformation.[35] Heat shock protein (*HSP86*) is involved in the cells response to stress, while glutathione reductase is involved in the antioxidant system.[36,37] Taken together, there are several genes which are either over or under expressed, which may help to promote the neoplastic transfromation of Bcr/Abl. Future studies on these genes may help to address the question of which genes help to promote this neoplastic transformation.

Although there is altered regulation of several genes observed in the $P190^{Bcr/Abl}$ transgenic mice, the genes that are of particular interest in the field of genomic instability are the DNA repair genes. Several groups have shown altered expression in DNA repair genes in human cancers.[19,38] Both *XPC* and *RAD23* are genes involved in DNA replication and repair.[16-21] The upregulation of these two genes may be though the direct induction of Bcr/Abl or perhaps due to a physical resistance to DNA repair. Although it is still unclear as to how and why these repair genes may be upregulated. Recent studies have shown altered regulation of replication factor C (RFCp140) in CML clinical samples and Bcr/Abl expressing cell lines.[39] RFCp140 plays a significant role in DNA recombination and repair as part of a larger protein complex by recruiting PCNA and attaching it to single stranded DNA.[39,40] Additional studies have shown that RFC is important for efficient binding of *PCNA* for catalyzed elongation of the singly primed DNA template but that RFC is not required for repair to occur. When mutants of *PCNA* were constructed for the binding regions of RFC, elongation occurs nevertheless, but with decreased efficiency.[41] Therefore, Bcr/Abl may directly affect or indirectly affect genes involved in DNA repair to promote this genomic instability during the progression of this disease.

These experiments clearly demonstrate that Bcr/Abl is the initial cause of the observed genomic instability in CML. These results also suggest that Bcr/Abl can confer genomic instability by affecting specific genes, including genes involved in mitogenic signaling and DNA replication and repair. Future experiments should focus upon what mutations are occurring and which are the most significant for the progression of this disease.

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Figure Legends

Figure 1: An example of a MicroArray membrane. This example is of a control mouse (BL6/CBA) kidney (N141K). The membrane probes for 588 mouse genes. Each black spot on this membrane is a separate gene.

Table 1: Observed genes expressed in control mouse (BL6/CBA) versus $P190^{Bcr/Abl}$ mouse kidney. Samples include control (Control,n=1) and pre-leukemic $P190^{Bcr/Abl}$ mice (n=3).

Table 2: Observed genes expressed in control mouse (BL6/CBA) versus $P190^{Bcr/Abl}$ mouse spleen. Samples include control (Control,n=1) and pre-leukemic $P190^{Bcr/Abl}$ mice (n=3).

Table 3: Summary and function of genes expressed in the kidney and spleen of pre-leukemic and leukemic $P190^{Bcr/Abl}$ mice.

Table 1:

Genes Turned On	Genes Over Expressed	Genes Under Expressed
Prolactin Receptor PRLR2	1. Plasma Glutathione per-oxidase precursor (*GSHPX-P*); *GPX3* 2. Granulocyte macrophage colony stimulating factor receptor low-affinity subunit precursor (*GM-CSF-R*) 3. Nuclear factor erythroid 2-related factor 2 (*NF-E2* related factor 2) 4. Non-muscle myosin light chain 3 5. Cathepsin D 6. RAD23 excision repair protein homolog B (*RAD23B*) 7. Xeroderma pigmentosum group C repair complementing 58-kDa protein (*XP-C* repair) 8. Lung Kruppel Like Factor (*LKLF*) 9. Cek5 Ligand	1. Tumor cell growth inhibiting factor-1 (*TIF-1*) 2. Ezrin; villin 2; *NF 2* (merlin) related filament/plasma membrane associated protein 3. Ornithine decarboxylase (*ODC*) 4. Prothymosin alpha (*PTMA*) 5. MAP kinase p38 6. Heat shock protein p86 (*HSP86*) 7. Glutathione S-transferase 5 8. Prothymosin alpha 9. Cytoplasmic beta actin 10. Glutathione reductase

Table 2:

Genes Turned On	Genes Over Expressed	Genes Under Expressed
None to Note	1. Paired Box Protein (*PAX5*) 2. B-cell specific transcription factor (*BSAP*) 3. 7S nerve growth factor alpha subunit (*alpha NGF*) 4. Protease kallikrein (*KLK4*)	None to Note

Table 3:

