Predictors of Imatinib Response in Patients with Chronic Myeloid Leukaemia

MD Thesis

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by

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Abstract

The majority of patients with chronic myeloid leukaemia (CML) achieve a complete cytogenetic response (CCR) on imatinib. However, we cannot predict which patients will have a suboptimal response. We set out to investigate ways of predicting poor cytogenetic response to imatinib in patients with CML.

We examined patients with CML for the possession of particular alleles of the rs2290573 and IL-1β +3953 polymorphisms because these polymorphisms had been linked with the rate of achievement of a major cytogenetic response (MCR). Following the discovery of a polymorphism (K247R) within the P-loop of BCR-ABL, we determined its frequency within both CML patients and healthy blood donors, and used *in vitro* biochemical and cellular assays to test its drug sensitivity. We examined patients with primary cytogenetic resistance to imatinib for the expression of genes associated with drug transport (hOCT1, MDR1, ABCG2, ABCC1, ABCA2, ABCC2, ABCC3, ABCC6 and MVP) and compared this to patients who achieved a complete cytogenetic response to imatinib. We used gene expression profiling of CML unselected white cells, and of CML CD34+ cells, to look for genes associated with poor cytogenetic response.

We could not find a correlation between the possession of the rs2290573 and IL-1β +3953 polymorphisms, and rate of MCR in our patients. The K247R polymorphism was rare, but 3 out of 5 patients with the arginine allele failed to achieve a major MCR. Despite its position in the P-loop, in *vitro* assays showed K247R to have a drug sensitivity phenotype highly similar to wild type BCR-ABL. Imatinib non-responders had significantly lower pre imatinib gene expression levels of hOCT1, and significantly higher levels of ABCC3, compared to responders. In addition, in imatinib non-responders we found that the expression level of a

variety of drug transport genes changed with time on imatinib, but these changes did not reach statistical significance. Gene expression profiling of CML total white cells revealed that imatinib responders and non-responders had highly similar gene signatures, and that the noise created by different sample source, handling and cell phenotype limited the detection of changes in gene expression. We successfully developed a technique for selecting CD34+ cells from cryopreserved CML mononuclear cells, and preliminary analysis of the CD34+ cell microarray data does not identify any genes that are significantly differentially expressed between cytogenetic responders and non-responders.

Possession of the rs2290573 and IL-1β +3953 polymorphisms did not aid prediction of achievement of MCR in our CML population. In patients with CML, possession of the arginine allele of K247R should not be confused with the development of a kinase domain mutation, and the failure of 3 out of 5 patients to achieve a MCR is likely to be a chance finding in a small cohort, but further collection of response data on patients with K247R is required. Differences in drug transport gene expression may influence patients' responses to imatinib, by potentially causing inadequate intracellular imatinib concentrations. Gene expression profiling of CML unselected WBC does not allow prediction of response to imatinib, and work is ongoing to see if the technique proves more useful when using RNA derived from CML CD34+ cells.

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List of Abbreviations

ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ANOVA	Analysis of Variance
AP	Accelerated Phase
ATP	Adenosine Triphosphate
BC	Blast Crisis
BM	Bone Marrow
CCR	Complete Cytogenetic Response
cDNA	Complementary Deoxyribonucleic Acid
CE	Clonal Evolution
CML	Chronic Myeloid Leukaemia
CP	Chronic Phase
CT	Threshold Cycle
DHPLC	Denaturing High Pressure Liquid Chromatography
DNA	Deoxyribonucleic Acid
FISH	Fluorescent In Situ Hybridisation
GST	Glutathione S-Transferase
IFN	Interferon alpha
IRIS	International Randomised Study of Interferon versus STI571
KM	Kinase Mutation
LB	Luria-Bertani Media
MCR	Major Cytogenetic Response
MDS	Multi-Dimensional Scaling

MMR	Major Molecular Response
MNC's	Mononuclear Cells
NR	Non Responder
OHSU	Oregon Health and Science University
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Ph	Philadelphia chromosome
R	Responder
RMA	Robust Multichip Analysis
RNA	Ribonucleic Acid
RQ-PCR	Real time Quantitative – Polymerase Chain Reaction
RT-PCR	Real Time quantitative – Polymerase Chain Reaction
SAM	Significance Analysis of Microarrays
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
SSCP	Single Strand Conformational Polymorphism
WBC	White Blood Cell
WT	Wild Type

Glossary of terms

Please note that any words or phrases within the body of this thesis that have been underlined have been included within a glossary of terms. The glossary can be found in section 10.

Published work arising from this thesis

L.C. Crossman, M. Loriaux, K. Vartanian, Y. Turpaz, R. Pillai, S.G. O'Brien, B.J. Druker, S.K. McWeeney, C.A. Harrington, M.W.N. Deininger. Gene Expression Profiling Of CML CD34+ Cells Prior To Imatinib Therapy Reveals Differences Between Patients With And Without Subsequent Complete Cytogenetic Response. **American Society of Hematology Annual Meeting**, December 2005

Lucy C. Crossman, Thomas O'Hare, Thoralf Lange, Stephanie G. Willis, Eric P. Stoffregen, Amie S. Corbin, Stephen G. O'Brien, Michael C. Heinrich, Brian J. Druker, Peter G. Middleton, Michael W.N. Deininger. A Gene Polymorphism Within The Kinase Domain Of BCR-ABL And Its Effects On Sensitivity To Tyrosine Kinase Inhibitors. **American Society of Hematology Annual Meeting**, December 2005

Lucy C. Crossman, Thomas O'Hare, Thoralf Lange, Stephanie G. Willis, Eric P. Stoffregen, Amie S. Corbin, Stephen G. O'Brien, Michael C. Heinrich, Brian J. Druker, Peter G. Middleton, Michael W.N. Deininger. A Single Nucleotide Polymorphism in the Coding Region of ABL and its Effects on Sensitivity to Imatinib. **Leukemia** (in press).

Lucy C. Crossman, Brian J. Druker, Michael W.N. Deininger. hOCT1 and resistance to imatinib (letter). **Blood** (in press).

Lucy C. Crossman, Motomi Mori, Yi-Ching Hsieh, Thoralf Lange, Peter Paschka, Christina A. Harrington, Knut Krohn, Dietger W. Niederwieser, Rüdiger Hehlmann, Andreas Hochhaus, Brian J. Druker, Michael W.N. Deininger. In chronic myeloid leukemia white cells from cytogenetic responders and non-responders to imatinib have very similar gene expression signatures. **Haematalogica** 2005; 90:459-464.

K.A. Vartanian, H. Paik, C. Runyan, B. Tompkins, **L. Crossman**, M. Deininger, C.A. Harrington. Factors influencing the optimization and standardization of the Affymetrix GeneChip expression assay small sample amplification protocol in the microarray core laboratory. Poster, **Annual Meeting of the Association of Biomolecular Resource Facilities**, February 2005.

Crossman LC, Heinrich M, Press R, Willis S, Deininger M, Druker B. Various mechanisms underlie cytogenetic refractoriness to imatinib. **Blood** 2004; 104 (11):2091 (Abstract)

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1 Introduction

1.1 Chronic myeloid leukaemia (CML)

Chronic myeloid leukaemia (CML) is a myeloproliferative disease that originates in an abnormal, pluripotent, bone marrow stem cell, and is consistently associated with the Philadelphia (Ph) chromosome and/or the BCR-ABL fusion gene[1-3]. The fusion gene is found in all cells of myeloid lineage, and may be found in some lymphoid cells too. The disease is biphasic or triphasic: the initial indolent chronic phase (CP) is followed by one or both of the aggressive transformed stages known as accelerated phase (AP) and blast crisis (BC)[4]. The worldwide incidence of CML is 1 to 1.5 cases per 100,000 population per year, with a slight male predominance, and an average age at diagnosis between the fifth and sixth decades of life[4]. Prior to the advent of imatinib, treatment with interferon alpha (IFN) based regimes led to a median survival of 5 years.

1.1.1 The Philadelphia (Ph) chromosome

The Ph chromosome is formed by a reciprocal translocation, t(9;22)(q34;q11), that creates a shortened 22q- (the Ph chromosome) and a derivative 9q+[5,6]. The fusion of the ABL gene on chromosome 9, with the break-point cluster region (BCR) gene on chromosome 22, results in the formation of the BCR-ABL fusion gene. The mechanisms behind the formation of the Ph chromosome are unknown. One theory is that chromosomal breaks, and consequent ligations, occur entirely at random and with relatively equal frequency between any two chromosomes in a cell. The "success" of the Ph chromosome is due to the selective growth advantage afforded to the cell by

BCR-ABL. An alternative idea is that the genome contains areas where chromatin is more sensitive to DNA damage, thus leading to non-random reciprocal translocations[7]. Indeed, ionising radiation is a known risk factor for CML[8,9].

1.1.2 The BCR-ABL fusion gene

The ABL gene is ubiquitously expressed, and appears to play a major role in the regulation of cell cycle progression[10]. When ABL is over-expressed, it induces cell cycle arrest and thus has a negative effect on cell growth[11], yet disruption of ABL expression in mice does not result in uncontrolled cell growth. ABL expression appears to be an essential requirement for normal growth and development, since mice homozygous for a null mutation in ABL are runted, lymphopenic and many die in the perinatal period[12,13].

The BCR gene is also ubiquitously expressed, but its functions remain unclear. It has been demonstrated that the first exon of BCR encodes a 160kD phosphoprotein with serine/threonine kinase activity[14], and that BCR knock-out mice have an increased neutrophil respiratory burst, but otherwise apparently normal haematopoiesis[15].

The BCR-ABL fusion gene encodes a BCR-ABL tyrosine kinase that is constitutively active, and can transform haematopoietic cells *in vitro* and *in vivo*[16,17]. The essential role of BCR-ABL for the pathogenesis of CML has become established during the past 20 years of research, and has been confirmed by the therapeutic success of the ABL-specific tyrosine kinase inhibitor, imatinib mesylate (hereafter referred to as imatinib).

1.2 Imatinib

Protein kinases are enzymes that transfer phosphate from adenosine triphosphate (ATP) to specific amino acids on substrate proteins. This protein phosphorylation leads to the activation of signal-transduction pathways, which play a critical role in a variety of biological processes, including cell growth, differentiation and death[18,19]. Protein kinases are made up of 2 subfamilies, the protein threonine kinases and the protein tyrosine kinases.

In the late 1980's, a project designed to identify compounds that might target protein kinase C discovered a 2-phenylaminopyrimidine derivative that inhibited both serine/threonine kinases and tyrosine kinases[20,21]. Derivative compounds were synthesised, and it was found that these compounds also inhibited the ABL tyrosine kinase. Imatinib (formerly CGP57148B and STI-571; Gleevec or Glivec, Novartis, Basel, Switzerland) had the highest selectivity for the growth inhibition of cells expressing BCR-ABL, and hence went on into clinical development. Imatinib has been shown to specifically recognise the inactive, and unphosphorylated, conformation of the kinase domain of ABL, and inhibits kinase activity by binding to the ATP-binding site of the kinase domain[22]. Enzyme kinetics studies suggest that imatinib is a competitive inhibitor of ATP binding[23], and that imatinib interacts closely with the kinase domain, engaging at least 21 amino acid residues[24].

1.2.1 *Activity of imatinib in vitro*

Biochemical assays of kinase activity have shown that imatinib potently inhibits the ABL tyrosine kinases, including cellular ABL, viral ABL and BCR-ABL[25-27]. Kinase assay results were confirmed in cell lines expressing constitutively active ABL, including $p210^{BCR-ABL}$, and also cell lines derived from patients with CML or Ph positive acute lymphoblastic leukaemia (ALL), and these showed 50% inhibitory concentration (IC_{50}) values ranging between 0.1 and 0.5μM[26-30]. Cell proliferation assay IC_{50} results were similar[27,30-32], with imatinib exposure leading to apoptotic cell death. Inhibition of cellular proliferation was confirmed in cells taken from patients with CML[30], and it was shown that there was selective inhibition of colony formation by committed progenitor cells, with minimal effect on normal haematopoiesis at imatinib concentrations up to 1μM[27,31].

Imatinib has also been shown to inhibit platelet-derived growth factor receptor (PDGFR) and c-KIT[25,33,34], but is inactive, or only weakly active, against the serine/threonine kinases, epidermal growth factor (EGF) receptor, vascular endothelial growth factor (VEGF) receptors, fibroblast growth factor (FGF) receptor, tyrosine kinases with immunoglobulin and EGF homology-2, c-MET, and non-receptor SRC family tyrosine kinases[25-27].

1.2.2 Activity of imatinib *in vivo*

Studies of the activity of imatinib, in CML models in mice, have shown that imatinib causes dose-dependent inhibition of tumour growth[27], and that continuous inhibition of BCR-ABL is required to achieve the greatest anti-tumour effect[35]. Mice with a

transduction-transplantation model of CML showed prolonged survival if they were treated with imatinib, but imatinib treatment within 48 hours of transplantation could not prevent the development of CML[36].

1.2.3 Phase I clinical trials

The phase I clinical trials of imatinib started in June 1998. Imatinib was studied in 83 patients with CP CML, in whom treatment with regimes containing IFN had failed[37]. Doses of imatinib ranged from 25mg to 1,000mg daily, with trends towards higher grades of adverse events at doses of 750mg or more. A clear dose-response relationship was evident, and those 54 patients taking 300mg or more had a 98% rate of complete haematological response (CHR) within 4 weeks. Of these patients, 31% had a major cytogenetic response (MCR) (i.e. 35% or less of the cells were Ph positive by cytogenetic analysis) and 13% had a complete cytogenetic response (CCR) (i.e. none of the cells were Ph positive by cytogenetic analysis). Side effects, including nausea, diarrhoea and periorbital oedema, were usually mild and myelosuppression was common, but this was felt to reflect effective reduction of CML haematopoiesis, rather than bone marrow toxicity. No maximum tolerated dose of imatinib was identified.

Pharmacokinetic studies found that at doses of 300mg or greater, plasma imatinib levels were achieved that were equivalent to the effective *in vitro* drug level of 1μM. Four hundred milligrams of imatinib was recommended as the standard dose for CP CML[37], since the steady state trough levels were approximately 2.13μM, and the half-

life was 19.3 hours, which meant that once daily dosing was enough to provide continuous kinase inhibition[38].

From April 1999 to March 2000, the trial was expanded to include a dose escalation study of imatinib in BC and Ph positive ALL[39]. Forty-eight patients with CML were enrolled, of which approximately half had received prior treatment for BC. Again, imatinib was well tolerated. With doses of 300mg to 1000mg daily, 11% of patients with myeloid BC achieved a CHR, while another 10% had a reduction in their bone marrow blasts to less than 5%. Patients with lymphoid BC had response rates of 20%, and 15%, respectively. Disappointingly, the majority of patients showed relapse of their disease within weeks to months.

1.2.4 Phase II clinical trials

In June 1999, three large multinational phase II trials were started. Imatinib, at a dose of 400mg, was given to 532 patients with late CP CML who had failed to respond to treatment with IFN[40] (Table 1.1). The other 2 trials evaluated imatinib 400mg and imatinib 600mg in 235 patients with AP[41] and in 260 patients with myeloid BC[42] (Table 1.1). Patients in CP, who had failed treatment with IFN, had a good response to imatinib with 41% achieving a CCR and 60% achieving a MCR[40]. Importantly, these responses appeared to be durable, with a progression-free survival of 89.2% at 18 months. As expected, the efficacy in AP patients was less than in CP patients, but better than in patients with BC[41,42]. These results led to the Food and Drug Administration (FDA) approving imatinib for the treatment of advanced phase CML, and treatment of CML after failure of IFN therapy, in May 2001. Two retrospective

studies have since compared the survival of historical controls managed with conventional IFN based therapy, with the survival of patients who received imatinib after failure of IFN treatment, and they both found a significant survival benefit for the imatinib treated patients[43,44], though one study found the survival benefit was only significant in the patients who achieved a cytogenetic response on imatinib[44].

Table 1.1 Phase II results with imatinib.

	Chronic phase (IFN failure)[40]	Accelerated phase[41]	Blast crisis[42]
CHR	95%	34%	8%
MCR	60%	24%	16%
CCR	41%	17%	7%
Disease progression	11%	40%	80%

CHR = complete haematological response, MCR = major cytogenetic response, CCR = complete cytogenetic response.

1.2.5 Phase III clinical trials

Between June 2000 and January 2001, 1,106 patients with CP CML, who were less than 6 months from diagnosis, were enrolled into the International Randomised Study of Interferon versus STI571 - the "IRIS" or 0106 study[45]. Patients were randomised to first-line imatinib 400mg (n = 533) or to standard treatment with IFN and low dose cytarabine (n = 533); they were allowed to cross over to the alternative treatment if stringent criteria for intolerance or treatment failure were met. After a median follow up of 19 months, imatinib showed significantly superior tolerability, haematological and cytogenetic responses and also reduced time to progression[45]. MCR and CCR rates at 18 months were 87.1% and 76.2% respectively in the imatinib arm and were

significantly superior to the MCR and CCR rates (34.7% and 14.5%, p = 0.001) in the IFN and cytarabine arm. At 18 months, the disease had not progressed in 92.1% of the imatinib treated patients, while only 73.5% of the combination treatment patients had failed to progress (p = 0.001). There were marked differences in the discontinuation and cross over rates between the two treatments. In those initially treated with imatinib, only 12.3% discontinued treatment and 2% crossed over to interferon. In those treated with IFN and cytarabine, 31.6% discontinued treatment and 57.5% crossed over to imatinib. As well as being clinically superior, a comprehensive quality of life study also demonstrated the superiority of imatinib over IFN[46]. Based on these results, the National Institute for Clinical Excellence approved imatinib for the first line therapy of CML in October 2003.

Largely due to the large numbers of patients crossing over to the imatinib group at an early stage in the trial, no difference in survival between the two groups, on an intention to treat basis, has been demonstrated. However, a retrospective comparison of the imatinib treated patients, with a historical control group of patients treated with IFN, showed superior survival for the imatinib treated patients[47].

A recent review of the 42 month follow up data, at the American Society of Hematology meeting 2004, confirmed that there had been a slight increase in imatinib response rates with further follow up: 91% MCR and 84% CCR. The overall progression-free survival was 94%. It was also clear that diagnostic Sokal scores had an impact on response rates, as shown in Figure 1.1, and on progression free survival, as shown in Figure 1.2. Achievement of a CCR was associated with a lower rate of disease progression at 24 months, compared to those patients who failed to have a

cytogenetic response, and that time to achievement of a CCR did not significantly affect the progression free survival (Figure 1.3). In patients who achieved a CCR, molecular monitoring by real time quantitative polymerase chain reaction (RQ-PCR) was performed. Patients who achieved a 3-log reduction in BCR-ABL transcripts by 12 months (major molecular response, MMR) had a progression-free survival of 100% at 24 months, compared to 95% in those patients without a MMR and 85% in those who failed to achieve a CCR[48]. After 2.5 years of follow up, the overall survival in the study was 95%. Only time will tell whether these excellent results will be maintained.

Figure 1.1 Estimated rates of CCR at 30 months for patients analysed by Sokal score – patients with the lowest score (best prognosis group) had the highest CCR rates. (Figure courtesy of Dr. S. G. O'Brien)

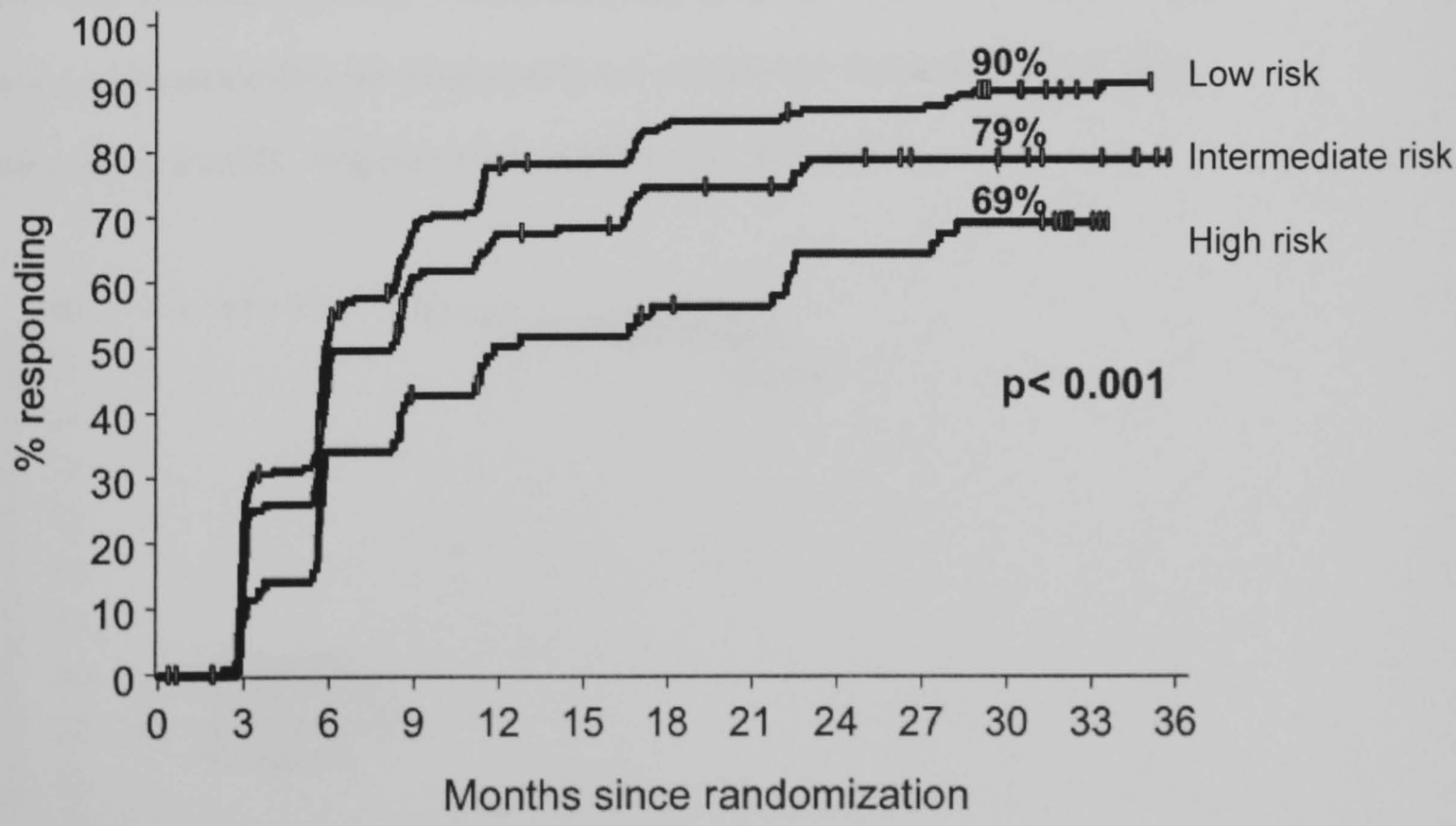


Figure 1.2 Estimated progression-free survival rates at 30 months for patients analysed by Sokal score – patients with the lowest score (best prognosis group) had the highest progression-free survival rates. (Figure courtesy of Dr. S.G. O'Brien)

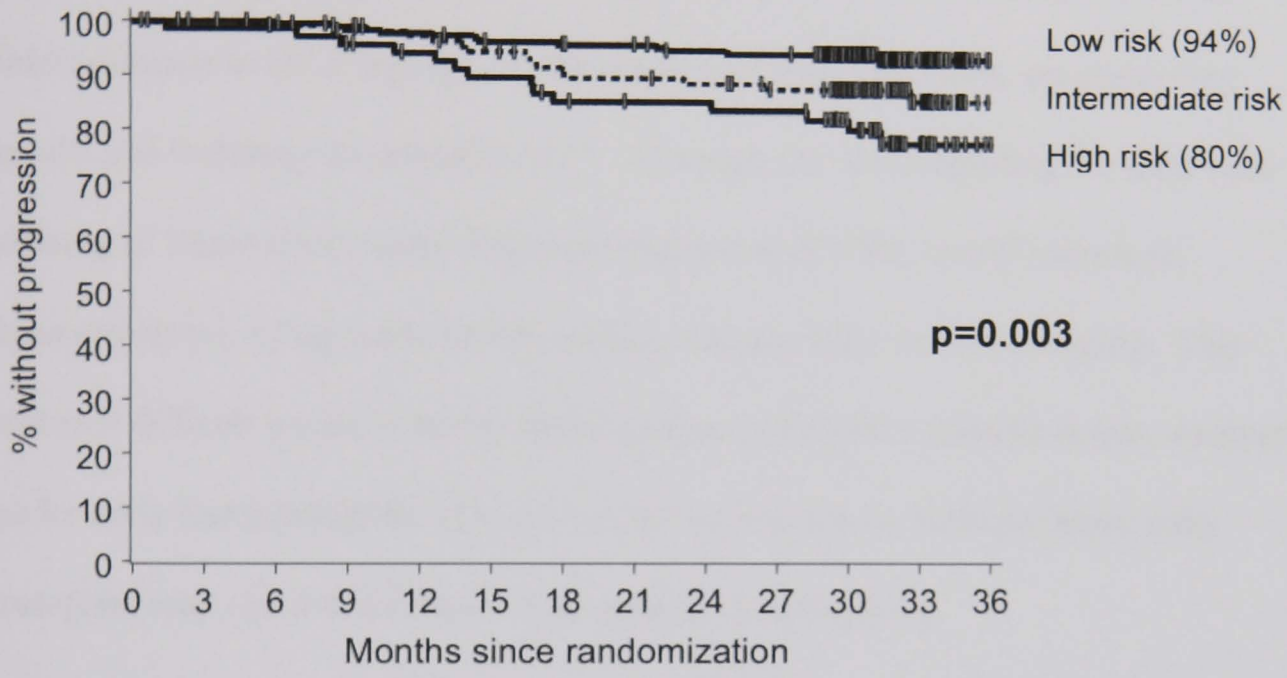
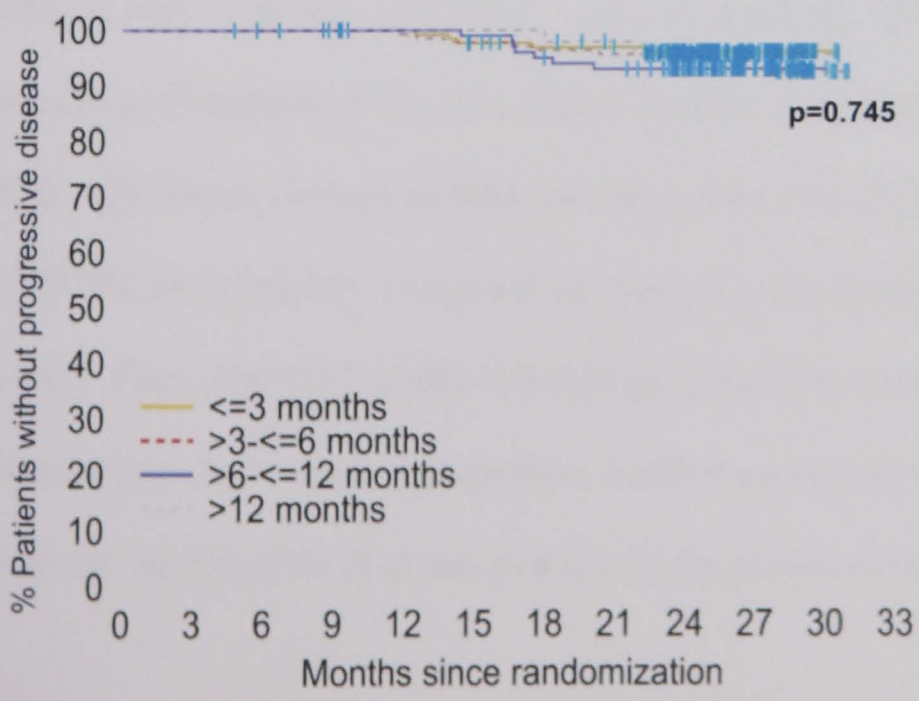


Figure 1.3 Progression-free survival with patients divided into cohorts based on their time to achieve a CCR. Patients taking more than 1 year to achieve a CCR have a progression-free survival that is not statistically different from patients that achieve a CCR. (Figure courtesy of Dr. S.G. O'Brien)



1.2.6 Imatinib and allogeneic transplantation

At the present time, allogeneic haematopoietic stem cell transplantation is the only known cure for CML, with its attendant morbidity and mortality[49,50]. Its use is limited by donor availability and the high toxicity of the procedure in older patients, but improvements in HLA typing, and the use of less toxic regimens, are improving results and widening its potential use[51]. Although the data regarding the long-term efficacy of imatinib in newly diagnosed patients with CML is still immature, haematological, cytogenetic and molecular response rates are encouraging. This makes it difficult to advise newly diagnosed patients with a suitable donor, whether to go for early transplantation, or to take imatinib and rely on having a more risky transplant later on, if their response to imatinib is suboptimal.

1.2.7 AMN107

AMN107 is a BCR-ABL tyrosine kinase inhibitor that has recently been developed from imatinib, resulting in a compound with 20 times imatinib's affinity for BCR-ABL, as well as greater specificity[52]. Just like imatinib, AMN107 binds to the inactive conformation of the ABL kinase domain. Cell lines expressing the common BCR-ABL kinase domain mutants (see below) are also 20 times as sensitive to AMN107, as to imatinib, except for cell lines expressing the T315I mutant BCR-ABL[53]. Thus, AMN107 would be predicted to have the potential to treat patients with refractoriness to imatinib, though there is still the potential for the development of resistance by selection of clones that favour the active conformation of the BCR-ABL

tyrosine kinase. Phase I clinical trials are in progress, and preliminary results are encouraging[54].

1.2.8 BMS-354825

BMS-354825 is a dual SRC/ABL inhibitor that has approximately 300 times the affinity of imatinib for the ABL kinase, and binds the kinase domain in both the active and inactive conformations. *In vitro* studies have shown that BMS-354825 inhibits all the imatinib resistant BCR-ABL mutants, except for T315I (see below)[55,56]. BMS-354825 would be predicted to be able to treat patients with imatinib resistance, and since there are fewer contact points with the kinase domain than with imatinib, it has the potential to be less likely to induce resistance[57]. However, BMS-354825 is less specific than imatinib and there is the potential for long-term toxicity due to SRC inhibition. Phase I trials are in progress, and preliminary results show that 73% of patients with imatinib-resistant CP CML have achieved a complete CHR, and that the drug appears to be well tolerated[58].

1.3 Imatinib resistance

In patients treated with imatinib, patients' response rates, and the durability of these responses, are highly dependent on the phase of the patient's disease when imatinib is started[37,39-42,45]. Primary resistance and secondary, or acquired, resistance to imatinib have been seen at all disease stages, but is a particular problem in more advanced disease. A number of mechanisms of resistance have been described, with most work having been done in patients with acquired resistance.

1.3.1 Kinase mutations (KM)

Point mutations within the ABL kinase domain have been reported as the most frequent mechanism for BCR-ABL reactivation within a clone of leukaemic cells[59-64]. KM lead to imatinib resistance by disrupting the critical contact points between imatinib and BCR-ABL, or by inducing a transition from the inactive state of the BCR-ABL tyrosine kinase, to the active state[65,66]. KM are found in 50% to 90% of patients with acquired resistance[23,59-61,63,67], but appear to be less frequent in patients with primary cytogenetic resistance to imatinib[59,62,68]. In some patients, KM detected at disease relapse on imatinib, have been found to be evident prior to the patient receiving imatinib[62,63,69], consistent with the theory that the selective pressure exerted by imatinib favours the outgrowth of pre-existing resistant cell clones. However, Willis *et al.*[70] recently detected KM at low levels in patients prior to their starting imatinib. With follow up, these patients did not invariably go on to show imatinib resistance, leading to the hypothesis that additional factors might be required to induce a fully drug resistant phenotype[70].

Characterisation of the mutants by biochemical and cellular *in vitro* assays generally reveals a decreased sensitivity of the mutants to imatinib, although the degree of resistance varies. KM are most commonly found within the ATP binding loop (P-loop), the A-loop, and at amino acid residues T315 and M351.

1.3.1.1 P-loop mutations

The P-loop encompasses the amino acids 244, through to 255, which interact with the phosphate groups of ATP. P-loop mutations are the most commonly identified KM,

and have been shown to be 70 to 100 times less sensitive to imatinib in kinase assays, and 10 times less sensitive to imatinib in cell proliferation assays[71]. They are associated with a particularly poor prognosis, as compared to patients with other types of mutation[67].

1.3.1.2 A-loop mutations

The A-loop is made up of amino acids 381 to 402 of ABL, and it regulates kinase activity. Mutations in the A-loop are thought to prevent the BCR-ABL kinase from adopting its inactive conformation, and so prevent imatinib from binding. A-loop mutations account for less than 10% of KM detected[72]. They exhibit moderate resistance in *in vitro* assays[64,71].

1.3.1.3 Mutations of T315

T315 forms a hydrogen bond with imatinib, and mutations at T315 generally cause almost complete insensitivity to imatinib[60,71]. T315 mutations account for just over 20% of KM detected[72]. T315I mutants are also resistant to the novel kinase inhibitors AMN107 and BMS-354825[52,53,55,56].

1.3.1.4 Mutations of M351

M351 helps to stabilise the auto-inhibited conformation of ABL, by its interaction with the ABL SH2 domain. Even though BCR-ABL is constitutively active, it still maintains some of the mechanisms responsible for auto-inhibition of ABL. Thus, mutations in M351 alter the interaction between the SH2 domain and the kinase domain, leading to a shift in the equilibrium towards the active conformation of the

BCR-ABL, to which imatinib cannot bind[72]. M351 mutations account for approximately 15% of KM detected, and cause mild imatinib insensitivity[72].

1.3.2 Amplification and over expression of BCR-ABL

Fluorescence in situ hybridisation (FISH) has been used to demonstrate the amplification of BCR-ABL, at the genomic DNA level, in CML patients resistant to imatinib[60]. In another report, increased BCR-ABL mRNA levels were seen in imatinib resistant patients[61]. Both these mechanisms are thought to lead to increased BCR-ABL protein expression, and hence imatinib resistance.

1.3.3 Clonal evolution

Although karyotypic abnormalities, in addition to the Ph chromosome, have been detected in patients at the time of disease relapse on imatinib[61,73,74], the pattern of clonal evolution (CE) does not appear to be different from that seen in patients having treatment with IFN[75]. Therefore, CE is more likely a sign of advancing disease, rather than a mechanism of resistance.

1.3.4 Drug transport mechanisms

Recent studies have examined drug transport as a potential mechanism for imatinib resistance. Imatinib has been shown to be a substrate for the ATP-binding cassette transporters which cause drug efflux, including P-glycoprotein (MDR1[76-83]), ABCG2[80,84,85] and ABCC1[77], and these transporters have been linked to imatinib resistance in CML cells[78,80,81,86]. Imatinib has also been shown to be a substrate for the

human organic cation transporter 1 (hOCT1)[83], which causes imatinib influx. Thomas *et al.*[83] proposed that imbalance of influx and efflux transport mechanisms, might lead to inadequate intracellular concentrations of imatinib, and hence a lack of response.

1.3.5 Quiescent stem cells

The normal stem cell population contains a large non-cycling G_0 fraction. Patients with CML also have a leukaemic population of these "quiescent stem cells"[87]. These BCR-ABL positive, CD34+ cells were able to spontaneously exit G_0 and enter a continuously cycling state with subsequent up regulation of IL-3 expression[88]. There is concern that imatinib will not be able to eliminate these malignant haemopoietic progenitor cells. It has been shown that imatinib does suppress growth of malignant CD34+ cells by inhibiting abnormally increased cell proliferation but it does not affect the apoptotic rate of these cells[89]. Further investigation of 15 patients in CCR showed that all still had evidence of BCR-ABL in their bone marrow CD34+ cells, as detected by FISH, and that the level of expression of BCR-ABL was higher in the CD34+ cells than in the bone marrow mononuclear cells. Also, serial evaluation of patients showed persistence of the BCR-ABL positive progenitors despite continued treatment with imatinib[90]. It has also shown that primitive, quiescent Ph positive progenitor cells from patients are insensitive to imatinib *in vitro*[91], and that CD34 positive, CD38 negative CML stem cells have BCR-ABL kinase activity despite the presence of imatinib[92]. These data strongly suggest that some malignant haemopoietic precursors are not eliminated by imatinib, and with this comes the risk of disease relapse[93]. Further research into therapeutic measures to eliminate these imatinib resistant stem cells is required.

1.4 Predicting response to imatinib

The data from the IRIS trial[45] demonstrated that at least three quarters of patients with newly diagnosed chronic phase CML achieved a CCR on imatinib, and 39% would have a MMR after 12 months of treatment[94]. However, we do not know which patients will have an optimal response to drug therapy or the durability of their responses. Allogeneic stem cell transplant is the only known curative treatment for CML, but is associated with a high risk of mortality. Being able to predict who will and who will not have an optimal response to imatinib would be of great value in helping clinicians decide which patients should preferably undergo allografting and which should consider drug therapy as first line treatment.

1.4.1 Sokal and Hasford scoring systems

The 'traditional' prognostic indicators, such as Sokal[95] and Hasford[96] scores, are mathematical calculations derived from clinical and laboratory parameters at diagnosis. The Sokal score was derived using patients treated with busulphan or hydroxyurea. The Hasford score was generated using patients only treated with interferon. Thus, these scores were not designed using data from patients treated with imatinib, and not all of their criteria, for example age, are as relevant to prognosis as they used to be[97]. However, it does appear that with increasing follow up of patients on first line imatinib, these scores are starting to divide patients into high, intermediate and low risk groups. The problem with these scores is that they do not identify an individual patient's prognosis, but rather give a prognosis for a group of patients. In patients where an allogeneic transplant is a potential option, one would

like to be able to predict that individual's response to imatinib, and so offer patients a well-informed choice about treatment risks and benefits. It would be a huge achievement to be able to accurately predict an individual patient's response to imatinib. Currently, the prognostic values of a number of techniques are under investigation.

1.4.2 Cytogenetic response

Data from the IRIS trial[45], which were presented at the American Society of Hematology meeting, were suggestive that knowledge of patients' cytogenetic responses after 6 or 12 months of imatinib treatment would allow the prediction of whether a patient was likely to achieve a CCR or not. They found that those patients with at least 95% Ph positive metaphases after 6 months of imatinib treatment were highly unlikely to achieve a CCR with 30 months of follow up. In addition, those patients who had achieved a MCR after up to 1 year of imatinib were very likely to achieve a CCR in the future. Patients without an MCR at 1 year were much less likely to achieve a CCR, though this was based on a much smaller number of patients.

1.4.3 Deletions of the derivative chromosome 9

The development of fluorescent in situ hybridisation (FISH) techniques in the mid 1990's to detect BCR-ABL led to the discovery of unexpected deletions in the derivative chromosome 9, in 10% to 15% of patients with CML[98,99]. These deletions were discovered to be adjacent to, and usually spanning, the translocation breakpoint of the derivative chromosome 9. They were very large, and varied considerably between patients[99]. The deletions appear to occur at the time of Ph translocation, as

different cohorts of patients have identical frequencies of deletions[100,101] and samples from individual patients, taken at diagnosis and then disease progression, show that not one of them acquires a deletion[100,101]. Deletions of the derivative chromosome 9 have been shown to be associated with a poor prognosis[99,101,102], with patients having a median survival approximately half that of other patients. These early studies included patients treated with hydroxyurea and IFN based regimes[99-103] and one study suggested that patients with deletions were more likely to relapse after allografting[102]. With regard to treatment with imatinib, the latest evidence suggests that treatment with imatinib overcomes the difference in survival seen between patients with and without deletions[104-106].

1.4.4 Telomere shortening

Reduction in telomere length has been demonstrated in some cancers, including CML[107], and evidence has suggested that it may prove to be a useful prognostic tool. In patients treated with interferon, those with longer telomeres had better cytogenetic responses, progression free survival and overall survival[108]; however, this did not hold true when this study was extended to include patients on other treatments. Telomere length was also found to correlate with reduced time from diagnosis to AP, but not to BC[109]. In patients treated with imatinib, the median telomere length of peripheral blood granulocytes was found to decrease as the fraction of Ph negative cells increased[110,111]. Interestingly, Drummond *et al.* found that of CML patients at diagnosis, patients with a high risk score had the greatest amount of telomere shortening, and that telomere shortening occurred rapidly during disease progression[112]. Thus, telomere shortening has the potential to act as a marker of

disease progression in CP[113], and in the future may find a role in helping to predict a patient's prognosis.

1.4.5 Gene polymorphisms

Polymorphisms in genes that are related to disease, drug target, absorption, distribution, metabolism and drug excretion may affect an individual's response to their disease treatment and hence may allow prediction of their response to that treatment. In order to investigate the potential of pharmacogenetic analysis to identify genetic markers associated with rate of MCR on imatinib, Dressman *et al.*[114] examined genotypes from 88 newly-diagnosed patients in the IRIS trial[45], and looked at 68 candidate polymorphic loci in 26 genes. The 26 genes included tyrosine kinases, cytokines and genes associated with drug transport. They found two polymorphisms that were significantly associated with achievement of a MCR on imatinib.

