THE UNIVERSITY OF SHEFFIELD

DIVISION OF GENOMIC MEDICINE

PATHOGENIC ROLE OF C-FMS AND FLT3 MUTATIONS IN ACUTE AND CHRONIC MYELOPROLIFERATIVE DISORDERS

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A thesis submitted for the fulfilment of the degree of PhD

December 2002

Dedication

I would like to give my great appreciations to my parents for their prayers and patience during my stay away from them and for instilling in me confidence and a drive for pursuing my PhD.

In addition, I would like to thank my father-in-law, Shaik Enad M. Al-Goraid, who encouraged me to achieve my goals.

I would also like to give my thankfulness to his Excellency Dr Ghazi Algosaibi for his great support in obtaining this PhD scholarship.

Acknowledgments

Completing a PhD is truly a marathon event, and I would not have been able to complete this journey without Allah's will and the aid and support of countless people over the past years. I would like to thank Prof. Ian Peake for giving me the opportunity to work in the Division of Genomic Medicine (DGM). I would also like to thank Dr Peter Winship who was always helpful with advice. For their hospitality I would like to thank the people in DGM since this PhD project profitted a lot from our interesting discussions and the many new impulses I received from them.

I would also like to express my gratitude towards my supervisor, Dr John Reilly who initiated this project, his insights and comments were invaluable over the years.

I am also grateful to my second supervisor, Dr Anne Goodeve, who was so kind to read the whole thesis thoroughly and correct the English spelling and grammar and contributed extremely to the scientific discussion. In addition, her leadership, support, attention to detail, and hard work have set an example I hope to match some day.

The friendly and supportive atmosphere inherent to the whole division contributed essentially to the final outcome of my studies. In this context I would like to thank everybody in the division and in the Molecular Haematology Department, the completion of this thesis would not have been possible without their help. I also wish to thank all my officemates in M floor and lately G floor. Apart from my colleagues, I would like to thank my family and friends who have never lost faith in this long-term study. In addition, I would like to thank my wife for her support and patience during her stay away from her parents and loved ones. My three sons, Hamza, Mohammed and Enad for their enjoyable atmosphere which was needed to break the tension of my PhD study. Finally, this work received the financial support from the Ministry of Higher Education of Saudi Arabia.

Declaration

Unless otherwise acknowledged, the experimental data hereinafter presented are the result of the author's own research and have not been submitted for any other degree or qualification at any university or institute of higher education

PUPLICATIONS ARISING FROM THIS THESIS

Papers

1: Abu-Duhier, F.M.; Goodeve, A.C.; Care, R.S.; Gari, M.; Wilson, G.A.; Peake, I.R. & Reilly, J.T. (2003) Mutational analysis of class III receptor tyrosine kinases (C-KIT, C-FMS, FLT3) in idiopathic myelofibrosis. *Br J Haematol,* **120,** 464-470.

2: Abu-Duhier, F.M.;Goodeve, A.C.;Wilson, G.A.;Carr, R.S.;Peake, I.R. & Reilly, J.T. (2002) FLT3 internal tandem duplication mutations are rare in agnogenic myeloid metaplasia. *Blood,* **100,** 364(letter)

3: Abu-Duhier, F.M.;Goodeve, A.C.;Wilson, G.A.;Care, R.S.;Peake, I.R. & Reilly, J.T. (2001) Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. *Br J Haematol,* **113,** 983-988.

4: Abu-Duhier, F.M.;Goodeve, A.C.;Wilson, G.A.;Care, R.S.;Peake, I.R. & Reilly, J.T. (2001) Genomic structure of human FLT3: implications for mutational analysis. *Br J Haematol,* **113,** 1076-1077.

5: Abu-Duhier, F.M.;Goodeve, A.C.;Wilson, G.A.;Gari, M.A.;Peake, I.R.;Rees, D.C.;Vandenberghe, E.A.;Winship, P.R. & Reilly, J.T. (2000) FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. *Br J Haematol,* **111,** 190-195.

Abstracts

1:Care, R.S.; Goodeve, A.C.; Abu-Duhier, F.M.; Geertsma-Kleinekoort, W.; Valk, P.J.M.; Lowenberg, B.; Wilson, G.A.; Gari, M.A.; Peake, I.R. & Reilly., J.T. (2002) Incidence and Prognosis of c-kit and FLT3 Mutations in Core Binding Factor (CBF) Acute Myeloid Leukaemias. *Blood*, **100**, 746a.

2:Abu-Duhier, F.M.;Goodeve, A.;Wilson, G.;Gari, M.;Peake, I. & Reilly, J. (2002) Mutational analysis of class III receptor tyrosine kinase (RTK III) genes do not play an important role in idiopathic myelofibrosis. *The Hematology Journal,* **3,** 302.

3: Abu-Duhier, F.M.;Goodeve, A.;Wilson, G.;Care, R.S.;Peake, I. & Reilly, J. (2001) Novel c-fms mutations in acute and chronic myeloproliferative disorders. *Blood,* **89,** 631a.

4: Abu-Duhier, F.M.;Goodeve, A.;Wilson, G.;Care, R.S.;Peake, I. & Reilly, J. (2001) The FLT3 gene: Determination of genomic structure and identification of novel Asp835 mutations in acute myeloid leukaemia (AML). *The Hematology Journal,* **1,** 83. (Oral presentation)

5: Abu-Duhier, F.M.; Goodeve, A.; Wilson, G.; Gari, M.; Peake, I.; Rees, D.C.; Vandenberghe, E.; Winship, P. & Reilly, J. (2000) Adverse prognostic significance of FLT3 internal tandem duplication mutations in acute myeloid leukaemia. *The Hematology Journal,* **1,** 56. (Oral presentation)

Summary

Myeloproliferative disorders describe a group of conditions characterised by clonal proliferation of one or more haematopoietic components in the bone marrow, liver or spleen. In this study, two types of patients were included in the analysis 1) idiopathic myelofibrosis (IMF) which is a clonal haematopoietic stem cell disorder which results in a chronic myeloproliferation and atypical megakaryocyte hyperplasia and 2) acute myeloid leukaemia (AML) which is a heterogeneous group of malignant neoplasms composed of clonal expansions of immature cells.

Receptor-type tyrosine kinases (RTKs) constitute a family of proteins which are important in growth and developmental processes. Class III RTKs are characterized by an extracellular region composed of five immunoglobulin-like domains and by a split tyrosine kinase domains. RTK class III genes share a common structural organization.

Some of the class III RTKs perform major functions in haematopoiesis. They are the colony-stimulating factor-1 (CSF-1R or *c-fms*), Steel factor (SLF or c-kit) receptors, and the product of the *FLT3* gene and *PDGFRβ*.

The aim of this study was to identify the type and occurrence of *c-fms* and *FLT3* mutations in IMF and AML. Therefore, the establishment of the conformation sensitive gel electrophoresis (CSGE) analysis for *c-fms* and *FLT3* gene was carried out in this study. Novel and previously identified polymorphic nucleotide alterations in the *c-fms* gene were investigated. In total, twelve different apparently polymorphic alterations were identified. These are five alterations that resulted in silent amino acid changes and 7 intronic nucleotide alterations. Furthermore, important mutations that have been previously identified in the *c-fms* gene (i.e. 301 and 969 mutation) were

also investigated. However, none of the patients analysed had the 301 or the 969 mutation. In addition, three novel nucleotide changes resulting in amino acid substitution were identified in exons 6, 8 and 9.

In the other part of the study, which concerns the *FLT3* gene, it was shown, together with the data of Agnes et al, (1994), that the entire *FLT3* gene comprises 24, rather than the previously assumed 21 exons. In addition, the homologous location for codon 301 of *the c-fms* gene was screened to identify possible pathologically important mutations in this location. None of the AML or the IMF patients showed any mutations in exon 9. In addition, the homologous location to the codon 413 mutation found in the *c-fms* gene was also investigated, similarly no mutation was identified in that part of the *FLT3* gene.

Furthermore, the previously identified, *FLT3* internal tandem duplication (ITD) was also investigated. The data from this study showed that *FLT3* ITD occurs in 12.6% of adults with AML at diagnosis. It was demonstrated that AML patients possessing a *FLT3* ITD should be regarded as having high risk disease, irrespective of cytogenetics. This study also identified the presence of an Asp835 mutation that occurs in approximately 7% of AML patients. None of the IMF patients, or normal individuals screened had the Asp835 mutation. However, the Asp835 mutation was not found to be of prognostic significance in this group of AML patients.

Abbreviations

µl	microlitres
aa	Amino acid
ALL	Acute lymphoblastic leukaemia
A-loop	Activation loop
AMM	Agnogenic myeloid metaplasia
APS	Ammonium Persulphate
ASA/H	Allele specific amplification/hybridisation
ASRA	Allele specific restriction analysis
ATP	Adenosine Triphpsphate
BDH	British Drug Houses
BFU-E	Erythroid burst-forming units
BFU-MK	Megakaryocytic burst-forming-units
BLAST	Basic logical alignment search tool
CCM	Chemical cleavage mismatch
cDNA	complementary DNA
CFU	Colony-forming units
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
CMPD	Chronic myeloproliferative diseases
CR	Complete remission
CREB	cAMP response element binding
CSFs	Colony stimulating factors
CSGE	Conformation Sensitive Gel Electrophoresis
CT	C-terminal
C-terminal	Carboxyl terminal
DFS	Disease free survival
DGGE	Denaturing gradient gel electrophoresis
EC	Extracellular
EDTA	Ethylenediaminetetra-acetic Acid
EFS	Event free survival
EGF	Epidermal growth factor
Eph	Ephrins
EPO	Erythropoietin
EtBr	Ethidium bromide
FAB	French-American-British

FL	FLT3 ligand
FLK2	Fetal liver kinase 2
FLT3	Fms-like tyrosine kinase 3
FMS	Feline McDonough sarcoma
G-6PD	Glucose-6-phosphate-dehydrogenase
GAP	GTPase-activated protein
GFX columns	Glass fibre matrix columns
GISTs	Gastrointestinal stromal tumours
Grb2	Growth factor receptor binding protein 2
HGF	Haematopoietic growth factors
HGP	Human genome project
HL60	Human leukaemia 60 cell line
HLH	Helix-loop-helix
Htgs	high throughput genomic sequence
IC	Intracellular
IGF1R	Insulin-like growth factor-1 receptor
Ig-like	Immunoglobulin like
ILs	Interleukins
IMF	Idiopathic myelofibrosis
ins/del	Insertion-deletion
ITD	Internal tandem duplication
JM	Juxtamembrane
KI	Kinase insert
KM	Kaplan-Meier
M	Molar
MAPK	Mitogen activated protein kinase
MDS	Myelodysplastic syndrome
Mg	miligram
$MgCl_2$	Magnesium chloride
Min	Minutes
MLL	Mixed-lineage leukaemia gene
mM	miliMolar
MPC	Multilineage progenitor cell
mRNA	Messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
NK	Natural killer
N-Terminal	Amino terminal
OLA	Oligonucleotide ligation assay

OS	Overall survival
PBS	Phosphate buffered saline
PDGFR	Platelet-derived growth factor receptor
PF4	Platelet factor 4
PI3k	Phospotidylinositol 3`-kinase
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
PNK	T_4-polynucleotide kinase
PTB	Phosphotyrosine binding
PV	Polycythaemia vera
RFLP	Restriction fragment length polymorphism
RHH	Royal Hallamshire Hospital
RNA	Ribose nucleic acid
rpm	Revolution per minute
RTKs	Receptor tyrosine kinase
SCF	Stem cell factor
SH2	Src homology2
SNL	Sinonasal lymphomas
SNPs	Single nucleotide polymorphisms
SSCP	Single strand conformational polymorphism
STI571	Signal transduction inhibitor 571
SYK	Spleen tyrosine kinase
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N,',N'-tetramethylethyenediamine
TGF-β	Transforming growth factor-β
TK	Tyrosine kinase
TM	Melting temperature
TM	Transmembrane
UPC	Unilineage progenitor cell
v-fms	Feline McDonough sarcoma virus

Table of Content

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

References

Appendices

Chapter 1

Introduction

1.1 Haematopoiesis

In humans, blood cells are programmed to live for only a few hours (granulocytes), or weeks (erythrocytes), before being destroyed. As a result, the body is required to produce approximately 10^{13} new myeloid cells to replace this loss. This activity takes place within the bone marrow, where blood cells in various stages of development can be identified morphologically, as well as more primitive cells. It is these premature cells that act as the precursors for the various mature cell lineages.

The pluripotent stem cell, thought to be a nondescript lymphocyte-like cell, is the producer of all these precursor cells and ultimately the native component found in the peripheral blood. Growth factors, including colony-stimulating factors and interleukins, can stimulate stem cell division, differentiation and maturation, to form the mature cellular elements of the blood. Mesenchymal cells in the yolk sac are the source of embryonic haematopoietic stem cells, while the fetal liver becomes the main site after 12 weeks. The bone marrow becomes an important site from week twenty, and becomes the main haematopoietic organ at the time of birth. During the first 2-3 years of life, active (red) bone marrow is found in the majority of skeletal bones, although a gradual replacement of active marrow by inactive (fatty) tissue occurs such that in adulthood active haematopoietic tissue is limited to the epiphyses of the long bones, the sternum, ribs, cranium, vertebrae and the pelvis.

The expansion of the haematopoietic tissue terminates after the third year of infancy such that, the volume of active marrow in an adult is similar to that in a 3 year old child. As stated, haematopoiesis derives from a pool of undifferentiated cells known as stem cells, which give rise to the more differentiated bone marrow cells by division and differentiation.

Stem cell have been shown to have the ability for self-renewal in mouse stem cells (Lu *et al*, 1988; Semenza *et al*, 1989).

A unique process of simultaneous balanced expansion and differentiation operates to maintain the stem cell pool throughout life while, at the same time, generating differentiating cells. *In vitro* experiments have shown that single undifferentiated blast cells divide repetitively, to produce clones of mature cells of specific lineages. The progenitor cell compartment encompasses the immediate progeny of stem cells, or multilineage progenitor cells (MPC), to cells committed to one differentiation lineage, the unilineage progenitor cell (UPC). After several divisions and lineage events, the MPC give rise to several UPCs, each of which is committed to a single lineage. Such progenitor cells have been named according to their product e.g. (CFU-GM, CFU-Eo, CFU-Bs, CFU-Meg, BFU-E). Cells whose progeny will be exclusively red cells have been termed erythroid burst-forming units (BFU-E). Similarly, megakaryopoiesis appears to progress in a fashion analogous to erythropoiesis, until the formation of a dedicated precursor cells termed megakaryocytic burst-forming-units (BFU-MK). Myeloid maturation is dependent on precursor cells called colony-forming units for granulocytes and monocytes (CFU-GM) (table 1.1).

Table 1.1. Haematopoietic progenitor cells

CFU-GEMM	Colony-forming unit-Granulocyte, erythrocyte, macrophage/monocyte, megakaryocyte.
CFU-GM	Colony-forming unit-granulocyte, macrophage/monocyte.
CFU-Eo	Colony-forming unit-eosinophil.
CFU-Bs	Colony-forming unit-basophil.
CFU-Meg	Colony-forming unit-megakaryocyte.
CFU-E	Colony-forming unit-erythrocyte.
BFU-E	Burst-forming unit-erythrocyte.

1.2 Haematopoietic growth factors and interleukins

Proliferation, differentiation and maturation of haematopoietic progenitor cells are mainly regulated by haematopoietic growth factors, or colony stimulating factors (CSFs), and interleukins (ILs) which have a role in survival and function regulation of mature blood cells. A significant advance in our understanding of haemopoiesis was made in the mid 1960s, with the introduction of the colony assay for haematopoietic progenitor cells (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). This assay demonstrated that under suitable conditions, individual haematopoietic stem, or progenitor cells, would divide and differentiate, giving rise to discrete colonies, each of which constituted the differentiated progeny of single ancestral cells.

Similarly, the growth factors, termed interleukins, were discovered using a variety of liquid culture systems designed to study T- and B-cell proliferation and differentiation (Quesenberry *et al*, 1989). The term 'interleukin' was initially applied to a series of polypeptide factors, on the basis that they were produced by, and acted upon, various leukocytes. The term was later extended, however, to include polypeptide factors released

during inflammatory responses, irrespective of whether they were also produced by, or acted upon, non-leukocytes. Twenty interleukins have been identified to date (table 1.2).

Table 1.2. Cytokines involved in haematopoietic blood cell development .

Name	Source	Target
Growth Factors		
Erythropiotin	Kidney, Liver	Erythroid progenitors
G-CSF	Macrophages, Endothelial cells, Fibroblasts.	Stem cells, Neutrophil precursors.
GM-CSF	T- and B-lymphocytes, monocyte-macrophage, Endothelial cells, Fibroblasts.	Progenitors for neutrophils, Eosinophils, monocytes.
M-CSF	Most tissues.	Monocyte-macrophage, granulocyte.
Thrombopoietin	Stromal cells	Megakaryocyte.
SCF	Stromal cell, endothelial cell, monocyte/macrophage.	Stem cell, megakaryocyte, mast cell, granulocyte, and eosinophil.
TGFβ	Platelets, mast cells	Fibroblast
FLT3 ligand		Stem cells, monocyte, macrophages, granulocyte, and erythrocyte
Interleukins		
IL-1	Monocyte-macrophage, endothelial cell, fibroblast.	T lymphocyte, stem cell, megakaryocyte.
IL-2	T lymphocyte.	T- and B-lymphocyte.
IL-3	T lymphocyte, mast cells.	Granulocyte, erythroblast, stem cell, mast cell eosinophil, megakaryocyte, monocyte-macrophage.
IL-4	T lymphocytes	B cells, mast cells, T cells.
IL-5	T lymphocyte, mast cells.	Eosinophil, B-lymphocyte.
IL-6	Monocyte-macrophage, megakaryocyte, eosinophil, B-lymphocyte, fibroblast, stromal cell.	Stem cell, .granulocyte, megakaryocyte, and monocyte-macrophage.
IL-7	Stromal cell.	Pre-B cells T-cells.
IL-8	Monocytes, T cells, fibroblasts	Neutrophils, T cells, basophils.
IL-9	T lymphocyte.	T lymphocyte, eosinophil, megakayocyte, mast cell.
IL-10	T cells, macrophages, B cells	B cells, macrophages, T cells, mast cells.
IL-11	Stromal cell, fibroblast.	Stem cell, megakarocyte, granulocyte, eosinophil, and mast cell.
IL-12	B cells, macrophages	T cells, NK cells
IL-13	T-cells	B cells.
IL-14	T-cells	Activated B cells.
IL-15	T-cells and epithelial cells	
IL-16	Eosinophils, $CD8^+$ T-cells	$CD4^+$ T-cells
IL-17	$CD4^+$ T-cells	Stimulates fibroblast to sustain $CD34^+$ proginators.
IL-18	Hepatocytes	Enhances NK cells activity
IL-19	Monocytes	induces production of IL-6 and TNF-alpha and results in cell apoptosis through TNF-alpha
IL-20	Keratinocytes	keratinocyte proliferation and acts as a paracrine or autocrine factor

Adapted from *(Harmening, 1992; Gallagher et al, 2000; Liao et al, 2002)*

Interaction between AML progenitor cells and the haematopoietic growth factors is required for cell proliferation in vitro. (Griffin and Lowenberg, 1986) demonstrated that AML colonies could be formed following incubation for 1-2 weeks with specific haematopoietic growth factors. This study highlighted the importance of haematopoietic growth factors for proliferation and survival of AML progenitor cells. Furthermore, leukaemic colonies can be induced and DNA synthesis stimulated in more than 80% of AML cells in the presence of interleukin-3 (IL3), GM-CSF and G-CSF (Miyauchi *et al*, 1987; Vellenga *et al*, 1987; Delwel *et al*, 1988; Pebusque *et al*, 1988)

A significant amount of research effort has been directed towards the investigation of this group of glycoproteins. At least 16 separate CSFs have been purified, characterised and subsequently molecularly cloned (Shurin *et al*, 1998). It has been shown that primitive progenitors are only able to proliferate *in vitro* when stimulated by multiple growth factors (Shurin *et al*, 1998). In contrast, however, single cytokines were able to stimulate committed progenitor cells, although the synergistic effect of a number of growth factors can cause enhanced growth (Metcalf, 1993; Ogawa, 1993).

In general, interaction of haematopoietic growth factors (HGF) with blood cells occurs at different levels in the cascade of cell differentiation, from multipotent progenitor cells to the mature cell found in peripheral blood (Groopman *et al*, 1989). Two important growth factor are GM-CSF and G-CSF, both categorised by their biologic activities on the haematopoietic system (Grosh and Quesenberry, 1992). GM-CSF is produced by T lymphocytes, monocytes, fibroblasts and endothelial cells and is encoded by a gene located on the long arm of chromosome 5 at band q21-q33 (Wong *et al*, 1985). Monocytes, fibroblasts and endothelial cells also produce G-CSF. The G-CSF gene has been mapped to the long arm of chromosome 17, at bands 17q11.2 to 17q11.21 (Gough *et al*, 1990). It is noteworthy that these myeloid growth factors have a high affinity for their cognate receptor, which is

typically present at low levels on the surface of the target cell (Nicola, 1989). Interestingly, activation of downstream signalling by these cytokines appears to require a low level of receptor binding (i.e. 5-10% of receptor occupancy) (Nicola, 1989).

GM-CSF has been found to act at several stages of myeloid differentiation, from the early stem cell to the mature cell. It is known to be specific for the development of both granulocytes and monocytes/macrophages, although, it also has a growth and differentiation effect on premature progenitors (Metcalf, 1990; Grosh and Quesenberry, 1992). Furthermore, its effect extends to the maintenance of mature cells, including macrophage and monocytes, although the amount needed for this function is less than that required to promote proliferation (Lau *et al*, 1996). The effect of GM-CSF on neutrophils includes enhancement of phagocytosis, induction of chemotaxis and migration of neutrophils to sites of inflammation (Lau *et al*, 1996). *In vivo* experiments, using animal models, has shown that GM-CSF also enhances the megakaryocyte number while, in an *in vitro* system, the addition of GM-CSF has been shown to support the growth of megakaryocytes (Hoffman, 1989). However, the effect of GM-CSF on megakaryocytopoiesis is not sufficient by itself, as GM-CSF interaction with other cytokines is needed (Ishibashi *et al*, 1990; Vannucchi *et al*, 1990).

In contrast, G-CSF is a late acting, lineage specific haematopoietin, acting mainly on committed progenitors of the neutrophil lineage (Grosh and Quesenberry, 1992), although it can interact with IL3, to enhance the proliferation of multipotent haematopoietic and megakaryocyte progenitors (Ikebuchi *et al*, 1988; McNiece *et al*, 1988). The activity of G-CSF is targeted toward regulating neutrophils function, including phagocytosis, superoxide release, antibody-dependent cellular cytotoxicity and migration (Groopman *et al*, 1989).

Erythropoietin (EPO) is a growth factor that is mainly produced in the kidneys and to a less extent the liver, in response to hypoxic conditions resulting from reduced atmospheric

oxygen (Goldberg *et al*, 1987; Goldberg *et al*, 1988). Erythropoietin was the first haematopoietic growth factor identified (Browne *et al*, 1986), and is encoded by a gene located on the long arm of chromosome 7 (Powell *et al*, 1986). EPO stimulates the growth and differentiation of erythroid progenitor cells (Browne *et al*, 1986) as well as enhancing the proliferation of more differentiated erythroid precursors.

FLT3 ligand (FL) is the ligand for the *FLT3* (fms-like tyrosine kinase 3) receptor. Expression of FL has been reported for bone marrow fibroblasts and haematopoietic cells of myeloid B-cell and T-cell lineages (Brasel *et al*, 1995). FL is believed to have a similar role in inducing proliferation of normal myeloid and lymphoid progenitors, although it is not as potent as other growth factors (Lyman *et al*, 1994). FL does, however, interact with other growth factors and interleukins to induce proliferation of myeloid and lymphoid progenitors (Lyman and Jacobsen, 1998). FL interestingly, has also been shown to stimulate the proliferation of primary AML cells that express *FLT3,* as well as myeloid and monocytoid leukaemic cell lines (Piacibello *et al*, 1995; Drexler, 1996). The importance of FL was highlighted as the result of its ability, together with other growth factors, to stimulate the expansion of $CD34^+$ haematopoietic progenitors. Indeed this function may be useful in stimulating marrow recovery after cytotoxic chemotherapy (Gilliland and Griffin, 2002). The use of FL as leukaemic therapy may not be without risk, as FL stimulation of *FLT3* can enhance the proliferation and survival of leukaemic blasts (Minden *et al*, 1996).

