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Investigations of FLT3 internal tandem duplications in patients with acute myeloid leukaemia

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A thesis submitted to the University of London for the degree of Doctor of Philosophy

2006

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WC1E 6HX
Abstract

In acute myeloid leukaemia (AML), further prognostic determinants are required in addition to cytogenetics to predict patients at increased risk of relapse. Recent studies have indicated an internal tandem duplication (ITD) in the FLT3 gene may adversely affect clinical outcome. The studies reported in this thesis evaluated the impact of a FLT3/ITD on outcome in 854 patients treated in UK MRC AML trials. A FLT3/ITD was present in 27% patients and was associated with leucocytosis and a high percentage of bone marrow blast cells. With respect to clinical outcome it predicted for increased relapse risk (RR), adverse disease-free survival (DFS), event-free survival (EFS) and overall survival (OS). In multivariate analysis, presence of a mutation was the most significant prognostic factor predicting for RR and DFS. In order to evaluate whether FLT3 mutations can be used as markers of minimal residual disease, paired presentation and relapse samples were studied. Twenty four patients were wild type FLT3 at diagnosis and 4 acquired a FLT3 mutation at relapse. Of 20 patients positive at diagnosis, 5 who were all originally ITD+, had no detectable mutation at relapse. Furthermore, another patient had a completely different ITD at relapse which could not be detected in the presentation sample. These results suggest that, at least in some patients, FLT3 mutations are secondary events in leukaemogenesis, are unstable and thus should be used cautiously for the detection of minimal residual disease. Given the high frequency of activating FLT3 mutations in patients with AML, FLT3 and its downstream pathway are attractive targets for directed inhibition. Several promising tyrosine kinase inhibitors have recently been identified and in vitro data reported here suggest that FLT3 inhibitors may have a role either as monotherapy or in combination with conventional cytotoxic agents in the treatment of AML.
Dedication

I dedicate this thesis to my energetic and bright son Dimitrios, (who was born while my experiments were about to be completed) and to my newborn baby Athanasios. I look forward to seeing their thesis in the distant future.

This thesis is dedicated to my wife Effie whose patience and tolerance are much appreciated.

This thesis is dedicated to my parents and my brother who have supported me all the way since the beginning of my life.

This work is dedicated to my supervisors Dr Rosemary Gale and Professor David Linch. Without them this thesis could not have been possible.

With them, I began my training in science. They were the ones who motivated me to continue and complete it. They will always be a source of continuous inspiration for me.

Last but not least, this thesis is dedicated to all those who believe in the richness of learning.

Acknowledgments

I would like to express sincere gratitude to the Leukaemia Research Fund for supporting this work.

I would like to thank the MRC and the UCLH DNA/RNA bank and their scientific and technical staff.

I would like to thank my scientific colleagues Dr Stephen Langabeer and Anthony Alan for their support in technical issues.
Table of Contents

Title page.................................................................1
Abstract............................................................................2
Dedication.........................................................................3
Table of Contents............................................................4
Abbreviations.................................................................8
List of Figures.................................................................10
List of Tables.................................................................12

Chapter 1........................................................................13
1.1 Acute myeloid leukaemia...........................................13
1.2 Classification and diagnosis of AML.........................13
  1.2.1 WHO classification of AML.................................16
1.3 Treatment of AML.....................................................17
  1.3.1 Induction chemotherapy.................................17
  1.3.2 Post induction chemotherapy............................17
  1.3.3 Autologous transplantation.............................17
  1.3.4 Allogeneic transplantation...............................18
  1.3.5 Treatment of relapse.......................................20
  1.3.6 Future direction in the treatment of AML...........20
1.4 Haemopoietic growth factor receptor abnormalities
  in acute leukaemias (excluding FLT3)...........................21
  1.4.1 M-CSF receptor................................................22
  1.4.2 G-CSF receptor................................................23
  1.4.3 C-kit receptor...................................................24
  1.4.4 PDGFRβ..........................................................24
  1.4.5 EPO-R..............................................................25
1.5 Aims of this thesis....................................................25

Chapter 2.......................................................................26
2.1 General materials and reagents.................................26
2.2 Extraction of DNA from PB or BM............................27
2.3 Reverse transcription (RT) .........................................................................27
2.4 Polymerase chain reaction (PCR) ..............................................................27
2.5 Semi-quantitative PCR ............................................................................28
2.6 Sequencing of PCR products ....................................................................28
2.7 Cloning of PCR products ..........................................................................29

Chapter 3 ...............................................................................................................31
3.1 Introduction ...................................................................................................31
3.2 FLT3 ................................................................................................................33
3.2.1 FLT3 expression in leukaemias .................................................................34
3.3 FLT3 mutations ............................................................................................35
3.3.1 Internal tandem duplications (ITDs) .........................................................35
3.3.2 Mutations in the second tyrosine kinase domain (TKDs) ..........................38
3.3.4 Additional mutations in the second tyrosine kinase domain (TKDs) ..........39
3.3.5 Novel FLT3 mutations .................................................................................40
3.4 Materials and methods .................................................................................40
3.4.1 PCR analysis of the FLT3/ITD mutation ...................................................41
3.4.2 Sensitivity and reproducibility of semi-quantitative PCR .......................41
3.5 Endpoints and Statistical methods .............................................................41
3.6 Results .............................................................................................................43
3.6.1 Evaluation of semi-quantitative PCR analysis .........................................43
3.6.2 Analysis of FLT3 gene (exon 14 and 15) in haematologically normal individuals .........................................................................................................................44
3.6.3 Patients’ characteristics .............................................................................45
3.6.4 Overall incidence of FLT3/ITD in 854 patients and in different FAB types and cytogenetic risk groups ......................................................47
3.6.5 Clinical and biological characteristics of the FLT3/ITD+ patients ..........47
3.6.6 FLT3/ITD mutation and clinical outcome as determined in univariate analysis .................................................................................................48
3.6.6.1 Response rate ..........................................................................................48
3.6.6.2 Survival outcome ...................................................................................49
3.6.7 Multivariate analysis of outcome including FLT3/ITD as a co-variable

3.6.8 Level of mutant and number of FLT3/ITD mutations

3.6.8.1 Number of FLT3/ITD mutations in relation to demographics and clinical outcome

3.6.8.2 Multivariate analysis of outcome including presence of a FLT/ITD and number of FLT3/ITD mutations as co-variables

3.6.8.3 Relative level of mutant in relation to demographics and clinical outcome

3.7 Discussion

Chapter 4

4.1 Introduction

4.2 Use of MRD in leukaemia

4.3 Methodologies for detecting MRD

4.3.1 Conventional cytogenetics

4.3.2 Fluorescence in situ hybridisation (FISH)

4.3.3 Flow cytometry

4.3.4 Nucleic acid amplification techniques

4.4 Application of MRD in ALL

4.5 Application of MRD in myeloid malignancies

4.5.1 CML

4.5.2 AML

4.6 Materials and Methods

4.6.1 Patients

4.6.2 Detection of mutations in the FLT3 gene

4.6.2.1 PCR analysis of the FLT3/ITD mutation

4.6.2.2 PCR analysis of the FLT3/D835 mutation and restriction enzyme digestion

4.6.3 Polymorphic marker analysis in order to confirm patients’ identity in samples taken at presentation and relapse

4.6.4 Cloning and sequencing of PCR products

4.7 Results

4.7.1 Paired presentation/remission samples
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>CBF</td>
<td>Core binding factor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation antigen</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CP</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease free survival</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxy nucleotide triphosphate</td>
</tr>
<tr>
<td>DTAB</td>
<td>Dodecyl trimethyl ammonium bromide</td>
</tr>
<tr>
<td>EFS</td>
<td>Event free survival</td>
</tr>
<tr>
<td>FAB</td>
<td>French American British</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft versus leukaemia</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>ID</td>
<td>Induction death</td>
</tr>
<tr>
<td>ITD</td>
<td>Internal tandem duplication</td>
</tr>
<tr>
<td>JM</td>
<td>Juxtamembrane</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MNC</td>
<td>Mononuclear cells</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>MRD</td>
<td>Minimal residual disease</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBSC</td>
<td>Peripheral blood stem cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RAEB-t</td>
<td>Refractory anaemia with excess of blasts in transformation</td>
</tr>
<tr>
<td>RD</td>
<td>Refractory disease</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAPol</td>
<td>Ribonucleic acid polymerase</td>
</tr>
<tr>
<td>RR</td>
<td>Relapse risk</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCT</td>
<td>Stem cell transplantation</td>
</tr>
<tr>
<td>Taq</td>
<td>Taq DNA polymerase</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA buffer</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>TRM</td>
<td>Transplant related mortality</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood count</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
List of Figures

Figure 3.1 Schematic representation of the functional domains of FLT3 .................................................................34
Figure 3.2 Schematic representation of FLT3 and FLT3/ITD activating mutations .....................................................35
Figure 3.3 Schematic representation of FLT3 and FLT3/TKD activating mutations .....................................................39
Figure 3.4 Evaluation of the sensitivity of the PCR technique using different mixtures of cells containing WT FLT3 and alleles with predominately FLT3/ITD .........................................................44
Figure 3.5 Semi-quantitative PCR demonstrating good agreement between observed and expected level of mutant using different mixtures from cells carrying WT and predominately ITD alleles .................................................................44
Figure 3.6 Relapse risk in patients with and without a FLT3/ITD ...........................................................................49
Figure 3.7 Disease free survival in patients with and without a FLT3/ITD ...............................................................50
Figure 3.8 Overall survival in patients with and without a FLT3/ITD ......................................................................50
Figure 3.9 Relapse rate in patients with or without a FLT3/ITD grouped according to risk category .......................52
Figure 3.10 Semi-quantitative PCR of FLT3/ITD in 12 patients with AML ...............................................................54
Figure 3.11 OS in patients with one and two or more FLT3/ITDs ............................................................................54
Figure 3.12 Level of mutant FLT3/ITD (% of total FLT3) ......................................................................................56
Figure 3.13 OS according to the mutant level of FLT3/ITD mutation .................................................................57
Figure 4.1 Identification of a D835 mutation in a patient with AML: The presence of the mutation abolishes the recognition of the cutting site by EcoRV endonuclease (GATATC), producing 3 size products ....................................................................................73
Figure 4.2 Polymorphic marker analysis in 2 AML patients at presentation (P) and relapse (R). Both patients presented with a FLT3/ITD which was lost at relapse ..........................................................77
Figure 4.3 Diagram to show the position of the different FLT3/ITDs detected in one patient at presentation and relapse.........................78

Figure 4.4 Kaplan Meier curves demonstrating OS according to FLT3 status at presentation and relapse (WT/WT: WT FLT3 at presentation and at relapse; MUT/MUT: FLT3/ITD at presentation and at relapse.................................79

Figure 5.1 Dose response curves of FLT3/ITD and non-ITD AML samples treated with AG1295.................................................................92

Figure 5.2 Dose response curves of FLT3/ITD and non-ITD AML samples treated with AG1296.................................................................92

Figure 5.3 Cytotoxic effect of AG1295, CEP-701, PKC-412 and cytarabine (Ara-C) in blast cells from patients with and without FLT3 mutations.................................................................95

Figure 5.4 Dose response curves of cells from an AML patient with FLT3 WT treated with Cytarabine (Ara C) alone and in combination with AG1295, CEP-701 or PKC-412.................96

Figure 5.5 Dose response curves of cells from an AML patient with FLT3 WT treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412.........................97

Figure 5.6 Dose response curves of cells from an AML patient with FLT3 WT treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412.................................98

Figure 5.7 Dose response curves of cells from an AML patient with FLT3/ITD treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412.........................99

Figure 5.8 Dose response curves of cells from an AML patient with FLT3/TKD treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412.........................100

Figure 5.9 Dose response curves of cells from an AML patient with FLT3/ITD treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412.................101
List of Tables

Table 3.1 Clinical and demographic characteristics of the 854 AML patients studied .................................................................46
Table 3.2 Incidence of FLT3/ITD+ patients in different cytogenetic risk groups and subgroups ..................................................48
Table 3.3 Clinical outcome in FLT3/ITD- and FLT3/ITD+ patients ........51
Table 3.4 Multivariate analysis of CR, RR, DFS, EFS and OS considering FLT3/ITD (yes or no) as the only FLT3 variable ..............51
Table 3.5 Clinical and demographic characteristics of patients grouped according the number of FLT3/ITDs and the relative level of mutation ..............................................................................................................56
Table 3.6 Incidence and frequency in different cytogenetic subgroups in adult and paediatric AML patients with a FLT3/ITD mutation ..........60
Table 4.1 Oligonucleotide primers used in PCR analysis ..................74
Table 4.2 FLT3 status and relative percentage of mutant in peripheral blood or bone marrow from 44 AML patients at presentation and relapse ..........................76
Table 5.1 Summary of FLT3 inhibitors .............................................88
Table 5.2 Patient demographics and FLT3 status .............................91
Table 5.3 Patient demographics and FLT3 status .............................93
Chapter 1

1.1 Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is characterised by an increase in the number of myeloid cells in the bone marrow and an arrest in their maturation. The abnormal proliferation of leukaemic cells gradually replaces the normal haemopoietic tissue, resulting in bone marrow insufficiency. This can be manifested either as anaemia, leucopenia, thrombocytopenia or combined cytopenia(s).

The incidence of AML increases with age and ranges from 0.7 to 3.9 cases per 100,000 between 0 and 60 years of age, and from 6.7 to 19.2 cases per 100,000 above 60 years (Ries et al, 1999). Although the aetiology is unknown, AML is strongly linked to radiation, prior exposure to alkylating agents and epipodophyllotoxins, and exposure to chemicals such as benzene. For example, there was a 20 fold increase of AML in Japanese people who survived the atomic bomb, and a 10 fold increase has been reported in workers exposed to benzene (Preston et al, 1996; Linet & Cartwright, 1996). Patients with certain congenital disorders such as Down’s syndrome, Fanconi’s anaemia, Klinefelter’s syndrome, Turner’s syndrome and Wiskott-Aldrich syndrome are also at higher risk of developing leukaemia.

1.2 Classification and diagnosis of AML

The clinical signs and symptoms of AML are very diverse and mainly related to insufficiency of normal haemopoiesis. Patients usually present with symptoms of anaemia (fatigue, breathlessness), thrombocytopenia (bleeding and bruising) or neutropenia (infections, septicaemia). A combination of the above symptoms is not unusual, and more rarely, other signs and symptoms related to leucocytosis, coagulopathy, skin (leukaemia cutis) or organ involvement can predominate at presentation.
Laboratory findings usually reveal pancytopenia, although any combination of anaemia, thrombocytopenia, and leucopenia or leucocytosis may exist. The diagnosis relies on the morphological identification of myeloblasts in preparations of peripheral blood and bone marrow stained with Wright-Giemsa stain. These cells are usually large, have distinct nucleoli and an increased nucleus to cytoplasm ratio. The cytoplasm may contain fine azurophilic granules and a variable number of Auer rods. Since 1976 AML has been classified according to the criteria of the French-American-British (FAB) group, which is based entirely on morphological and cytochemical criteria. Morphologic criteria are based on two factors: the direction of cell line differentiation and the degree of maturation of the proliferating cells. Eight sub-classifications are recognised from M0 to M7: M0 with minimal differentiation, M1 without maturation, M2 with maturation, M3 acute promyelocytic leukaemia, M4 acute myelomonocytic leukaemia, M5 acute monocytic leukaemia, M6 erythroleukaemia and M7 acute megakaryocytic leukaemia. For many years FAB classification has facilitated the correlation of clinical and laboratory findings and responses to treatment between clinical trials.

Cytochemistry allows the demonstration of specific enzymes or other substances in individual cells and identifies cells across the myeloid, monocytic, erythroid, megakaryocytic and lymphoid lineages, complementing the Wright-Giemsa stain. Certain cytochemical reactions are essential in distinguishing AML from acute lymphoblastic leukaemia (ALL). The peroxidase, Sudan Black B and chloracetate esterase reactions reveal granulocytic differentiation in the M1, M2, M3 and M4 types of AML, whilst the non-specific esterases and the acid phosphatase and lysozyme reactions demonstrate monocytic differentiation in M4 and M5. In erythroleukaemia (M6), the Periodic Acid Schiff (PAS) reaction may be strongly positive. In megakaryoblastic leukaemia (M7), the cytochemical profile shows positive reactions with PAS, acid phosphatase and α-naphthyl acetate esterase (ANAE).
Immunophenotypic analysis by flow cytometry has become a powerful tool for accurate identification of myeloid and lymphoid lineage leukaemias. An important use of flow cytometry is in the identification of AML in minimally differentiated and megakaryoblastic leukaemias, where blasts can be small and resemble lymphoblasts. The most commonly used monoclonal antibodies in use for the differentiation of myeloid lineage leukaemias are against HLA DR, CD33 (myeloid cells), CD13, CD14, CD15 (myelomonocytic antigens), CD41, CD61 (megakaryocytic antigens), glycophorin and transferrin receptor antigens (i.e. CD71 in erythroleukaemia) and CD2, CD3, CD4, CD8, CD19, CD20 (T and B cell antigens). However, leukaemic blasts may aberrantly express some antigens of another lineage or lack expression of an expected antigen.

Cytogenetic analysis is an essential component of the diagnosis of AML, with approximately 55% to 80% of newly diagnosed patients presenting with an abnormal karyotype. Primary chromosome aberrations are frequently found as the sole karyotypic abnormality and are often specifically associated with a particular AML subtype. Secondary aberrations are thought to play an important role in the progression of the disease and lead to genomic imbalances such as gains or losses of whole chromosomes, deletions or unbalanced translocations. Secondary aberrations may already be present at diagnosis or may appear for the first time at relapse. Two major types of primary chromosome abnormalities can be identified in AML: Balanced structural abnormalities usually leading to a leukaemia-specific fusion transcript, and unbalanced aberrations leading to gain and/or loss of genetic material. The most common balanced chromosome abnormalities are t(8;21), t(15;17), inv(16)/t(16;16), and 11q23 abnormalities. The most common unbalanced abnormalities are deletion 5q, monosomy 7, deletion 7q, deletion 9q, trisomy 8, 11, 13, 21, 4.

The 4th international workshop on chromosomes in leukaemia was the first large prospective multi-centre study to establish the role of cytogenetics as an independent prognostic factor in AML (Bloomfield et al, 1984). Since then, many studies have confirmed that pre-treatment karyotype constitutes an independent prognostic determinant for attainment of complete remission (CR),
risk of relapse and survival. Three large collaborative studies have each proposed a system that assigns AML patients to one of 3 risk groups, favourable, intermediate or adverse. These 3 cytogenetic systems assign many karyotypic abnormalities to same risk group, however, there are some differences. For example, in the MRC classification, any abnormality that is not classified as favourable or adverse is classified in the intermediate risk group. In contrast, both the Southwest Oncology group/Eastern Cooperative Oncology group (SWOG/ECOG) and cancer and Leukaemia group B (CALGB) classify particular abnormalities into risk groups, and leave as “not classified” aberrations too infrequent to be analysed. Despite these differences, the inv(16)/t(16;16), t(8;21) and t(15;17) have been categorised in the favourable group by all three cytogenetic risk systems. They all also agree that prognosis of patients with inv(3) or t(3;3), -7 and a complex karyotype is poor. The definition of complex karyotype differs, though, with the MRC characterising this category as the presence of more than 4 unrelated cytogenetic abnormalities, where as the others use a cut off point of three abnormalities. In the MRC study, the outcome of patients with del(7q) that was not part of a complex karyotype and was not accompanied by -5/del(5q) or abn(3q) did not differ significantly from outcome of patients with normal karyotype, and therefore these patients are classified as having standard risk disease (Grimwade et al, 1998). Patients with a normal karyotype are the single largest cytogenetic subset of adult AML. They are classified in the intermediate prognostic category, as the outcome is inferior to those with favourable risk disease, but superior to poor risk disease.

1.2.1 WHO classification of AML

In 1999 the World Health Organisation (WHO) proposed a classification system for haemopoietic and lymphoid neoplasms (Vardiman et al, 2002). In an attempt to define biologic entities that have clinical relevance, morphologic, immunophenotypic, genetic and clinical features were incorporated in the classification of AML. Four major categories are now recognised: 1) AML with recurrent genetic abnormalities, 2) AML with multilineage dysplasia, 3) AML
and myelodysplasia (MDS) therapy related and 4) AML not otherwise
categorised.

1.3 Treatment of AML

1.3.1 Induction chemotherapy

Although, AML was a fatal disease 30 years ago, improvements have been
made since then as a consequence of a more intensive approach to treatment.
With current protocols in adult patients below the age of 60 years, 80% of
patients will enter complete remission (CR) and a substantial number will be
cured (Grimwade et al., 1998). The backbone of induction treatment relies on
the combination of cytarabine with one of the anthracyclines (i.e. daunorubicin,
idarubicin, mitoxantrone). The value of a third drug is controversial, however
well established in some studies. For example, the Australian Leukaemia study
group showed a benefit in the arm of patients treated with etoposide as a third
drug (Bishop et al., 1990). The use of high dose of cytarabine during induction
chemotherapy has failed to increase the rate of remission (Bishop et al., 1996;
Weick et al., 1996), however, it can favourably affect relapse and overall
survival (Bishop et al, 1996).

1.3.2 Post induction chemotherapy

Once patients achieve CR, further treatment is essential to prevent subsequent
relapse, and increase survival rate. In younger patients this treatment can be
administered in the form of either chemotherapy, autologous transplantation, or
allogeneic transplantation using sibling or alternative donors.

1.3.3 Autologous transplantation

Several studies in Europe and the United States have been conducted aiming to
evaluate the potential benefit of autologous transplantation over conventional
chemotherapy (EORTC-GIEMMA, GOELAM and US Intergroup) (Zittoun et
al, 1997; Harousseau et al, 1997; Cassileth et al, 1998), or in addition to
chemotherapy (MRC) (Burnett et al, 1998). Generally, all studies have shown a reduction in relapse risk, however, this could not be translated into a survival benefit (Zittoun et al, 1997; Harousseau et al, 1997; Cassileth et al, 1998; Burnett et al, 1998). This can be explained by the fact that some patients in the chemotherapy group were able to be salvaged with a transplant in second complete remission (CR2). It is worth mentioning that both the GOELAM and the US Intergroup studies demonstrated a better survival in the chemotherapy arm, and it is of interest that in both studies high dose cytarabine was included in the treatment protocols (Harousseau et al, 1997; Cassileth et al, 1998). The MRC AML 10 trial addressed a different question, namely the role of autologous transplantation in addition to what was considered to be completed induction and consolidation chemotherapy (Burnett et al, 1998). Like all the other trials, the relapse risk was reduced, however, this was counterbalanced by a high risk of death during the procedure, so that the overall survival was not improved. Nevertheless, a survival advantage did become apparent when the follow up exceeded the 2nd year, as patients die from transplant related toxicity mainly the first 2 years following the transplant, which compromises survival benefit. Despite the evidence of reduced relapse risk in recipients of autologous transplantation, relapse remains a major cause of treatment failure. Stem cell contamination by the tumour cells as well as residual disease may account for this increased risk of relapse. As a result, much energy has been devoted to finding methods to purge the bone marrow/peripheral blood stem cells of contaminated cells, and techniques involving in vitro purging have been applied (Gorin et al, 1990). Despite some encouraging results, this method has not been widely accepted, especially when identical regimens using un-purged marrow produced similar results.