Genes Turned On	*Function*	Genes Over Expressed	*Function*	Genes Under Expressed	*Function*
Prolactin Recpeter	*Mammary development*[26]	GPX3	*Reduction of lipid hydroproxides*[52]	TIF-1	*Inhibits Growth of Tumor Cell Lines*[31]
		GM-CSF-R	*Cytokine receptor*[53]	NF 2 (merlin)	*Tumor Supressor*[32]
		NF-E2 related factor 2	*DNA binding transcription factor*[54]	ODC	*Enzyme/Synthesis of Polyamines*[33]
		non-muscle myosin light chain 3	*Cellular movement and shape changes*[30]	PTMA	*Cell Proliferation*[34]
		Cathepsin D	*Proteolytic enzyme*[27]	MAP kinase p38	*Mitogenic Signaling*[35]
		RAD23B	*Nucleotide excision repair*[55]	HSP86	*Stress Response Protein*[37]
		XPC repair	*Nucleotide excision repair*[56]	glutathione S-transferase 5	*Metabolic Enzyme*[57]
		LKLF	*Angiogenesis*[28]	prothymosin alpha	*Cell proliferation*[58]
		Cek5 Ligand	*Cell Adhesion*[59]	cytoplasmic beta actin	*Celll Structure*[60]
		PAX5	*B Cell lymphopoiesis*[22,23]	glutathione reductase	*Antioxident, Metabolic Enzyme*[36]
		BSAP	*B cell activator protein*[24]		
		NGF-alpha	*Nerve Growth Factor*[61]		
		KLK4	*Protease, Cellular Proliferation*[29]		

remains one of the classic examples demonstrating the role of genomic instability in tumor progression. This is evidenced by the increased frequency of cytogenetic, genetic and epigenetic alterations that accompany the transition from the chronic phase of the disease to its invariably fatal blast crisis. During the transition from chronic to blast crisis, a high frequency of secondary chromosomal abnormalities have been reported, including the loss of the tumor suppressor genes p53 and the retinoblastoma (Rb) genes.[6;8] These chromosomal abnormalities suggest that genetic events may be critical for the progression to blast crisis. Both the expression of the dominant oncogene *BCR/ABL* and the concurrent loss of an allele of the tumor suppressor gene *c-Abl* could potentially promote genomic instability in Ph+ leukemias. It is still unclear however, as to whether *Bcr/Abl* is the only genetic event that is required for the transition of this disease. A major interest therefore, is to examine the role of Bcr/Abl in genomic instability as it occurs during the course of this disease.

2. Observed Genomic Instability in Pre-Leukemic $P190^{Bcr/Abl}$ Transgenic Mice

Previously, our laboratory has examined genomic instability using cell lines in which we have demonstrated that the retroviral transduction of $P210^{Bcr/Abl}$ into 32D cl3(G) ceils results in the inhibition of apoptosis, altered cell cycle regulation, and induces rapid numerical and structural chromosomal abnormalities.[9] More recently, we measured the basal point mutation rate in heterozygote $P190^{Bcr/Abl}$/LIZ transgenic mice. The chromosomally-integrated λ bacteriophage shuttle vector (LIZ) contains a bacterial *lacI* gene as a target for mutation and *alacZ* as a reporter gene (ie. Big Blue® mutation

detection system, Stratagene). The line of $P190^{Bcr/Abl}$ mice used express Bcr/Abl ubiquitously before developing B-cell lymphoma/leukemia with a latency of approximately 100 days.[10; 11] In this pre-leukemic period, when there is no sign of cellular transformation, we observed a 2-3 fold steady-state increase in the frequency of point mutations in $P190^{Bcr/Abl}$/LIZ mice as compared to control.[7] Although these studies demonstrated that the expression of Bcr/Abl can directly induce a mutator phenotype before leukemic onset, the experimental system used restricted characterization of this mutator phenotype to the study of point mutations only.

In chapter II, I therefore examined the question of Bcr/Abl induced genomic instability by using a system to observe a different type of mutation, seen as insertions and deletions. In order to measure insetion and deletion mutations, I utilized Inter Simple Sequence Repeat Polymerase Chain Reaction (Inter-SSR PCR) using primers consisting of a set of eight CA repeats.[12; 13] Using this technique, I compared the $P190^{Bcr/Abl}$ pre-leukemic mice and $P190^{Bcr/Abl}$ leukemic mice to control mice (BL6/CBA), and found an increase in the number of altered bands in the pre-leukemic and leukemic mice versus control. I also discovered that the frequency of altered bands can be decreased using the c-Abl specific kinase inhibitor, STI571. A partial reversal of the mutations was observed in pre-leukemic kidney but not in the spleen. This may be in part due to the high expression of Bcr/Abl noted in the kidney as compared to the spleen *(Laneuville, unpublished data)*. These results confirmed previous observations in our laboratory, which suggest that Bcr/Abl can directly induce a mutator phenotype before leukemic transformation. Furthermore, here I have extended the type of mutations that occur to include insertions and deletions.

restricted oncogenicity is playing a role, meaning that the environment in the hematopoietic system is favored by Bcr/Abl for tumor formation. Unfortunately, the current set of animal models for CML are limited by the fact that all mice manifest with a lymphoid type of leukemia, with no myeloid involvement. It is important to note that since there is presently no true mouse model for CML, in the studies performed on the $P190^{Bcr/Abl}$ transgenic mice in our laboratory, we have looked at Bcr/Abl *in vivo* before leukemic transformation and are not using these mice to model for CML per se.