CSF-1, or M-CSF, is synthesized by mesenchymal cells and is thought to be able to stimulate the survival, proliferation and differentiation of haematopoietic cells within the monocyte-macrophage lineage (Stanley *et al*, 1983). CSF-1 is encoded by a gene located on the long arm of chromosome 5 (Ralph *et al*, 1986). Expression of CSF-1 on premature haematopoietic cell can be considered as an early marker of commitment to the monocytes-macrophage series (Bartelmez *et al*, 1985). CSF-1 play an important autocrine and/or

paracrine role in cancer of the ovary, endometrium and breast, as well as myeloid and lymphoid malignancies (Stanley *et al*, 1997). CSF-1 has been shown to participate in the regulation of mononuclear phagocyte production, since the injection of recombinant human CSF1 into mice causes an increase in circulating monocytes, from 3% to 30% (Stanley *et al*, 1994). Although CSF-1 has an obvious effect on the function of mature monocytes and macrophages, it does not appear to affect premature progenitor cells of other cell lineages (Stanley *et al*, 1997).

Stem cell factor (SCF) is encoded by a gene located on chromosome 12 in humans (Anderson *et al*, 1991) and has an effect on the development of colony-forming unit-granulocyte/macrophage (CSF-GM) (Heyworth *et al*, 1992). Its effect is weak, however, on myeloid colony formation (Heyworth *et al*, 1992). When combined with GM-CSF, G-CSF, IL3 or EPO it can enhance colony growth of myeloid and erythroid lineage (McNiece *et al*, 1991). SCF also has an enhancing effect on megakaryocytopoiesis, since it acts in synergy with IL3 and GM-CSF (Avraham *et al*, 1992).

1.3 Receptor tyrosine kinases (RTKs)

Receptor tyrosine kinase (RTKs) are an important group of genes. Up to 90 tyrosine kinase genes have been identified so far, of which 58 are of the receptor type (Reilly, 2002). RTKs are transmembrane proteins that are involved in ligand binding and signal transduction (Gupta *et al*, 2002). They catalyse the transfer of the γ-phosphate of ATP to tyrosine residues of protein substrates. Recently, RTKs have been grouped into 20 subfamilies based on kinase domain sequence (Robertson *et al*, 2000; Robinson *et al*, 2000). These subfamilies are characterised by an extracellular ligand binding domain, a transmembrane (TM) domain and an intracellular tyrosine kinase domain (Robinson *et al*, 2000) (figure 1.1)

The extracellular domain has the least homology between the RTKs. The TM domain is believed to play a critical role in receptor activation, due to the presence of a common alpha helical structure which is believed to anchor the molecule in the cell membrane (Gupta *et al*, 2002). The cytoplasmic kinase domain is separated from the TM by the juxtamembrane region (JM), which is highly conserved in each family of RTKs. However, the kinase domains are the most conserved domains among RTKs (Gupta *et al*, 2002).

The most important domains in RTKs are the activation loop (A-loop), the nucleotide-binding loop and the catalytic loop, which are located in the IC part of the receptor. All of these domains have a crucial role in phosphorylation and hence down-stream signalling. The A-loop in the inactive state, prevents phosphorylation, by either blocking the substrate or the ATP binding site of the receptor (Mohammadi *et al*, 1996). However, in the active state, the A-loop, by repositioning and contacting specific residues in the c-terminal domain of the receptor, exposes the kinase domain (Mohammadi *et al*, 1996) (see figure 1.4). It is believed that the catalytic loop of the protein kinase contains an invariant aspartate residue that serves as the catalytic base in the phosphotransfer reaction (Johnson *et al*, 1996).

Another consistent feature of RTK structure is the kinase insert (KI) domain which is present in variable length and sequence within the kinase domain. Mutational analysis of several kinases has revealed that this part of the receptor is not essential for intrinsic kinase activity, however, it does contains tyrosine residues that are sites for autophosphorylation (Heidaran *et al*, 1991). Similarly, downstream of the kinase domain, the C-terminal tail contains target sites for autophosphorylation (Schlessinger, 2000).

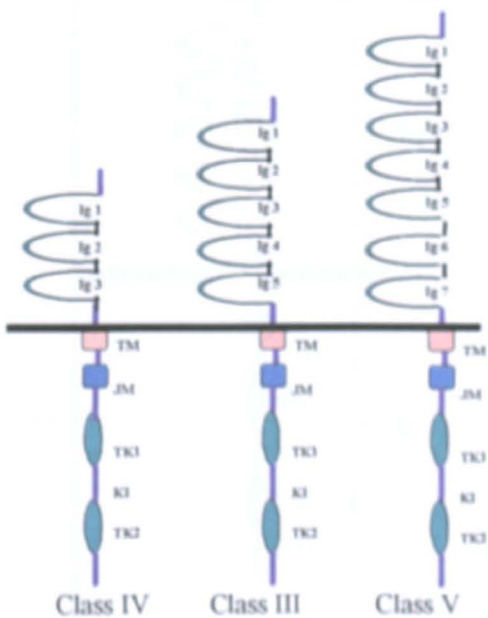


Figure1.1. Schematic representations of class III, IV and V RTKs. Class III have five Ig-like domains, class IV have three Ig-like domains and class V have seven Ig-like domains in their extracellular part of the receptor.

1.3.1 Class III receptor tyrosine kinase

Class III RTKs are characterized by an extracellular ligand-binding domain that contains five immunoglobulin like domains (figure 1.2). Class III RTKs like many other RTKs have a TM, JM, two intracellular tyrosine kinase (TK1 and TK2) domain divided by a KI, and a C-terminal domain (Yarden *et al*, 1987; Ullrich and Schlessinger, 1990).

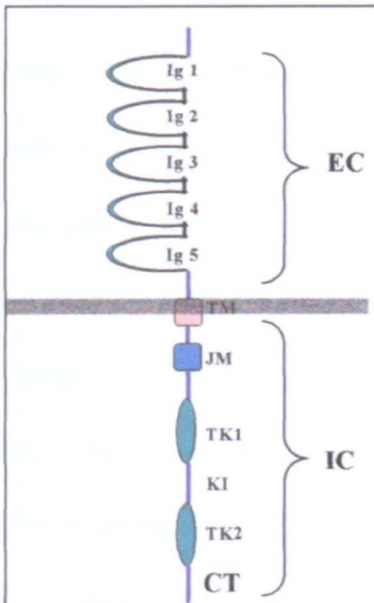


Figure 1.2 schematic representation of class III tyrosine kinase receptor with an extracellular domain, EC (with 5 immunoglobulin- like domains, Ig); transmembrane domain, TM; intracellular domain, IC; juxtamembrane domain, JM; tyrosine kinase domains, TK1 & TK2; kinase insert domain, KI. C-terminus; CT.

Five receptors of RTKs belong to the class III RTKs, these are *c-fms* (Coussens *et al*, 1986), *c-kit* (Yarden *et al*, 1987), *FLT3* (Rosnet *et al*, 1993a), *PDGFRα* (Claesson-Welsh *et al*, 1989) and *PDGFRβ* (Yarden *et al*, 1987). All class III RTKs except *PDGFRα*, have an important role in haematopoiesis. A close evolutionary relationship was suggested for class III RTKs due to their close genomic structure and paired chromosomal location (Rosnet *et al*, 1991b; Andre *et al*, 1992; Rosnet *et al*, 1993b). Chromosome 4 contains two class III RTKs; the *c-kit* and *PDGFRα* gene, which are located in tandem on bands q11-q13 (Gronwald *et al*, 1990; Giebel *et al*, 1992). Similarly, both the PDGFRβ and *c-fms* genes lie in tandem on chromosome 5 at q31-q33 (Groffen *et al*, 1983; Roberts *et al*, 1988). The

FLT3 and *FLT1* (a class V RTK) genes both map to 13q12, were *FLT3* is located in a head-to-tale fashion to *FLT1* (Rosnet *et al*, 1993b; Imbert *et al*, 1994).

1.3.1.1 Colony stimulating factor 1 receptor CSF-1R

The actions of CSF-1 are mediated through its binding to a single class of high-affinity receptors (CSF-1R), expressed on monocytes, macrophages, and their committed progenitors (Sherr *et al*, 1988). The *c-fms* proto-oncogene encodes the cell surface receptor for the macrophage colony stimulating factor, CSF-1 (Sherr *et al*, 1985). *c-fms* was cloned and sequenced in 1989, and was shown to consist of 42 kb of DNA, including 1.7 kb 5′ to the first *c-fms* coding exon, 33 kb of interrupting *c-fms* coding sequences, and 7 kb of DNA 3′ to the *c-fms* mRNA polyadenylation site. It has been shown that the *c-fms* gene contains 22 exons, however, exon 1 is non coding, therefore the coding exons are exon 2-22 (Hampe *et al*, 1989). The *c-fms* coding exons are short, with length ranging from 89 base pairs (bp) (exon 16) to 285bp (exon 4); exon 22 consists of 153bp encoding the *c-fms* C-terminus, as well as 777 bp of 3′ non-coding sequence before to the polyadenylation site (Hampe *et al*, 1989). Intron sizes are variable, ranging from 6355 bp (intron 11) to as short as 81 bp (intron 18) (Hampe *et al*, 1989). Initial analysis was performed using primer extension and nuclease protection experiments for c-fms promoter region. It was concluded that the upstream non-coding exon 1 is transcribed, however, sequences corresponding to known mammalian promoter motifs have not yet been identified, nor has a *c-fms* promoter been functionally demonstrated (Hampe *et al*, 1989). However in murine tumour cell line the expression of the *c-fms* is regulated by two distinct promoters: distal and proximal. The distal promoter is active in trophoblasts during embryogenesis and the proximal promoter directs expression to the cells of myeloid lineage (Favot *et al*, 1995).

In relation to myeloid neoplasms, two *c-fms* mutations have been reported in several myeloproliferative and myelodysplastic disorders, for instance Ridge et al, (1990), reported in human myeloid malignancies, an incidence of 12.7% (14/110) for mutations of codon 969 and 1.8% (2/110) for mutations of codon 301. Codon 969 mutations are located in the C-terminal tail region. Although that codon 969 mutation occurred in heterogeneous disorders, it has been found to be associated with poor outcome in the myelodysplastic syndrome (MDS) group. The codon 301 mutations are located in the fourth Ig-like domain of *c-fms*, and involve a highly conserved amino acid. Furthermore, the same genetic alteration was found in *v-fms* (feline McDonough sarcoma virus) (Roussel *et al*, 1988; Woolford *et al*, 1988). In addition, allelic loss of *c-fms* has been detected in both AML and MDS (Ridge *et al*, 1990; McGlynn *et al*, 1997).

1.3.1.2 FMS-like tyrosine kinase (FLT3)

The search for additional members of the RTK family led to two groups discovering a RTK termed fetal liver kinase 2 (FLK2) in mice (Matthews *et al*, 1991a; Matthews *et al*, 1991b) and in humans, the FMS-like tyrosine kinase (*FLT3*) (Rosnet *et al*, 1991a; Rosnet *et al*, 1991b). *FLT3* has been shown to be expressed in a variety of human and murine cell lines of both myeloid and B-lymphoid lineage (Brasel *et al*, 1995; Turner *et al*, 1996). In normal bone marrow, the expression of *FLT3* appears to be restricted to early progenitors, including $CD34^+$ cells with high expression of CD117 (c-kit) (Rasko *et al*, 1995; Rosnet *et al*, 1996). The amino acid sequence of FLK2 was found to be nearly identical to *FLT3* except for two amino acids in the extracytoplasmic domain and 31 amino acids in the C-terminus in the cytoplasmic domain (Lyman *et al*, 1993). Therefore, it was suggested that they are encoded by one gene and share the same ligand (Lyman *et al*, 1993). The human *FLT3* cDNA was initially cloned from a pre-B-cell line and shows a great similarity with the corresponding mouse FLT3/FLK2 protein (Rosnet *et al*, 1993a). Human *FLT3* is encoded by a gene

located on chromosome 13q12 and shares structural homology with other subclass III RTKs. It has also been demonstrated that the human *FLT3* extracellular domain exhibits 18% and 19% homology with c-kit and *c-fms*, respectively, which increases to 63% and 64% for the tyrosine kinase domain (Agnes *et al*, 1994).

FLT3 is expressed in $CD34^+$ bone marrow cells in humans (Small *et al*, 1994). Recently the human *FLT3* receptor has attracted attention due to fact that it is highly expressed in many types of leukaemia, including AML, B-ALL and T-ALL (Birg *et al*, 1992; Rosnet *et al*, 1993a; Turner *et al*, 1996). Interestingly, an internal tandem duplication (ITD) has been documented in more than 20% of AML and in most cases the duplication occurs in the JM domain and correlates with a poor prognosis (Nakao *et al*, 1996; Horiike *et al*, 1997; Abu-Duhier *et al*, 2000). Furthermore, an Asp835 mutation has been identified in the activation loop of the *FLT3* gene, and is present in 7% of adult AML (Abu-Duhier *et al*, 2001; Yamamoto *et al*, 2001). Taking into account the ITD and the Asp835 mutation, about a third of AML have a *FLT3* mutation. It is noteworthy that the Asp835 mutation is analogous to the Asp816 alteration of the *c-kit* gene (Abu-Duhier *et al*, 2001; Yamamoto *et al*, 2001).

1.3.1.3 c-kit

The extracellular domain of *c-kit* consists of five Ig-like domains as in all other class III RTKs. As stated earlier, *c-kit* and its ligand (SCF) have a critical role in normal haematopoiesis. *c-kit* is expressed by 70% of $CD34^+$ cells in bone marrow, including lineage-restricted haematopoietic progenitor cells (Ashman *et al*, 1991; Papayannopoulou *et al*, 1991) and by primitive cells capable of establishing long-term *in vitro* haematopoiesis (Simmons *et al*, 1994). Human *c-kit* (CD117) consists of 975 amino acids: twenty three form the signal sequence at the N-terminus, while the five Ig-like domains consist of 497 amino acids and contain nine potential N-glycosylation sites (Blechman *et al*, 1993). The Ig-

like domains are characterised by a primary sequence of 70-100 amino acids residues with disulfide bridges spanning 40-60 amino acids (reviewed by Blechman *et al* 1993). The three amino terminal Ig-like domains contain the stem cell factor (SCF) binding site, while the fourth Ig-like domain is essential for receptor dimerisation (Lev *et al*, 1993; Blechman *et al*, 1995). The function of the fifth Ig-like domain, however, has not been determined (Blechman *et al*, 1995). It is worth mentioning that *c-kit* mutations have been associated with AML exhibiting either an inv(16) or t(8;21) karyotype, i.e. the core binding factor leukaemias. For instance, an AML patient with t(8;21) has been reported having Asp816Tyr (Beghini *et al*, 1998). Later the same group reported Asp816 mutation in 4/9 cases with t(8;21) and 2/6 cases with inv(16) (Beghini *et al*, 2000). Moreover, around a third of the AML-M4Eo patients analysed in a study carried out by Gari *et al* (1999) have been reported to have a novel exon 8 deletion/insertion mutation with consistent loss of Asp419 located in the fifth Ig-like domain of the *c-kit* receptor (Gari *et al*, 1999). Deletions in the extracellular domain can lead to ligand-independent activation due to the removal of the negative regulatory constraints (Khazaie *et al*, 1988; Uren *et al*, 1997). It was suggested, therefore, that the discovery of Asp419 mutations could be the first evidence to the importance of the fifth Ig-like domain (Gari *et al*, 1999). *c-kit* mutations have also been reported in exon 2 in two patients with idiopathic myelofibrosis and one with chronic myeloid leukaemia (CML) (Nakata *et al*, 1995). Although it was not determined if such mutation resulted in a constitutively activated receptor, it was documented that this acquired abnormality of the extracellular domain resulted in enhanced sensitivity of the patients stem cells to SCF (Kimura *et al*, 1997).

1.3.1.4 Platelet-derived growth factor receptor (PDGFRβ)

PDGFRβ, a class III RTK is a 170-190 KDa single transmembrane glycoprotein (CD140b), expressed on fibroblasts, smooth muscle cells, glial cells and chrondrocytes. PDGFRβ has also been identified on different types of haematopoietic cells, including B and T lymphocytes (Goustin *et al*, 1990; Tsai *et al*, 1994), NK cells (Gersuk *et al*, 1991), cultured monocytes (Inaba *et al*, 1993), HL60 myelomonocytic cells (Pantazis *et al*, 1990), platelets and megakaryocytes (Yang *et al*, 1997).

Golub et al, (1994) showed that an infrequent cytogenetic abnormality in atypical CML, namely t(5;12) (q31;p13), results in a TEL/ PDGFRβ fusion gene. The NH-2 terminus of the receptor is replaced by the first 154 amino acids of the transcription factor TEL, which contains a putative helix-loop-helix (HLH) domain, a motif that enables the fusion receptor to dimerize in the absence of ligand (Golub *et al*, 1994).

Furthermore, the oncogenic capacity of TEL/PDGFRβ was confirmed *in vivo* by showing that expression of the chimeric gene, when under the control of the CD11b promoter in transgenic mice, can cause a chronic myeloprliferative syndrome characterized by leucocytosis, megakaryocytic hyperplasia and splenomegaly due to an extramedullary haematopoiesis (Ritchie *et al*, 1999).

Class III RTKs (figure 1.3) are therefore of great interest due to the association with an increasing number of haematological malignancies (Reilly, 2002) (see table 1.3).

Table 1.3 Example of dominant class III RTKs oncogenes

RTK (proto-oncogene)	**Viral oncogene** (viral oncoprotein)	**Oncogenic alteration**	**Tumour/cancer types**
PDGFR-β		Tel-PDGR-β (t(5;12 translocation fusing Ets-like Tel with PDGR-β PTK domain) Overexpression	Tel-PDGR-β; chronic myelomonocytic luekaemia. Tel-PDGR-β; glioma
CSF-1R (c-fms)	v-fms from FeSV	v-fms; truncated CSF-1R PTK with mutant C-terminal tail constitutively active c-fms; GOF point mutations overexpression	v-fms; felinefibrosarcoma c-fms; acute and chronic myelomonocytic leukaemias, monocytic tumour, malignant histocytosis, endometrial cancer, glioma
Kit/SCFR (c-kit)	v-kit from FeSV	v-kit; truncated Kit/SCF-1R PTK with mutant C-terminal tail Constitutively active c-kit; GOF point mutations and small deletions over expression	v-kit; feline fibrosarcomas c-kit; malignant gastrointestinal stromal tumours, acute myeloid luekaemias, myelodysplastic syndromes, mast-cell leukemia/systemic mastocytosis, seminomas/dysgerminomas, small-cell lung cancer and other carcinomas
Flk2/Flt3		Overexpression of Internal tandem gene duplications in JM region Asp835 mutation in TK2	Haematopoietic malignancies

Adapted from Jensen and Hunter, 2001

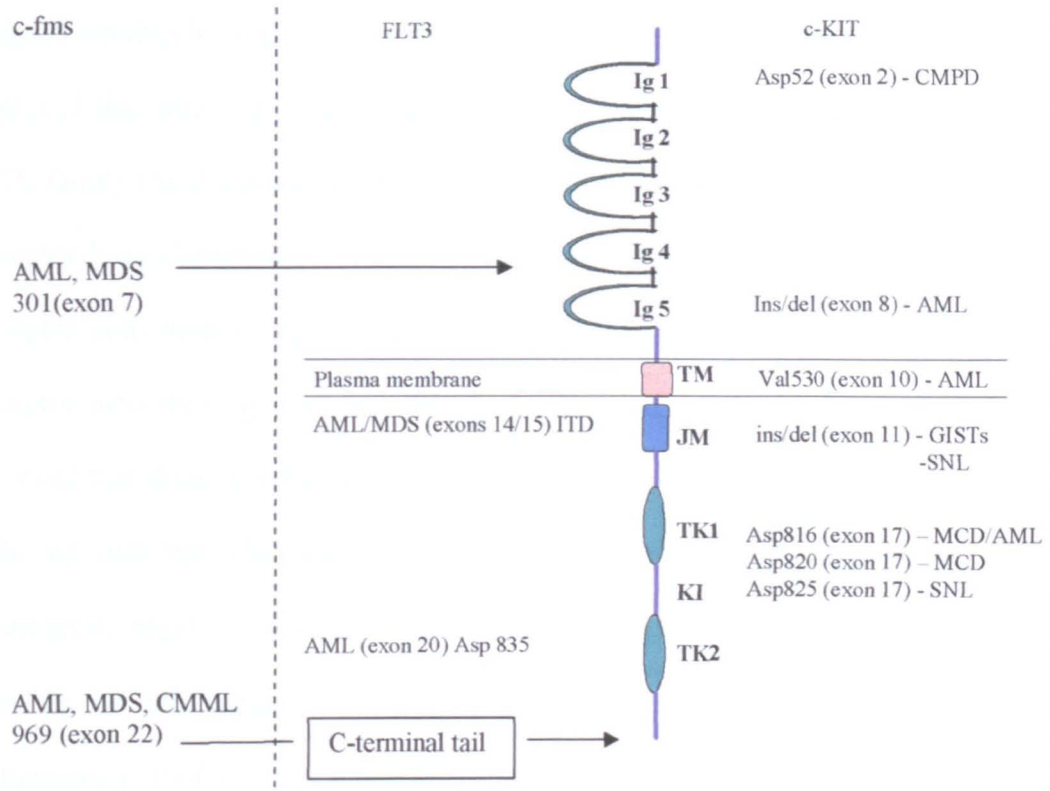


Figure 1.3 A Schematic representation of some class III RTKs highlighting the position of reported mutations and their disease associations of *c-fms*, *FLT3* and *c-kit*. CMPD, chronic myeloproliferative diseases; AML, acute myeloid leukaemia; GISTs, gastrointestinal stromal tumours; SNL, sinonasal lymphomas; MCD, mast cell disease; MDS, myelodysplastic syndrome; CMML, chronic myelomonocytic leukaemia; ins/del, insertion-deletion mutations.

Adapted from(Reilly, 2002)

1.3.2 Activation of RTKs by ligand binding

Ligand binding is considered as the main step for receptor activation (Heldin, 1995). It is believed that this step is the major cause for dimerization in monomeric receptors of the RTK family (Schlessinger, 2000). It is noteworthy to mention that ligand binding can induce receptor homodimerization or heterodimerization (Schlessinger, 2000). The second step of receptor activation is signal transduction. Although this step is considered essential for receptor activation, it does not directly lead to increased kinase activity. It is therefore, believed that there is a requirement for further conformational changes in the receptor. It is believed that the TM domain has an exceptional role in this process. A conserved hydrophilic region of several residues has been noticed within the alpha helices such that they lie on the same face of the domain (Sternberg and Gullick, 1990; Ullrich and Schlessinger, 1990). As an effect of the ligand binding, these will lead to coupled rotation of the paired RTKs within a receptor-ligand complex. Therefore, the hydrophilic residues located in the TM (and other) domains of each receptor would be able to form hydrogen bonds that hold them in fixed position. As a result, the kinase domain of each receptor would be in a contact with that of its partner and therefore allow transphosphorylation of one by the other (Bell *et al*, 2000).

In general, there are two theories as to how receptor transphosphorylation is initiated. The first is that the highly mobile A-loop (see section 1.3) will swap between an active and an inactive conformation and therefore, would provide the kinase domain of the unphosphorylated receptor with a low-level of activity. Furthermore, the ligand binding simply amplifies the local concentration of kinase domain (i.e. enzyme and substrate) consequently, this will increase the likelihood of transphosphorylation (Hubbard *et al*, 1998). The second suggestion is that the dimerization transiently stabilizes the A-loop allowing the

substrate binding and phosphotransfer to occur (Johnson *et al*, 1996) (figure 1.4). It is worth mentioning that tyrosine phosphorylation of activated RTKs both promotes intrinsic kinase activity and generates sites of interaction for a variety of downstream phosphotyrosine binding signalling protein (Hubbard *et al*, 1998).