1.3.4 Allogeneic transplantation

Autologous transplantation is a relatively safe procedure, however, its value is limited by the lack of a graft-versus-leukaemia (GVL) effect. In contrast, during the last 2-3 decades, data from single institutions or registries have demonstrated that allogeneic transplantation introduces a powerful GVL effect. Further evidence that a GVL effect exists comes from the higher relapse risk in
patients who receive a T-cell depleted transplant and in transplants involving identical twins (Gale et al, 1994). However, the strongest evidence, which has been documented by almost every study, is the association of acute or chronic graft-versus-host disease (GVHD) with a decreased risk of relapse (Ringden et al, 2000). Undoubtedly, allogeneic transplantation is the most active anti-leukaemic treatment offering substantial reduction in relapse, nevertheless its value still remains controversial. Firstly, increased toxicity associated with the procedure compromises potential benefits associated with allogeneic transplantation. Secondly, patients who undergo transplantation are selected and may represent patients with more favourable disease compared to those who never receive the transplant as a result of early relapse.

In an effort to overcome several sources of bias, investigators have tried to evaluate potential efficacy of the transplant with an intention-to-treat analysis. Four major prospective trials have been conducted in adult patients with AML and all of them have demonstrated a reduced relapse risk for the transplant arm compared to chemotherapy (Keating et al, 1998; Harousseau et al, 1997; Cassileth et al, 1998; Burnett et al, 1998). However, this reduction in relapse risk did not translate into a real survival benefit in all studies. In fact, in one of the studies (US Intergroup study) patients in the chemotherapy arm showed a modest superior outcome compared to the transplant arm (Cassileth et al, 1998). Nevertheless, small number of patients and poor compliance with the transplant allocation may account for these differences. Current practice in the UK is that in good risk disease, transplantation in first remission is not indicated (Burnett et al, 1998). In poor risk disease, in the absence of effective chemotherapy, allogeneic transplantation is fully justifiable and effort should be made for the transplant to be given without delay, once remission has been achieved. Given the fact that relapse risk remains high even after the transplant, novel approaches will be needed in this group to enhance a potential survival benefit. Finally, in patients with standard risk disease, there is no convincing data to support the routine use of an allogeneic transplant in CR1. As it appears that chemotherapy continues to produce improved outcome with time, the role of allograft in standard risk disease will become more controversial. Nevertheless, the limits of conventional chemotherapy might have been
reached, and therefore novel approaches would probably be needed to increase survival rate with a minimum cost in terms of toxicity. The newly introduced reduced intensity conditioning regimens have already been shown to be associated with low toxicity, and further studies are warranted to establish their efficacy in AML (Martino et al, 2002).

1.3.5 Treatment of relapse

Once relapse has occurred, survival depends on the length of first remission and the age of the patient. There is no standard treatment for relapsed disease and various protocols are being used. The aim of re-induction is to bring the patient back to remission before receiving a bone marrow transplant. In fact, if a transplant can be given in 2nd remission, approximately 25%-30% of patients can be salvaged. Nevertheless, even with a transplant, subsequent relapse remains a major cause of treatment failure and this group of patients are candidates for more experimental treatments.

1.3.6 Future direction in the treatment of AML

There is no doubt that a new era has emerged for cancer targeted therapy and immunotherapy. The metabolic and enzymatic pathways affected by the genetic aberrations are potential targets for treatment. Acute promyelocytic leukaemia (APL) treated by all trans retinoic acid (ATRA) is the first model of a human malignant disease reverted by a differentiating agent. A pharmacologic dose of ATRA overcomes the blockage of exchange between corepressors and coactivators caused by the fusion protein PML-RARA. Several investigations have reported clinical benefits during clinical trials of ATRA in the treatment of APL, all showing evidence for high efficacy and low toxicity (Fenaux et al, 1993; Frankel et al, 1994). In the initial report from Huang et al (1998), 23 of 24 patients with APL achieved either partial remission (PR) or complete remission without developing bone marrow hypoplasia.
Mutations and dysregulation of Ras has been associated with the development of myeloid leukaemias (Reuter et al, 2000). Farnesyl transferase inhibitors, which target the post-translational modification of ras to prevent the subcellular localisation necessary for signal transduction, are another example of targeted therapy in the treatment of AML (Lancet et al, 2003).

Cell surface antigens represent another target for novel therapies. The first of these new targeted agents to be approved by the US food and drug administration is gemtuzumab ozogamicin. This agent is an immunoconjugate of an anti-CD33 antibody chemically linked to potent cytotoxic agent, calicheamicin (van Der Velden et al, 2001). Clinical studies are also being conducted in Europe and Japan using WT1 (Wilms tumour) peptides specific for the HLA-A2 and A24 alleles as vaccines, although results have not yet been reported.

1.4 Haemopoietic growth factor receptor abnormalities in acute leukaemias (excluding FLT3)

Clear evidence from animal models has indicated that mutations in growth factor receptors can cause constitutive activation of the receptor which leads to oncogenic transformation. The capacity of mutant haemopoietic growth factor receptors to induce factor-independent cell proliferation and leukaemogenesis was initially appreciated in studies of acute leukaemia viruses of mice, several of which contain mutated receptor genes as viral oncogenes. For example, the acute transforming Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV) contains the designated oncogene v-kit, which displays partial homology with tyrosine-specific protein kinase oncogenes (Besmer et al, 1986). As haemopoietic growth factor receptors carry signals controlling blood cell development, there is considerable interest in the possibility that receptor abnormalities might contribute to leukaemia. To date, activating mutations have been reported in the receptors for macrophage colony stimulating factor (M-CSF), stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF) in human leukaemias, however, their frequency is extremely low, raising
the possibility of additional operating mutations in the same or different pathways in order to produce a leukaemic phenotype.

1.4.1 M-CSF receptor

The c-fms gene encodes the cell surface receptor for M-CSF, where interaction of ligand with the receptor stimulates proliferation and maturation of cells in the monocyte/macrophage lineage. The transforming capacity of c-fms was first discovered through analysis of the Susan McDonough feline sarcoma virus (SM-FeSV) (McDonough et al, 1971). The SM-FeSV genome contains a mutated, constitutively active form of the receptor called v-fms. Cells expressing this mutant receptor display constitutive tyrosine phosphorylation. Its tumouriogenic capacity has also been demonstrated in animal models, where transfected bone marrow cells infused into lethally irradiated mice can produce erythroleukaemia or B-cell lymphoma (Heard et al, 1987). In human leukaemias, c-FMS expression has been reported in 30% of AML cases of monocytic lineage (Ashmun et al, 1989; Rambaldi et al, 1988). Activating point mutations at codons Leu301 and Tyr969 of c-FMS have been reported in patients with AML and myelodysplastic syndromes (MDS) (Ridge et al, 1990; Tobal et al, 1990), although confirmation by enzyme digestion or sequencing was not done. Allelic loss of one (hemizygous) or both alleles (homozygous) of the c-FMS gene has also been reported in patients with myelodysplasia, chromosome 5 deletion and AML (Boulwood et al, 1991; McGlynn et al, 1997). McGlynn et al (1997), using a semi-quantitative Southern blotting technique, found that loss of one allele occurred in 5/18 cases of AML M4 and 8/27 cases of AML M1, M2 and M3. In addition, loss of both FMS alleles occurred in 3/18 cases of AML M4.
1.4.2 G-CSF receptor

Mutations in the G-CSF receptor gene have been detected in approximately 20% of patients with severe congenital neutropenia (SCN). These mutations are mainly located in a critical cytoplasmic region (spanning nucleotides 2384-2429), and lead to truncation of the distal cytoplasmic region of the receptor, with subsequent disruption in downstream signalling. When expressed in myeloid murine L-GM cells, a truncated G-CSF receptor transduced a proliferative signal in response to G-CSF, but was unable to signal differentiation (Dong et al, 1993). These data implicate G-CSF receptor truncation as a possible leukaemogenic mechanism, because cells carrying a mutant receptor may have a growth advantage due to prolonged activation of the receptor in the presence of the ligand. This is also consistent with the natural history of the disease, as patients with SCN carrying these mutations have a strong predisposition to AML (Dong et al, 1997). However, in mice these mutations have not caused leukaemia (Hermans et al, 1998; Mc Lemore et al, 1998).

In AML, mutations in the G-CSF receptor are rare events. Bernard et al (1996) using SSCP analysis in 40 patients with AML identified a substitution at nucleotide 2088 which changes a threonine to asparagine at amino acid 617 in the transmembrane domain. However, it was not clear whether this base change was a leukaemia-specific mutation or an infrequent polymorphism because no remission blood or marrow, constitutive DNA or family members were available for study. Subsequently, the same group, in order to determine whether it was a leukaemia-specific mutation, screened this nucleotide of the G-CSF receptor in genomic DNA from a further 515 patients with AML at presentation and identified the same mutation in 1 patient (Forbes et al, 2002). This mutation was not present in remission bone marrow from this patient, indicating that it was leukaemia-specific. In vitro experiments showed that the mutant receptor conferred growth factor independence on factor dependent Ba/F3 cells, and retroviral transduction of the mutant receptor into primary CD34+ cells induced myeloid differentiation in the absence of G-CSF. Nevertheless, it should be pointed that the screening method was specifically
for this mutation and would not pick up other potentially activating mutations in the same region.

1.4.3 C-kit receptor

C-kit is a member of the type III receptor tyrosine kinase family and plays a crucial role in normal haemopoiesis. Somatic mutations causing constitutive activation of the receptor have been associated with a number of different neoplastic conditions such as systemic mastocytosis, sinonasal natural killer T-cell lymphomas, human gastrointestinal stromal tumours and myelofibrosis (Hirota et al, 1998; Kimura et al, 1997; Nagata et al 1995).

In AML, c-kit is expressed by myeloblasts in approximately 60%-80% of cases, as assessed by surface immunostaining or expression of c-kit messenger RNA (Ikeda et al, 1991). In AML, activating mutations in the second tyrosine kinase domain (Asp 816) have been reported in patients with t(8;21) and inv(16) (Beghini et al, 2000). Gari et al (1999) reported in-frame deletion plus insertion mutations in exon 8, corresponding to the fifth immunoglobulin-like domain in the extracellular region of the receptor, in 7/21 patients with inv(16) and 1/23 patients with t(8;21), all of which the codon for Asp419 was involved in all of these. One patient with inv(16) showed a Val530Ile substitution in exon 10. More recently, Kohl et al (2005) showed that exon 8 c-kit mutations induced receptor hyperactivation in response to stem cell factor (SCF), stimulation in terms of proliferation, and resistance to apoptotic cell death (Kohl et al, 2005).

1.4.4 PDGFRβ

Rearrangement of the platelet derived growth factor receptor β (PDGFRβ) with a number of different partner genes is a frequent finding in myeloproliferative syndromes and chronic myelomonocytic leukaemia (CMML) (Cross NC & Reiter, 2002). PDGFRβ gene rearrangement with formation of a fusion gene was first recognised in association with t(5;12)(q31;p12), the fusion gene formed being ETV6- PDGFRβ (Golub et al, 1994). Subsequently, PDGFRβ
was found to contribute to other fusion genes in patients with myeloproliferative disorders. However, in AML such rearrangements are extremely rare with only two reported cases in the literature, one in transformed atypical CML with t(5;12) (ETV6- PDGFRβ) (Wessels et al, 1993) and the other in relapsed AML with t(5;14) (CEV14- PDGFRβ) (Abe et al, 1997).

1.4.5 EPO-R

Mutations with truncated EPO-R have been reported in benign human erythrocytosis and primary familiar polycythaemia but no pathogenic mutations in human erythroleukaemias have been identified so far (Le Couedic et al, 1996).

1.5 Aims of this thesis

Although examples exist in which receptor mutations appear to contribute to pre-malignant or overt disease in AML patients, these mutations occur rarely. Recently an internal tandem duplication of the juxtamembrane domain coding sequence of the FLT3 gene has been described in AML and has been associated with adverse outcome. This thesis presents results of studies to investigate the incidence, biological and clinical characteristics associated with FLT3 mutations in a large cohort of AML patients; whether these mutations can be used as markers of minimal residual disease; and the response to FLT3 inhibitors in samples from patients with newly diagnosed AML with and without FLT3 mutations, either as monotherapy or in combination with conventional cytarabine.
Chapter 2

2.1 General materials and reagents

All chemicals were provided from Sigma Chemical Co or BDH/Merck unless otherwise stated.

Buffers/solutions

10x TBE Buffer (pH 8.3). For 1L

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<tr>
<td>Tris</td>
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</tr>
<tr>
<td>Orthoboric acid</td>
<td>55.7g</td>
</tr>
<tr>
<td>EDTA</td>
<td>7.4g</td>
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</table>

2) DTAB (dodecyltrimethylammonium bromide) (8%). For 50ml

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<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>EDTA</td>
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</tr>
<tr>
<td>Tris</td>
<td>0.61g</td>
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</table>

3) L-Broth medium plates. For 1L

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</tr>
<tr>
<td>Agar</td>
<td>15g (Calbiochem)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1ml 10mg/ml (Gibco)</td>
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4) SOC medium. For 100ml

<table>
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<tr>
<td>Tryptone</td>
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</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
</tr>
<tr>
<td>1M NaCl</td>
<td>1ml</td>
</tr>
<tr>
<td>1M KCl</td>
<td>1ml</td>
</tr>
<tr>
<td>2M Mg^{2+}</td>
<td>1ml</td>
</tr>
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</table>

(pH 7.0)
2.2 Extraction of DNA from PB or BM

Method: Approximately 10-50 x 10^6 leukocytes were obtained from buffy coats, either from BM or PB and re-suspended in 2.4ml PBS (Gibco). DTAB (dodecyl-trimethyl ammonium bromide) reagent (4.8ml) was added, mixed thoroughly and incubated at 68°C for 5 minutes. An equal volume of chloroform (7.2ml) was then added to the lysed cells, mixed thoroughly and centrifuged at 3500rpm for 20 minutes. The aqueous layer was decanted into a new 15ml centrifuge tube and the DNA precipitated with an equal volume of ethanol (100%). The sample was then centrifuged at 3000rpm for 5 minutes to pellet the DNA which was transferred to a 1.5ml tube. The DNA was washed in 1ml 75% ethanol, re-suspended in an appropriate volume of water, allowed to dissolve thoroughly and stored at 4°C.

2.3 Reverse transcription (RT)

Method: In a 20μl reaction volume, the final concentrations were 1x reaction buffer, 5.25 mM MgCl2, 1 mM each dNTP, 20 units RNAse inhibitor, 3-5 units AMV (avian myeloblastosis virus) reverse transcriptase, 250ng oligo dT (Promega, UK) and 1μg RNA. Reaction mixtures were incubated at 42°C for 1 hour followed by 95°C for 5 minutes to denature the enzyme. The resulting cDNA was stable for several weeks when stored at 4°C.

2.4 Polymerase chain reaction (PCR)

Method: Approximately 100ng DNA was added to a reaction mix containing 1x buffer [16mM (NH₄)₂SO₄, 67mM Tris HCl pH 8.8, 0.01% Tween 20], 1.0mM MgCl₂, 200μM dNTPs (Bioline, London UK) and 10 pmols each primer (Oswel/Eurogentec Ltd, Hampshire, UK) in a total volume of 19μl.
mix was heated to 95°C for 5 minutes and held at 85°C whilst 1 µl containing 0.5 units BIOTAQ™ DNA polymerase (Bioline Ltd. London, UK) was added, then 30 cycles each of 95°C for 30 seconds, 60°C for 30 seconds (60°C for FLT3/ITD and 63°C for FLT3/D835 mutations) and 72°C for 30 seconds performed, followed by 5 minutes at 72°C. Amplified products were electrophoresed through 2% agarose gels and visualized under UV light with ethidium bromide staining.

2.5 Semi-quantitative PCR

Method: PCR was carried out as described above with the addition of 1 pmol of primer end-labelled with γ-[³²P]-ATP (3000Ci/mmol, Amersham Pharmacia Biotech UK Ltd., Little Chalfont, UK). Ten pmols primer was incubated with 10 units of T4 polynucleotide kinase (Promega, UK) in the manufacturer’s buffer and 3 µl γ-[³²P]-ATP for 30 minutes at 37°C. PCR was performed as described above, however, the number of amplification cycles was reduced to 25. An aliquot of 2 µl of the PCR product was added in 2 µl of stop solution (0.5M EDTA/glycerol/bromophenol blue), heated to 95°C for 5 minutes, was immediately placed on ice and subsequently loaded on to the polyacrylamide gels. Products were then electrophoresed through denaturing polyacrylamide gels (7M urea, 6% polyacrylamide crosslinker ratio 37.5:1, 0.5 x Tris-Borate-EDTA), the gels were dried and exposed to Hyperfilm-MP (Amersham). Autoradiographic signals were quantified using densitometry (Hoefer Scientific Instruments, San Francisco, CA).

2.6 Sequencing of PCR products

Method: PCR products were cleaned using a PCR purification kit (Wizard PCR preps DNA purification system, Promega, UK) and run on an agarose gel for approximate quantification. Sequencing PCR was performed by adding approximately 10 ng cleaned PCR product to a single reaction mix containing the dye terminators, DNA polymerase, MgCl₂ and buffer (BigDye™ Terminator cycle sequencing kit, Perkin Elmer, Boston MA, USA). The products were subsequently precipitated with 2.0 µl Na acetate (3M, pH 4.6)
and 50μl ethanol (95%) and washed in 75% ethanol. The samples were then dried, re-suspended in 15μl Template Suppression Reagent™ (TSM), denatured at 95°C for 5 minutes and placed on ice before loading on the 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.7 Cloning of PCR products

Method: PCR products were cloned using the TA cloning system which utilises the single deoxyadenosine overhang at the 3' end of PCR products. Two μl of PCR product was incubated with 1μl of the T-vector, 1μl T4 DNA ligase and 5μl ligation buffer (TA cloning kit, Promega, UK) in a final volume of 10μl at 4°C overnight. Competent bacteria (Top10F', E. coli from in house stock) were removed from -70°C and thawed on ice for 5 minutes. Two μl of 5% beta-mercaptoethanol was added to the competent bacteria together with 2 μl of the ligation reaction, incubated on ice for 20 min and subsequently placed at 42°C for 30 seconds. Transformed bacteria were then placed in SOC, then were shaken at 37°C, 225rpm for 60 minutes.

During this period the LB-agar/ampicillin petri dishes were removed from 4°C and coated with 40μl (100mM) isopropyl-1-thio-β-D-galactoside (IPTG), and 20μl (50mg/ml) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). After incubation at 37°C for 60 minutes, 100 μl of transformed bacteria were aliquoted to individual X-Gal/IPTG/ampicillin/LB-agar petri dishes. The petri dishes were then inverted and placed at 37°C overnight.

Selection principle: The TA cloning system involves plasmids that have been engineered to contain a multiple cloning site (MCS) which has a lac-Z gene attached. If DNA (the fragment being incorporated) ligates successfully into the MCS, the lac-Z gene is disrupted, and the host bacteria appear as white colonies, whereas a functional Lac-Z gene will produce beta-galactosidase which produces blue colonies. Transformed clones (white colonies) were picked with a pipette tip and dipped into a PCR master mix containing all the reagents for the PCR. PCR was performed as described above directly on the
bacteria and clones containing the ITDs were identified by running the PCR product on an agarose gel. Mutant clones were sequenced as described above.
Chapter 3

3.1 Introduction

The past twenty to thirty years have seen significant improvements in therapy for AML such that complete remission (CR) can now be achieved in about 80% of adult patients under the age of 55 years. (Grimwade et al, 1998) This achievement is mainly related to the intensification of post-remission treatment and advanced supportive care of critically ill patients. For example, in the United Kingdom Medical Research Council (MRC) trials for the treatment of AML, a progressive improvement in survival has been observed in 3 consecutive studies carried out over the last 18 years. In the MRC AML 9 trial completed in 1990, the overall survival (OS) in patients below the age of 60 years induced by DAT 3+10 (daunorubicin for 3 days, cytarabine and 6-thioguanine for 10 days) was 25% at 10 years (Rees et al, 1996). In contrast, in the more recent MRC AML 12 trial for patients below the age of 60 years of age, which recruited a total of 3,459 patients over 7 years, the OS had nearly doubled to 45% at 5 years (Burnett et al, 2003). However, despite this progress, relapse remains a major cause of failure in a substantial number of patients, and approximately 50% will relapse and die from their disease. Therefore, to improve survival further, it will be necessary to identify those patients who are at increased risk of relapse, and to determine more appropriate therapy for them.

Certain biological markers such as age, WBC at presentation, antecedent myelodysplasia (MDS), lactate dehydrogenase (LDH) levels, expression of the multiple drug resistance (MDR) phenotype, karyotype, and response to course 1 of induction chemotherapy have been used over the years to identify patients at increased risk of relapse (Buchner et al, 1999; Wheatley et al, 1999). Among them, cytogenetic abnormalities have emerged as an independent prognostic factor, predicting not only those patients who are likely to achieve CR but also those who are at risk of relapse (Grimwade et al, 1998; Slovak et al, 2000). Apart from their predictive value in disease outcome, cytogenetics have
significantly increased our knowledge of the basic genetic mechanisms involved in the process of leukaemogenesis.

Three major cytogenetic risk groups have been proposed and integrated into a prognostic index applicable in risk-directed therapy. Patients with favourable cytogenetic disease [i.e. t(8;21), t(15;17), inv(16)] are not recommended for a BMT in CR1 and long-term disease free survival (DFS) can be achieved with chemotherapy alone (Burnett et al, 2002). In patients with poor risk disease as defined by the MRC group [i.e. -7, -5, del(5q), 3q abnormalities, complex karyotype with more than 4 unrelated abnormalities], the survival rate is less than 20% and these patients are considered candidates for more aggressive or experimental treatment (Grimwade et al, 1998). Between these two groups, a high proportion of newly diagnosed AML patients (approximately two thirds) are classified as having standard or intermediate risk disease. These patients have either normal cytogenetics or abnormalities that are not included in the definition of the other subgroups [i.e. +8, 11q23, +21, del(7q), del(9q), +22 (according to the MRC classification)] (Grimwade et al, 1998). Although the remission rate in this group of patients is similar to that observed in favourable risk disease, the relapse risk is in excess of 50%. Moreover, they lack other biological or molecular markers that discriminate them as “carriers” of that risk. Therefore, additional prognostic factors are required, in particular to identify those patients with standard risk disease who are at risk of relapse. As the clinical value of intensive protocols and high dose chemotherapy followed by stem cell transplant has now been firmly established, thorough understanding of the individual prognostic factors has become of significant importance for treatment choice when therapeutic decisions have to be taken. For example, it was not uncommon some years ago to transplant patients with t(8;21) in CR1, but this option can now be reserved for those patients who might relapse (Burnett, 2002). Similarly, in the future, one may anticipate that the emergence of genetically defined subgroups within patients with standard risk disease may differentiate between those who will need a BMT in CR1 and those who will need to save such approach for subsequent relapses.
Recently, the emergence of FLT3 mutations as potential independent prognostic factors in AML has generated much interest at the clinical, molecular and possibly therapeutic level.

3.2 FLT3

FLT3 (fms-like tyrosine kinase 3), also known as stem cell tyrosine kinase-1 (STK1) or fetal liver tyrosine kinase-2 (FLK-2), is one of the Class III tyrosine kinase (TK) receptors that share sequence homology and structural characteristics. The latter include five immunoglobulin-like domains in the extracellular region, an intracellular juxtamembrane (JM) domain, two TK domains interrupted by a kinase insert, and a C-terminal tail (Agnes et al, 1994) (Figure 3.1). The gene is located at chromosome 13q12 and consists of 24 exons (previously reported as 21) (Rosnet et al, 1991; Abu-Duhier et al, 2001a).