The rs2290573 polymorphism maps to the putative tyrosine kinase gene DKFZP434C131, and the effect of the rs2290573 polymorphism on the function of DKFZP434C131 is unknown. Dressman *et al.*[114] found a significant correlation between the presence of the "CC" genotype of the rs2290573 gene polymorphism and reduced rate of MCR on first line imatinib. A "CC" genotype was linked to a MCR rate of 47%, compared to a "CT" or "TT" genotype that had a MCR rate of 88% ($p<0.05$)[115].

IL-1β is a member of the IL-1 family and acts as an agonist of the pro-inflammatory response. Both the IL-1β +3953 and IL-1β -511 polymorphisms have been linked to increased IL-1 production *in vitro*[116,117], and the IL-1β +3953 polymorphism has been associated with adult periodontitis[118,119] and inflammatory bowel disease[120-122]. These 2 polymorphisms have been shown to be in linkage disequilibrium with each other[117,123]. Dressman *et al.* showed a significant correlation between the "CC" genotype of the IL-1ß -511 polymorphism and achievement of a MCR; however this correlation was not sustained with further patient follow up[114].

The TP53 gene encodes the p53 tumour-suppressor protein. Bergamaschi *et al.*[124] recently found that possession of the A1 allele, of the TP53 gene (codon 72) polymorphism, was significantly more frequent in patients with CML than controls. Interestingly, they found that the highest frequency of this allele was seen in patients with a high risk Sokal score, and in patients who remained at least 65% Ph positive during the first 6 months of imatinib therapy. Dressman *et al.*[114] also studied this polymorphism in their patients, but they failed to see a correlation with achievement of a MCR.

Amirzargar *et al.*[125] examined the genotype frequency of the T helper types 1 and 2 cytokine polymorphisms in patients with CML, and compared this with normal controls. They found several genotypes were significantly more common in CML patients, and this led them to postulate, based on their knowledge of the functional effects of these polymorphisms, that CML patients might have higher TGF-β levels, and lower IL-4 and IL-10 levels, than normal controls. We note that Dressman *et*

al.[114] also studied polymorphisms in the TNF, IL1-α and IL-1β genes, finding no links with rate of MCR, other than for the IL-1β -511 polymorphism.

1.4.6 Gene expression profiling

Microarray gene expression profiling allows for the simultaneous assessment of the gene expression levels of tens of thousands of genes. Some of the earliest clinical studies using gene expression profiling took place in acute leukaemia, since access to pure tumour cell populations was relatively easy. After examining the gene expression profiles of 38 patients with either acute myeloid leukaemia (AML) or ALL, Golub *et al.*[126] developed a class predictor using the 50 genes that were most differentially expressed between AML and ALL. This predictor correctly assigned 36 out of 38 samples to their correct diagnosis, and was able to accurately assign a diagnosis of AML or ALL to 29 out of 34 samples from an independent set of unknown leukaemia samples. Although there are more direct, and less costly, ways of classifying acute leukaemia, this study proved that microarray technology was capable of identifying underlying biological categories in patient samples[127].

Gene expression profiling has been shown to be a useful technique that enables the prediction of response to treatment in several haematological malignancies, including ALL[128-130], AML[131,132] and non-Hodgkins lymphoma (NHL)[133,134]. Indeed, in diffuse large B-cell lymphoma this has led to the identification of 6 genes whose expression, as measured by RQ-PCR, accurately predicts survival[135]. It would therefore appear that gene expression profiling could potentially be used for predicting response to imatinib in patients with CML, and that ultimately a simpler, RQ-PCR method could

be used to predict response to the same therapy regime, based on the expression levels of a few select genes.

1.4.7 Gene expression profiling in patients with CML

Studies on the gene expression profile of CML patient cells have found that their expression profile differs from healthy cells[136], and that it changes with advancement of the disease[136,137]. It has also been shown that cells with deletions of the derivative 9q+, have a different gene expression pattern from those without these deletions[100]. Imatinib sensitive and resistant CML cell lines, derived from KCL22 cells, have different gene profiles, and the expression pattern of resistant cells changes with culture in the presence of imatinib[138]. In patients with Ph positive ALL, gene expression profiling has been shown to allow prediction of patients' sensitivity to imatinib[129], and similar work has been published for patients with CML[139-142].

A study by Kaneta *et al.*[143] identified 79 genes that were expressed differentially between responders and non-responders to imatinib, where response was classed as a MCR after 5 months therapy and non-response as remaining at least 65% Ph positive by FISH. On the basis of the expression patterns of 15 or 30 genes they formulated a scoring system that accurately predicted response in a further 4 test cases, though follow up of the test cases was short at less than 200 days.

Work by McLean and colleagues[141] also examined gene expression profiles on whole blood samples taken from 66 CML patients in the IRIS study prior to treatment with imatinib. Patients with a CCR after 12 months of imatinib treatment were compared

with non-responders (>65% Ph positive by FISH after 12 months treatment) and a set of 31 genes was identified which was predictive of cytogenetic response in 94% of patients (62 of 66). However, this group did not attempt to show the reproducibility of their results by testing them in an independent set of patients.

Yong *et al.* presented their work at the American Society of Hematology meeting, 2003. They looked at the gene expression profile of CD34+ cells, taken from leukopheresis samples, from 19 CML patients taken within 3 months of diagnosis, to examine whether patients had "indolent" disease (minimum CP duration of 7 years) or "aggressive" disease (maximum CP duration of 3 years). They identified a predictive set of 28 genes, with a number of genes involved in myeloid differentiation being up regulated in responders but down regulated in non-responders. They confirmed the expression of their discriminating genes by RQ-PCR and stated that they were hoping to validate their results in an independent group of CML patients.

1.5 Overall aims of our studies

The treatment of patients with CML has been dramatically changed by the advent of imatinib therapy. It would be very useful to predict which patients will not achieve a CCR on imatinib, so that these patients could be targeted for more aggressive initial therapy. We set out to identify genetic markers that associated with patients' responses to imatinib, with the hope of using these to predict an individual patient's response to treatment. We approached this in two ways: firstly by examining individual candidate genes and polymorphisms, and secondly by examining overall gene expression patterns.

2 General methods

2.1 Introduction

I have included here a description of all the methods we have used for DNA extraction, RNA extraction, cDNA synthesis, polymerase chain reactions (PCR), real time quantitative PCR (RQ-PCR) and western blotting.

2.2 DNA Extraction

2.2.1 Phenol/Chloroform extraction of DNA from cell pellets

Cell pellets were thawed at room temperature and the cells were then resuspended in 900µl TNE buffer (50mM Tris-HCl (pH7.5), 150mM NaCl and 10mM EDTA). Next, 10µl 20% SDS and 10µl proteinase K (10mg/ml) were added and mixed by inverting the sample 2 or 3 times. Samples were incubated at 37^{0}C overnight. The next day, 900µl neutralised, water saturated phenol was added to each sample at room temperature, and the sample inverted at a rate of one inversion per second for 10 minutes to ensure a good emulsion was made. Samples were then centrifuged for 1min at 500g and the upper phase removed to a fresh tube. Next, 900µl of chloroform:isoamyl alcohol (24:1) was added and the sample was inverted at a rate of one inversion per second for 5 minutes, prior to centriugation at 500g for 1minute. The upper phase was removed to a 15ml tube, and 450µl (half volume) 7.5M ammonium acetate was added and the mixture gently inverted 6 times. Three millilitres of absolute alcohol was added to each tube and the sample was inverted 2

or 3 times. The sample was then incubated at room temperature for a minimum of 20 minutes to allow the DNA to precipitate.

2.2.2 Spooling of DNA

The end of a sterile glass Pasteur pipette was heated in a bunsen flame to seal its tip. The glass pipette was used to 'spool' the DNA precipitate, which was then air dried for a few seconds and the DNA was then unwound into a fresh tube containing 500µl TE buffer (10mM Tris-HCl (pH8.0) and 1mM EDTA). The DNA was either stored at room temperature for a few days, or left overnight at 37°C if required the next day, to allow the high molecular weight DNA to re-dissolve.

2.3 RNA Extraction

2.3.1 RNeasy® Mini Kit (Qiagen, Valencia, CA)

RNA was extracted according to the manufacturer's instructions (http://www1.qiagen.com/literature/handbooks/INT/RNAStabilizationAndPurificationFromClinicalSamples.aspx). In brief, cells for RNA extraction were lysed and homogenised in the presence of guanidine isothiocyanate containing buffer, and either stored at -80°C or used immediately. Ethanol was added to provide the correct binding conditions, and the sample was then placed onto an RNeasy mini column. Here, the total RNA was bound to the membrane, and any contaminants were washed away with Buffer RW1 and Buffer RPE (supplied by the manufacturer). RNA was then eluted into 30µl of water. The RNA was used immediately, or stored at -80°C.

2.3.2 PicoPure™ RNA Isolation Kit (Arcturus, Mountain View, CA)

RNA was extracted according to the manufacturer's instructions (http://www.arctur.com/research_portal/resources/download.htm) and included a step for DNase treatment (User Guide, Appendix A). This protocol was used in preference to the RNeasy® Mini Kit when we were using an input of less than 5 x 10^6 cells; pilot studies had shown that we could achieve higher RNA yields with the PicoPure™ RNA Isolation Kit, than with RNeasy®, at cell numbers ≤5 x 10^6. In brief, the cells for RNA extraction were lysed in the presence of guanidine isothiocyanate containing buffer, at 42°C for 30 minutes, and centrifuged. The RNA containing supernatant was either stored at -80°C, or used immediately. Ethanol was added to provide the correct binding conditions, and the sample then placed onto a purification column. Here, the total RNA was bound to the membrane, and the sample washed once with Wash Buffer 1 (W1). The sample was then treated with the RNase-Free DNase kit (Qiagen), according to the manufacturer's instructions, then washed a second time with W1. The sample was then washed twice with Wash Buffer 2 (W2) and then the RNA was eluted into 11µl of water. The RNA was either used immediately or stored at -80°C until required.

2.3.3 Ethanol precipitation of RNA

When required, we precipitated RNA in order to concentrate it. RNA was made up to a volume of 100µl with water, and 5µl 5M sodium chloride and 200µl 100% ethanol were added. The sample was placed in liquid nitrogen until it froze solid, and then it was centrifuged at 16,000g, at 4°C, for 20 minutes. The RNA pellet was then washed

twice with 500µl 70% ethanol, and then residual ethanol was removed and the sample allowed to air dry for 10 minutes. The RNA was then suspended in 10µl RNase free water and either used immediately, or stored at -80°C.

2.4 cDNA Synthesis

2.4.1 SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA)

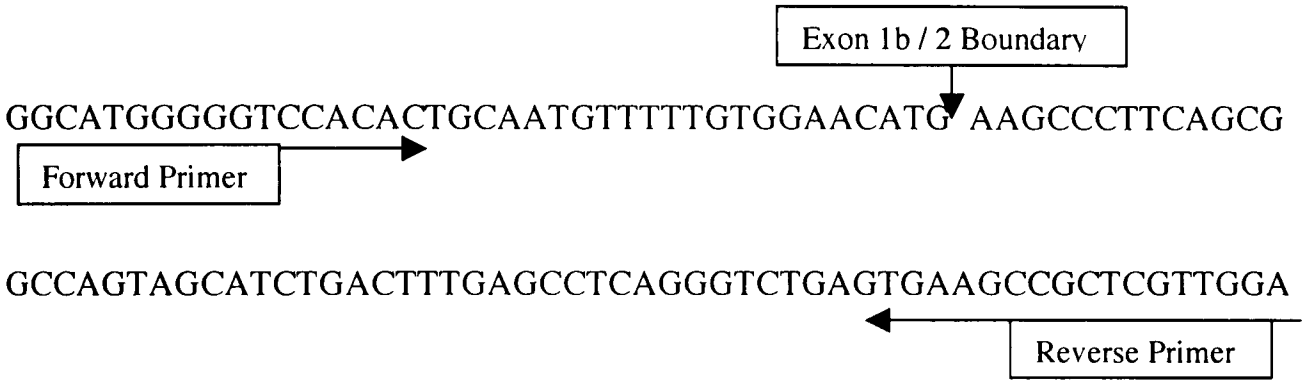
In order to manufacture single stranded cDNA from RNA extracted using the RNeasy® Mini Kit, we used the Invitrogen kit, SuperScript™ III First-Strand Synthesis System for RT-PCR, according to the manufacturer's instructions (http://www.invitrogen.com/content.cfm?pageid=10745#sensitive). In brief, we used up to 4µg of total RNA made up to 16µl with RNase free water, and added 2µl 50ng/µl random hexamers and 2µl of 10mM dNTP mix. This mixture was incubated at 65°C for 5 minutes, before the addition of 4µl 10x RT buffer (manufacturer supplied), 25mM magnesium chloride, 0.1M dithiothreitol (DTT), 2µl (40U/µl) RNase OUT and 2µl of 200U/µl SuperScript™ III reverse transcriptase. Samples were then incubated at 25°C for 10 minutes, 50°C for 50 minutes and 85°C for 5 minutes. Next, 2µl (2U/µl) *E. coli* RNase H was added and the sample incubated at 37°C for 20 minutes. The final reaction volume was 42µl. The quality of the resulting cDNA was then assessed by PCR for ABL, and the cDNA was either used immediately or stored at -80°C.

2.5 Polymerase Chain Reactions (PCR)

2.5.1 PCR for ABL

We designed a PCR to detect the exon 1/2 boundary of the ABL gene (Figure 2.1) so that we could not only assess the quality of any cDNA that we made, but also to go on and develop the assay for use as a standard in RQ-PCR experiments.

Figure 2.1 Sequence of ABL (5' – 3') and situation of primers for the PCR of the exon 1/2 boundary.



Forward primer = 5' – ggcatgggggtccacact – 3'

Reverse primer = 5' - tccaacgagcggcttcac – 3'

We used a DYAD™ DNA engine PCR machine and included a positive control (ABL-b plasmid 0.1ng/μl) and a negative control (water) with each experiment. The master mix and temperature protocol that we used are shown below:

Table 2.1 Master mix

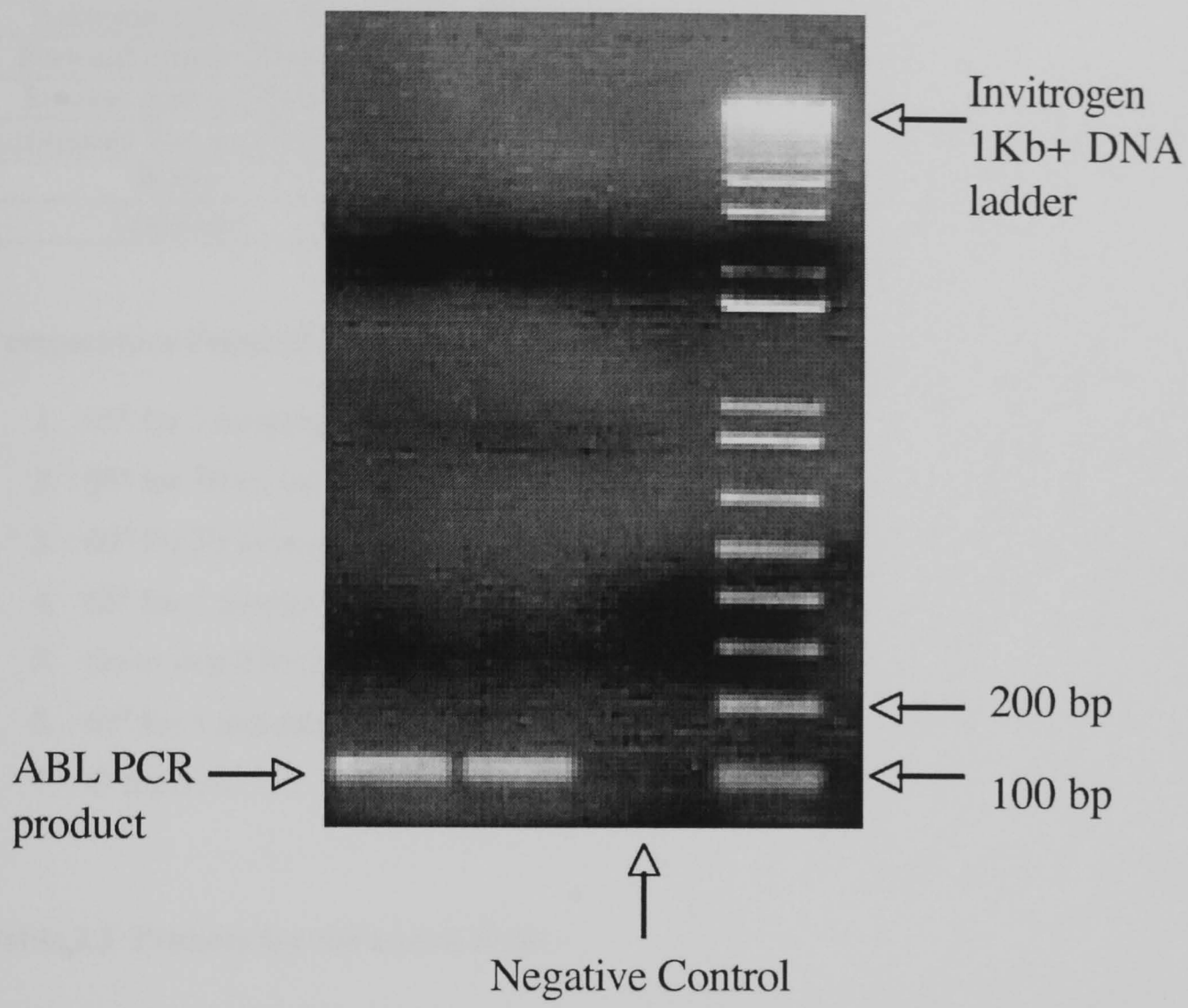
Reagent	Volume for 20μl Reaction
10 x PCR Buffer (Roche)	2μl
dNTP's	1μl
Forward Primer	1μl
Reverse Primer	1μl
$MgSO_4$	0.8μl
Deionised Water	14μl
Taq DNA polymerase (Roche)	0.2μl
Template cDNA To Add	1μl
Total Volume	**21μl**

ABL PCR- Temperature Programme

1. 95°C for 5 minutes
2. 95°C for 15 seconds
3. 60°C for 20 seconds
4. 72°C for 20 seconds
5. Cycle to step 2 for 34 more times
6. Incubate at 72°C for 3 minutes
7. Incubate at 4°C forever

The PCR product underwent electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and this was then inspected for the presence of a band of 105 base pairs in length (Figure 2.2).

Figure 2.2 1% Agarose gel electrophoresis of ABL PCR product.



2.5.2 Nested PCR for the kinase domain of ABL or BCR-ABL

In order to perform sequencing of the kinase domain of ABL or BCR-ABL, we needed to perform nested PCR to amplify this region. We used the AccuPrime™ *Taq* DNA Polymerase System (Invitrogen). We used the following master mix (Table 2.2) and temperature protocol for the 2 rounds of PCR, combined with the appropriate ABL or BCR-ABL primers (Table 2.3).

Table 2.2 Master mix for the nested PCR reaction.

Reagent	Volume (μl)
Accuprime Buffer II	5
Forward primer 10μM	2
Reverse primer 10μM	2
Accuprime *Taq polymerase*	0.5
Water	39.5
Template	1

Temperature Protocol for the nested PCR

1. 95° for 2 minutes
2. 95° for 30 seconds
3. 60° for 30 seconds
4. 68° for 1 minute 30 seconds
5. Go to step 2 for 35 more cycles
6. 68° for 5 minutes
7. 4° forever

Table 2.3 Primers for the nested PCR.

Primers	BCR-ABL	ABL
1st round nested PCR –	5'-TTCAGAAGCTTCTCCCTGACAT-3'	5'-GCTTGCCTGCCCTGCA-3'
1st round nested PCR –	5'-CCTTCTCTAGCAGCTCATACACCTG-3'	5'-CCTTCTCTAGCAGCTCATACACCTG-3'
2nd round nested PCR –	5'-ACAGCATTCCGCTGACCATC-3'	5'-GGCATGGGGGTCCACACT-3'
2nd round nested PCR –	5'-GCCATAGGTAGCAATTTCCC-3'	5'-GCCATAGGTAGCAATTTCCC-3'

2.5.3 Sequencing of the kinase domain of ABL or BCR-ABL

Nested PCR products were run on a 1% agarose gel, stained with ethidium bromide, and the PCR product of the correct length (approximately 1,400 base pairs) was cut out of the gel (Figure 2.3). The DNA was extracted from the gel pieces using the Montage™ DNA Gel Extraction Kit (Millipore, Billerica, MA) according to the manufacturer's instructions (http://www.millipore.com/publications.nsf/docs/pf1230en00). The DNA was precipitated by the following method: 5% 5M ammonium acetate and 200% ethanol were added to the sample and the mixture was then placed in liquid nitrogen until frozen solid; the mixture was centrifuged at 16,000g, at 4°C, for 30 minutes, and then the DNA pellet was washed twice with 70% ethanol and air dried before suspending in 10µl water. The precipitated DNA was then prepared using the master mix shown in Table 2.4 and the primers shown in Table 2.5, and sent for automated sequencing at the Vollum Institute, Oregon Health and Science University.

Figure 2.3 1% Agarose Gel Electrophoresis of BCR-ABL nested PCR product.

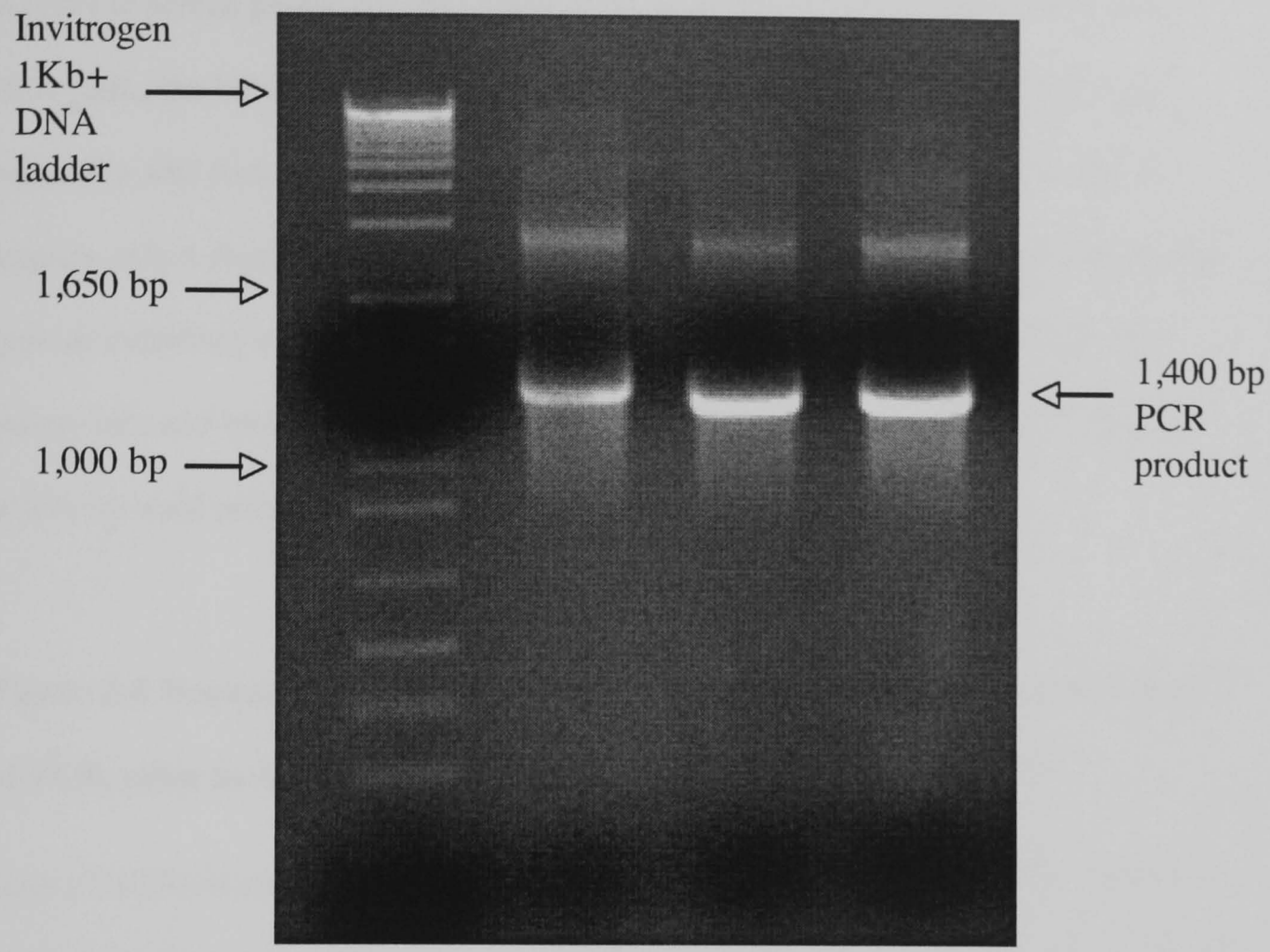


Table 2.4 Sequencing master mix

Reagent	Volume (µl)
Template	5µl
Water	4.8µl
Primer 1µM	3.2

Table 2.5 Primers used for automated sequencing.

Primers	BCR-ABL	ABL
Sequencing	5'-TGGTTCATCATCATTCAACGG-3'	5'-TGGTTCATCATCATTCAACGG-3'

2.5.4 *AluI* Restriction enzyme digestion

In order to screen people for the alleles of the K247R polymorphism in ABL and BCR-ABL, the kinase domain was amplified by nested PCR. However, there are numerous *AluI* restriction sites within the kinase domain, and we therefore had to amplify only a short segment of the kinase domain during the second PCR reaction to provide ourselves with a clear result (Figure 2.4). We therefore used a different master mix and temperature protocol for the second round of nested PCR, from that which we used prior to sequencing (Table 2.6).

Figure 2.4 Sequence (5' to 3') for the PCR product following the second round of PCR, prior to AluI restriction enzyme digestion (AluI cuts AG↓CT).

tggttcatcatcattcaacggtggccgacgggctcatcatcaccacgctccattatccagccccaaagcgcaacaagccca
ctgtctatggtgtgtccccaactacgacaagtgggagatggaacgcacggacatcaccatgaagcacaagctgggcgg
gggccagtacggggaggtgtacgagggcgtgtggaagaaatacagcctgacggtggccgtgaagaccttgaaggagg
acaccatggaggtggaagagttcttga

Codon 247 of the kinase domain is shown in red and the primers are in green. The more common lysine allele of K247R is shown; the arginine allele of K247R has the sequence agg at codon 247.

Temperature protocol for the second round of PCR, prior to *AluI* digestion.

1. 95° for 2 minutes
2. 95° for 30 seconds
3. 60° for 30 seconds

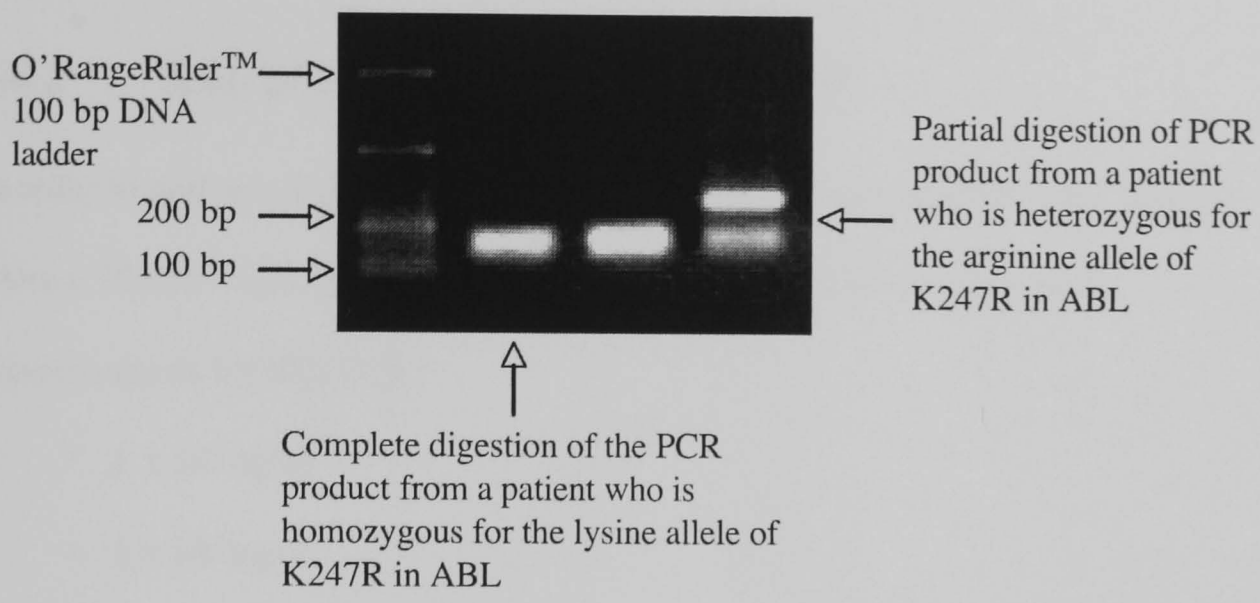
4. 68° for 1 minute
5. Go to step 2 for 35 more cycles
6. 68° for 5 minutes
7. 4° forever

Table 2.6 Master mix for second PCR reaction, prior to AluI digestion.

Reagent	Volume (µl)
Accuprime Buffer I	5
Forward primer 10μM	2
Reverse primer 10μM	2
Accuprime *Taq* polymerase	0.5
Water	39.5
Template	1

Ten microlitres of the nested PCR product was incubated with 1µl (10,000U/ml) *AluI* (New England Biolabs, Beverly, MA), 5µl 10x NEBuffer 2 and 34µl water, at 37°C for 2 hours; the product was then run on a 1% agarose gel, stained with ethidium bromide, and the results viewed under ultraviolet lighting. Since *AluI* cuts AG↓CT, the PCR product from people in possession of the arginine allele of K247R would remain undigested (263 base pairs), while the more common lysine allele of K247R would be digested (151 base pairs + 112 base pairs) (Figure 2.5).

Figure 2.5 1% Agarose gel electrophoresis of ABL nested PCR products following AluI Digestion.



2.6 Real time quantitative PCR (RQ-PCR)

2.6.1 ABL plasmid

In order to develop a RQ-PCR system for assessment of gene expression levels, we needed a plasmid containing the exon 1/2 ABL sequence that we could detect by PCR. We used TOPO TA Cloning® version K2 (Invitrogen) to insert the ABL PCR product (described above) into the pCR®2.1-TOPO vector; this reaction was then transformed into chemically competent One Shot® *E. coli*. *E. coli* were incubated overnight on plates containing imMedia™ Amp Blue for *lacZ*+ Amp^R recombinant *E. coli* strains. White colonies were selected and ABL PCR was performed to check that the plasmid in these colonies contained the correct sequence. The ABL plasmid was then transformed into DH5α *E. coli* (Invitrogen). Plasmid-containing colonies were selected, and grown on in 200ml LB, at 37°C in a large rotating incubator, overnight.

The plasmid was then harvested using the Plasmid Midi Kit (Qiagen), and the plasmid checked for the correct sequence by ABL PCR.

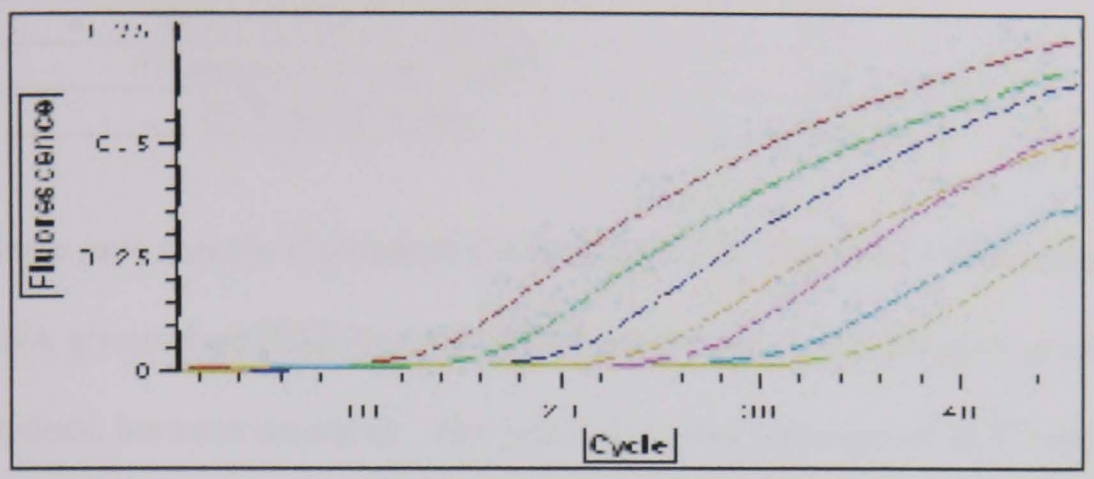
2.6.2 ABL plasmid dilutions for RQ-PCR

In order to perform RQ-PCR we needed 7 standard dilutions of the ABL plasmid. From a 10ng/μl ABL plasmid solution, we made the following standard concentrations for RQ-PCR:

- 1×10^{-1}ng/μl
- 1×10^{-2}ng/μl
- 1×10^{-3}ng/μl
- 1×10^{-4}ng/μl
- 1×10^{-5}ng/μl
- 1×10^{-6}ng/μl
- 1×10^{-7}ng/μl

A graph of the resulting fluorescence with cycle number for these standards is shown in Figure 2.6.

Figure 2.6 Graph showing fluorescence emitted with increasing cycle number, during RQ-PCR of the standard concentrations of ABL plasmid.



2.6.3 RQ-PCR protocol

RQ-PCR was performed on a 96 well plate, using the MJ Research Opticon™ 2.0 DNA Engine. We used the Assays-on-Demand™ Gene Expression Products (Applied Biosystems, Foster City, CA) to supply us with primer and Taqman probe sets for the genes whose expression level we wished to measure (test genes). We designed a Taqman probe, labelled with VIC reporter dye, for our ABL PCR (sequence 5' - CAGCGGCCAGTAGC – 3'), which was made for us by Applied Biosystems. In order to provide good quality RQ-PCR data, we used 3 replicates of each standard dilution, 3 replicates of ABL RQ-PCR for each patient sample, and 2 replicates of RQ-PCR for each test gene for each patient. For the RQ-PCR, we used 18µl of master mix (Table 2.7 and Table 2.8) plus 2µl of template (ABL plasmid or patient cDNA diluted 1 in 3 with water).

Table 2.7 ABL RQ-PCR master mix.

Component	Volume x 1
Platinum® Quantitative PCR SuperMix-UDG	10μl
Forward Primer, 10μM	1μl
Reverse Primer, 10μM	1μl
Fluorogenic Probe, 10μM	1μl
PCR grade water	5μl

Please note that the Platinum® Quantitative PCR SuperMix-UDG contains uracil DNA glycosylase (UDG) and dUTP to prevent the reamplification of carryover PCR products between reactions. The principle is that inclusion of dUTP ensures that any amplified DNA will contain uracil, and the UDG removes uracil residues from single or double stranded DNA and so prevents dU-containing DNA from serving as a template in future PCRs.

Table 2.8 Test gene RQ-PCR master mix.

Component	Volume x 1
Platinum Quantitative PCR SuperMix-UDG	10μl
Assays-on-Demand™ primer and probe mix	1μl
PCR grade water	7μl

The temperature protocol used was as follows:

1. 50°C for 2 minutes
2. 95°C for 2 minutes
3. 95°C for 15 seconds
4. 60°C for 30 seconds
5. 72°C for 1 second
6. Read plate
7. Cycle steps 3 to 6 for 45 cycles

2.7 Western Blotting

2.7.1 Protein Lysates

Protein lysates were made from 5 x 10^6 cells. Cells were washed twice with cold PBS and then placed in 100µl NP40 lysis buffer (1% NP40, 150mM sodium chloride, 20mM Tris/HCl (pH 8.0), 10% glycerol and 1mM EDTA) containing the protease inhibitors, 1mM 4-(2-Aminoethyl)-bezenesulfonylfluoride.HCl (AEBSF) and 10µg/ml aprotinin, and the phosphatase inhibitor, 1mM sodium vanadate. The sample was then rocked for 30 minutes at 4°C. Following centrifugation at 14,000g for 10 minutes at 4°C, the supernatant was removed to a fresh microcentrifuge tube. The sample was immediately placed into liquid nitrogen until frozen solid, and then stored at -80°C.

2.7.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer

We used the Criterion™ Precast Gel System (Bio-Rad Laboratories, Hercules, CA) for our SDS-PAGE and protein transfer. Equal volumes of protein lysates, suspended in equal volumes of 1x SDS gel-loading buffer (50 mM Tris/HCl (pH 6.8), 2% SDS, 2% β-Mercaptoethanol, 0.1% bromophenol blue and 10% glycerol), were heated to 100°C for 4 minutes, then centrifuged to bring the liquid to the bottom of the microcentrifuge tube, immediately prior to loading onto a Criterion™ Precast Gel (10% Tris-HCl) (Bio-Rad). The gel underwent electrophoresis at 200V for 1 hour

and the proteins were then transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA), at 100V for 1 hour and 10 minutes.

2.7.3 Western blotting of Abl

In order to detect expression of Abl, membranes were placed in 5% non-fat milk in Tris-buffered saline Tween-20 (TBST) solution (10mM Tris-base (pH8.0), 0.875% sodium chloride and 0.05% Tween-20) and rocked for 1 hour. The membrane was then washed twice with TBST, and placed in a 1 in 1000 solution of CST 2862 rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) in TBST. The membrane was rocked in the primary antibody solution at room temperature for 2 hours, and then washed 3 times with TBST. The membrane was next placed in a solution of TBST containing 2μl of goat anti-rabbit horseradish peroxidase conjugated antibody (Promega, Madison, WI), per 10ml TBST, and rocked for 1 hour at room temperature. The immunoblot was then washed 3 times in TBST to remove excess antibody, placed into a 1:1 mixture of peroxide and luminol for 30 seconds, and then chemiluminescence was detected using the Lumi-Imager system (Roche, Indianapolis, IN).

2.7.4 Western blotting of tyrosine phosphorylated proteins

The protocol for the western blotting of tyrosine phosphorylated proteins was identical to that used for detecting Abl, except for initially placing the membranes in 5% bovine serum albumin in TBST, rather than using milk, and for the antibodies used. We used the 4G10 primary antibody (kindly provided by Dr. Brian Druker) and a goat anti-mouse horseradish peroxidase conjugated secondary antibody (Promega).

3 rs2290573 and IL-1β +3953 gene polymorphisms and response to imatinib

3.1 Introduction

Dressman et al[114]. set out to use pharmacogenetic analysis, on a subset of 88 patients from the IRIS trial[45], to identify genetic markers associated with achievement of a MCR. They studied a panel of 68 single nucleotide polymorphisms, in 26 genes, but they did not state why these particular genes were chosen. After adjustment for multiple comparisons, they found a significant correlation between the presence of the "CC" genotype of the rs2290573 gene polymorphism and reduced rate of major cytogenetic response (MCR) on first line imatinib. Dressman *et al.* also showed a significant correlation with the "CC" genotype of the IL-1ß -511 polymorphism and achievement of a MCR, however this correlation was not sustained with further patient follow up[114]. IL-1β is a member of the IL-1 family and acts as an agonist of the pro-inflammatory response. The IL-1β -511 and IL-1β +3953 polymorphisms have been shown to be in linkage disequilibrium with each other[117,123]. We set out to test their findings in our population of patients with CML, being treated in Newcastle upon Tyne.

3.2 Aims

1. To test the hypothesis that the presence of the "CC" genotype of the rs2290573 gene polymorphism is associated with a reduced rate of MCR, in patients with CML on imatinib.

2. To test the hypothesis that achievement of a MCR, by patients with CML on imatinib, correlates with the presence of the IL-1β +3953 gene polymorphism.

3.3 Key Points

- In a population of CML patients being treated with imatinib in Newcastle upon Tyne, we found that the genotypes of the rs2290573 polymorphism, and the IL-1β +3953 polymorphism, did not correlate with the rate of MCR.
- In order to test Dressman *et al.*'s findings[114], we would need to examine CML patients of identical ethnic and racial background who have received imatinib as first line therapy, and to directly assess the IL-1β -511 genotype

3.4 Methods

3.4.1 Patients

One hundred and twenty two patients with CML, who had been treated with at least 6 months of imatinib and on whom we had follow up data available, were identified at the Royal Victoria Infirmary, Newcastle upon Tyne (Table 3.1). All patients had peripheral blood total white cells available for DNA extraction, and gave consent for entry into this study (Appendix 1). One hundred and three healthy blood donors from the Newcastle upon Tyne region of the UK, who had had DNA extracted by phenol / chloroform extraction from peripheral blood total white cells, were used as controls. All donors had given their informed consent for their DNA to be used for the determination of allele frequencies as part of a control population.

Table 3.1 Characteristics of 122 imatinib treated CML patients in Newcastle upon Tyne.

Characteristic	Value
Age at start of imatinib therapy (years):	
Median	52
Range	21 - 77
Disease phase at start of imatinib therapy:	
Chronic phase (early chronic phase)	97 (23)
Accelerated phase	12
Blast crisis	13
Time from diagnosis to start of imatinib therapy (months):	
Median	28.7
Range	0 - 1083.2
Number of patients having treatment for CML, before starting imatinib:	10
Length of patient follow up (months):	
Median	23.6
Range	6.1 – 37.9

3.4.2 DNA extraction

Total genomic DNA was extracted from the cells by SDS lysis, proteinase K digestion, phenol / chloroform extraction and ethanol precipitation.