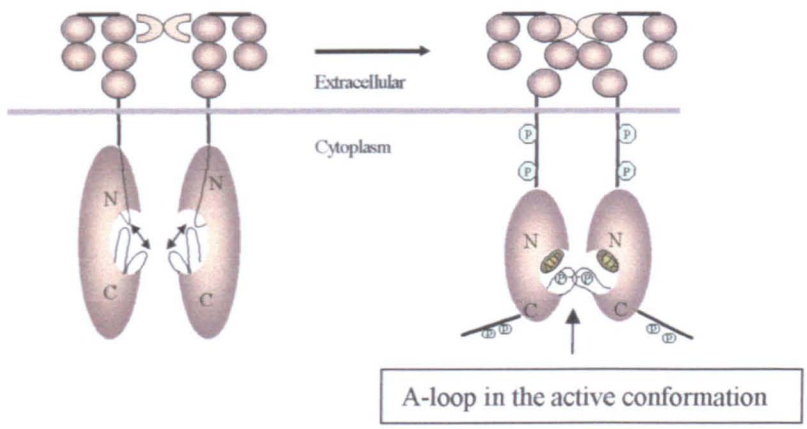


Figure 1.4. Schematic diagram showing the swap of the A-loop between an active and an inactive conformation. N, N-terminus; C, C-terminus; P, Phosphorylation.

Adapted from (Blume-Jensen and Hunter, 2001)

The autophosphorylation of RTKs has an essential role in controlling the protein kinase activity plus its role in the enrolment and activation of a variety of signalling proteins. The sites of tyrosine autophosphorylation are located in the non catalytic regions of the receptor molecule which also work as binding sites for SH2 (Src homology2) or PTB (phosphotyrosine binding) domains of a variety of signalling proteins. Therefore, it is believed that the SH2 domain-mediated binding of signalling proteins to tyrosine

autophosphorylation sites provides a mechanism for assembly and recruitment of signalling complex by activated receptor tyrosine kinases (Schlessinger, 2000).

1.4 Myeloproliferative disorders

In 1951 the concept of myeloproliferative disorders was introduced by Dameshek et al,(1951) to describe a group of conditions characterised by clonal proliferation of one or more haematopoietic components in the bone marrow, liver or spleen (i.e. chronic myeloid leukaemia (CML), polycythaemia vera (PV), essential thrombocythaemia, myelofibrosis (MF) and acute myeloid leukaemia (AML)). The fact that these disorders are closely related to each other accounts for the occurrence of transitional forms in a number of cases and, in addition, evolution from one entity into another may occur during the course of the disease (figure 1.5). It is generally believed that each of these disorders is a clonal haematopoietic neoplasm

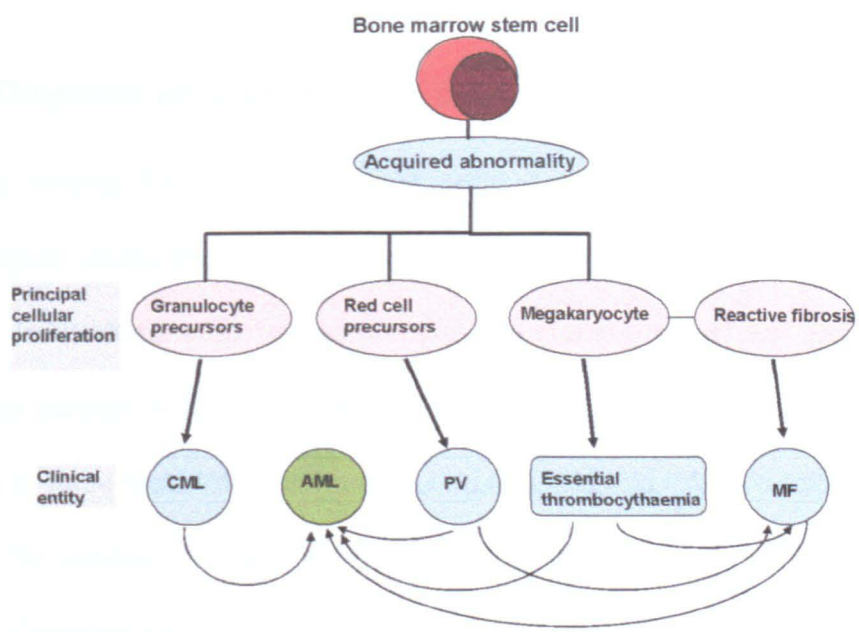


Figure 1.5. Schematic representation showing the relationship of various myeloproliferative disorders to each other.
Chronic myeloid leukaemia, CML; polycythaemia vera, PV; myelofibrosis, MF; acute myeloid leujkaemia, AML.
Adapted from Hoffbrand and pettit, (1993).

1.4.1 Leukaemias

Accumulation of abnormal white cells in the bone marrow is the main feature of leukaemia. The accumulation of these abnormal cells may cause bone marrow failure, a raised circulating white cell count and infiltration of organs. Leukaemias are classified in two main groups; acute and chronic leukaemia. The two main types of chronic leukaemias are chronic myeloid leukaemia (CML) and chronic lymphocytic leukaemia (CLL). Acute leukaemia, a heterogeneous group of malignant neoplasms composed of clonal expansions of immature cells, which is characterised by the presence of more than 30% myeloblasts or lymphoblasts in the bone marrow at clinical presentation, is divided into two groups, acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) on the basis of morphology and cytochemistry.

1.4.1.1 Diagnosis of Acute Myeloid Leukaemia (AML)

The main criteria for the diagnosis of AML is dependent on an examination of the morphological characterization of leukemic myeloblasts in peripheral blood and bone marrow. Infiltrations with leukaemic cells and marrow failure are the main blood film features for patients with AML. The number of leukaemic blasts present in blood can vary from zero to more then 200×10^9/L with a median count of $15\text{-}20 \times 10^9$/L which is directly related to the number of cells in the bone marrow. However, attempts to classify leukaemia from the characteristics of blood blast should not be made. Myelodysplastic syndrome (MDS) can be distinguished from AML by the presence of less than 30% of myeloblasts in the bone marrow aspirate (Catovsky *et al*, 1991). ALL can be distinguished from AML by the morphological picture, as sometimes myeloid characteristics of the cells are obvious (Catovsky *et al*, 1991), however, immunological and immunohistochemical methods are still

required for distinguishing AML from ALL in the majority of cases (Matutes *et al*, 1988; Ryan, 1992).

1.4.1.2 Classification of acute myeloid leukaemia

A classification system for AML was introduced by the French-American-British (FAB) co-operative group in 1976 (Bennett *et al*, 1976), and has been updated and included new diagnostic criteria (Bennett *et al*, 1985b; 1991). AML is divided into eight types M0-M7 (Bennett *et al*, 1985b; a; 1991) based on the morphological appearance of the blast cells and their reactivity with histochemical stains (table 1.4). Although this classification was initially proposed to define the subtypes of AML morphologically, it has been shown to be clinically and prognostically important.

Table 1.4. French-American-British (FAB) classification of AML

FAB subtype	Common name (% of cases)	Results of cytochemical staining		
		Myeloper-oxidase	Sudan black	Non-specific esterase
M0	Acute myeloblastic leukaemia with minimal differentiation (3%)	-	-	-
M1	Acute myeloblastic leukaemia without maturation (15-20%)	+	+	-
M2	Acute myeloblastic leukaemia with maturation (25-30%)	+	+	-
M3	Acute promyelocytic leukaemia (5-10%)	+	+	-
M4	Acute myelomonocytic leukaemia (20%)	+	+	+
M4Eo	Acute myelomonocytic leukaemia with abnormal eosinophils (5-10%)	+	+	+
M5	Acute monocytic leukaemia (2-9%)	-	-	+
M6	Erythroleukaemia (3-5%)	+	+	-
M7	Acute megakaryocytic leukaemia (3-12%)	-	-	+

Reviewed by Löwenberg et al, 1999.

1.4.1.3 Incidence and pathogenesis

According to the Leukaemia Research Fund report (2000), 1,600 cases of AML are diagnosed each year in the UK. However, it appears that the incidence of the disease increases gradually with age especially after 55 years after which it tends to increase

progressively (Stevens, 1996). In childhood, AML is responsible for a minor fraction (10-15%) of leukaemias.

1.4.1.4 Cytogenetics and molecular genetics

Several studies have highlighted the strong association between specific AML subtypes and karyotypes. These chromosomal abnormalities are seen at diagnosis but are not detectable cytogenetically if complete remission is achieved, however, they can reappear at relapse (Walker *et al*, 1994).

In AML, cytogenetic abnormalities are either numerical or structural. For instance, trisomy 8 is the most frequent numerical abnormality seen in AML and, although not typically associated with a specific FAB type, it is typical of FAB types M1, M4 and M5. In 1973, the t(8;21) translocation was described, interestingly more than 90% of identified cases were M2, however, not all M2 FAB type have t(8;21) translocation as only 40% of M2 have t(8;21) translocation (Rowley, 1973). The t(8;21) translocation has been described at molecular level, the genes involved being *ETO* on chromosome 8 and *AML1* on chromosome 21,which results in a fusion gene *AML1/ETO* (Downing *et al*, 1993).

1.4.2 Idiopathic myelofibrosis (IMF)

Idiopathic myelofibrosis (IMF) was first described by Heuck et al, (1879) who reported the presence of marrow fibrosis and extramedullary haematopoiesis in the liver and spleen of two patients (Heuck, 1879). The disease is described as a chronic, malignant haematological disorder characterised by splenomegaly, a leukoerythroblastic blood picture, teardrop poikilcytosis, a varying degree of marrow fibrosis and extramedullary haematopoiesis (Varki *et al*, 1983).

Table 1.5. Conditions associated with Myelofibrosis

Malignant diseases	Non-malignant diseases
Acute megakaryoblastic leukaemia.	Renal osteodystrophy.
Idiopathic Myelofibrosis.	Vitamin D deficiency.
Chronic Granulocytic leukaemia.	Hypoparathyroidism.
Acute myeloid leukaemia.	Hyperparathoidisim.
Acute lymphoblastic leukaemia.	Grey platelet syndrome.
Hairy cell leukaemia.	Systemic lupus erythematosis.
Transitional myeloproliferative syndrome.	Systemic sclerosis.
Polycythaemia rubra vera.	Thorium dioxide administration.
Systemic mastocytosis.	
Hodgkin's disease.	
Myeloma.	

Reviewed by McCarthy, 1985.

Different terms have been used to describe the disease, these include, myelofibrosis, myelosclerosis, osteosclerosis, idiopathic myeloid metaplasia and agnogenic myeloid metaplasia (AMM) (Silverstein *et al*, 1967; Silverstein, 1970). It is noteworthy to mention that fibrosis of the bone marrow is not specific to IMF and it may appear with other disorders (table 1.5). The marrow fibrosis in IMF is thought to be a response to a clonal proliferation of haematopoietic stem cells, which mainly leads to progressive marrow failure (Jacobson *et al*, 1978).

1.4.2.1 Incidence, epidemiology and aetiology

Earliest studies show no epidemiological data to estimate the actual incidence of IMF. An overall annual incidence of 0.5-1.3 per 100,000 was reported for IMF in Australia (Dougan *et al*, 1981). In Japan, IMF is considered a rare disorder with 0.084 per 100,000 (Reiter *et al*, 1997). However, the incidence of myelofibrosis was 18 times greater among survivors who were 10,000m or less from the hypocentre of the atomic bomb explosion at Hiroshima (Anderson *et al*, 1964). These data highlighted the strong link between excessive radiation exposure and development of IMF. Furthermore, the disease has been also associated with

exposure to benzene (Hu, 1987), petroleum product (Honda *et al*, 1995), and thorium (Visfeldt and Andersson, 1995).

Ward and Block et al, (1971) reported that the disease is one-quarter as common as CML (Ward and Block, 1971). The male to female ratio is 2:1 (Dougan *et al*, 1981). Similar to other myeloproliferative disorders, most IMF cases occur in middle aged and elderly people, i.e. between 50 and 70 years (Boxer *et al*, 1975).

1.4.2.2 Pathogenesis

A number of earlier studies have proposed that haematopoiesis in IMF is clonal, resulting from the malignant proliferation of a pluripotent stem cell. This conclusion was first proposed after the analysis of glucose-6-phosphate-dehydrogenase (G-6PD) isoenzyme in a black female patient who was heterozygous for this X-linked gene (Jacobson et al, 1978). Subsequently, this result was confirmed using cytogenetic analysis (Wang et al, 1992) and fibroblast proliferation kinetics (Castro-Malaspina et al, 1982). It is believed that the megakaryocyte cell lineage plays an important role in the pathogenesis of IMF (Groopman, 1980; Castro-Malaspina, 1984). Castro-Malaspina (1984), hypothesized that the intramedullary death of large numbers of megakaryocyte is due to a defective maturation of megakaryocytes which is accompanied with an ensuing abnormal release of megakaryocyte components, including platelet-derived growth factor (PDGF), a mitogen for human bone marrow fibroblasts, and platelet factor 4 (PF4) which inhibits collagenase activity (Castro-Malaspina et al, 1981). It was suggested, therefore, that the excessive accumulation of collagen within the marrow stroma is due to the imbalance between increased collagen production and decreased collagen degradation (Castro-Malaspina, 1984). In addition, the growth factors present in platelet alpha granules are thought to be pathologically important, including epidermal growth factor (EGF) and transforming growth factor-β (TGF-β)

(Kimura et al, 1988). The above hypothesis concerning the pathogenesis of IMF has been supported by several observations, which suggests that the release of the mitogens from the megakaryocyte cell lineage may be pathologically involved. For instance, the accumulation of collagen fibre has been found to be closely associated with clusters of dysplastic megakaryocytes (Hasselbalch, 1990). In addition, the findings of decreased PDGF activity in the circulating platelet or increased urinary platelet factor 4 are consistent with the idea that an abnormal release of PDGF occurs from platelets or megakaryocytes in the bone marrow microenvironment (Burstein et al, 1984; Baglin et al, 1988). Plasma and urinary PDGF excessive release is associated with severe myelofibrosis characteristic of patients with acute megakaryoblastic leukaemia (Gersuk et al, 1989; Reilly et al, 1996). The increased bone marrow macrophage population might be due to the increased release of PDGF (Thiele et al, 1992) and the reduced natural killer cell activity (Gersuk et al, 1993) that characterise the disease.

Furthermore, bone marrow fibroblasts from patients with myeloproliferative disorders have been found to exhibit an increased sensitivity to various mitogens, which might enhance fibroblast proliferation and accumulation of collagen in the bone marrow (Gay et al, 1984; Reilly et al, 1985).

1.5 Aim of the study

Mutations in receptor tyrosine kinases (RTKs) have been linked to an increasing number of human diseases. Interestingly, mutations observed in RTK, result in constitutive receptor activation. Mutations in *c-kit,* a class III RTKs, have been reported in exon 2 in idiopathic myelofibrosis and chronic myeloid leukaemia patients. Furthermore, a novel exon 8 deletion/insertion mutations have been identified with consistent loss of Asp419 located in the fifth Ig-like domain of the *c-kit* receptor in a third of the AML-M4Eo patients analysed. As a result, class III RTKs were of great interest due to the association with an increasing number of haematological malignancies. It is also believed that many novel mutations remain to be identified. Furthermore, two *c-fms* mutations have been reported in several myeloproliferative and myelodysplastic disorders. However, IMF patients have not been investigated for this mutation. In addition, *c-fms* has not been under full mutational analysis to confirm the presence or absence of any other mutation within the gene. Furthermore, *FLT3*, a member of RTK class III has been shown to be preferentially expressed on the surface of a high proportion of AML and B-lineage ALL cells in addition to haematopoietic stem cells, brain, placenta and liver. An interaction of *FLT3* and its ligand has been shown to play an important role in the survival, proliferation and differentiation of not only normal haematopoietic cells but also leukaemia cells. Recently, a *FLT3* ITD and Asp835 mutations were reported in AML. In addition, activation of the tyrosine kinase receptors was also reported in human leukaemias. This study aimed to perform systemic screening of the genetic alterations of *c-fms* and *FLT3* genes in AML and IMF patients. Structural analysis for the *FLT3* gene was also required to facilitate the screening for genetic alterations. This study could help in the diagnosis and treatment of *c-fms* and *FLT3* genes mediated diseases in particular AML and IMF.

Chapter 2

Materials and Methods

2.1 Patients and control subjects

2.1.1 Acute myeloid leukaemia (AML) patients

Samples of genomic DNA were obtained from the bone marrow at presentation of all the AML cases studied. Sixty cases of AML were initially studied (group A). These cases were entered into the Medical Research Council (MRC) AML X Trial. According to French-American-British (FAB) criteria (Bennett *et al*, 1976) they were morphologically classified as having AML and they were sub classified as: M0 (n=4), M1 (n=8), M2 (n=10), M3 (n=10), M4 (n=12), M5 (n=10), M6 (n=6). Standard cytogenetic analysis demonstrated inv(16) (n=7), t(8;21) (n=2), t(15;17) (n=10), other chromosomal abnormalities (n=25) and normal karyotype (n=16).

Due to the study requirements, 43 further AML cases were obtained with either inv(16) (n=30) or t(8;21) (n=13) (group B). These patients were entered into the MRC AML XII trial. Morphological diagnoses, cytogenetic analysis and DNA extraction were performed on AML X and XII samples by University College London (UCL) staff (Department of Haematology).

Two small groups of local AML patients (numbering 8 and 12 patients) were also entered into the study (group C1, C2). Furthermore, 185 new AML samples from patients entered into AML X and XII trails were entered at the end of the study (group D) in order to establish the result obtained in a large number of AML patients (cytogenetic and FAB classification are not provided).

2.1.2 Idiopathic myelofibrosis (IMF) patients

Forty cases of IMF were studied following informed consent. All cases of IMF fulfilled the following criteria: a leucoerythroblastic blood picture, tear drop poikilocytosis, absence of monocytosis, marked bone marrow fibrosis and lack of the Philadelphia chromosome. Peripheral blood samples were obtained from these cases and DNA was extracted from each sample using the Nucleon™ BACC2 extraction kit (see section 2.3). Patients with the closely related disorders, post-polycythaemic myelofibrosis, myelodysplasia with myelofibrosis and transitional myelodysplasia myelofibrosis (Reilly and Dolan, 1991) were excluded from the study. All IMF samples were referred to Royal Hallamshire Hospital (RHH), Sheffield from all over the United Kingdom.

2.1.3 Normal controls

EDTA tubes were used to collect 5 ml of peripheral blood from seventy normal unrelated individuals and DNA was extracted using the Nucleon™ BACC II method (Nucleon Biosciences). Subsequently, 130 further DNA samples were obtained from normal unrelated individuals.

2.1.4 Ethical approvals

AML samples used in this study were obtained from the MRC (Medical Research Council) DNA bank. All patients consented for samples to be stored at the time of diagnosis for future studies. Samples from patients with IMF were obtained with informed consent, the study having been approved by the Northern General Hospital Research Ethics Committee (first 20 samples) and CSUH Trust's Research Ethics Committee (last 20 samples) (SSREC02/287).

2.2 Materials

2.2.1 Chemicals, reagents and plastic-ware

Reagents and buffers were prepared using chemical components supplied by Sigma® Chemical Company Ltd, British Drug Houses (BDH) Chemicals Ltd or Merck® Ltd unless otherwise noted. DNA markers for agarose gel electrophoresis were obtained from MBI Fermentas. All plastic-ware was purchased from Sarsted Ltd and Sterilin Ltd.

2.2.2 Radioactive isotope

γ-^{32}P ATP with specific activity of (3000Ci/mMol; 111Btq/mMol) was purchased from Amersham Life Sciences, Ltd, UK.

2.3 Methods

2.3.1 Genomic DNA extraction from whole blood

Nucleon™ BACC2 DNA extraction kit (Nucleon Biosciences) was used to extract genomic DNA from whole blood. 5-10 ml of whole blood with EDTA-anticoagulant was mixed and lysis buffer (solution A; 10 mM Tris-HCl, 320 mM sucrose, 5 mM $MgCl_2$, 1% Triton X-100, pH 8.0) was added up to a final volume of 45ml in 50 ml polypropylene tubes and incubated for 5 minutes at room temperature. The supernatant was removed and the cell pellet collected after centrifugation (Beckman J6 centrifuge) at 1300 g for 4 minutes. If using a swabbing technique (using a plastic spatula or wire brush), place the swab containing the cells in a 2ml tube. Add 1.5ml PBS and centrifuge for 1 min. If extracting from saliva, collect the saliva in a sterile universal tube. Put the IsoCod (ABgene) (to absorb the saliva) stick in the tube containing the saliva and incubate for 1 min at room temperature Remove the IsoCod stick and put it in a 2ml tube and centrifuge to collect the cell pellet. The cell pellet was washed in 20 ml (5ml in case of saliva or buccul swab) of lysis buffer and centrifuged at 800 g for 5 minutes. 2ml of nuclear lysis buffer (solution B; 400 mM Tris-HCl pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% SDS) was used to resuspend the cell pellet. 0.5ml of 5 M sodium perchlorate was used to deproteinise the sample. After inverting the tube seven times, the solution was transferred to a 15ml screw-capped polypropylene centrifuge tubes. After adding 2 ml of pre-cooled chloroform, the solution was mixed by inverting the tube at least seven times. The solution was centrifuged at 800g for 1 minute and 0.3 ml of Nucleon Silica suspension was added above the solution. The tubes were centrifuged at 1300 g for 3 minutes after that the upper aqueous layer was removed into a universal container containing 5 ml of ice-cold absolute ethanol, to precipitate the DNA. After inverting the tube gently several

times the DNA was removed using a sealed glass Pasteur pipette. The DNA was air dried for 10-15 minutes and resuspended in 100-200 µl of sterile water. DNA samples were quantitated as described in section 2.5 and then stored at –20 °C.

2.3.2 Cell Separation using VarioMACS system

This method was used to extract and store DNA from myeloid and lymphoid cells from the blood of patients with IMF. It is suitable for 5-30 ml of whole blood in EDTA. Separation of 30 ml of fresh whole blood (less than 8 hours old) was carried out in twelve 15 ml centrifuge tubes per patient.

5 ml Histopaque 1119 was added to a 15 ml conical centrifuge tube using sterile syringe and needle. 5 ml of Histopaque 1077 was carefully layered over the Histopaque 1119 as above. The whole blood was diluted 1:2 with phosphate buffered saline (PBS) in a separate 15 ml tube. 5 ml of diluted blood was carefully layered over the Histopaque 1077. The tube was centrifuged at 700g in a MSE Mistral 2000 for 30 minutes at room temperature. Two layers of cells were seen (A and B in figure 2.1).

The upper fluid was aspirated and discarded to within 0.5 cm of layer A. Cells were transferred from this layer to a tube marked "A" (MNCs). Similarly, the histopaque fluid was aspirated and discarded to within 0.5 cm of layer B and cells were transferred from this layer to a tube labelled "B" (granulocytes). Each aliquot (A and B) was washed twice in 10 ml of PBS, centrifuged at 800g for 5-10 minutes and the supernatant was discarded the each time. The cells were resuspended in 2 ml PBS by gentle aspiration with a Pasteur pipette. Each aliquot (A and B) was diluted 1:10 in PBS (100 µl of A or B cell suspension in 900 µl of PBS) and the cell count for both A and B suspension and for the original whole blood sample was established using an automated haematology analyser (Bechman Coulter GenS System 2) in the Haematology Department.

The percentage of T-cells ($CD3^+$) and neutrophils ($CD15^+$) in each aliquot (A and B) and whole blood was determined by flowcytometry (see section 2.4.2). Cell numbers were calculated as follows:

Example:

WBC=7.3×10^9 /l (count from the analyser) total white blood count in 30 ml = 7.3 × 30/1000 =2.19×10^8 cells.

CD 3 +ve cells from whole blood = 5.24% × 2.19/100 = 1.147×10^7 T cells.

CD 15 +ve cells from whole blood = 86.09% × 2.19/100 = 1.88×10^8 neutrophils.

Count for A suspension in 2 ml = 5.1×10^9/l = $5.1 \times 10^9 \times 2/1000 = 10.2 \times 10^6$ cells

CD3 +ve cells from A cell suspension = 90.2%

Purity for T-cells = 90.2% (T-cells)

$$\text{Yield \%} = \frac{10.2 \times 10^6}{1.147 \times 10^7} = 88.9\% \text{ (T-cells)}$$

2.3.2.1 Magnetic Cell Sorting (MACS)

Each aliquot of cells (A and B) was pelleted by spinning at 800g for 10 minutes, the supernatant was completely removed and each cell pellet was resuspended in 80 µl of cold PBS per 10^7 cells. If the cells clumped together a 30µm nylon mesh was placed over the column and the cell suspension was added onto the top of the nylon mesh and the cell suspension was allowed to pass through.