FLT3 is predominantly expressed on haemopoietic progenitor cells in the bone marrow, thymus and lymph nodes (Rosnet et al, 1993), but is also found on other tissues such as placenta, brain, cerebellum and gonads (Maroc et al, 1993). Interaction with its ligand (FL) results in receptor dimerization, autophosphorylation, and subsequent phosphorylation of cytoplasmic substrates that are involved in signalling pathways regulating the proliferation of pluripotent stem cells, early progenitor cells and immature lymphocytes (Lyman, 1995). This interaction is influenced by other cytokines such as Kit ligand (KL). In fact, when primitive human progenitor cells are stimulated in vitro with either FL or KL alone they show little or no proliferative response, but both ligands together synergistically enhance growth (Hannum et al, 1994). Further evidence for the importance of FLT3 in early haemopoiesis has come from FLT3 knockout mice. They are healthy with normal peripheral blood counts, but they have reduced numbers of bone marrow early B-cell precursors, plus a defect in primitive cells as measured by long term competitive repopulation assays and a reduced ability to reconstitute B-cell, T-cell and myeloid lineages when transplanted into irradiated hosts (Mackarehtschian et al, 1995).
3.2.1 FLT3 expression in leukaemias

Expression of FLT3 has been reported at the mRNA and/or protein level in 93% of AML patients, 87% of T-cell acute lymphoblastic leukaemia (ALL) and up to 100% of B cell ALL patients (Drexler, 1996). It has not been detected in chronic myeloid leukaemia in chronic phase but appears to be highly expressed during disease transformation, irrespective of the phenotype (Birg et al, 1992). In leukaemic cell lines it is expressed in approximately 90% of pre B cell lines, but less frequently (40% - 80%) in myeloid and monocytic cell lines (Drexler, 1996). Studies have also demonstrated very high levels of FLT3 mRNA and protein in AML patients, leading to the suggestion that its over-expression may play a role in the survival and proliferation of leukaemic cells (Carow et al, 1996). This is in line with the finding that FL can induce proliferation in some FLT3-expressing primary leukaemic cells and cell lines (Dehmel et al, 1996). Furthermore, chronic exposure of mice to FL by transplantation with primary haemopoietic cells constitutively expressing the FL gene has been shown to induce leukaemia with a long latency period, indicating a possible autocrine mechanism for maintaining the survival of a leukaemic clone (Hawley et al, 1998).
3.3 FLT3 mutations

3.3.1 Internal tandem duplications (ITDs)

The first FLT3 mutations to be identified were serendipitously detected during an investigation into the incidence and distribution of FLT3 mRNA in samples from adult AML and childhood ALL patients (Nakao et al., 1996). Unexpectedly long fragments were detected in PCR products of the JM domain in 5 out of 30 AML patients. They were also found using genomic DNA from the same patients, excluding the possibility of aberrant alternative splicing. Further analysis showed that they all contained a tandemly duplicated sequence, sometimes with insertion of additional nucleotides. The duplicated region was variable in both size and location in different individuals but always fell within the JM domain encoded by exons 14 and 15 (previously 11 and 12) (Abu-Duhier et al., 2001a). The resulting transcripts were always in-frame and would therefore be expected to produce functional FLT3 chains (Figure 3.2).

![Figure 3.2 Schematic representation of FLT3 and FLT3/ITD activating mutations](image_url)
Preliminary *in vitro* analysis of FLT3/ITDs transfected into Cos7 cells showed that they induced ligand-independent receptor dimerization and phosphorylation, irrespective of the location and length of the ITD, and led to phosphorylation of wild type (WT) FLT3 expressed in the same cell (Kiyoi *et al.*, 1998). They have been shown to confer growth factor independence on factor-dependent cell lines such as Ba/F3 and 32D cells, and to induce constitutive activation of downstream signalling molecules such as STAT5, MAP kinase, Akt, SHC, CBL, VAV and SHP2 (Hayakawa *et al.*, 2000; Mizuki *et al.*, 2000; Tse *et al.*, 2000; Kiyoi *et al.*, 2002). However, comparable constitutive activation is not always observed in primary leukaemic blasts. Of 27 AML samples studied by Fenski *et al.* (2000), 18 had ligand-dependent FLT3 phosphorylation and 3 of these had a FLT3/ITD. Conversely, 3 samples had constitutive FLT3 phosphorylation, but only 1 had a FLT3/ITD. Similarly, Birkenkamp *et al.* (2001) reported that of 12 samples with both FLT3 and STAT5 constitutive phosphorylation, only 8 had a FLT3/ITD, and of 5 samples with constitutive STAT5 but not FLT3 phosphorylation, 2 had a FLT3/ITD. It is likely that, at least to some extent, these results indicate the redundancy of signalling pathways within the cell, and the multiple ways in which they can become activated.

A striking feature of the FLT3/ITDs identified in AML patients is their diversity, in both size and location. In general the length of the duplication varies between 12 and 204 bps but it has been reported to be as short as 3 bps and as long as >400bps (Schnittger *et al.*, 2002). Most ITDs occur at the 5' end of the JM domain, in exon 14, and the TK1 domain is minimally involved, but they differ in the precise sequence duplicated and its starting point. Several inserted sequences of unknown origin varying between 9 and 36bps have also been detected (Kiyoi *et al.*, 1998; Frohling *et al.*, 2002; Thiede *et al.*, 2002). There is no sequence which is common to all reported duplications, but the involved region does contain a tyrosine-rich stretch of sequence and most ITDs include at least 1 of the tyrosines 589, 591, 597 or 599 which form part of the motifs YFYV and YEYDLK. Since the latter are homologous to autophosphorylation sites in the JM domains of other TK receptors, e.g. the platelet derived growth factor β receptor (PDGFβR), it was initially thought that the
mechanism by which duplication might lead to enhanced growth was through the gain of additional SH2 binding domains (Yokota et al., 1997). However, neither substitution of all 4 tyrosines with phenylalanine, nor deletion of between 1 (ΔY599) and 4 (ΔY589-599) tyrosines, alters the in vitro constitutive phosphorylation state and ability to induce growth factor independence of a FLT3/ITD, although these residues are clearly important for ligand-dependent activation of the WT receptor (Kiyoi et al., 2002). Instead, current models suggest that the JM domain has a negative regulatory role which is disrupted by the ITD elongation (Gilliland & Griffin, 2002; Kiyoi et al., 2002). In the WT receptor, the JM domain takes up an α-helical conformation which blocks activation of the kinase and may inhibit self-dimerization. Ligand binding overcomes the inhibitory effect by inducing a conformational change and/or phosphorylation of key tyrosine residues. The ITDs may therefore prevent the protective association between JM domain and kinase, exposing the latter to constitutive activation. They may further allow recruitment of molecules that could stabilize this conformation or alter downstream signalling.

The in vivo tumourigenic potential of a FLT3/ITD has been demonstrated by injection of 32D cells carrying a FLT3/ITD into syngeneic mice, which led to rapid development of a leukaemia-like disease (Mizuki et al., 2000). Furthermore, AML cells with an ITD showed an increased ability to repopulate bone marrow in NOD/SCID mice (Rombouts et al., 2000). Transplantation of bone marrow cells retrovirally transduced with FLT3/ITDs into recipient mice led to an oligoclonal myeloproliferative disorder, but, this was not sufficient to cause leukaemia (Kelly et al., 2002a). However, retroviral transduction of FLT3/ITD into bone marrow cells obtained from PML/RARα transgenic mice results in a short latency acute promyelocytic leukaemia like disease with complete penetrance, suggesting that FLT3 signaling can cooperate with PML/RARα to give the complete differentiation block. This indicates the requirement for additional co-operating mutations for a fully transformed phenotype in this model. (Kelly et al., 2002b).

It can be hypothesized that in such murine models only '2 hits' are required to generate leukaemia, the mutation in the transcription factor (e.g. PML/RARα)
producing a block in differentiation and the mutant growth factor receptor providing a proliferative or survival signal (Deguchi & Gilliland, 2002). Interestingly, three recent publications have presented data suggesting that FLT3/ITD mutations may also contribute to a block in differentiation. Firstly, expression of a FLT3/ITD in 32D cells inhibited the G-CSF induced expression of myeloid maturation markers such as myeloperoxidase, lysozyme and C/EBPα (Zheng et al, 2002). Secondly, microarray expression analysis of 32D cells transfected with either (WT) or ITD FLT3 demonstrated that certain markers associated with myeloid differentiation, e.g. PU-1 and C/EBPα, were suppressed in FLT3/ITD expressing cells (Mizuki et al, 2003). Evidence of suppression of C/EBPα has come from the use of FLT3 inhibitors, where, the ability of the FLT3 tyrosine kinase inhibitor CEP-701 to restore induction of C/EBPα expression in 32D/FLT3/ITD cells in response to G-CSF indicates that the suppression of C/EBPα depends on the tyrosine kinase activity of FLT3/ITD (Zheng et al, 2004). It should be noted, however, that reduced differentiation is frequently a feature of an increased proliferation rate. In the FDCP-mix cell line, for instance, high concentrations of IL-3 block or reduce the usual differentiation induced by other growth factors (Heyworth et al, 1990).

3.3.2 Mutations in the second tyrosine kinase domain (TKDs)

Mutations at aspartic acid 835 (D835) and isoleucine 836 (I836) in exon 20, in the second TK domain, were first independently identified in AML patients by Yamamoto et al (2001) and Abu-Duhier et al (2001b). They include at least 6 different substitutions within the D835 codon leading to missense mutations, predominantly tyrosine and histidine, less frequently valine, glutamate and asparagine, and mutation of I836 to methionine (Figure 3.3). Complete deletion of I836, insertion of nucleotides, and complex changes have also been detected, but the sequence always remains in frame. Occasional cases have been reported in ALL (2.8%) and MDS (3.4%) (Yamamoto et al, 2001). The mutations were found to cause constitutive tyrosine phosphorylation of the receptor when transfected into Cos 7 cells, and to confer IL-3 independent growth on 32D
cells, indicating their gain-of-function property (Yamamoto et al, 2001). The amino acids are part of the activation loop which blocks access of ATP and substrate to the kinase domain when the receptor is in an inactive state. Ligand-induced activation leads to phosphorylation within the loop causing it to take up an active configuration and allow kinase activity. The TKD mutations are thought to mimic the latter by interfering with the inhibitory effect of the loop, resulting in constitutive kinase activation. Their effect is therefore similar to that of FLT3/ITD mutations in disrupting the auto-inhibitory mechanisms that normally protect the cell from unregulated signalling through FLT3. However, possible differences in the functional consequences of the various TKD mutations have not been investigated.

![Schematic representation of FLT3 and FLT3/TKD activating mutations](image)

**Figure 3.3 Schematic representation of FLT3 and FLT3/TKD activating mutations**

### 3.3.4 Additional mutations in the second tyrosine kinase domain (TKDs)

A further constitutively activating FLT3 mutation in exon 20 has been reported by Spiekermann et al (2002) who detected an additional 6 bps leading to the insertion of glycine and serine between amino acids 840 and 841 in 2 out of 359 (0.5%) *de novo* AML patients. Functional analysis demonstrated that the mutant receptor was constitutively phosphorylated and induced factor
independence in Ba/F3 cells. Like the D835/I836 mutations, it is probable that this mutation also interferes with the inhibitory role of the activation loop.

3.3.5 Novel FLT3 mutations

Novel Flt3 mutations have been reported in exon 14 (point mutations in codon 579, 590-91 and 592) (Stirewalt et al, 2004), in exon 16 (point mutation in the first catalytic domain) (Piccaluga et al, 2004) and exon 20 (point mutation, Y842C) (Kindler et al, 2004). However, at this time, the importance of these novel mutations remain unknown and additional studies will be needed to determine potential functional and clinical impact.

This chapter reports studies to determine the impact of FLT3/ITD mutations on clinical outcome in a large group of well-documented patients, mostly less than 60 years of age, treated intensively in the UK MRC AML 10 and 12 trials.

3.4 Materials and methods

DNA was available from peripheral blood (PB) or bone marrow (BM) from a total of 854 eligible adult patients at diagnosis who had been entered into either the MRC AML 10 (n = 406) or AML 12 (n = 448) trials. The DNA samples were obtained from the UCLH DNA/RNA bank. All patients had karyotypic analysis which was performed either in local UK cytogenetic laboratories or, where no such service was available, at the MRC AML trials cytogenetic centre at University College Hospital, London (UCLH). Of the 854 patients studied in total, 746 patients were screened for FLT3/ITD mutations at UCLH and 108 patients were screened by MF and DB in Dundee. DNA from PB of 100 haematologically normal individuals who routinely attended the antenatal clinic at UCLH was also studied for FLT3/ITD mutations.
3.4.1 PCR analysis of the FLT3/ITD mutation

Exons 14 and 15 (previously 11 and 12) and the intervening intron of the FLT3 gene were amplified from DNA using previously described primers 11F (5'-GCAATTAGGTATGAAAGCCAGC-3') and 12R (5' GCTTTGACATTGGACGGAACC-3') (Kiyoi et al, 1998).

PCR was performed as described in Chapter 2.4. DNA from the cell line TF-1 was used as the WT control. A fragment of 328bp was produced from WT alleles. A repeat analysis was carried out on all samples with an additional band (FLT3/ITD+).

The same primers were used to screen cDNA, but the fragment produced was 90bps smaller. Semi-quantitative PCR was performed on all FLT3/ITD+ samples as described in chapter 2.5 using the primers as above. The level of mutant FLT3 expressed as a percentage of the total signal.

3.4.2 Sensitivity and reproducibility of semi-quantitative PCR

In order to evaluate sensitivity of the technique, HL-60 cell line blasts with WT FLT3 were mixed with blast cells from a patient carrying predominantly ITD alleles (94%) to create nine different mixtures containing between 100% and 0.01% patient cells (50:50, 80:20, 90:10, 95:5, 99:1, 99.5:0.5, 99.9:0.1, 99.95:0.05, 99.99:0.01). DNA was extracted and semi-quantitative PCR was performed as described in chapter 2.5. Reproducibility was assessed by 5 separate analyses of 3 patient samples with high, intermediate or low level of mutant FLT3.

3.5 Endpoints and Statistical methods

Statistical analysis was performed by the Clinical Trial Service Unit, University of Oxford. Standard MRC criteria were followed to define complete remission (CR), remission failure, resistant disease (RD), induction death (ID), overall survival (OS), disease free survival (DFS), event free survival (EFS) and relapse risk (RR) as follows: CR was defined as a normocellular BM containing
<5% blasts and showing evidence of normal maturation of other marrow elements. PB regeneration was not a requirement, but 97% of cases defined as CR achieved a neutrophil count of 1 x 10^9/L and a platelet count of 100 x 10^9/L. Remission failures were classified by the clinicians as either partial remission (PR, defined as 5 – 15% blasts or <5% blasts but a hypocellular BM), resistant disease (RD, >15% blasts in the BM) or induction death (ID, i.e. related to treatment or hypoplasia). Where the clinicians' evaluation was not available, deaths within 30 days of entry were classified as ID and deaths later than 30 days after entry as RD. OS was defined as the time from diagnosis to death, and EFS as the time from diagnosis to an event (either failure to achieve remission, death in first CR or relapse), with patients not achieving remission being counted as having an event on day 1. For patients achieving CR, DFS was the time from the date of first CR to an event (death in first CR or relapse) and RR was the cumulative probability of relapse, censoring at death in CR. The Wilcoxon two sample test (for continuous data), Mantel Haenszel test for trend (for grouped continuous data) and Fisher's exact test (in 2 x 2 tables) were used to test for differences in clinical and demographic data by FLT3/ITD positivity, number of mutations (one versus two or more) and percentage mutation (<60% versus ≥ 60%). Kaplan-Meier life-tables were constructed for survival data and were compared by means of the log rank test, with surviving patients being censored at 1 May 1999 (AML 10 patients) or 1 June 2000 (AML 12 patients). A complete follow-up of all surviving patients to elucidate their current status was undertaken in May 1999 (AML 10) and June 2000 (AML 12) so information on relevant events is available to the above censoring dates for the vast majority of patients, with the small number (n=11) of patients lost to follow-up being censored at the date they were last known to be alive. Median follow up was 52 months (range 11-127 months). Odds ratios were calculated from the observed minus expected number of events (O-E) and its variance (O-E) and odds ratio plots were used to illustrate differences in the relative effect of FLT3/ITD between prognostic factor subgroups. Multivariate logistic regression analysis with a logit link function was used to find the factors most closely associated with CR rate and multivariate Cox models were used to analyse OS, EFS, DFS and RR. Models were fitted using stepwise selection, with variables added to the model if they had a P value < 0.01 but
removed if they had a P value > 0.05. Because of multiple testing, the level of significance was set at P = 0.01. All P values are two-tailed.

3.6 Results

3.6.1 Evaluation of semi-quantitative PCR analysis

Sensitivity of the method was determined from analysis of mixtures of cells with WT FLT3 and cells from a patient with 94% FLT3/ITD alleles. The mutant could be detected when ≥0.5% of total FLT3 (Figure 3.4). Quantification of the different mixtures gave results which were in good agreement with the expected values: expected 47% mutant, observed 48%; 19% and 23%; 9.4% and 12.8%; 4.7% and 5%; 1% and 1.4% respectively. The results were also highly reproducible: 5 analyses of 3 samples with different FLT3/ITD levels gave mean ± SD values of 94.4% ± 0.5% (range 93%-95%), 53.5% ± 1.1% (52%-55%) and 12.8% ± 0.8% (12-14%) respectively (Figure 3.5).
Figure 3.4 Evaluation of the sensitivity of the PCR technique using different mixtures of cells containing WT FLT3 and alleles with predominately FLT3/ITD

Figure 3.5 Semi-quantitative PCR demonstrating good agreement between observed and expected level of mutant using different mixtures from cells carrying WT and predominately ITD alleles

3.6.2 Analysis of FLT3 gene (exon 14 and 15) in haematologically normal individuals
DNA from PB of 100 haematologically normal individuals was studied for FLT3/ITD mutations and all were found to have only WT alleles (FLT3/ITD negative).

3.6.3 Patients' characteristics

Details of the patients' age, sex, FAB type, PB white cell count (WBC) and % BM blasts are given in Table 3.1. The majority of patients (792/854, 93%) had de novo AML. Four hundred and thirty two patients were male (51%) and 422 female (49%). Median age at entry was 41 years and only 4 patients were more than 60 years of age (61-63 years). The median WBC count at presentation was 18.3x10^9/L, while 116 patients (14%) had >100x10^9/L. The median percentage of bone marrow blasts at presentation was 80%.

Cytogenetic analysis revealed that 242 patients (28%) had favourable risk disease, 434 patients (51%) intermediate risk and 79 (9%) adverse risk disease, as defined according to the MRC criteria. All patients were treated according to the MRC AML 10 and 12 protocols, details of which are given in appendix 1.
Table 3.1 Clinical and demographic characteristics of the 854 AML patients studied

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>FLT3/ITD- (% total ITD-)</th>
<th>FLT3/ITD+ (% total ITD+)</th>
<th>% FLT3/ITD+</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>854</td>
<td>627</td>
<td>227</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>AML 10</td>
<td>406 (48%)</td>
<td>301 (48%)</td>
<td>105 (46%)</td>
<td>26%</td>
<td>0.7</td>
</tr>
<tr>
<td>AML 12</td>
<td>448 (52%)</td>
<td>326 (52%)</td>
<td>122 (54%)</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>De novo</td>
<td>792 (93%)</td>
<td>582 (93%)</td>
<td>210 (93%)</td>
<td>27%</td>
<td>0.9</td>
</tr>
<tr>
<td>Secondary</td>
<td>62 (7%)</td>
<td>45 (7%)</td>
<td>17 (7%)</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>FAB type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>14 (2%)</td>
<td>14 (2%)</td>
<td>0 (0%)</td>
<td>0%</td>
<td>0.02</td>
</tr>
<tr>
<td>M1</td>
<td>148 (17%)</td>
<td>108 (17%)</td>
<td>40 (18%)</td>
<td>27%</td>
<td>1.0</td>
</tr>
<tr>
<td>M2</td>
<td>210 (25%)</td>
<td>161 (26%)</td>
<td>49 (22%)</td>
<td>23%</td>
<td>0.2</td>
</tr>
<tr>
<td>M3</td>
<td>159 (19%)</td>
<td>101 (16%)</td>
<td>58 (26%)</td>
<td>36%</td>
<td>0.004</td>
</tr>
<tr>
<td>M4</td>
<td>172 (20%)</td>
<td>121 (19%)</td>
<td>51 (22%)</td>
<td>29%</td>
<td>0.4</td>
</tr>
<tr>
<td>M5</td>
<td>81 (10%)</td>
<td>61 (10%)</td>
<td>20 (9%)</td>
<td>25%</td>
<td>0.7</td>
</tr>
<tr>
<td>M6</td>
<td>15 (2%)</td>
<td>14 (2%)</td>
<td>1 (&lt;1%)</td>
<td>7%</td>
<td>0.08</td>
</tr>
<tr>
<td>M7</td>
<td>11 (1%)</td>
<td>11 (2%)</td>
<td>0 (0%)</td>
<td>0%</td>
<td>0.04</td>
</tr>
<tr>
<td>Bilineage</td>
<td>1 (&lt;1%)</td>
<td>0 (0%)</td>
<td>1 (&lt;1%)</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>RAEB-t</td>
<td>8 (1%)</td>
<td>7 (1%)</td>
<td>1 (&lt;1%)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>35 (4%)</td>
<td>29 (5%)</td>
<td>6 (3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>432 (51%)</td>
<td>322 (51%)</td>
<td>110 (48%)</td>
<td>25%</td>
<td>0.5</td>
</tr>
<tr>
<td>Female</td>
<td>422 (49%)</td>
<td>305 (49%)</td>
<td>117 (52%)</td>
<td>28%</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15</td>
<td>5 (1%)</td>
<td>3 (&lt;1%)</td>
<td>2 (1%)</td>
<td>40%</td>
<td>0.3</td>
</tr>
<tr>
<td>15-34</td>
<td>290 (34%)</td>
<td>222 (35%)</td>
<td>68 (30%)</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>35-54</td>
<td>477 (56%)</td>
<td>342 (55%)</td>
<td>135 (59%)</td>
<td>28%</td>
<td></td>
</tr>
<tr>
<td>≥ 55</td>
<td>82 (10%)</td>
<td>60 (10%)</td>
<td>22 (10%)</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>300 (35%)</td>
<td>251 (40%)</td>
<td>49 (22%)</td>
<td>16%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10-19</td>
<td>132 (16%)</td>
<td>98 (16%)</td>
<td>34 (15%)</td>
<td>26%</td>
<td></td>
</tr>
<tr>
<td>20-49</td>
<td>168 (20%)</td>
<td>125 (20%)</td>
<td>43 (19%)</td>
<td>26%</td>
<td></td>
</tr>
<tr>
<td>50-99</td>
<td>120 (14%)</td>
<td>76 (12%)</td>
<td>44 (19%)</td>
<td>37%</td>
<td></td>
</tr>
<tr>
<td>≥ 100</td>
<td>116 (14%)</td>
<td>63 (10%)</td>
<td>53 (23%)</td>
<td>46%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>18.3</td>
<td>14.3</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% BM blasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;80%</td>
<td>349 (41%)</td>
<td>285 (45%)</td>
<td>64 (28%)</td>
<td>18%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥ 80%</td>
<td>417 (49%)</td>
<td>278 (44%)</td>
<td>139 (61%)</td>
<td>33%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>80</td>
<td>79</td>
<td>87</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P values are for the Mantel Haenszel test for trend in age, WBC, % BM blasts and Fisher's exact test for AML 10 versus AML 12, de novo versus secondary AML and each individual FAB type versus all other known FAB types.
3.6.4 Overall incidence of FLT3/ITD in 854 patients and in different FAB types and cytogenetic risk groups

In the total group of 854 patients, 227 (27%) had a FLT3/ITD mutation. There was a slight difference in the incidence observed in DNA from BM (104/330, 32% FLT3/ITD+) and PB (103/391, 26% FLT3/ITD+) but this was not significant (p = 0.1). FLT3/ITD was found in all FAB subgroups except M0 (0/14, p = 0.02) and M7 (0/11, p = 0.04), and was found in only 1 out of 15 patients with M6 (p = 0.08), although the numbers of patients in these groups were small. It was significantly more common in M3 (58/159, 36%, p = 0.004) and in accord with this finding, the incidence of FLT3/ITD in patients with t(15;17) was 37% (p = 0.02). Lower frequencies were found in patients with t(8;21) and inv(16), (9%, p = 0.0004 and 7%, p = 0.003 respectively). It is noteworthy that a high frequency of mutations was also observed in patients with normal cytogenetics (34%, p = 0.0001). Particularly low levels of mutations were found in 11q23 (0%, p = 0.006), del 5q (0%, p = 0.006) and complex karyotypes (2%, p = 0.00003) (Table 3.2).