3.4.3 PCR and single strand conformational polymorphism (SSCP) analysis of the rs2290573 polymorphism

Novartis provided the sequence of the rs2290573 polymorphism, and primers were designed (Table 3.2) using the genomic sequence obtained from the NCBI website and mac vector™ software. Template DNA was amplified by PCR; the components for each individual PCR reaction were as follows: 10µl of 10x KCl buffer, 16µl of dNTP's, 5µl 20µM forward primer, 5µl 20µM reverse primer, 0.5µl AmpliTaq® (Applied Biosystems, Warrington, UK), 63.5µl water and 1µl template DNA. The PCR temperature protocol was as follows: denature at 94°C for 30 seconds, 30 cycles

of 60°C for 1 minute then 72°C for 1 minute, extend at 72°C for 10 minutes to terminate the reaction. PCR products underwent electrophoresis on a 1.5% agarose gel, in the presence of ethidium bromide, to check both their size and specificity. SSCP analysis of the PCR product was performed on 10% polyacrylamide gels (ProtoFLOWgel; Flowgen, Ashby de la Zouch, UK) at 20°C. Silver staining revealed single stranded conformers (Figure 3.1). Three examples of each homozygote were sequenced to determine which was homozygote for the C allele and which for the T allele, and also to confirm the accuracy of the SSCP analysis. (Assistance in establishing the SSCP assay for the rs2290573 polymorphism was given by Miss Julie Gardner.)

Figure 3.1 Silver stained polyacrylamide gel demonstrating SSCP analysis of the rs2290573 polymorphism.

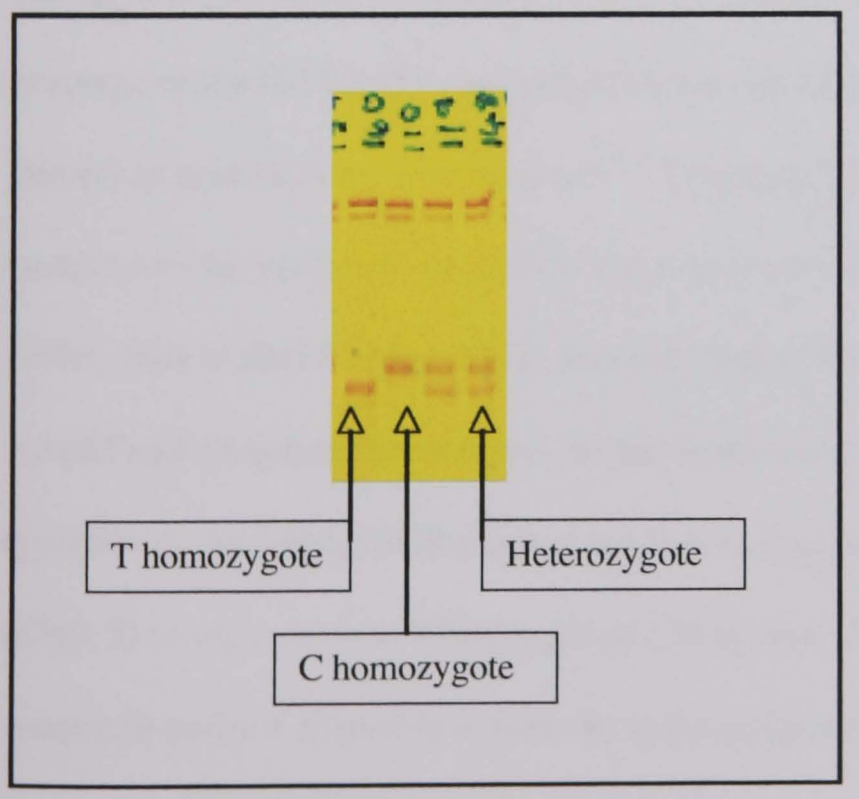


Table 3.2 Primers used for PCR of the rs2290573 polymorphism.

Primer Direction	Primer Sequence
Forward	5'-CCTCCCTTACCAAAGACCTGAGAC-3'
Reverse	5'-GCACTGTCCATCCAAGAAGCC-3"

3.4.4 PCR and *Taq1* restriction enzyme digestion analysis of the IL-1β +3953 polymorphism

Work previously done in our laboratory by Cullup *et al.*[123,144] examined the allele frequency of the IL1ß-3953 and IL1ß-511 in 229 normal blood donors, in the North East of England. We chose to examine the IL-1β +3953 polymorphism in our patients, since the requirements for the IL-1β -511 polymorphism assay were unavailable at the time, and the IL-1β +3953 polymorphism had been shown to be in linkage disequilibrium with IL-1β -511[117,123]. To investigate the allele frequency and genotype of the IL1ß-3953 gene polymorphism in 84 patients with CML, we used primers as described by Santtila *et al.*[117]. Template DNA was amplified by PCR. The components for each individual PCR reaction were as follows: 10µl of 10x KCl buffer, 16µl of dNTP's, 5µl 20µM forward primer, 5µl 20µM reverse primer, 0.5µl AmpliTaq® (Applied Biosystems), 63.5µl water and 1µl template DNA. *Taq1* digestion of the 249 bp PCR product resulted in fragments that either remained intact (allele 2) or were cut into 2 fragments of 135 bp and 114 bp (allele 1). PCR and restriction enzyme digestion conditions were as described previously[144]. Digestion products were analyzed on a 2% agarose gel, stained with ethidium bromide.

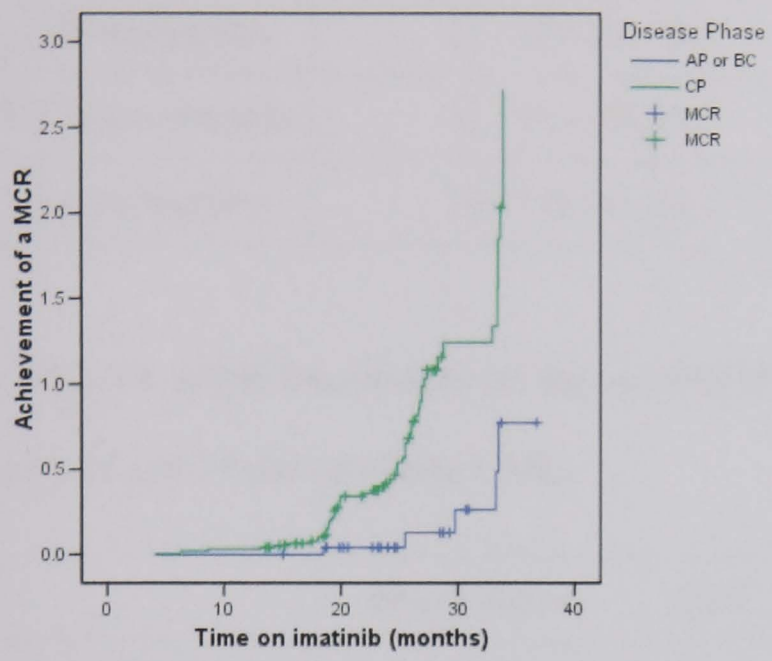
3.4.5 Statistical analysis

Statistical analysis was performed using SPSS version 12 software. The Hardy-Weinberg equation was used to calculate expected allele frequencies.

3.5 Results

In order to verify the integrity of our clinical dataset, we examined our patient population for achievement of a MCR over time from starting imatinib, after stratifying for those patients in chronic phase and those with advanced disease (Figure 3.2). We were able to detect expected differences in our patient population over time, i.e. that chronic phase patients were more likely to achieve a MCR (Log rank p=0.0002), than those with more advanced disease[40-42].

Figure 3.2 Achievement of a MCR with time on imatinib, in 123 patients stratified for disease phase at time of starting imatinib.



MCR = major cytogenetic response, AP = accelerated phase, BC = blast crisis, CP = chronic phase

The allele frequencies of the rs2290573 polymorphism were examined in 103 healthy blood donors and results are shown in Table 3.3. We noted that the observed and expected genotype frequencies were very close, consistent with the alleles being inherited in a co-dominant manner. The allele frequencies of the rs2290573 and IL-1β +3953 polymorphisms in patients with CML were found to be very similar to the allele frequencies seen in healthy blood donors from the North East of England[123] (Table 3.4 and Table 3.5), suggesting that the patients had been drawn from the same ethnic population. No selection bias was evident in recruiting patients for the study, nor any effect of genotype on the incidence of CML or entry into the study.

Table 3.3 Observed and expected genotype frequencies for the rs2290573 polymorphism in 103 blood donors.

	Observed genotype frequency	Expected genotype frequency
C homozygotes	17 / 103 (16.5%)	17.5 / 103 (17.0%)
CT heterozygotes	52 / 103 (50.5%)	50.5 / 103 (49.0)%
T homozygotes	34 / 103 (33.0%)	35.0 / 103 (34%)

Table 3.4 Allele frequencies for the rs2290573 polymorphism in 103 blood donors and 74 patients with CML.

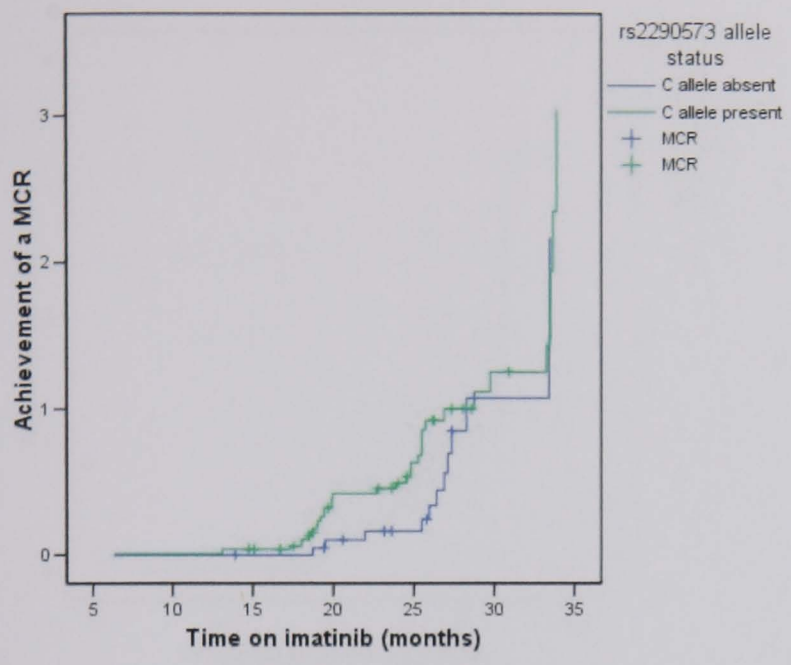
	Blood donors	CML patients
C allele frequency	86 / 206 (41.7%)	68 / 148 (45.9%)
T allele frequency	120 / 206 (58.3%)	80 / 148 (54.1%)

Table 3.5 Allele frequencies for the IL-1β +3953 polymorphism in 229 blood donors and 84 patients with CML.

	Blood donors	CML patients
Allele 1 frequency	344 / 458 (75.0%)[123]	129 / 164 (78.6%)
Allele 2 frequency	114 / 458 (25.0%)[123]	35 / 164 (21.4%)

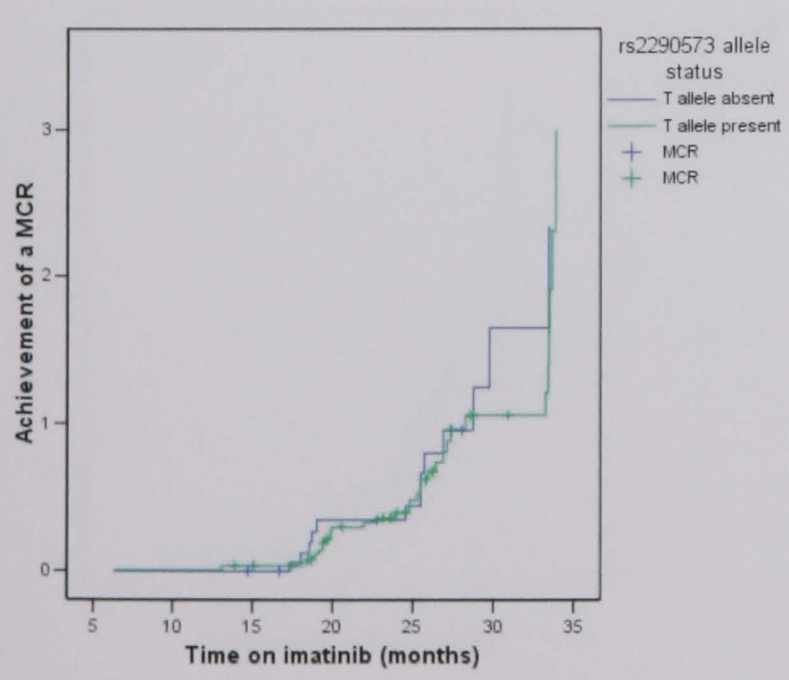
In order to assess whether possession of particular alleles of the rs2290573 or IL-1β +3953 polymorphism were associated with the incidence of MCR in our patients, we used Kaplan-Meier analysis, plotting MCR response as a time dependent variable, stratified by genotype, as shown in Figures 3.3 to 3.6. We found that no significant difference in response could be detected when comparing patients with different alleles, even when the analysis was restricted to patients in chronic phase (not shown).

Figure 3.3 Achievement of a MCR with time on imatinib, with 74 patients stratified for possession of the rs2290573 polymorphism allele C.



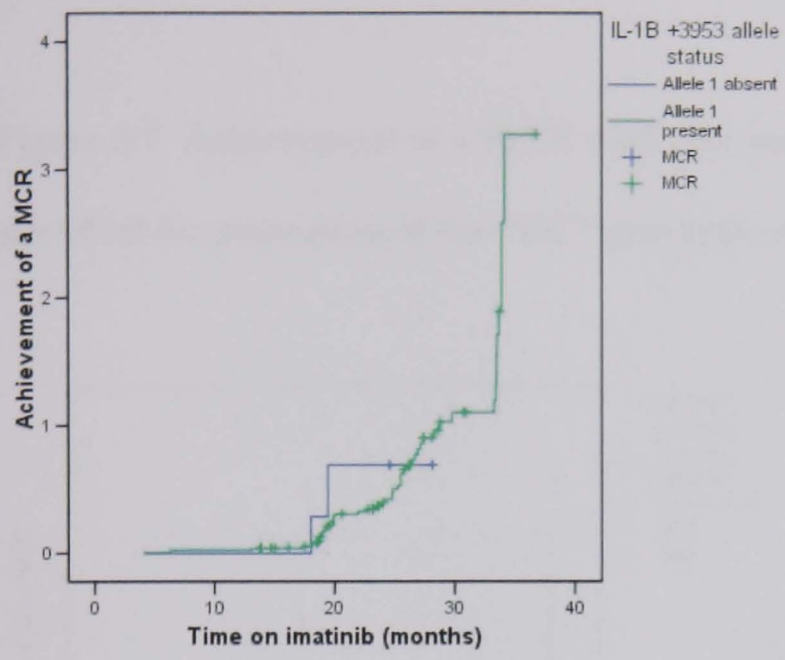
Log Rank p = 0.27

Figure 3.4 Achievement of a MCR with time on imatinib, with 74 patients stratified for possession of the rs2290573 polymorphism allele T.



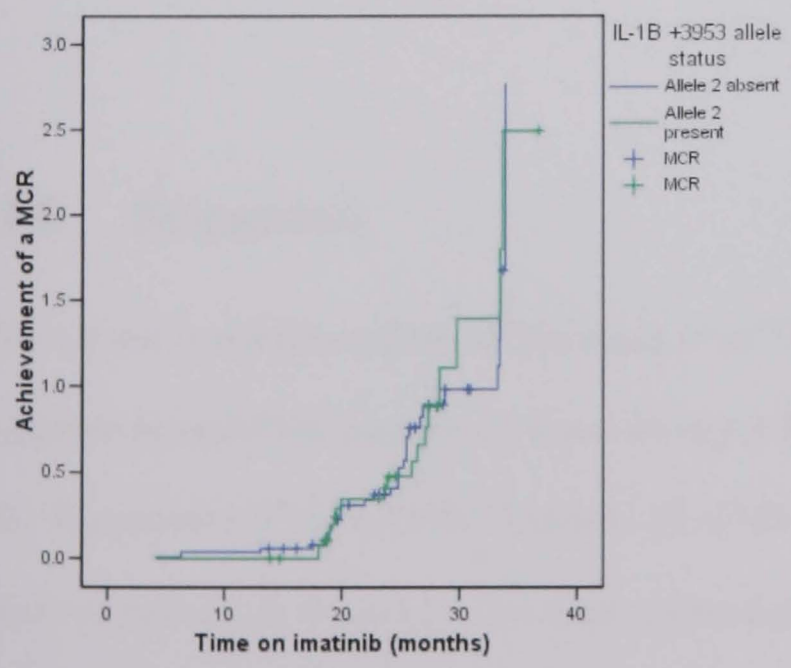
Log Rank p = 0.64

Figure 3.5 Achievement of a MCR with time on imatinib, with 84 patients stratified for possession of the IL-1β +3953 polymorphism allele 1.



Log Rank p = 0.76

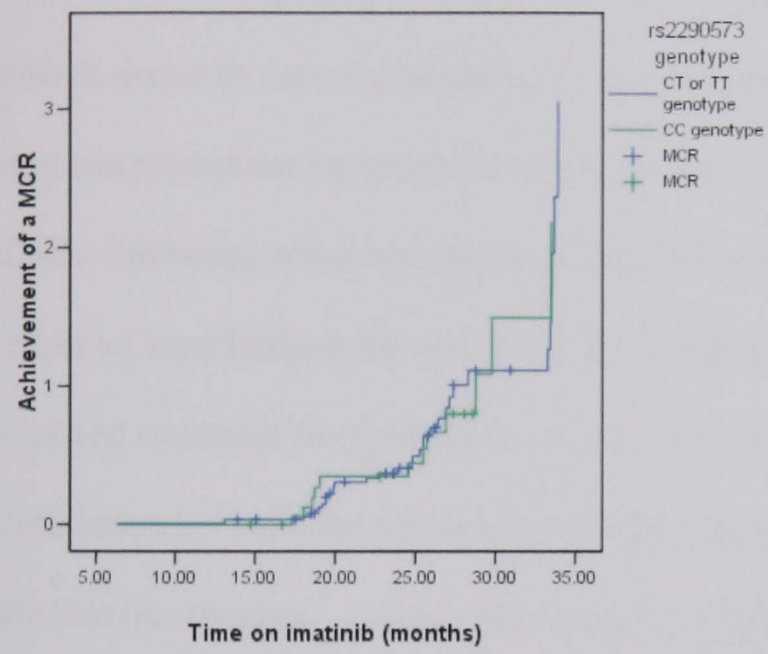
Figure 3.6 Achievement of a MCR with time on imatinib, with 84 patients stratified for possession of the IL-1β +3953 polymorphism allele 2.



Log rank p = 0.96

Dressman *et al.*[114] found an association between the "CC" genotype of the rs2290573 polymorphism and a reduced rate of MCR; however, we could not confirm the association between genotype and rate of MCR in our patient population (Figure 3.7).

Figure 3.7 Achievement of a MCR with time on imatinib, with 74 patients stratified for possession of the "CC" genotype of the rs2290573 polymorphism.



Log rank p = 0.96

3.6 Discussion

We set out to test the findings of Dressman *et al.*[114] by examining a population of imatinib treated CML patients in Newcastle upon Tyne. We genotyped our patients for the presence of the rs2290573 and IL-1β +3953 gene polymorphisms, and found them to have allele frequencies for these polymorphisms that were highly similar to that of healthy local blood donors. However, we were unable to confirm the correlation between possession of a particular genotype and rate of MCR that had been found by Dressman *et al.*[114].

Aside from a simple non-verification of Dressman's work, although it has been found that most gene association studies fail to be verified in subsequent studies[145], there are a number of features of our study which differ from that of Dressman, and might contribute to a different result. Firstly, our patient populations are different. Dressman et al[114]. studied chronic phase CML patients who received imatinib as first line therapy, whilst the majority of our patients were at a more advanced stage of their illness with most patients being in late chronic phase. It may well be that we were not able to detect an association between the polymorphisms and MCR because the polymorphisms are associated with effects that are only seen in the early stages of CML. However, when we examined only chronic phase patients, we still could not detect an association with rate of MCR. Secondly, the majority of our patients had received treatment for their CML prior to receiving imatinib and it may be that the association between the polymorphisms and MCR only holds true when imatinib is the first line therapy – indeed, Dressman *et al.* failed to detect the association when looking at 20 patients who had received imatinib after failing interferon[114].

Thirdly, Dressman *et al.*[114] found that after dividing the patients by racial group, the correlation of the "CC" genotype of the rs2290573 polymorphism with reduced rate of MCR was only upheld in Caucasian patients. Indeed, even though the frequency of the "CC" genotype in our patients (25.7%) was similar to the frequency seen in all Dressman *et al.*'s patients (23%), it was significantly higher (chi-square test, $p = 0.047$) than that seen in their Caucasian population (13.0%). The majority of our population of patients with CML were Caucasian, but our CML population was derived from a different ethnic background than Dressman *et al.*'s population. It is

thus possible that this difference in ethnicity of the populations made us unable to detect the effects that Dressman *et al.* found.

Fourthly, we did not examine our patients for the IL-1β -511 polymorphism, but examined them for the IL-1β +3953 polymorphism that is in linkage disequilibrium with IL-1β -511[117,123]. In addition, the IL-1β -511 polymorphism was associated with rate of MCR, but not after a further 6 months of patient follow up, and so any weak correlation with genotype and rate of MCR may have been lost with our indirect method of analysis.

We have failed to confirm Dressman *et al.*'s findings[114], which may be due to the differences in our studies, or it may be that their findings were due to type I errors, despite statistical correction for multiple comparisons. We therefore believe that to truly test Dressman *et al.*'s findings[114], we would need to examine CML patients of identical ethnic and racial background who have received imatinib as first line therapy, and to directly assess the IL-1β -511 genotype.

4 A single nucleotide polymorphism in ABL and imatinib sensitivity

4.1 Introduction

Acquired resistance to imatinib is frequently associated with kinase domain mutations (KM) that interfere with drug binding[60,61,63,64,73]. KM cluster in certain regions of the BCR-ABL kinase and confer varying levels of drug resistance[72]. Additionally, it has been observed that mutations within the ATP binding loop (P-loop) are associated with a poor prognosis compared to other mutation types, irrespective of their degree of imatinib resistance[67]. Whilst analyzing patients for KM, who were being treated at the Department of Haematology of the University of Leipzig for CML, we identified a single nucleotide polymorphism (SNP) at codon position 247 (numbering according to ABL type 1a[146]) within the P-loop domain of BCR-ABL, leading to the substitution of arginine for lysine (K247R). Since 3 out of 4 patients with this P-loop polymorphism had an inadequate response to imatinib, we hypothesized that the arginine allele may modulate imatinib sensitivity, or to one of the novel BCR-ABL tyrosine kinase inhibitors, AMN107 and BMS-354825.

4.2 Aims

1. To determine the incidence of the arginine allele, of the K247R polymorphism, within a population of patients with CML being treated at the Department of Hematology of the University of Leipzig, Germany.

2. To determine the incidence of the arginine allele, of the K247R polymorphism, within a healthy population of blood donors from the North East of England.
3. To use *in vitro* biochemical and cellular assays to examine the drug sensitivity of BCR-ABL expressing the arginine allele of K247R.

4.3 Key Points

- The arginine allele of K247R was found to be rare in both patients with CML being treated in Leipzig, and in healthy blood donors from the North East of England.
- *In vitro* biochemical and cellular assays showed that BCR-ABL expressing the arginine allele of the K247R polymorphism had an imatinib, AMN107 and BMS-354825 sensitivity that was very similar to that seen with wild type BCR-ABL.
- When screening patients with CML for the development of KM, clinicians should not mistake the presence of the arginine allele, of the K247R polymorphism, for a functional KM.
- Continued collection of clinical data would be required on patients with CML who express the arginine allele of K247R in BCR-ABL, to confirm that our *in vitro* findings are true *in vivo*.

4.4 Methods

4.4.1 Patients

Whilst screening patients with CML for KM, we identified 4 patients treated at the Department of Haematology of the University of Leipzig, Germany, who possessed the arginine allele of the K247R SNP, within BCR-ABL. Clinical features of these patients are shown in Table 4.1. Presence of the arginine allele, found in samples taken both before and after commencement of imatinib treatment, was confirmed by repeated nested PCR and automated sequencing, using BCR-ABL and ABL specific primers (Table 4.2).

Table 4.1 The clinical characteristics of all the patients with BCR-ABL expressing the arginine allele of the K247R polymorphism.

Patient ID	CHR	Best Cytogenetic Response	Time to Best Cytogenetic Response	Length of Follow Up
EL	Yes	100% Ph+	6 months	6 months
KE	Yes	4% Ph+	6 months	12 months
PN	Yes	36% Ph+	21 months	24 months*
CR	Yes	96% Ph+	15 months	15 months

CHR = complete haematological response

* = 88% Ph+ at 24 months

Table 4.2 Table of primers used in the nested PCR, sequencing and single strand conformational polymorphism (SSCP) analysis.

Primers	BCR-ABL	ABL
1st round nested PCR –	5'-TTCAGAAGCTTCTCCCTGACAT-3'	5'-GCTTGCCTGCCCTGCA-3'
1st round nested PCR –	5'-CCTTCTCTAGCAGCTCATACACCTG-3'	5'-CCTTCTCTAGCAGCTCATACACCTG-3'
2nd round nested PCR –	5'-ACAGCATTCCGCTGACCATC-3'	5'-GGCATGGGGGTCCACACT-3'
2nd round nested PCR –	5'-GCCATAGGTAGCAATTTCCC-3'	5'-GCCATAGGTAGCAATTTCCC-3'
Sequencing	5'-TGGTTCATCATCATTCAACGG-3'	5'-TGGTTCATCATCATTCAACGG-3'
SSCP - forward	-	5'–TGGTTCATCATCATTCAACGG-3'
SSCP – reverse	-	5'-AGGCTGTATTTCTTCCACACG-3'

4.4.2 Sequence analysis of BCR-ABL and ABL in CML patients

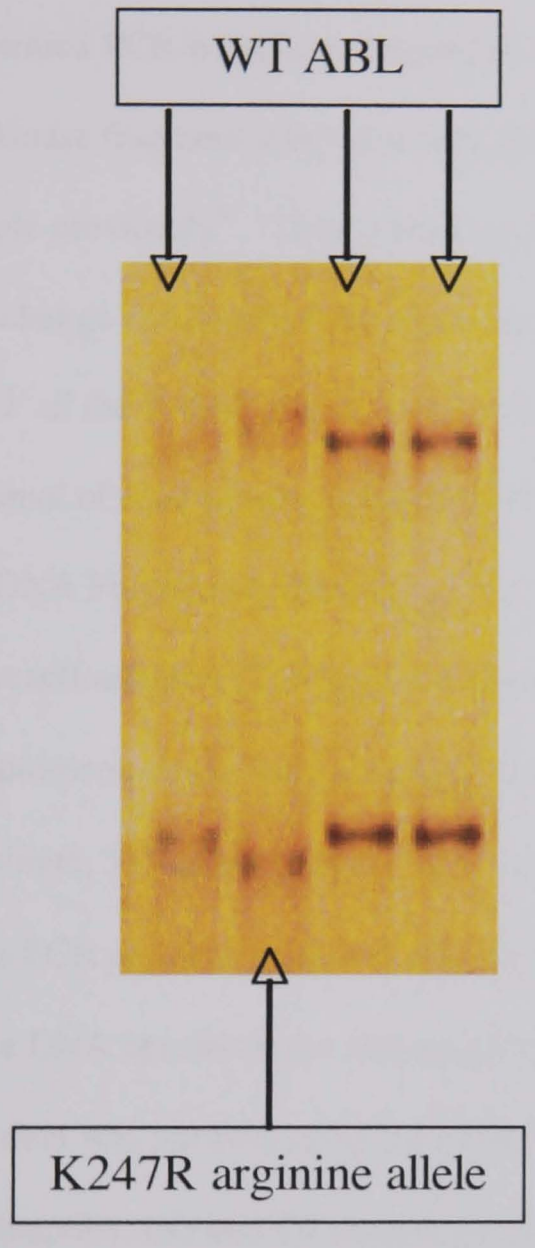
In order to determine the frequency of the arginine allele within the Leipzig based CML population, all CML patients from the Department of Haematology of the University of Leipzig with available cDNA (n = 167) underwent screening. RNA was extracted from bone marrow total white cells and reverse transcribed into cDNA as described[31]. All patients gave their consent for entry into this study (Appendix 2). BCR-ABL and ABL were specifically amplified by nested PCR, followed by automated sequencing. The presence of the arginine allele was confirmed by repeat sequencing, and by *AluI* (New England BioLabs, Beverly, MA) digestion of the

nested PCR product, as the nucleotide exchange (AAG to AGG) eliminated the *AluI* restriction site.

4.4.3 Screening for the arginine allele of K247R in healthy blood donors

In order to examine the allele frequency of K247R in Western Europeans, Dr. Peter Middleton screened 213 blood donors from the Newcastle upon Tyne region of the UK, who had given informed consent for their DNA to be used for the determination of allele frequencies as part of a control population. Genomic DNA was extracted by phenol, chloroform and ethanol precipitation from the buffy coat of a 4.5ml EDTA blood sample from each individual. Screening for the arginine allele of K247R was performed by single strand conformational polymorphism (SSCP) analysis[147]. Primer sequences (Table 4.2) flanking the K247R site were predicted for the genomic DNA sequence using Macvector Version 7.2 analytical software and allowed amplification of a 205 bp region spanning the K247R polymorphism; annealing temperature 57°C, final primer concentration 0.2μM. The polymorphism was demonstrated by SSCP analysis on 12% polyacrylamide gels (ProtoFLOWgel; Flowgen, Ashby de la Zouch, UK) at room temperature. Silver staining revealed single stranded conformers (Figure 4.1). Presence of the arginine allele was confirmed by *AluI* digestion of the PCR product.

Figure 4.1 Silver stained polyacrylamide gel demonstrating the single stranded conformers seen with the WT ABL allele and with the K247R arginine allele.



WT ABL = ABL expressing the lysine allele of the K247R polymorphism, K247R arginine allele = ABL expressing the arginine allele of the K247R polymorphism

4.4.4 Statistical analysis of the allele frequency

The frequency of the arginine allele, in Leipzig CML patients, and in normal blood donors, was compared by the chi-square test using Yates' correction.

4.4.5 Mutagenesis of the AatII/KpnI ABL kinase

To introduce the K247R arginine allele single base change into full-length $p210^{BCR-ABL}$, we performed PCR-based site-directed mutagenesis of the *AatII/KpnI* wild type (WT) ABL kinase fragment subcloned into pGEM7[148] (Promega, Madison, WI) which had been made previously[71]. Thus, a forward primer was designed that contained the A to G base change and spanned the kinase at position 247, while the reverse primer was 9 bases 3' of the SNP (Table 4.3). The PCR reaction was set up with 100ng of plasmid, 50pmol of each primer, 0.2mM dNTP's, 1.5mM $MgCl_2$, 5% DMSO, 2.5U *PfuTurbo*® DNA Polymerase (Stratagene, La Jolla, CA), 1x Cloned *Pfu* DNA Polymerase reaction buffer (Stratagene) and made up to 100µl with water. The temperature protocol was as follows: denature at 95°C for 2 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 5 minutes; extend at 72°C for 7 minutes. The PCR products underwent electrophoresis on a 1.2% agarose gel at 110V, and the DNA species in the size range 3000 - 4000 base pairs was excised. The PCR product was purified using the High Pure PCR Product Purification Kit (Roche, Indianapolis, IN) and T4 phosphorylation was done with T4 polynucleotide kinase (Fermentas, Hanover, MD). DH5α cells (Invitrogen, Carlsbad, CA) were transformed with the phosphorylated DNA, after ligation with T4 DNA ligase (Roche), and then plated overnight on imMedia™ Amp Agar (Invitrogen, Carlsbad, CA). Colonies were selected and the DNA extracted using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The DNA was sent for conventional sequencing to confirm correct insertion of the desired base change.

Table 4.3 Primers required for PCR-based site directed mutagenesis of the AatII/KpnI ABL kinase fragment.

Direction	Primer
Forward	5'-ATGAAGCACA**G**GCTGGGCGGG-3'
Reverse	5'-GGTGATGTCCGTGCGTTCCATC-3'

G = base change from adenine to guanine

4.4.6 Mutagenesis of full-length $p210^{BCR-ABL}$ and generation of vectors

Following *AatII/KpnI* digestion of the K247R kinase fragment in pGEM7, the K247R ABL kinase fragment was subcloned into full-length $p210^{BCR-ABL}$ in pGEM5[148] (Stratagene) (pGEM5-$p210^{BCR-ABL}$) as described above. Following *EcoRI* (New England BioLabs) digestion of pGEM5-$p210^{BCR-ABL}$, the full-length $p210^{BCR-ABL}$ fragment containing the K247R base change ($p210^{BCR-ABL-K247R}$) was then subcloned into the *EcoRI* site of the mammalian expression vector pSRα. The mammalian expression vector pSRα containing $p210^{BCR-ABL}$ and $p210^{BCR-ABL-T315I}$ were generated in an identical fashion, as previously described[71].

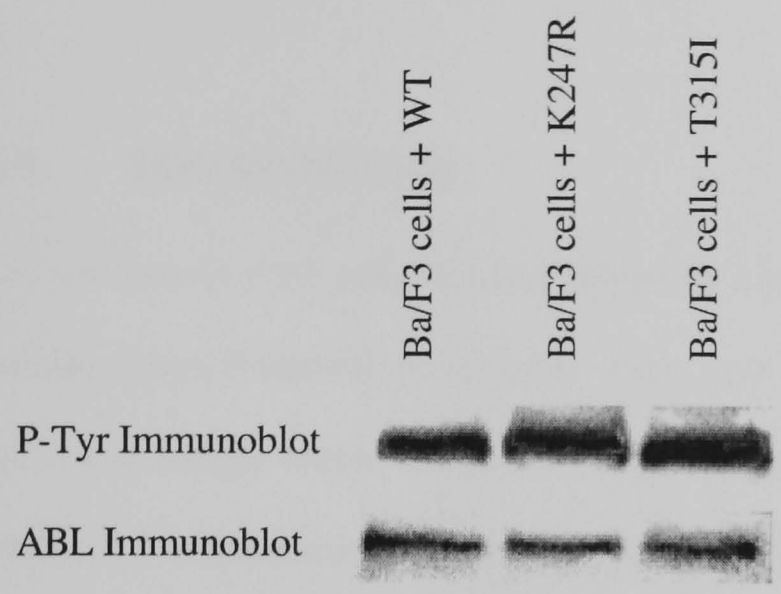
4.4.7 Infection of murine haemopoietic cells

Bosc23 cells[149] were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1unit/ml penicillin, 1μg/ml streptomycin and 4mM L-glutamine. For the production of cells expressing $p210^{BCR-ABL-K247R}$, Bosc23 cells were transiently

transfected with pSRα containing $p210^{BCR\text{-}ABL\text{-}K247R}$, and the viral supernatant was harvested 48 hours later. Immunoblotting of Bosc23 cell lysates confirmed expression of BCR-ABL.

Ba/F3 cells were incubated with the filtered Bosc23 viral supernatant containing 8μg/ml polybrene and 15% WEHI conditioned media as a source of IL-3. Stable transfectants were selected by maintaining cells in RPMI 1640 supplemented with 10% FBS, 1unit/ml penicillin, 1μg/ml streptomycin, 4mM L-glutamine, 15% WEHI and 0.75mg/ml G418 sterile solution (Stratagene). Cells with stable BCR-ABL expression were further selected by removal of WEHI. Ba/F3 cells expressing $p210^{BCR\text{-}ABL}$ and $p210^{BCR\text{-}ABL\text{-}T315I}$ had been generated previously, in an identical fashion[71]; cryopreserved samples of these cells were thawed and maintained in RPMI 1640 supplemented with 10% FBS, 1unit/ml penicillin, 1μg/ml streptomycin, 4mM L-glutamine. Equivalent BCR-ABL expression of the cell lines was confirmed by immunoblot analysis (Figure 4.2).

Figure 4.2 Immunoblot demonstrating tyrosine phosphorylated BCR-ABL and expression of BCR-ABL in the Ba/F3 cell lines expressing p210BCR-ABL, p210BCR-ABL-K247R and p210BCR-ABL-T315I.



P-Tyr Immunoblot = tyrosine phosphorylated BCR-ABL detected by mouse monoclonal phosphotyrosine antibody, ABL Immunoblot = BCR-ABL expression detected by rabbit monoclonal ABL antibody, Ba/F3 cells + WT = Ba/F3 cells expressing $p210^{BCR\text{-}ABL}$, Ba/F3 cells + K247R = Ba/F3 cells expressing $p210^{BCR\text{-}ABL\text{-}K247R}$, Ba/F3 cells + T315I = Ba/F3 cells expressing $p210^{BCR\text{-}ABL\text{-}T315I}$

4.4.8 Cell proliferation assays

Exponentially growing parental Ba/F3 cells (supplemented with IL-3) and Ba/F3 cells expressing $p210^{BCR\text{-}ABL}$, $p210^{BCR\text{-}ABL\text{-}K247R}$ and $p210^{BCR\text{-}ABL\text{-}T315I}$ were plated in quadruplicate at 5000 cells/well in 96-well plates. Cells were incubated in the presence of imatinib (0 - 1μM), AMN-107 (0 - 1μM) (kindly provided by Novartis Pharmaceuticals) and BMS-354825 (0 - 32nM) (kindly provided by Bristol-Myers

Squibb). Cell proliferation was measured with a methanethiosulfonate (MTS) based viability assay (CellTiter 96 Aqueous One Solution Reagent; Promega, Madison, WI), as previously described[71]; results from day 3 were used to construct the best-fit curves, and calculate the cellular IC_{50} in Microsoft Excel.

4.4.9 Immunoblotting

Ba/F3 cell lines (1 x 10^6 cells) were incubated for 3 hours in 1.25ml media containing escalating doses of imatinib (0 - 6.4μM). Cells were collected by centrifugation and lysed in SDS sample buffer. Lysates were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) for immunoblotting. Tyrosine phosphorylated BCR-ABL was detected with mouse monoclonal phosphotyrosine antibody 4G10; BCR-ABL expression was detected using the rabbit polyclonal ABL antibody CST 2862 (Cell Signaling Technology, Beverly, MA).

4.4.10 Generation of GST-ABL fusion proteins

The ABL kinase domain (KD), consisting of ABL amino acids 220-498, was subcloned into the *Bam*HI site of pGEX KG (Amersham Biosciences, Buckinghamshire, UK) as described previously[65]. Single base changes within the KD, i.e. K247R and the mutation T315I, were constructed using the GeneTailor™ Site Directed Mutagenesis System (Invitrogen, Carlsbad, CA). We generated glutathione S-transferase (GST) fusion proteins of WT, K247R and T315I by inducing exponentially growing transformed DH5α™-T1^R E. *Coli* with 0.5mM isopropyl-1-thio-β-D. The cells were lysed via French pressing and sonication, after addition of phosphate buffered saline (PBS) containing 0.8mM 4-(2-aminoethyl)-benzenesulfonyl

fluoride hydrochloride, 0.8mM phenylmethylsulfonyl fluoride, aprotinin 16μg/ml, 0.4% Triton X-100 and 1 complete protease inhibitor cocktail tablet (Roche). Binding to glutathione-sepharose overnight at 4°C, purified the GST-ABL fusion proteins. Bound proteins were washed thrice with PBS, once with 0.5M LiCl and once again with PBS. Proteins were then eluted in 1.5 ml of elution buffer containing 50mM Tris, pH 8.0, 150mM NaCl and 30mM glutathione, and then diluted 1:1 with 80% glycerol. A Bradford assay was used to determine the concentration of each protein.

4.4.11 Kinase autophosphorylation assays

Dr. Thomas O'Hare carried out kinase autophosphorylation assays in the presence of imatinib (0-5μM) as described previously[150]. In brief, 500ng of fusion protein in 30μl of kinase buffer (20mM Tris, pH 7.5, 10mM $MgCl_2$, 10μM sodium vanadate, 1μM dithiothreitol, 1% dimethyl sulfoxide (Me_2SO)), was incubated in the presence of imatinib for 10 minutes, after which 5μM [γ-^{32}P] ATP was added and the kinase reaction allowed to proceed for 30 minutes. The reactions were terminated by addition of SDS loading dye and boiling; the samples were analysed by SDS-PAGE. ABL autophosphorylation signal intensity was quantified with a PhosphorImager (Molecular Dynamics). ABL immunoblots to demonstrate equal protein loading were performed with α-Abl Ab-2 (Oncogene Science, Cambridge, MA).