20 µl of well mixed CD3 MicroBeads was added to aliquot A (T-cells). 20 µl of well mixed CD15 MicroBeads was added to aliquot B (neutrophils) per 10^7 cells, both tubes were mixed well separately and incubated for 15 minutes at 6-12°C. Both aliquots were washed by adding 5 ml of PBS, centrifuged at 800g for 10 minutes, the supernatants were completely removed and the cell pellets were resuspended in 500 µl of cold PBS per 10^8 cells.

For aliquot A, one of the positive selection column was chosen according to the expected number of positive cells; RS^+ (for up to 10^7 positive cells), or VS^+ (for up to 10^8 positive cells) and the column was placed in the magnetic field of the VarioMACS (see fig 2.2).

The column (RS^+ or VS^+) was prepared by pipetting 500µl of cold PBS for RS^+ and 3 ml of cold PBS for VS^+ onto the top of column, the buffer allowed to pass through the column into the collection tube (waste), another collection tube was prepared. 500µl of the cell suspension (A) was added to the column, the unbound cells were allowed to pass through and then rinsed with cold PBS; 4 ×500 µl for RS^+ column and 4 × 3 ml for VS^+ column. The effluent was collected as the negative fraction. The column was removed from separator, then placed on a collection tube supplied with the column. Cold PBS was added; 1 ml to RS^+ column or 5 ml to VS^+ column. Bound cells was flushed out by pushing down the plunger supplied with the column, the collection tube contains the positive fraction A (see figure 2.2).

The procedure was repeated for aliquot B. The collection tube contained the positive fraction B. The cell count was performed for each fraction as well as for $CD3^+$ and $CD13^+$ (see section 2.3.2) as before to establish the purity and yield. DNA was extracted from T-cells (A) and neutrophils (B) using the nucleon protocol (section 2.3.1).

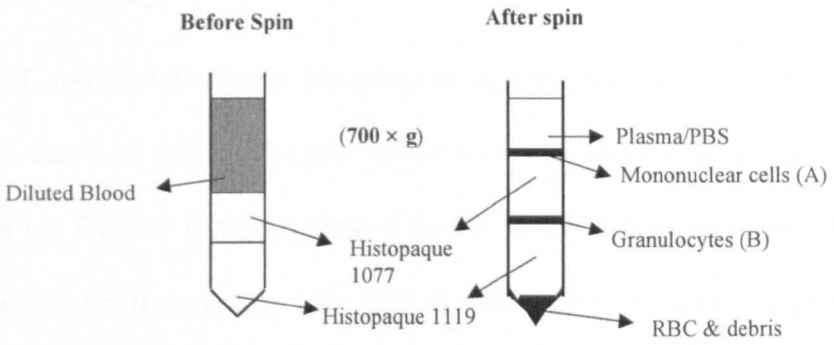


Figure 2.1. Cell separation using histopaque 1119 and histopaque 1077

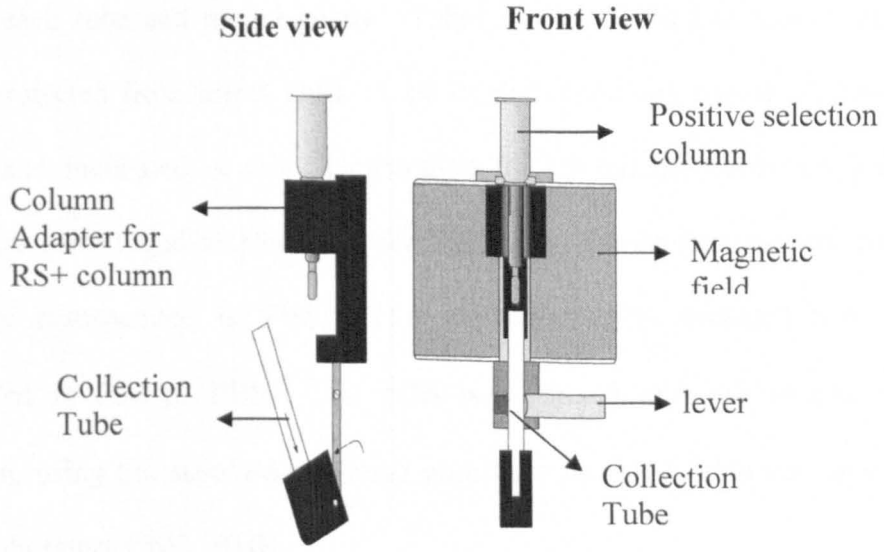


Figure 2.2. Representative diagram showing positive selection column plus adapter in Vario MACS.

2.3.2.2 Flow cytometry

Peripheral blood, mononuclear cells (A cells) or neutrophils (B cells) may have high cell counts in some cases of IMF. The cell count was obtained by using the automated cell analyser (Bechman Coulter GenS System 2) in the Haematology Department in the RHH. The sample was diluted if necessary with PBS to adjust the cell count to approximately 10×10^9/l.

3 falcon tubes were labelled as WB, A and B. 50 µl of cells was added to each tube. 5 µl of CD3 phycoerythrin (PE) and 5 µl of CD13 fluorescein isothiocyanate isomer (FITC) were added to each tube and mixed gently. Tubes were incubated at room temperature for 15 minutes protected from direct light. 1 ml of Facs Lyse solution was added to each tube, vortexed and incubated at room temperature for 15 minutes protected from direct light. Tubes were centrifuged at 1800 rpm for 5 minutes, the supernatant was removed and the cells were resuspended in PBS. The supernatant was decanted and the cells were resuspended in 200 µl PBS. The cells were mixed and refrigerated until ready for acquisition, using the standard operating procedure for acquisition and analysis in the Cell Marker Laboratory CML, RHH.

2.3.3 Determination of DNA concentration

Two methods are commonly used for determination of DNA (genomic, plasmid and primers) or RNA concentration, quantitative and qualitative methods. The quantitative method is based on the optical density measurement while; the qualitative method is based on the estimation against a standard DNA ladder of known concentration. Only the quantitative method was used in this study.

2.3.3.1 Optical density

The DNA concentration in the solution was measured by reading the absorbance at a wavelength of 260 nm. 1:100 dilution with sterile water was performed in a 1.5 ml tube and transferred to a 1 ml Quartz cuvette. The cuvette was then placed into a UV spectrophotometer (Beckman). One absorbance unit at 260 nm wavelength (OD_{260}) corresponds to a concentration 50 μg/ml for double stranded DNA, 25 μg/ml for single stranded DNA and 40 μg/ml for RNA. DNA concentration was calculated using the above equation. Afterwards the DNA was diluted to the concentration required.

2.3.4 DNA amplification

Polymerase chain reaction (PCR) was the selected method to carry out DNA amplification for *c-fms* (2-22) and *FLT3* (9, 11, 14-15, 20). All 21 exons of *c-fms*, exons 9, 11, 14-15, 20 of *FLT3* and all intron/exon boundaries were amplified independently.

2.3.4.1 Designing primers and synthesis

Primer 3, a computer software available over the Internet was used to design all the primers for *c-fms* (see table 2.1) and *FLT3* (see table 2.2) gene amplification (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), except for the ITD for which previously designed primers were initially used (Nakao *et al*, 1996). Primers for the *c-fms* gene were synthesised using a 381A DNA synthesiser (Applied Biosystems) by Hazel Holden (Division of Genomic Medicine, University of Sheffield, UK). Primers for the *FLT3* gene were synthesised by Abgene Ltd, UK.

All PCR reactions comprised the following; 500 ng of genomic DNA, 16.6 mM $(NH_4)_2SO_4$, 67 mM tris HCl (pH 8.8), 10 mM β-mercaptoethanol, 100 μg bovine serum albumin (BSA), 300 ng of each primer, 200 μM dNTPs (Pharmacia), $MgCl_2$ 1-1.5, and 1 U Taq DNA

polymerase (Bioline) in a final volume of 50 µl. Samples were initially denatured at 95°C for 5 minutes. DNA amplification was performed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 52-60 °C for 30 seconds and extension at 72°C for 30 seconds. 5 µl of each PCR product was loaded onto a 4% polyacrylamide gel (section 2.3.6.2) to confirm that the amplification had occurred. Each PCR product was then screened for mutations using conformation sensitive gel electrophoresis (2.3.5).

Table 2.1 Primer sequence and annealing temperatures required for PCR amplification of the *c-fms* gene.

Exon number	Primer name	Sequence 5'-3'	Location*	Size/bp	AT^{0}C
2	2F 2R	GTCTCTTCTCCCAAGACCCC TCACAGGGGTCTTCTCCATC	1562-1581 2005-2024	463	52
3	3F 3R	TGAGCAGGTTGAGGGTTAGG TCTTCCAGGTCCTTGCTCAT	7138-7157 7683-7702	565	52
4	4F 4R	AGGGCAGTTGTAACCCTGTG CCATTGAGGGGAAGAAGTGA	7966-7985 8406-8425	460	56
5	5F 5R	ATCATATTCAGGGAGCCTGG GAGATCTCGGGTGAGTCTGC	10040-10060 10362-10381	341	56
6	6F 6R	AAAGGCTGAGGATTGGAACC AAGGGAAAGCTCCTGGAGAG	10824-10843 11213-11182	409	56
7	7F 7R	GGTGGATGACAAAATGGAC CTTGCTGAAGCATACCCCAT	14767-14785 15077-15096	331	56
8	8F 8R	TCACCAACACCACCTGATTCT CATCCCTCCAGGCAGTCC	17736-17756 17923-17940	205	56
9	9F 9R	AGGGCACAGGGAAGTAGGTA CATGTCCCTCCCACTCACA	17955-17974 18207-18225	271	58
10	10F 10R	AGTGGGAGGGACATGCTG GCTAGGATCTGCTCCAAAGG	18211-18228 18573-18592	382	56
11	11F 11R	CCACTGTGTTCCAGGCAGT CCATCCAAATCTGGCTCACT	19919-19937 20158-20177	259	52
12	12F 12R	CTCTTGGGGGTCAGAAACAA CAAAGGGCCTCTGTCCAAG	26402-26421 26677-26695	294	56
13	13F 13R	ATGGGCCCTTGGACAGAG TGTGTGTGATGCCTCTTGTG	26670-26687 26906-26925	256	56
14	14F 14R	CCCCATGTATCTGTGTGGTG CCTGGGGCCCTGAGATTC	27283-27302 27511-27528	246	52
15	15F 15R	CCCAGTCTCATGCTCCTGTT TGGCTTTGAAGACAGACTCG	28391-28410 28677-28696	306	56
16	16F 16R	TGAGCAGTGCAGTGATGATG AGCAGCCCCTTCTCCTTTT	30678-30697 30818-30886	209	56
17	17F 17R	AAAAGGAGAAGGGGCTGCT ACAGACCTGGGTGGCTATGA	30868-30886 31063-31082	215	60
18	18F 18R	CCTCAGGCTCAGGTAGGAGA GGGATGACAGTCCCCAGTTAT	31930-31949 32166-32186	257	56
19	19F 19R	GCCCAAAATAACTGGGGACT CACCAAACAGCTTTGTCCAC	32159-32178 32361-32380	222	56
20	20F 20R	GGGAATGGGGAGAAGACAAT AAAGCCTGGGGTGTCCTTT	32936-32955 33117-33135	200	60
21	21F 21R	GTGTTAATGGCCCCTGGAC CAGCCCAACGTGCTTTACC	33836-33854 34037-34055	220	60
22	22F 22R	GAGAGAGCGGGTGAGTGG GCCGAGCTGTTGAGTGAAAT	33996-34013 34358-34377	382	52

Table 2.2 Primer sequence and annealing temperatures required for PCR amplification of the *FLT3* gene.

Exon number	Primer name	Sequence 5'-3'	Size/bp	AT ^{0}C
9	9F 9R	CTCCTGGGTTTTCACTTGGA TCCTTCTCAAGGGCAACAAG	400	58
11	11F 11R	CCCAGCCAGTGAGCTTATTT GGTCAGAGAGTTTTATGTTCTTCCA	201	58
14-15	14F 15R	GCAATTTAGGTATGAAAGCCAGC CTTTCAGCATTTTGACGGCAACC	328	52
20	20F 20R	CCAGGAACGTGCTTGTCA TCAAAAATGCACCACAGTGAG	194	56

2.3.5 Conformation sensitive gel electrophoresis

Conformation sensitive gel electrophoresis (CSGE) was the method chosen for mutation detection. CSGE analysis was used to identify the alterations in electrophoretic movement of double stranded DNA that resulted from the bending of heteroduplexes at sequence mismatches (Ganguly *et al* 1993). Heating the PCR products at 95°C for 5 minutes and then incubating it at 65°C for 30 minutes was carried out to denature the DNA and to create heteroduplexes of mutant and wild-type complementary DNA strands. 40 well castle combs were used to create the wells of the 10% polyacrylamide gels (41×33×0.1 cm) prepared as 99:1 acrylamide: 1,4 *bis*-acrolypiperazine (BAP; Fluka), 10% ethelyene glycol, 15% formamide in 0.5×TTE buffer (1×TTE = 89 mM tris, 28.5 mM taurine, 0.2 mM EDTA), were pre-electrophoresed for 20 min at 750 volts. 5-7 µl of heteroduplexed PCR products were then mixed with 2 µl loading dye (50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) and loaded onto the gel. The gel was electrophoresed for 16-18 hours at 400 volts and stained in 0.5×TTE buffer containing 1 µg/ml ethidium bromide for 5 minutes at room temperature, destained for 10 minutes in sterile water and the DNA bands were visualised under UV light.

2.3.6 DNA electrophoresis

Electrophoresis is used to separate molecules by some property. In the case of DNA the molecules can be separated based on their size. DNA has a negative charge in solution, so it will migrate to the positive pole in an electric field.

Two types of gel were used for identification and purification of DNA fragments by electrophoresis, namely agarose and polyacrylamide gel. Different concentrations were used for separation depending on the size of the DNA fragment and the resolution required (Sambrook *et al* 1989). DNA samples were mixed with 6×gel loading buffer (50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol,) prior to loading onto the gel. Ethidium bromide (EtBr) was used to localise the DNA fragments within the gel and visualised by illuminating with UV light on a transilluminator (UVP, 302 nm). The size of DNA fragments was estimated by comparison with known molecular size standards.

2.3.6.1 Agarose gel electrophoresis

In agarose gel electrophoresis the DNA is forced to move through a sieve of molecular proportions that is made of agarose. The end result is that large pieces of DNA move slower than small pieces of DNA. The place in the gel that the DNA migrates to is observable under ultraviolet light when the current is turned off and the gel is stained with EtBr. Although agarose gels have lower resolving power than polyacrylamide gels, they have a greater range of separation. Solid agarose (0.7-1.0%) was melted by boiling in 1× electrophoresis buffer (1×TBE; 0.089 M tris-borate pH 8.0, 2 mM EDTA), and after cooling EtBr was added to a concentration of 0.5 μg/ml. The agarose was poured into a plastic mould and allowed to set.

2.3.6.2 Polyacrylamide gel electrophoresis

Another type of gel was also used (polyacrylamide gel) due to it's higher resolving power than agarose gels and for being able to separate fragments that differ in size by as little as 0.2%. Furthermore, polyacrylamide gel electrophoresis is sufficient for the separation of small fragments of DNA (less than 1 kb in length). 4-8 % polyacrylamide gels were prepared from stock solutions containing acrylamide and bisacrylamide at a ratio of 19:1 Depending on the type of DNA analysis, two types of polyacrylamide gels are commonly used; non-denaturing and denaturing gels.

Only the non-denaturing polyacrylamide gels were used for the separation of double stranded DNA fragments. Gels were prepared in 1×TBE with 0.1% (w/v) ammonium persulphate (APS) and 0.001% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) using BioRad mini-protean II vertical electrophoresis equipment. After polymerisation of the gel, the wells were washed with buffer to remove any unpolymerised material. DNA samples were mixed with 6× loading buffer and loaded onto the gels. 8×10 cm gels were electrophoresed for 25 minutes at 200 volts. Gels were stained with ethidium bromide (EtBr) and visualised under UV light.

2.3.6.3 Silver staining of polyacrylamide gels

In some cases, more sensitive method than EtBr staining is needed to visualise the DNA fragments separated by polyacrylamide gel electrophoresis. Therefore, silver staining was used following electrophoresis. The gel was placed in fixing solution (0.5% acetic acid, 10% ethanol) for 5 min. The gel was then submerged in 10% $AgNO_3$ solution for 20 min, washed twice with H_2O and transferred into fresh formaldehyde NaOH solution (0.1% formaldehyde, 1.5 NaOH) and left until stained. When the bands were visualised the gel was

submerged in 0.75 Na_2CO_3 for 10 min, drained and sealed in plastic for documentation and storage.

2.3.7 DNA purification

This step was undertaken in order to prepare the DNA to be suitable for DNA sequencing, restriction enzyme analysis and other analytical techniques. Gel electrophoresis can be used as a preparative procedure for the isolation of individual species of DNA molecules.

2.3.7.1 Agarose gel DNA purification

In some cases a clean PCR product was not achieved, therefore, other DNA purification technique is recommended. Pharmacia Biotech DNA purification kit was used to purify the DNA band from agarose gel. Using a clean scalpel the DNA band of interest was excised from the gels under UV illumination. Excised DNA band was cut into several small pieces and transferred into 1.5 ml tube. 10µl/10µg of capture buffer was added to the tube (maximum 300µl / 0.3 g gel slice), mixed by vortexing several times. The tube was incubated in a water bath at 60°C for 5-15 min until the gel was completely dissolved. The sample was transferred to GFX column (provided with the kit), incubated at RT for 1 min, centrifuged (MSE) mistral 2000 at full speed for 30 second. The flow-through was discarded. The GFX column was placed inside a new collection tube and 500µl of wash buffer was added to the column, centrifuged at full speed (MSE) mistral 2000 for 30 second. After discarding the collection tube the GFX column was transferred to a fresh 1.5 ml tube, 20-40 µl of DW was added to the top of the glass fibre matrix in the GFX column to elute the DNA. The GFX column was incubated at RT for 1 min, centrifuged at full speed for 1 min to recover the purified DNA.

2.3.7.2 Enzymatic purification of PCR product for DNA sequencing

PCR product had to be treated to remove any unincorporated dNTPs and primers, this was achieved by pre-treatment with the Thermo Sequenase Cycle Sequencing Kit (AmershamTM). Purification was simply performed by breaking down the primers and the dNTPs using two hydrolytic enzymes active in the PCR reaction buffer. 5 µl of PCR product was mixed with 10 units of exonuclease I and 2 units of shrimp alkaline phosphatase and incubated at 37°C for 15 minutes. Enzymes were inactivated by further incubation at 80°C for 15 minutes.

2.3.8 DNA sequencing

Thermo-Sequenase cycle sequencing kit (Amersham™) was used to perform direct DNA sequencing. The manufacturers protocol was followed with some alterations if needed.

2.3.8.1 DNA Sample preparation

The PCR fragment required for sequencing was purified using the enzymatic purification method (sections 2.3.7.2). The purified PCR fragment was then electrophoresed on a polyacrylamide mini gel (section 2.3.6.2) and the DNA concentration was estimated using the method in section 2.3.3.1.

2.3.8.2 End labelling of primers

T_4-polynucleotide kinase (PNK) was used to label the 5′ end of the sequencing primer as described by the manufacturer. 1µl of the primer (70 ng/µl) was added into a 0.5 ml eppendorf tube containing 1µl of PNK buffer (1×PNK buffer), 1µl of PNK enzyme (10 units) and 3 µl of γ-^{32}P ATP (approximately 1.11 MBq). The tube was then vortexed, pulse-

spun and incubated at 37°C for 30-45 minutes. To inactivate the residual PNK enzyme, the mixture was incubated at 80°C for 10 minutes.

2.3.8.3 DNA sequencing protocol

A 0.5 ml tube was used to prepare the reaction mix by adding 1-10 µl of the purified template DNA (20-100 ng), 2 µl reaction buffer (1×reaction buffer), 2 µl Thermo-Sequenase DNA polymerase (8 Units) and 1µl labelled primer (1.5 pmoles). The final volume was made up to 17.5µl with sterile water. The tube was mixed and spun. 4µl of the reaction mix was then added to four 0.5 ml tubes each containing 4 µl of 150 µM (di-deoxy NTPs) each of ddATP (A tube), ddCTP (C tube), ddGTP (G tube) and ddTTP (T tube). Each tube was then mixed by vortexing, pulse-spun and overlaid with one drop of mineral oil. Tubes were placed in a thermal cycler (Perkin Elmer Cetus 480) and cycled through 50 cycles of 30 seconds at 95°C (denaturing), 30 seconds at 50°C (annealing) and 2.0 minutes at 72°C (extension). At the end of the 50 cycles, reactions were stopped by the addition of 4µl of stop solution (95% formamide in 0.05% bromophenol blue, 0.05% xylene cyanol, 20mM EDTA pH 8.0) to each tube and the mixture was vortexed and pulse-spun. Tubes were incubated at 90°C for 5min prior to loading on 6% denaturing polyacrylamide gel.

2.3.8.4 Denaturing electrophoresis of sequencing reactions

All sequencing reactions (A, C, G and T tubes) were electrophoresed though a 6% denaturing polyacrylamide gel containing 7 M urea (section 2.3.6.2). The gels, 50 cm in length, 20 cm width, 0.35 cm in thickness with 24 lanes were electrophoresed for 15-30 minutes at 38 watts. 4 µl of each sample was loaded onto the gel and electrophoresed using electrophoresis tanks (Scotlab) at a constant power of 38 watts until the desired separation had occurred, usually until the bromophenol blue dye had reached the bottom of the gel for

sequence close to the primer (up to a maximum of 2 hours). The sequencing gel was fixed in 10% acetic acid and 10% ethanol for 10-15 minutes to remove the urea, transferred to 3 MM Whatman filter paper and covered by Saran Wrap (Genetic Research Instrumentation). The gel was then dried for 2 hours using a Biorad Gel dryer followed by autoradiography (section 2.3.9).

2.3.9 Autoradiography

Due to light sensitivity, all steps had been performed in the dark room. Gels were exposed to Cronex X-ray film (Du Pont) in lightproof cassettes, containing intensifying screens, before being developed using Cronex developer and fixer. The exposure time for most gels was overnight, depending on the level of the radioactivity emitted from the gel. Pre-flashing of the films (flashgun, Amersham) was performed when a low level of radioactivity was detected, to increase the film sensitivity and also by leaving them during the exposure time at -70°C (Laskey and Mills 1977).

2.3.10 Automated sequencing

DNA samples were prepared as described in section 2.3.8.1 diluted in 1/10 in 10µl of water. Automated sequencing was then performed using ABI 377 automated gene sequencer (DGM).

2.3.11 Statistical analysis

2.3.11.1 Kaplan-Meier Survival Analysis

The objective of the Kaplan-Meier (KM) methodology is to estimate the probability of survival of a defined group at a designated time interval (conditional probability). KM uses a non-parametric survival function for a group of patients (in other words their survival

probability after the time *t*) and therefore does not make assumptions about the survival distribution. Stat-view software (Abacus Concepts, Inc) available in the Division of Genomic Medicine was used to measure the survival analysis using Kaplan-Meier method.

2.3.12 Numbering schemes

Alterations were numbered according to the cDNA position unless it was located in the intronic part of the gene. In case if the alteration is located in the intronic part, the number of the nearest exonic nucleotide from either end of the exon i.e. 5` or 3`will be used, however, the distance (number of nucleotides) from that end to the alteration site will be also included as + if it was in the 3` end and as – (minus) if it is located in the 5` of the exon (see figure 2.3).