3.6.5 Clinical and biological characteristics of the FLT3/ITD+ patients

Presence of the mutation was not related to sex or age. FLT3/ITD+ patients altogether had significantly higher PB white cell counts at diagnosis (median count for FLT3/ITD- patients was $14.3 \times 10^9/L$ and for FLT3/ITD+ patients $38.0 \times 10^9/L$, p < 0.001), and this was also the case for patients with FAB type M3 (median count for FLT3/ITD- patients $3.3 \times 10^9/L$ and for FLT3/ITD+ patients $11.4 \times 10^9/L$, p < 0.0001). The percentage of BM blast cells was also higher in the FLT3/ITD+ patients (median for FLT3/ITD- patients 79%, FLT3/ITD+ 87%, p < 0.001).
Table 3.2 Incidence of FLT3/ITD+ patients in different cytogenetic risk groups and subgroups

<table>
<thead>
<tr>
<th>Cytogenetics</th>
<th>Total</th>
<th>FLT3/ITD-</th>
<th>FLT3/ITD+</th>
<th>% FLT3/ITD+</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Favourable</strong> risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t(15;17)</td>
<td>133</td>
<td>84</td>
<td>49</td>
<td>37%</td>
<td>0.002</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>67</td>
<td>61</td>
<td>6</td>
<td>9%</td>
<td>0.0004</td>
</tr>
<tr>
<td>inv(16)</td>
<td>42</td>
<td>39</td>
<td>3</td>
<td>7%</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Intermediate</strong> risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>281</td>
<td>185</td>
<td>96</td>
<td>34%</td>
<td>0.0001</td>
</tr>
<tr>
<td>del(7q)</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td>10%</td>
<td>0.1</td>
</tr>
<tr>
<td>11q23</td>
<td>18</td>
<td>18</td>
<td>0</td>
<td>0%</td>
<td>0.006</td>
</tr>
<tr>
<td>+8</td>
<td>74</td>
<td>53</td>
<td>21</td>
<td>28%</td>
<td>0.7</td>
</tr>
<tr>
<td>+22</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td>17%</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Adverse risk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>44</td>
<td>43</td>
<td>1</td>
<td>2%</td>
<td>0.00003</td>
</tr>
<tr>
<td>del(5q)</td>
<td>19</td>
<td>19</td>
<td>0</td>
<td>0%</td>
<td>0.006</td>
</tr>
<tr>
<td>-5</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td>0%</td>
<td>0.02</td>
</tr>
<tr>
<td>-7</td>
<td>30</td>
<td>28</td>
<td>2</td>
<td>7%</td>
<td>0.01</td>
</tr>
<tr>
<td>abn(3q)</td>
<td>23</td>
<td>19</td>
<td>4</td>
<td>17%</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Unknown</strong></td>
<td>99</td>
<td>68</td>
<td>31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P values are from Fisher's exact test for each individual cytogenetic abnormality versus all other known karyotypes.

3.6.6 FLT3/ITD mutation and clinical outcome as determined in univariate analysis

The median follow-up of surviving patients included in this study was 52 months, with some patients followed up for over 10 years, allowing long-term analysis of relapse rates and survival.

3.6.6.1 Response rate

The CR rate for the 854 patients was 82%. Details of the relationship between the presence of a FLT3/ITD mutation and clinical outcome, as determined by
univariate analysis, are given in Table 3.3. There was borderline significance to
the association between the presence of a FLT3/ITD mutation and a lower CR
rate \( (p = 0.05) \) and a higher ID rate \( (p = 0.04) \), but no significant relationship
with RD \( (p=0.4) \).

3.6.6.2 Survival outcome

The overall actuarial survival at 5 years from diagnosis was 41\% (SE 1.8) for
the entire cohort of 854 patients. Presence of the mutation had a significant
impact on survival, including OS, EFS, DFS and RR (Table 3.3). The RR in
patients with a FLT3/ITD at 5 years was 62\% (SE 4.2) (176 patients) as
opposed to 44\% (SE 2.6) (524 patients) in those without the mutation \( (p<0.001) \)
(Figure 3.6). Similarly, DFS (Figure 3.7), EFS and OS (Figure 3.8) were 32\%
(SE 3.8) (176 patients), 25\% (SE 3.1) (226 patients) and 35\% (SE 3.4) (227
patients) respectively in patients with a FLT3/ITD as opposed to 46\% (SE 2.4)
(524 patients), 39\% (SE 2.1) (625 patients) and 43\% (SE 2.2) (627 patients) in
those without the mutation \( (p<0.001 \) for all of them).

\[ \text{Figure 3.6 Relapse risk in patients with and without a FLT3/ITD} \]
3.6.7 Multivariate analysis of outcome including FLT3/ITD as a co-variable

A Cox multivariate analysis was performed for RR, DFS, EFS and OS, the variables considered being the presence of a FLT3/ITD mutation, the cytogenetic risk group (favourable versus intermediate versus adverse), presentation WBC, percentage BM blasts at diagnosis, age, sex, and FAB type. In addition, for analyses of outcome after CR, the response status post course 1
of induction chemotherapy was included, as this parameter would always be used clinically in decisions about treatment intensification. The presence of a FLT3/ITD was the most significant factor adversely affecting RR (p<0.0001, risk ratio 2.19) and DFS (p<0.0001, risk ratio 1.97). For RR, cytogenetic risk (p<0.0001, risk ratio 1.83) and status post course 1 (p=0.001, risk ratio 2.03) were also relative risks, however less powerful (Table 3.4).

### Table 3.3 Clinical outcome in FLT3/ITD- and FLT3/ITD+ patients

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>FLT3/ITD-</th>
<th>FLT3/ITD+</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>854</td>
<td>627</td>
<td>227</td>
<td></td>
</tr>
<tr>
<td>Complete remission</td>
<td>82%</td>
<td>84%</td>
<td>78%</td>
<td>0.05</td>
</tr>
<tr>
<td>Induction death</td>
<td>8%</td>
<td>7%</td>
<td>11%</td>
<td>0.04</td>
</tr>
<tr>
<td>Resistant disease</td>
<td>10%</td>
<td>9%</td>
<td>11%</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Outcome at 5 years (with SE)

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RR</td>
<td>49%</td>
<td>44%</td>
<td>62%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DFS</td>
<td>42%</td>
<td>46%</td>
<td>32%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EFS</td>
<td>35%</td>
<td>39%</td>
<td>25%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OS</td>
<td>41%</td>
<td>44%</td>
<td>35%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

P values are for Mantel Haenszel test for trend for CR and reasons for failure, and log rank test for outcome at 5 years. Figures in parentheses are standard errors.

### Table 3.4 Multivariate analysis of CR, RR, DFS, EFS and OS considering FLT3/ITD (yes or no) as the only FLT3 variable

<table>
<thead>
<tr>
<th>CR</th>
<th>RR</th>
<th>DFS</th>
<th>EFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=655</td>
<td>N=524</td>
<td>N=524</td>
<td>N=656</td>
<td>N=656</td>
</tr>
<tr>
<td>Cytogenetic risk (OR=4.78, p&lt;0.0001)</td>
<td>FLT3 (Risk R=2.19, p=0.0001)</td>
<td>FLT3 (Risk R=1.77, p&lt;0.0001)</td>
<td>Cytogenetic risk (Risk R=1.92, p&lt;0.0001)</td>
<td>Cytogenetic risk (Risk R=1.97, p&lt;0.0001)</td>
</tr>
<tr>
<td>AML M3 (OR=7.02, p=0.0001)</td>
<td>Cyto genetic risk (Risk R=1.83, p&lt;0.0001)</td>
<td>Status post course 1 (Risk R=1.97, p=0.0002)</td>
<td>FLT3 (Risk R=1.34, p=0.003)</td>
<td>Gender (Risk R=0.76, p=0.01)</td>
</tr>
<tr>
<td>Initial WBC (OR=0.30, p=0.0002)</td>
<td>Status post course 1 (Risk R=2.03, p=0.001)</td>
<td>AML M0 (Risk R=1.73, p=0.001)</td>
<td>Gender (Risk R=0.75, p=0.005)</td>
<td>Age (Risk R=1.01, p=0.01)</td>
</tr>
<tr>
<td>Gender (OR=0.30, p=0.004)</td>
<td>AML M3 (Risk R=0.52, p=0.01)</td>
<td>Cyto genetic risk (Risk R=1.54, p=0.001)</td>
<td>Initial WBC (Risk R=1.002, p=0.006)</td>
<td>FLT3 (Risk R=1.31, p=0.03)</td>
</tr>
<tr>
<td>Age (OR=1.03, p=0.01)</td>
<td>AML M0 (Risk R=1.66, p=0.01)</td>
<td>AML M3 (Risk R=0.59, p=0.01)</td>
<td>Age (Risk R=1.01, p=0.03)</td>
<td>Initial WBC (Risk R=1.002, p=0.03)</td>
</tr>
</tbody>
</table>

OR: odds ratios, Risk R: risk ratios
OR and RR are given in order of most to least important
Cytogenetic risk group was more significant than the presence of a FLT3/ITD for EFS (p<0.0001, risk ratio 1.92) and OS (p<0.0001, risk ratio 1.97), reflecting the additional influence of cytogenetics on the CR rate, but a FLT3/ITD still added prognostic information to the other prognostic factors (p =0.009 for OS and p= 0.002 for EFS). Figure 3.9 shows the relapse rate in patients with or without a FLT3/ITD grouped according to risk category. The Kaplan Meier curves clearly demonstrated that in all risk categories patients with a FLT3/ITD do worse compared to those without the mutation. However, the biggest difference in relapse rate was observed in patients with standard risk disease and FLT3/ITD as compared to those with standard risk disease and wild type gene (74% versus 48% at 5 years). This striking difference may have tremendous therapeutic implications as will be discussed later on in this chapter.

![Figure 3.9 Relapse rate in patients with or without a FLT3/ITD grouped according to risk category](image)

**3.6.8 Level of mutant and number of FLT3/ITD mutations**

Semi-quantitative radioactive PCR was carried out on 224 of the 227 FLT3/ITD+ samples. This was more sensitive than agarose gel electrophoresis...
at separating bands with small size differences. Moreover, this technique allowed the relative level of the mutant to be quantified.

3.6.8.1 Number of FLT3/ITD mutations in relation to demographics and clinical outcome

Semi-quantitative radioactive PCR showed that a significant proportion of the FLT3/ITD+ patients (51/224, 23%) had more than one FLT3/ITD mutation present (Figure 3.10). The majority of these patients (45/51, 88%) had two mutations but up to four were observed. In most patients one mutation was predominant and the other(s) were minor components, but in 15 patients the mutations were present in approximately equal proportions. There was a suggestion that patients with two or more mutations presented with a higher WBC (median WBC for patients with one FLT3/ITD 34.4 x 10^9/L, two or more 51.5 x 10^9/L, p=0.07) and a higher percentage of blasts in the BM at diagnosis (median 86% for patients with one FLT3/ITD, 90% for two or more, p=0.07) (Table 3.5). Patients with two or more mutations had a similar RR (64% at 5 years, SE 8.7) to those with a single mutation (64%, SE 4.6, p=0.6). There was, however, a suggestion that patients with two or more mutations had a worse OS, 36% (SE 3.7) at 5 years, for one mutation, 21% (SE 5.8) for two or more, p=0.04) (Figure 3.11). This was due to a significantly higher number of deaths in remission in those patients with two or more mutations (34% versus 13%, p=0.04), which may have occurred by chance.
3.6.8.2 Multivariate analysis of outcome including presence of a FLT/ITD and number of FLT3/ITD mutations as co-variables

Interestingly, when the number of mutations (0 versus 1 versus 2 or more) was added into the multivariate analysis as well as the presence or absence of the mutation, then the number of mutations replaced the latter as the most important independent variable predicting for RR ($p<0.0001$, risk ratio 1.86) and DFS ($p<0.0001$, risk ratio 1.59). In addition, the number of mutations
became the second most important factor predicting for EFS (p= 0.0007, risk ratio 1.32) and OS (p =0.0007, risk ratio 1.27), with cytogenetic risk being the most important factor predicting for EFS (p<0.0001, risk ratio 1.92) and OS (p<0.0001, risk ratio 1.98).

3.6.8.3 Relative level of mutant in relation to demographics and clinical outcome

The level of mutant detected relative to WT varied between 0.5% and 97% of total FLT3 signal (Figure 3.10). From previous evaluation of the relative intensity of X-chromosome alleles using a similar technique, it was found that the proportion of signal from one allele where there are two equally represented alleles was 54% ± 4% (Gale et al, unpublished observations). A technical cut-off of 60% (50% ± 2SDs) was therefore used to represent the presence of two abnormal alleles in a cell or the presence of a single mutant allele plus deletion of the other allele in at least some cells. Most patients (201/224, 90%) had <60% mutant. In a significant proportion of patients (59, 26%) the level was consistent with a heterozygous mutation in all or the majority of cells, i.e. 40 - 60% mutant (Figure 3.12). However, in 23 patients (10%) there was ≥ 60% mutant, suggesting bi-allelic mutation or loss of WT alleles. This was associated with an older age at diagnosis: the median age at presentation in the group with <60% mutant was 42 years and with ≥ 60% mutant 49 years, p=0.04 (Table 3.5). It was also associated with leukocytosis: median WBC at diagnosis was 35.2 x 10^9/L with <60% mutant, 63 x 10^9/L with ≥ 60% mutant (p=0.03), poorer cytogenetic risk group (p=0.03) and with FAB type M4 (p=0.02). No patients with ≥ 60% mutant had FAB type M3 (p<0.001). There was no difference in achievement of CR in the two groups, but there was a suggestion of worse outcome in patients with ≥ 60% mutant, with a marginally increased RR in this group [85% (SE 11.9) versus 62% (SE 4.3) at 5 years, (p =0.06)] (Figure 3.13). When the percentage mutation (0% versus <60% versus ≥ 60%) was added as a parameter to the multivariate analysis that already included the number of mutations, then no further prognostic information was obtained.
### Table 3.5 Clinical and demographic characteristics of patients grouped according to the number of FLT3/ITDs and the relative level of mutation

<table>
<thead>
<tr>
<th></th>
<th>One FLT3/ITD</th>
<th>More than one FLT3/ITD</th>
<th>P value</th>
<th>&lt;60% FLT3/ITD</th>
<th>&gt;60% FLT3/ITD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>One FLT3/ITD</td>
<td>( % of total with one FLT3/ITD)</td>
<td>( % of total with more than one)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>173</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAB type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>31 (19%)</td>
<td>8 (16%)</td>
<td>.8</td>
<td>33 (14%)</td>
<td>6 (25%)</td>
<td>.3</td>
</tr>
<tr>
<td>M2</td>
<td>33 (20%)</td>
<td>14 (27%)</td>
<td>.2</td>
<td>44 (22%)</td>
<td>5 (22%)</td>
<td>1.0</td>
</tr>
<tr>
<td>M3</td>
<td>45 (26%)</td>
<td>12 (24%)</td>
<td>.9</td>
<td>57 (28%)</td>
<td>0 (0%)</td>
<td>.008</td>
</tr>
<tr>
<td>M4</td>
<td>39 (23%)</td>
<td>11 (22%)</td>
<td>1.0</td>
<td>40 (20%)</td>
<td>10 (43%)</td>
<td>.02</td>
</tr>
<tr>
<td>M5</td>
<td>17 (10%)</td>
<td>3 (6%)</td>
<td>.6</td>
<td>18 (9%)</td>
<td>2 (9%)</td>
<td>1.0</td>
</tr>
<tr>
<td>M6</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>1.0</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Cytogenetic risk group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favourable</td>
<td>44 (25%)</td>
<td>12 (24%)</td>
<td>.3</td>
<td>56 (28%)</td>
<td>0 (0%)</td>
<td>.03</td>
</tr>
<tr>
<td>Intermediate</td>
<td>103 (61%)</td>
<td>27 (53%)</td>
<td></td>
<td>116 (58%)</td>
<td>16 (70%)</td>
<td></td>
</tr>
<tr>
<td>Adverse</td>
<td>2 (1%)</td>
<td>4 (8%)</td>
<td></td>
<td>6 (3%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>22 (13%)</td>
<td>8 (10%)</td>
<td></td>
<td>23 (11%)</td>
<td>7 (30%)</td>
<td></td>
</tr>
<tr>
<td>Age at entry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15</td>
<td>1 (1%)</td>
<td>1 (2%)</td>
<td>.8</td>
<td>2 (1%)</td>
<td>0 (0%)</td>
<td>.5</td>
</tr>
<tr>
<td>15-34</td>
<td>50 (29%)</td>
<td>15 (29%)</td>
<td></td>
<td>61 (30%)</td>
<td>4 (17%)</td>
<td></td>
</tr>
<tr>
<td>35-54</td>
<td>105 (61%)</td>
<td>30 (19%)</td>
<td></td>
<td>117 (58%)</td>
<td>18 (78%)</td>
<td></td>
</tr>
<tr>
<td>≥55</td>
<td>17 (10%)</td>
<td>5 (10%)</td>
<td>.3</td>
<td>23 (11%)</td>
<td>7 (30%)</td>
<td>.04</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>40 (23%)</td>
<td>7 (14%)</td>
<td>.1</td>
<td>46 (23%)</td>
<td>1 (4%)</td>
<td>.3</td>
</tr>
<tr>
<td>10-19</td>
<td>29 (17%)</td>
<td>5 (10%)</td>
<td></td>
<td>31 (15%)</td>
<td>3 (13%)</td>
<td></td>
</tr>
<tr>
<td>20-49</td>
<td>32 (19%)</td>
<td>10 (20%)</td>
<td></td>
<td>38 (19%)</td>
<td>4 (17%)</td>
<td></td>
</tr>
<tr>
<td>50-99</td>
<td>32 (19%)</td>
<td>12 (24%)</td>
<td></td>
<td>34 (17%)</td>
<td>10 (43%)</td>
<td></td>
</tr>
<tr>
<td>≥100</td>
<td>39 (23%)</td>
<td>14 (27%)</td>
<td>.07</td>
<td>48 (24%)</td>
<td>5 (22%)</td>
<td>.03</td>
</tr>
<tr>
<td>Median</td>
<td>34.4</td>
<td>51.5</td>
<td>.07</td>
<td>35.2</td>
<td>63.0</td>
<td>.03</td>
</tr>
<tr>
<td>% BM blasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;80%</td>
<td>54 (31%)</td>
<td>10 (20%)</td>
<td>.2</td>
<td>56 (28%)</td>
<td>8 (35%)</td>
<td>.6</td>
</tr>
<tr>
<td>≥80%</td>
<td>104 (60%)</td>
<td>33 (65%)</td>
<td></td>
<td>123 (61%)</td>
<td>14 (61%)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>86</td>
<td>90</td>
<td>.07</td>
<td>86</td>
<td>87.5</td>
<td>.5</td>
</tr>
</tbody>
</table>

P values are Mantel Haenszel test for the trend in cytogenetic risk, age, WBC, % BM blasts and Fisher's exact test for each individual FAB type versus all other known FAB types.

![Figure 3.12 Level of mutant FLT3/ITD (% of total FLT3)](image-url)
3.7 Discussion

At the time this work was initiated, 2 studies had been published showing the prognostic significance of a FLT3/ITD mutation in adult patients with AML. The first report by Kiyoi et al (1999) of 201 adult patients with newly diagnosed AML except M3, showed that the incidence of a FLT3/ITD was 22.8% and demonstrated, in a multivariate model analysis, that a FLT3/ITD was an independent prognostic factor predicting for OS in patients below the age of 60 years. The second study a year later by Rombouts et al (2000) of 81 patients with AML confirmed the increased relapse risk in patients with FLT3/ITD, indicating the prognostic importance of this mutation.

The present study was conducted on a homogeneous group of adult patients below the age of 60, where 93% of patients had de novo AML and were treated with 2 similar protocols according to the UK MRC 10 and 12 trials. It aimed to explore possible associations of the mutation with adverse clinical outcome, and at the same time to look at the biological, molecular and clinical features associated with the mutation. The incidence of FLT3/ITD mutations was found to be 27%, which was marginally higher than that found in the Japanese (23%) (Kiyoi et al, 1999) and Dutch (22%) (Rombouts et al, 2000) studies. It is
possible that all studies based on DNA banks slightly overestimate the true incidence of FLT3/ITD mutations as there may be a bias towards availability of DNA from patients with higher peripheral WBC. In addition, 19% of patients had FAB type M3, which had the highest incidence of FLT3/ITD mutations (36%) of any FAB type. Indeed, the proportion of M3 cases in this study (19%) slightly exceeds the expected incidence of about 15% in this age group, reflecting the thoroughness of DNA collection in this disease subtype. Despite these minor reservations, it is clear that FLT3/ITD mutations are the most common single mutation described in AML to date.

Certain biological and clinical features associated with the presence of a FLT3/ITD were identified. The incidence of the mutation varied across different FAB types with the highest incidence found in AML M3 (36%, p=0.004). Conversely, a low incidence was observed in AML M6 (1/15 patients, p=0.08), although the number of patients studied was small. FLT3/ITD positive cases were not found either in 14 patients with AML M0 (p=0.02) or in 11 patients with AML M7 (p=0.04). The significance of this variation among different FAB types remains obscure and no explanation can be given why the mutation occurs more frequently in certain FAB types and not others. Nevertheless, the most interesting variation in the frequency of the mutation was found in different cytogenetic abnormalities. High incidence (37%) was observed in patients with t(15;17) while the frequency in core binding factor leukaemias was low, 9% and 7% in t (8;21) and inv(16) respectively. High incidence was also found in patients with normal cytogenetics (34%) and in patients with standard cytogenetic risk disease (30%), as defined by MRC criteria (p=0.0001 for both). This observation may have important clinical implications as patients with standard risk disease represent 1/3 of newly diagnosed cases of AML. Furthermore, the lack of additional prognostic factors in this group of patients may well define FLT3/ITD+ patients as a distinct subgroup with adverse clinical outcome, where more intensive treatment may be needed compared to FLT3/ITD- patients.
At the time of writing there are more than 25 published series reporting incidence of FLT3/ITD mutations in adult, paediatric and elderly patients with AML (Table 3.6). Several additional studies have been reported in abstract form. The results appear relatively diverse, as certain factors such as age and cytogenetic risk subgroup differ from study to study. For example, the incidence of the mutation in adult patients ranges from 13% to 34%. However, there are a number of common features which are evident in most studies. The incidence of the mutation is higher in patients with t(15;17), normal cytogenetics and standard risk disease, and significantly lower in patients with core binding factor leukaemias and poor risk cytogenetics. In paediatric patients the incidence varies between 4.3% and 16.5% and there is a trend to increase with age as it has been demonstrated in 2 different studies (Zwaan et al, 2003; Liang et al, 2002). Finally, incidence of 33.6% and 18% has been reported in elderly patients with AML (Stirewalt et al, 2001; Andersson et al, 2004).