4.4.12 In vitro peptide substrate phosphorylation assays with GST-ABL kinase domains

The effects of imatinib (0 - 5μM), AMN-107 (0 - 5μM) and BMS-354825 (0 - 32nM) on GST-ABL kinase activity were examined using a synthetic peptide substrate (biotin-EAIYAAPFAKKK-amide; Upstate Biotechnology, Charlottesville, VA), as described previously[150]. Immediately prior to the phosphorylation assay, all fusion proteins were dephosphorylated by incubation for 1 hour, at 30°C, in the presence of LAR phosphatase (New England BioLabs); the reaction was terminated by the addition of 2mM sodium vanadate. Dr. Thomas O'Hare performed peptide substrate phosphorylation assays at 30°C for 15 minutes in a 25μl reaction mixture: 8mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7, 0.2mM ethylenediaminetetraacetic acid (EDTA), 50μM Abletide, 30mM $MgCl_2$, 10mM β-glycerol phosphate, 1mM ethylene glycol tetraacetic acid (EGTA), 0.002% Brij-35, 0.4mM dithiothreitol (DTT), 0.2mg/ml BSA, 0.4mM sodium orthovanadate, 10nM WT, K247R or T315I GST-ABL kinase, and 100μM [γ-^{32}P] ATP (5000cpm/pmol). Reactions were terminated by placing a fraction of the reaction mixture onto a p81 phosphocellulose filter and immersing in 0.75% phosphoric acid. Filters were washed 3 times in 0.75% phosphoric acid, rinsed in acetone and air dried. Phosphate incorporation was determined by scintillation counting, with correction for background binding, as determined by omitting peptide substrate from the kinase reaction. The WT and K247R GST-ABL fusion proteins underwent kinase assays in the presence of imatinib, AMN-107 and BMS-354825; the T315I GST-ABL fusion protein was examined only in the presence of imatinib.

4.5 Results

4.5.1 The arginine allele of the K247R polymorphism in patients with CML

Since only cDNA was available from the CML patients, we sequenced both BCR-ABL and ABL-specific PCR products. Amplification of ABL was successful in 167 patients and in 157 of these BCR-ABL could also be amplified. The arginine allele was identified in BCR-ABL PCR products from 4 patients. ABL PCR products from these 4 patients were WT in 3 patients with 100% Ph+ metaphases, whilst 1 case exhibited both the WT and arginine alleles. As the latter patient was in partial cytogenetic remission at the time of the sample, this likely reflected amplification of paternal and maternal alleles from normal cells, while the template in the other 3 patients was derived from leukemic cells and thus reflected the non-translocated WT ABL allele. The arginine allele (50%) was also found in the ABL PCR product of one additional patient. *AluI* digestion confirmed the sequencing results in all cases. Since there is no evidence of allele specific variation in ABL expression, such as imprinting[151], the allelic frequency in the CML population is 5/167 x 2 = 1.5%.

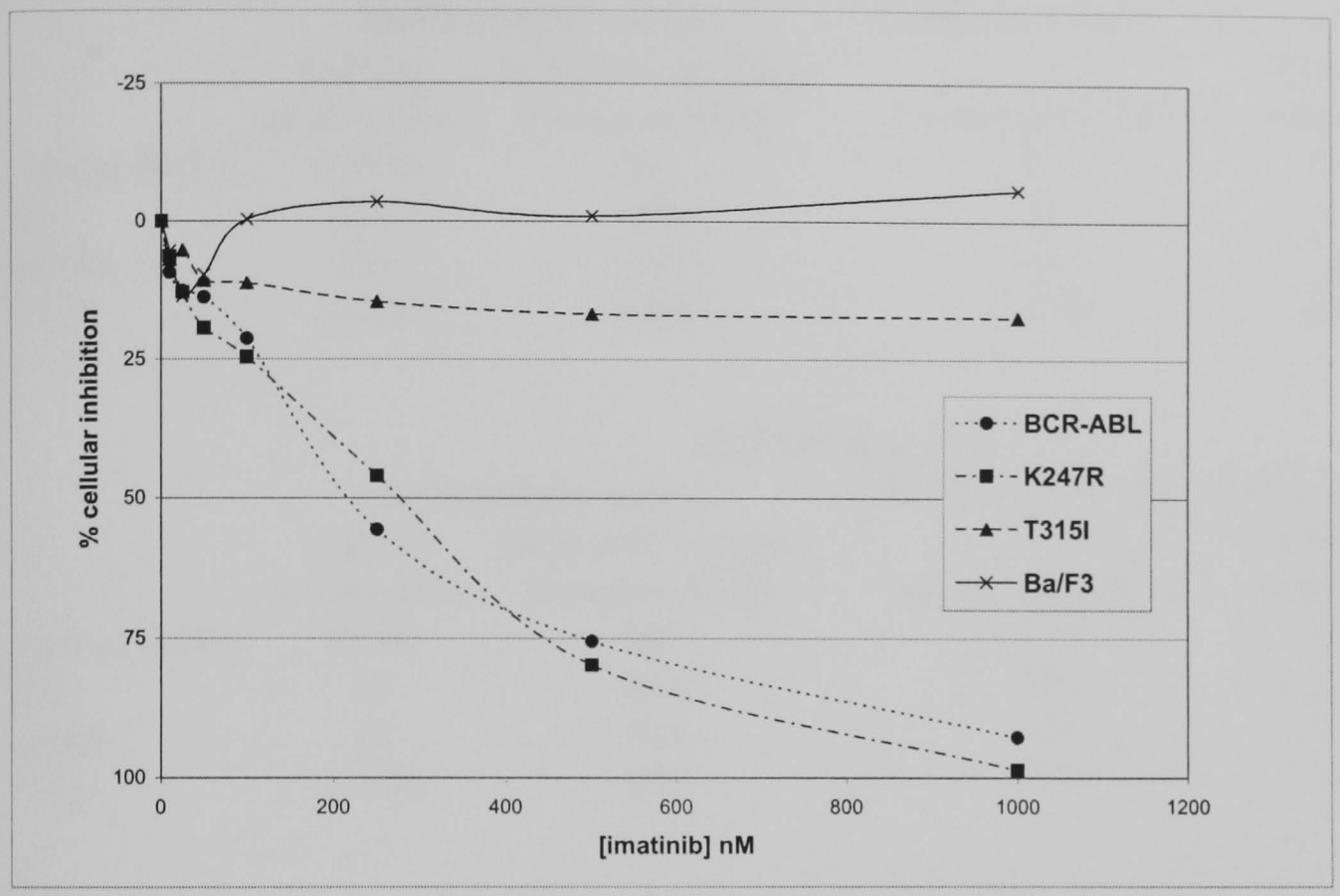
Two hundred and thirteen Western European blood donors, recruited from the North East of England, were examined for the presence of the arginine allele in ABL by SSCP analysis. One out of 213 individuals was found to be heterozygous for this allele (allele frequency 1/213 x 2 = 0.2%). The frequency of the arginine allele in ABL in controls was thus different from that of the CML patients, but not significantly ($p = 0.12$, chi-square test with Yates' correction). The frequency of the arginine allele within a healthy German population has recently been found to be 1%

to 2%, which is equivalent to the frequency in the German CML population (personal comment from Dr. Jan Cornelissen).

4.5.2 Cellular assays

MTS assays were performed in order to assess the sensitivity of Ba/F3 cell lines expressing $p210^{BCR\text{-}ABL}$, $p210^{BCR\text{-}ABL\text{-}K247R}$ and $p210^{BCR\text{-}ABL\text{-}T315I}$ to imatinib, AMN-107 and BMS-354825. All 3 drugs showed a concentration-dependent, anti-proliferative effect on Ba/F3 cells expressing $p210^{BCR\text{-}ABL}$, $p210^{BCR\text{-}ABL\text{-}K247R}$ and $p210^{BCR\text{-}ABL\text{-}T315I}$ (Figure 4.3, Table 4.4). The IC_{50}'s for the $p210^{BCR\text{-}ABL}$ cell line, and the $p210^{BCR\text{-}ABL\text{-}K247R}$ cell line, were similar. As expected, parental Ba/F3 cells and Ba/F3 cells expressing $p210^{BCR\text{-}ABL\text{-}T315I}$ were insensitive to all 3 drugs, at all concentrations (Table 4.4).

Figure 4.3 Graph showing the effect of increasing concentrations of imatinib on cell proliferation.



Parental Ba/F3 cells (Ba/F3), and Ba/F3 cells expressing $p210^{BCR\text{-}ABL\text{-}WT}$ (BCR-ABL), $p210^{BCR\text{-}ABL\text{-}K247R}$ (K247R) and $p210^{BCR\text{-}ABL\text{-}T315I}$ (T315I) were cultured in the presence of increasing concentrations of imatinib (0-1μM). The number of viable cells was measured by MTS assay after 3 days.

Table 4.4 Results of the cellular and biochemical assays of drug sensitivity.

	Imatinib IC_{50} (nM)			
	Ba/F3 cellular assays		**Purified GST-ABL kinase assays**	
	Cellular proliferation	BCR-ABL tyrosine Phosphorylation	Autophosphorylation	Peptide substrate
parental Ba/F3	>1000	NA	NA	NA
WT	215	440	300	90
K247R	275	360	450	90
T315I	>1000	>6400	>5000	>5000

	AMN107 IC_{50} (nM)			
	Ba/F3 cellular assays		**Purified GST-ABL kinase assays**	
	Cellular proliferation	BCR-ABL tyrosine phosphorylation	Autophosphorylation	Peptide substrate
parental Ba/F3	>1000	ND	ND	NA
WT	30	ND	ND	25
K247R	35	ND	ND	50
T315I	>1000	ND	ND	ND

	BMS-354825 IC_{50} (nM)			
	Ba/F3 cellular assays		**Purified GST-ABL kinase assays**	
	Cellular proliferation	BCR-ABL tyrosine phosphorylation	Autophosphorylation	Peptide substrate
parental Ba/F3	>32	ND	ND	NA
WT	1.9	ND	ND	3.2
K247R	2	ND	ND	2.1
T315I	>32	ND	ND	ND

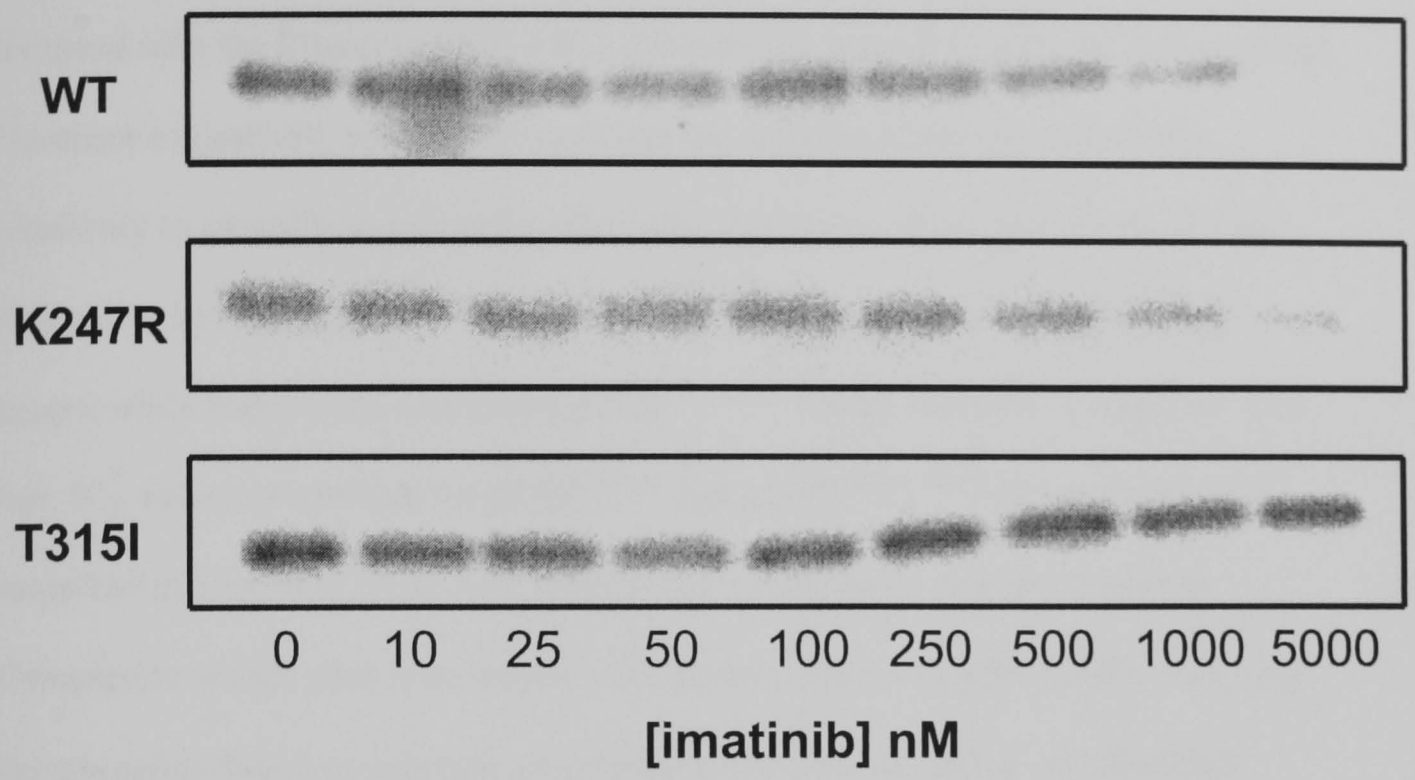
ND = not done, NA = not applicable, WT = Ba/F3 cells expressing $p210^{BCR-ABL}$ in the cellular assays and WT GST-ABL fusion protein in the kinase assays, K247R = Ba/F3 cells expressing $p210^{BCR-ABL-K247R}$ in the cellular assays and K247R GST-ABL fusion protein in the kinase assays, T315I = Ba/F3 cells expressing $p210^{BCR-ABL-T315I}$ in the cellular assays and T315I GST-ABL fusion protein in the kinase assays. Most assays were performed more than once, with cell lines being plated in quadruplicate for the cellular proliferation assays and then measured in duplicate, BCR-ABL tyrosine phosphorylation and GST-ABL kinase activity were observed in triplicate, while kinase autophosphorylation assays were performed only once.

BCR-ABL tyrosine phosphorylation assays on Ba/F3 cells expressing $p210^{BCR\text{-}ABL}$, $p210^{BCR\text{-}ABL\text{-}K247R}$ and $p210^{BCR\text{-}ABL\text{-}T315I}$, were performed in the presence of imatinib. Imatinib inhibited tyrosine phosphorylation, in a concentration-dependent manner, in Ba/F3 cells expressing $p210^{BCR\text{-}ABL}$ and $p210^{BCR\text{-}ABL\text{-}K247R}$; the IC_{50}'s were similar for these 2 cell lines (Table 4.4). Since the tyrosine phosphorylation IC_{50}'s for $p210^{BCR\text{-}ABL}$ and $p210^{BCR\text{-}ABL\text{-}K247R}$ in the presence of imatinib were so similar, we did not repeat these experiments in the presence of AMN-107 or BMS-354825.

4.5.3 Biochemical assays

To determine the ability of imatinib, AMN-107 and BMS-354825 to directly inhibit kinase activity of purified ABL-kinase in a cell-free system, we carried out biochemical experiments with the use of GST-ABL kinase fusion proteins. All 3 drugs inhibited WT and K247R GST-ABL kinases in a dose-dependent manner, with these kinases showing similar IC_{50}'s with each drug (Table 4.4). T315I GST-ABL kinase was used as a positive control and was only examined in the presence of imatinib. As we expected, T315I GST-ABL fusion protein showed no reduction in kinase activity even at the highest imatinib concentrations (Table 4.4). In addition, we found that imatinib inhibited autophosphorylation of WT and K247R GST-ABL kinases in a dose-dependent fashion, but was unable to inhibit T315I autophosphorylation (Figure 4.4, Table 4.4). Due to the similarity of the autophosphorylation results of the WT and K247R GST-ABL kinases in the presence of imatinib, these experiments were not repeated in the presence of AMN-107 or BMS-354825.

Figure 4.4 Figure illustrating the effect of increasing concentrations of imatinib on GST-ABL kinase autophosphorylation.



Kinase autophosphorylation assays were performed on WT, K247R and T315I GST-Abl kinase proteins, in the presence of increasing concentrations of imatinib (0-5μM).

4.6 Discussion

We have identified a rare single nucleotide polymorphism (K247R) within the P-loop domain of ABL. It would appear that the incidence of the arginine allele in patients with CML being treated in Leipzig, is similar to that of the healthy German population, and close to that seen in blood donors from the North East of England. While this work was in progress, Irving *et al.* reported one healthy control heterozygous for the arginine allele and suspected that K247R may be a rare polymorphism, in accordance with our data[152].

While all 4 patients with the arginine allele expressed within BCR-ABL achieved a complete hematological response, 3 failed to achieve a major cytogenetic response to imatinib (median follow up 13.5 months, range 6 to 24 months). Since K247R is localized with the P-loop of BCR-ABL, a mutational hotspot in patients with acquired resistance to imatinib, we hypothesized that the arginine allele may reduce the sensitivity to imatinib and possibly other ABL inhibitors. However, no significant differences between $p210^{BCR-ABL}$ and $p210^{BCR-ABL-K247R}$ were noted in proliferation assays, while BaF/3 cells expressing $p210^{BCR-ABL-T315I}$ were resistant. Consistent with this, IC_{50} values of imatinib for $p210^{BCR-ABL}$ and $p210^{BCR-ABL-K247R}$ were similar with respect to inhibition of BCR-ABL tyrosine phosphorylation in cellular lysates. Comparable results were seen with *in vitro* kinase assays using GST-ABL KD, with autophosphorylation or substrate phosphorylation as the endpoint. Proliferation assays carried out in the presence of AMN-107 and BMS-354825 also showed no difference in sensitivity between $p210^{BCR-ABL}$ and $p210^{BCR-ABL-K247R}$.

The similar imatinib sensitivity of the lysine and arginine alleles of the K247R polymorphism is not entirely unexpected since the exchange of arginine for lysine is conservative. In contrast, the mutation L248R/V, in immediate proximity to K247R, confers a high level of resistance[153]. These data suggest that the failure of 3 out of 4 patients, with the arginine allele of the K247R polymorphism expressed in BCR-ABL, to achieve a MCR may be a chance finding in a small cohort. It remains formally possible, though unlikely, that the arginine allele may modulate disease biology, perhaps over months to years, irrespective of imatinib sensitivity in *in vitro* assays. To ascertain this, it would be necessary to collect clinical data on more CML patients in possession of the arginine allele of K247R expressed in BCR-ABL.

The importance of our data at the present time is that when screening for the development of KM in CML patients on imatinib, patients in possession of the arginine allele of K247R expressed in BCR-ABL should not be confused with patients who have P-loop mutations. The development of a P-loop mutation is a cause for concern because these mutations cause imatinib resistance[71], are associated with a very poor prognosis,[67] and hence a change in therapeutic strategy for these patients is often considered. The arginine allele of K247R does not affect sensitivity to imatinib *in vitro*, and without other evidence of inadequate response to imatinib, or emergence of imatinib resistance, should not be seen as a trigger to change patients' management.

5 Mechanisms of primary cytogenetic resistance to imatinib

5.1 Introduction

Acquired resistance to imatinib is most frequently due to the development of either a point mutation within the BCR-ABL kinase domain[59-61,63,67], or over expression of the BCR-ABL protein[60,61]. Less is known about the mechanisms behind cytogenetic refractoriness (i.e. primary cytogenetic resistance) to imatinib; we therefore decided to investigate a population of patients with CML, treated at Oregon Health and Science University (OHSU), for the role that kinase mutations (KM), and the expression of genes associated with drug transport, might play in primary cytogenetic resistance to imatinib.

5.2 Aims

1. To examine the prevalence of BCR-ABL kinase domain mutations, in patients with CML who have primary cytogenetic resistance to imatinib.
2. To examine the expression level of a set of genes associated with drug transport, in patients with CML who have primary cytogenetic resistance to imatinib.

5.3 Key Points

- BCR-ABL kinase domain mutations are less frequent among patients with primary cytogenetic resistance to imatinib, than those patients with acquired cytogenetic resistance.
- Prior to starting imatinib, significantly lower expression of hOCT1 and significantly higher expression of ABCC3 are seen in patients who go on to show primary cytogenetic resistance to imatinib, than in patients who go on to have a complete cytogenetic response to imatinib.
- It would appear that a variety of drug transport mechanisms might underlie primary cytogenetic resistance in our population of patients with CML.
- Further work is required to investigate whether hOCT1 allele status influences CML patients' responses to imatinib.
- Further work is required to ascertain whether imatinib is a substrate for ABCC3.

5.4 Methods

5.4.1 Patients

At OHSU we identified 28 patients with CML who had attained a complete haematological response (CHR) to imatinib, but who had failed to achieve even a minor cytogenetic response, i.e. had remained at least 65% Philadelphia chromosome (Ph) positive as assayed by cytogenetic analysis of at least 10 metaphases. These patients with primary cytogenetic resistance to imatinib were categorised as non-responders (NR). All of these patients had had treatment for their CML, prior to

imatinib. All 28 NR had a cryopreserved sample of bone marrow (BM) mononuclear cells (MNC's) taken at between 9 and 15 months from starting imatinib therapy. Fifteen of the NR also had a sample of BM MNC's available that had been stored immediately prior to imatinib commencement.

We also identified 15 patients with CML, based at OHSU, who had attained a CHR and who had also achieved a complete cytogenetic response (CCR) to imatinib within the first year of treatment. These patients were defined as responders (R) and they all had had BM MNC's stored immediately prior to starting imatinib treatment. The 15 R, and the 15 NR with pre and post imatinib samples, were of similar age, disease phase at time of starting imatinib and male to female ratio (Table 5.1), however, the 15 R had a significantly shorter time from diagnosis to starting imatinib (median 20 months, range 5.3 to 55.5 months) than the 15 NR (median 41.7 months, range 6.8 to 101.8 months) ($p = 0.04$) (Table 5.1). These 15 R and 15 NR patients were used for our real time quantitative PCR (RQ-PCR) experiments, since they had samples allowing us to compare the groups pre-imatinib. All patients consented to enter this study (Appendix 2).

Four samples of healthy BM MNC's were obtained from AllCells (Berkeley, CA), to act as normal controls.

Table 5.1 Characteristics of the 15 NR and the 15 R used for RQ-PCR.

Patient ID	Sex	Responder / Non Responder	Disease Phase at Start of Imatinib	Age at Diagnosis (years)	Time until Start of Imatinib (days)
CS01	M	R	Late CP	50.4	599
CS03	F	R	Late CP	51.2	423
CS05	M	R	Early CP	30.2	253
CS07	F	R	Late CP	31.5	472
CS08	F	R	Early CP	33.2	262
CS09	M	R	Late CP	68.8	1664
CS11	M	R	Early CP	71.8	1071
CS23	M	R	Accelerated (CE)	45.7	1410
CS19	M	R	Accelerated (CE)	76.3	160
CS27	M	R	Accelerated	53.7	1269
CS28	M	R	Late CP	64.9	636
CS33	M	R	Early CP	40.4	277
CS34	M	R	Late CP	51.0	693
CS35	F	R	Early CP	57.7	314
CS37	F	R	Late CP	65.8	1290
LCC01	M	NR	Late CP	50.1	680
LCC02	M	NR	Late CP	59.7	1377
LCC04	F	NR	Accelerated	55.8	1425
LCC09	M	NR	Late CP	76.8	1330
LCC11	F	NR	Late CP	60.0	597
LCC13	F	NR	Late CP	66.9	3054
LCC14	M	NR	Late CP	46.1	1084
LCC15	F	NR	Early CP	57.3	204
LCC16	M	NR	Late CP	58.2	1250
LCC17	M	NR	Accelerated (CE)	23.5	2012
LCC18	F	NR	Late CP	64.0	1271
LCC19	M	NR	Late CP	69.7	1682
LCC24	F	NR	Late CP	41.5	1246
LCC27	M	NR	Early CP	61.3	270
LCC28	M	NR	Accelerated (CE)	63.3	386

M = male, F = female, R = imatinib responder, NR = imatinib non-responder, CP – chronic phase, Accelerated (CE) = accelerated phase with cytogenetic clonal evolution being the sole criteria

5.4.2 Sample preparation

At the time of BM aspiration, BM samples were enriched for MNC's by density gradient centrifugation with Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden). BM MNC's were suspended in 60% RPMI Medium 1640 (Invitrogen, Carlsbad, CA), 30% Foetal Bovine Serum (Hyclone, Utah) and 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen. When required, BM MNC's samples were thawed at 37°C, and immediately washed in 24ml Dulbecco's phosphate buffered saline (PBS) containing 0.1% human albumin (Baxter Healthcare Corporation, Glendale, CA) and 1% recombinant DNase solution (Pulmozyme®, Genentech, San Francisco, CA). RNA was extracted using the RNeasy® Mini Kit (QIAGEN, Valencia, CA) and quantified by spectrophotometry. Those samples that were more dilute than 400μg/ml underwent ethanol precipitation to concentrate them. Where possible, 4μg of RNA was used for cDNA synthesis, however, if the RNA yield had been less than 4μg then all available RNA was used. The cDNA was synthesised using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Samples of cDNA were deemed to be of good quality if the ABL exon 1/2 transcript could successfully be amplified by PCR.

5.4.3 Mutation screening

The cDNA from post imatinib samples was screened for KM by Dr. Michael Heinrich, using denaturing high-pressure liquid chromatography (DHPLC)[152,154]. We confirmed the presence of KM by using BCR-ABL specific primers to perform nested PCR and automated sequencing.

5.4.4 Real time quantitative PCR (RQ-PCR)

5.4.4.1 Use of Taqman technology to assess mRNA levels

In order to measure gene expression levels in the 15 R and 15 NR with pre imatinib samples, RQ-PCR was performed as described in Chapter 2. The genes investigated (Table 5.2) were chosen based on the knowledge of their ability to actively assist imatinib entry into cells (hOCT1)[83], cause imatinib efflux from cells (MDR1[76-81], ABCG2[80,84,85] and ABCC1[77]) or to have been linked to resistance of chemotherapeutic drugs (ABCA2[155,156], ABCC2[157-160], ABCC3[161-163], ABCC6[164,165], MVP[166-168]). Taqman Assays-on-Demand™ Gene Expression Products (Applied Biosystems, Foster City, CA) were used to provide primer and probe sets for these genes. The ABL exon 1/2 transcript was used as a control target, as described in Chapter 2.

Table 5.2 Candidate genes: symbol and name

Gene symbol	Gene name
hOCT1	human organic cation transporter 1
MDR1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
ABCA2	ATP-binding cassette, sub-family A (ABC1), member 2
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
ABCC6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6
MVP	major vault protein

5.4.4.2 Use of LightCycler technology to assess mRNA levels

Clinical monitoring of BCR-ABL expression at OHSU is done using LightCycler technology with G6PD as a control target[169]. Dr. Richard Press kindly quantified

BCR-ABL expression, on all R and NR samples undergoing RQ-PCR, by LightCycler technology for us.

5.4.5 Calculation of the magnitude of change in gene expression level

The magnitude of change in gene expression level, or fold change (FC), was calculated as follows, where CT is the threshold cycle or cycle number at which the fluorescence emission exceeds the fixed threshold (i.e. the point during the reaction at which a sufficient number of amplicons have accumulated in that well, to be at a statistically significant point above baseline):

- Pre imatinib fold change = $2^{n(pre)}$

n(pre) = pre imatinib control gene mean CT - pre imatinib test gene mean CT

- Post imatinib fold change = $2^{n(post)}$

n(post) = post imatinib control gene mean CT - post imatinib test gene mean CT

- Fold change in gene expression with time on imatinib = $2^{n(post - pre)}$

n(post - pre) = n(post) - n(pre)

5.4.6 Statistical analysis

Statistical comparisons were made by use of the non-parametric Mann-Whitney test in SPSS version 12, since we could not assume a normal distribution of gene expression levels. Multiple comparison adjustment of the p-value was performed by use of the Bonferroni correction.

5.5 Results

Forty-three patients with CML were studied, of which 28 had primary cytogenetic resistance to imatinib and were defined as NR. With follow up, 14 NR went on to show disease progression; 1 patient lost their CHR, 11 developed accelerated phase (AP) CML (with 9 having cytogenetic clonal evolution (CE) as the sole criteria for AP) and 2 developed myeloid blast crisis (BC). The median time to progression was 19.2 months (range, 2.9 to 49.8 months) with a median follow up of 21 months (range, 10 to 58 months). The 15 R showed no evidence of disease progression with follow up (median follow up was 39 months; range, 12 to 45 months).

All NR were examined for the presence of KM at between 9 and 15 months after starting imatinib therapy. Four patients were found to each have a single KM: 3 mutations were within the p-loop (Y253H, Y253F and G250E) and 1 mutation was within the activation loop (M351T). As shown in Table 5.2, all patients that had a KM showed disease progression during follow up, with new CE, AP or BC.

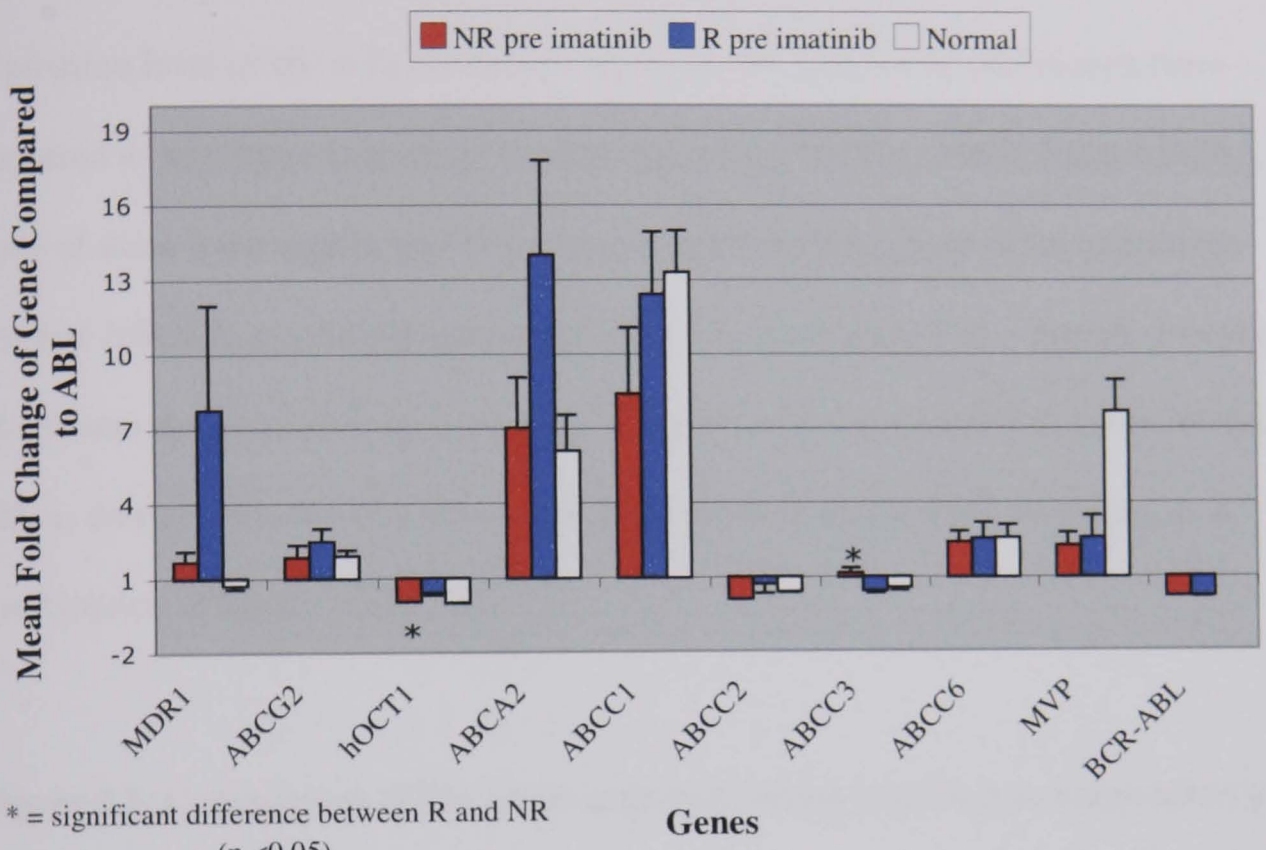
Table 5.3 Characteristics of the 4 NR with BCR-ABL KM at 9 to 15 months from starting imatinib.

Patient ID	**25**	**28**	**12**	**13**
Sex	F	M	F	F
Age at Diagnosis (years)	47	63	56	66
Months from Diagnosis to Start of Imatinib	90	13	44	102
Disease Phase when Started Imatinib	CP + CE	CP + CE	CP	CP
KM	**Y253H**	**Y253F**	**M351T**	**G250E**
Disease Progression	BC	CE	CE	AP
Months to Progression	16	36	39	6
Months of Follow Up	14	36	46	12

CP = chronic phase, CE = cytogenetic clonal evolution, KM = kinase domain mutation, BC = blast crisis, AP = accelerated phase

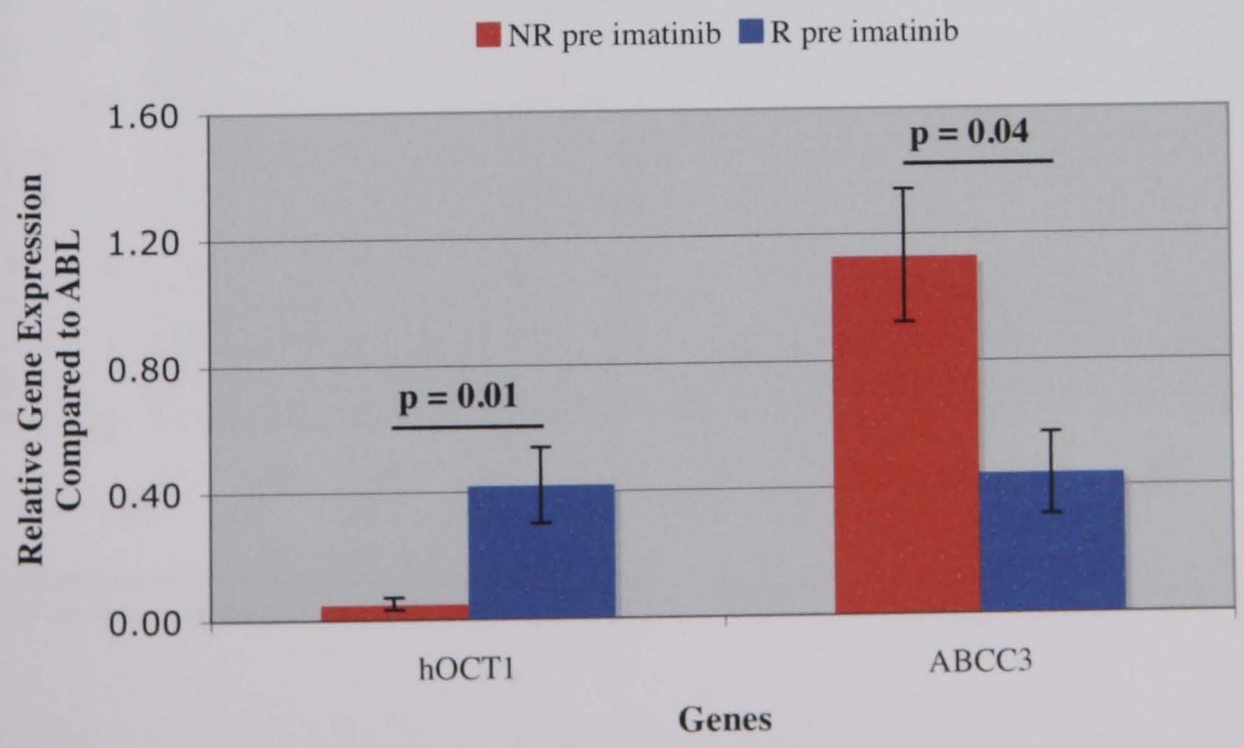
Prior to treatment with imatinib, BCR-ABL expression levels in R and NR were not significantly different. Mean drug transporter expression levels in R and NR prior to imatinib were compared, and after multiple comparison adjustments, hOCT1 and ABCC3 were the only genes that were significantly differentially expressed between R and NR ($p = 0.01$ and $p = 0.04$ respectively) (Figure 5.1). The expression level of hOCT1 in NR was one eighth that seen in R, while ABCC3 expression was two and half times that seen in R (Figure 5.2). Gene expression levels in CML patients did not vary significantly from that seen in normal bone marrow.

Figure 5.1 Comparisons of the pre-imatinib bone marrow mean gene expression levels from non-responders (NR), responders (R) and normal donors.



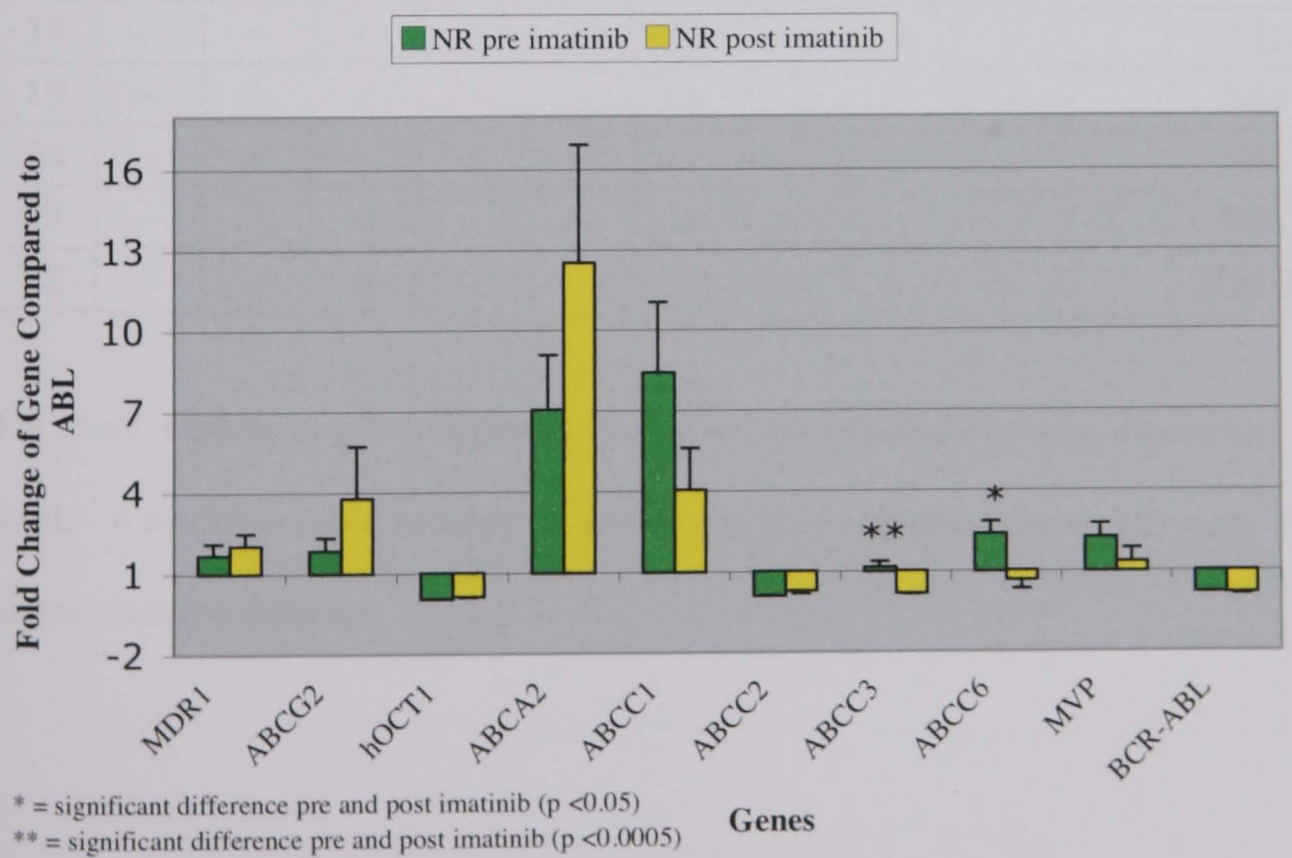
* = significant difference between R and NR ($p < 0.05$)

Figure 5.2 The mean pre-imatinib bone marrow hOCT1 and ABCC3 expression levels in non-responders (NR) and responders (R).



After 9 to 15 months of imatinib treatment, there was no significant change in the BCR-ABL expression level seen in NR. We compared the pre and post imatinib expression level of the drug transporter genes in NR, and found that though there appeared to be a trend to increase in some genes, Eg. MDR1, ABCG2 and ABCA2, none of these were significant. There were significant decreases in the expression level of ABCC3 ($p = 0.0003$) and ABCC6 ($p = 0.01$) (Figure 5.3). Overall, 14 out of 15 patients showed either up-regulation of a gene with the potential to cause imatinib efflux, down-regulation of a gene with the potential to cause imatinib influx, or a combination of these (Table 5.3).

Figure 5.3 Comparison of the mean gene expression level in non-responders pre and post imatinib.



* = significant difference pre and post imatinib ($p < 0.05$)
** = significant difference pre and post imatinib ($p < 0.0005$)

Table 5.4 Two fold increases (+), and two fold decreases (-), in the gene expression level of drug transporter genes and BCR-ABL, as seen in NR following 9 to 15 months treatment with imatinib.