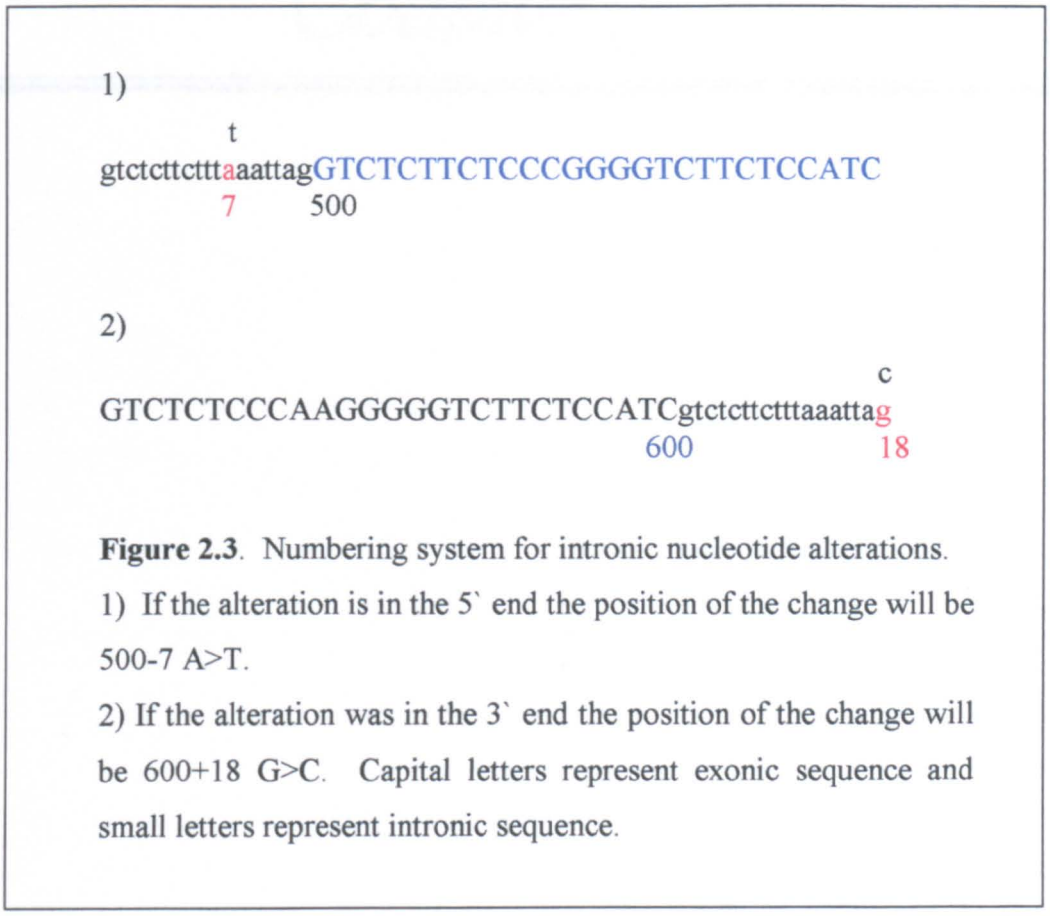


Figure 2.3. Numbering system for intronic nucleotide alterations.
1) If the alteration is in the 5` end the position of the change will be 500-7 A>T.
2) If the alteration was in the 3` end the position of the change will be 600+18 G>C. Capital letters represent exonic sequence and small letters represent intronic sequence.

Chapter 3

ESTABLISHMENT OF SCREENING OF THE *C-FMS* GENE AND ANALYSIS OF POLYMORPHIC SEQUENCE ALTERATION.....54

Establishment of screening of the *c-fms* gene and analysis of polymorphic sequence alteration

3.1 Introduction

c-fms or CSF1R is a class III RTK that is encoded by the *c-fms* proto-oncogene (Sherr *et al,* 1985), it is located on the long arm of chromosome 5 at q33.3 (Groffen *et al,* 1983). *c-fms* and other class III RTK consist of an extracellular part that consists of five immunoglobulin repeats, a transmembrane domain (TM), a juxtamembrane domain (JM), an intracellular tyrosine kinase domain, composed of two tyrosine kinase domain (TK1 and TK2) separated by a kinase insert, and a c-terminal domain (Yarden and Ulrich, 1988).

c-fms is about 75kb in length and consists of 22 exons with exon 1, however, being a non-coding exon, located 26kb upstream from the receptor-coding sequence (exon 2) (Hampe *et al*, 1989; Visvader and Verma, 1989). All the *c-fms* coding exons are short, with lengths ranging from 89bp (exon 16) to 285bp (exon 4) (Hampe *et al,* 1989). Intron sizes, however, are variable, ranging from 81bp (intron 18) to 6355bp (intron 11) (Hampe *et al,* 1989).

Exons 2-10 and the 5′ portion of exon 11 encode the extracellular domain of the *c-fms* receptor, while the intracellular domain is encoded by the 3′ end of exon 11 and by exons 12-22 (Hampe *et al,* 1989). The ATG initiation codon is located in exon 2, which encodes the signal peptide for translocation of the polypeptide chain into the cavity of the endoplasmic reticulum (Wheeler *et al,* 1986). Exon 3 to the 5′ end of exon 11 encode the remainder of the extracellular domain. Yet, exon 11 also contains the complete hydrophobic membrane-spanning segment that attaches the polypeptide within the membrane of the endoplasmic reticulum during its synthesis (Rettenmier *et al,* 1985). Exon 13 encodes the predicted site

of ATP binding (Lysine 616). The 3′ region of exon 17 to exon 21 encode the most closely homologus sequence to other class III RTK, namely *c-kit*, which shows the closest similarity to *c-fms* of other known genes (Besmer *et al,* 1986; Yarden *et al,* 1986). *c-kit* has the greatest degree of similarity in exon 19, decreases in exon 20 and is the weakest in exon 21 of the *c-fms* receptor (Hampe *et al,* 1989). Yet, the kinase insert (KI) sequence is found between the ATP binding site in exon 13 and the most highly conserved region of the tyrosine kinase domain (Hampe *et al,* 1989). Exon 22 contain the TGA termination codon and the 3′ untranslated sequence of 777 bp length 5′ to the site of polyadenylation (Hampe *et al,* 1989).

A number of different biological questions are raised when a new gene is identified underlining the relationship of the gene structure to function and the possible pathological relevance of mutations or polymorphisms. Polymorphism refers to the simultaneous occurrence in the population of allelic variation. A variant that occurs in greater than 1% of the normal population and has no pathological effect on any expressed gene product can be considered as a polymorphism (Peake and Winship, 1991). Mutations can be distinguished from polymorphisms by the fact that "any putative mutation must demonstrate a functional consequence to eliminate the possibility of a polymorphism" (Trent, 1997). Generally, mutation refers to the identification of a change in the DNA that produces disease, or dysfunction (Trent, 1997). Small nucleotide deletions can be identified by PCR based techniques e.g. the common three nucleotide deletion affecting codon Phe508 in cystic fibrosis. However, in terms of causing genetic disorders, point mutations occur more frequently than deletions. In the practical world, point mutations affecting a single nucleotide are more difficult to identify because they are small and heterogeneous; in the case of inherited mutations, it is not unusual for each family to have its own specific mutation (Trent, 1997).

In the last few years, there has been an increased necessity to screen amplified DNA to identify gene fragments likely to contain a mutation. In some diseases, there are a number of point mutations, which have a great effect on the circumstances of the disease. DNA sequencing technologies are one of the main approaches that now are being used to sequence small regions of interest in the human genome. For unknown mutations however, in some cases, time and cost considerations could limit the use of this approach as a mutation detection technique.

A number of mutation detection techniques have been developed to screen DNA for point mutations. Methods can be divided into two categories according to whether the mutation is previously known or not. Some of the commonly used methods for detection of known mutations include allele specific amplification/hybridisation (ASA/H) (Okayama *et al,* 1989) and the oligonucleotide ligation assay (OLA) (Nickerson *et al,* 1990). However, these methods are unable to detect mutations outside the chosen area and are not useful for screening a given gene for novel mutations. Therefore, these methods will not be described in this study. In contrast, if the position or nature of a mutation is unknown, then screening can be achieved using one of the several techniques including single strand conformational polymorphism (SSCP) (Orita *et al,* 1989); denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman, 1979) and conformation sensitive gel electrophoresis (CSGE) (Ganguly *et al,* 1993).

3.1.1 DNA Sequencing

The technical innovation for DNA sequencing was established in 1977, when Maxam and Gilbert described a method for sequencing using base specific chemical degradation and Sanger and his colleagues described a method for enzymatic sequencing using chain terminators (Maxam and Gilbert, 1977; Sanger *et al*, 1977). These basic techniques of DNA

sequencing, established 1977, are still in use. DNA sequencing methods have been further refined to enable automation.

The basis of both techniques of DNA sequencing (Maxam and Gilbert, 1977; Sanger *et al*, 1977) is the generation of a set of single stranded DNA fragments, which are separated by size on a polyacrylamide gel. However, the Sanger chain termination method has been under further development and has become more widely used. Automated chain termination DNA sequencing was introduced in 1986 (Ansorge *et al*, 1986) with fluorescent labelling, decreasing the time of detection and excluding the use of radioactive material.

In general, using sequencing as a mutation detection technique is the most sensitive and accurate technique available. However, the disadvantage of using the automated sequencing in screening a large number of patients, is that it is an expensive approach. Using manual DNA sequencing would be an impractical and time-consuming approach.

3.1.2 Single Strand Conformational Polymorphism (SSCP) analysis

SSCP is a scanning technique that is widely used because of its simplicity. The principle of this method is the ability of a nucleotide alteration to change the electrophoretic mobility of a single DNA strand in a non-denaturing polyacrylamide gel. Unlike double-stranded DNA, single stranded DNA is flexible and will adopt a conformation determined by intramolecular interactions (Orita *et al*, 1989). The presence of any sequence change, due to mutation or polymorphism between mutated and wild type DNA, will alter the differential migration pattern. This can be detected as an alteration in the electrophoretic mobility of the single-stranded DNA in non-denaturing polyacrylamide gel (Orita *et al*, 1989).

The sensitivity of SSCP analysis can reach up to a 95% detection level in certain model systems (Michaud *et al*, 1992; Sheffield *et al*, 1993). Achieving such high detection rates by

SSCP may require running the gels under specific conditions, because the conformation of single-stranded DNA is sensitive to a number of parameters, including ionic strength, the type of gel matrix, fragment length, and temperature. When the SSCP technique was originally introduced, radioactive nucleotides were used and SSCP was originally performed on large sequencing gels. It was performed in a cold room to control the temperature, which makes the technique difficult and not always reproducible (Orita *et al,* 1989). However, the simplicity and reproducibility of this method was increased when the technique was modified by the development of a non-radioactive technique and introducing temperature controlled electrophoretic units (Ainsworth *et al*, 1991; Yap and McGee, 1993). SSCP, however, is less effective if DNA fragments of more than 200bp are analysed, since sensitivity of PCR-SSCP tends to decrease as fragment length increases. For example, if a 400 bp fragment is screened for a single-base change, then the sensitivity falls to approximately 80% compared to 95% sensitivity for a 200 bp fragment (Hayashi and Yandell, 1993).

3.1.3 Denaturing Gradient Gel Electrophoresis (DGGE)

In 1979, denaturing gradient gel electrophoresis (DGGE) was introduced as an alternative method to distinguish any segment of DNA containing a single base pair mutation from its corresponding wild-type segment (Fischer and Lerman, 1979). The separation principle of this technique is based on the melting properties of DNA in solution. Thus, when the temperature or the denaturant concentration is raised, this will cause the DNA molecules to melt in discrete segments called the melting domains. It is noteworthy to mention that the melting temperature (TM) of a melting domain depends on its nucleotide sequence. The separation of a DNA double strand into single-strand segments increases if the DNA fragment is electrophoresed through a linear gradient of increasing denaturant concentration.

Furthermore, a single base substitution in the DNA molecule can cause differential mobility during DGGE. This causes the DNA segment to form a less uniform three-dimensional structure that moves through a polyacrylamide matrix at a reduced rate. At a certain position, the DNA molecule will start branching and slowing down when it enters the concentration of denaturant where its lowest temperature domain melts. This results in separation of different fragments by the end of the run. A computerised program has been developed to determine the theoretical melting domains of the DNA fragment of interest (Lerman and Silverstein, 1987). However, the DGGE method is not very popular since it requires a significant time for preparation and analysis when compared to SSCP and CSGE.

3.1.4 Conformation Sensitive Gel Electrophoresis (CSGE)

One of the most commonly used methods for mutation detection is heteroduplex analysis. Heteroduplexes are formed by mixing wild type and mutant DNA amplified by PCR. By heating and cooling, the samples are denatured and re-annealed (Ganguly *et al,* 1993). In a non-denaturing and for some applications, a mildly denaturing system, mutations can be detected by heteroduplex analysis based on the decreased mobility of the heteroduplex compared with the corresponding homoduplex (Ganguly *et al,* 1993). Due to an expanded open double-strand pattern surrounding the mismatched bases, the heteroduplexes tend to migrate more slowly than their parallel homoduplexes.

One of the heteroduplex detection methods is CSGE. As stated earlier, in the presence of a single mismatch a differentiation can occur between heteroduplexes and homoduplexes using polyacrylamide gels in a mildly denaturing solvent system. In an early study, it was shown that DNA conformational changes can be created below the concentration required for total denaturation at moderate temperatures in the presence of mildly denaturing solvents like ethylene glycol and formamide (Orosz and Wetmur, 1977; Lee *et al*, 1981). Therefore, this

conformational change will expand the differential migration of DNA heteroduplexes and homoduplexes during gel electrophoresis (figure 3.1).

CSGE is a highly sensitive mutation detection system and, according to a recent study can reach up to 94% sensitivity (Korkko *et al,* 2002). Furthermore, a recent report demonstrated that CSGE can be adopted for use on a fluorescent platform (F-CSGE) and results in a higher throughput and sensitivity (Ganguly *et al,* 1998). The simplicity of this method and the direct use of PCR product without the need for any extra purification are the main advantages of this method. Therefore, many laboratories have adopted this method for scanning genes for sequence alterations including mutations and single nucleotide polymorphisms (SNPs).

For this part of the study, the main aim was to screen the *c-fms* gene for mutations in two different groups of patients having AML or IMF. CSGE was the method of choice for this study due to several factors. This technique was previously used and established in our laboratory for the analysis of the factor VIII gene in patients affected by haemophilia A (Williams *et al,* 1998; Goodeve *et al,* 2000). It was also used in further genes such as the von Willbrand factor gene (Abuzenadah, 1998), factor IX (Hinks *et al,* 1999) and produced reliable results in all of these studies. In the factor IX gene, ten previously reported mutations and eleven novel mutations were identified in 21 patients with haemophilia B using CSGE demonstrating 100% sensitivity in this patient cohort (Hinks *et al,* 1999). Therefore, the CSGE method was selected on basis of laboratory experience, sensitivity and simplicity.

The establishment of CSGE analysis for the *c-fms* gene is described in this chapter. Furthermore, the novel and previously identified fragments of *c-fms* exhibiting polymorphic nucleotide alteration are presented in this chapter. Newly identified and previously reported mutations will be presented in chapter 4 of this study.

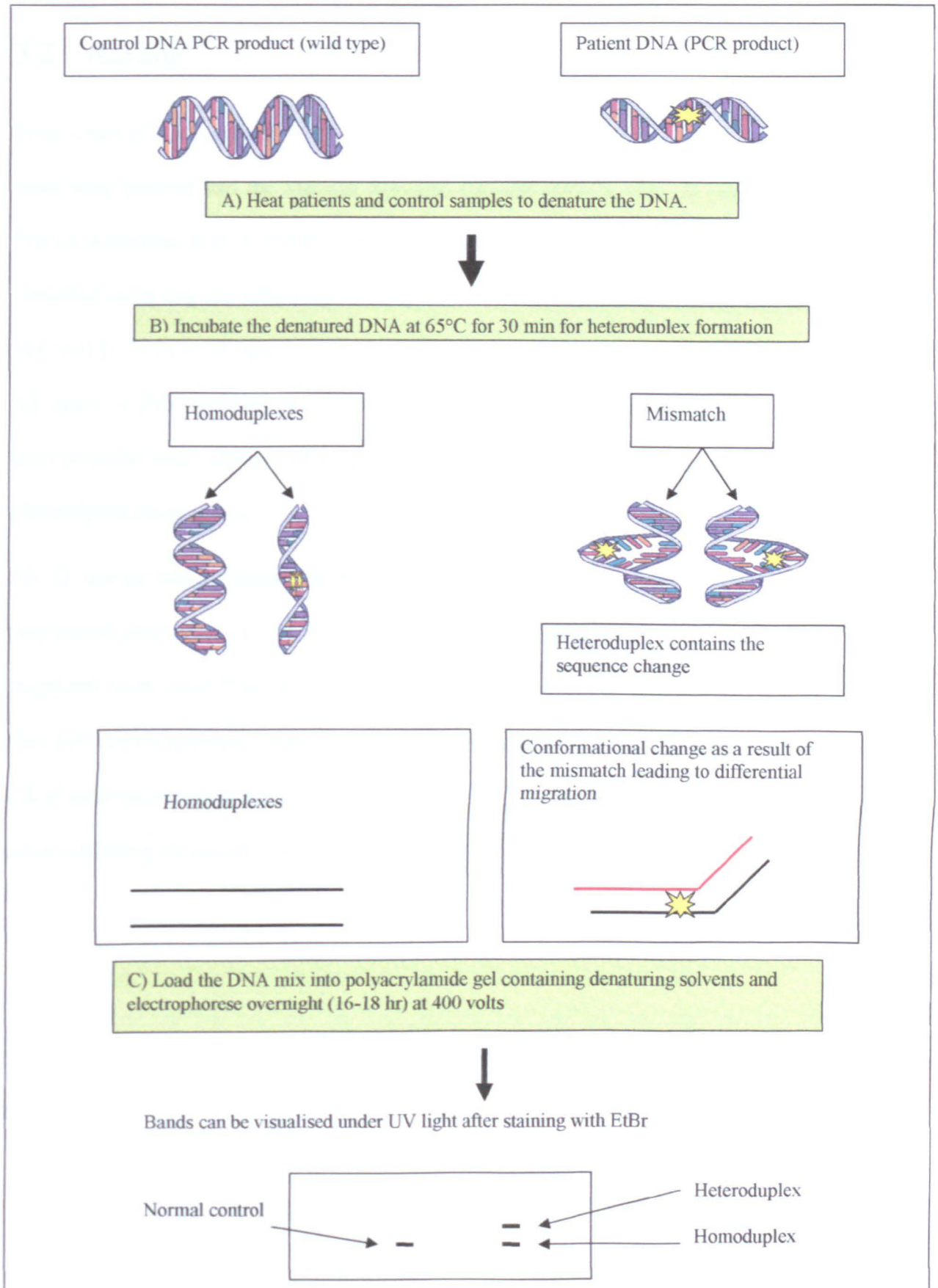


Figure 3.1. Schematic representation of CSGE analysis of heteroduplexed DNA sample with a single base substitution demonstrating the principle of the technique

3.2 Results

Sixty cases of AML (group A) were initially screened for changes in the *c-fms* gene. These cases were entered into the Medical Research Council (MRC) AML X Trial. According to French-American-British (FAB) criteria (Bennett *et al*, 1976) they were morphologically classified as having the following AML types: M0 (n=4), M1 (n=8), M2 (n=10), M3 (n=10), M4 (n=12), M5 (n=10) and M6 (n=6). Furthermore, forty cases of IMF were also studied. All cases of IMF fulfilled the following criteria: a leucoerythroblastic blood picture, tear drop poikilocytosis, absence of monocytosis, marked bone marrow fibrosis and lack of the Philadelphia chromosome.

All 21 exonic and flanking intronic regions of the *c-fms* gene were amplified using the polymerase chain reaction (PCR) and the primers listed in table 2.1 (section 2.3.4.1). DNA fragments were amplified individually using PCR and specific primers for each exon and then self-heteroduplexed (heated to denature DNA and then cooled to re-anneal the DNA). CSGE analysis was then performed for all samples, DNA sequencing was then performed for cases exhibiting abnormal CSGE patterns.

3.2.1 Polymorphism in exon 3

Exon 3 was amplified from the genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of self-heteroduplexed PCR product of each patient demonstrated that 47 patients (24 AML and 23 IMF) had abnormal CSGE patterns (fig 3.2). DNA sequence analysis of patients with aberrant patterns revealed that patients had a C→T substitution at position 384 of the c-fms cDNA (numbering after (Coussens *et al,* 1986)) and were heterozygous for a silent change at codon 28 (Pro28Pro; CCC→CCT; figure 3.3). Further analysis of 70 normal individuals by CSGE followed by DNA sequencing showed that seven individuals were heterozygous for the same silent change in exon 3.

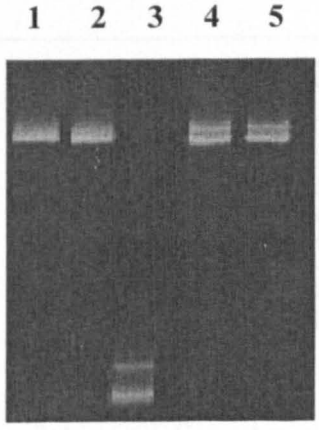


Figure 3.2 CSGE analysis of exon 3 PCR fragment demonstrating a polymorphism in the *c-fms* gene. The gel shows the normal pattern in lanes 1and 2 and a positive control in lane 3 and two samples with positive patterns in lanes 4 and 5.

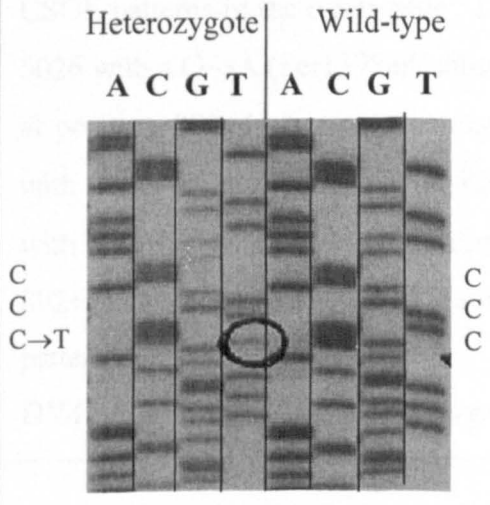


Figure 3.3 Direct DNA sequence analysis showing the novel C→T polymorphism at codon 28 in exon 3.

3.2.2 Polymorphism in exon 4 and the surrounding intronic sequence

Exon 4 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR product from each patient demonstrated that 15 patients (11 AML and 4 IMF) showing 3 aberrant CSGE patterns. One AML sample had a G→A (Ser139Ser) substitution at position 717 plus a G→A substitution at position 892+41 with CSGE pattern as shown in figure 3.4 (lane 1). The pattern shown in lane 2 was seen in 13 patients (9 AML and 4 IMF) who had a G→A substitution at position 892+41. A further AML patient had a C→G substitution at position 892+54 plus a T→C substitution at position 892+65 with a different pattern as in figure 3.3 lane 3. Seventy normal individuals were screened using CSGE, 8 out of 70 had a band shift as shown in lane 2 indicating heterozygosity for the G to A substitution at position 892+41.

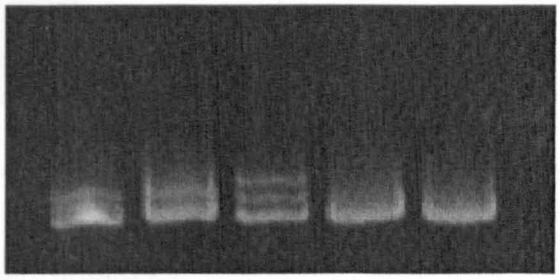


Figure 3.4. CSGE analysis of exon 4 PCR fragment demonstrating 3 different CSGE patterns in the *c-fms* gene. Lane 1 shows the CSGE pattern of AML patient 5026 with a G→A (Ser139Ser) substitution at position 717 plus a G→A substitution at position 892+41 (intronic non coding region). Lane 2 shows an AML patient with the G→A substitution at position 892+41 alone, lane 3 shows an AML patient with a C→G substitution at position 892+54 plus a T→C substitution at position 892+65 (both intronic). Lanes 4 and 5 show two AML patients with normal CSGE patterns.

DNA sequences are shown in figure 3.5.