The presence of a FLT3/ITD mutation was not found to predict for the attainment of CR, which is in accord with the study by Kiyoi et al (1999), although not the Dutch (Rombouts et al, 2000) study. The lack of effect on the CR rate may be because a FLT3/ITD mutation does not significantly affect chemosensitivity of the majority of blast cells present at diagnosis, although it must be noted that as the vast majority of patients achieve CR during induction chemotherapy, any subtle effects on chemosensitivity might not be clinically apparent as determined by the CR rate. Subsequently, the lack of effect on CR rate has been confirmed in most studies in adult patients with AML. In contrast, almost every paediatric study has shown FLT3/ITD to affect adversely the CR rate (Kondo et al, 1999; Meshinchi et al, 2001; Zwaan et al, 2003).

The presence of a FLT3/ITD mutation did, however, have a major impact on long-term outcome in the cohort of 854 patients studied. In particular, it was the most important factor predicting for relapse from CR (p<0.0001), and in the presence of a FLT3 mutation the actuarial RR at 5 years was 64% (SE 4.1), compared to 44% (SE 2.4) in patients without a mutation. The relative effect of FLT3/ITD on RR was not found to differ significantly between risk groups.
Table 3.6 Incidence and frequency in different cytogenetic subgroups in adult and paediatric AML patients with a FLT3/ITD mutation

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of patients</th>
<th>Incidence of FLT3/ITD (%)</th>
<th>Frequency in different cytogenetic subgroups</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unspecified</td>
<td>201</td>
<td>22.8</td>
<td>Kiyoi et al (1999)</td>
<td></td>
</tr>
<tr>
<td>74 (all M3)</td>
<td>20.3</td>
<td></td>
<td>Kiyoi et al (1997)</td>
<td></td>
</tr>
<tr>
<td>90 (all M3)</td>
<td>37.0</td>
<td></td>
<td>Noguera et al (2002)</td>
<td></td>
</tr>
<tr>
<td>979</td>
<td>20.4</td>
<td>t(8;21): 5%</td>
<td>Thiede et al (2002b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>inv(16): 2.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>t(15;17): 31%, P=0.001</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Normal karyotype: 29.6%, P=0.001</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>t(6;9): 90%, P=0.01</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Complex karyotype: 2.2%, P=0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1003</td>
<td>23.5</td>
<td>t(8;21): 8.7%, P=0.006 Absent in inv(16), P=0.0002</td>
<td></td>
<td>Schnittger et al (2002b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t(15;17): 35.3%, P=0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11q23: 0.4%, P=0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complex karyotype: 2.5%, P&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100*</td>
<td>26.0</td>
<td></td>
<td>Kainz et al (2002)</td>
<td></td>
</tr>
<tr>
<td>&lt; 60 years</td>
<td>81</td>
<td>22.2</td>
<td>Rombouts et al (2000)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>112</td>
<td>20.0</td>
<td>Yokota et al (1997)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82**</td>
<td>28.0</td>
<td>Whitman et al (2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>523</td>
<td>23.0</td>
<td>Frohling et al (2002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>t(8;21): 11%</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>inv(16): 2%</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>t(15;17): 39%</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>11q23: 7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 60 years</td>
<td>140</td>
<td>34.0</td>
<td>Stirewalt et al (2001)</td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>94</td>
<td>5.3</td>
<td>Iwai et al (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>13.8</td>
<td>Xu et al (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>11.0</td>
<td>Kondo et al (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>16.5</td>
<td>Meshinchi et al (2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>11.3</td>
<td>Liang et al (2002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>234</td>
<td>11.5</td>
<td>Normal karyotype: 54.5% P=0.003</td>
<td>Zwaan et al (2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11q23: 0.9%, p=0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 (non M3)</td>
<td>22.2</td>
<td></td>
<td>Arrigoni et al (2003)</td>
<td></td>
</tr>
<tr>
<td>29 (all M3)</td>
<td>34.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All intermediate and favourable risk disease; ** All normal karyotype

The effect was most reliably demonstrable in the intermediate risk group, whether defined by cytogenetics alone or by the combination of cytogenetics and response to the first cycle of induction chemotherapy, which is the very
group in whom improved prognostic stratification is most required. In fact, when relapse risk was analysed in patients with standard risk disease, it was found to be 74% in FLT3/ITD patients as compared to 48% in those without the mutation (Figure 3.9). Interestingly, the relapse risk in patients with standard risk disease and a FLT3/ITD was similar to that observed in patients with poor risk disease, suggesting the possibility of treating these patients with more experimental therapies. In accord with being the major factor predicting for relapse, the presence of a FLT3/ITD was also the major factor predicting for DFS (p<0.0001). Furthermore, it had an adverse effect on EFS (p<0.001) and OS (p<0.001) (Figure 3.8).

Cox multivariate analysis was performed for RR, DFS, EFS and OS, the variables considered being the presence of a FLT3/ITD mutation, the cytogenetic risk group, presentation WBC, percentage BM blasts at diagnosis, age, sex, FAB type and the response status post course 1 of induction chemotherapy. The analysis showed that a FLT3/ITD was the most significant independent factor predicting for risk of relapse (p<0.0001, risk ratio 2.19) and for DFS (p<0.0001, risk ratio 1.97). This finding has now been confirmed by several studies, which agree that the presence of a FLT3/ITD is associated with increased risk of relapse and reduced DFS and EFS (Kiyoi et al, 1999; Rombouts et al, 2000; Frohling et al, 2002). Since patients with AML who relapse have limited chances for salvage, OS in these patients will also be affected.

However, two studies are of particular interest as they failed to show a FLT3/ITD as an independent prognostic factor predicting for disease outcome. In a study of 640 patients, Thiede et al (2002) found that, despite an increased RR and reduced DFS, there was only a trend for reduced OS with an ITD (p=0.09). Similarly, Schnittger et al (2002) reported that an ITD affected EFS in their study of 563 patients, but not OS. The difference in outcome in these 2 studies may have been influenced by a number of factors such as the short follow up of less than one year, and the heterogeneity of patients studied with respect to age and type of disease (de novo and secondary AML and antecedent MDS). They do, however, raise the possibility that different treatment regimens
might have contributed towards a better outcome in their patients, especially the inclusion of very high dose cytarabine. In the present study, patients were treated according to the UK MRC protocols with a cumulative dose of cytarabine up to 11.6g/m², where in the 2 studies mentioned above, the cumulative cytarabine dose was up to 8 fold higher, ranging from 22.4 to 92.8g/m². Nevertheless, this may depend on the subgroup of AML as Frohling et al (2002) found that intensive chemotherapy, including post-remission cytarabine up to doses of 42g/m², was of no benefit in FLT3/ITD patients with normal cytogenetics. This group also fared badly in the study by Boissel et al (2002) comparing 3 different reinforced induction regimens, although they did find some suggestion of drug intensification improving outcome in FLT3/ITD patients. It is clear that evaluation of treatment protocols during the induction or consolidation phase in patients with a FLT3/ITD requires further investigation, preferably prospective within randomised trials, in order to further refine risk-adapted therapy.

The observation that 23% of FLT3/ITD+ patients had more than one FLT3/ITD mutant of varying levels, suggests that, at least in some patients, the mutations were present in different clones. Patients with two or more mutants did not have a significantly higher RR than those patients with just one mutation (p=0.6). Patients with more than one mutation had a significantly higher mortality in remission (p=0.04) and this translated into a marginally reduced EFS (p=0.07) and OS (p=0.04). The reason why patients with more than one FLT3/ITD should have had a higher treatment-related mortality is not known. It is possible that patients who develop FLT3/ITD mutations may have other defects in their DNA repair mechanisms and fail to tolerate the deleterious effect of cytotoxic chemotherapy. Nevertheless, this might be a spurious association due to the play of chance. On a multivariate model where presence of the mutation and the number of mutations (none, one or more than one) were considered as co-variables, the latter replaced the former as the most powerful predictor of RR and DFS. This observation is difficult to explain and a number of possible explanations can be hypothesised. The number of mutations may have a “dosing effect” on relapse risk, exerting the greatest functional effect on the small population of clonogenic cells presumed to be responsible for
leukaemic relapse. Alternatively, the development of additional ITDs may take place in a cell which has already acquired a malignant phenotype, implying secondary evolutionary mechanisms, that may develop clones resistant to chemotherapy. Irrespective of mechanism, this finding will need further exploration and confirmation in order to be able to be incorporated into clinical practice.

In addition to the frequent detection of more than one FLT3/ITD mutation, quantitative analysis demonstrated that the level of mutant was ≥ 60% of the total FLT3 signal in approximately 10% of patients with a FLT3/ITD. This finding implies either bi-allelic mutations or deletion of WT alleles, however, this 10% value is undoubtedly an underestimate, as the presence of residual normal cells in some samples (especially from PB) would tend to decrease the ratio of the mutant to WT FLT3 allele. Subsequent analysis revealed that there was a suggestion of higher RR in those patients with ≥ 60% mutant (p=0.06), but in a multivariate analysis that already included the number of mutations, the presence of ≥ 60% mutant was not an independent prognostic factor. This finding has also been explored by 2 different groups (Whitman et al, 2001; Thiede et al, 2002). In a study by Whitman et al (2001) of 8/23 FLT3/ITD+ AML cases lacking a FLT3 WT allele (FLT $^{ITD^-}$) the outcome was worse as compared to FLT3/ITD cases carrying a WT allele (FLT $^{ITD^+/WT}$) (P=0.008). In another large study, patients with a mutant to wild type ratio of 0.78 or greater were noted to have a significantly worse outcome than FLT3/ITD+ patients with a ratio less than 0.78 (Thiede et al, 2002). The mechanism causing the altered ratio is attributed in some cases to loss of heterozygosity (LOH) (Whitman et al, 2001) but this has not been a consistent finding. Thiede et al (2001) were unable to detect LOH of the WT allele using FISH assays in 9 patients with FLT3/ITD, suggesting that an increased ITD/wild type ratio might be due to homologous recombination of the FLT3/ITD allele, which essentially creates more copies of the FLT3/ITD allele than of the wild type allele. Since a mutant FLT3 in a mutant/WT heterodimer can trans-phosphorylate the WT chain, it implies that a mutant homodimer has some gain-of function more than simply activating the kinase. Alternatively, formation of the homodimer may
reflect an underlying mechanism of genetic instability which has other unknown genomic consequences that may, in turn, influence clinical outcome. In AML, relapse remains the principal cause of treatment failure for the majority of patients. The outcome following relapse is extremely poor and only a small fraction of patients can be salvaged. The fate of patients with a FLT3/ITD at presentation who relapse remains unknown. This study, which aimed to investigate the significance of FLT3/ITD in newly diagnosed patients with AML, was not able to explore their outcome post relapse.

In conclusion, in this large cohort of patients, the presence of a FLT3/ITD is the most common molecular abnormality in AML. The mutations are especially common in t(15;17) and patients with normal cytogenetics but relatively rare in core-binding factor leukaemias and those cases with poor cytogenetics. Knowledge of how FLT3/ITD interacts with other cytogenetic abnormalities might provide biological insight into leukaemogenesis and clarify the uncertainties of risk stratification. FLT3/ITD is an important factor predicting for relapse and DFS and is, as a consequence, an independent risk factor for EFS and OS. The laboratory test to identify the presence or absence of a FLT3/ITD mutation is simple, quick and reproducible. The present results suggest that even further prognostic stratification may be achieved by taking into account the number of mutations and the relative ratio of the mutant and WT alleles. Taken together, these results suggest that FLT3 mutations confer an adverse outcome and this information should be used prospectively in order to define effective therapeutic strategies in patients with AML.
Chapter 4

4.1 Introduction

Despite the progress in the treatment of AML, unfortunately, in many patients a temporary phase of clinical remission is followed by relapse. Indeed, approximately 70% of patients relapse, mainly within the first 2 years of completion of treatment, and die of refractory disease, with the probability of 3-year survival ranging between 8% and 29% (Leopold LH & Willemze R, 2002). Chemotherapy has increased in intensity in recent years but is perceived to have reached the limit of toxicity. Allogeneic BMT, either human leukocyte antigen (HLA)-sibling matched or matched unrelated donor, is the only treatment to offer long-term DFS and possible cure, however the increased toxicity associated with the procedure restricts its use to those patients who are young and fit to tolerate such approaches (Drobyski, 2004). Nevertheless, relapse remains a major cause of failure, even in those patients who receive a BMT and especially for those with poor risk disease features. At present, little is known about the kinetics of leukaemia relapse, the development of drug resistance and other host factors that might affect the progression of the disease (Ferrant et al, 1997). It is thought that in leukaemia the estimated tumour burden of $10^{12}$ malignant cells may still be as high as $10^{10}$ after achievement of CR. This so-called minimal residual disease (MRD) remains morphologically undetectable as microscopic analysis identifies, at best, a sensitivity of 1-5 leukaemic cells in 100 normal cells. Considerable effort has therefore been directed at identifying markers that can be used to detect residual disease at an earlier stage and lead to therapeutic intervention before overt haematological relapse occurs. This technology is becoming increasingly applicable, not only during induction chemotherapy, but also during consolidation and post-BMT. In clinical practice, MRD evaluation is emerging as an integral part of the modern management of patients with leukaemia.
4.2 Use of MRD in leukaemia

The first MRD studies in patients with leukaemia were carried out soon after antibodies for leukocyte differentiation antigens became available. Expression of the common antigen (CD10) and terminal deoxynucleotidyl transferase (TdT) in ALL cells suggested that these antigens could potentially be used as markers to identify persistent leukaemia. Similarly, expression of TdT with T-lymphoid antigens in T-ALL allowed the use of this combination of markers to detect MRD in T-ALL (Janossy et al, 1980; Bradstock et al, 1981). Since then, several methods to study MRD have been developed and the most reliable include flow cytometric profiling of aberrant immunophenotypes, PCR amplification of fusion transcripts, and amplification of antigen receptor genes.

4.3 Methodologies for detecting MRD

4.3.1 Conventional cytogenetics

Karyotypic analysis at the time of diagnosis is an essential test, as it allows the detection of structural and numerical changes specific to the leukaemic clone. The identification of clonal abnormalities can also provide valuable information related to disease prognosis. However, its value for the detection of MRD is limited by its insensitivity. This is due to the relatively small number of cells examined, inadequate chromosome morphology, and the lack of metaphases.

4.3.2 Fluorescence in situ hybridisation (FISH)

FISH is an advantageous technique compared to conventional cytogenetics due to its enhanced power to detect structural rearrangements which may be missed with standard banding methods. It requires chromosome specific or locus specific probes and can detect aberrations in non-dividing interphase cells. However, the sensitivity of the technique approaches 1%, which is markedly below that desired for MRD detection.
4.3.3 Flow cytometry

MRD monitoring using flow cytometry is based on the observation that leukaemic cells frequently display aberrant phenotypic features that allow their distinction from normal cells. Cells labelled with a panel of fluorescent antibodies can be detected and quantified by a fluorescence activated cell sorter (FACS). The advantages of flow cytometric analysis include its relatively high sensitivity for detecting leukaemic cells, ranging from 1 leukaemic cell in 100 to 10,000 normal cells, its ability to analyse a large number of cells and its rapidity. However, optimisation of the technique requires the use of several combinations of antibodies and technical expertise.

4.3.4 Nucleic acid amplification techniques

Breakpoint fusion regions of chromosomal aberrations, aberrant genes, and rearranged immunoglobulin and T-cell receptor genes have been used with success in molecular studies of MRD. PCR is highly sensitive, detecting up to 1 leukaemic cell in 1,000,000 normal cells, however it remains technically demanding and susceptible to false positives due to contamination. Initial studies of MRD relied on the RT-PCR assay being either positive or negative. However, it has become apparent that estimation of the level of MRD by quantitating target genes can provide a more sensitive tool for monitoring MRD. Quantification of the level of transcripts of a target gene can be carried out either by competitive RT-PCR or the cycle-cycle (real time) techniques. Competitive RT-PCR involves adding a known amount of PCR-amplifiable standard into an RNA sample and then amplifying the standard and target RNAs in the same reaction. The exogenous standard and the endogenous target use the same primers for amplification, thus a competition for amplification components including primers, dNTPs, and polymerase ensues. If the exogenous standard is designed to be amplified at the same rate as the target sequence, then the ratio of products obtained from the endogenous and exogenous targets at the end of the amplification reflects the initial
ratio of target to standard. Since the amount of exogenous standard added to the RT-PCR is known, the amount of endogenous target in the RNA sample can be determined. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (Livak et al, 1995). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

4.4 Application of MRD in ALL

In ALL and generally in malignancies of lymphoid origin, clonal rearrangements of either immunoglobulin or T-cell receptor genes are suitable for MRD analysis (Goulden et al, 2001). They occur in up to 90% of patients, can be detected using PCR techniques, and reappear at relapse, although development of secondary rearrangements has been noted in some cases (Cave et al, 1998). Early studies investigating the clinical relevance of MRD in ALL produced conflicting results. This was largely because of poor study design, however prospective blinded studies have provided much greater consensus, demonstrating conclusively the clinical significance of MRD. In these studies, MRD during conventional chemotherapy was found to be an independent predictor of outcome, as the presence and level of residual leukaemia correlated with the risk of early relapse (Cave et al, 1998). In the BMT setting in children with ALL, MRD detection by PCR amplification of antigen receptor genes immediately before conditioning was also predictive of subsequent relapse. For example in the study by Knechtli et al (1998) of 39 children who underwent a T-cell depleted allogeneic transplant for ALL, the detection of high level MRD (> than 1 leukaemic cell in 1000 normal cells) before transplantation was associated with 0% EFS at 2 years, whereas the EFS for those who had undetectable levels of MRD was 73% (p<0.001) (Knechtli et al, 1998). In ALL therefore, the collective evidence for a correlation between presence of MRD
and increased risk of relapse is very strong and several protocols have recently incorporated this knowledge into treatment adapted therapy. For example, children who remain strongly positive for MRD after induction or consolidation therapy may well benefit from transplantation approaches while in morphologic remission, rather than await overt haematological relapse and transplantation in 2nd CR.

4.5 Application of MRD in myeloid malignancies

4.5.1 CML

The BCR/ABL oncoprotein is a constitutively activated tyrosine kinase which now considered to be the principal cause of the chronic phase of CML. Monitoring of the BCR/ABL fusion protein using approaches such as FISH or RT-PCR has been used to correlate this marker with disease status and outcome, especially after BMT (Serrano et al, 2000; Hochhaus et al, 2000; Olavarria et al, 2001).

4.5.2 AML

In AML, cytogenetic analysis, FISH, multiparametric flow cytometry and PCR are currently being used for detecting MRD. Conventional cytogenetics can only be used in patients with numerical and structural abnormalities to assess remission status post induction chemotherapy. An additional benefit of this technique is its ability to detect the acquisition of new clonal aberrations at disease progression. However, as mentioned above, the value of cytogenetics as marker of MRD is very limited due to the insensitivity of the technique. MRD monitoring using flow cytometry in AML has extensively been applied in recent years. Although studies of MRD are still limited as compared to ALL, several studies have been published demonstrating that this technique is a useful approach for predicting outcome in patients with AML treated with intensive regimens (San Miguel et al, 1997; Venditti et al, 2000). The study of PCR based techniques is limited by the availability of suitable molecular markers. Three molecular markers that have been used are the PML/RARα fusion transcript.
arising from the t(15;17) translocation, AML1/ETO arising from t(8;21) and CBFB/MYH11 from inv(16) (Lo Coco et al, 1999; Tobal & Yin 1996; Marcucci et al, 2001; Laczika et al, 1998). However, the qualitative detection of MRD does not necessarily indicate subsequent relapse (Kusec et al, 1994, Tobal et al, 1995) and therefore quantification of the tumour burden is necessary. For example, it has been shown that patients who are positive for AML1/ETO using sensitive RT-PCR assays can remain in morphological and clinical remission for many years. Persistence of the hybrid gene has been detected not only in patients treated with chemotherapy but also in recipients of autologous or allogeneic BMT (Kusec et al, 1994; Jurlander et al, 1996). Nevertheless, reappearance of the marker, or persistence at a certain level post-induction chemotherapy, does generally signify an increased risk of relapse (Diverio et al, 1998; Tobal et al, 2000).

In the BMT setting, recent studies have also suggested a role for molecular monitoring in AML. MRD monitoring in patients who undergo an autologous or allogeneic BMT has demonstrated that the presence of residual disease 3 months post-transplant is predictive of relapse (Meloni et al, 1997; Roman et al, 1997). For example, 2 cases of APL with evidence of residual disease post ABMT were successfully treated with ATRA, maintaining continuous morphological and molecular remission even after discontinuation of retinoic acid treatment (Grimwade et al, 1998b). The successful eradication of residual disease in these patients suggests that MRD monitoring should be routinely performed post-transplant in order to identify patients who would be suitable for tailored therapeutic approaches including donor lymphocyte infusion (DLI) and molecularly targeted strategies.

However, to date, t(8;21), t(15;17) and inv(16) together account for approximately one quarter of adult AML patients, (Grimwade et al, 1998a) and therefore other markers are required to extend the potential application of MRD to more AML patients. Recent studies have suggested that expression of the Wilms Tumour gene (WT1) gene might be used as a marker of MRD in AML. Using quantitative analysis, the WT1 expression is at least 10 times lower in normal progenitor cells compared with leukaemic cells (Inoue et al, 1996; Inoue
et al, 1997). Several studies using a qualitative assay for WT1 in AML found no correlation between WT1 expression at diagnosis and achievement of CR. For example, in a study by Gaiger et al (1998) of 44 patients, there was no difference in the DFS and OS from remission between WT1-positive and negative patients (p>0.1). The authors concluded that the monitoring of WT1 gene expression by qualitative RT-PCR during treatment and CR is of very limited value. In contrast, more recent data suggest that using sensitive quantitative assays, WT1 can be used as a marker of MRD in order to predict patients with AML at early risk of relapse. However, the clinical use of WT1 as a marker of MRD in AML will need to be evaluated in prospective studies involving large number of patients. This knowledge has currently being incorporated into the MRC 15 trial, where the value of quantitative monitoring by REAL time PCR will be assessed prospectively.

As mutations in the FLT3 gene occur in about one third of adult AML patients, the studies in this chapter explored their suitability as markers for MRD, looking for the presence of ITDs and D835 alterations in paired samples from patients at presentation and relapse.