Patient ID	MDR1	ABCG2	hOCT1	ABCA2	ABCC1	ABCC2	ABCC3	ABCC6	MVP	BCR-ABL	Kinase Mutation
1	~	~	-	-	-	-	-	-	-	~	ND
2	+	+	-	-	-	-	-	-	-	~	ND
4	+	~	-	-	-	-	-	-	+	-	ND
9	~	+	-	-	-	-	-	-	-	~	ND
11	~	~	-	-	-	-	-	-	-	+	ND
13	~	+	+	~	~	~	-	-	-	-	**G250E**
14	~	-	-	-	-	-	-	-	-	+	ND
15	-	-	+	-	-	-	-	-	-	-	ND
16	-	~	~	+	~	~	-	-	-	-	ND
17	+	+	+	+	~	+	-	-	+	~	ND
18	~	~	+	+	~	+	-	-	~	-	ND
19	~	~	+	+	+	+	~	~	~	~	ND
24	~	+	~	+	-	~	-	-	-	~	ND
27	~	~	~	~	-	+	-	-	~	-	ND
28	~	~	+	+	~	+	-	+	~	-	**Y253F**

+ = 2 fold increase in gene expression level, **-** = 2 fold decrease in gene expression level, ~ = neither a 2 fold increase or decrease in gene expression level, ND = no kinase mutation detected

5.6 Discussion

We identified 28 NR who were treated with imatinib following failure or intolerance of interferon-α (IFN), and who achieved a CHR yet failed to achieve even a minor cytogenetic response to imatinib. The majority of patients were in late chronic phase prior to receiving imatinib and, in keeping with their poor cytogenetic responses, half the patients progressed during follow up. Since KM are found in 50% to 90% of patients with acquired resistance to imatinib[59-61,63,67], we hypothesised that they might also play a role in primary cytogenetic resistance. At between 9 and 15 months from starting imatinib, we detected KM in only 4 out of 28 NR (14%), in keeping with observations by Branford *et al.*[59] who found that only 1 out of 10 patients with primary cytogenetic resistance, had evidence of a KM. All of our patients with a KM showed progression of their disease during follow up. Since only a minority of our patients with primary cytogenetic resistance showed evidence of a KM, we do not believe that KM can explain the majority of primary cytogenetic resistance in our patient population.

Genes that affect drug transportation have been implicated in resistance to many chemotherapeutic drugs, and imatinib has been shown to be a substrate of a number of drug transporter proteins[76-81,83-85]. Therefore we used RQ-PCR to explore the hypothesis that the expression of drug transporter genes might correlate with primary cytogenetic resistance to imatinib. When comparing pre-imatinib gene expression in 15 NR, with 15 R, we found that NR had a significantly lower level of hOCT1, and a significantly higher expression of ABCC3, than R. Since hOCT1 actively transports imatinib into cells, patients with low baseline expression of hOCT1 may be unable to achieve adequate intracellular concentrations of imatinib, and hence fail to achieve a

cytogenetic response. We do not know whether imatinib is a substrate for ABCC3, and further work is required to investigate this before we can make any conclusions about the potential of ABCC3 to cause imatinib resistance.

The Applied Biosystems Taqman gene expression assay we used for hOCT1 and ABCC3 had primers and probe designed to cover the exon 4/5 boundary of the functional transcript of hOCT1, and the exon 8/9 boundary of the full length transcript of ABCC3. However, we note that there are 2 single nucleotide polymorphisms (SNP's) (P283L and R287G) within the exon 4/5 region of hOCT1, and that these have been linked to significantly diminished transport activity in cell lines, as compared to wild type hOCT1[170]. No SNP's have been reported around the region covered by the ABCC3 assay. Although the Taqman gene expression assays were designed such that polymorphisms should not affect performance of the assay[171], potentially we may not have been as efficient at detecting hOCT1 mRNA transcribed from the leucine or glycine alleles, and so have underestimated the expression of hOCT1 in patients with these polymorphisms. If this were to be the case, we would have been indirectly detecting the alleles associated with diminished transport activity. The allele frequencies of the P283L and R287G polymorphisms are not known, though several other hOCT1 polymorphisms associated with reduced function have been shown to have allele frequencies of between 0.6 and 9.1 in a small study of Caucasians[172]. Unfortunately, there is no more patient material to allow us to assess the allele status of our patients, but it would be interesting to perform further work to investigate the hypothesis that hOCT1 allele status influences cytogenetic response to imatinib in patients with CML.

When examining the change in drug transporter gene expression level over time, we found that though there was a trend towards increasing expression of genes associated with imatinib efflux (MDR1, ABCA2, ABCG2), none of these were significant. In order to have least 80% power to detect a significant difference between the expression of MDR1, ABCA2 and ABCG2 in R and NR, we would need to examine at least 88 patients (44 R and 44 NR), 72 patients (36 R and 36 NR) or 8820 patients (4410 R and 8820 NR) respectively. The only genes to show a significant change in expression were ABCC3 and ABCC6, both of which were down regulated. We would predict that if ABCC3 or ABCC6 were to transport imatinib it would be by drug efflux. ABCC3 was expressed at a high level in NR bone marrow at baseline, and if it were a cause of persisting imatinib resistance in our NR we would have expected its expression level to remain high. We think that it is unlikely that ABCC3 expression, or ABCC6 expression, are a major cause of cytogenetic resistance in our patients. Ideally we would have liked to measure the changes in gene expression in control BM, with time on imatinib, to allow comparison with the changes seen in NR. However, by the very nature of our imatinib responding patients, they had all achieved a CCR at 12 months, and therefore the majority of their BM MNC's post imatinib were no longer BCR-ABL positive, i.e. they would have had a fundamentally different cell population from that of our NR, making them unsuitable controls.

Overall, our patients showed changes in expression in a variety of genes associated with drug transport, with some genes (E.g. MDR1, ABCG2, hOCT1, ABCA2, and ABCC2) being more commonly affected than others. We therefore believe, that in our patient population, a variety of drug transport mechanisms may play a role in cytogenetic refractoriness to imatinib, and that different levels of expression and

activity of transporters may be important. We believe that further work is warranted to explore the interaction of drug transporters, including hOCT1, as potential causes of primary cytogenetic resistance to imatinib.

6 Gene expression profiling of CML white cells

6.1 Introduction

In a landmark study, achievement of a complete cytogenetic response (CCR) was associated with progression-free survival, while patients who failed to attain a CCR had a significant risk of relapse[45]. Predicting failure to achieve a CCR would be clinically relevant, as patients unlikely to respond to standard treatment may benefit from more aggressive initial therapy, such as higher doses of imatinib. As the mechanisms underlying primary cytogenetic resistance are less well understood, we decided to explore gene expression profiling for identification of genes associated with failure to achieve a CCR. By developing a predictive algorithm from the expression profiling data of a "training" cohort of patients, studies in several haematological malignancies have managed to predict the response of patients in a similar "test" cohort; this has included being able to identify Philadelphia chromosome positive acute lymphoblastic leukaemia cells, from patients that are either sensitive or resistant to imatinib[128,129,135,173,174]. We therefore chose to explore the use of this gene expression profiling in CML, as a tool for predicting cytogenetic response to imatinib.

6.2 Aims

1. To test whether gene expression profiling, of RNA extracted from the white blood cells of patients with CML, allows us to develop a tool for predicting response to imatinib.

6.3 Key points

- Gene expression profiling, of the RNA from the unselected white cells of 28 patients with CML, reveals that cytogenetic responders and non-responders have similar gene expression profiles.
- Our study did not allow us to develop a tool for predicting patients' cytogenetic responses to imatinib.
- Using unselected cells from either blood or bone marrow, and using different RNA extraction protocols within the same experiment, introduced unwanted gene signal (noise) into our analysis, which reduced the power of this study to identify relevant significantly differentially expressed genes.

6.4 Methods

6.4.1 Patients

A training set of 14 patients with CML in first chronic phase (CP) was selected from several hundred CML patients who had been enrolled in consecutive Novartis-sponsored trials at the Department of Hematology of the University of Leipzig, Germany. Selection was based on each patient's response to imatinib and the availability of sufficient material (cellular lysates for RNA extraction stored prior to imatinib therapy). An independent test set of 15 patients was selected, using the same criteria, from the coordinating centre of the German CML Study Group at the Faculty of Clinical Medicine Mannheim of the University of Heidelberg, Germany. Local

institutional review boards approved these studies. All patients consented to entry into this study (Appendix 2).

One patient from the training set was excluded due to an apparent physical defect of the chip, which was identified by the pseudo-image of the .cel file (N.B. All underlined words or phrases may be found in the Glossary of terms, section 10). Details on the remaining patients are given in Table 6.1. There were no significant differences in median age [Leipzig: 46.3 years (range, 29 to 58.1 years), Mannheim: 51.6 years (range, 34 to 72.9), p = 0.11] and duration of disease [Leipzig: 1.2 years (range, 4 days to 7.6 years), Mannheim: 4 years (range 87 days to 11.3 years), p = 0.12]. Fifteen patients were female and 13 were male. All patients studied achieved a complete haematological response to imatinib. Patients were defined as responders (R) to imatinib if they achieved a CCR within nine months or less (n = 16), while non-responders (NR) were defined as those who had failed to achieve a major cytogenetic response (MCR) within one year of treatment (n = 12).

Table 6.1 Patient characteristics

Patient identity	Treatment centre	Training / Test Set	Disease category	Material	Response to imatinib	Ph+ metaphases prior to starting imatinib [%]	Months until CCR	Ph+ metaphases at 12 months [%]
1399	M	Test	ND	PB	R	100	3	0
2205	M	Test	ND	PB	R	100	6	0
201	M	Test	IFN (R)	BM	R	92	6	0
11008	M	Test	IFN (R)	PB	R	72	6	0
1229	M	Test	IFN (R)	PB	R	100	3	0
1035D	M	Test	IFN (R)	PB	R	92	3	0
962	M	Test	IFN (I)	PB	R	100	3	0
2023	M	Test	IFN (I)	PB	R	100	6	0
695696	L	Training	ND	BM	R	100	9	0
481	L	Training	ND	BM	R	100	3	0
518	L	Training	ND	BM	R	100	3	0
998	L	Training	IFN (R)	PB	R	100	9	0
1169	L	Training	IFN (R)	PB	R	100	3	0
96	L	Training	IFN (R)	BM	R	47	6	0
988	L	Training	IFN (R)	BM	R	100	9	0
40	L	Training	IFN (I)	BM	R	98	9	0
2028	M	Test	IFN (R)	PB	NR	Dry tap*	-	88
1866	M	Test	IFN (I)	PB	NR	100	-	100
2115	M	Test	IFN (I)	BM	NR	100	-	100
92239	M	Test	IFN (I)	BM	NR	96	-	98
1844	M	Test	IFN (I)	PB	NR	100	-	100
2210	M	Test	IFN (I)	PB	NR	100	-	92
1	M	Test	IFN (I)	BM	NR	100	-	100
316	L	Training	IFN (R)	BM	NR	100	-	100
70	L	Training	IFN (R)	BM	NR	100	-	88
1290	L	Training	IFN(R)	PB	NR	100	-	100
1258	L	Training	IFN (R)	PB	NR	100	-	100
67	L	Training	IFN (I)	PB	NR	100	-	100

BM - bone marrow, CP - chronic phase, IFN (I) - interferon intolerant, IFN (R) - interferon resistant, L – Leipzig, M - Mannheim, ND - newly diagnosed, NR - non-responder, PB - peripheral blood, R - responder.

*Results at 3 months from starting imatinib showed 90% Ph-positive metaphases.

6.4.2 RNA extraction and microarray analysis

RNA samples for the microarray analysis were isolated from total bone marrow (BM) white cells, or peripheral blood (PB) white cells, using guanidine-thiocyanate / phenol-chloroform extraction[175] with RNA purification by RNeasy (Qiagen, Hilden, Germany) (Leipzig cohort) or cesium chloride-gradient purification[176] (Mannheim cohort). RNA was processed by the DNA Core of the IZKF (Interdisciplinary Center for Clinical Research) at the University of Leipzig (Leipzig cohort) or by the Affymetrix Microarray Core at Oregon Health & Science University (Mannheim cohort). In the latter core, each sample of RNA underwent quality assessment on the RNA 6000 LabChip using the 2100 Bioanalyzer (Agilent, Palo Alto, CA). Those samples whose electropherogram showed the presence of discrete 18S and 28S ribosomal RNA peaks and the absence of irregularly-sized low molecular weight RNA species (i.e., degraded RNA) were considered to be of good quality and were used for microarray analysis.

Microarray assays were performed according to the standard protocol described in the Affymetrix GeneChip Expression Analysis Technical Manual, rev.3 (http://www.affymetrix.com/support/index.affx). Messenger RNA was amplified and labelled from 5μg (Leipzig) or 4μg (Mannheim) of total RNA. An aliquot (200ng) of the resulting cRNA underwent quality assessment on the RNA 6000 LabChip using the 2100 Bioanalyzer (Agilent, Palo Alto, CA). Samples that produced sufficient cRNA yield and had cRNA electropherograms that showed a size distribution pattern predictive of acceptable microarray assay performance were considered to be of good quality. These samples were fragmented and combined with array hybridization

controls consisting of biotinylated cRNAs for four bacterial genes (Affymetrix, Santa Clara, CA) in hybridization buffer. Ten micrograms of cRNA target were then hybridized with the GeneChip HG_U95Av2 array (Affymetrix, Santa Clara, CA) and scanned using the GeneArray laser scanner (Affymetrix, Santa Clara, CA). The HG_U95Av2 array contains 12,625 transcripts, including 67 control genes. The array image scans were processed with Affymetrix Microarray Suite software, version 5.0 (MAS 5.0). All GeneChip expression arrays contain control probe sets for both spiked and endogenous RNA transcripts (e.g., bacterial genes BioB, BioC, BioD, CreX and species-specific actin and GAPDH). Following image processing and single array analysis of the array pattern with the MAS statistical expression analysis algorithm[177,178], six values are examined: background, noise, average signal, percent present and ratio of signal values for probe sets representing the 5' and 3' ends of actin and GAPDH transcripts. All assays in this study met standard performance thresholds for background (less than 90) and RNA integrity ("housekeeping" control gene 3'/5'ratios less than 2).

6.4.3 Data Analysis

Initial image analysis, signal quantification and intra-chip normalisation (i.e., global scaling) was done with MAS 5.0[177,178]. An unsupervised hierarchical clustering of samples based on MAS 5.0 signal data showed a noticeable difference between the two sites. In order to reduce the site difference, Robust Multichip Analysis (RMA)[179] implemented in the Bioconductor (http://www.bioconductor.org) was used as an alternative method of signal quantification, pre-processing, and intra- and inter-chip normalization. Significance Analysis of Microarrays (SAM)[180] was performed on the

training set to identify differentially regulated genes associated with a lack of response to imatinib. Genes that met a q value of ≤10% were selected for further validation in the test set. The unadjusted t-test, p value and fold change of these genes were computed using the test set. As the test set failed to replicate the findings from the training set, we performed Analysis of Variance (ANOVA) on all patients (n = 28) in training and test sets in order to identify sources of variation. For each gene, the ANOVA model included the site (Leipzig vs. Mannheim), source of samples (PB vs. BM), the response status (R vs. NR) and two-way and three-way interaction terms. The p values from the ANOVA F statistics for each effect were adjusted for multiple comparisons using the False Discovery Rate (FDR) of Benjamini and Hochberg[181]. Data visualisation was performed using a hierarchical clustering analysis and Multidimensional Scaling (MDS). Gene annotation was obtained through Affymetrix NetAffx site (http://www.affymetrix.com/analysis/index.affx).

6.4.4 Real time quantitative PCR measurement of gene expression

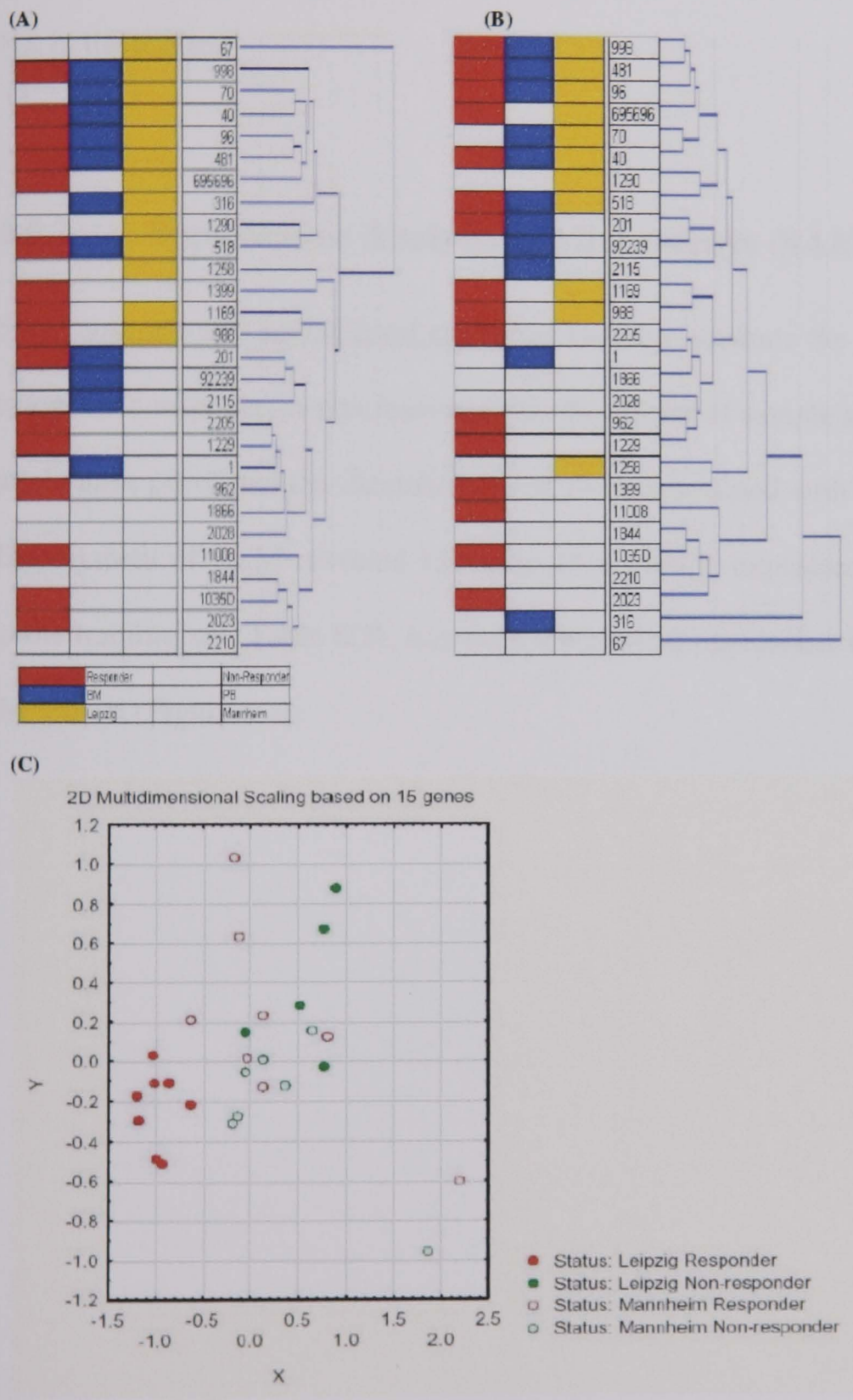
In order to confirm the gene expression seen on the oligonucleotide arrays, a selection of genes that were differentially expressed in the training set, prior to multiple comparison adjustment, had their gene expression levels measured by real time quantitative PCR (RQ-PCR) using primer and probe sets supplied by Applied Biosystems.

6.5 Results

6.5.1 Gene expression profiling of unselected white cells

Oligonucleotide microarrays were used to explore the gene expression profiles of unselected white blood or bone marrow cells from 29 patients with CML in first CP. Unsupervised hierarchical clustering of samples, based on log 2 transformed MAS 5.0 signal values, revealed clustering of patients based on the site of sample processing and sample source but not by cytogenetic response (Figure 6.1). The site effect was substantially attenuated after RMA normalisation (Figure 6.1) and thus RMA was considered to provide better normalisation of the data than MAS 5.0. Our RMA normalised data are available for review at: http://www.ncbi.nlm.nih.gov/geo, accession number GSE2535. With both methods, one patient sample (67) was found to be an outlier. No reason was identified by review of clinical parameters, image file or sample quality parameters; therefore, this sample continued on into further analysis.

Figure 6.1 Two dendrograms (A, B) showing unsupervised hierarchical clustering of all 28 patient samples and one 2D multidimensional scaling graph (C) of all patients, based on the 15 most significantly differentially expressed genes between R and NR in the training set.



(A) Unsupervised hierarchical clustering, based on log 2 transformed MAS5.0 signal values, showing clustering of patients by site of sample origin and sample source, but

not by cytogenetic response. (B) Unsupervised hierarchical clustering, based on RMA signal values, showing no obvious clustering by any parameter. (C) 2D Multidimensional scaling of patients from both training (Leipzig patients) and test sets (Mannheim patients). Using the 15 genes most significantly differentially expressed between R and NR in the training set, we were unable to separate R from NR in the test set.

6.5.2 Significance Analysis of Microarrays (SAM)

Since unsupervised hierarchical clustering failed to separate the patients according to cytogenetic response, it was reasoned that the effects of sample source and processing might have prevented the identification of genes associated with response to imatinib. Employment of SAM revealed 15 genes differentially expressed between R and NR in the training set (Table 6.2), however, differential expression was not confirmed in the test set (Figure 6.1).

Table 6.2 The top 15 differentially expressed genes, with a q value <0.1, identified for responders and non-responders in the training set, together with the fold change and level of significance of these genes when examined in the test set.

Training Set				Test Set	
Probe Set ID	**Gene Title**	**Public Database (Locus Link) Number**	**Fold Change**	**Fold Change**	**Unadjusted T Test P-Value**
40215_at	UDP-glucose ceramide glucosyltransferase	7357	+2.1	-1.4	>0.05
37985_at	lamin B1	4001	+1.9	-1.3	>0.05
38402_at	lysosomal-associated membrane protein 2	3920	+1.8	-1.1	>0.05
2065_s_at	BCL2-associated X protein	581	+1.4	1.0	>0.05
39064_at	5,10-methenyltetrahydrofolate synthetase	10588	+1.4	+1.1	>0.05
1360_at	X-ray repair complementing defective repair in Chinese hamster cells 4	7518	+1.3	1.0	>0.05
138_at	mitogen-activated protein kinase kinase kinase kinase 1	11184	-1.3	+1.1	>0.05
33134_at	adenylate cyclase 3	109	-1.3	-1.1	0.043
39709_at	selenoprotein W, 1	6415	-1.4	1.0	>0.05
36811_at	lysyl oxidase-like 1	4016	-1.6	-1.2	>0.05
36757_at	histone 1, H3h	8357	-1.6	-1.1	>0.05
41337_at	amino-terminal enhancer of split	166	-1.7	1.0	>0.05
41743_i_at	optineurin	10133	-1.8	+1.2	>0.05
39081_at	no gene title given	4502	-1.9	+1.1	>0.05
36780_at	clusterin	1191	-2.6	-1.9	>0.05

The fold change (FC) is defined as: $\pm 2^{|\Delta|}$, where $|\Delta|$ is the absolute value of the difference in mean log (signal) between R and NR groups. The positive sign (+) indicates up-regulation of R compared to NR, while the negative sign (-) indicates down-regulation. Note that a fold change of 1.0 is equivalent to no difference in gene expression.

6.5.3 Analysis of variance

Since there was no overlap in the differentially expressed genes between the two patient cohorts, analysis of variance was performed in order to identify sources of variability. All samples were combined, and we performed gene-by-gene ANOVA to evaluate the impact of the site and sample source, in addition to the response status. Using a false discovery rate of 0.10 or less, ANOVA identified genes differentially expressed between the two sites (4421 genes) and between BM and PB samples (439 genes). None of the genes met the statistical significance criteria for response status or for two or three-way interactions between the sites, source and response status.

6.5.4 Sample size and power considerations

Since the results indicated that the differences between R and NR were subtle, the observed differences were used to estimate the theoretical number of samples required to identify genes significantly associated with cytogenetic refractoriness. Using gene-specific mean squared errors (MSE) from the ANOVA model, the average sample size required to detect a 1.5-fold change between R and NR, using 80% power and a 1% significance level to adjust for multiple comparisons, was 258 patients (129 responders and 129 non-responders). An even larger sample size would be required to develop a robust test set for validation of a classification algorithm.

6.5.5 Real time quantitative PCR

We confirmed the microarray findings that responders had either a higher or lower level of gene expression, compared to non-responders, in 5 out of the 6 genes tested.

RQ-PCR of the dCMP deaminase gene (631_g_at) could not confirm the microarray findings, however, the difference in expression level between R and NR was only small (R:NR = 1.5:1). The magnitudes of the fold differences in gene expression between R and NR for all 6 genes, as measured by both microarray analysis and RQ-PCR, were not significantly different from eachother (Student's paired t-test, p = 0.53).

6.6 Discussion

Predicting cytogenetic response prior to imatinib would be clinically useful, since it would allow for early risk stratification. The patients for our study were selected from patient populations treated at two different centres, based on cytogenetic response and availability of sufficient RNA. Most of the NR did not achieve even a minor cytogenetic response, whilst 46.7% of R with available data were in a CCR at 3 months. Our patient population therefore represented, for most part, the extremes of the spectrum of responses. Nonetheless, we found that the pre-therapeutic gene expression profiles of unselected white cells did not allow us to identify genes consistently associated with lack of cytogenetic response to imatinib.

Our results fail to confirm two recently published studies. Using cDNA arrays on mononuclear cells from 22 CML patients (18 in CP), Kaneta *et al.*[139] reported 71 genes associated with MCR. McLean *et al.*[140] analyzed the transcriptional profile of total PB from 66 newly diagnosed patients in CP. From a list of 55 genes, with at least 1.7 fold difference between patients with CCR and patients with >65% Ph-positive metaphases after 13 months of treatment, they selected an optimal list of

identified 31 predictive genes using a "leave one out" strategy. Remarkably, the discriminating genes did not overlap between the two studies, or with the 15 most significant genes in our training set. Using RMA and the published genes (with the exception of eight genes of Kaneta *et al.* for which the probe IDs were missing in the published manuscript[139]), we could not distinguish between R and NR in our data set.

There are several potential explanations for the discrepant results. Firstly, different cells were analysed. RNA extracted from whole blood, as in the study by McLean *et al.*[140] contains large amounts of globin transcripts, and yields significantly less present calls than RNA extracted from selected white or mononuclear cells[182]. Secondly, our study included patients in late chronic phase pre-treated with various drugs and the cytogenetic response in such patients may be influenced by factors different from newly diagnosed patients. In addition, since we do not have data on patients' serum imatinib concentrations, it may be that some patients did not achieve adequate drug levels. Most importantly, we used an independent control group for validation, and applied rigorous statistical methods for data analysis.

Significant differences were evident between samples from Leipzig and Mannheim, likely reflecting different methods of sample preparation, and between samples derived from PB vs. BM, confirming previously published data[136]. However, regardless of these potential confounding factors, the differences between R and NR were extremely subtle in either cohort, and associated with very high false discovery rates. In addition, our power calculations indicate that although it would theoretically still be possible to identify a more robust set of predictive genes, this would require a very large patient cohort.

In addition to these considerations, CML may differ from other haematological malignancies, such as acute myeloid leukaemia[131,173,183] or acute lymphoblastic leukaemia[128-130], where gene expression signatures have been shown to correlate with response to chemotherapy and survival. In these disorders resistance to therapy may be a quality of all the blast cells, and thus its signature may be detectable in this population. In CML, this may be more comparable to haematologic resistance, rather than to cytogenetic resistance. Refractory progenitor cells, the transcriptional signature of which may be undetectable in the noise of unselected white cells, may mediate the latter. Thus, it is conceivable that enriching for progenitor cells (e.g. CD34+ cells) may sufficiently reduce the noise to uncover the transcriptional signature associated with cytogenetic resistance. We therefore believe that, if gene expression profiling is going to be able to help us to predict patients' responses to imatinib, analysing expression profiles of CML CD34-postive progenitor cells may be the best method.

7 Isolating CML CD34+ cells

7.1 Introduction

In order to perform gene expression profiling on CML CD34+ cells, we needed to develop a protocol that would allow us to isolate CD34+ cells from CML mononuclear cells. We needed a protocol that would be suitable for CD34+ cell isolation from cryopreserved cells, and one that would be suitable for CD34+ cell isolation from the peripheral blood of newly diagnosed patients. For the latter we chose to use immunomagnetic separation of CD34+ cells, using the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Auburn, CA), because we had experience of using this kit[184] and we needed a simple technique that would allow us to select cells as soon as possible after phlebotomy, even when the time of arrival of a patient's sample was unpredictable. For the CD34+ cell selection from cryopreserved cells, we elected to base our method on a flow cytometry technique since this study allowed us more time to plan CD34+ isolation procedures and so allowed us to make use of this technique's ability to give us a highly pure cell population. Since we had no previous experience in using this method of CD34+ cell isolation, we needed to develop and test a protocol, before proceeding to the samples we wished to use for the microarray experiment.

7.2 Aims

1. To develop and test a technique of cell sorting CD34+ cells from CML mononuclear cells stored in liquid nitrogen.

2. To design a protocol for CD34+ cell isolation from the peripheral blood of newly diagnosed patients with CML.

7.3 Key Points

- We have developed a technique for isolating highly pure samples of CD34+ cells from CML mononuclear cells stored in liquid nitrogen.
- We have designed a simple protocol for isolating CD34+ cells from the peripheral blood of newly diagnosed patients with CML.

7.4 Methods

7.4.1 Isolation of CD34+ cells from CML mononuclear cells stored in liquid nitrogen

7.4.1.1 Patient material

In order to develop the protocol for isolation of CD34+ cells, we identified a large number of samples of mononuclear cells (MNC's) that had been separated by density gradient centrifugation from one CML patient's peripheral blood leukopheresis sample. Samples consisting of 5 x 10^7 MNC's had been suspended in 50% Iscove's modified dulbecco's media (IMDM), 45% fetal bovine serum (FBS) and 5% dimethyl sulfoxide (DMSO), and these had then been cryopreserved in liquid nitrogen.

7.4.1.2 Sample thawing and enrichment for viable cells

When required, an aliquot of MNC's was thawed at 37°C and immediately suspended in Dulbecco's phosphate buffered saline (PBS) containing 0.1% human albumin (Baxter Healthcare Corporation, Glendale, CA), 1% recombinant DNase solution (Pulmozyme®, Genentech, San Francisco, CA) and 2.5mM magnesium chloride and then centrifuged at 980rpm for 5 minutes at 5°C. The sample was enriched for viable cells using the Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions.

7.4.1.3 Cell staining

The viable cells were suspended in Hanks' balanced salt solution (HBSS) with 3% FBS, 2% HEPES and 1% recombinant human DNase (Genentech), and incubated with 20µl CD34-fluorescein isothiocyanate (FITC) and 20µl CD45-PerCP-Cy5.5 monoclonal antibodies (BD Biosciences, San Jose, CA) for every 1 x 10^6 cells. Excess fluorochrome was washed off and the cells resuspended in HBSS containing 0.5% FBS, 2% HEPES and 1% recombinant human DNase (Genentech). For the identification of dead cells, propidium iodide (PI) (Roche, Indianapolis, IN) was added to the cell solution immediately prior to sorting.

7.4.1.4 Cell sorting CD34+ cells

A BD FACSAria™ (BD Biosciences, San Jose, CA) was used to sort the CD34+ cells. The BD FACSAria™ is an electrostatic cell sorter that works by ejecting a focused stream of cells through a nozzle into air. A laser beam passes through the cuvette flow cell and then, depending on the fluorescent signal and scatter results, charged

droplets containing cells are deflected into the appropriate collection tubes. In order to select CML CD34+ cells, gates were set to select viable mononuclear cells by forward scatter (FSC) and side scatter (SSC) (Figure 7.1), and then cell debris and clumped cells were excluded by limiting the FSC-width (FSC-W) and FSC-height (FSC-H) of the selected cells (Figure 7.2). Next, gates were set to select only PI negative cells to ensure that only viable cells were selected (Figure 7.3). Finally, on the CD34-FITC and CD45-PerCP-Cy5.5 histogram, CD45-PerCP-Cy5.5 dim cells that brightly co-expressed CD34-FITC were selected (Figure 7.4). We chose our final gating to allow us to isolate reasonable numbers of stem cells, since all but the very earliest stem cells express the CD34 antigen, and to put a limit on the maturity of our selected cells, since cells progressively express more CD45 antigen as they mature. (N.B. The software attached to the BD FACSAria™ only allowed us to save scatter plots in a black background format.) After sorting a fraction of the selected cells underwent flow cytometry, with gates as previously described, to assay the percentage of CD34+ cells within the sample.

7.4.1.5 RNA extraction, quantitation and quality assessment

Remaining selected CD34+ cells were placed in PicoPure™ extraction buffer (Arcturus, Mountain View, CA) and stored at -80°C. RNA was later extracted from 3 samples and sent to the Affymetrix Microarray Core, at Oregon Health and Science University, for quantitation using the NanoDrop® ND-1000 UV-vis spectrophotometer, and assessment of quality on the RNA 6000 LabChip using the 2100 Bioanalyzer (Agilent, Palo Alto, CA).

Figure 7.1 Scatter plot showing mononuclear cells, as measured by forward scatter (FSC-A) and side scatter (SSC-A), with gate P1 covering the region of mononuclear cells, and avoiding the region of cell debris.

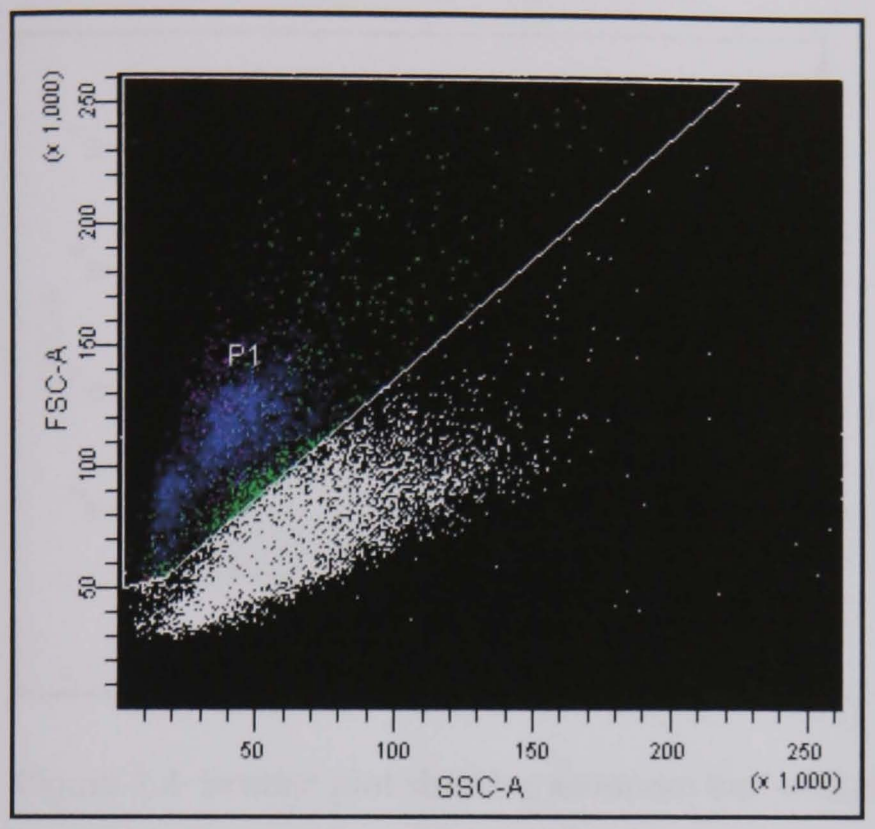


Figure 7.2 Scatter plot showing mononuclear cells, as measured by forward scatter width (FSC-W) and forward scatter height (FSC-H), with gate P2 placed over the region of intact single mononuclear cells.

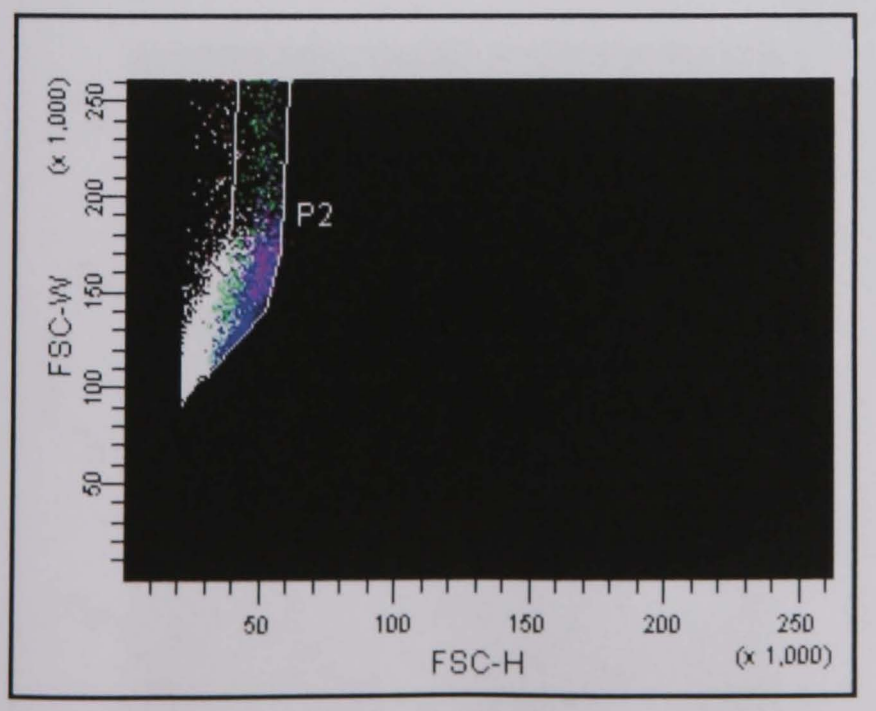


Figure 7.3 Scatter plot showing mononuclear cells, as measured by expression of CD34-FITC (FITC-A) and propidium iodide (PI-A), with gate P3 over the region of viable cells.

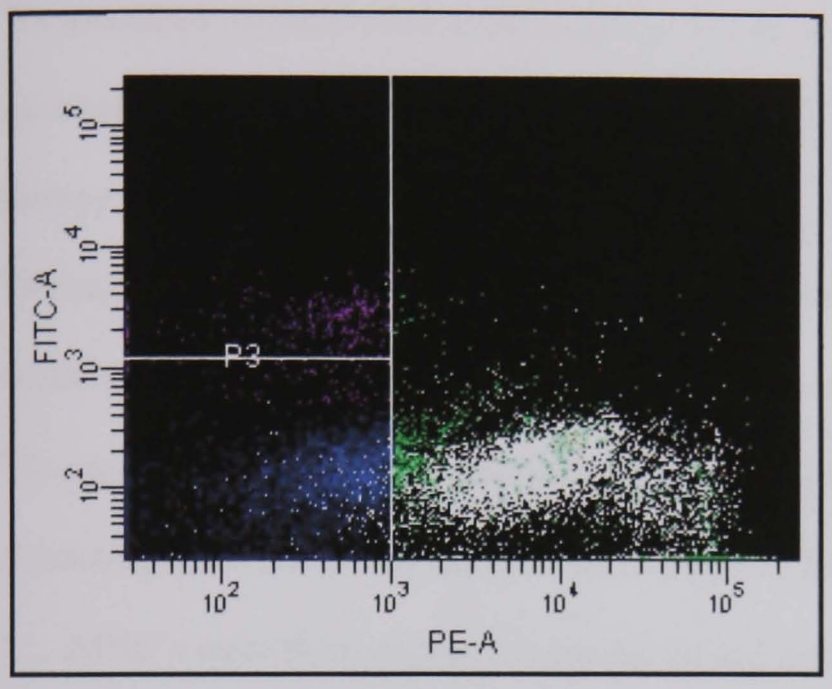
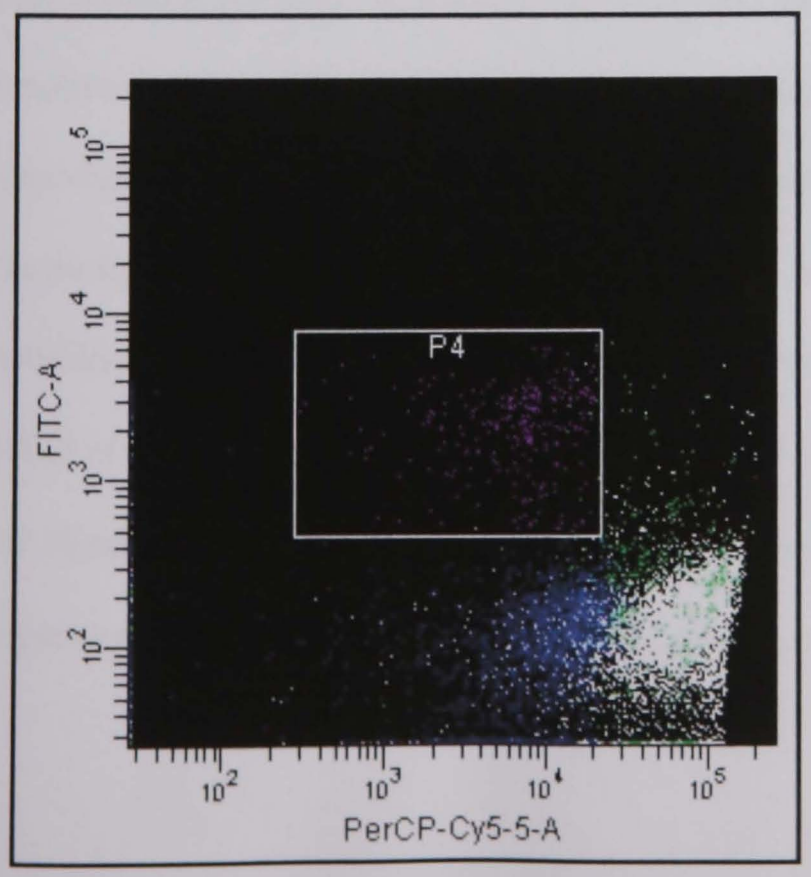


Figure 7.4 Scatter plot showing mononuclear cells, as measured by expression of CD34-FITC (FITC-A) and CD45-PerCP-Cy5.5 (PerCP-Cy5.5-A), with gate P4 over the region of CD45-PerCP-Cy5.5 dim cells that brightly co-express CD34-FITC.