4. The Effects of STI571 on c-Abl and Bcr/Abl

The development and recent approval of STI571 (Gleevec) by the Food and Drug Administration in the United States is a major step forward in cancer treatment and the beginning of a drug discovery revolution that has been predicted to follow. STI571 has an impressive therapeutic effect in the treatment of CML patients. It is too early, however, to know as to what extent the beneficial effects observed upon short term treatment for chronic phase patients can be sustained over time. The drug has been reported to have only partial effects in the treatment of acute patients[14]. The reversibility of Bcr/Abl associated point mutagenesis I observed in the $P190^{Bcr/Abl}$ transgenic mouse system with STI571 treatment suggests that disease progression arising from ongoing point mutagenesis should be decreased. Bcr/Abl induced mutagenesis in $P190^{Bcr/Abl}$ transgenic mice, however, is not limited to point mutations alone but also includes an increased frequency of insertions and deletions. The observations that I have made to date do not exclude the possibility that Bcr/Abl may be associated with yet additional mechanisms of mutagenesis, or that all of these would necessarily be reversible with

observed an increase in insertions and deletions when the tetracycline was withdrawn from the $P210^{Bcr/Abl}$ transgenic mice and, therefore, Bcr/Abl expressed. The observed genomic instability was shown to be irreversible. Most likely, the irreversibility of genomic instability in this model is due to secondary effects caused by the initial expression of Bcr/Abl or by a residual Bcr/abl clone lingering even after Bcr/Abl expression is discontinued. These results, however, confirmed the previous results seen in $P190^{Bcr/Abl}$ transgenic mice and reconfirm that Bcr/Abl is required for the induction and maintenance of Ph+ leukemias. These results further demonstrate that Bcr/Abl itself is responsible for the genomic instability seen upon disease progression.

6. Gene Expression in $P190^{Bcr/Abl}$ Transgenic Mice

In chapters II-IV, I demonstrated that genomic instability occurs in Bcr/Abl transgenic mice. In Chapter V, I have taken these studies one step further by identifying potential genes responsible for this observed instability. In order to measure Bcr/Abl mediated changes in gene expression, I utilized cDNA expression arrays in both $P190^{Bcr/Abl}$ pre-leukemic and leukemic mice. When I compared the kidneys of pre-leukemic $P190^{Bcr/Abl}$ mice to the kidneys of control mice (BL6/CBA), I noted an upregulation in several genes, including the lung Kruppel-like Factor and the Cek5 ligand and two DNA repair genes, *RAD23* and *XPC*. Several genes were also shown to be down regulated, including p38 MAP kinase, heat shock protein p86 (*HSP86*), glutathione S-transferase 5, prothymosin alpha, cytoplasmic beta-actin and glutathione reductase. When we compared the spleen of the pre-leukemic $P190^{Bcr/Abl}$ mice to the spleen of the control mice (BL6/CBA), we noted an upregulation in paired box protein (*PAX5*) and B

cell transcription factor (*BSAP*). These results suggest that Bcr/Abl alone can induce genomic instability before the onset of leukemia. The results from this chapter also show that Bcr/Abl can confer this genomic instability by affecting specific genes, including genes involved in mitogenic signaling and DNA replication and repair. The mechanisms whereby Bcr/Abl mediates these effects may be through a physical resistance to DNA repair or through direct gene induction by Bcr/Abl.

The observed regulation of these genes, and in particular genes involved in DNA repair and tumor suppression, may help to explain results from previous chapters. I noted an upregulation in the repair genes *RAD23* and *XPC*, both of which are involved in nucleotide excision repair.[16;17] The over expression of these genes may be caused by Bcr/Abl induced genetic changes which require DNA repair to occur. The exact role of Bcr/Abl in DNA repair certainly merits future study.

7. Mechanisms Involved in Genomic Instability

An important question in cancer biology concerns the mechanisms whereby cancer cells become genetically unstable. Cancer cell susceptibility to genomic changes may in part be due to the inappropriate expression of certain genes including those involved in the protection of the genome. Although it is still unclear how this process works, many genes have been identified such as tumor suppressor genes or proto-oncogenes.[1] It is now clear that several genetic events are required for the development of a tumor. Therefore, a major interest is to determine what genes are responsible for tumor development.