4.6 Materials and Methods

4.6.1 Patients

Paired PB or BM samples at presentation and first relapse were available from 44 patients with de novo AML who were treated with protocols from the MRC AML 10 and 12 trials. A further sample at second relapse was obtained from one patient. Eleven of these patients had AML FAB type M1, 7 M2, 5 M3, 13 M4, 7 M5 and 1 patient had RAEB-t. Median age at presentation was 42 years (range 12 - 61 years), and median white cell count was 50.1 x 10⁹/L (range 0.8 - 541 x 10⁹/L). DNA samples were used for analysis in 42 patients, in the remaining 2 patients only RNA samples were available. Paired DNA samples from BM at presentation and first CR following induction chemotherapy were also studied in 13 AML patients who all had a mutation in the FLT3 gene at diagnosis.
4.6.2 Detection of mutations in the FLT3 gene

4.6.2.1 PCR analysis of the FLT3/ITD mutation

PCR and semi-quantitative PCR analysis of the FLT3/ITD mutation was performed as described in Chapter 2.4, 2.5 and 3.4.1. DNA from the cell line TF-1 was used as the wild type control.

4.6.2.2 PCR analysis of the FLT3/D835 mutation and restriction enzyme digestion

PCR analysis was performed as described in Chapter 2.4. Amplification of exon 20 of the FLT3 gene was performed using the primers: 19F, 5'-CCGCCAGGAACGTGCTTG-3', and 19R, 5'-GCAGCCTCACATTGCCCCC-3', as previously described by Yamamoto et al (2001). Amplified products were digested with EcoRV (Invitrogen, UK) for 4 hours at 37°C, then electrophoresed through 4% agarose gels and visualized under UV light with ethidium bromide staining. D835 and I836 amino acids are encoded by GATATC, which is the recognition sequence for EcoRV. In alleles containing a D835 or I836 mutation the 114bp PCR fragment remained uncut but in WT alleles it was digested to fragments of 68 and 46bps (Figure 4.1).
4.6.3 Polymorphic marker analysis in order to confirm patients’ identity in samples taken at presentation and relapse

Tandemly repeated DNA sequences are widespread throughout the human genome and show sufficient variability among individuals. These tandemly repeated regions of DNA are typically classified into several groups depending on the size of the repeat region. Minisatellites (variable number of tandem repeats, VNTRs) have core repeats with 9-80 bp, while microsatellites (short tandem repeats, STRs) contain 2-5 bp repeats. The variety of alleles present in a population is such that a high degree of discrimination among individuals in the population may be obtained when multiple STR loci are examined. For the purpose of this study, in cases where differences were observed in the pattern of FLT3 mutations at presentation and relapse, 3 polymorphic markers were used to confirm that the DNA samples were from the same patient: KB9 on chromosome 19, D21S270 and D21S65 on chromosome 21 (Preudhomme et al, 2000; Horwitz et al, 1999). One primer from each pair (Table 4.1) was $^32$P end-labelled and semi-quantitative PCR performed as described in Chapter 2.5. The annealing temperatures were 58°C for D21S270 and D21S65, and 65°C for KB9. Products were electrophoresed through denaturing polyacrylamide gels.
(7M urea, 6% polyacrylamide, crosslinker ratio 37.5:1, 0.5 x Tris-Borate-EDTA), the gels were dried and exposed to Hyperfilm (Amersham).

Table 4.1. Oligonucleotide primers used in PCR analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB9</td>
<td>5'-TGCAAGGGCTTGGAGGCTGATG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ATCTCGACAACAGGTCCTTG-3'</td>
</tr>
<tr>
<td>D21S270</td>
<td>5'-GAATGTTTTAATAATGGTGTTA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ACAAAAAGTTATGGTCAGG-3'</td>
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<tr>
<td>D21S65</td>
<td>5'-CCGAAAACCTTACTGGAACAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GATCATCCAGGAATCCAAA-3'</td>
</tr>
</tbody>
</table>

4.6.4 Cloning and sequencing of PCR products

This was performed as described in Chapter 2.6 and 2.7.

4.7 Results

4.7.1 Paired presentation/remission samples

Remission samples (marrow n=9, peripheral blood n=4) from 13 patients who had a FLT3 mutation at presentation were studied. All 13 patients had an ITD, median mutant level 44% of total FLT3 (range 2% - 90%), and 1 patient also had a D835 mutation (D835Y). In remission all patients lost their mutation(s) confirming that FLT3 mutations are leukaemia-specific.

4.7.2 Paired presentation/relapse samples

4.7.2.1 Patients without a FLT3 mutation at presentation

Of the 24 patients studied who had only WT FLT3 alleles at presentation, 20 patients remained WT at first relapse and 4 patients acquired a FLT3 mutation (Table 4.2). Two of these patients acquired a D835 mutation, both G → T leading to substitution of tyrosine for aspartate, and 2 patients gained a
FLT3/ITD. The D835 mutant accounted for approximately half of the total FLT3 in the samples, and in the patients with ITDs the mutant levels were 38% and 42% respectively, suggesting that the majority of cells at relapse were heterozygous for the mutant alleles. No ITD was detected in presentation samples from the latter 2 patients using the more sensitive radioactive PCR and all 4 patients had high blast counts at diagnosis (patients 21-24, Table 4.2). These results suggested that a FLT3 mutation had not been missed at diagnosis and that its acquisition at relapse was evidence of clonal progression. In 3 of these 4 patients, sufficient DNA was available at both presentation and relapse to study the alleles at 3 loci known to be polymorphic for repeat sequences. The size of the alleles was the same at presentation and relapse, confirming that the 2 samples came from the same individual. One patient who had only WT FLT3 alleles at presentation and first relapse acquired a FLT3/ITD at second relapse with a mutant level of 45%. Polymorphic markers indicated that all 3 samples were from the same patient.
Table 4.2. FLT3 status and relative percentage of mutant in peripheral blood or bone marrow from 44 AML patients at presentation and relapse

<table>
<thead>
<tr>
<th>PRESENTATION</th>
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</tr>
<tr>
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<td>WT</td>
</tr>
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<td>WT</td>
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</tr>
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<td>44**</td>
<td>ITD+</td>
</tr>
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MNC indicates mononuclear cells; BM, bone marrow; PB, peripheral blood; NA, not available
* Information in brackets relates to second relapse
** Different mutations were detected at presentation and relapse

4.7.2.2 Patients with a FLT3 mutation at presentation

Twenty patients were studied who had a FLT3 mutation at presentation, 18 were ITD+ with median mutant level 28% (range 5% - 91%) and 2 had D835Y mutations at about the 50% mutant level (Table 4.2). The median time to relapse for these 20 patients with a FLT3 mutation at presentation was 218 days (range 38 – 716), which was significantly less than in those patients who had not had a
FLT3 mutation at presentation ($P = 0.008$, Student's t-test). Five patients (25%) lost their FLT3 mutation at relapse. All had presented with an ITD and the mutant levels were 6%, 11%, 20%, 28% and 44% respectively. Polymorphism analysis demonstrated that the paired samples were from the same individual in 4 cases from whom DNA samples were available. Representative gels from two patients are shown in Figure 4.2. Only RNA was available in the remaining patient. The loss was confirmed by radioactive PCR. In 4 of the 5 patients, the percentage of blast cells was similar in the presentation and relapse samples (#25-28, Table 4.2) and therefore it is unlikely that the mutation had been missed at relapse. In the remaining patient (#29), the blast cell count was lower at relapse (44%) than at presentation (95%). However, at diagnosis all cells appeared to be heterozygous for the mutation (mutant level 44%), and therefore, even with the lower blast cell count, a heterozygous mutation would still have been well within the limits of detection of the technique.

<table>
<thead>
<tr>
<th>Marker</th>
<th>KB9</th>
<th>D21S65</th>
<th>D21S270</th>
</tr>
</thead>
<tbody>
<tr>
<td>Status</td>
<td>P</td>
<td>R</td>
<td>P</td>
</tr>
<tr>
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<td>Pt 2</td>
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</table>

Figure 4.2 Polymorphic marker analysis in 2 AML patients at presentation (P) and relapse (R). Both patients presented with a FLT3/ITD which was lost at relapse

Fifteen patients had a FLT3 mutation at both presentation and relapse. Two had D835Y mutations at both stages of their disease and 13 had ITDs. Sequencing and semi-quantitative PCR showed that 6 patients relapsed with the same ITD at approximately the same level (#32-37, Table 4.2). The median difference between presentation and relapse in the level of mutant was 0% (range -3% to +3%). Three of these patients had levels of mutant which were consistent with
homozygosity or hemizygosity in most blast cells at both presentation and relapse (#35-37). Six patients relapsed with the same ITD but at an increased level, where the median difference between presentation and relapse in the level of mutant was 29.5% (range 13.5% to 45%) (#38-43, Table 4.2). In 1 of these patients, 3 different ITDs were detected at diagnosis (mutant levels 28%, 3%, 1% respectively), but only the predominant mutation was present at relapse (#42, Table 4.2). Another patient (#41, Table 4.2) had 2 different ITDs at diagnosis, mutant levels 26% and 2%, and both were increased at relapse, 56% and 14% respectively. The remaining patient who was ITD+ at presentation, mutant level 44%, relapsed with a different ITD, mutant level 60% (#44, Table 4.2). The relapse mutation was not detectable in the presentation sample using radioactive PCR analysis. At presentation the ITD was 33 bps, nucleotides 36503 - 36535 from the DNA sequence (Genbank accession number 13628652) whereas at relapse the ITD was 60 bps, 51 bps from nucleotides 36481 - 36531 plus an additional insertion of 9 bps (Figure 4.3). Polymorphic markers confirmed that the samples were from the same individual.

Figure 4.3 Diagram to show the position of the different FLT3/ITDs detected in one patient at presentation and relapse

4.7.2.3 Survival analysis

Twenty patients who remained wild type FLT3 at both time points were compared with 15 patients who presented and relapsed with the same
FLT3/ITD. The OS in the latter group was worse and this was statistically significant (p=0.01) (Figure 4.4). The majority of patients with a FLT3 mutation at presentation and relapse, relapsed and died within the first 400 days from diagnosis. Patients who changed the pattern of the mutation at relapse were not included in this analysis as the numbers were too small for any meaningful interpretations.

Figure 4.4 Kaplan Meier curve demonstrating OS according to FLT3 status at presentation and relapse (WT/WT: WT FLT3 at presentation and at relapse; MUT/MUT: FLT3/ITD at presentation and at relapse)

4.8 Discussion

It is now more than a decade since techniques capable of detecting residual leukaemia at levels below the threshold of light microscopy have been used to improve clinical outcome. For example, the detection of MRD in children with ALL during induction chemotherapy has been shown to be of significant prognostic value, thus permitting the stratification of children with ALL to less toxic or more intensive arms of chemotherapy (Van Dongen et al, 1998). Similarly, in patients with CML who undergo an allogeneic transplant, DLI before the onset of haematologic relapse has been shown to be associated with an increased likelihood of anti-leukaemic response and a return to complete cytogenetic and molecular remission.(van Rhee F, 1994) In acute promyelocytic leukaemia, conversion to PCR positivity for PML/RARα in 2 successive bone
marrow samples post-consolidation chemotherapy is being used as a trigger to initiate salvage treatment. (Diverio et al, 1998) However, the clinical potential for such analysis in AML is restricted by the paucity of suitable markers. The recent identification of activating mutations in the FLT3 gene as the most common mutation in AML, occurring in up to one third of adult patients, (Kiyoi et al, 1999; Yamamoto et al, 2001) suggested that the mutations may be a relevant marker for MRD. Results presented in this chapter, which aimed to investigate whether FLT3 mutations can be used as markers of MRD, first confirmed that the mutation is only found in leukaemic cells. Analysis of a small group of 13 patients in morphological remission demonstrated that neither the presentation ITDs nor D835 mutation could be detected in bone marrow or peripheral blood samples obtained after induction chemotherapy. Eight of the patients had had mutant levels of 40% - 46% of total FLT3, indicating that the majority of cells carried the mutation (assuming heterozygosity), and 2 of the patients had had evidence for biallelic mutations or loss of one allele, with mutant levels of 74% and 90% respectively.

For a leukaemic marker to be clinically useful as an early predictor of relapse, it is important that the marker consistently reappears at relapse. However, 5 of the 20 patients (25%) who were positive for a FLT3 mutation at diagnosis lost their mutation at relapse. All had had ITDs and the level of mutant suggests that in 3 of them at least half of the cells in the sample analyzed carried the mutation. The inability of the mutation to be detected at relapse cannot be attributed to insensitivity of the technique used, as in all 5 cases the negative results from the cold PCR used bone marrow samples and were confirmed by the more sensitive radioactive PCR, which can detect ITDs at least at the 0.5% level (see chapter 3). Furthermore, in 4 of the 5 patients the blast cell counts were similar to those at presentation.

The results presented in this chapter are consistent with 2 studies, one by Nakano et al (1999) and the other by Shih et al (2002), in which 1 out of 6 ITD+ (16%) and 1 out of 17 ITD+ (6%) patients at presentation respectively were FLT3/WT at relapse, but it is in contrast to Schnittger et al (2002) who reported that 25 patients with a mutation at presentation all relapsed with the same
marker. However, more recently Schnittger et al (2004) have updated their previous study, assessing 97 paired samples at diagnosis and relapse, and found a gain of a FLT3/ITD in 4/55 cases (7.2%) and loss of the mutation in 4/42 (9.5%). Fifty one patients were negative at both time points, and 38 cases were positive for a FLT3/ITD at both time points.

A number of other issues arise that are relevant for MRD detection, particularly for ITDs. Two patients had evidence of more than 1 mutation at diagnosis, and in 1 of these patients only 1 of the 3 ITDs was detected at relapse. This indicated that the different mutants were present in separate subclones, only 1 of which survived or was selected after chemotherapy. In the other patient both mutants were detected at relapse at increased levels, though in different relative proportions, and it is not possible to determine whether the mutants were in separate clones which both survived, or whether the minor mutant was acquired on the other allele in a cell which was already heterozygous for an ITD and neither clone was completely eliminated by the treatment. In addition, 1 patient with an ITD at diagnosis relapsed with a completely different ITD, which would present as a false negative result if mutation sequence-specific primers were utilized to improve the sensitivity of the assays used. In fact, Stirewalt et al (2001) reported nested quantitative real time PCR assays for 4 AML samples with FLT3 ITDs using patient-specific primers, where they detected between 0.01 and 0.001% of FLT3/ITD positive sample in a background of WT DNA. However, these assays are laboratory intensive requiring the development of patient-specific primers and are only appropriate in cases where patients present and relapse with AML cells containing the same FLT3/ITDs. Analysis of FLT3 mutations as an early indicator of relapse should therefore be used with caution.

The results presented in this chapter also shed light on the role of FLT3 mutations in the pathogenesis of AML. The findings show that these mutations may be present in only a minority of blast cells at presentation, and at relapse the ratio of mutant to WT allele frequently increases, as seen in 6 out of 12 patients with the same ITD mutation at presentation and relapse (#38-43, Table 4.2). Results in 3 of these patients were consistent with a greater proportion of ITD+ blast cells at relapse (#39-41), and in 1 patient (#39) relapse appeared to
be associated with the development of homozygosity or hemizygosity for the mutant in at least some of the cells. This is in accordance with the recent study published by Schnittger et al (2004), where 24.5% of cases sustained the same level of mutant to WT type allele, but the majority of cases (63%) showed an increased level. This indicates that the FLT3 alterations can be secondary mutations arising in an already malignant clone, with selection of the subclone containing the FLT3 mutation because of the growth or survival advantage it confers. Five out of 24 patients (21%) who were WT at presentation acquired FLT3 mutations for the first time in either first or second relapse, which is similar to the frequency found at presentation (Chapter 3; Kiyoi et al, 1999). This is fully in accord with the fact that FLT3 mutations are secondary events. It also indicates that the FLT3 pathway is active in myeloid cells at the stage of differentiation equivalent to the clonogenic leukaemic cell, as the presence of these random mutations occurring in an already transformed clone lead to its further selection. The observation that at relapse 1 out of 20 patients had a different mutation and 5 had lost the FLT3 mutation is also compatible with this model. For this to occur, another subclone would need to develop from a leukaemic cell that had been present at diagnosis but did not contain the FLT3 mutation. To outgrow the mutant FLT3+ cells, this subclone must have acquired alternative mutation(s) imparting a greater survival/growth advantage than that provided by the original FLT3 mutation. One possibility is a secondary mutation in N-Ras as such mutations have been documented to arise in relapse when not present at diagnosis (Bartram et al, 1989; Nakano et al, 1999). In the 5 patients whom we studied who lost FLT3 mutations at relapse, only one, however, acquired an N-Ras mutation. (Ras analysis was performed in Dr Bowen’s lab in Ninewells Hospital, Dundee). This is a potential target as FLT3 activates N-Ras, (Dosil et al, 1993) and the rarity of FLT3 mutations and N-Ras mutations in the same blast cells suggests that both mutations are predominantly using the same pathways (Kiyoi et al, 1999). Consequently, in future it may be informative to screen for mutations in other candidate genes. These data also demonstrate that patients who present and relapse with a FLT3/ITD have a very poor outcome compared to patients with wild type allele (Figure 4.4). However, this finding will need to be validated further in studies incorporating a large number of patients.
In summary this study of paired diagnosis/relapse samples indicates that the majority of patients with FLT3/ITD mutations relapse with an equal or higher level of the same mutation. A smaller but not insignificant number of patients lose the mutation at relapse or relapse with a new one. For this small group of patients it seems that the FLT3/ITD is not present within the leukaemic stem cell that leads to disease relapse. It is also suggests that these mutations are late hits during the process of leukaemogenesis and might have implications for the therapeutic use of FLT3 kinase inhibitors in patients whose cells express FLT3 mutations. (Zhao et al, 2000; Tse et al, 2001, Levis et al, 2001) As these mutations are secondary events, there will always be a high probability that leukaemic subclones not containing the mutant will be present which will have a selection advantage in the presence of a FLT3 kinase inhibitor. These findings have important clinical implications for studies evaluating the efficacy of FLT3 inhibitors in patients with AML and should be considered when clinical resistance to these compounds occurs.
Chapter 5

5.1 Introduction

Conventional cancer chemotherapy relies on the premise that anti-cancer drugs will preferentially kill rapidly dividing tumour cells rather than normal cells. In order to eradicate tumour cells and maintain a complete remission status, large doses of drugs are typically used. However, this may cause profound toxicity, increasing the rate of mortality and morbidity. One of the most important goals therefore of modern anti-cancer research is to develop drugs with better discrimination between malignant cells and normal cells. In recent years, tremendous efforts have been focused on the development of kinase targeted therapies, as aberrant protein kinases are thought to be critical molecules for the development of human cancer. In this field, the most successful examples include the small molecule kinase inhibitor STI571 (Imatinib/Gleevec, Novartis) for the treatment of chronic myeloid leukaemia (CML), and the monoclonal antibody Trastuzumab (Herceptin) directed against the RTK HER2 for the treatment of breast cancer.

CML is a clonal stem cell disorder in which the reciprocal translocation t(9;22) generates two novel fusion genes: BCR-ABL on the derivative 22q-chromosome and ABL-BCR on chromosome 9q+. The fusion protein BCR-ABL has constitutive kinase activity that deregulates signal transduction pathways. Imatinib occupies the ATP-binding site of the molecule and prevents phosphorylation of downstream substrates that are involved in regulating the cell cycle. This results in inhibition of cell proliferation and induction of apoptosis in BCR-ABL positive cells. In colony-forming assays that used mononuclear cells from peripheral blood or bone marrow from patients with CML, imatinib produced a 92%-98% reduction in the number of BCR-ABL positive colonies (Druker et al, 1996). Imatinib has synergistic or additive activity with interferon-alpha, hydroxyurea, daunorubicin, doxorubicin, etoposide, cytarabine, cyclophosphamide, and vincristine against BCR-ABL positive cells (Fang et al, 2000; Thiesing et al, 2000; Kano et al, 2001).
recent years, the introduction of imatinib has completely changed the therapeutic algorithm in the management of CML, and several studies have shown its efficacy in patients either in the chronic phase or blastic crisis of CML. In a retrospective study comparing the progression free survival (PFS) of 143 patients who received imatinib after failure of interferon-based therapy with 264 historical controls managed with conventional therapy (as per MRC CML3 trial), a significant benefit was demonstrated for patients treated with imatinib (p=0.0065). However, an overall survival benefit was confined to those patients who achieved a cytogenetic response (Martin et al., 2003). In a phase III randomised study, imatinib and the combination of interferon plus cytarabine were compared, and imatinib was found to be superior with respect to complete haematologic response and major or complete cytogenetic response (O'Brien et al., 2003). Since a large fraction of patients crossed from interferon/cytarabine to imatinib, no difference in OS was observed. In addition to its superior efficacy, imatinib offered clear quality of life advantages compared with interferon and cytarabine, including in those patients who crossed over during the study period (Hahn et al., 2003). In an effort to improve PFS and OS, several investigators are currently exploring the use of imatinib in combination with other drugs such as interferon and cytarabine.

However, the development of resistance to this drug is a frequent setback, particularly in patients in advanced phases of the disease (Reviewed by Deininger et al., 2005). Several mechanisms of resistance have been described, the most frequent are amplification and/or mutations of the BCR-ABL gene. To overcome resistance, several approaches have been studied in vitro and in vivo. They include dose escalation of imatinib, combination of imatinib with chemotherapeutic drugs, alternative BCR-ABL inhibitors, inhibitors of kinases downstream of BCR-ABL, farnesyl and geranylgeranyl transferase inhibitors, histone deacetylase, proteasome and cyclin-dependent kinase inhibitors, arsenic trioxide, hypomethylating agents, troxacitabine, targeting BCR-ABL messenger RNA, and immunomodulatory strategies (Deininger et al., 2005). It is important to understand that these approaches differ in efficiency, which is often dependent on the mechanism of resistance. Further investigation into the molecular mechanisms of disease and how to specifically target the abnormal
processes will guide the design of new treatment modalities in future clinical trials.

In addition to small molecule inhibitors, monoclonal antibodies have been developed that target cell surface receptor molecules. The humanised monoclonal anti-HER2 antibody Trastuzumab was the first genomic-research-based targeted anti-cancer therapeutic to be approved by the FDA for the treatment of breast cancer (Shepard et al, 1991). HER2 is a member of the EGF receptor family and was first identified as a transforming gene in chemically-induced rat neuroblastomas. The HER2/NEU gene is amplified in approximately 30% of patients with breast cancer and is a prognostic marker for adverse outcome (Slamon et al, 1987; Carter et al, 1992). Several clinical studies have demonstrated objective response rates in previously treated and untreated patients with metastatic breast cancer (Tan et al, 2003). For example, in a randomised study of 469 patients by Salmon et al (2001) in women with metastatic breast cancer overexpressing HER2, the addition of trastuzumab to chemotherapy was associated with a longer time to disease progression (median 7.4 vs. 4.6 months; p<0.001), a longer duration of response (median 9.1 vs. 6.1 months; p<0.001), a lower rate of death at 1 year (22% vs. 33%, p=0.008), and longer survival (median survival 25.1 vs. 20.3 months; p=0.046).