7.4.2 Isolation of CD34+ cells from the peripheral blood of newly diagnosed patients with CML

We identified patients with newly diagnosed CML who were yet to receive any treatment for their disease, and who were expected to receive imatinib as first line therapy. All patients gave their consent for inclusion in this study (Appendix1). Twenty millilitres of peripheral blood were taken and placed into a sample tube containing preservative free heparin, and this was then transported to the laboratory. The sample was diluted with minimacs buffer (phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2mM EDTA), according to Table 7.1. MNC's were then separated from the diluted cell solution by density gradient centrifugation, washed 3 times in minimacs buffer, and resuspended in 300µl minimacs buffer per 1 x 10^8 cells. A maximum of 2 x 10^8 cells were labelled with FcR blocking reagent and Hapten-Antibody, according to the instructions in the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec). Labelled cells were placed onto a MS column (Miltenyi Biotec) and CD34 cells were immunomagnetically separated from the mononuclear cells, according to the manufacturer's instructions. Up to 1 x 10^5 cells underwent flow cytometry to assess the purity of the cells, and up to 2 x 10^4 cells were stored for later fluorescent in situ hybridisation (FISH) analysis of BCR-ABL. The remaining cells were placed in 100µl of PicoPure Extraction Buffer (Arcturus) per 5 x 10^6 cells, incubated at 42°C for 30 minutes, centrifuged at 3,000 rpm for 2 minutes and then the RNA containing supernatant was placed into a fresh microcentrifuge tube and stored at -80°C.

Table 7.1 Table of the volumes required for dilution of the peripheral blood samples with buffer.

Peripheral blood white cell count (x 10^9/l)	Volume of sample (ml)	Volume of minimacs buffer (ml)
10	20	20
10-25	20	40
25-50	20	60
50-100	20	80
100-300	20	100
>300	20	120

7.5 Results and Discussion

In order to develop and test a protocol for isolating CD34+ cells from cryopreserved CML MNC's, we chose to use MNC's from a single patient with CML so that we could directly compare the protocols we designed, and not be concerned that differences in the purity of the selected cells were simply a reflection of using samples from different patients. The final successful protocol is described in the methods section above, and it was repeated on 4 separate occasions to check its reliability.

In the design of the protocol for separation of CD34+ cells from fresh peripheral blood, we had previous experience of using the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec)[184] and therefore did not perform any testing of our protocol, but proceeded to directly acquiring patient samples. The results of this are discussed in Chapter 8.

7.5.1 Sample thawing and enrichment for viable cells

The percentage of viable MNC's immediately after thawing was low, with a median viability prior to centrifugation being only 14.6% (range, 12.6% to 30.6%). In order to maximise the viability of the cells, we thawed the MNC's rapidly in a 37°C water bath and immediately washed them in an albumin containing solution. As a consequence of the poor cell viability, there was free DNA within the solution that the MNC's were suspended in, and this resulted in cell clumping. We therefore used DNase in all our solutions, and this successfully reduced the problem of cell clumping, and hence maximised our cell yield.

In order to remove as many apoptotic and dead cells as possible prior to sorting, we used the Dead Cell Removal Kit (Miltenyi Biotec) which immunomagnetically removed phosphatidylserine-positive cells. Immediately prior to sorting, the kit improved the median viability of the MNC's to 21.7% (range, 12.6% to 24.8%). Before using the Dead Cell Removal kit, the BD FACSAria™ tended to underestimate CD34+ cell purity by miscounting a proportion of cell debris as cells. Although the improvement in viability with the kit was only small, minimising the cell debris made a 10% - 20% improvement in the accuracy of our measurements of cell purity.

We had initially attempted to use the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec) to enrich the cryopreserved MNC's for CD34+ cells prior to carrying out cell sorting; however, we lost a large number of cells without significantly enhancing the CD34+ cell purity of the cells prior to sorting. Although we did not formally record the viability of the MNC's extracted from the peripheral blood of newly diagnosed patients with CML, we noted that their viability was almost

always greater than 90%. Thus, it may be that the large difference in viability of the MNC's obtained from frozen patient samples, as compared to fresh patient samples, resulted in the poor results we saw with the immunomagnetic method of separating CD34+ cells from cryopreserved MNC's.

7.5.2 Cell staining

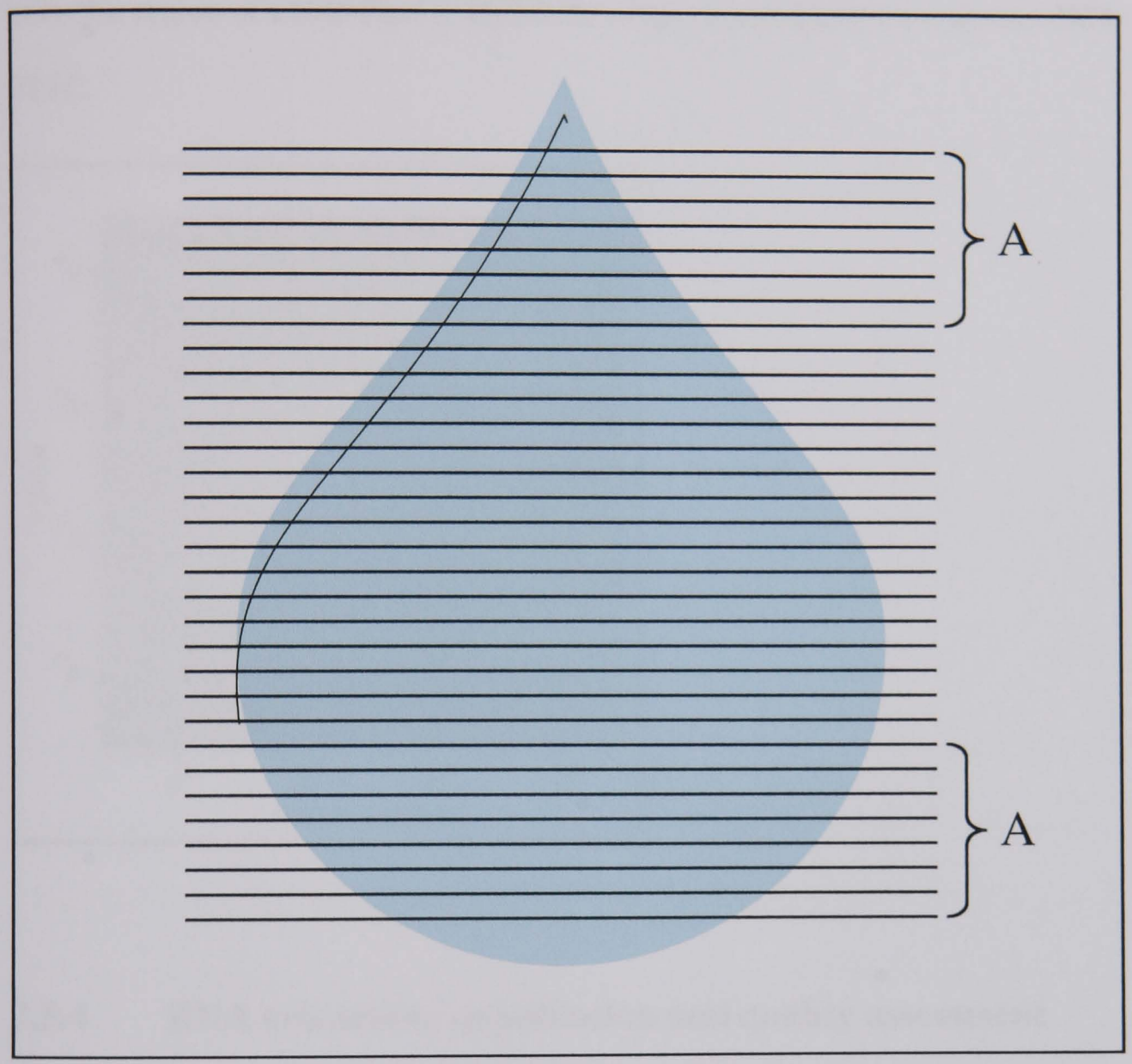
Whilst the MNC's were staining, they were kept in a solution containing 3% FBS, however, immediately prior to sorting they were placed in a solution containing less protein. This reduction in protein reduced the viscosity of the cell sorting stream, and hence reduced unwanted spraying of the stream. We chose to use antibodies labelled with FITC and PerCP-Cy5.5 since we found these fluorochromes gave us the most distinct compensation settings, when used in conjunction with PI.

7.5.3 Cell sorting CD34+ cells

In order to maximise our cell viability during sorting, we used the largest available nozzle (100 microns) to reduce the pressure placed on cells within the sorting stream. To maximise the purity of the sorted cells, we used the "single cell" sort setting (Figure 7.5). Our gating strategy was designed to firstly select viable cells that would include CD34+ cells, then gate out debris and clumped cells. Next, we picked out alive and non-apoptotic cells, and then finally selected our CD34+ cell population. Flow cytometry was used to assess the purity of the sorted CD34+ cells (Figure 7.6). From a median of 3.06×10^6 stained cells (range, 8.25×10^5 to 5.95×10^6), with a median CD34+ cell purity of 2.2% (range, 1.0% to 2.3%), we sorted a median of 2.83×10^4 CD34+ cells (range, 7.95×10^3 to 3.92×10^4), with a median CD34+ cell purity

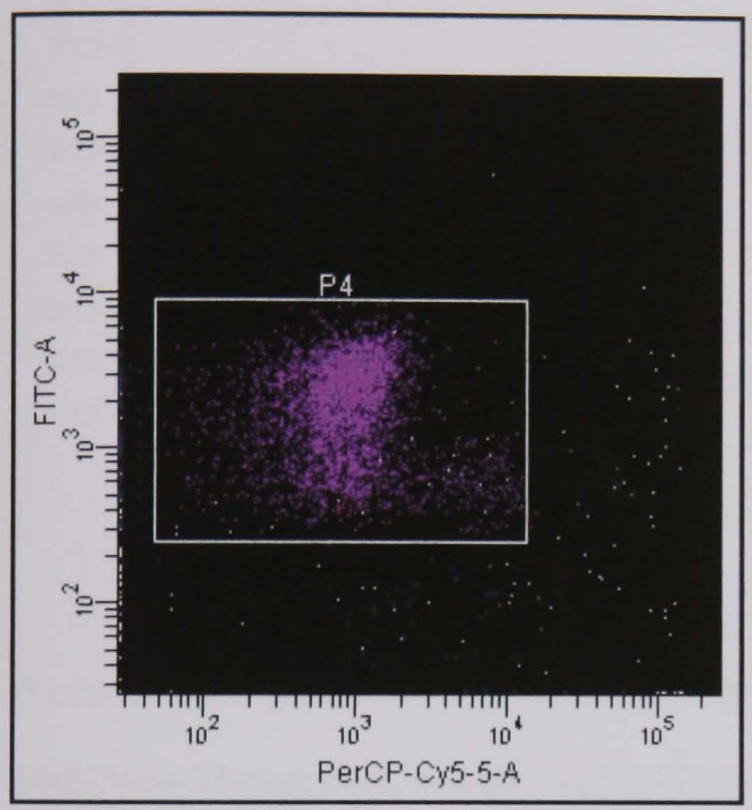
of 96.9% (range, 93.4% to 98.0%). We therefore felt confident that our protocol could be used to select viable CD34+ cells from cryopreserved CML MNC's, and provide a sample of highly pure CD34+ cells that would be suitable for RNA extraction.

Figure 7.5 Diagram showing how the masks on the BD FACSAriaTM divide a cell containing drop into 32 segments (not to scale).



The "single cell" sort option is designed to maximise purity, even at the expense of cell yield. The phase mask is set to 16 so that if the desired cell is in either the top or bottom quarters of the drop (regions marked A), that drop is aborted. In addition, the purity mask is set to 32 so that if the desired cell is within either the top or bottom half of the drop, the following or proceeding drops are respectively aborted from the sort.

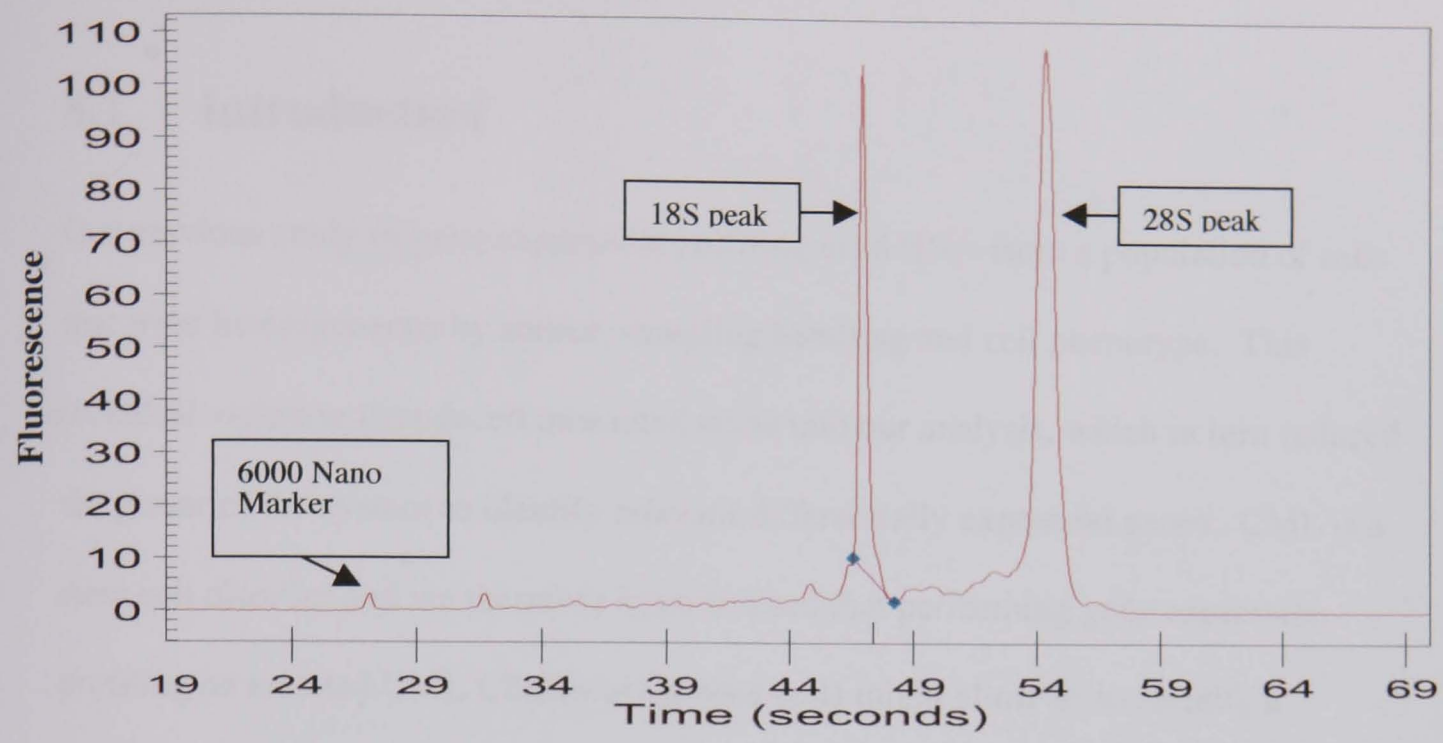
Figure 7.6 Scatter plot showing sorted CD34+ cells, as measured by expression of CD34-FITC (FITC-A) and CD45-PerCP-Cy5.5 (PerCP-Cy5.5-A), with gate P4 over the region of CD45-PerCP-Cy5.5 dim cells that brightly co-express CD34-FITC.



7.5.4 RNA extraction, quantitation and quality assessment

RNA was extracted from 3 samples of sorted CD34+ cells, quantitated and assessed for quality. All samples were found to be of good quality by the 2100 Bioanalyzer (Agilent) with electropherograms showing the presence of discrete 18S and 28S ribosomal RNA peaks and the absence of irregularly sized low molecular weight RNA species (i.e. degraded RNA) (Figure 7.7). The number of CD34+ cells isolated was less than 4000, and consequently the RNA yield was low, with a median of 14.95ng RNA extracted (range, 3.66ng to 56.46ng).

Figure 7.7 Example of an electropherogram resulting from RNA extracted from sorted CD34+ cells.



An electropherogram showing fluorescence against time, and the identification of the 6000 Nano Marker peak, and the 18S and 28S ribosomal peaks. As shown, RNA likely to perform well in a microarray assay has 18S and 28S peaks, and the absence of a spiky distribution of increased fluorescence between the 6000 Nano Marker and the 18S peak.

In conclusion, despite the poor viability of MNC's post thawing, we were able to select a pure population of CD34+ cells and extract good quality RNA from these cells. The number of CD34+ cells that we could isolate limited our RNA yields. We therefore proposed that in order to achieve enough RNA for our planned microarray experiment, we would need to maximise the number of cells thawed per patient, and also to use a second round of amplification in the RNA labelling step of the array hybridisation protocol.

8 Gene expression profiling of CML CD34+ cells

8.1 Introduction

Our previous study of gene expression profiling used RNA from a population of cells that were heterogeneous by source, sampling handling and cell phenotype. This technical variation introduced unwanted noise into our analysis, which in turn reduced the power of the system to identify relevant differentially expressed genes. CML is a stem cell disorder and we therefore hypothesised that performing gene expression profiling on isolated CML CD34+ progenitor cells might allow us to identify a transcriptional profile associated with resistance to imatinib, by eliminating the noise created by cells of different phenotypes.

8.2 Aims

1. To determine whether gene expression profiling of CML CD34+ cells, isolated from bone marrow cells stored in liquid nitrogen, would allow us to develop a tool for predicting patients' responses to imatinib.

8.3 Key points

- At present, it is uncertain whether gene expression profiling of CML CD34+ cells, isolated from bone marrow cells stored in liquid nitrogen, is going to be useful in developing a tool for predicting patients' responses to imatinib.

- We experienced difficulties in accurately measuring low concentrations of RNA, which may have led to insufficient RNA input into RNA quality assessment and amplification.
- Future work should consider using RNA extracted from CD34+ cells that has been freshly prepared from newly diagnosed and untreated patients with CML, in order to compare the gene profiles of imatinib responders and non-responders.

8.4 Methods

8.4.1 Patients

At Oregon Health and Science University (OHSU), 51 patients with CML were identified who had had bone marrow (BM) mononuclear cells stored just prior to their commencement on imatinib (Table 8.1). All patients had either achieved a complete cytogenetic response (CCR) during their first year of imatinib therapy and were defined as responders (R, n = 35), or they had remained at least 65% Ph positive by cytogenetic analysis during their first year of imatinib treatment and were defined as non-responders (NR, n = 16). All patients consented to entry into this study (Appendix 2).

Table 8.1 Clinical characteristics of the fifty-one patients with CML.

Characteristic	Value
Sex – no. (%)	
Male	28 (54.9)
Female	23 (45.1)
Age – years	
Median	57
Range	29 - 75
Hemoglobin – g/dl	
Median	12.4
Range	9.5 – 16.3
White cell count – $x10^3/l$	
Median	12.8
Range	2.5 – 116.0
Platelet count – $x10^3/l$	
Median	323
Range	19 - 1372
Peripheral blood basophil count - %	
Median	5
Range	0 - 31
Peripheral blood blast count - %	
Median	0
Range	0 - 11
Bone marrow blast count - %	
Median	1.75
Range	0.0 – 18.0
Disease Phase – no. (%)	
Chronic phase	34 (66.7)
Early chronic phase	9
Late chronic phase	25
Chronic phase + cytogenetic clonal evolution	12 (23.5)
Accelerated phase	5 (9.8)
Days from diagnosis to starting imatinib – no.	
Median	1059
Range	71 - 5492
Treatment prior to imatinib – no. (%)	
Hydroxyurea	45 (88.2)
Alone	5
Interferon alpha	45 (88.2)
Cytarabine	8 (15.7)
Anagrelide	5 (9.8)
Stem cell transplant	
Autologous	2 (3.9)
Matched unrelated donor	2 (3.9)
Other*	5 (9.8)

* Other treatments were busulphan (n = 3), etoposide (n = 1), interleukin-2 (n = 1) and topotecan (n = 1).

8.4.2 Samples and CD34+ cell isolation

Mononuclear cells were purified from BM by density gradient centrifugation and cryopreserved in liquid nitrogen. CD34+ cells were isolated according to methods described in Chapter 7. After sorting, CD34+ cells were placed in PicoPure™ extraction buffer, according to the manufacturer's instructions (Arcturus, Mountain View, CA) and stored at -80°C until time for completion of RNA extraction. Small samples of CD34+ cells were also stored for fluorescence in-situ hybridization (FISH) to assess the presence of BCR-ABL.

8.4.3 RNA extraction and gene expression profiling

RNA extraction, including a step for DNase treatment to reduce DNA interference, was completed once all cell sorting had been finished; we used the PicoPure™ RNA Isolation Kit (Arcturus) and the QIAGEN® RNase-Free DNase Set (QIAGEN, Valencia, CA) according to the manufacturers' instructions. The samples of RNA were then sent to the Affymetrix Microarray Core at Oregon Health and Science University. Here, Dr. Christina Harrington and Mrs. Kristina Vartanian quantified the RNA using the NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies,Wilmington, DE), and assessed its quality using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples with electropherograms showing a size distribution pattern predictive of acceptable microarray assay performance[142] were considered to be of good quality. Samples containing 5 to 10ng (median 9ng) of good quality total RNA underwent amplification and labelling using the two-cycle cDNA synthesis and IVT labelling protocol (Affymetrix, Santa Clara, CA) (www.affymetrix.com/support/technical/manual/expression_manual.affx).

Following successful amplification, 5μg of labelled target cDNA was hybridized to HG-U133 Plus 2.0 GeneChip arrays (Affymetrix). Arrays were scanned using a laser confocal scanner (Agilent) and the image processing and expression analysis were performed using Affymetrix GCOS v1.2 software. For each analysis, scaled or unscaled, the parameters α_1 and α_2 were set to 0.05 and 0.065 (Affymetrix defaults) respectively. These parameters, or p-value cut-offs, set the point at which a probe set was called present (P) ($p < 0.05$), absent (A) ($p > 0.065$) or marginal (M) (p value between 0.05 and 0.065). Minimal quality control parameters for inclusion in the study included P>30%, average signal in keeping with the average signal of other samples within that hybridization group, and an actin 3'/5' ratio of <17. The 36 microarrays included in the study had an average present call of 41.5% (range, 38.8% to 47.1%).

8.4.4 Statistical analysis

Robust Multichip Analysis (RMA)[179] implemented in Bioconductor (http://www.bioconductor.org) was used for signal quantification, pre-processing, and intra- and inter-chip normalisation. Levene's test was used to test all genes for equal variance in gene expression across R and NR. Analysis of variance, using a pooled-variance formula, was used to identify genes that were differentially expressed between R and NR, except for those genes identified as having unequal variance across R and NR where a separate-variance formula was used. Adjustment for multiple comparisons was made using the False Discovery Rate (FDR) of Benjamini and Hochberg[181]. Unsupervised hierarchical clustering of all probe sets, and probes sets with an adjusted p value of <0.51 (n = 1019), was executed using Cluster 3.0

software[185], and results viewed using Java TreeView 1.0.7 software[186]. Sample size and power calculations were performed in Microsoft Excel, and significance analysis of microarrays (SAM)[180]. Gene annotation was obtained through Affymetrix NetAffx site (http://www.affymetrix.com/analysis/index.affx). (Please note that the statistical analysis, apart from the unsupervised hierarchical clustering, was kindly done by Miss Solange Mongoue-Tchokote.)

8.5 Results

8.5.1 CD34+ cell isolation

The CD34+ cell isolation procedure was performed on cryopreserved bone marrow mononuclear cells from 51 patients with CML. The procedure was regarded as a success if greater than 1,000 CD34+ cells were isolated, with a purity of greater than 80% CD34+ by flow cytometry. The procedure was repeated in 17 patients (twice in 2 patients) in an attempt to obtain even higher numbers of cells for analysis. Thus, 64 out of 70 procedures were deemed successful (91.4%), with 5 procedures being inadequate due to a resulting CD34+ purity of less than 80% and only 1 procedure failing due to a low resulting number of CD34+ cells. Overall we isolated a median of 1.0×10^4 CD34+ cells, with 95.9% median purity, from a median of 1.4×10^7 BM mononuclear cells (Table 8.2). Although the viability of the mononuclear cells was generally low, the viability of the resulting CD34+ cells was good, since only PI negative CD34+ cells were selected. Dr. Yassmine Akkari and Dr. Susan Olson are currently doing the FISH analysis of the isolated CD34+ cells.

Table 8.2 Results from all 73 CD34+ cell isolation procedures.

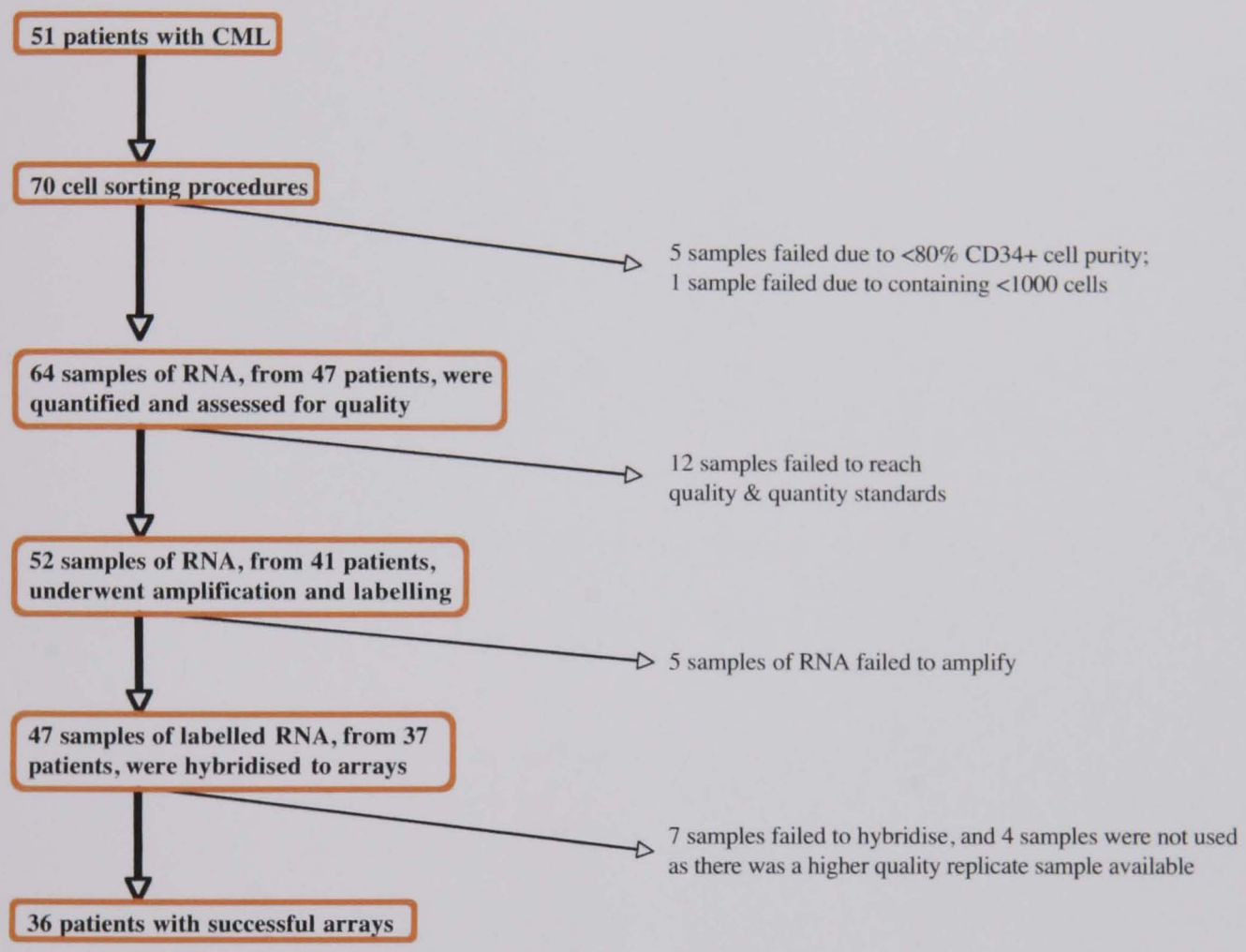
Parameter	Value
Total number of BM mononuclear cells immediately post thaw	
Median	1.4×10^{7}
Range	$1.5 \times 10^{5} - 4.2 \times 10^{7}$
Viability of BM mononuclear cells immediately post thaw - %	
Median	21.7
Range	1.4 – 86.2
Number of viable BM mononuclear cells immediately prior to sorting	
Median	1.9×10^{6}
Range	$7.2 \times 10^{4} - 1.1 \times 10^{7}$
Viability of BM mononuclear cells immediately prior to sorting- %	
Median	42.5
Range	6.2 – 91.7
Percentage of BM CD34+ cells immediately prior to sorting - %	
Median	10.9
Range	1.7 – 68.6
Number of CD34+ cells isolated and placed into RNA lysis buffer	
Median	1.0×10^{4}
Range	$8.1 \times 10^{1} - 9.1 \times 10^{4}$
Purity of CD34+ cells isolated and placed into RNA lysis buffer - %	
Median	95.9
Range	17.5 – 100

8.5.2 RNA Extraction and Gene Expression Profiling

Sixty-four samples of RNA were assessed for quantity and quality at the Affymetrix Microarray Core – a flow chart of the scheme for RNA processing can be seen in Figure 8.1. Unfortunately, 12 samples failed to meet the required quality and quantity of RNA. As one would expect from using samples of less than 9.1×10^{4} cells, we were only achieving low concentrations of RNA, with a median concentration of 5.5ng/μl (range, 1.04ng/μl to 33.6ng/μl). Fifty-two samples of RNA underwent 2 rounds of amplification, of which only 5 failed to amplify. Thus, 47 samples of RNA,

from 37 patients with CML, were hybridised to the HG-U133 Plus 2.0 GeneChip arrays, resulting in good quality gene expression profiling data on 36 patients.

Figure 8.1 Flow chart of the steps from cell sorting samples from 51 patients with CML, through to successful array hybridisation in 36 patients.



8.5.3 Statistical Analysis

The signal intensity log ratios across all arrays were successfully normalised using RMA (Figure 8.2 and Figure 8.3). Levene's test revealed equal variance in gene expression across R and NR in 54544 probe sets, with only 131 probe sets having unequal variance. Unsupervised hierarchical clustering of the 36 arrays, by use of all probe sets, showed no clustering by response status (Figure 8.4). After selecting

probe sets that were differentially expressed between R and NR (n = 1019), even though the probe sets did not reach statistical significance after multiple comparison adjustment, we found that the data clustered by response status (Figure 8.5). Only 10 of the probe sets found to be differentially expressed between R and NR had an adjusted p value of <0.5, and all of these probe sets had a higher signal in R than NR (Table 8.3).

Figure 8.2 Boxplot of the signal intensity log ratios for all 36 arrays prior to normalisation.

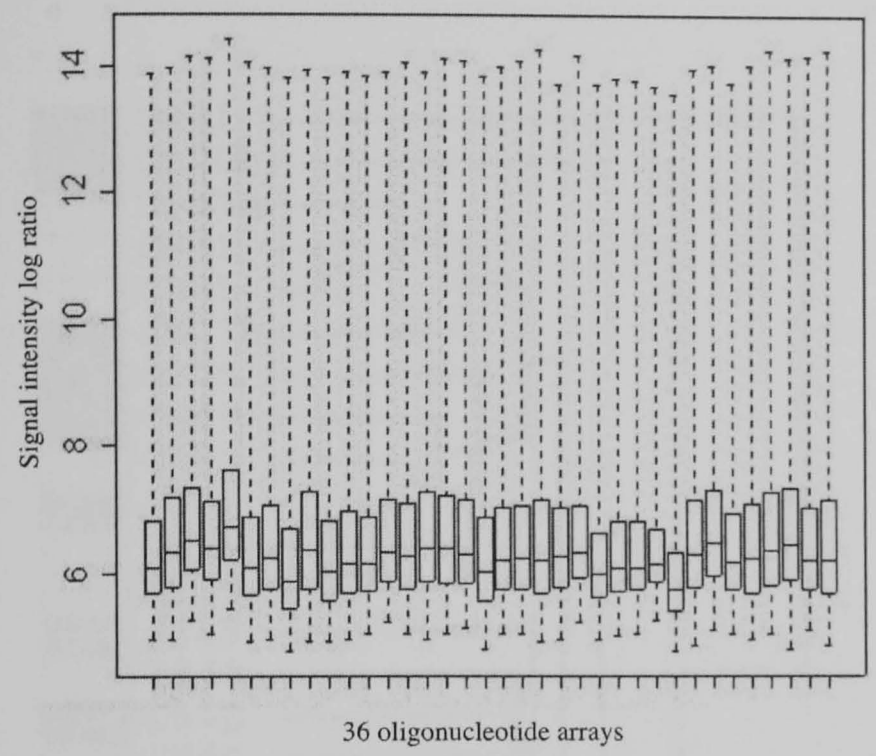


Figure 8.3 Boxplot of the signal intensity log ratios for all 36 arrays after normalisation with RMA.

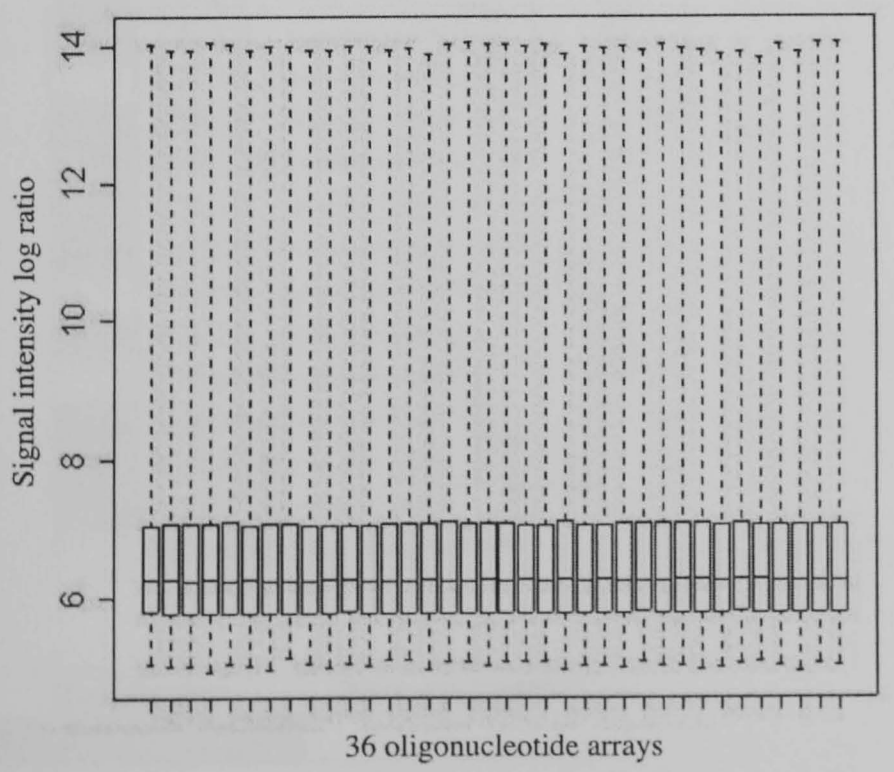
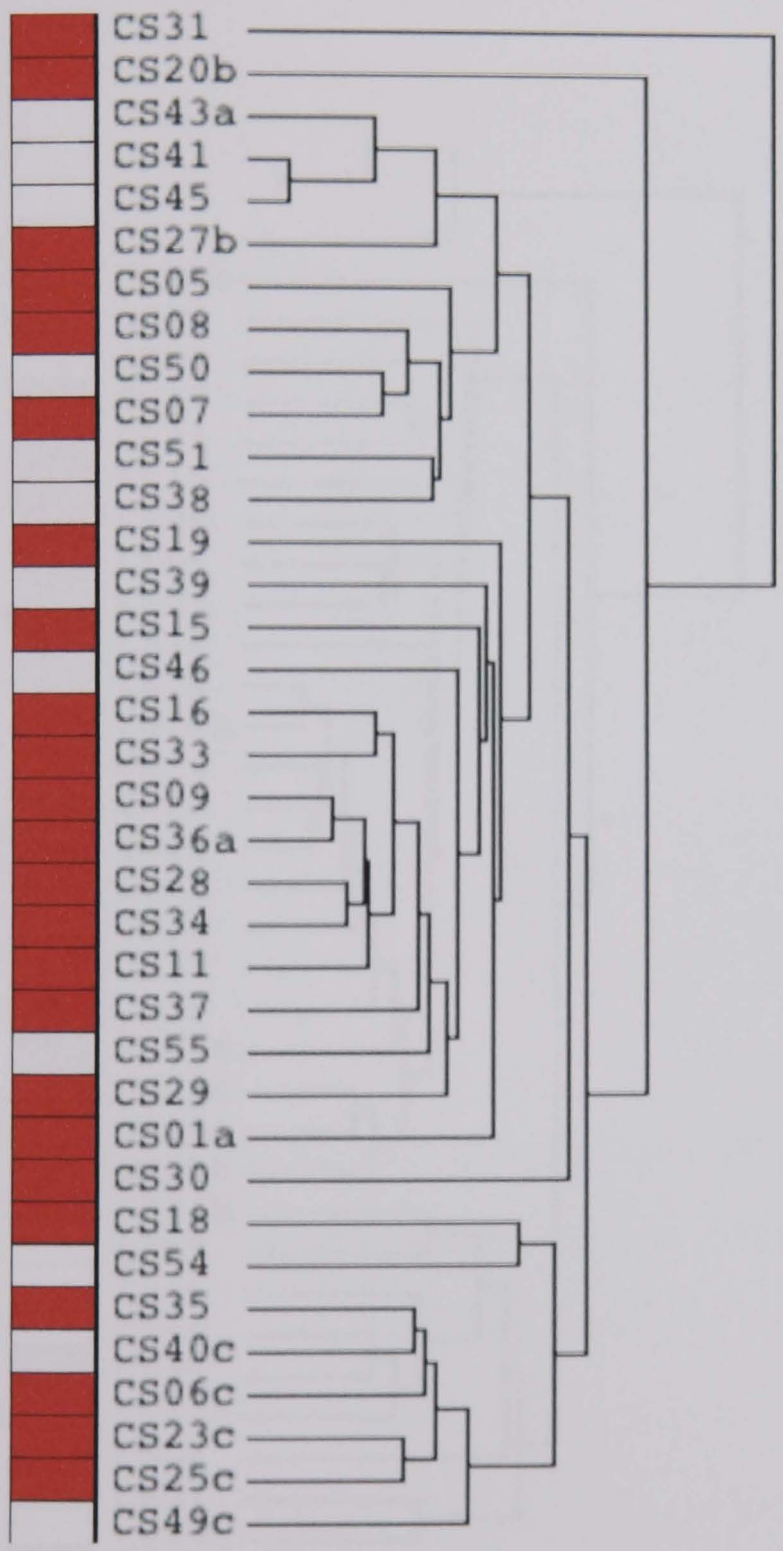


Figure 8.4 Dendrogram showing unsupervised hierarchical clustering of all 36 patient samples based on RMA signal values, showing no obvious clustering by response status.



Responder	Non-responder

Figure 8.5 Dendrogram showing unsupervised hierarchical clustering of all 36 patient samples based on RMA signal values from probe sets identified as being differentially expressed between R and NR, and having an adjusted p value of <0.51, showing clustering by response status.