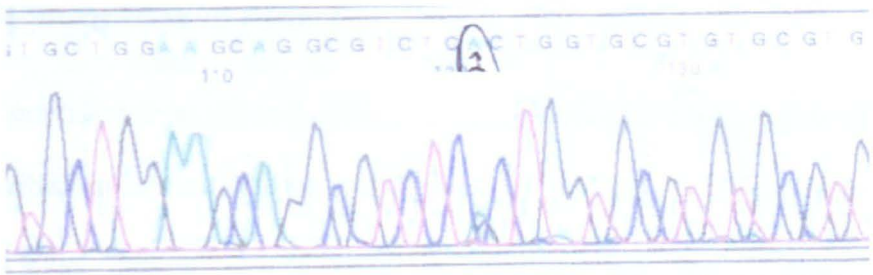


Figure 3.5A Automated sequence analysis showing the novel 717 TCG→TCA (Ser139Ser) silent change in exon 4 of the *c-fms* gene (position 121 on this trace).

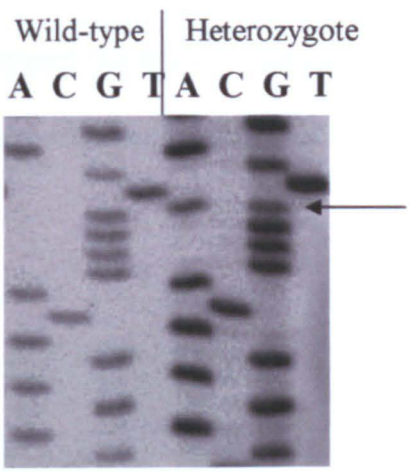


Figure 3.5B Direct DNA sequence analysis showing novel 892+41 G→A polymorphism in intron 4.

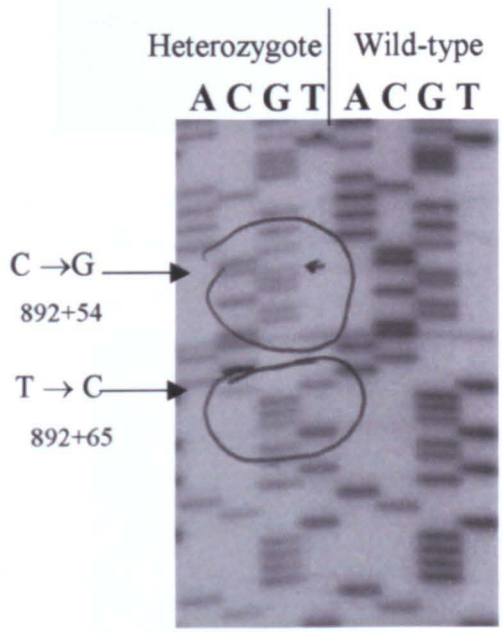


Figure 3.5C. Direct DNA sequence analysis showing novel 892+54 C→G and 892+65 T→C polymorphisms.

3.2.3 Polymorphism in exon 5

Exon 5 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR products from each patient demonstrated that 51 patients (28 AML and 23 IMF) had abnormal CSGE patterns as seen in figure 3.6A. Direct DNA sequence analysis of patients with different patterns than normal revealed that all these patients had a C→T substitution at position 1026 and were heterozygous for a silent change at codon 242 (Thr242Thr; ACC→ACT; figure 3.6B). Further analysis of 70 normal individuals showed that 20 (28.6%) of them were also heterozygous for a C→T substitution at position 1026. This polymorphism was previously identified after sequencing cDNA clones from different normal individuals (Hampe *et al*, 1989).

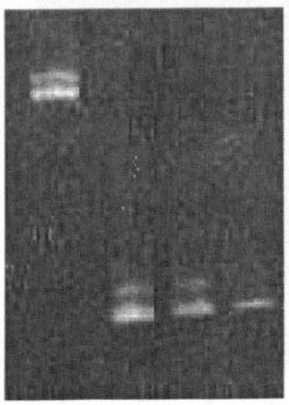


Figure 3.6A. CSGE analysis of exon 5 PCR fragment demonstrating a polymorphism in the *c-fms* gene. Lane 1 shows a positive control. Lanes 2 and 3 show AML samples positive for the previously reported change in exon 5. Lane 4 shows an AML sample with wild type sequence for the exon 5 fragment.

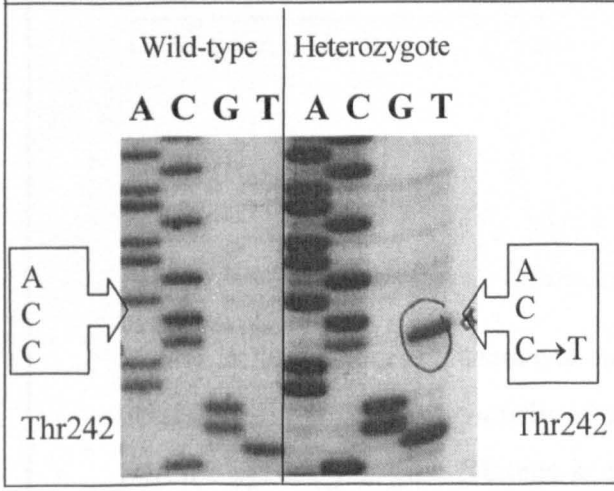


Figure 3.6B Direct DNA sequence analysis showing the Thr242Thr silent change in exon 5 of the *c-fms* gene (Hampe *et al*, 1989).

3.2.4 Polymorphism in intron 6

Exon 6 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR product of each patient demonstrated that 49 patients (26 AML and 23 IMF) had abnormal CSGE patterns (figure 3.7A). DNA sequence analysis of patients with different patterns than normal revealed that patients have a TT→AG substitution at position 1189+67-68 in intron 6 (figure 3.7B). Further analysis of 70 normal individuals showed that 16 individuals had the same change in intron 6 (22.9%). Hampe et al, (1989) previously identified this polymorphism after sequencing different clones from different normal individuals. A further intronic change; a C→T at position 1189+28 was identified in two AML patients. This change is described in chapter 4.

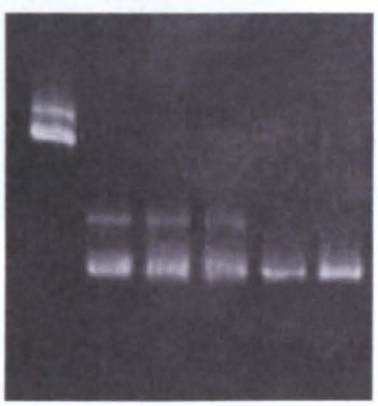


Figure 3.7A CSGE analysis of intron 6. Lane C shows a positive control. Lanes 1-3 show abnormal CSGE profiles in three AML patients. Lanes 4 and 5 show a normal CSGE pattern.

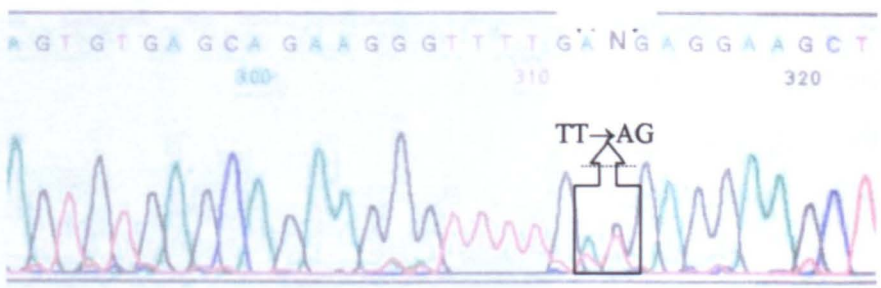


Figure 3.7B Automated sequence analysis showing an individual heterozygous for the previously identified TT→AG change in intron 6 of the *c-fms* gene (Hampe *et al*, 1989).

3.2.5 Polymorphism in Intron 11

Exon 11 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR product of each patient demonstrated that four patients (2 AML and 2 IMF) had abnormal CSGE patterns (figure 3.8A). DNA sequence analysis of patients with different patterns than normal revealed that patients have a novel intronic C→T substitution at position 1926+6 (figure 3.8B). Further analysis of 70 normal individuals by CSGE showed that only one individual had the same change in intron 11. The novel change was classified as polymorphic with a low frequency.

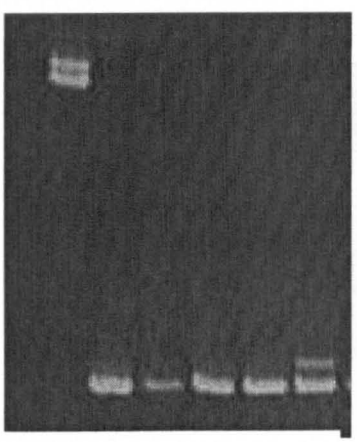


Figure 3.8A. CSGE analysis of intron 11 PCR fragment demonstrating polymorphism in the *c-fms* gene. Lane C shows a positive control. Lanes 1-4 show IMF samples with wild type sequence for the exon 11 fragment. Lane 5 shows an IMF sample positive for a C→T substitution in intron 11 of the *c-fms* gene.

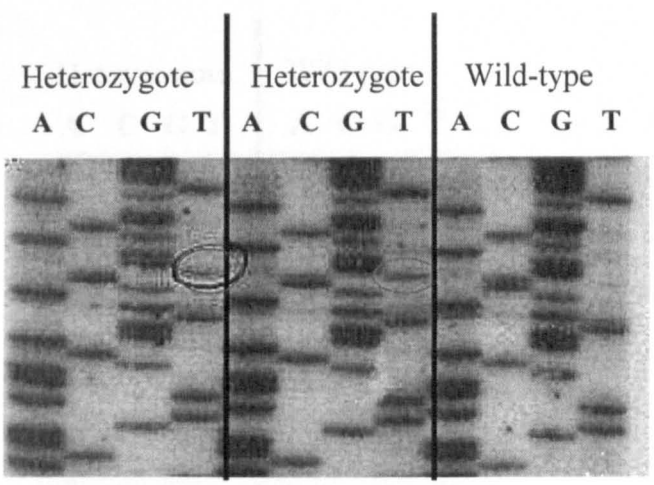


Figure 3.8B. Direct DNA sequence analysis showing the novel C→T polymorphism in two IMF patients and the wild type sequence of one normal individual.

3.2.6 Polymorphism in exon 19

Exon 19 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR product of each patient demonstrated that 10 patients (6 AML and 4 IMF) had abnormal CSGE patterns (figure 3.9A). DNA sequence analysis of patients with different patterns than normal revealed that patients had a novel C→G substitution at position 2835, and were heterozygotes for a silent change at codon 846 (Leu846Leu) (figure 3.9B). Further CSGE analysis of 70 normal individuals showed that three individuals (4.3%) were heterozygous for the same novel change in exon 19.

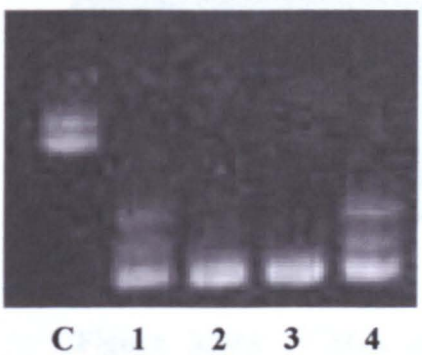


Figure 3.8A CSGE analysis of exon 19 PCR fragment demonstrating a silent change in the *c-fms* gene. Lane C shows a positive control. Lanes 1 and 4 show AML samples positive for a C→G substitution. Lanes 2 and 3 show wild type sequence for the exon 19 fragment of the *c-fms* gene.

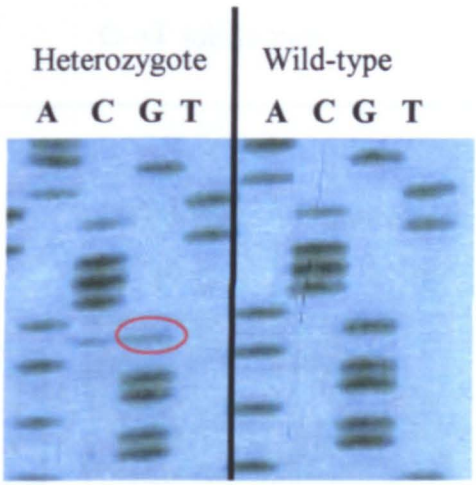


Figure 3.9B. Direct DNA sequence analysis of exon 19 fragment showing the novel C→G substitution in one AML patients and the wild type sequence of one normal individual.

3.2.7 Polymorphism in exon 21

Exon 21 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR product of each patient demonstrated that one IMF patient had an abnormal CSGE pattern (figure 3.10A, lane 11). DNA sequence analysis of the positive IMF patient revealed that this patient has a novel C→T substitution at position 3009, and was heterozygous for a silent change at codon 904 (Phe904Phe) (figure 3.10B). None of the 70 normal individuals analysed by CSGE has this novel change.

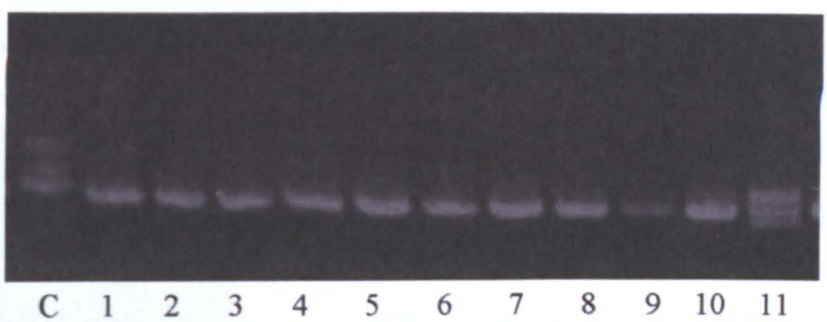


Figure 3.10A CSGE analysis of exon 21 PCR fragment demonstrating a silent change in the *c-fms* gene. Lane C shows a positive control. Lanes 1-10 show IMF samples with a normal pattern on CSGE, lane 11 shows an IMF sample positive for a C→T substitution

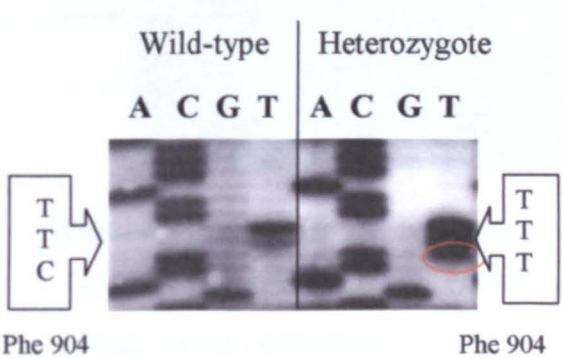


Figure 3.10B. Direct DNA sequence analysis of exon 21 fragment showing the wild type sequence of one normal individual and the novel C→T substitution in one IMF patient.

3.2.8 Polymorphism in the 3` non coding region of the *c-fms* gene

Exon 22 fragment was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of the PCR product of each patient demonstrated that 31 patients (17 AML and 14 IMF) had abnormal CSGE patterns (figure 3.11A). DNA sequence analysis of patients with different patterns than normal revealed that patients have a novel TC→CA substitution at 3254-3255 in the 3` non coding region of the *c-fms* gene (figure 3.11B-C). Further analysis of 70 normal individuals by CSGE showed that eleven individuals (15.7%) had the same change in the 3` non coding region of the *c-fms* gene. In addition, this change was confirmed recently by another group (Romashchenko *et al,* 2002)

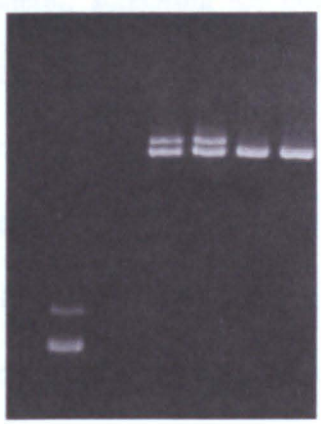


Figure 3.11A CSGE analysis of exon 22. Lane C shows a positive control. Lane B shows a PCR blank. Lanes 1-2 show abnormal CSGE profiles in two AML patients. Lanes 3 and 4 show a normal CSGE pattern.

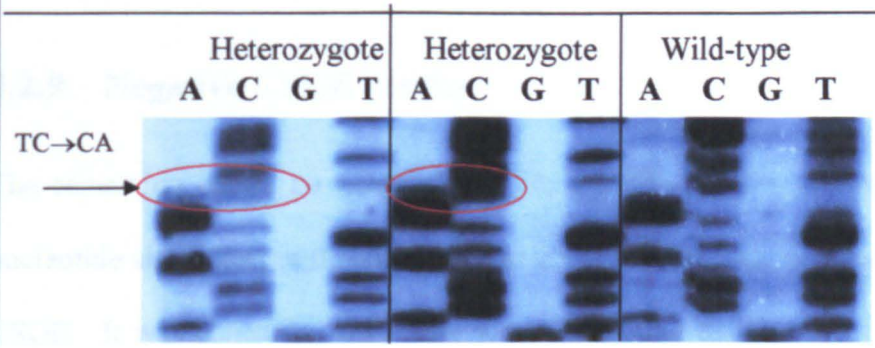


Figure 3.11B. Direct DNA sequence analysis showing the novel TC→CA polymorphism in two AML patients and the wild type sequence of one normal individual.

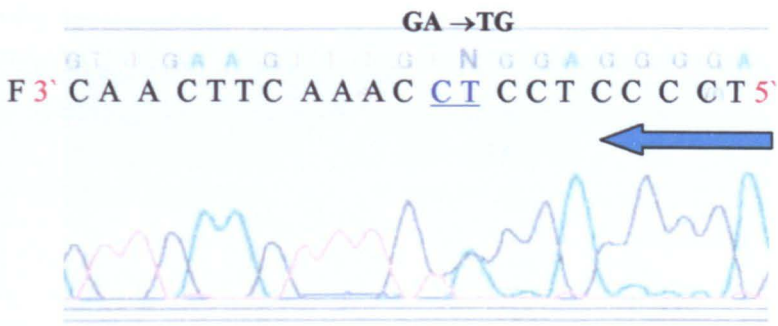


Figure 3.11C Automated sequence analysis of showing the identified TC→CA change in the 3′ non coding region of the *c-fms* gene. The arrow indicates 5′ to 3′ direction.
F= Forward sequence

In general, screening for polymorphisms in this chapter shows that there is an increase heterozygocity in both AML and IMF patients compare to the normal individuals for all the *c-fms* polymorphisms except the alteration within intron 13 where no heterozygote was identified in the IMF patients (table 3.2). Furthermore, the TT→AG substitution at position 1189+67-68 in intron 6 could create a new splicing site, according to the consensus of the donor and acceptor sequence (Mount, 1982), within intron 6 that could modify the structure of the mRNA and perhaps the protein of the *c-fms* gene.

3.2.9 Negative CSGE results

The remaining exons i.e. exons 2, 7, 10, 14, 15, 16, 17, 18 and 20 were analysed for nucleotide alterations in the same way as above. None has presented an aberrant pattern on CSGE. It was concluded that there were no detectable alteration in these fragments. The changes identified throughout *c-fms* are summarized in Table 3.1.

Table 3.1A Spectrum and percentages of polymorphic nucleotide alterations identified within the *c-fms* gene by CSGE.

Exon/Intron No.	Nucleotide change*[R]	Amino acid change*[R]	No. AML patients n=60 (%)	IMF patients n=40 (%)	normal controls n=70 (%)
Exon 3	384 C>T	Pro 28 Pro	24 (40)	23 (57.5)	8 (11.4)
Exon 4	717 G>A	Ser 139 Ser	1 (1.6)	0	0
Intron 4	892+41 G>A 892+54 [1]C>G 892+65 [2]T>C	NA NA	9 (15) 1 (1.6) 0	4 (10) 0 0	8 (11.4) 0 0
Exon 5	1026 C>T	Thr 242 Thr	28 (46.6)	23 (57.5)	20 (28.5)
Intron 6	1189+28 C>T 1189+67-68 TT>AG	NA	2 (3.3) 26 (43.3)	0 23 (57.5)	0 16 (22.9)
Intron 11	1926+6 C>T	NA	2 (3.3)	2 (5)	1 (1.4)
Intron 13	Not possible to sequence	NA	18 (30)	NT	23 (32.9)
Exon 19	2835 C>G	Leu 846 Leu	6 (10)	4 (10)	3 (4.3)
Exon 21	3009 C>T	Phe 904 Phe	0	1 (2.5)	0
3`UTR	3254-3255 TC>CA	NA	17 (28.3)	14 (35)	11 (15.7)

*[R] = Numbering according to (Coussens *et al*, 1986).
[1, 2] Both changes were found in the same patient

Table 3.1B. Difference in the frequency of heterozygote between different groups

Exon/Intron No.	Nucleotide change	AML/IMF p-value	AML/control p-value	IMF/control p-value
Exon 3	384 C>T	NS	0.0002	<0.0001
Exon 5	1026 C>T	NS	NS	0.0028
Intron 6	1189+28 C>T 1189+67-68 TT>AG	NS NS	NS 0.0128	NS 0.0003
Intron 13	Not possible to sequence	<0.0001	NS	<0.0001
3`UTR	3254-3255 TC>CA	NS	NS	0.0202

NS= not statistically significant.
Only significant heterozygosity difference were included in the table

3.2.10 Screening for restriction sites

To be able to detect the presence or absence of a homozygous version of the identified alteration in this study, a further step was introduced. All novel and previously identified alterations were analysed for the consequence of any alteration at restriction enzyme sites using the Webcutter 2.0 programme provided via the internet by Yale University (http://www.firstmarket.com/cutter/cut2.html).

The sequence of PCR fragments with and without alterations were screened for a restriction site using all the enzymes in the programme database (Table 3.2) to identify the presence of specific enzymes that could cut the PCR fragment with or without the alteration. Therefore, this approach was used to identify the exact occurrence and frequency of the changes identified in the *c-fms* gene during this study. Furthermore, this approach may help other researchers to screen for using restriction endonuclease analysis.

Table 3.2 Restriction endonuclease site analysis within the *c-fms* gene.

Exon/ Intron No.	Fragment size/bp	Nucleotide change	Enzyme	Product size/bp	
				Wild type allele	Abnormal allele
Exon 3	565	384 C>T	AvaI	209, 356	565
Exon 4	460	717 G>A	BcgI	146, 314	460
Intron 4	460	892+41 G>A	NEC*	-	-
		892+54 C>G	BssAI	34, 87, 163, 176	121, 163, 176
		892+65 T>C	NEC	-	-
Exon 5	341	1026 C>T	StyI	63, 96, 182	96, 245
Intron 6		1189+28 C>T	NEC		
		1189+67-68 TT>AG	NEC		
Intron 11	259	1926+6 C>T	PleI	259	48, 211
Exon 19	222	2835 C>G	BseRI	34, 65, 123	34, 188
Exon 21	220	3009 C>T	NEC		
3′ UTR	382	3254-3255 TC>CA	NEC	-	-

NEC = No Enzyme Cut identified

3.3 Discussion

This study presents, for the first time, polymorphism analysis of the complete coding and flanking sequence of the *c-fms* gene. Previous studies have sought a small number of mutations based on investigations designed to specific locations in the *c-fms* gene (Roussel *et al,* 1987; Roussel *et al,* 1988; Woolford *et al,* 1988). Polymorphisms have been also reported in a few studies which have compared different genomic or cDNA clones in each study (Coussens *et al,* 1986; Hampe *et al,* 1989).

This study identified 12 different apparently polymorphic alterations, five alterations that result in silent amino acid changes and 7 intronic nucleotide alterations. Of these, one of the silent changes is a C→T at position 1026 (Thr242Thr in exon 5), one intronic alteration (TT→AG at position 1189+67-68 in intron 6) and the TC→CA substitution at position 3254-3255 in the 3` non coding region of the *c-fms* gene have been previously reported (table 3.1) (Hampe *et al,* 1989; Romashchenko *et al,* 2002). The TT→AG change at position 1189+67-68 in intron 6 could have a potential functional relevance as this change could create a consensus 3` acceptor splicing site within intron 6 that could modify the structure of the mRNA and protein of the *c-fms* gene. However, to confirm this observation, the mRNA should be analysed further to identify the possible splicing forms. The TC→CA substitution was 34 bp downstream of the translation stop codon. Furthermore, according to a recent study carried out by Romashchenko *et al*, (2002) the polymorphic dinucleotide appeared to be located immediately upstream of an octamer showing 100% homology to the cis element -CAAACTTC-, which is responsible for controlled instability of mRNAs of several genes (Romashchenko *et al,* 2002). Based on these data, functional significance was assumed for this polymorphism of the *c-fms* gene. Hence, this polymorphism could affect the half life of the *c-fms* mRNA because this region is involved in the stability of the mRNA

Therefore, all of the previously identified polymorphisms within the screened part of the *c-fms* gene were detected using CSGE (Table 3.1). All the polymorphisms that were identified in this study were included in this chapter.