The mechanisms responsible for the Bcr/Abl associated mutator phenotype that I have described are unknown but could involve functional disruption of DNA repair

proteins or pathways. Our laboratories results suggest a DNA repair defect as the mutations observed include point mutations, insertions and deletions. Recent studies have shown altered regulation of replication factor C (RFCp140) in CML clinical samples and Bcr/Abl expressing cell lines.[18] RFCp140 plays a role in DNA recombination and repair as part of a larger protein complex by recruiting PCNA and attaching it to single stranded DNA.[19; 20] Recent studies have demonstrated that RFC is important for the efficient binding of PCNA for the catalyzed elongation of the singly primed DNA template, but RFC is not required for repair to occur. When mutants of PCNA were constructed within the binding regions of RFC, elongation still occurred but was not as efficient.[21] Other studies indicate that c-Abl may play an important role in the regulation of the cell cycle and cell response to genotoxic stress. DNA damage causes activation of ataxia-telangiectasia mutated (ATM) kinase which phosphorylates c-Abl and p53 on serine residues and results in their activation.[2;3;22] Activated c-Abl in turn phosphorylates the recombination repair associated protein Rad51, and the p53 homologous protein p73.[4; 23-27] Therefore, interference of normal c-Abl by Bcr/Abl, or the loss of one *c-abl* allele in cells bearing the translocation t(9;22) could impair the cellular response to DNA damage and compound the effects of Bcr/Abl expression. The inactivation of one c-Abl allele and the functional disruption of a DNA repair protein or pathway may very well be the cause of the observed genomic instability.

8. Future Research in CML

The approval of STI571 by the Food and Drug Administration gives new hope for the treatment of CML. However, the treatment of acute Ph+ patients is still a cause for

major concern. Furthermore, as STI571 is a c-Abl inhibitor, it is still unclear what the long term effects of c-Abl inhibition may be. Recently Wang et. al (2001), have shown that once Bcr/Abl is treated with STI571 it loses its ability to bind to F-actin and translocates into the nucleus until treatment is discontinued.[28] After treatment, Bcr/Abl moves back into the cytoplasm to again render its oncogenic activities. This may be a mechanism for resistance and will need to be further addressed. Effective treatment of Ph+ leukemias may require STI571 in combination with a drug to block the nuclear export of Bcr/Abl, such as leptomycin B.

The precise effects of STI571 and the complete signal transduction mechanism of Bcr/Abl still remains to be defined. The specific protein functions which STI571 inhibits, and how long treatment needs to be administered for these proteins to remain dormant, still needs to be fully examined. I have demonstrated that STI571 has the ability to increase the number of insertions and deletions in control mice, possibly due to the inhibition of normal c-Abl. Therefore, STI571 may cause long term side effects through the inhibition of normal c-Abl.

Another issue remaining to be addressed in CML is why the P210 length of Bcr/Abl gives rise to CML and the P190 length renders ALL in humans. To date, all of the *in vivo* mouse models have demonstrated that no matter what length of protein is expressed, an ALL type of leukemia is manifested. Clearly, future work should be aimed at developing an animal model for CML.

My work and others have demonstrated a potential role for DNA repair in the evolution of CML. However, the exact role that Bcr/Abl plays in affecting DNA repair remains to be determined. Future research should be aimed towards elucidating the repair mechanisms involved in the transition of this disease. Since the initial discovery of the

Ph chromosome, our understanding of Ph positive leukemias has improved tremendously. Not only has the protein itself, and its signal transduction mechanisms, been described, but a kinase inhibitor has been developed that blocks the oncogenic effects of Bcr/Abl. Major questions still need to be investigated however, including the long term effects of STI571 on patients, treatment options for acute patients, the differences between the P190kDa and P210kDa molecular weights of the protein, and the precise mechanism of genomic instability as it pertains to the progression of this disease.

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Chapter VII Contributions to Original Research

1. The mutation frequencies previously noted in the $P190^{Bcr/Abl}$ x Big Blue transgenic mice can be reversed using a c-Abl specific kinase inhibitor, STI571.

2. The $P190^{Bcr/Abl}$ Transgenic mice develop large mutations in the genome (observed as insertions and deletions) before leukemic onset. These insertions and deletions can be reduced by using the c-Abl specific kinase inhibitor, STI571.

3. The $P210^{Bcr/Abl}$ inducible transgenic mice develop gross abnormalities in the genome (observed as insertions and deletions) upon expression of Bcr/Abl. These observed insertions and deletions are therefore Bcr/Abl dependent.

4. The $P190^{Bcr/Abl}$ transgenic mice over express and under express several genes in the pre-leukemic stage compared to normal, control mice. Some of these genes include genes involved in DNA repair and tumor suppressor genes.