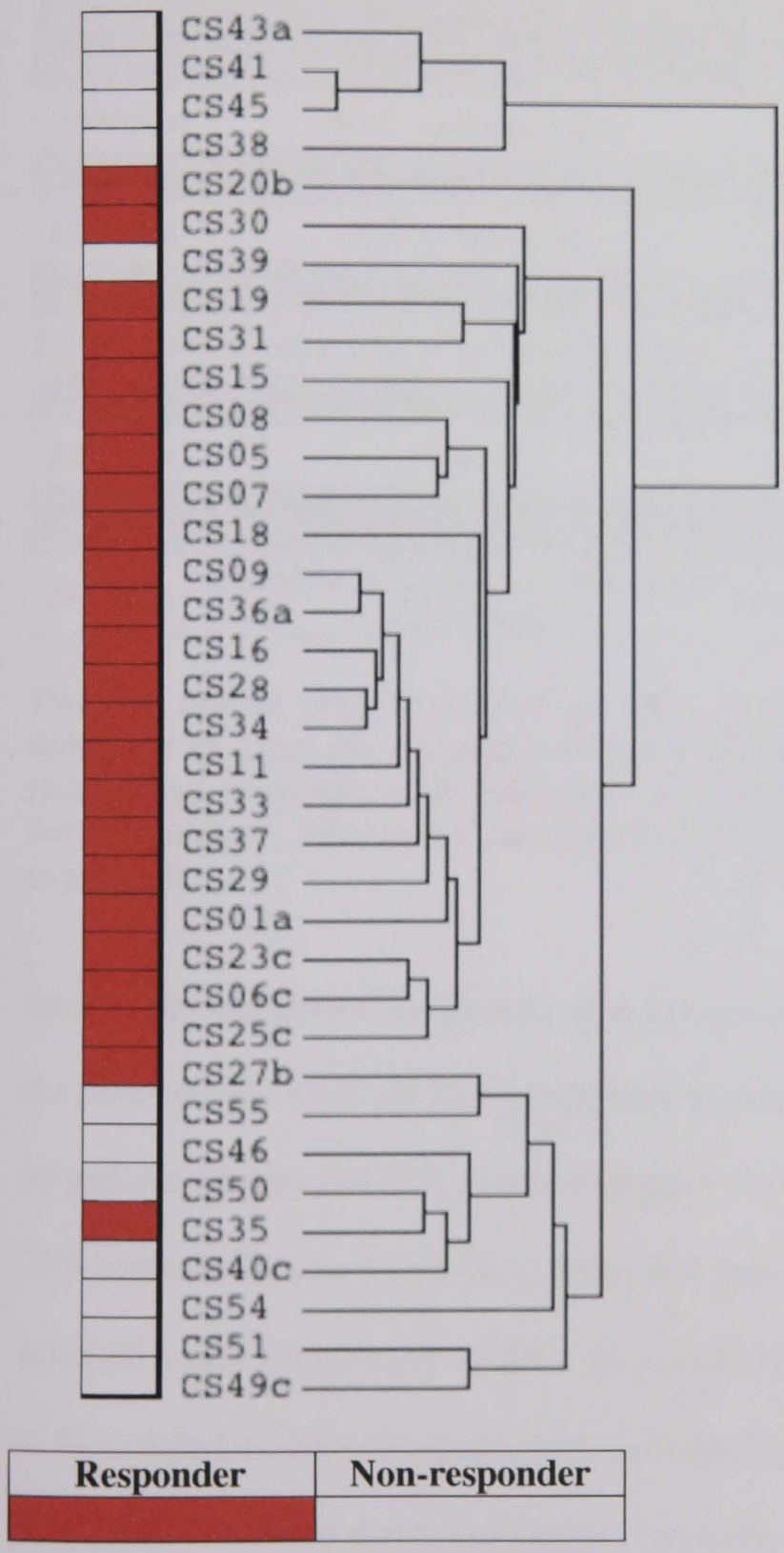


Table 8.3 Description of the probe sets identified as being differentially expressed between R and NR, and having a p value of <0.5 after multiple comparison adjustment.

Probe Set ID	Gene Title	Gene Symbol	Unadjusted p value	Adjusted p value	Fold Change (R-NR)
1554882_at	Excision repair cross-complementing rodent repair deficiency, complementation group 8	ERCC8	0.02	0.38	+1.2
1559025_at	MLL septin-like fusion	MSF	0.01	0.38	+1.18
210492_at	Microfibrillar-associated protein 3-like	MFAP3L	0.02	0.38	+1.16
219710_at	KIAA1985 protein	KIAA1985	0.02	0.38	+1.14
220230_s_at	Cytochrome b5 reductase b5R.2	CYB5R2	0.02	0.38	+1.11
224045_x_at	Chromosome 18 open reading frame 2	C18orf2	0.02	0.38	+1.1
231137_at	Unknown	---	0.02	0.38	+1.03
235188_at	Unknown	---	0.03	0.42	+1.25
235449_at	Leucine rich repeat and sterile alpha motif containing 1	LRSAM1	0.03	0.42	+1.2
243105_at	Suppressor of variegation 4-20 homolog 1 (Drosophila)	SUV420H1	0.03	0.43	+1.18

The fold change (FC) is defined as: $\pm 2^{|\Delta|}$, where $|\Delta|$ is the absolute value of the difference in mean log (signal) between R and NR groups. The positive sign (+) indicates up-regulation of R compared to NR, while the negative sign (-) indicates down-regulation. Note that a fold change of 1.0 is equivalent to no difference in gene expression.

Sample size and power calculations were performed using an alpha of 0.05, and used the gene-specific mean squared errors from the ANOVA model. We found that with 36 patients we only had 51% power to detect a 1.5 fold change in gene expression, 75% power to detect a 2 fold change and 98% power to detect a 3 fold change. If we accepted a false positive rate of 20%, we would need to increase our patient number to 41 to detect a 2 fold change in gene expression, or to 73 to detect a 1.5 fold change. Thus, if we wanted to divide our patients into a training set and a test set we would ideally want at least 73 patients in each group.

8.6 Discussion

We set out to test whether gene expression profiling of CD34+ cells, from patients with CML, would enable us to predict patients' responses to imatinib. Following on from the work we did on the gene expression profiling of unselected CML white cells, we deliberately tried to minimise sources of technical variation within our experiment. Not only did we select cells of a distinct immunophenotype, and with a high threshold of purity, we also only allowed one individual to execute each stage of the experiment and we used the same kits and reagents throughout. We chose patients with the same criteria as for the unselected white cell experiment, in an attempt to polarise our population to the extremes of good and poor responses to imatinib, and we deliberately chose to use more patients than the previous experiment, to try and maximise the power of this study.

We found that the CD34+ cell isolation procedure was highly successful in achieving a highly pure sample of CD34+ cells from cryopreserved BM mononuclear cells, increasing the median percentage of BM CD34+ cells from 10.9% prior to sorting, to 95.9% after sorting. However, this was at the expense of cell yield, partly because of the poor viability of the cells after thawing, and partly because of the need to use the "single cell" sort setting on the BD FACSAria™ (BD Biosciences). The median number of CD34+ cells that we were able to isolate from patients' cryopreserved BM mononuclear cells was only 1×10^4, resulting in low yields of RNA.

Twelve out of 64 RNA samples failed to reach our thresholds for quality and quantity. Even though we had found that the NanoDrop® could accurately measure control mouse brain RNA down to a concentration of 1ng/μl, we found that in this study

RNA concentrations <3ng/μl led to difficulties with accurate measurement of RNA quantity, and hence a tendency to overestimate the true quantity of RNA. This may have led to putting forward too little RNA for quality assessment for some of the samples that failed, and so being given a falsely low measurement of RNA quality (personal communication from Dr. Christina Harrington). However, in general the quality of the RNA was found to be good, and those samples of RNA derived from >2.0 x 10^4 CD34+ cells were consistently found to pass our quality and quantity thresholds.

We needed to perform 2 rounds of RNA amplification in order to achieve the required 5μg of RNA to hybridise to the arrays. We used the two-cycle cDNA synthesis and IVT labelling protocol that was released by Affymetrix in 2003, as we had shown that mouse brain total RNA inputs as low as 1.5ng produced sufficient cRNA for array hybridisation, with relatively good array performance[187]. For this study, we found that we could amplify samples of control human brain RNA, ranging in size from 5ng to 10ng, to between 58.2μg and 125.25μg, and that CD34+ cell RNA inputs of less than 5ng gave us informative, but less sensitive, array data[187]. For samples of RNA derived from CML CD34+ cells, we achieved successful amplification of RNA in 47 out of 52 samples. The failure of 5 samples to amplify may be due in part to insufficient RNA input, due to inaccurate measurement of initial RNA quantity, and also that inexplicably one round of labelling and amplification appeared to under perform.

Previous work on rat brain RNA had shown that the 2 rounds of amplification reduced the sensitivity of the array by 5 to 10%, as measured by the percentage of

present calls, and that 3'/5' actin ratios increased with RNA inputs of <20ng, but that other performance metrics (%P, average signal, background, noise) were good[187]. We therefore accepted a relatively high 3'/5' actin ratio in this study. Hybridisation of RNA to the arrays was successful in 40 samples. Seven samples failed quality assessment of the array, due to a higher than acceptable actin 3'/5' ratio and / or an average signal out of keeping with the other samples within that hybridisation group.

We successfully normalised the 36 arrays, and then examined unsupervised hierarchical clustering of all the data. This revealed no clustering of the arrays by response status, sex, purity of the CD34+ cells (≥95% pure versus <95% pure), or by disease phase at diagnosis (not shown). Although there was not any clustering of the arrays by response to imatinib, we were reassured that there was not any unexpected clustering of the arrays as we had seen in the unselected CML white cell study. We next examined the data for probe sets that were differentially expressed between R and NR. After multiple comparison adjustment, none of the probe sets met an adjusted p value of <0.05. However, by including only those probe sets with an adjusted p value of <0.51 in the hierarchical clustering, we were able to see clustering of the arrays by response status. Thus, although the probe sets may not reach statistical significance, as a group they were able to divide the patients into R and NR. The top 10 differentially expressed probe sets do not identify any genes that are currently known to be involved in CML biology, but the MSF gene has been reported as being the fusion partner of MLL in a single case of therapy-related acute myeloid leukaemia[188]. However, no conclusions should be made at present about the usefulness of these probe sets in distinguishing R and NR, since these probe sets did

not meet statistical significance after multiple comparison adjustment and further analysis of the data are required.

There are a number of reasons why we may not have seen significant differences in the gene expression profiles of CD34+ cells derived from R and NR. Firstly, the majority of patients had received treatment for their CML prior to receiving imatinib, and this may well have altered the genes that their stem cells expressed. Since most patients had treatment with interferon and hydroxyurea, and few had other treatments, it would be hard to see this influence on the unsupervised hierarchical clustering dendrogram, and might explain the lack of separation of R and NR. Secondly, all the CD34+ cells were derived from cryopreserved mononuclear cells and had undergone a quite intensive series of events leading to their isolation. It would be surprising if the freezing and isolation had not influenced the gene expression of the CD34+ cells in some way, and this may explain why the gene expression of R and NR are so similar. Thirdly, our patients all had high levels of Philadelphia chromosome positive metaphases in their bone marrow prior to starting on imatinib, and it may be that we are simply seeing the influence of overwhelming expression of BCR-ABL from the CD34+ cells, and that the changes that differentiate R from NR are so subtle that we cannot pick them up with this technology. Fourthly, we were using small amounts of input RNA and relying on 2 rounds of RNA amplification, both of which may have reduced the sensitivity of the arrays to pick up differences in gene expression. In addition, our power calculations reveal that we were underpowered and we would have needed 73 patients to reliably detect genes with a 1.5 fold difference in gene expression between R and NR, though this is a much smaller number of patients than we would have required when using the data from the gene expression profiling of

CML white cells. Lastly, we may be searching for a difference in gene expression profile that simply does not exist.

We shall continue our analysis of the data and see if we are able to use different statistical methods, and pathway analysis, to develop a robust algorithm predictive of response to imatinib. We had originally planned to randomly divide our patients into a training and test set, as in the unselected cell experiment, however, due to loss of patients along the way if we were to continue along this route we would most likely have to use a separate cohort of patients to test our results, potentially introducing more unwanted variation / noise into our analysis. Although it would appear that we have reduced the noise within this experiment (as compared to our CML white cell microarray study) by virtue of requiring less patients to detect a 1.5 fold change in differential gene expression between R and NR, we still have a long way to go before we can conclude whether these data are going to be more useful in developing a tool predictive of response to imatinib.

8.7 Future work

We originally set out to explore the question of whether gene expression profiling could be a tool for predicting patients' responses to imatinib, by designing an experiment using RNA from fresh CD34+ cells, taken from newly diagnosed and completely untreated patients with CML. We had planned to recruit patients from the STI571 Prospective International Randomised Trial (SPIRIT) (www.spirit-cml.org), which had been anticipated to start in early 2003, however, due to problems with obtaining funding, SPIRIT only started to recruit patients in May 2005. Thus there

has been a significant delay in patient recruitment, despite our best efforts to recruit patients in the meantime.

We have isolated CD34+ cells from patients' peripheral blood, by immunomagnetic separation (Chapter 7), and placed them into RNA lysis buffer. A sample of each patient's CD34+ cells was also stored for FISH for BCR-ABL. We anticipated performing gene expression profiling, using 90 HG-U133 Plus 2.0 GeneChip arrays (Affymetrix) (kindly donated to us by Novartis, Basel, Switzerland), on the patients who went on to achieve a CCR at 1 year from starting imatinib, and compare this to the patients who remained at least 65% Philadelphia chromosome positive at 1 year.

At present, Miss Jean Norden has collected CD34+ cell samples from 29 CML patients and the median number of CD34+ cells collected is 4×10^5 (range, 2×10^5 to 3×10^6). From the 17 samples with flow cytometry results of CD34+ cell purity, the median purity is 75% (range, 15% to 96%). These 29 samples were sent to Oregon Health and Science University, in anticipation of undergoing RNA amplification and hybridisation to arrays, but unfortunately 18 samples thawed in transit. Since one trial shipping, containing control samples, had allowed the samples to thaw en route, and the quality assessment of these samples was consistent with degraded RNA, we do not anticipate being able to use these 18 samples for our work. Thus, we are left with only 11 potential samples, and are currently debating the practicality of continuing this project.

8.7.1 Potential strengths

We believe that by using RNA extracted from isolated CD34+ cells, taken from fresh patient material before the patient receives any drug treatment, we shall gain our best chance of obtaining gene expression levels that are due to disease, rather than confounding factors. By using immunomagnetic columns to obtain the CD34+ cells, we are minimising the handling of the cells, compared to cell sorting, and also using a simple technique that could potentially be translated to the clinical laboratory should we find that gene expression in CD34+ is able to predict patients' responses to imatinib. If we can recruit patients as part of a clinical trial, then we shall have access to the good quality follow up data that is essential for this study. We have the advantage of having worked closely with the Affymetrix Microarray Core at Oregon Health and Science University on our two other CML microarray projects, and would have the benefit of their experience in using small amounts of RNA for amplification, labelling and hybridisation.

8.7.2 Disadvantages

The main problem with this approach is obtaining high numbers of consistently pure CD34+ cells. From 20ml peripheral blood we have been obtaining a median of 4 x 10^5 CD34+ cells, with a median purity of 75%. We know from the data we have on RNA extracted from cell sorted CD34+ cells, that 4 x 10^5 CD34+ cells is going to give us approximately 500ng to 1μg of RNA; we shall therefore still have to perform 2 rounds of RNA amplification in order to hybridise the RNA to arrays, and have the associated 3' bias in the amplification, 5% to 10% drop in sensitivity of the array and cost. The purity of the cells, after using one immunomagnetic column, is highly

variable and would result in approximately half of the samples being rejected because of low CD34+ cell purity. Another option to consider would be using more than 2 $x10^8$ input cells and run these through 2 larger columns, however, the increased purity tends to be at the cost of cell yield, and it is unlikely that we are going to have access to greater than 2 x 10^8 cells in the majority of patients.

The SPIRIT trial has 3 arms and only a third of patients will receive the standard dose of imatinib (400mg once daily); this would slow down our patient recruitment since we could only compare patients receiving identical treatment. We were planning on using the Affymetrix Microarray Core to perform RNA amplification and array hybridisation; however, we will have to run the risk of shipping precious samples that might thaw en route. Finally, based on our experiences with the 2 other microarray projects, we may simply be chasing differences that are not there, or cannot be detected by microarray technology.

9 Discussion

The treatment of patients with CML has been revolutionised by the advent of imatinib. Prior to imatinib, an average of 13% of CML patients in chronic phase (CP) had a complete cytogenetic response (CCR) on treatment with interferon alpha (IFN)[189]. Results of the IRIS trial[45] have shown that 84% of patients on imatinib achieve a CCR, that this usually occurs within the first year of treatment, and it is associated with a reduced rate of disease progression. In addition, with the advent of AMN107 and BMS-354825 there is hope of patients achieving a CCR even if they fail to respond, or lose their response to imatinib. However, the potentially toxic treatment of allogeneic haematopoietic stem cell transplantation remains the only known cure for patients with CML[49,50]. At diagnosis it would be very useful to be able to predict which patients will, or will not, have a cytogenetic response to imatinib, so that in those patients with a suitable donor, one could give patients accurate and individual advice about the likely success of their treatment options. We therefore set out to test candidate markers that associated with CML patients' responses to imatinib, with the hope of using these to predict an individual patient's response to treatment. We approached this in two ways: firstly by examining individual candidate genes and polymorphisms, and secondly by examining overall gene expression patterns.

9.1 Is the possession of a particular genotype associated with response to imatinib?

Dressman *et al.*[114] showed an association between the "CC" genotype of the rs2290573 polymorphism and reduced rate of MCR, while Bergamaschi *et al.*[124] found an association between the possession of the A1 allele of the TP53 gene polymorphism, and CML patients remaining at least 65% Ph positive on imatinib. However, we genotyped our Newcastle population of CML patients for the rs2290573 polymorphism and could find no correlation with rate of major cytogenetic response (MCR), or rate of CCR, on imatinib. Dressman *et al.*[114] had examined their patients for an association between rate of MCR and genotype of the TP53 gene polymorphism, and also could find no association. Although these single associations may be true for one population, it would appear that these findings are hard to validate in an independent population of patients with CML.

There are a number of reasons why these findings could be hard to validate, including that there is variation of the underlying association between genotype and response between populations, the populations consists of a mix of subpopulations with different allele frequencies and disease risks, inadequate power leading to false negative results, and false positive results leading to failure to attribute the results to chance[145]. However, if genotypes shown to be associated with response to treatment can be validated in a large and independent population of patients, then genotyping polymorphisms would be an attractive method for identifying predictive markers, since it has the advantage of being a simple technique that could easily be translated to the clinical laboratory.

At the present time, K247R is the only known polymorphism within the coding region of ABL. We investigated the effects on drug sensitivity of translocation of the arginine allele, of the K247R polymorphism, into BCR-ABL. Although K247R is located within the P-loop domain of the BCR-ABL kinase, we did not find any *in vitro* evidence for reduced sensitivity of this variant to imatinib, AMN107 or BMS-354825. However, since point mutations in the kinase domain can so easily alter imatinib sensitivity, one could readily envisage that a single nucleotide polymorphism could do the same. As more patients with CML are screened for kinase domain mutations, we may well pick up more rare polymorphisms situated within ABL and it would be interesting to see if these could influence patients' responses to treatment.

Imatinib has been shown to be a substrate for a number of genes associated with drug transport, including the human organic cation transporter 1 (hOCT1)[76-83,85,188]. Our results from a small study of patients with primary cytogenetic resistance to imatinib, suggest that expression of drug transport genes, in particular the hOCT1 gene, may be associated with lack of response to imatinib. The hOCT1 gene contains several polymorphisms, where the possession of a particular allele has been associated with alteration of its functional activity. Our RQ-PCR assay of hOCT1 covered a region containing the P283L and R287G polymorphisms. The leucine and glycine alleles are associated with diminished hOCT1 transport activity[170], and although our assay was designed such that polymorphisms should not affect its performance, we potentially may not have been as efficient at detecting hOCT1 mRNA transcribed from these alleles, as from the proline and arginine alleles. Thus, we may have been indirectly detecting the alleles associated with reduced hOCT1 activity. It would therefore be

very interesting to explore the hypothesis that possession of particular alleles of the hOCT1 gene polymorphisms are associated with a patient's response to imatinib.

9.2 Does genotyping allow the prediction of imatinib response?

Currently we are a long way from determining whether genotyping will allow us to predict a patient's response to imatinib. In the future, if one could identify robust associations between the possession of particular genotypes and patients' responses to imatinib, then one could envisage identifying a pattern of genotypes that might be predictive of an individual patient's response to imatinib.

9.3 Do gene expression profiles prior to imatinib associate with future cytogenetic response?

In order to achieve a visual overview of our microarray data, we used the conventional approach of unsupervised hierarchical clustering to group together patients based on the similarity of their gene expression profiles. When considering all the probes on the arrays, we did not see any clustering of patients based on their future cytogenetic responses to imatinib. However, by filtering the CD34+ cell data for the top most differentially expressed probes between cytogenetic responders (R) and non-responders (NR), we then saw division of our patients by response. Thus, there would appear to be differences in the pattern of gene expression between R and NR when considering a subset of probes, but these differences are subtle and can easily be overwhelmed by the influences of sample source and handling. Indeed, our

top most differentially expressed genes from either the white cell data, or CD34+ cell data, were not significantly differentially expressed after adjustment for the multiple comparisons were made; this may in part be due to both studies being underpowered to detect a 1.5 fold change in gene expression, or less, between R and NR.

9.4 Does gene expression profiling allow the prediction of imatinib response?

Kaneta *et al.*[139] and McClean *et al.*[140] have both published work that suggests that this is possible, but only one of these studies was validated in a test set of patients, albeit small. We had designed our gene expression profiling studies to incorporate both a training set and a test set of patients, so that we could validate any algorithm for prediction that we developed. With the white cell expression profiling data, we found that an algorithm that predicted response in our training set, did not predict response in our test set; explanations for this included lack of power and confounding factors, but also the serious issue of whether there truly were robust enough differences, between the gene expression profile of R and NR, to allow us to develop an algorithm to predict response.

The statistical analysis of the gene expression profiling data from CML CD34+ cells is currently ongoing, but we are concerned that though we may have been able to minimise systematic variation due to sample handling and cell phenotype, we have not been able to significantly improve on the power of our white cell gene expression profiling study. Although there appear to be differences in the transcriptional signatures of CD34+ cells from R and NR, we may be underpowered to use these

differences to develop and test an algorithm predictive of imatinib response. At the moment, it appears unlikely that with our gene expression profiling data will allow us to predict an individual patient's response to imatinib.

9.5 Is gene expression profiling going to be a useful technology for predicting CML patients' responses to imatinib?

Gene expression profiling has shown that it allows the prediction of patients' responses to drug therapy in several haematological malignancies[128-135], and one might therefore assume that CML would be no different. However, in the diseases where gene expression profiling has shown itself to be useful, the malignant cells that have been profiled tend to have a clearly identifiable histological phenotype, whereas in CML the malignant cells are of many different phenotypes, unless one simply selects one cell phenotype, E.g. CD34+ cells. In the acute leukaemias, remission is defined as a reduction in bone marrow blast cells to less than 5%, whereas in CML response is measured at the haematological, cytogenetic and molecular levels; perhaps asking gene expression profiling to identify the differences between patients who do or do not achieve a cytogenetic response is too subtle a question to be asked by this technology, unless one restricts oneself to CML stem cells. Indeed, the difference between CML and many of the non-Hodgkins lymphomas and acute leukaemias, is that in the latter most cells are at a single or similar stage of maturation (arrested at this stage by the malignant process) while within CML cells, every stage of maturation between pluripotent blasts and mature erythrocytes, neutrophils and platelets may be represented. Concentrating on the gene expression profile of

selected CML stem cells would overcome the wide diversity of expression profiles seen from cells of different phenotypes.

At the current time, our experience leads us to have some serious concerns about the future usefulness of gene expression profiling in predicting CML patients' responses to imatinib. Not only is it an expensive and complicated technology to use, it requires a multidisciplinary team of scientists, statisticians and clinicians to produce and interpret the data, and results so far in CML have not been hopeful. We still believe that our initial ideas of examining the gene expression profiles of fresh CD34+ cells, from the peripheral blood of newly diagnosed and untreated patients with CML, would be our best source of material to answer the question of whether gene expression profiling is going to be useful for predicting response to imatinib; but one would ideally want unlimited access to funds and patients, so that one could use RNA from at least 200 patients (100 R and 100 NR) to maximise the power of the study, and to have enough RNA not to need to perform 2 rounds of RNA amplification prior to array hybridisation, and so not risk reduction in sensitivity of the arrays.

9.6 Summary

From our studies it would appear that investigating the influences of target genes, in particular genes associated with drug transport, and the effect of polymorphisms on imatinib response, is more likely to yield dividends in the search for methods that might allow us to predict patients' responses to imatinib, rather than using overall gene expression patterns.

10 Glossary of terms

I have included here a glossary of the words, or phrases, that have been underlined within the body of this thesis.

.dat file

This is the image data file that is generated by the scanner – a single intensity value is generated for each probe cell on the array.

.cel file

This is the cell intensity file, which contains the cell intensities and coordinates from the primary image in the .dat file.

Pseudo-image

This is a feature-level view of the Affymetrix GeneChip and it is generated by use of the .cel file data. To examine the overall quality of the array, the pseudo-image can be checked for obvious anomalies (bright or dark patches, edge defects, debris, etc.) and be used to make sure the gridding was successful[190].

Background

Microarray Analysis Suite (MAS) 5.0 is part of Affymetrix's GeneChip Operating System (GCOS), and it manipulates the probe intensity data. It calculates the average background signal on the array by dividing the chip into 16 rectangular regions, selecting the lowest 2% of signal in those regions, and averaging these values.

Noise

Noise is a measure of the variability of the pixel values within a probe cell, averaged over all probe cells on an array. MAS 5.0 divides the chip into 16 rectangular sections, and takes the standard deviation of the lowest 2% of signal in that section. and averages these values.

Average signal

This is the average of all the signal intensities, from all of the probes on the array.

Present, marginal and absent

The detection calls of present, marginal or absent are generated by MAS5.0. They are based on a Wilcoxon signed rank test intended to test the difference in signal intensities between the perfect match probe set and its mismatch probe set. Perfect match probe sets with a p value <0.04 are called present, those with a p value between 0.04 and 0.06 are called marginal, and those with a p value >0.06 are called absent.

Percent present

Percent present is the percentage of perfect match probe sets that are detected as "present" on that array.

Normalisation

Normalisation is the attempt to compensate for systematic technical differences between chips, to see more clearly the systematic, biological differences between samples[191]. MAS 5.0 normalisation uses a linear regression model, where a baseline array is selected against which all other arrays are compared. Robust multichip analysis (RMA)[179] uses quantile normalisation that assumes that though there may be differences between arrays in the expression of individual probes, the distribution of the expression values does not vary dramatically between arrays. Signal values on each array are ranked. Signal values for each rank are averaged across all the arrays, so that after normalisation the arrays share the same set of signal values, but the probes associated with those signal values will vary from array to array.

Unsupervised hierarchical clustering

Hierarchical clustering is an agglomerative technique where arrays with similar expression profiles are joined to form groups, and these groups are further joined until a complete single hierarchical tree is formed. Unsupervised hierarchical clustering does not use any biological information about functionally related genes to "guide" the clustering algorithm[192].

Multi-dimensional scaling

Multi-dimensional scaling is a form of principal component analysis. It is a complex mathematical method of visualising data in 2 or 3 dimensions, where the distance separating data points is representative of the dissimilarity of the data points to each other.

11 Appendix 1: Newcastle University consent documents

All patients gave their consent for these studies, in according to the guidelines set out by the Declaration of Helsinki. Ethical approval was obtained from the Newcastle and North Tyneside Local Research Ethics Committee.

11.1 Patient information sheet

Research Study at the Medical School, University of Newcastle upon Tyne

Pharmacogenomic Investigations into Response and Resistance to Imatinib in Chronic Myeloid Leukaemia

You are being invited to take part in a research study for which we would need a small extra sample of blood and/or a mouthwash sample. This would be taken with the other blood samples which are taken each time you visit the clinic. If you do not wish to take part, your care will not be affected in any way. You will continue to be followed up as normal in the clinic, whether you take part or not.

Before you decide, you will need to understand why the research is being done and what it will involve. Please take your time to read the following information carefully. Please ask us if there is anything you do not understand or if you would like more information.

We are investigating whether we can predict the response that patients with Chronic Myeloid Leukaemia (CML) will have when they are given the drug imatinib (STI571, Glivec, Gleevec). We are going to try and do this by looking at patients' DNA. This area of research is called "pharmacogenomics" because we are striving to understand how genes influence the different responses people have to the same drug. Genes contain the instructions for making living organisms and are made up of DNA. Our bodies use the DNA to make messages known as RNA. Most DNA is identical among human beings, but the small variations we all have in our DNA may explain why different people have different responses to the same drug.

Recent research has suggested that understanding more about individual's DNA may allow us to predict who will and will not respond to imatinib. We would like to try and confirm these findings. We believe that this would be very helpful because although imatinib is a highly effective treatment for CML it doesn't work for everyone. If doctors could predict who would not respond then alternative treatments,

such as bone marrow transplant, could be offered earlier.

This is how you could help. We need blood and/or mouthwash samples from people with CML who are taking or are about to start taking the drug imatinib. In a few people, we may take a couple of samples over the following year. In our laboratory, we will examine the genetic material (either DNA or RNA) in your blood and/or mouthwash sample and compare this to your response to imatinib. We will see if we can predict your response to treatment. We hope to use what we learn to predict people's responses to imatinib in the future.

If a blood sample is needed, this will be taken at the same time as your routine clinic blood tests and so no extra venepuncture should be necessary. In exceptional circumstances, it may be necessary to take an extra blood test just for the research. Risks associated with taking blood may include pain, bruising or penetration of the needle through the vein leading to bleeding from the site of the needle.

Confidentiality

You will not be identified in any reports or publications resulting from this research. Results from this research will not be placed in your medical records. Information about your clinical details and the results of analysis of your samples will be held on a computer. We may share this information, with limited access, with colleagues in other research groups in the UK and abroad.

We will consider your sample as a gift to us and our research. In the future, we may think of additional research questions where it would be very valuable to go back and analyse your samples again. We would like to ask your permission to do this.

We do not intend to test any of your family members and so we do not forsee that the findings of our research will have any wider implications than the prediction of response to treatment.

If you would like more information please do not hesitate to contact:

Dr Stephen O'Brien
Department of Haematology
Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
NE1 4LP

Tel: 0191 2820568

11.2 Patient consent form

Pharmacogenomic Investigations into Response And Resistance to Imatinib in Chronic Myeloid Leukaemia

Name of Researchers: Dr Stephen O'Brien and Dr Pete Middleton

I have read the information sheet on the above study and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done.

I agree to give a sample my blood and/or mouthwash. I understand how the sample will be collected, that giving this sample for research is voluntary and I am free to withdraw my approval for collection of the sample at anytime.

I agree to the storage, on a secure computer system, of information about my clinical details and the analysis of my research samples and that this may be shared with certain research workers in the UK and abroad.

I understand that my samples will be treated as gifts from me to the research team.

I agree to the future use of my samples for research into improving the treatment of Chronic Myeloid Leukaemia.

I know how to contact the research team if needed.

Name of patient:

Signature of patient:

Name of person taking consent: **Date of consent:**

Signature of person taking consent:

12 Appendix 2: Oregon Health and Science University consent documents

All patients gave their consent for these studies, in according to the guidelines set out by the Declaration of Helsinki. Ethical approval was obtained from the Institutional Review Board.

12.1 Patient information sheet

IRB# 6384
Approved:
Revised: 4/5/2004

OREGON HEALTH & SCIENCE UNIVERSITY
GENETIC RESEARCH CONSENT AND AUTHORIZATION FORM

TITLE: Mechanism of Imatinib Mesylate Resistance

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SPONSOR: Leukemia and Lymphoma Society

PURPOSE:

You have been invited to participate in this study because you have Chronic Myelogenous Leukemia (CML), or Philadelphia chromosome positive Acute Lymphoblastic Leukemia. You may or may not have begun taking Imatinib Mesylate (Gleevec). We are collecting blood and bone marrow samples to identify any relationship between your individual genetic markers and your treatment outcome, including improvement with Imatinib treatment or failure to respond to treatment with Imatinib.

Some patients have already donated blood and bone marrow samples for a former study examining the effect of Imatinib on leukemia cells. If you have donated samples to that former study, we would like your permission to use the blood and bone marrow samples you previously donated for a new study to identify any relationship between your individual genetic markers and your treatment outcome, including improvement with Imatinib treatment or failure to respond to treatment with Imatinib.

Your personal involvement in the study will take approximately 10 additional minutes of your time during appointments when routine blood tests or bone marrow procedures are done as part of your regularly scheduled medical care. The use of individual genetic markers from your blood and bone marrow together with information about how you are doing during your treatment with Imatinib will be used for this research.

The DNA/RNA (genetic blueprints) taken from your samples of blood and bone marrow may be stored for future use to research scientific questions related to leukemia, oncology, other disease processes or other drug research and development.

SUBJECT ACCESS TO GENETIC INFORMATION:

Results of these studies are for research purposes only and since they are not expected to benefit you directly or to alter your treatment course, the results will not be made available to you, members of your family, your personal physician, or other third parties.

PROCEDURES:

As part of this study, your medical records will be reviewed for past disease history, and future information collected about your disease. In addition, your routine clinical laboratory results, research laboratory results and clinical information will be entered into a research database that is password-protected and secure.

Samples of your blood and bone marrow will be obtained for use in this study. We will obtain the samples at the same time routine blood tests or bone marrow procedures are done as part of your regularly scheduled medical care. You may refuse to donate the blood or bone marrow aspirate at any time.

For the blood samples, an additional one to two teaspoons of blood is needed. Blood is collected through a needle attached to a syringe after placing the needle through the skin into your vein. A small bandage will be applied and the procedure will then be completed.

Bone marrow is obtained by a bone marrow aspirate procedure. A local anesthetic (pain killer) will be given to numb a small area on the back of your hip. Then, a needle will be placed into the hipbone and about 1 additional teaspoon of bone marrow will be taken out for the purpose of this study. This additional sample will be collected each time you have a bone marrow aspirate procedure as part of your regularly scheduled medical care. The procedure lasts about 10 minutes.

We are also requesting permission to use your previously donated blood and bone marrow samples for use in this study in order to identify any relationship between your individual genetic markers and your Imatinib treatment outcome.

RISKS AND DISCOMFORTS:

Blood drawing will cause some pain and carries a small risk of bleeding, bruising or infection at the puncture site.

A bone marrow biopsy will cause some pain and carries a small risk of bleeding, bruising or infection at the biopsy site. An allergic reaction to the numbing medicine is rare (less than 1 in 10,000). Significant bleeding from a bone marrow biopsy is rare. Infection of the biopsy site may occur in up to 1 in 100 cases.

If the results of these studies of your genetic makeup were to be released through a breach of confidentiality, this could affect your ability to get insurance or to get or keep a job. However, because the study results will not be entered into your medical record, there should be no risk to you that your insurance or employment will be affected in any way.

There are no foreseeable risks to you for giving us permission to use your previously collected blood and bone marrow samples.

BENEFITS:

You will not benefit from participating in this study. However, by serving as a subject, you may contribute new information, which may benefit patients in the future.

ALTERNATIVES:

You may choose not to participate in this study.

CONFIDENTIALITY:

Confidentiality of information relating to your sample(s) will be protected to the extent permitted by law. To protect you against the risk of loss of confidentiality all samples as well as your medical information will be marked with a code number only

and will not identify you by name. Only the investigators named on this consent form will be authorized to link the code number to you. Research records may be reviewed and/or copied by the study sponsor (Leukemia and Lymphoma Society), the National Cancer Institute, the US Food and Drug Administration, and the OHSU Institutional Review Board.

All other parties including employers, insurance companies, personal physicians, and relatives will be refused access to the information or to the samples, unless you provide written permission, or unless we are required by law to do so.

Neither your name nor your identity will be used for publication or publicity purposes.

COSTS:

There will be no extra costs to you for the **additional** blood or bone marrow samples being taken for purposes of this study; however, you or your insurance will still bear the costs associated with your routine blood draws and bone marrow aspirate and biopsies as part of your standard and usual care for CML.

COMMERCIAL DEVELOPMENT:

Under Oregon law, the cell lines and purified DNA derived from your blood or bone marrow samples are yours to do with as you determine. By consenting to participate, you authorize the use of your samples for the research described in the PURPOSE and PROCEDURES sections of this document. In addition, you acknowledge that OHSU may make any lawful use of your samples, including, but not limited to, future research studies, destroying them, or transferring them to a public or private entity.

Samples obtained from you in this research may be used to make a discovery that could be patented or licensed to a company. There are no plans to provide financial compensation to you should this occur. However, should OHSU ever provide your samples to anyone else for research or commercial use, it will do so in such a way as to protect your privacy and confidentiality as stated in the CONFIDENTIALITY section of this document. Further, you will have no responsibility or liability for any use that may be made of your samples.

LIABILITY:

It is not the policy of the U.S. Department of Health and Human Services, or any federal agency funding the research project in which you are participating to compensate or provide medical treatment for human subjects in the event the research results in physical injury.

The Oregon Health & Science University is subject to the Oregon Tort Claims Act (ORS 30.260 through 30.300). If you suffer any injury and damage from this research

project through the fault of the University, its officers or employees, you have the right to bring legal action against the University to recover the damage done to you subject to the limitations and conditions of the Oregon Tort Claims Act. Oregon Health & Science University is also subject to the Oregon Genetic Privacy law (ORS 192.531 through ORS 192.549) and its requirements concerning confidentiality and the legal remedies provided by that law for breach of its requirements. You have not waived your legal rights by signing this form. For clarification on this subject, or if you have further questions, please call the OHSU Research Integrity Office at (503) 494-7887.

PARTICIPATION:

If in the future you decide you no longer want to participate in this research, we will destroy all your blood or bone marrow samples. However, if your genetic samples are already being used in an ongoing research project and if their withdrawal jeopardizes the success of the entire project, we may ask to continue to use them until the project is completed.

Dr. Brian Druker, (503) 494-5596 has offered to answer any other questions you may have about this study. If you have any questions regarding your rights as a research subject, you may contact the OHSU Research Integrity Office at (503) 494-7887.

You do not have to join this or any research study. If you do join, and later change your mind, you may quit at any time. If you refuse to join or withdraw early from the study, there will be no penalty or loss of any benefits to which you are otherwise entitled.

Please initial only one of the options below.

_____ I give my consent for my blood/bone marrow samples, **including my previously donated samples, to be stored and used for this study only**. I reserve the right to withdraw my samples unless the withdrawal would jeopardize this study.

_____ I give my consent for my blood/bone marrow samples to be used for this study and stored for possible future use to research scientific questions related to leukemia, oncology, other disease processes or other drug research and development, **but I wish to be contacted for permission prior to any future use.** I also reserve the right to withdraw my samples in the future.

_____ I give my consent for my blood/tissue samples to be used for this study and future studies to research scientific questions related to leukemia, oncology, other disease processes or other drug research and development. **I do not need to be contacted for permission in the future**. However, I reserve the right to withdraw my samples in the future.

12.2 Patient consent form

You will receive a copy of this consent form. Your signature below indicates that you have read the foregoing and agree to participate in this study, and agree to allow us to use your previously collected blood and bone marrow samples.

_____	_____
Signature of Subject	Date Signed
_____	_____
Signature of Investigator	Date Signed

copies to:	Subject
	Inpatient Chart
originals to:	Clinic chart/Research chart

13 References

1. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature. 1985;315:550-554.

2. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, Geurts van Kessel A, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, et al. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature. 1983;306:277-280.

3. Jaffe ES, Harris NL, Stein H, Vardiman JW eds. World Health Organization of Tumours: Pathology and Genetics of Tumours of Haemopoietic and Lymphoid Tissues. Lyon: IARC Press; 2001.

4. Vardiman JW, Pierre R, Thiele J. Chronic myeloproliferative diseases. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. World Health Organization of Tumours: Pathology and Genetics of Tumours of Haemopoietic and Lymphoid Tissues. Lyon: IARC Press; 2001:352.

5. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature. 1973;243:290-293.

6. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science. 1960;132:1497. Abstract.

7. Surralles J, Puerto S, Ramirez MJ, Creus A, Marcos R, Mullenders LH, Natarajan AT. Links between chromatin structure, DNA repair and chromosome fragility. Mutat Res. 1998;404:39-44.

8. Corso A, Lazzarino M, Morra E, Merante S, Astori C, Bernasconi P, Boni M, Bernasconi C. Chronic myelogenous leukemia and exposure to ionizing radiation--a retrospective study of 443 patients. Ann Hematol. 1995;70:79-82.

9. Tanaka K, Takechi M, Hong J, Shigeta C, Oguma N, Kamada N, Takimoto Y, Kuramoto A, Dohy H, Kyo T. 9;22 translocation and bcr rearrangements in chronic myelocytic leukemia patients among atomic bomb survivors. J Radiat Res (Tokyo). 1989;30:352-358.

10. Van Etten RA. Cycling, stressed-out and nervous: cellular functions of c-Abl. Trends Cell Biol. 1999;9:179-186.

11. Sawyers CL, McLaughlin J, Goga A, Havlik M, Witte O. The nuclear tyrosine kinase c-Abl negatively regulates cell growth. Cell. 1994;77:121-131.

12. Schwartzberg PL, Stall AM, Hardin JD, Bowdish KS, Humaran T, Boast S, Harbison ML, Robertson EJ, Goff SP. Mice homozygous for the ablm1 mutation show poor viability and depletion of selected B and T cell populations. Cell. 1991;65:1165-1175.

13. Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell. 1991;65:1153-1163.

14. Maru Y, Witte ON. The BCR gene encodes a novel serine/threonine kinase activity within a single exon. Cell. 1991;67:459-468.