Herceptin and imatinib are powerful examples of the potential clinical utility of kinase targeted therapy and therefore broad application of this treatment strategy to other cancers is expected over the next few years. Since FLT3 is expressed on the surface of blast cells in the majority of AML patients and approximately one quarter of these patients carry an activating mutation conferring a worse prognosis, targeting FLT3 using specific inhibitors may establish new treatment options for the treatment of AML. Indeed, several tyrosine kinase inhibitors which were not originally developed with FLT3 as the intended target have been reported to inhibit FLT3 in cell lines and primary leukaemic blasts. At the time the work outlined in this chapter was started, a number of inhibitors had become available, and a dozen studies have since demonstrated their in vitro and in vivo efficacy in cell lines, primary blast cells and animal models (Table 5.1). Four inhibitors were used in the studies.
reported here. AG1295 (6,7-Dimethyl-2-phenylquinoxaline) and AG1296 (6,7-Dimethoxy-2-phenylquinoxaline) are compounds of the tyrphostin class which potently inhibit both PDGF and SCF receptor activation in intact cells with a high degree of specificity (Gazit et al., 1996; Kovalenko et al., 1994; Banai et al., 1998; Ueda et al., 2002). Tyrphostins are not very soluble and are not amenable to use in patients. CEP-701, an indolocarbazole derivative, is a relatively selective FLT3 inhibitor with an IC$_{50}$ of 3 nM in in vitro kinase and cell-based FLT3 autophosphorylation assays (Levis et al., 2002). The staurosporin derivative PKC-412 was originally identified as a protein kinase C inhibitor (Meyer et al., 1989) and subsequently shown to inhibit members of the class III RTKs, including vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), KIT and FLT3 (Weisberg et al., 2002). PKC-412 was identified as a FLT3 inhibitor based on its selective inhibition of Ba/F3 cells transfected with FLT3/ITD. The IC$_{50}$ for inhibition of proliferation and phosphorylation was in the order of 10nM (Weisberg et al., 2002).

5.2 Material and methods

5.2.1 Samples from AML patients

Patient samples were obtained from peripheral blood or bone marrow of adults with newly diagnosed or relapsed AML. For fresh samples, mononuclear cells were recovered by Ficoll-Hypaque density centrifugation, washed thoroughly, suspended in growth medium [RPMI 1640 tissue culture medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS) and 5% amoxicillin] and incubated in a humidified 5% CO$_2$ atmosphere at 37°C. No cytokines were added. For frozen samples, bone marrow or peripheral blood mononuclear cells were thawed slowly and cultured in Iscove’s solution (Gibco, UK) supplemented with 10% FCS, 5% amoxicillin, for 4 hours in 5% CO$_2$ at 37°C. Subsequently, viability was assessed by trypan blue dye exclusion assay which relies on an alteration in membrane integrity, as determined by the
uptake of dye by dead cells, thereby giving a direct measure of cell viability. Only samples with greater than 80% viability were used.

Table 5.1 Summary of FLT3 inhibitors

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<th>RESULTS</th>
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<tr>
<td>Herbimycin A</td>
<td>PDGFR, EGFR</td>
<td>FLT3/ITD cDNA transfected 32D &amp; Cos7 cells</td>
<td>Inhibition of proliferation</td>
<td>Zhao et al, 2000</td>
</tr>
<tr>
<td>AG1295</td>
<td>PDGFR, KIT</td>
<td>Mice transplanted with transfected 32D/ITD cells</td>
<td>Prolonged latency of disease or prevented leukaemia</td>
<td>Levis et al, 2001</td>
</tr>
<tr>
<td>AG1296</td>
<td>PDGFR, KIT</td>
<td>Primary blasts from patients with AML (n=23)</td>
<td>Cytotoxic to FLT3/ITD cells</td>
<td>Tse et al, 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chimeric TEL/FLT3 transfected Ba/F3 cells</td>
<td>Inhibition of phosphorylation (IC_{50} 0.3 μM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibition of proliferation (IC_{50} 1.5 μM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increase in apoptosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FLT3/ITD transfected Ba/F3 cells</td>
<td>Inhibition of auto phosphorylation (IC_{50} 1 μM)</td>
<td>Tse et al, 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibition of proliferation (IC_{50} 0.5-0.8 μM)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Primary blasts from patients with AML</td>
<td>Inhibition of proliferation of FLT3/ITD blasts</td>
<td></td>
</tr>
<tr>
<td>CEP-701</td>
<td>PDGFR, KIT, FMS,</td>
<td>FLT3/ITD cDNA transfected Ba/F3 cells (BaF3/ITD)</td>
<td>Inhibition of phosphorylation (IC_{50} 2nM) Inhibition of proliferation of BaF3/ITD cells (IC_{50} 5nM)</td>
<td>Levis et al, 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Primary blasts from patients with AML (n=8)</td>
<td>Induced cytotoxicity in 3/4 with FLT3/ITD</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Balb/c mice injected with BaF3/ITD cells</td>
<td>Prolonged survival of mice</td>
<td></td>
</tr>
<tr>
<td>SU5416</td>
<td>PDGFR, KIT, FMS,</td>
<td>FLT3/ITD cDNA transfected Ba/F3 cells</td>
<td>Inhibition of proliferation (IC_{50} 250nM ) Inhibition of phosphorylation (IC_{50} 100nM)</td>
<td>Yee et al, 2002</td>
</tr>
<tr>
<td>SU11248</td>
<td>PDGFR, KIT, FMS,</td>
<td>RS411 cell line (WT FLT3 cell line)</td>
<td>Inhibition of phosphorylation of STAT5 and MAP kinase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV411 cell line (FLT3/ITD cell line)</td>
<td>Inhibition of apoptosis</td>
<td></td>
</tr>
<tr>
<td>PKC-412</td>
<td>Protein kinase C,</td>
<td>BaF3/ITD cells</td>
<td>Inhibition of proliferation (IC_{50} 10nM )</td>
<td>Weisberg et al, 2002</td>
</tr>
<tr>
<td></td>
<td>PDGFR, KIT</td>
<td>BaF3/D835 cells</td>
<td>Inhibition of proliferation (IC_{50} &lt; 10nM )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BaF3/ITD cells</td>
<td>Induction of apoptosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balb/c mice injected with BaF3/ITD cells</td>
<td>Prevention of leukaemia</td>
<td></td>
</tr>
</tbody>
</table>

RTK: receptor tyrosine kinase; PDGFR: platelet-derived growth factor receptor; EGFR: epidermal growth factor receptor; VEGFR: vascular endothelial growth factor receptor
5.2.2 Mutational analysis

Mutational analysis of FLT3 was performed on all AML samples as described in chapters 3.4.1 and 4.6.2.2.

5.2.3 Inhibitors

PKC-412 was obtained from Novartis Pharma AG, Basel Switzerland, the indolocarbazole CEP-701 from Cephalon Incorporation, USA, and tyrphostins AG1295 and AG1296 from LC Laboratories, Woburn, MA, USA. All these compounds were dissolved in dimethyl sulfoxide (DMSO) to create 4mM stock solutions which were frozen at -20°C in glass (CEP-701, PKC-412) or plastic vials (tyrphostins) and diluted appropriately before each experiment. Final concentration of DMSO in growth medium did not exceed 0.1%.

5.2.4 Cell culture and MTS assay

Mononuclear cells from patients were plated in triplicate at 2\times10^4 cells/100\mu l per well in 96-well plates with growth medium and in the presence of varying concentrations of inhibitors. Inhibitor-free controls contained the same amount of DMSO as in the experimental groups. Cells were incubated in a humidified 5% CO₂ atmosphere at 37°C for 48 (thawed cells) or 72 hours (fresh cells). Cell viability was measured using the CellTiter96® non-radioactive cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturers’ instructions. This is a colorimetric method for determining the number of viable cells which uses a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS) and an electron coupling reagent (phenazine methosulfate, PMS). MTS is bioreduced by viable cells into a formazan product that is soluble in tissue culture medium. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product, as measured by the absorbance at 562 nm, is directly proportional to the number of living cells in the sample. At the
indicated time point (48 hours for thawed cells or 72 hours for the fresh cells), 20μl of the combined MTS/PMS solution was added to each well and the plates incubated for a further 4 hours. The plates were then read in an ELISA microplate reader at 562 nm. The absorbances were corrected for blank readings obtained from culture medium without cells.

5.2.5 Statistical analysis

The Student’s t-test was utilised in order to compare results obtained in the MTS assays.

5.3 Results

5.3.1 Proliferation assays using tyrphostins AG1295 and AG1296

Twelve fresh samples from patients with de novo AML were studied using the tyrphostins AG1295 and AG1296. Three were female and 9 male. The median age at presentation was 41 years (range 15-60). Patient characteristics including the cytogenetic risk group are provided in Table 5.2. Nine patients were FLT3/ITD negative and 3 FLT3/ITD positive. None of them harboured a FLT3/TKD mutation. AG1295 and AG1296 were used at the following concentrations: 0.5, 1, 2, 3, 4, 5, 7.5 and 10 μM according to previous published data (Levis et al, 2001). Figures 5.1 and 5.2 show the dose response curves obtained. The data were grouped according to FLT3/ITD status and a mean response for each AG1295 and AG1296 concentration was calculated and expressed as a percentage of the mean optical density (OD) of the control wells. Errors bars represent the standard deviation (SD) of the mean OD of the treatment points. The sensitivity towards the tyrphostin AG1295 differed between cells harbouring the wild type and mutant receptor (Figure 5.1). The cytotoxicity was more apparent in blast cells with the mutation, however, when the t-test was used to compare the mean cytotoxicity between wild type and mutant blasts at each concentration of the inhibitor, there was no statistically
significant difference. There was a slight cytotoxic effect with AG1296, but this was not significantly different from the control (Figure 5.2).

Table 5.2 Patient demographics and FLT3 status

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Cytogenetic risk group</th>
<th>FLT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39</td>
<td>M</td>
<td>Standard</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>M</td>
<td>Standard</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>M</td>
<td>Favourable</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>M</td>
<td>Standard</td>
<td>WT</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>F</td>
<td>Standard</td>
<td>WT</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>M</td>
<td>Favourable</td>
<td>WT</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>M</td>
<td>Favourable</td>
<td>WT</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
<td>M</td>
<td>Poor</td>
<td>WT</td>
</tr>
<tr>
<td>9</td>
<td>54</td>
<td>F</td>
<td>Standard</td>
<td>WT</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>M</td>
<td>Standard</td>
<td>ITD</td>
</tr>
<tr>
<td>11</td>
<td>41</td>
<td>M</td>
<td>Standard</td>
<td>ITD</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>F</td>
<td>Poor</td>
<td>ITD</td>
</tr>
</tbody>
</table>
5.3.2 Proliferation assays using AG1295, CEP-701 and PKC-412 combined with cytarabine

In order to determine whether the addition of standard cytotoxic agents might potentiate the effects of tyrosine kinase inhibitors on primary AML blast cells, proliferation assays were performed using a fixed dose of AG1295, CEP-701
or PKC-412 incubated with varying doses of cytarabine. The two agents were added simultaneously to the cells and cytarabine was used at three different concentrations: 0.001, 0.01 and 0.1mg/ml. Cells were incubated in a humidified 5% CO₂ atmosphere at 37°C for 48 hours and the MTS assay was performed as described in Material and Methods (5.2.4). Thawed blast cells were chosen from three patients with FLT3 mutations (2 FLT3/ITD, 1 FLT3/TKD) and 3 patients with WT FLT3 protein. The patients' characteristics are given in Table 5.3.

### Table 5.3 Patient demographics and FLT3 status

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Cytogenetic risk group</th>
<th>FLT3 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>M</td>
<td>Poor</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>M</td>
<td>Favourable</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>M</td>
<td>Standard</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>M</td>
<td>Standard</td>
<td>ITD</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>M</td>
<td>Standard</td>
<td>TKD</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>M</td>
<td>Standard</td>
<td>ITD</td>
</tr>
</tbody>
</table>

TKD: tyrosine kinase domain mutation

Figure 5.3 shows the dose response of each sample treated with either AG1295, CEP-701, PKC-412, or cytarabine at a concentration of 0.1mg/ml. In accordance with the results reported above using AG1295 in fresh primary blast cells, a similar effect was observed in thawed blast cells. The cytotoxic effect of the inhibitor was more apparent in blast cells harbouring a FLT3 mutation than in those with WT FLT3 (Figure 5.3). AG1295 at a concentration of 2μM produced a strong cytotoxic effect in blast cells from 2 of the 3 patients with a mutation compared to little/no effect in cells from the 3 patients with WT FLT3. However, this difference was not statistically significant. AG1295 at a concentration of 5μM produced statistically significant cytotoxicity (p=0.02) in mutant compared to WT samples (Figure 5.3). CEP-701 was also more cytotoxic to blast cells harbouring a FLT3 mutation (Figure 5.3), and this
difference was statistically significant (p=0.02) at 20nM CEP-701, but not at 5nM (p=0.1). Of note, 20nM CEP-701 was also slightly cytotoxic to blast cells with WT FLT3 (median cytotoxicity 27%), and the difference in the cytotoxic effect at 5 and 20nM CEP-701 in these cells was statistically significant (p=0.03). PKC-412 was also cytotoxic to blast cells with FLT3 mutations, however, there was some effect in blast cells without FLT3 mutations. For example, in Patient 2 (WT FLT3), PKC-412 at 5 and 20nM produced 47% and 42% cytotoxicity, respectively. Even though there was a trend for PKC-412 to be more toxic to cells harbouring FLT3 mutations, this effect was not statistically significant at either concentrations (5 or 20nM). Paradoxically, the maximum cytotoxic effect with PKC-412 was achieved at the minimum concentration (5nM rather 20 nM) (Figure 5.3).

When cytarabine was added with the inhibitors, different patterns of cytotoxicity were observed for each patient sample. In samples from patient 1 (WT FLT3), cytarabine produced a 35% cytotoxic effect at the maximum concentration of 0.1mg/ml, but this was not augmented by adding AG1295 or PKC-412 (Figure 5.4 A, C). However, when CEP-701 was added the cytotoxic effect of cytarabine was slightly enhanced, with 55% killing at 0.1mg/ml cytarabine and 20nM CEP-701 (Figure 5.4 B). In patient 2 (WT FLT3), neither cytarabine, AG1295, CEP-701 nor the two combined produced any major effect (Figure 5.5A, B). However, PKC-412 produced 40% cytotoxicity on its own which was not augmented by cytarabine (Figure 5.5C). Samples from patient 3 (WT FLT3) showed only 20% cytotoxicity with cytarabine alone at the maximum concentration of 0.1mg/ml, although this was augmented when combined with AG1295 or CEP-701 (Figure 5.6A, B). PKC-412 had little cytotoxic effect when used alone, and this was not enhanced by the addition of cytarabine (Figure 5.6C). In patient 4 (FLT3/ITD), cytarabine alone produced nearly 40% cytotoxicity at 0.01mg/ml concentration. Even though all three inhibitors showed considerable cytotoxic effect when used alone, combination with cytarabine did not enhance the cytotoxicity (Figure 5.7A, B, C). In patient 5 (FLT3/D835), cytarabine produced a major cytotoxic response, with 80% cytotoxicity at 0.1mg/ml. Similarly, as single agents all three inhibitors showed considerable cytotoxicity, and there was therefore, little or no additional effect.
when they were combined with cytarabine (Figure 5.8A, B, C). In patient 6 (FLT3/ITD), cytarabine at the minimum concentration of 0.001 mg/ml produced a 55% cytotoxic effect which remained unchanged at 0.01 and 0.1 mg/ml. All three inhibitors produced a major cytotoxic effect, which was not enhanced by cytarabine (Figure 5.9A, B, C).
Patient 1

**A**

0 0.001 0.01 0.1

OD (% Control)

Cytarabine (mg/ml)

- Ara-C
- Ara-C+ 2μM AG1295
- Ara-C+ 5μM AG1295

**B**

0 0.001 0.01 0.1

OD (% Control)

Cytarabine (mg/ml)

- Ara-C
- Ara-C+ 5nM CEP-701
- Ara-C+ 20nM CEP-701

**C**

0 0.001 0.01 0.1

OD (% Control)

Cytarabine (mg/ml)

- Ara-C
- Ara-C+ 5nM PKC-412
- Ara-C+ 20nM PKC-412

Figure 5.4 Dose response curves of cells from an AML patient with FLT3 WT treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412
Figure 5.5 Dose response curves of cells from an AML patient with FLT3 WT treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412
Figure 5.6 Dose response curves of cells from an AML patient with FLT3 WT treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412
Figure 5.7 Dose response curves of cells from an AML patient with FLT3/ITD treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412
Figure 5.8 Dose response curves of cells from an AML patient with FLT3/TKD treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412
Patient 6

Figure 5.9 Dose response curves of cells from an AML patient with FLT3/ITD treated with Cytarabine (Ara-C) alone and in combination with AG1295, CEP-701 or PKC-412
5.4 Discussion

Identification of the key role of protein kinases as potential oncoproteins has led to the emergence of a new era of targeted therapies. The introduction of imatinib, an agent targeting the causative molecular event in CML, has been heralded as a major advance in the treatment of cancer. Certainly, the early phase I/II and more recent clinical trials with imatinib have validated the concept that a precise understanding of the pathogenesis of a cancer can lead to more effective and less toxic therapies.

The high incidence of activating mutations of FLT3 (see chapter 3) in AML suggested that inhibitors of mutant FLT3 might have potential therapeutic activity in these patients. The preliminary data in this chapter showed that the inhibitors AG1295, CEP-701 and PKC-412 could induce a greater in vitro cytotoxic effect in blast cells of patients with FLT3/ITDs when compared to cells with the WT of the receptor. This effect was apparent in samples from patients with FLT3/ITDs, and was also observed in a patient with a FLT3/TKD mutation (Patient 5, Figure 5.8). Even though this later observation relied on a sample from a single patient, these agents may also have a role in patients with FLT3/TKD mutations. However, large studies will be required in order to assess potential applicability. The cytotoxicity was dose dependent for AG1295 and CEP-701, but not for PKC-412, where the inhibitor at 5nM concentration was more potent compared to 20nM (Figure 5.3). At present there is no clear explanation for this observation.

These data are in agreement with other studies which have explored potential cytotoxicity of FLT3 inhibitors in primary AML blasts. In a study by Levis et al (2001) using AG1295 on primary blasts from 23 patients with AML, cytotoxic responses were observed in 8 FLT3/ITD compared to 15 FLT3/WT specimens (p=0.001). The difference was statistically significant at each concentration point (0.5, 1, 2, 3, 4 and 5 µM). In another study by the same group using CEP-701 on primary AML samples from 8 patients, 3 of 4 FLT3/ITD samples displayed a cytotoxic response to CEP-701, as did a single
non-ITD sample (Levis et al, 2002). One of the FLT3/ITD samples, along with the D835 mutant sample and the other 2 WT samples, did not respond to CEP-701. A cytotoxic effect could be seen at approximately 5 nM and was maximal at 50nM (Levis et al, 2002). PKC-412 has been found to be cytotoxic to Ba/F3 cells transfected with FLT3/ITD but non-toxic in parental Ba/F3 cells at concentrations up to 100nM (Weisberg et al, 2002). Furthermore, survival was significantly prolonged in PKC-412 treated Balb/c mice transplanted with cells transduced with a FLT3/ITD expressing retrovirus. The majority of animals treated with placebo developed characteristic features of FLT3/ITD myeloproliferative disorder, whilst none of those treated with PKC-412 did (Weisberg et al, 2002).

However, if FLT3 mutations are secondary events (see chapter 4), there will always be a high probability that leukaemic subclones not containing the mutant will be present which will have a selection advantage in the presence of a FLT3 kinase inhibitor. Clinically, this may result in relapse with a predominant FLT3 WT clone, suggesting that FLT3 inhibitors should probably be used in combination with conventional chemotherapy. Experience in antineoplastic treatment has shown over the years that monotherapy with single agents is unlikely to be curative, and the development of resistance to chemotherapeutic drugs is a ubiquitous problem in the treatment of haematological and non-haematological cancer. This applies not only to conventional cytotoxics but also to inhibitors, as it is becoming increasingly apparent that resistance to tyrosine kinase inhibitors is an emerging problem with therapeutic implications. For example, as clinical experience with imatinib grows in CML, some patients become resistant, failing to achieve cytogenetic response. Several mechanisms have been implicated such as amplification of BCR/ABL or mutations in the BCR/ABL protein altering imatinib binding characteristics, or through indirect modulation of kinase function (Deininger et al, 2005). Therefore in the future, treatment with conventional cytotoxics and inhibitors is more likely to be offered as combination rather than monotherapy.

As cytarabine remains the backbone of modern anti-leukaemic treatment, it was therefore used with inhibitors in combination experiments. The results of this
type of approach, as presented in this chapter, suggest that inhibitors combined with conventional cytotoxic agents can provide some benefit. However, what it became apparent was the variability in the cytotoxic effect from patients’ sample to sample. These different patterns of cytotoxicity suggest that treatment might have to be individualised using in vitro studies with patients’ samples in order to assess potential cytotoxicity prior to introducing the combination into clinical practice. However, such approach would be practically difficult to apply to each patient as it remains laborious and difficult to standardise. More importantly, patients presenting with AML require immediate treatment without delay. Furthermore, the interpretation would be difficult as in vitro models do not necessarily represent in vivo behaviour (Smith et al, 2004). Undoubtedly, results of in vitro experiments should be interpreted with caution when transferred to clinical practice.

Since the work presented in this chapter was completed, several studies have been published looking at the “combination model” using conventional cytotoxic drugs with inhibitors. SU11248 was found to synergistically inhibit the proliferation of primary AML myeloblasts with a FLT3/ITD but not WT FLT3 when used with cytarabine (Yee et al, 2004). CEP-701 also induced in vitro cytotoxicity in a synergistic fashion when used simultaneously or immediately following exposure to chemotherapeutic agents, either cytarabine, daunorubicin, mitoxantrone or etoposide (Levis et al, 2004). Interestingly, the sequence in which the chemotherapy and CEP-701 were administered appeared to be important because treatment of cells with CEP-701 prior to the addition of chemotherapy seemed to antagonise the cytotoxic effects of agents such as cytarabine and etoposide. This may be because inhibitors block cells from cycling and therefore, cycle specific cytotoxic agents (i.e. cytarabine) lose their potency.

At present, phase I and II trials using these inhibitors as monotherapy in patients with AML have failed to show major clinical responses. For example, in a phase I/II trial using CEP-701 as salvage treatment in 14 patients with refractory, relapsed or poor risk AML expressing FLT3 activating mutations, minor clinical efficacy was observed. In 5 out of 14 patients, CEP-701
effectively lowered peripheral blast cell counts from a range of 27%-94% to a range of 1%-10% in 4 patients, and one patient showed a decrease in the percentage of bone marrow blasts to less than 5% (Smith et al, 2004). However, none of the responses were durable (2 weeks to 3 months). In another study by Stone et al (2004) using PKC-412 as monotherapy in 20 patients with relapsed/refractory AML, the peripheral blast count decreased by 50% in 14 patients, but only 6 patients showed a 50% reduction in the blast count in the bone marrow. Of note, 2 patients achieved less than 5% blasts in the bone marrow. Again, none of the patients sustained a durable response. In a third Phase II study by Giles et al (2003) of 55 patients with refractory AML/MDS, modest clinical activity was observed using SU5416. This was restricted to only 4 patients (7%) who achieved either a partial or haematological improvement, whilst, disappointingly, the overall median survival for the entire group of patients with AML was 12 weeks. This study also raised the possibility of unacceptable toxicity when inhibitors were combined with conventional chemotherapy. For example, 7% of patients developed thromboembolic events while on SU5416 as monotherapy. However, thromboembolic events in the range of 42% have been reported for patients with solid tumours treated with SU5416 plus gemcitabine (Kuenen et al, 2002) and cisplatin, and 22% when combined with 5-fluorouracil and leucovorin (Rosen et al, 2000).