It was reported recently that interaction of different components involved in mRNA synthesis, maturation, transport, translation or degradation might take place due to nucleotide alteration at some SNP sites (Shen *et al,* 1999). Still, SNPs without an obvious effect on the RNA consensus and protein sequence could lead to phenotypic effect, mainly by non-consensus-dependent mechanisms (Shen *et al,* 1999). Furthermore, Shen and his colleagues demonstrated that SNPs could cause significant structural variation in allelic forms of human mRNA. Therefore, this structural variation can affect the mRNA-structure-dependent mechanisms consequently causing allele-specific biological consequence (Shen *et al,* 1999).

Screening for restriction sites was also performed for all the changes to facilitate any future analysis for any of those specific alterations that was identified in the *c-fms* gene. Furthermore, allelic frequency can be obtained from restriction enzyme analysis for any specific alteration identified earlier in this chapter. It is noteworthy to mention that identification of restriction enzyme sites was the method of choice for the first systematic studies of single base variants (Botstein *et al,* 1980). Restriction fragment length polymorphism (RFLP) is the method of distinguishing alleles or variants based on its fragment sizes, simply by digestion of a piece of DNA containing the relevant site with an appropriate restriction enzyme (Botstein *et al,* 1980). Furthermore, SNPs are the most abundant type of DNA sequence variation in the human genome (Cooper *et al,* 1985). Still, if the SNP were found within a small, unique segment of DNA, it would also serve as a physical landmark or as a genetic marker whose transmission can be traced from parent to child (Kwok and Gu, 1999). Therefore, identifying novel polymorphisms and the

identification of suitable enzymes to be used as a differentiation tool between the two allelic variants between those novel polymorphisms would be very beneficial for future studies.

As stated in chapter 2, CSGE was the technique of choice for the mutational analysis of the *c-fms* gene in this study. This technique is able to detect mutations in double stranded DNA due to the fact that single-base mismatches can produce conformation changes in the double helix that in turn will cause different migration speeds between the heteroduplexes and homoduplexes (Ganguly *et al,* 1993).

The high sensitivity of this technique was one of the advantages of this technique. Several studies showed that CSGE is able to detect up to 95% of nucleotide mismatches in a PCR product of 200-800bp in length (Ganguly *et al*, 1993; Ganguly and Prockop, 1995). Higher sensitivity was reported afterwards in another study carried out by Korkko et al, (1998) when CSGE detected all 76 previously identified single-base changes in a large series of PCR products from collagen genes that contain multiple exons with highly repetitive and GC-rich sequences.

The usefulness of this technique was confirmed by several studies, for instance CSGE was able to identify nucleotide alterations in seven out of seven patients (100%) with severe haemophilia A (Williams *et al,* 1998). Furthermore 10 previously reported and 11 newly identified mutations were identified by CSGE in 21 patients with haemophilia B patients (Hinks *et al,* 1999).

It is noteworthy to mention that sensitivity is not the only advantage of this technique. Safety for example, is another advantage for using CSGE. Abuzenadah et al, (1998) reported that CSGE does not require the use of toxic and radioactive materials such as those used in the chemical cleavage mismatch (CCM) technique (Abuzenadah, 1998). Furthermore, a special consideration for the cost of applying this technique in a large-scale

study would obviously be in favour of using CSGE due to its low cost in comparison to other techniques. For instance, a cost comparison between direct sequencing and CSGE in the analysis of collagen *COL2A1* gene demonstrated the huge difference in the cost of applying CSGE ($365/patient) and direct DNA sequencing ($1,583/patient) due to the fact that only aberrant fragment are sequenced when CSGE is used (Ganguly and Williams, 1997). Nevertheless, the large number of samples that can be analysed in one CSGE run (40 samples/CSGE gel) demonstrated that not only the quality but also the quantity of the analysis is improved in comparison to direct DNA sequencing. Normally 4-6 hours of preparation is required on the first day and the result can be obtained on the following day. In addition, no special preparation of the PCR product is required and up to four CSGE tanks can be handled in one run.

As stated earlier, a number of novel and previously identified polymorphisms were identified in this study. It is well established that polymorphisms can be found throughout the genome, e.g. in exons, introns, promoter region or enhancer (Krawczak *et al,* 1992; Drazen *et al,* 1999; Schork *et al,* 2000). Furthermore, polymorphisms can have an important effect in some cases, for instance, it can have a direct effect on splicing or gene expression (Krawczak *et al,* 1992). However, in this study, none of the polymorphisms identified were likely to have an effect at an important site (i.e. promoter, enhancer or splicing sites). All the identified polymorphisms were analysed for an obvious effect on splicing sites, according to consensus sequence of the 5` donor and 3` acceptor sites of the gene (Mount, 1982). Except for the C→T at position 1026 and the TC→CA substitution at position 3254-3255 alterations, no direct effect was predicted for other alterations identified in the *c-fms* gene. Thus, none of the alteration identified have been shown to create a new splicing site. However, further analysis at the RNA level would be required to exclude this event. Furthermore, as has been suggested recently, large-scale epidemiological studies might be

to be biologically inert (i.e. neutral) or have unknown function" (Schork *et al,* 2000). Therefore, a combination of other information from genomic structure analysis and protein level studies, which examine the molecular physiology and pathology of the gene, may enlighten us about more useful information regarding the effect of such alteration within the *c-fms* gene or other class III RTK.

Chapter 4

SEQUENCE ALTERATIONS THAT PREDICT AMINO ACID CHANGES IN THE *C-FMS* GENE

Sequence alterations that predict amino acid changes in the *c-fms* gene

4.1 Introduction

c-fms, *c-kit*, *FLT3* and *PDGFRβ* are all members of class III RTK family (Ullrich and Schlessinger, 1990). Class III RTK (Ullrich and Schlessinger, 1990) are closely related proteins that are characterised by five immunoglobulin-like domains in the extracellular part, a single transmembrane domain (TM), a juxtamembrane domain (JM), two intracellular kinase domains (TK1 and TK2) separated by a kinase insert domain (KI), and have a C-terminal domain in their 3` part. Ligand-dependent activation is initiated by ligand binding causing receptor dimerization and subsequent activation of the intrinsic tyrosine activity catalysing transphosphorylation of specific tyrosine residues (Ullrich and Schlessinger, 1990; Weiss and Schlessinger, 1998).

Previous studies revealed that class III RTK have a crucial role in normal haematopoiesis (Coussens *et al*, 1986; Yarden *et al*, 1987; Rosnet *et al*, 1993a). An important role for the c-fms receptor in monocytic differentiation has been established since 1985, when c-fms was detected in peripheral blood monocytes and on blast cells from certain patients with myelomonocytic leukaemia (Sariban *et al*, 1985). Further studies highlighted the role of this receptor in growth and differentiation of the monocyte-macrophage-osteoclast lineage (Sherr, 1990). Other RTK class III, like *c-kit* and *FLT3*, are also important in haematopoiesis. *c-kit* and *FLT3* are essential for the survival, proliferation and differentiation of haematopoietic progenitor cells (Lyman and Jacobsen, 1998). Until now, the *PDGFRβ* relationship to haematopoiesis has not been well defined, however, it has been reported that it might have a significant role in megakaryocytopoiesis (Yang *et al*, 1997b).

Earlier studies reported the presence of a number of important mutations in some of the class III RTK (Roussel *et al,* 1987; Roussel *et al,* 1988; Woolford *et al,* 1988). In the human haematopoietic system, 70% of CD34+ cells in the bone marrow express c-kit (Ashman *et al,* 1991). A pathogenic role of *c-kit* was highlighted in mastocytosis when two point mutations Val560Gly and Asp816Val in the juxtamembrane and the phosphotransferase domain respectively, were identified in the mast cell leukaemia cell line, HMC-1 (Furitsu *et al,* 1993). Additionally, the presence of the *c-kit* Asp816Val mutation was identified in the peripheral blood mononuclear cells of patients with myelofibrosis and in patients with myelodysplasia associated with mastocytosis (Nagata *et al,* 1995). It is noteworthy that *c-kit* mutations have also been identified in AML. An Asp 816Tyr mutation was identified in AML-M2 patients with t(8;21) (Beghini *et al,* 2000a). Gari et al (1999) identified other mutations in exon 8 (deletion-plus-insertion) that consistently affect the Asp419 codon.

Several mutations have also been reported in *FLT3*, another class III RTK. Internal tandem duplication (ITD) mutations were the first reported somatic mutation in the FLT3 gene (Nakao *et al,* 1996). The ITD mutations affect the juxtamembrane (JM) domain and are mainly present in exon 14 of the *FLT3* gene (see chapter 5). The location and length of the ITD mutation varies from case to case, although, the gene was always transcribed in frame and encoded mutant *FLT3* with a long JM domain (Nakao *et al,* 1996). Several studies reported the presence of *FLT3* ITD mutations in a high proportion of AML cases (20-24 %) (Yokota *et al,* 1997; Xu *et al,* 1999; Rombouts *et al,* 2000; Kottaridis *et al,* 2001). The *FLT3* ITD has been associated with poor prognosis in AML (Kiyoi *et al,* 1999; Abu-Duhier *et al,* 2000; Rombouts *et al,* 2000). So far it is unclear what is the underlying mechanism of the receptor constitutive activation, although it is known that the mutated receptor is dimerized and phosphorylated in the absence of ligand (Kiyoi *et al,* 1998). Another interesting *FLT3* mutation are the novel changes seen at codon Asp835 that were recently identified by two separate groups (Abu-Duhier *et al,* 2001b; Yamamoto *et al,* 2001) in approximately 7% of

AML cases. The mutation was independent of the *FLT3* ITD, and suggest that *FLT3* is the most commonly mutated gene in AML.

Another receptor of the class III RTK, *c-fms*, was found to be a cellular homologue of the *v-fms* gene of the Susan McDonough strain of the feline sarcoma virus (Donner *et al,* 1982). Interestingly, the *v-fms* gene product was found to possess constitutive kinase activity in the absence of ligand (Sacca *et al,* 1986). Critical genetic alterations resulting in the induction of cell transformation were identified by comparing the structure of the *c-fms* and *v-fms*-coded glycoprotiens (Woolford *et al,* 1988). Comparison of the *c-fms* sequence with that of *v-fms* shows that the proteins encoded by these two genes differs by nine amino acids, as a result of substitutions and the replacement of 50 C-terminal amino acids present in c-fms by 11 unrelated residues in *v-fms*. Using chimeric *c-fms* genes and site-directed mutagenesis, Woolford et al, (1988) were able to determine that the C-terminal modification, present in *v-fms*, is sufficient to generate a partially transforming phenotype, but that mutations at amino acid positions 301 and 374 are also required (in addition to the C-terminal modification) to generate a fully transforming *c-fms* gene (Woolford *et al,* 1988). Furthermore, other sites crucial for transforming activity have been identified in the extracellular part (i.e. 218, 267, 312, 329, 335, 337 and 343) and the intracellular part (i.e. 802) of the c-fms receptor using a cassette mutagenesis technique (van Daalen Wetters *et al,* 1992; Morley *et al,* 1999).

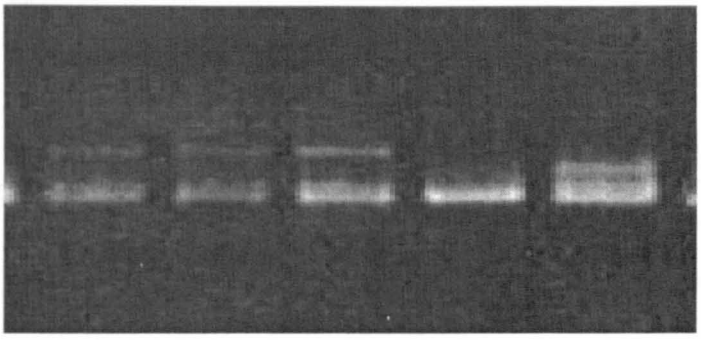
In 1987, a leukaemogenic role for the *c-fms* gene was suggested by the finding that over-expression of c-fms in mice leads to the development of myeloblastic leukaemia (Gisselbrecht *et al,* 1987). Mutations at codon 301 of the human c-fms was found to induce ligand-independent transformation of the mouse NIH3T3 cultured cells (Roussel *et al,* 1988). Furthermore, later studies identified *c-fms* point mutations at codon 301 and 969 in AML and MDS patients (Ridge *et al,* 1990; Tobal *et al,* 1990). However, in a study of 110 patients with AML or MDS, the mutations at codon 969 were more frequent (12.7%) than those at codon 301 (1.8%) (Ridge *et al,* 1990). *c-fms* mutation at codon 969 was also identified in 11

out of 70 patients in remission from lymphoma (Baker *et al,* 1995a). Another *c-fms* mutation at codon 965 was identified in a single patient with B-cell malignancy (Baker *et al,* 1995b). Mutational analysis of the *c-fms* gene will be described in this chapter. Furthermore, important mutations that have been previously identified in the *c-fms* gene (i.e 301 and 969 mutation) will be analysed in this selected group of myeloproliferative patients. Previously identified polymorphisms and silent changes were discussed in chapter 3.

4.2 Results

4.2.1 Change in Exon 6

Exon 6 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR product of each patient demonstrated that two AML patients had abnormal CSGE patterns (figure 4.1A). DNA sequence analysis of patients with different patterns than normal revealed that two AML patients (number 5026 and 5308) each had three changes; a G→T substitution at position 1033 which predicts for an amino acid change at codon 245 (Ala245Ser); a C→T intronic change at position 1189+28 and the previously identified intronic polymorphism TT→AG at position 1189+67-68 (see section 3.2.1.4), (figure 4.2B). None of the 40 IMF and the 70 normal individuals analysed by CSGE had the G→T substitution (Ala245Ser) at position 1033.



1 2 3 4 5

Figure 4.1A CSGE analysis of exon 6 PCR fragment demonstrating a change in the *c-fms* gene. Lanes 1-3 AML patients with an intronic change TT→AG (see section 3.2.1.4). Lane 4 shows an AML patient with a normal CSGE profile, lane 5 shows an AML patient 5026 with an abnormal CSGE profile due to a G→T (Ala245Ser) substitution at position 1033.

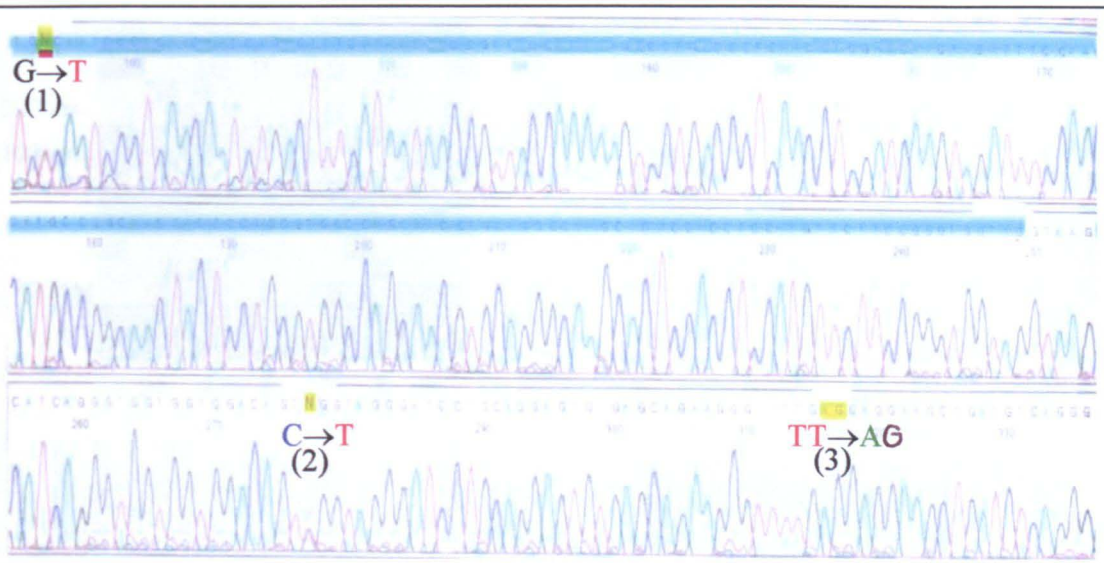


Figure 4.1B Automated sequence analysis of exon 6 showing (1) nucleotide substitution G→T at position 1033 which predicts for the Ala245Ser amino acid change, (2) C→T novel nucleotide substitution at position 1189+28 in the intronic part of the PCR fragment, (3) the previously identified nucleotide substitution TT→AG at position 1189+67-68. The blue highlighted sequence indicates the exonic part of the fragment. Yellow highlighted sequence indicates the position of nucleotide substitution for each change.

4.2.1.1 Exon 6 restriction enzyme analysis

The genomic sequence of the amplified exon 6 fragment was analysed for the consequence of the identified alteration at any restriction enzyme sites using Webcutter 2.0 programme provided via the Internet by Yale University (http://www.firstmarket.com/cutter/cut2.html). The *Cac8I* restriction enzyme was found to cut four times in the wild type sequence and three times in the case of a G→T change at position 1033 (figure 4.1C) (see table 4.2). Only two AML patients have this change, none of the 40 IMF and the 70 normal individuals analysed by restriction enzyme had the G→T substitution (Ala245Ser) at position 1033.

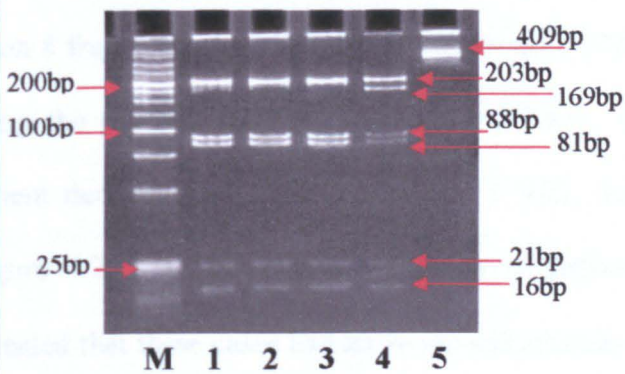


Figure 4.1C Cac8I digest of exon 6 PCR fragment (size 409bp). Lane M shows a marker, lanes 1, 2 and 3 show AML patients with wild type alleles, lane 4 show a heterozygote for the G→T change at position 1033, lane 5 show undigested PCR product.

4.2.1.2 Codon 245 cross-species comparison

Cross-species comparison of amino acid sequence of the *c-fms* and *c-kit* gene was performed around codon 245 located in the third Ig-like domain. However, the result of the analysis shows that this codon is not highly conserved (figure 4.1D).

c-fms	**Human**	HNNT----KLAIPQQ
		245
c-FMS	**Rat**	RGDT----KLEIPLN
		245
c-kit	**Human**	RENS--QTKLQEKYN
		256
c-KIT	**Rat**	KMNPQPQHIAQVKHN
		259

Figure 4.1D BLAST alignment showing cross-species comparison of amino acid sequence of c-fms and c-kit in human and rat showing an alignment of the amino acid Ala245. Red letters represent the amino acid which is mutated in human c-fms, blue letters represent the homologous amino acid in the different proteins.

4.2.2 Change in Exon 8

Exon 8 fragment was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. CSGE analysis of PCR product of each patient demonstrated that 13 patients (5 AML and 8 IMF) had abnormal CSGE patterns (figure 4.2A). DNA sequence analysis of patients with a different pattern than normal revealed that these cases had an A→G substitution at position 1385 which predicts an amino acid change at codon 362 (His362Arg) (figure 4.2B).

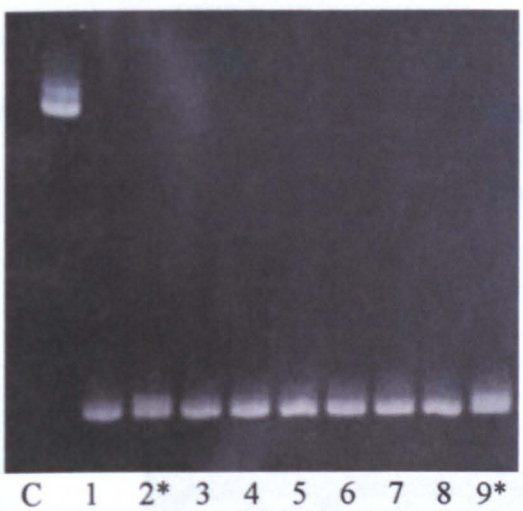


Figure 4.2A CSGE analysis of exon 8 PCR fragment demonstrating a change in the *c-fms* gene. Lane C shows a positive control. Lanes 1 and 3-8 show IMF with a normal pattern. Lanes 2* and 9* show two IMF patients with abnormal CSGE profiles.

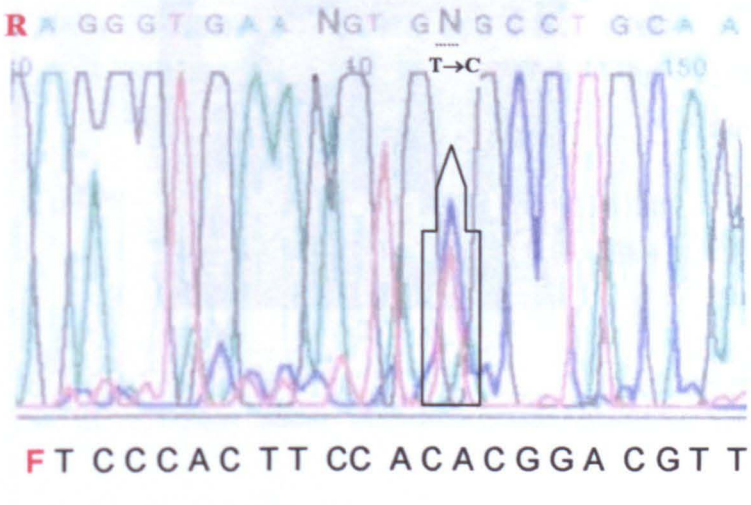


Figure 4.2B Automated sequence analysis of exon 8 using the reverse primer showing the nucleotide substitution T→C (A→G in forward sequence).
F= forward sequence
R= reverse sequence

Seventy normal individuals were screened for the change using CSGE, six were identified (8.5%) with A→G substitution at position 1385. Therefore, this change was considered to be polymorphic.

4.2.2.1 Exon 8 restriction enzyme analysis

The genomic sequence of the amplified exon 8 fragment was analysed for the consequence of the identified alteration at restriction enzyme sites using Webcutter 2.0 programme provided via the Internet by Yale University (www.firstmarket.com/cutter/cut2.html). The *Hin6I* restriction enzyme was found to cut once in the wild type sequence and twice in the case of an A→G change at position 1385 (figure 4.2C) (see table 4.2). In total five AML patients and 8 IMF patients analysed using restriction enzyme digestion had the A→G change at position 1385. Seventy normal individuals were also analysed using restriction enzyme, six individuals (8.5%) were heterozygous for the A→G change at position 1385.

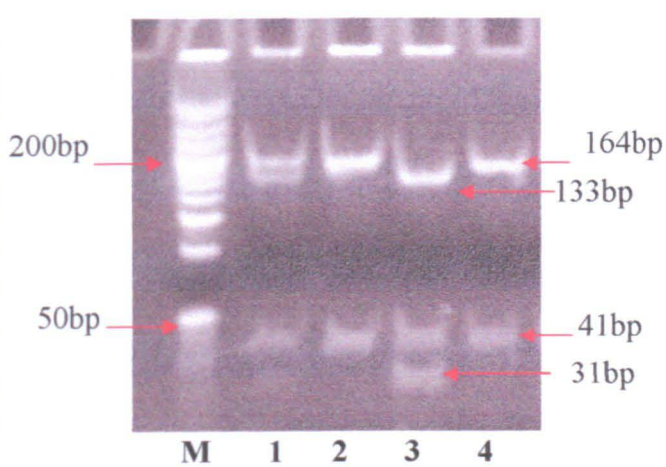


Figure 4.2C *Hin6I* digest of exon 8 PCR fragment (size 205bp). Lane M shows a marker, lane 1 show a heterozygote for the A→G change at position 1385, lanes 2 and 4 show IMF patients with wild type alleles, lane 3 shows an IMF patient homozygous for the A→G change at position 1385.