15. Voncken JW, van Schaick H, Kaartinen V, Deemer K, Coates T, Landing B, Pattengale P, Dorseuil O, Bokoch GM, Groffen J, et al. Increased neutrophil respiratory burst in bcr-null mutants. Cell. 1995;80:719-728.

16. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science. 1990;247:824-830.

17. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science. 1990;247:1079-1082.

18. Druker BJ, Lydon NB. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. J Clin Invest. 2000;105:3-7.

19. Kolibaba KS, Druker BJ. Protein tyrosine kinases and cancer. Biochim Biophys Acta. 1997;1333:F217-248.

20. Zimmermann J, Buchdunger E, Mett H, Meyer T, Lydon N. Potent and selective inhibitors of the Abl kinase - phenylamino-pyrimidine (PAP) derivatives. Bioorganic and Medicinal Chemistry Letters. 1997;7:187-192.

21. Zimmermann J, Buchdunger E, Mett H, Meyer T, Lydon N, Traxler P. Phenylamino-pyrimidine (PAP) — derivatives: a new class of potent and highly selective PDGF-receptor autophosphorylation inhibitors. Bioorganic and Medicinal Chemistry Letters. 1996;6:1191-1289.

22. Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. Science. 2000;289:1938-1942.

23. Cowan-Jacob SW, Guez V, Fendrich G, Griffin JD, Fabbro D, Furet P, Liebetanz J, Mestan J, Manley PW. Imatinib (STI571) resistance in chronic myelogenous leukemia: molecular basis of the underlying mechanisms and potential strategies for treatment. Mini Rev Med Chem. 2004;4:285-299.

24. Nagar B, Bornmann WG, Pellicena P, Schindler T, Veach DR, Miller WT, Clarkson B, Kuriyan J. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). Cancer Res. 2002;62:4236-4243.

25. Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, Lydon NB. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction

mediated by c-kit and platelet-derived growth factor receptors. J Pharmacol Exp Ther. 2000;295:139-145.

26. Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, Lydon NB. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. Cancer Res. 1996;56:100-104.

27. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med. 1996;2:561-566.

28. Beran M, Cao X, Estrov Z, Jeha S, Jin G, O'Brien S, Talpaz M, Arlinghaus RB, Lydon NB, Kantarjian H. Selective inhibition of cell proliferation and BCR-ABL phosphorylation in acute lymphoblastic leukemia cells expressing Mr 190,000 BCR-ABL protein by a tyrosine kinase inhibitor (CGP-57148). Clin Cancer Res. 1998;4:1661-1672.

29. Deininger MW, Vieira S, Mendiola R, Schultheis B, Goldman JM, Melo JV. BCR-ABL tyrosine kinase activity regulates the expression of multiple genes implicated in the pathogenesis of chronic myeloid leukemia. Cancer Res. 2000;60:2049-2055.

30. Gambacorti-Passerini C, le Coutre P, Mologni L, Fanelli M, Bertazzoli C, Marchesi E, Di Nicola M, Biondi A, Corneo GM, Belotti D, Pogliani E, Lydon NB. Inhibition of the ABL kinase activity blocks the proliferation of BCR/ABL+ leukemic cells and induces apoptosis. Blood Cells Mol Dis. 1997;23:380-394.

31. Deininger MW, Goldman JM, Lydon N, Melo JV. The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL-positive cells. Blood. 1997;90:3691-3698.

32. Dan S, Naito M, Tsuruo T. Selective induction of apoptosis in Philadelphia chromosome-positive chronic myelogenous leukemia cells by an inhibitor of BCR - ABL tyrosine kinase, CGP 57148. Cell Death Differ. 1998;5:710-715.

33. Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Regenass U, Lydon NB. Selective inhibition of the platelet-derived growth factor signal transduction pathway by a protein-tyrosine kinase inhibitor of the 2-phenylaminopyrimidine class. Proc Natl Acad Sci U S A. 1995;92:2558-2562.

34. Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. Blood. 2000;96:925-932.

35. le Coutre P, Mologni L, Cleris L, Marchesi E, Buchdunger E, Giardini R, Formelli F, Gambacorti-Passerini C. In vivo eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. J Natl Cancer Inst. 1999;91:163-168.

36. Wolff NC, Ilaria RL, Jr. Establishment of a murine model for therapy-treated chronic myelogenous leukemia using the tyrosine kinase inhibitor STI571. Blood. 2001;98:2808-2816.

37. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344:1031-1037.

38. Peng B, Hayes M, Resta D, Racine-Poon A, Druker BJ, Talpaz M, Sawyers CL, Rosamilia M, Ford J, Lloyd P, Capdeville R. Pharmacokinetics and pharmacodynamics of imatinib in a phase I trial with chronic myeloid leukemia patients. J Clin Oncol. 2004;22:935-942.

39. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001;344:1038-1042.

40. Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B, Goldman J, O'Brien SG, Russell N, Fischer T, Ottmann O, Cony-Makhoul P, Facon T, Stone R, Miller C, Tallman M, Brown R, Schuster M, Loughran T, Gratwohl A, Mandelli F, Saglio G, Lazzarino M, Russo D, Baccarani M, Morra E. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med. 2002;346:645-652.

41. Talpaz M, Silver RT, Druker BJ, Goldman JM, Gambacorti-Passerini C, Guilhot F, Schiffer CA, Fischer T, Deininger MW, Lennard AL, Hochhaus A, Ottmann OG, Gratwohl A, Baccarani M, Stone R, Tura S, Mahon FX, Fernandes-Reese S, Gathmann I, Capdeville R, Kantarjian HM, Sawyers CL. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. Blood. 2002;99:1928-1937.

42. Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB, Ottmann OG, Schiffer CA, Talpaz M, Guilhot F, Deininger MW, Fischer T, O'Brien SG, Stone RM, Gambacorti-Passerini CB, Russell NH, Reiffers JJ, Shea TC, Chapuis B, Coutre S, Tura S, Morra E, Larson RA, Saven A, Peschel C, Gratwohl A, Mandelli F, Ben-Am M, Gathmann I, Capdeville R, Paquette RL, Druker BJ. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood. 2002;99:3530-3539.

43. Kantarjian H, O'Brien S, Cortes J, Giles F, Shan J, Rios MB, Faderl S, Verstovsek S, Garcia-Manero G, Wierda W, Kornblau S, Ferrajoli A, Keating M, Talpaz M. Survival advantage with imatinib mesylate therapy in chronic-phase chronic myelogenous;eukemia (CML-CP) after IFN-alpha failure and in late CML-CP, comparison with historical controls. Clin Cancer Res. 2004;10:68-75.

44. Marin D, Marktel S, Szydlo R, Klein JP, Bua M, Foot N, Olavarria E, Shepherd P, Kanfer E, Goldman JM, Apperley JF. Survival of patients with chronic-phase chronic myeloid leukaemia on imatinib after failure on interferon alfa. Lancet. 2003;362:617-619.

45. O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, Cornelissen JJ, Fischer T, Hochhaus A, Hughes T, Lechner K, Nielsen JL, Rousselot

P, Reiffers J, Saglio G, Shepherd J, Simonsson B, Gratwohl A, Goldman JM, Kantarjian H, Taylor K, Verhoef G, Bolton AE, Capdeville R, Druker BJ. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2003;348:994-1004.

46. Hahn EA, Glendenning GA, Sorensen MV, Hudgens SA, Druker BJ, Guilhot F, Larson RA, O'Brien SG, Dobrez DG, Hensley ML, Cella D. Quality of life in patients with newly diagnosed chronic phase chronic myeloid leukemia on imatinib versus interferon alfa plus low-dose cytarabine: results from the IRIS Study. J Clin Oncol. 2003;21:2138-2146.

47. Kantarjian HM, O'Brien S, Cortes J, Giles FJ, Rios MB, Shan J, Faderl S, Garcia-Manero G, Ferrajoli A, Verstovsek S, Wierda W, Keating M, Talpaz M. Imatinib mesylate therapy improves survival in patients with newly diagnosed Philadelphia chromosome-positive chronic myelogenous leukemia in the chronic phase: comparison with historic data. Cancer. 2003;98:2636-2642.

48. Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, Gathmann I, Bolton AE, van Hoomissen IC, Goldman JM, Radich JP. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med. 2003;349:1423-1432.

49. Clift RA, Storb R. Marrow transplantation for CML: the Seattle experience. Bone Marrow Transplant. 1996;17 Suppl 3:S1-3.

50. Horowitz MM, Rowlings PA, Passweg JR. Allogeneic bone marrow transplantation for CML: a report from the International Bone Marrow Transplant Registry. Bone Marrow Transplant. 1996;17 Suppl 3:S5-6.

51. Radich JP, Olavarria E, Apperley JF. Allogeneic hematopoietic stem cell transplantation for chronic myeloid leukemia. Hematol Oncol Clin North Am. 2004;18:685-702, x.

52. O'Hare T, Walters DK, Deininger MW, Druker BJ. AMN107: tightening the grip of imatinib. Cancer Cell. 2005;7:117-119.

53. Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A, Huntly B, Fabbro D, Fendrich G, Hall-Meyers E, Kung AL, Mestan J, Daley GQ, Callahan L, Catley L, Cavazza C, Azam M, Neuberg D, Wright RD, Gilliland DG, Griffin JD. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell. 2005;7:129-141.

54. Giles F, Kantarjian H, Wassmann B, Cortes J, O'Brien S, Tanaka C, Rae P, Mietlowski W, Romano A, Alland L, Dugan M, Albitar M, Ottmann O. A phase I/II study of AMN107, a novel aminopyrimidine inhibitor of Bcr-Abl, on a continuous daily dosing schedule in adult patients (pts) with imatinib-resistant advanced phase chronic myeloid leukemia (CML) or relapsed/refractory philadelphia chromosome (Ph+) acute lymphocytic leukemia (ALL). Blood. 2004;104:10a.

55. Deininger MW, Druker BJ. SRCircumventing imatinib resistance. Cancer Cell. 2004;6:108-110.

56. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science. 2004;305:399-401.

57. Nelson L. New generation leukaemia drugs are on their way. Drug Discov Today. 2004;9:732-733.

58. Sawyers CL, Shah NP, Kantarjian HM, Donato N, Nicoll JM, Bai SA, Huang F, Clark E, DeCillis AP, Talpaz M. Hematologic and cytogenetic responses in imatinib-resistant chronic phase chronic myeloid leukemia patients treated with the dual SRC/ABL kinase inhibitor BMS-354825: results from a phase I dose escalation study. Blood. 2004;104:4a.

59. Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, Herrmann R, Lynch KP, Hughes TP. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood. 2002;99:3472-3475.

60. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science. 2001;293:876-880.

61. Hochhaus A, Kreil S, Corbin AS, La Rosee P, Muller MC, Lahaye T, Hanfstein B, Schoch C, Cross NC, Berger U, Gschaidmeier H, Druker BJ, Hehlmann R. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia. 2002;16:2190-2196.

62. Roche-Lestienne C, Soenen-Cornu V, Grardel-Duflos N, Lai JL, Philippe N, Facon T, Fenaux P, Preudhomme C. Several types of mutations of the Abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. Blood. 2002;100:1014-1018.

63. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell. 2002;2:117-125.

64. von Bubnoff N, Schneller F, Peschel C, Duyster J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. Lancet. 2002;359:487-491.

65. Corbin AS, Buchdunger E, Pascal F, Druker BJ. Analysis of the structural basis of specificity of inhibition of the Abl kinase by STI571. J Biol Chem. 2002;277:32214-32219.

66. Bohmer FD, Karagyozov L, Uecker A, Serve H, Botzki A, Mahboobi S, Dove S. A single amino acid exchange inverts susceptibility of related receptor tyrosine kinases for the ATP site inhibitor STI-571. J Biol Chem. 2003;278:5148-5155.

67. Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Szer J, Taylor K, Herrmann R, Seymour JF, Arthur C, Joske D, Lynch K, Hughes T. Detection of BCR-

ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. Blood. 2003;102:276-283.

68. Soverini S, Martinelli G, Rosti G, Bassi S, Amabile M, Poerio A, Giannini B, Trabacchi E, Castagnetti F, Testoni N, Luatti S, de Vivo A, Cilloni D, Izzo B, Fava M, Abruzzese E, Alberti D, Pane F, Saglio G, Baccarani M. ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likelihood of progression to blast crisis and shorter survival: a study by the GIMEMA working party on chronic myeloid leukemia. Journal of Clinical Oncology. 2005;23:4100-4109.

69. Roche-Lestienne C, Lai JL, Darre S, Facon T, Preudhomme C. A mutation conferring resistance to imatinib at the time of diagnosis of chronic myelogenous leukemia. N Engl J Med. 2003;348:2265-2266.

70. Willis S, Lange T, Demehri S, Otto S, Crossman L, Niederwieser D, Stoffregen E, McWeeney S, Kovacs I, Park B, Druker BJ, Deininger MW. High sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. Blood. 2005.

71. La Rosee P, Corbin AS, Stoffregen EP, Deininger MW, Druker BJ. Activity of the Bcr-Abl kinase inhibitor PD180970 against clinically relevant Bcr-Abl isoforms that cause resistance to imatinib mesylate (Gleevec, STI571). Cancer Res. 2002;62:7149-7153.

72. Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. Blood. 2005;105:2640-2653.

73. Al-Ali HK, Heinrich MC, Lange T, Krahl R, Mueller M, Muller C, Niederwieser D, Druker BJ, Deininger MW. High incidence of BCR-ABL kinase domain mutations and absence of mutations of the PDGFR and KIT activation loops in CML patients with secondary resistance to imatinib. Hematol J. 2004;5:55-60.

74. Marktel S, Marin D, Foot N, Szydlo R, Bua M, Karadimitris A, De Melo VA, Kotzampaltiris P, Dazzi F, Rahemtulla A, Olavarria E, Apperley JF, Goldman JM. Chronic myeloid leukemia in chronic phase responding to imatinib: the occurrence of additional cytogenetic abnormalities predicts disease progression. Haematologica. 2003;88:260-267.

75. Schoch C, Haferlach T, Kern W, Schnittger S, Berger U, Hehlmann R, Hiddemann W, Hochhaus A. Occurrence of additional chromosome aberrations in chronic myeloid leukemia patients treated with imatinib mesylate. Leukemia. 2003;17:461-463.

76. Mahon FX, Belloc F, Lagarde V, Chollet C, Moreau-Gaudry F, Reiffers J, Goldman JM, Melo JV. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood. 2003;101:2368-2373.

77. Hegedus T, Orfi L, Seprodi A, Varadi A, Sarkadi B, Keri G. Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. Biochim Biophys Acta. 2002;1587:318-325.

78. Illmer T, Schaich M, Platzbecker U, Freiberg-Richter J, Oelschlagel U, von Bonin M, Pursche S, Bergemann T, Ehninger G, Schleyer E. P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate. Leukemia. 2004;18:401-408.

79. Widmer N, Colombo S, Buclin T, Decosterd LA. Functional consequence of MDR1 expression on imatinib intracellular concentrations. Blood. 2003;102:1142.

80. Burger H, Nooter K. Pharmacokinetic resistance to imatinib mesylate: role of the ABC drug pumps ABCG2 (BCRP) and ABCB1 (MDR1) in the oral bioavailability of imatinib. Cell Cycle. 2004;3:1502-1505.

81. Mukai M, Che XF, Furukawa T, Sumizawa T, Aoki S, Ren XQ, Haraguchi M, Sugimoto Y, Kobayashi M, Takamatsu H, Akiyama S. Reversal of the resistance to STI571 in human chronic myelogenous leukemia K562 cells. Cancer Sci. 2003;94:557-563.

82. Hamada A, Miyano H, Watanabe H, Saito H. Interaction of imatinib mesilate with human P-glycoprotein. J Pharmacol Exp Ther. 2003;307:824-828.

83. Thomas J, Wang L, Clark RE, Pirmohamed M. Active Transport of Imatinib into and Out of Cells: Implications for Drug Resistance. Blood. 2004.

84. Ozvegy-Laczka C, Hegedus T, Varady G, Ujhelly O, Schuetz JD, Varadi A, Keri G, Orfi L, Nemet K, Sarkadi B. High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter. Mol Pharmacol. 2004;65:1485-1495.

85. Burger H, van Tol H, Boersma AW, Brok M, Wiemer EA, Stoter G, Nooter K. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood. 2004;104:2940-2942.

86. Lange T, Gunther C, Kohler T, Krahl R, Musiol S, Leiblein S, Al-Ali HK, van Hoomissen I, Niederwieser D, Deininger MW. High levels of BAX, low levels of MRP-1, and high platelets are independent predictors of response to imatinib in myeloid blast crisis of CML. Blood. 2003;101:2152-2155.

87. Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. Blood. 1999;94:2056-2064.

88. Holyoake TL, Jiang X, Jorgensen HG, Graham S, Alcorn MJ, Laird C, Eaves AC, Eaves CJ. Primitive quiescent leukemic cells from patients with chronic myeloid leukemia spontaneously initiate factor-independent growth in vitro in association with up-regulation of expression of interleukin-3. Blood. 2001;97:720-728.

89. Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ, Bhatia R. Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. Blood. 2002;99:3792-3800.

90. Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, Sawyers CL, Arber DA, Slovak ML, Forman SJ. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood. 2003;101:4701-4707.

91. Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, Holyoake TL. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 2002;99:319-325.

92. Elrick LJ, Hamilton A, Deininger MW, Holyoake TL. Imatinib mesylate does not inhibit BCR-ABL kinase activity in CML stem cells in vitro. Blood. 2004;104:546a (abstract).

93. Elrick LJ, Jorgensen HG, Mountford JC, Holyoake TL. Punish the parent not the progeny. Blood. 2005;105:1862-1866.

94. Hughes T, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley M, Gathmann I, Bolton AE, van Hoomissen I, Goldman JM, Radich J. Frequency of major molecular responses to imatinib or interferon alpha plus cytarabine in newly-diagnosed chronic myeloid leukemia. N Engl J Med. 2003;349:1423-1432.

95. Sokal JE, Baccarani M, Russo D, Tura S. Staging and prognosis in chronic myelogenous leukemia. Semin Hematol. 1988;25:49-61.

96. Hasford J, Pfirrmann M, Hehlmann R, Allan NC, Baccarani M, Kluin-Nelemans JC, Alimena G, Steegmann JL, Ansari H. A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon alfa. Writing Committee for the Collaborative CML Prognostic Factors Project Group. J Natl Cancer Inst. 1998;90:850-858.

97. Roberto L, Massimo B, Ida C, Chiara S, Enrico M, Marco M, Antonio C, Rosa de C, Eleonora R, Giacomo MS, Francesca B, Antonio S, Franco M, Giuliana A, Fiammetta N. Elderly patients with Ph+ chronic myelogenous leukemia (CML): results of imatinib mesylate treatment. Leuk Res. 2005;29:287-291.

98. Grand F, Kulkarni S, Chase A, Goldman JM, Gordon M, Cross NC. Frequent deletion of hSNF5/INI1, a component of the SWI/SNF complex, in chronic myeloid leukemia. Cancer Res. 1999;59:3870-3874.

99. Sinclair PB, Nacheva EP, Leversha M, Telford N, Chang J, Reid A, Bench A, Champion K, Huntly B, Green AR. Large deletions at the t(9;22) breakpoint are common and may identify a poor-prognosis subgroup of patients with chronic myeloid leukemia. Blood. 2000;95:738-743.

100. Cohen N, Rozenfeld-Granot G, Hardan I, Brok-Simoni F, Amariglio N, Rechavi G, Trakhtenbrot L. Subgroup of patients with Philadelphia-positive chronic myelogenous leukemia characterized by a deletion of 9q proximal to ABL gene: expression profiling, resistance to interferon therapy, and poor prognosis. Cancer Genet Cytogenet. 2001;128:114-119.

101. Huntly BJ, Reid AG, Bench AJ, Campbell LJ, Telford N, Shepherd P, Szer J, Prince HM, Turner P, Grace C, Nacheva EP, Green AR. Deletions of the derivative chromosome 9 occur at the time of the Philadelphia translocation and provide a powerful and independent prognostic indicator in chronic myeloid leukemia. Blood. 2001;98:1732-1738.

102. Kolomietz E, Al-Maghrabi J, Brennan S, Karaskova J, Minkin S, Lipton J, Squire JA. Primary chromosomal rearrangements of leukemia are frequently accompanied by extensive submicroscopic deletions and may lead to altered prognosis. Blood. 2001;97:3581-3588.

103. Storlazzi CT, Specchia G, Anelli L, Albano F, Pastore D, Zagaria A, Rocchi M, Liso V. Breakpoint characterization of der(9) deletions in chronic myeloid leukemia patients. Genes Chromosomes Cancer. 2002;35:271-276.

104. Quintas-Cardama A, Kantarjian H, Talpaz M, O'Brien S, Garcia-Manero G, Verstovsek S, Rios MB, Hayes K, Glassman A, Bekele BN, Zhou X, Cortes J. Imatinib mesylate therapy may overcome the poor prognostic significance of deletions of derivative chromosome 9 in patients with chronic myelogenous leukemia. Blood. 2005;105:2281-2286.

105. Yoong Y, VanDeWalker TJ, Carlson RO, Dewald GW, Tefferi A. Clinical correlates of submicroscopic deletions involving the ABL-BCR translocation region in chronic myeloid leukemia. Eur J Haematol. 2005;74:124-127.

106. Huntly BJ, Guilhot F, Reid AG, Vassiliou G, Hennig E, Franke C, Byrne J, Brizard A, Niederwieser D, Freeman-Edward J, Cuthbert G, Bown N, Clark RE, Nacheva EP, Green AR, Deininger MW. Imatinib improves but may not fully reverse the poor prognosis of patients with CML with derivative chromosome 9 deletions. Blood. 2003;102:2205-2212.

107. Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S. Telomerase activity in normal leukocytes and in hematologic malignancies. Blood. 1995;85:2315-2320.

108. Iwama H, Ohyashiki K, Ohyashiki JH, Hayashi S, Kawakubo K, Shay JW, Toyama K. The relationship between telomere length and therapy-associated cytogenetic responses in patients with chronic myeloid leukemia. Cancer. 1997;79:1552-1560.

109. Boultwood J, Peniket A, Watkins F, Shepherd P, McGale P, Richards S, Fidler C, Littlewood TJ, Wainscoat JS. Telomere length shortening in chronic myelogenous leukemia is associated with reduced time to accelerated phase. Blood. 2000;96:358-361.

110. Brummendorf TH, Ersoz I, Hartmann U, Balabanov S, Wolke H, Paschka P, Lahaye T, Berner B, Bartolovic K, Kreil S, Berger U, Gschaidmeier H, Bokemeyer C, Hehlmann R, Dietz K, Lansdorp PM, Kanz L, Hochhaus A. Normalization of previously shortened telomere length under treatment with imatinib argues against a preexisting telomere length deficit in normal hematopoietic stem cells from patients with chronic myeloid leukemia. Ann N Y Acad Sci. 2003;996:26-38.

111. Brummendorf TH, Ersoz I, Hartmann U, Bartolovic K, Balabanov S, Wahl A, Paschka P, Kreil S, Lahaye T, Berger U, Gschaidmeier H, Bokemeyer C, Hehlmann R, Dietz K, Lansdorp PM, Kanz L, Hochhaus A. Telomere length in peripheral blood granulocytes reflects response to treatment with imatinib in patients with chronic myeloid leukemia. Blood. 2003;101:375-376.

112. Drummond M, Lennard A, Brummendorf T, Holyoake T. Telomere shortening correlates with prognostic score at diagnosis and proceeds rapidly during progression of chronic myeloid leukemia. Leuk Lymphoma. 2004;45:1775-1781.

113. Holyoake DT. Recent advances in the molecular and cellular biology of chronic myeloid leukaemia: lessons to be learned from the laboratory. Br J Haematol. 2001;113:11-23.

114. Dressman MA, Malinowski R, McLean LA, Gathmann I, Capdeville R, Hensley M, Polymeropoulos MH. Correlation of major cytogenetic response with a pharmacogenetic marker in chronic myeloid leukemia patients treated with imatinib (STI571). Clin Cancer Res. 2004;10:2265-2271.

115. Malinowski R, Dressman M, McLean LA, Gathmann I, Capdeville R, International Randomised Study of Interferon vs. Glivec (IRIS) Study Group, Polymeropoulos MH. Pharmacogenetic analysis of cytogenetic response in chronic myelogenous leukemia patients treated with imatinib (Glivec/Gleevec™, STI571). American Society of Hematology (abstract). 2002.

116. Pociot F, Molvig J, Wogensen L, Worsaae H, Nerup J. A TaqI polymorphism in the human interleukin-1 beta (IL-1 beta) gene correlates with IL-1 beta secretion in vitro. Eur J Clin Invest. 1992;22:396-402.

117. Santtila S, Savinainen K, Hurme M. Presence of the IL-1RA allele 2 (IL1RN*2) is associated with enhanced IL-1beta production in vitro. Scand J Immunol. 1998;47:195-198.

118. Gore EA, Sanders JJ, Pandey JP, Palesch Y, Galbraith GM. Interleukin-1beta+3953 allele 2: association with disease status in adult periodontitis. J Clin Periodontol. 1998;25:781-785.

119. Kornman KS, Crane A, Wang HY, di Giovine FS, Newman MG, Pirk FW, Wilson TG, Jr., Higginbottom FL, Duff GW. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol. 1997;24:72-77.

120. Heresbach D, Alizadeh M, Dabadie A, Le Berre N, Colombel JF, Yaouanq J, Bretagne JF, Semana G. Significance of interleukin-1beta and interleukin-1 receptor

antagonist genetic polymorphism in inflammatory bowel diseases. Am J Gastroenterol. 1997;92:1164-1169.

121. Nemetz A, Kope A, Molnar T, Kovacs A, Feher J, Tulassay Z, Nagy F, Garcia-Gonzalez MA, Pena AS. Significant differences in the interleukin-1beta and interleukin-1 receptor antagonist gene polymorphisms in a Hungarian population with inflammatory bowel disease. Scand J Gastroenterol. 1999;34:175-179.

122. Nemetz A, Nosti-Escanilla MP, Molnar T, Kope A, Kovacs A, Feher J, Tulassay Z, Nagy F, Garcia-Gonzalez MA, Pena AS. IL1B gene polymorphisms influence the course and severity of inflammatory bowel disease. Immunogenetics. 1999;49:527-531.

123. Cullup H, Dickinson AM, Cavet J, Jackson GH, Middleton PG. Polymorphisms of interleukin-1alpha constitute independent risk factors for chronic graft-versus-host disease after allogeneic bone marrow transplantation. Br J Haematol. 2003;122:778-787.

124. Bergamaschi G, Merante S, Orlandi E, Galli A, Bernasconi P, Cazzola M. TP53 codon 72 polymorphism in patients with chronic myeloid leukemia. Haematologica. 2004;89:868-869.

125. Amirzargar AA, Bagheri M, Ghavamzadeh A, Alimoghadam K, Khosravi F, Rezaei N, Moheydin M, Ansaripour B, Moradi B, Nikbin B. Cytokine gene polymorphism in Iranian patients with chronic myelogenous leukaemia. Int J Immunogenet. 2005;32:167-171.

126. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science. 1999;286:531-537.

127. Ebert BL, Golub TR. Genomic approaches to hematologic malignancies. Blood. 2004;104:923-932.

128. Chiaretti S, Li X, Gentleman R, Vitale A, Vignetti M, Mandelli F, Ritz J, Foa R. Gene expression profile of adult T-cell acute lymphocytic leukemia identifies distinct subsets of patients with different response to therapy and survival. Blood. 2004;103:2771-2778.

129. Hofmann WK, de Vos S, Elashoff D, Gschaidmeier H, Hoelzer D, Koeffler HP, Ottmann OG. Relation between resistance of Philadelphia-chromosome-positive acute lymphoblastic leukaemia to the tyrosine kinase inhibitor STI571 and gene-expression profiles: a gene-expression study. Lancet. 2002;359:481-486.

130. Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, Behm FG, Raimondi SC, Relling MV, Patel A, Cheng C, Campana D, Wilkins D, Zhou X, Li J, Liu H, Pui CH, Evans WE, Naeve C, Wong L, Downing JR. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell. 2002;1:133-143.

131. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, Beverloo HB, Moorhouse MJ, van der Spek PJ, Lowenberg B, Delwel R. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004;350:1617-1628.

132. Lacayo NJ, Meshinchi S, Kinnunen P, Yu R, Wang Y, Stuber CM, Douglas L, Wahab R, Becton DL, Weinstein H, Chang MN, Willman CL, Radich JP, Tibshirani R, Ravindranath Y, Sikic BI, Dahl GV. Gene expression profiles at diagnosis in de novo childhood AML patients identify FLT3 mutations with good clinical outcomes. Blood. 2004;104:2646-2654.

133. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J, Jr., Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503-511.

134. Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC, Gaasenbeek M, Angelo M, Reich M, Pinkus GS, Ray TS, Koval MA, Last KW, Norton A, Lister TA, Mesirov J, Neuberg DS, Lander ES, Aster JC, Golub TR. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med. 2002;8:68-74.

135. Lossos IS, Czerwinski DK, Alizadeh AA, Wechser MA, Tibshirani R, Botstein D, Levy R. Prediction of survival in diffuse large-B-cell lymphoma based on the expression of six genes. N Engl J Med. 2004;350:1828-1837.

136. Nowicki MO, Pawlowski P, Fischer T, Hess G, Pawlowski T, Skorski T. Chronic myelogenous leukemia molecular signature. Oncogene. 2003;22:3952-3963.

137. Ohmine K, Ota J, Ueda M, Ueno S, Yoshida K, Yamashita Y, Kirito K, Imagawa S, Nakamura Y, Saito K, Akutsu M, Mitani K, Kano Y, Komatsu N, Ozawa K, Mano H. Characterization of stage progression in chronic myeloid leukemia by DNA microarray with purified hematopoietic stem cells. Oncogene. 2001;20:8249-8257.

138. Tipping AJ, Deininger MW, Goldman JM, Melo JV. Comparative gene expression profile of chronic myeloid leukemia cells innately resistant to imatinib mesylate. Exp Hematol. 2003;31:1073-1080.

139. Kaneta Y, Kagami Y, Katagiri T, Tsunoda T, Jin-nai I, Taguchi H, Hirai H, Ohnishi K, Ueda T, Emi N, Tomida A, Tsuruo T, Nakamura Y, Ohno R. Prediction of sensitivity to STI571 among chronic myeloid leukemia patients by genome-wide cDNA microarray analysis. Jpn J Cancer Res. 2002;93:849-856.

140. McLean LA, Gathmann I, Capdeville R, Polymeropoulos MH, Dressman M. Pharmacogenomic analysis of cytogenetic response in chronic myeloid leukemia patients treated with imatinib. Clin Cancer Res. 2004;10:155-165.

141. Yong ASM, Tipping AJ, Goldman J, Melo JV. The Gene Expression Profile of CML CD34+ Cells Can Predict the Duration of Chronic Phase. American Society of Hematology (abstract). 2003.

142. Crossman LC, Mori M, Hsieh YC, Lange T, Paschka P, Harrington CA, Krohn K, Niederwieser DW, Hehlmann R, Hochhaus A, Druker BJ, Deininger MW. In chronic myeloid leukemia white cells from cytogenetic responders and non-responders to imatinib have very similar gene expression signatures. Haematologica. 2005;90:459-464.

143. McLean LA GI, Capdeville R, International Randomised Study of Interferon vs. Glivec (IRIS) Study Group, Polymeropoulos MH, Dressman M. Correlation of Cytogenetic Response with Gene Expression Profiles in Chronic Myelogenous Leukaemia Patients Treated with Imatinib (Gleevec™/Glivec). American Society of Hematology (abstract). 2002.

144. Cullup H, Dickinson AM, Jackson GH, Taylor PR, Cavet J, Middleton PG. Donor interleukin 1 receptor antagonist genotype associated with acute graft-versus-host disease in human leucocyte antigen-matched sibling allogeneic transplants. Br J Haematol. 2001;113:807-813.

145. Colhoun HM, McKeigue PM, Davey Smith G. Problems of reporting genetic associations with complex outcomes. Lancet. 2003;361:865-872.

146. Nardi V, Azam M, Daley GQ. Mechanisms and implications of imatinib resistance mutations in BCR-ABL. Curr Opin Hematol. 2004;11:35-43.

147. Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics. 1989;5:874-879.

148. Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene. 1985;33:103-119.

149. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci U S A. 1993;90:8392-8396.

150. O'Hare T, Pollock R, Stoffregen EP, Keats JA, Abdullah OM, Moseson EM, Rivera VM, Tang H, Metcalf CA, 3rd, Bohacek RS, Wang Y, Sundaramoorthi R, Shakespeare WC, Dalgarno D, Clackson T, Sawyer TK, Deininger MW, Druker BJ. Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent ATP-based oncogenic protein kinase inhibitor: implications for CML. Blood. 2004;104:2532-2539.

151. Melo JV, Yan XH, Diamond J, Goldman JM. Lack of imprinting of the ABL gene. Nat Genet. 1994;8:318-319.

152. Irving JA, O'Brien S, Lennard AL, Minto L, Lin F, Hall AG. Use of denaturing HPLC for detection of mutations in the BCR-ABL kinase domain in patients resistant to Imatinib. Clin Chem. 2004;50:1233-1237.

153. Azam M, Latek RR, Daley GQ. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. Cell. 2003;112:831-843.

154. Soverini S, Martinelli G, Amabile M, Poerio A, Bianchini M, Rosti G, Pane F, Saglio G, Baccarani M. Denaturing-HPLC-based assay for detection of ABL mutations in chronic myeloid leukemia patients resistant to Imatinib. Clin Chem. 2004;50:1205-1213.

155. Luciani MF, Denizot F, Savary S, Mattei MG, Chimini G. Cloning of two novel ABC transporters mapping on human chromosome 9. Genomics. 1994;21:150-159.

156. Vulevic B, Chen Z, Boyd JT, Davis W, Jr., Walsh ES, Belinsky MG, Tew KD. Cloning and characterization of human adenosine 5'-triphosphate-binding cassette, sub-family A, transporter 2 (ABCA2). Cancer Res. 2001;61:3339-3347.

157. Taniguchi K, Wada M, Kohno K, Nakamura T, Kawabe T, Kawakami M, Kagotani K, Okumura K, Akiyama S, Kuwano M. A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res. 1996;56:4124-4129.

158. Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res. 1999;59:2532-2535.

159. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res. 1997;57:3537-3547.

160. Minemura M, Tanimura H, Tabor E. Overexpression of multidrug resistance genes MDR1 and cMOAT in human hepatocellular carcinoma and hepatoblastoma cell lines. Int J Oncol. 1999;15:559-563.

161. Young LC, Campling BG, Cole SP, Deeley RG, Gerlach JH. Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. Clin Cancer Res. 2001;7:1798-1804.

162. Young LC, Campling BG, Voskoglou-Nomikos T, Cole SP, Deeley RG, Gerlach JH. Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. Clin Cancer Res. 1999;5:673-680.

163. Zeng H, Bain LJ, Belinsky MG, Kruh GD. Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. Cancer Res. 1999;59:5964-5967.

164. Longhurst TJ, O'Neill GM, Harvie RM, Davey RA. The anthracycline resistance-associated (ara) gene, a novel gene associated with multidrug resistance in a human leukaemia cell line. Br J Cancer. 1996;74:1331-1335.

165. O'Neill GM, Peters GB, Harvie RM, MacKenzie HB, Henness S, Davey RA. Amplification and expression of the ABC transporters ARA and MRP in a series of multidrug-resistant leukaemia cell sublines. Br J Cancer. 1998;77:2076-2080.

166. Kitazono M, Okumura H, Ikeda R, Sumizawa T, Furukawa T, Nagayama S, Seto K, Aikou T, Akiyama S. Reversal of LRP-associated drug resistance in colon carcinoma SW-620 cells. Int J Cancer. 2001;91:126-131.

167. Kitazono M, Sumizawa T, Takebayashi Y, Chen ZS, Furukawa T, Nagayama S, Tani A, Takao S, Aikou T, Akiyama S. Multidrug resistance and the lung resistance-related protein in human colon carcinoma SW-620 cells. J Natl Cancer Inst. 1999;91:1647-1653.

168. Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, van Heijningen TH, van Kalken CK, Slovak ML, de Vries EG, van der Valk P, et al. Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res. 1993;53:1475-1479.

169. Neumann F, Herold C, Hildebrandt B, Kobbe G, Aivado M, Rong A, Free M, Rossig R, Fenk R, Schneider P, Gattermann N, Royer-Pokora B, Haas R, Kronenwett R. Quantitative real-time reverse-transcription polymerase chain reaction for diagnosis of BCR-ABL positive leukemias and molecular monitoring following allogeneic stem cell transplantation. Eur J Haematol. 2003;70:1-10.

170. Takeuchi A, Motohashi H, Okuda M, Inui K. Decreased function of genetic variants, Pro283Leu and Arg287Gly, in human organic cation transporter hOCT1. Drug Metab Pharmacokinet. 2003;18:409-412.

171. Applied-Biosystems. The Design Process for a New Generation of Quantitative Gene Expression Analysis Tools: TaqMan ®Probe-Based Assays for Human, Mouse, and Rat Genes.; 2004.

172. Kerb R, Brinkmann U, Chatskaia N, Gorbunov D, Gorboulev V, Mornhinweg E, Keil A, Eichelbaum M, Koepsell H. Identification of genetic variations of the human organic cation transporter hOCT1 and their functional consequences. Pharmacogenetics. 2002;12:591-595.

173. Bullinger L, Dohner K, Bair E, Frohling S, Schlenk RF, Tibshirani R, Dohner H, Pollack JR. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. N Engl J Med. 2004;350:1605-1616.

174. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI, Gascoyne RD, Muller-Hermelink HK, Smeland EB, Giltnane JM, Hurt EM, Zhao H,

Averett L, Yang L, Wilson WH, Jaffe ES, Simon R, Klausner RD, Powell J, Duffey PL, Longo DL, Greiner TC, Weisenburger DD, Sanger WG, Dave BJ, Lynch JC, Vose J, Armitage JO, Montserrat E, Lopez-Guillermo A, Grogan TM, Miller TP, LeBlanc M, Ott G, Kvaloy S, Delabie J, Holte H, Krajci P, Stokke T, Staudt LM. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002;346:1937-1947.

175. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156-159.

176. Cross NC, Feng L, Chase A, Bungey J, Hughes TP, Goldman JM. Competitive polymerase chain reaction to estimate the number of BCR-ABL transcripts in chronic myeloid leukemia patients after bone marrow transplantation. Blood. 1993;82:1929-1936.

177. Hubbell E, Liu WM, Mei R. Robust estimators for expression analysis. Bioinformatics. 2002;18:1585-1592.

178. Liu WM, Mei R, Di X, Ryder TB, Hubbell E, Dee S, Webster TA, Harrington CA, Ho MH, Baid J, Smeekens SP. Analysis of high density expression microarrays with signed-rank call algorithms. Bioinformatics. 2002;18:1593-1599.

179. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003;4:249-264.

180. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 2001;98:5116-5121.

181. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B Methodological. 1995;57:289-300.

182. Affymetrix. An Analysis of Blood Processing Methods to Prepare Samples for GeneChip® Expression Profiling: Affymetrix Technical Note; 2003.

183. Schoch C, Kohlmann A, Schnittger S, Brors B, Dugas M, Mergenthaler S, Kern W, Hiddemann W, Eils R, Haferlach T. Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. Proc Natl Acad Sci U S A. 2002;99:10008-10013.

184. Irving JA, Lennard A, Storey N, Conn J, Dunn J, Oliver K, Proctor SJ, Dickinson AM. Analysis of CD34 populations in mobilised peripheral blood stem cell harvests and in bone marrow by fluorescent in situ hybridisation for the bcr/abl gene fusion in patients with chronic granulocytic leukaemia. Leukemia. 1999;13:944-949.

185. de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. Bioinformatics. 2004;20:1453-1454.

186. Saldanha AJ. Java Treeview--extensible visualization of microarray data. Bioinformatics. 2004;20:3246-3248.

187. Vartanian KA, Paik H, Runyan C, Crossman L, Deininger M, Harrington C. Factors influencing the optimization and standardization of the Affymetrix GeneChip expression assay small sample amplification protocol in the microarray core laboratory. Annual Meeting of the Association of Biomolecular Resource Facilities (Poster); 2005.

188. Osaka M, Rowley JD, Zeleznik-Le NJ. MSF (MLL septin-like fusion), a fusion partner gene of MLL, in a therapy-related acute myeloid leukemia with a t(11;17)(q23;q25). Proc Natl Acad Sci U S A. 1999;96:6428-6433.

189. Bonifazi F, de Vivo A, Rosti G, Guilhot F, Guilhot J, Trabacchi E, Hehlmann R, Hochhaus A, Shepherd PC, Steegmann JL, Kluin-Nelemans HC, Thaler J, Simonsson B, Louwagie A, Reiffers J, Mahon FX, Montefusco E, Alimena G, Hasford J, Richards S, Saglio G, Testoni N, Martinelli G, Tura S, Baccarani M. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. Blood. 2001;98:3074-3081.

190. Simon RM, Korn EL, McShane LM, Radmacher MD, Wright GW, Zhao Y. Design and analysis of DNA microarray investigations. New York: Springer-Verlag; 2003.

191. Reimers M. Statistical analysis of microarray data. Addict Biol. 2005;10:23-35.

192. Quackenbush J. Computational analysis of microarray data. Nat Rev Genet. 2001;2:418-427.