In conclusion, the growing evidence for an important role of FLT3 mutations in AML suggests a potential for the use of FLT3 inhibitors in the treatment of this disease. However, as in at least some patients, FLT3 mutations are likely to be secondary events (chapter 4), and there is the potential of developing drug resistance to FLT3 inhibitors, it is more likely that multi-agent combination regimens will be used in the treatment of AML in the near future. The use of these drugs has already raised several questions which will need to be answered prior to potential incorporation into clinical practice. As in vivo and in vitro efficacy has been shown in patients with AML irrespective of their FLT3 status, should the use of inhibitors be restricted to patients with FLT3 mutations, in patients over-expressing FLT3, or used in all patients with AML who enter these trials? At which stage of the disease should these inhibitors be
incorporated into the treatment protocols (induction, consolidation, relapse, refractory disease)? Should patients above the age of 60, where experimental treatments are needed more, be considered for this type of treatment as first line therapy? Is there a role for inhibitors targeting minimal residual disease? As several questions remain to be answered, patients offered this type of therapeutic modality should only be treated as part of large clinical trials.
Chapter 6

Conclusions and future directions

The tyrosine kinase receptor FLT3 is expressed on early haemopoietic progenitors, B-cell precursors and macrophage precursors. FLT3 plays an important role in early haemopoietic progenitor proliferation and survival, as well as in macrophage and dendritic cell differentiation. In AML, FLT3 is highly expressed in approximately 90% of cases. Nakao et al (1996) first identified FLT3 mutations in AML in 1996 during an investigation into the incidence and distribution of FLT3 mRNA in samples from adult AML and childhood ALL patients. Unexpectedly long fragments were detected in PCR products of the JM domain in 5 out of 30 (16%) AML patients. Further analysis showed that they all contained a tandemly duplicated sequence, sometimes with insertion of additional nucleotides. The duplicated region was variable in both size and location in different individuals but always fell within the JM domain encoded by exons 14 and 15. The resulting transcripts were always in-frame and would therefore be expected to produce functional FLT3 chains. These internal tandem duplications (ITDs) have also been detected in 3% of patients with myelodysplastic syndromes (Horiike et al, 1997; Yokota et al, 1997; Xu et al, 1999), and occasional patients with ALL, although some of the latter patients had biphenotypic characteristics (Xu et al, 1999; Nakao et al, 2000). They have not been found in patients with CML, chronic lymphoid leukaemia, non-Hodgkin's lymphoma or multiple myeloma (Yokota et al, 1997), nor in normal individuals (Ishii et al, 1999). FLT3 mutations have also been found in exon 20 of the TKD. They include point mutations, deletions or insertions, and the most common nucleotide substitution changes an aspartic acid to tyrosine at amino acid 835. TKD mutations are found in patients with AML (approximately 7%), MDS (2%-5%) (Horiike et al, 1997; Yokota et al, 1997; Xu et al, 1999), and ALL (1-3%) (Xu et al, 1999; Nakao et al, 2000).

Preliminary studies showed that FLT3/ITD mutations confer an adverse clinical outcome in patients with AML (Kiyoi et al, 1999). However, this was a
relatively small study with heterogeneous patients including those above and below the age of 60. Therefore, studies presented in this thesis aimed to assess the clinical significance of FLT3/ITD mutations in a large cohort of adult patients below the age of 60, homogeneously treated according to MRC 10 and 12 trial protocols (chapter 3). In the total group of 854 patients, 227 (27%) had a FLT3/ITD. Mutations were found in all FAB subgroups except M0 (0/14, p=0.02) and M7 (0/11, p=0.04), and in only 1 out of 15 patients with M6 (p=0.08). They were significantly more common in M3 (36%, p=0.004) and, in accord with this finding, the incidence of FLT3/ITD in patients with t(15;17) was 37% (p=0.02). A high frequency of mutations was also observed in patients with normal cytogenetics (34%, p=0.0001). Low frequencies were found in patients with t(8;21) and inv(16), (9%, p=0.0004 and 7% respectively, p=0.003). FLT3/ITD+ patients had significantly higher PB white cell counts and percentage of BM blast cells at diagnosis (p<0.0001). Presence of the mutation had a significant impact on clinical outcome, including OS, EFS, DFS and RR. The RR in patients with a FLT3/ITD at 5 years was 62% as opposed to 44% in those without the mutation (p<0.001). DFS, EFS and OS were 32%, 25% and 35% respectively in patients with a FLT3/ITD as opposed to 46%, 39% and 43% in those without the mutation (p<0.001 for all of them). In multivariate analysis, the presence of a FLT3/ITD was the most significant factor adversely affecting RR and DFS (p<0.0001 for both). For RR, cytogenetic risk (p<0.0001) and status post course 1 (p=0.001) were also relative risks, however less powerful.

Semi-quantitative radioactive PCR showed that a significant proportion of the FLT3/ITD+ patients (51/224, 23%) had more than one FLT3/ITD mutation present. The majority of these patients (45/51, 88%) had two mutations but up to four were observed. In most patients, one mutation was predominant and the other(s) were minor components. There was a suggestion that patients with two or more mutations presented with a higher WBC count and a higher percentage of blasts in the BM at diagnosis. Patients with two or more mutations had the same RR as those with a single mutation. There was, however, a suggestion that patients with two or more mutations had a worse OS, 36% at 5 years for one mutation, 21% for two or more (p=0.04). When the number of mutations (0
versus 1 versus 2 or more) was added into the multivariate analysis as well as
the presence or absence of the mutation, then the number of mutations replaced
the latter as the most important independent variable predicting for RR and
DFS. This finding has not been investigated by other groups and will require
further clinical and biological exploration in the future to determine its
significance.

The level of mutant detected varied between 0.5% and 97% of total FLT3
signal. Most patients (201/224, 90%) had <60% mutant. In a significant
proportion of patients (59, 26%), the level was consistent with a heterozygous
mutation in all or the majority of cells. However, in 23 patients (10%) there
was ≥60% mutant, suggesting bi-allelic mutation or loss of WT alleles. This
was associated with an older age at diagnosis, leucocytosis, poorer cytogenetic
risk group and with FAB type M4. Survival analysis revealed that there was a
suggestion of worse outcome in patients with ≥60% mutant, with a marginally
increased RR in this group (85% versus 62%, p =0.06). Since these studies
were completed, several other groups have demonstrated the adverse outcome
of FLT3/ITDs in adult, paediatric and elderly patients with AML, particularly
where there is loss of the wild type allele (Whitman et al, 2001; Frohling et al,
2002; Stirewalt et al, 2001; Iwai et al, 1999; Meshinchi et al, 2001; Zwaan et
al, 2002). As the presence of mutations in the FLT3 gene is a frequent finding
in patients with AML, studies were then performed to explore their suitability
as markers for MRD (chapter 4). Samples from 13 patients who presented with
a FLT3 mutation were studied at the time of morphological remission (<5%
blasts in the bone marrow). These samples revealed that in remission all
patients lost their mutation(s), confirming that FLT3 mutations are leukaemia-
specific. Paired PB or BM samples at presentation and first relapse were
available from 44 patients with de novo AML. Of the 24 patients studied who
had only WT FLT3 alleles at presentation, 20 patients (83%) remained WT at
first relapse and 4 patients (17%) acquired a FLT3 mutation. Two of these
patients acquired a D835 mutation, and 2 patients gained a FLT3/ITD.

In 20 patients who had a FLT3 mutation at presentation, 18 were ITD+ and 2
had D835Y mutations. Five patients (25%) lost their FLT3 mutation at relapse.
Fifteen patients had a FLT3 mutation at both presentation and relapse. Two had
D835Y mutations at both stages of their disease and 13 had ITDs. Six patients relapsed with the same ITD at approximately the same level. Six patients relapsed with the same ITD but at an increased level, where the median difference between presentation and relapse in the level of mutant was 29.5%. In 1 patient, 3 different ITDs were detected at diagnosis (mutant levels 28%, 3%, 1% respectively), but only the predominant mutation was present at relapse. Another patient had 2 different ITDs at diagnosis with mutant levels 26% and 2%. Both were increased at relapse, 56% and 14% respectively. The remaining patient who was ITD+ at presentation, with mutant level 44%, relapsed with a different ITD, mutant level 60%. This study of paired diagnosis/relapse samples indicates that the majority of patients with FLT3/ITD mutations relapse with a similar or higher level of the same mutation. A smaller (25%) but not insignificant number of patients lose the mutation at relapse or relapse with a new one (5%). For this latter group of patients it seems that the FLT3/ITD is not present within the leukaemic stem cell that leads to disease relapse. It is also suggests that these mutations may be late hits during the process of leukaemogenesis, are unstable and thus should be used cautiously for the detection of minimal residual disease.

Given the high frequency of FLT3 mutations in patients with AML, FLT3 and its downstream signalling pathway are attractive targets for directed inhibition. Several promising tyrosine kinase inhibitors have recently been identified and studies were therefore performed to explore the cytotoxic effect of 4 different inhibitors on primary cells from patients with AML using a proliferation assay (chapter 5). Initially, 12 fresh samples from patients with de novo AML were studied using the tyrphostins AG1295 and AG1296. Nine patients were FLT3/ITD negative and 3 FLT3/ITD positive. A cytotoxic effect was more apparent in cells harbouring a mutant receptor compared to WT FLT3, however, the difference was not statistically significant. There was a slight cytotoxic effect with AG1296 in cells with FLT3/ITDs, but again this was not different from the control WT cells.

Subsequently, in order to determine whether the addition of standard cytotoxic agents might potentiate the effects of tyrosine kinase inhibitors on primary
AML blast cells, proliferation assays were performed using fixed doses of AG1295, CEP-701 or PKC-412 incubated with varying doses of cytarabine. Thawed blast cells were chosen from 3 patients with FLT3 mutations (2 FLT3/ITD, 1 FLT3/TKD) and 3 patients with WT FLT3. AG1295, CEP-701 and PKC-412, used as single agents, had a greater cytotoxic effect in blast cells harbouring a FLT3 mutation than in those with WT FLT3. This difference was statistically significant when 5μM AG1295 and 20nM CEP-701 were used. Even though inhibitors were primarily cytotoxic to cells harbouring FLT3 mutations, CEP-701 and PKC-412 were also cytotoxic to blast cells with WT FLT3. When cytarabine was added, different patterns of cytotoxicity were observed for each patient sample. In some patients’ samples inhibitor or cytarabine alone produced a major cytotoxic effect, whereas, in others an additive effect was shown. Despite the wide variability in cell cytotoxicity from patient to patient, the in vitro data reported in chapter 5 suggest that FLT3 inhibitors may have a role in the treatment of AML.

The studies presented in this thesis and results reported by other groups have shown that FLT3 ITDs play a major role in disease outcome, both in adult and paediatric AML (Kiyoi et al., 1999; Thiede et al., 2002; Kondo et al., 1999; Meshinchi et al., 2001). Nevertheless, many questions remain. For example, is the size of the ITD predictive of worse outcome as has recently been suggested? Stirewalt et al. (2006) reported that with increasing size of ITD there is a more significant loss of autoinhibitory function of FLT3, translating into inferior prognosis. However, large studies should address this observation as Kusec et al. (2006) failed to confirm Stirewalt’s findings.

At the moment the molecular mechanism that generates a FLT3/ITD remains unclear. Study of the accumulated sequences has revealed that ITD mutations essentially cluster in the tyrosine rich stretch from Y589 through Y599. It has been suggested that if a lagging strand makes a hairpin at this position during DNA replication and the mismatch repair system is impaired, a FLT3/ITD would be generated by replication slippage (Kiyoi et al., 2006).
A further question is whether the apparently homozygous pattern of the mutation in at least a proportion of cells in those cases with more than 50% mutant is due to hemizygosity resulting from deletion of the wild allele, or homozygosity due to mitotic recombination. To date, strong evidence suggests that the latter is more likely to occur. FISH analysis in chromosome 13 failed to reveal allelic loss at 13q12 (Thiede et al, 2002), while recent studies have shown that the high degree of association between uniparental disomy and homozygous mutation suggest that uniparental disomy might account for a substantial proportion of cases exhibiting a lack of the wild type allele (Fitzgibbon et al, 2005; Raghavan et al, 2005).

Although FLT3 is expressed on the surface of a high proportion of AML blast cells, little is known about the biologic and clinical significance of the level of FLT3 expressed in acute leukaemia. It has been suggested that gene amplification might be a possible mechanism leading to overexpression of the transcript (Armstrong et al, 2003), however, this has only been demonstrated in an MLL-rearranged ALL cell line and not in primary AML cells. Some studies have evaluated the clinical relevance of the FLT3 transcript level and differences in FLT3 expression in specific AML subgroups, and have suggested that overexpression of FLT3 is an unfavourable prognostic factor that might distinguish a novel disease entity in AML without FLT3 mutations (Ozeki et al, 2004; Kuchenbauer et al, 2005). However, as different techniques have been used (i.e. Northern blotting, RT-PCR), and the expression level has not been quantitatively determined, the clinical interpretation remains unclear. Moreover, the surface expression level of FLT3 protein has not been related to the expression level of the FLT3 transcript. Nevertheless, it has been shown that the autophosphorylation of WT FLT3 induced by its overexpression can be inhibited by FLT3 kinase inhibitors (Armstrong et al, 2003), and therefore cases with overexpressed FLT3 may benefit from treatment with FLT3 inhibitors.

The data on paired presentation-relapse samples in this thesis suggests that FLT3 mutations may be secondary events, as a small but consistent proportion of patients with AML initially harboring FLT3/ITD mutations lacked these
mutations at relapse. This would suggest that at least in some cases, the mutations occurred at a later stage of leukaemic transformation, and that chemotherapy was successful in eradicating the clones expressing the mutant FLT3. Furthermore, some samples were found to contain multiple different FLT3/ITD mutations, again suggesting that they were present in subclones of cells. However, this will require further exploration as studies have suggested that in some patients FLT3/ITDs can be found in the leukaemic stem cell (Levis et al., 2005). They sorted primary AML samples into stem cell-enriched CD34+/CD38' fractions and then analysed the sorted and unsorted cells for the FLT3 mutant:wild-type ratio. In each case, the FLT3 mutant:wild-type ratio was not changed by selection of CD34+/CD38' cells, implying that the mutations were present in the leukaemic stem cells.

The biology of FLT3 mutations and the role of these mutations in promoting leukaemogenesis remain unclear. The constitutive activation of the receptor induced by FLT3/ITDs promotes in vitro ligand independent proliferation and blocks myeloid differentiation of early haemopoietic cells (Kiyoi et al., 2002; Zheng et al., 2002). Wild type FLT3 appears to transduce its signalling cascade via the phosphatidylinositol-3 kinase (PI3K) and Ras pathways, leading to activation of AKT, signal transducer and activator of transcription-5 (STAT), and extracellular signal regulated kinase-1 and 2 (ERK1/2) (Hayakawa et al., 2000; Mizuki et al., 2000; Spiekermann et al., 2003). Presence of a FLT3/ITD confers growth factor independent proliferation on haemopoietic cells via the Ras and STAT5 pathways (Gilliland & Griffin, 2002). However, STAT5 might play a more critical role in FLT3/ITD signalling compared to the WT receptor (Mizuki et al., 2000). The identification of proteins that co-operate in FLT3-mediated signalling will improve the understanding of how constitutive activation of FLT3 induced by the presence of an ITD participates in leukaemogenesis. Microarray data might also help to bring further insight into the role of activated FLT3 in leukaemogenesis and its interaction with other proteins. Animal data suggest that transplantation of FLT3/ITD transduced bone marrow cells into irradiated Balb/c mice rapidly leads to a fatal myeloproliferative syndrome characterised by marked neutrophil leucocytosis and extramedullary haemopoiesis (Kelly et al., 2002a). FLT3 mutations appear
to co-operate with the PML/RARA fusion gene, accelerating disease onset in a mouse model of leukaemia (Kelly et al, 2002b). It has therefore been hypothesised that the development of leukaemia requires the co-operation of at least two classes of genetic abnormalities, one which impairs differentiation and a second which promotes proliferation. Thus, a FLT3/ITD may co-operate with for example, AML1/ETO, CBFb/MYH11, or PML/RARA to cause AML. However, caution must be exercised in extrapolating results of cell lines or murine models into human haemopoiesis. For example, enforced expression of a FLT3/ITD in human CD34+ cells conferred properties of self-renewal and enhanced erythropoiesis, but not a leukaemic phenotype (Chung et al, 2005). Hence, it seems likely that in order for activated tyrosine kinases to contribute to the development of frank leukaemia, they must work in concert with other mutations that confer self-renewal to the blood cell.

Very recently, Falini et al (2005) reported that in a high proportion of adults with AML the nucleophosmin (NPM1) gene is mutated resulting in an aberrant cytoplasmic localisation of the product. These mutations are found almost exclusively in patients with a normal karyotype and are highly associated with FLT3/ITDs, suggesting that this group may define distinct disease entity. Several groups have tried to correlate the presence of the mutation with clinical outcome and it has become apparent that presence of NPM1 mutation is associated with favourable outcome, except in the presence of a FLT3/ITD (Suzuki et al, 2005; Dohner et al, 2005; Schnittger et al, 2005). Taken together, these data indicate that further work will be needed to unfold the mechanisms by which such mutations co-operate and contribute to leukaemogenesis.

In relation to treatment, large prospective trials will be required in order to determine therapeutic strategies which potentially can negate the adverse clinical outcome associated with FLT3/ITDs. Published data has suggested that protocols incorporating a high dose of cytarabine might improve outcome. For example, in the studies presented in this thesis on patients treated in the MRC AML10 and 12 trials, the dose of cytarabine in the protocols ranged from 5.6 to 11.6 g/m², whilst in the German trials which did not observe an impact of a FLT3/ITD on OS (AML-96, AMLCG92 and AMLCG99), the cytarabine dose
was up to 8 fold higher, with the lowest dose at 22.4 g/m^2 and the highest at 92.8 g/m^2 (Schnittger et al, 2002). Another study has supported the idea that intensive chemotherapy during induction and consolidation may abrogate the adverse outcome in FLT3/ITD patients (Boissel et al, 2002). They evaluated outcome in patients randomised to receive higher than conventional doses of classical 3+7 induction regimes, with protocol arms containing double induction and timed sequential chemotherapy. They found no difference in relapse-free survival between FLT3/ITD positive and negative patients, although there was a trend for worse OS in the FLT3/ITD patients (p = 0.09). Although the cumulative doses of cytarabine and anthracycline did not differ from those used in the MRC protocol, this potential drug intensification may have produced profound cell toxicity able to overcome any chemoresistance of remaining leukaemic cells.

However, these suggestions rely on retrospective data and small numbers of patients with heterogeneous characteristics in relation to age and cytogenetic risk group. Therefore, it will be important for clinicians to design well controlled randomised studies incorporating FLT3/ITDs in risk assessment and stratification. For example, the BFM study group has decided to stratify patients with FLT3/ITDs to the high risk arm in their AML-2004 trial in paediatric patients (Zwaan et al, 2004). This study involves a first randomisation between cytarabine, daunorubicin, etoposide (ADE) and cytarabine, idarubicin, etoposide (AIE), with subsequent risk assignment between high and standard risk disease. Patients with FLT3/ITDs are assigned to the high risk arm and receive high dose cytarabine with mitoxantrone, with a second randomisation between cytarabine/idarubicin or cytarabine/idarubicin in combination with 2-chloro-2-deoxyadenosine (2-CDA). Another possibility is that strategies incorporating high dose procedures might have to play a role in patients with FLT3 ITDs. This has been evaluated in 1135 adult patients treated according to UK MRC AML 10 and 12 protocols. In 141 patients who received an autograft in CR1, FLT3/ITDs remained a poor prognostic factor for relapse. In the allograft setting, the impact of a FLT3/ITD on the relapse rate was less marked, but in a donor-versus-no donor analysis, there was no significant difference in the benefit of a donor with regard to relapse in FLT3/ITD+ and
FLT3/ITD- patients (Gale et al, 2005). Currently, a Spanish trial is awaiting ethical approval in order to randomise patients with standard risk disease and FLT3 mutations either to receive or not a stem cell transplant in CR1 (personal communication, Dr R Martino).

The role of FLT3 inhibitors in AML needs to be explored in future studies. *In vitro* studies have shown that FLT3 inhibitors can induce cytotoxicity in cell lines and primary AML cells. Several Phase I and II trials have been published, however, the efficacy of these agents in disease outcome remains unimpressive (Smith et al, 2004; Stone et al, 2004). Even though FLT3 inhibitors can reduce the number of blast cells in the peripheral blood/marrow, complete remissions rarely occur and are not durable. As studies have suggested that FLT mutations in some patients might represent secondary events, the use of FLT3 inhibitors as monotherapy is unlikely to be effective. However, presuming that FLT3 inhibition can be incorporated into standard chemotherapy protocols, large clinical studies will be required to explore potential efficacy. It should be noted that the development of resistance to tyrosine kinase inhibitors can be anticipated, however, it might be possible to overcome such resistance by using alternative inhibitors with different chemical structures. The structure of FLT3 has been described by Griffith et al and may provide insight into the mechanism by which the JM domain in the wild type receptor inhibits the catalytic activity of the kinase domain. Since this interaction is disrupted in FLT3/ITDs, this may open a window of opportunity for the design of specific FLT3 inhibitors. Finally, AML patients whose leukaemic blasts are shown to over-express WT FLT3 that is constitutively activated by FL comprise an additional population that should be considered for future trials.

In conclusion, despite some insight into the biology of FLT3, its leukaemogenic properties remain largely unknown and will require further exploration. Ultimately these studies should reveal novel therapeutic targets for the treatment of AML.
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127


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Appendix

Treatment protocols

AML 10 trial

In AML 10 trial patients were randomised to receive 2 courses of induction therapy of either DAT (daunorubicin, cytarabine, 6-thioguanine): 3+10 for course 1 and 3+8 for course 2, or ADE (cytarabine, daunorubicin, etoposide): 10+3+5 for course 1 and 8+3+5 for course 2. Both arms then received 2 courses of consolidation therapy of MACE (amsacrine, cytarabine, etoposide) and MIDAC (mitozantrone, cytarabine). Patients achieving CR were allowed to proceed to an allogeneic transplant if a suitable HLA-matched donor was available or were eligible to be randomized to receive either an autologous transplant or no further treatment.

AML 12 trial

Patients entered into the AML 12 trial were randomized to receive one course of induction therapy of either ADE (10+3+5) or MAE (mitozantrone, cytarabine, etoposide: 3+10+5). BM remission status was then assessed and patients were assigned to one of three risk groups, good, standard or poor based on the cytogenetics and response to the first cycle of therapy. Patients in the good and standard risk categories received a second course of induction chemotherapy as before, either ADE (8+3+5) or MAE (3+8+5), then a third course of MACE consolidation therapy. Good risk patients were then randomized to receive either just one further course of chemotherapy (MIDAC) or a fourth course of ICE (idarubicin, cytarabine, etoposide) plus a fifth course of MIDAC. Standard risk patients were randomized to receive either MIDAC, or ICE then MIDAC, or ICE followed by a transplant, or a transplant only, in a 4 versus 5 courses and transplant versus chemotherapy 2 x 2 design. Patients in the transplant groups received an allogeneic transplant if a suitable matched sibling donor was available or, if not, an autologous transplant. Patients
assigned to the poor risk group were entered into the MRC trial for refractory/relapsed AML and were randomized to receive re-induction chemotherapy with either standard (10+3+5 then 8+3+5) or continuous ADE, and then further randomized to receive either cyclosporin A or not. For those patients with a clinical diagnosis of acute promyelocytic leukemia, all-trans retinoic acid, either as a short or extended course, was given in addition to the chemotherapy described above.
MRC AML 10 protocol
(Age <55 years)

MRC AML 12 protocol
(Age <60 years)
Publications arising from work in this thesis