4.2.2.2 Codon 362 cross-species comparison

Furthermore, cross-species comparison of amino acid sequence of the c-fms and c-kit gene was performed around codon 362 which is located in the fourth Ig-like domain. The result of the analysis shows that this amino acid is highly conserved across the other class III RTKs except in the human c-fms protein (figure 4.2D).

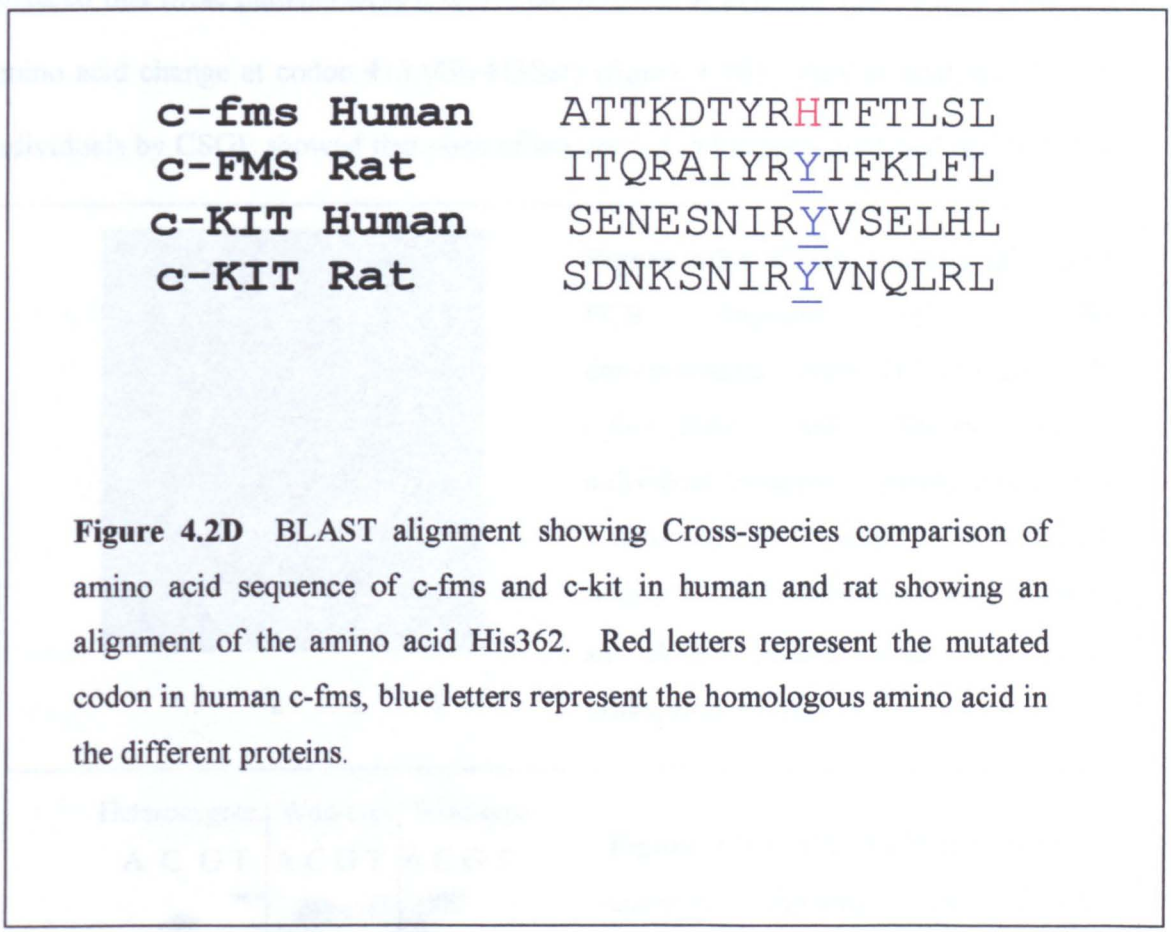


Figure 4.2D BLAST alignment showing Cross-species comparison of amino acid sequence of c-fms and c-kit in human and rat showing an alignment of the amino acid His362. Red letters represent the mutated codon in human c-fms, blue letters represent the homologous amino acid in the different proteins.

4.2.3 Change in Exon 9

Exon 9 was amplified from genomic DNA of 100 patients (60 AML and 40 IMF) using the primers described in section 2.3.4.1. Initial CSGE analysis of the PCR product of each patient demonstrated that three patients (1 AML and 2 IMF) had abnormal CSGE patterns (figure 4.3A). DNA sequence analysis of patients with different patterns than normal revealed that these patients have a G→A substitution at position 1537 which predicts for an amino acid change at codon 413 (Gly413Ser) (figure 4.3B). Further analysis of 70 normal individuals by CSGE showed that none of the normal individuals analysed had this change.

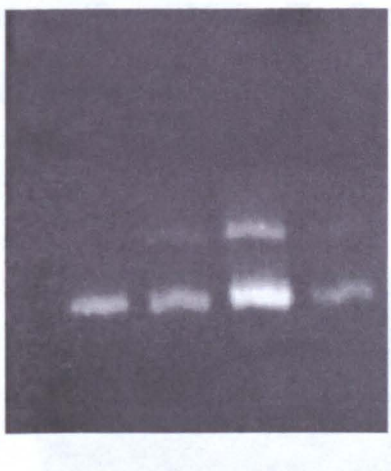


Figure 4.3A CSGE analysis of exon 9 PCR fragment (size 271bp) demonstrating codon 413 change in the *c-fms gene*. Lane 1 shows a normal individual (negative control), lanes 2 and 3 show two IMF patients (54 and 02D) with a G→A substitution, lane 4 shows an AML patient with the G→A substitution (5104).

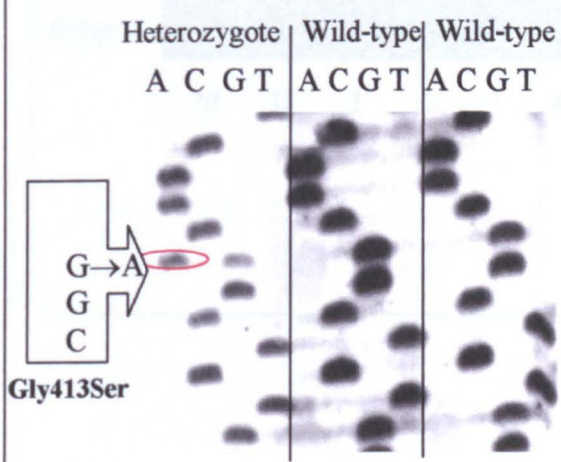


Figure 4.3B Direct DNA sequence analysis showing the G→A nucleotide substitution which predict for an Gly413Ser amino acid change in one AML patient (5104), the wild type sequence in another AML patient and a normal individual.

4.2.3.1 Exon 9 restriction enzyme analysis

Analysis of the sequences involved in the nucleotide alterations affecting codon 413 demonstrated that the G→A substitution at position 1537 would create a restriction enzyme site for the *AluI* enzyme in the 271bp fragment and produce two fragments for the abnormal allele (151 and 120 bp). Therefore, in order to screen the same 100 patients samples and the 70 normal individuals for a possible heterozygous or homozygous G→A substitution at position 1537, the 271bp fragment was digested with *AluI* enzyme (see chapter 2). Restriction enzyme analysis confirmed the presence of the G→A substitution in the same three patients (figure 4.3C). The G→A substitution at position 1537 was not identified in any other patients or in any of the 70 normal individuals.

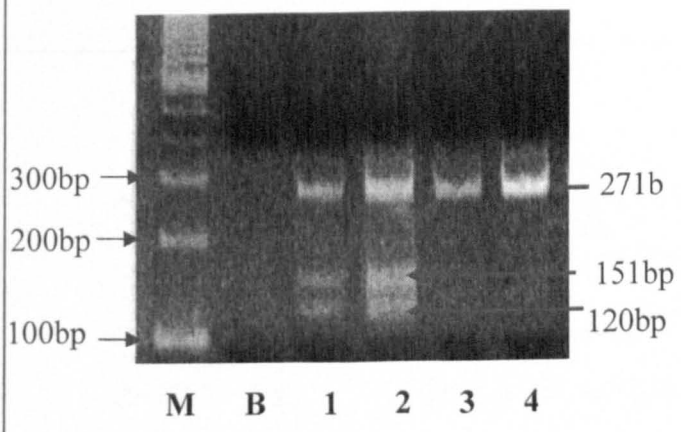


Figure 4.3C *AluI* digest of exon 9 PCR fragment. Lane M shows a 100bp marker, lane B shows negative control, lanes 1 and 2 show two IMF patients positive for the G→A substitution, lanes 4 and 5 show two normal individuals without the G→A substitution at codon 413.

In order to confirm the percentage of the G→A substitution at position 1537, forty three more AML patients (group B) were screened from different FAB groups. Another AML patient was identified to have codon 413 mutation among the 43 new AML (number 5341). Similarly, the number of normal individual screened was also increased but none of the extra 130 normal individuals screened by CSGE has this change (total number of normal individuals screened was 200).

4.2.3.2 Codon 413 cross-species comparison

Cross-species comparison of the amino acid sequence of the c-fms and c-kit proteins was performed around codon 413, located in the fifth Ig-like domain, and the result of the analysis highlighted that this amino acid is highly conserved, furthermore, it also shows the short distance between codon 413 in c-fms and the previously identified mutation at codon 419 in exon 8 of the *c-kit* gene (Gari *et al*, 1999) (figure 4.3D).

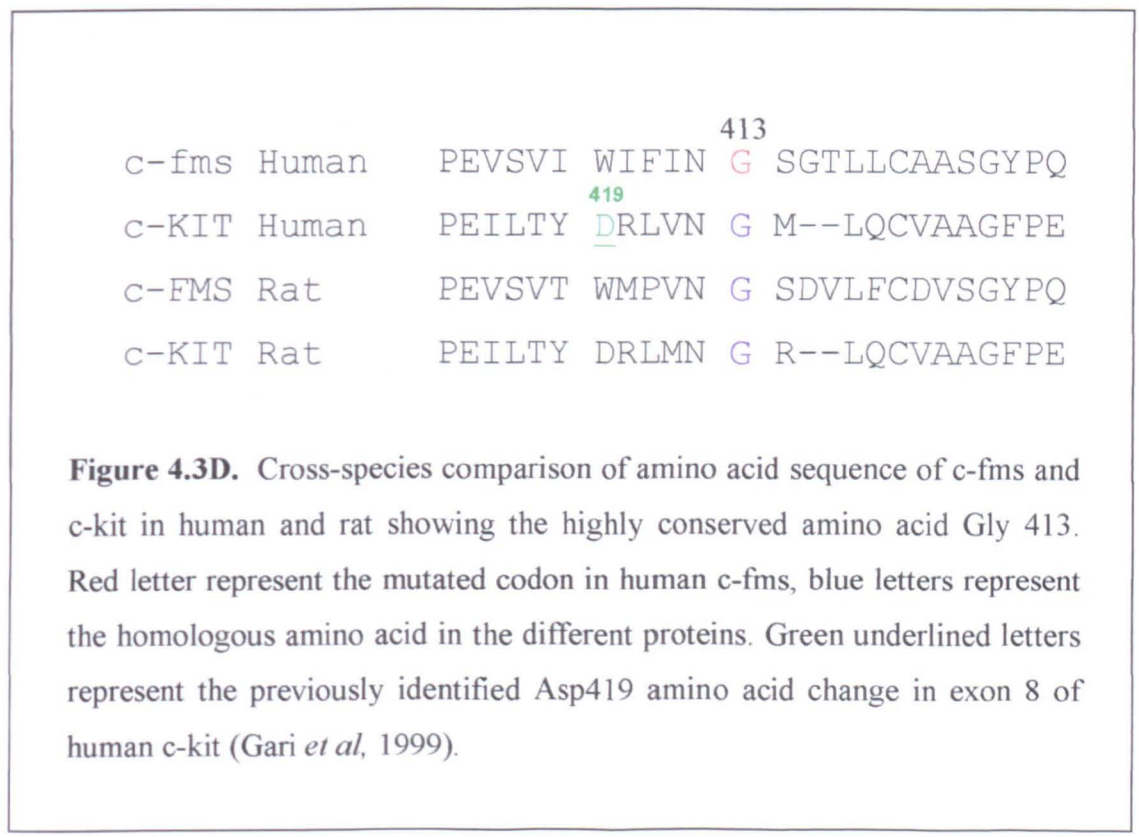


Figure 4.3D. Cross-species comparison of amino acid sequence of c-fms and c-kit in human and rat showing the highly conserved amino acid Gly 413. Red letter represent the mutated codon in human c-fms, blue letters represent the homologous amino acid in the different proteins. Green underlined letters represent the previously identified Asp419 amino acid change in exon 8 of human c-kit (Gari *et al*, 1999).

4.2.3.3 *Analysis of germ line cells*

In order to clarify whether the codon 413 mutation is an acquired or inherited mutation, remission samples were necessary for patients with the *c-fms* mutation. However, this was not achievable due to the fact that patients were deceased or did not achieve a remission state. Therefore, DNA from normal somatic cells was the second option to carry out the analysis. A buccal swab and a saliva sample were requested from patient 02D. DNA was extracted from both samples using the same technique in section 2.3.1 (after centrifugation of each swab to collect the cells) followed by PCR and CSGE analysis. However, due to the fact that the patient had IMF and had bleeding gums, contamination with blood was not prevented. Therefore, a definite result was not achieved concerning the presence or absence of exon 9 mutation at codon 413 of the *c-fms* gene in the somatic cells of this patient. The result of the CSGE analysis showed that the exon 9 mutation is present in both samples (buccal swab and saliva sample) (figure 4.3E).

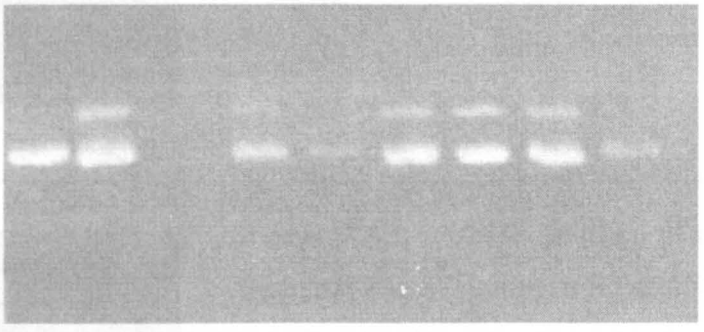
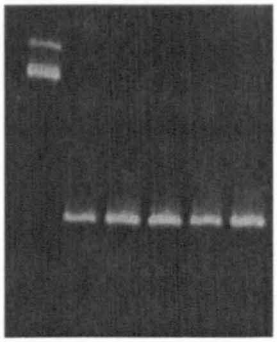


Figure 4.3E CSGE analysis of exon 9 PCR fragment demonstrating codon 413 change in the *c-fms* gene. Lane N shows a normal individual (negative control), lane +C positive control (sample with exon 9 mutation), lane B a blank, lane 1 patient 02D sample (buccal swab), lane 2 normal individual (buccal swab) lanes 3, 4 and 5 show two IMF patients (54 and 02D) and an AML patient (5341) with the G→A substitution, lane 6 shows patient 02D sample (saliva).

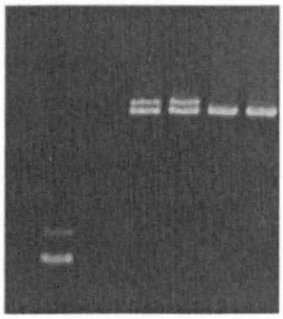
4.2.4 Analysis of the previously identified mutations affecting codons 301 and 969

Two earlier studies reported the presence of two different mutations in the *c-fms* gene namely codon 301 and 969 (in exon7 and exon 22 respectively). In this study, none of the 143 patients (40 IMF and 103 AML) analysed using CSGE had the 301 change, due to the absence of bandshifts in exon 7 (figure 4.4A). Similarly, none of the 160 patients (40 IMF and 110 AML) analysed using CSGE had the 969 change after sequencing patients with bandshifts in exon 22 fragment. However, a TC→CA substitution was identified at 3254-3255 in the 3` non coding region of the *c-fms* gene in 31 patients (14 IMF and 17 AML) as shown in figure 4.4B (see chapter 3 section 3.2.1.8 for more details).



C 1 2 3 4 5

Figure 4.4A CSGE analysis of exon 7 PCR fragment demonstrating the negative result for this fragment. Lane C shows a positive control, lanes 1-5 show normal CSGE patterns in 5 IMF patients



C B 1 2 3 4

Figure 4.4B CSGE analysis of exon 22 PCR fragment demonstrating a bandshift due only to an intronic change. Lane C shows a positive control. Lane B show a blank. Lanes 1-2 show abnormal CSGE profiles in two AML patients. Lanes 4 and 5 show normal CSGE patterns. The alteration in patients was shown to result from TC→CA substitution at 3254-3255 in the 3` non coding region of the *c-fms* gene.

Although the result of the analysis was satisfactory due to the sensitivity and the use of gel controls in each run of CSGE a further step was preferred to confirm this result. Therefore, a positive control for both of the 301 and the 969 mutation was sought to confirm the sensitivity of the CSGE technique. However, a positive control for the codon 301 mutation was not obtainable. A supposed positive control for the 969 mutation was received from a research group in the United Kingdom. Unfortunately, no mutation at codon 969 was detected after screening this samples using CSGE. Therefore, this control was sequenced using automated sequencing to clarify the presence or absence of the 969 mutation in this sample. However, the result of the sequencing confirmed our initial finding and shows that this control sample does not contain any change at codon 969 of the *c-fms* gene (figure 4.4D).

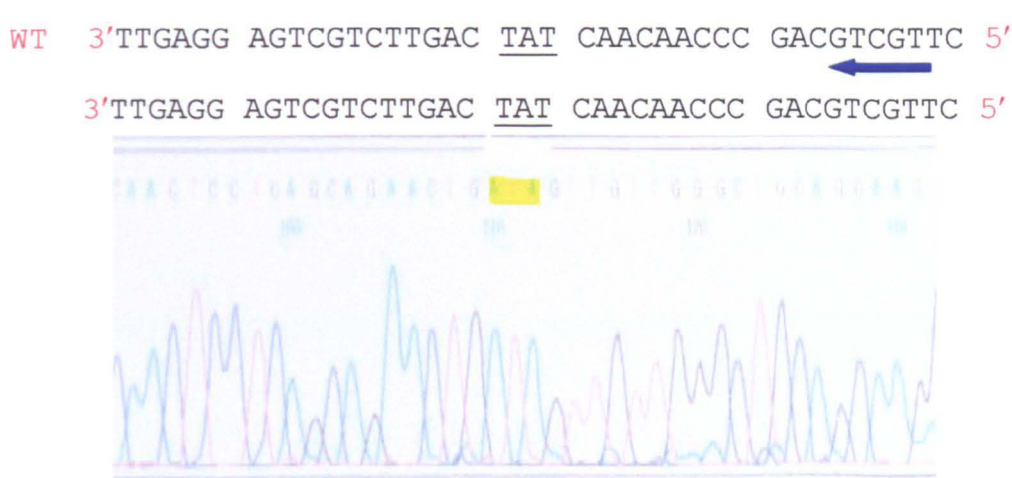


Figure 4.4D Automated sequence analysis of exon 22 showing the nucleotide sequence using the reverse primer for the "positive control" for codon 969 mutation. Bold letters represent the forward sequence around codon 969 in the *c-fms* gene. WT = wild-type sequence.

4.2.5 Novel mutations of the c-fms gene

Mutations identified by this study within the *c-fms* gene are summarized in table 4.1. Table 4.2 shows the analysis of restriction endonuclease sites for novel identified mutations within the *c-fms* gene.

Table 4.1 Spectrum and percentage of amino acid alterations identified within the *c-fms* gene.

Exon/Intron No.	Nucleotide change	Amino acid change	AML patients n=60	IMF patients n=40	Normal controls n=70
Exon 6	1033 G>T	Ala 245 Ser	2 (3.3%)	0	0
Exon 8	1385 A>G	His 362 Arg	5 (8.3%)	8 (20%)	6 (8.5%)
Exon 9	1537 G>A	Gly 413 Ser	2* (1.9%)	2 (5%)	0

* Note: only one AML patient (out of the 60) had the Gly413Ser change in exon 9. The second AML patient was identified in the second patients group (group B) screened (see section 4.2.3.1)

Table 4.2 Restriction endonuclease site analysis within the *c-fms* gene

Exon/ Intron No.	Fragment size/bp	Nucleotide change	Enzyme	Product size/bp	
				Wild type allele	Abnormal allele
Exon 6	409	1033 G>T	Cac8I	16, 21, 81, 88, 203	16, 21, 169, 203
Exon 8	205	1385 A>G	Hin6I	41, 164	31, 41, 133
Exon 9	271	1537 G>A	AluI	271	120, 151

4.3 Discussion

c-fms or CSF-1R is the receptor for CSF1, a homodimeric glycoprotein produced primarily by messenchymal cells that stimulates the proliferation and enhances the viability of monocytes, macrophages, and their committed bone marrow progenitors (Stanley et al, 1983). As stated earlier, c-fms is one of the class III RTK that is characterised by five repeated Ig-like domains in the extracellular part as well as the presence of a particular kinase insert domain between two tyrosine kinase domains (Coussens et al, 1986).

A number of previously identified and novel polymorphisms that were identified in the c-fms receptor in AML and IMF patients were discussed in chapter 3. This chapter however, demonstrates that a number of nucleotide changes were identified that predicted amino acid changes in the *c-fms* gene. In total, three novel nucleotide changes were identified in exons 6, 8 and 9. In exon 6, a G→T substitution that predicts an amino acid change at codon 245 (Ala245Ser) was identified in two AML patients. The change is located in the extracellular part of the c-fms receptor. None of the IMF or the normal individuals screened had this change. The result of the cross-species comparison of the Ala245 aa shows that this aa is not conserved. In addition, the Ala245Ser change is unlikely to modify the structure of the protein since Ala and Ser have similar degree of hydrophobicity. Exon 8 showed an A→G substitution in 13 patients (5 AML and 8 IMF) at position 1385, which predicts an amino acid change at codon 362 (His362Arg) and was also located in the extracellular part of the receptor. However, normal individuals were also identified with the same change in exon 8 of the *c-fms* gene. It was noticed that there is significant increase of the Arg362 in IMF patients compared to AML and normal individuals. Despite that the aa are positively charged, they have different degree of hydrophobicity. In addition, this aa change (His362Arg) could also affect the three dimensional structure of the protein. Therefore, these observation suggesting that the Arg could be involved in the pathogenesis of IMF.

In exon 9 a novel G→A substitution at codon 413 (Gly413Ser), was identified in four patients (2 AML and 2 IMF) out of the 143 patients (103 AML and 40 IMF) screened for exon 9 (2.8%). None of the 200 normal individual screened had this change.

It is noteworthy to mention that all these changes were identified in the extracellular part of the c-fms receptor. The exon 9 (Gly413Ser) mutation for instance, is located in the fifth immunoglobulin-like domain in the extracellular part of *c-fms*. This part of the class III RTKs have been suggested to be important as a negative regulatory constraint and its removal (i.e. by deletion of the receptor) can lead to ligand-independent activation (Khazaie *et al,* 1988; Uren *et al,* 1997). Furthermore, gene transfer experiment using an experimentally mutated version of *c-fms* showed that an extracellular mutation at codon 301 resulted in the activation of the tyrosine kinase activity and was sufficient to transform mouse 3T3 cells *in vitro* (Roussel *et al,* 1988). Although the codon 301 activating mutation, located in the fourth Ig- like domain, does not affect the CSF-1 binding site in the receptor's extracellular domain, it must induce a conformational change that mimics the effect of ligand binding, resulting in c-fms independent signals for cell growth (Roussel *et al,* 1988). Furthermore, novel activating mutations were previously identified, using random chemical mutagenesis, within sequence separating the third and fourth immunoglobulin-like domains, as well as within non-covalently stabilized loop 4 of the c-fms extracellular domain (van Daalen Wetters *et al,* 1992). Therefore, it was suggested that mutations able to activate the c-fms receptor are not restricted to the previously identified mutation at codon 301 of the *c-fms* gene (van Daalen Wetters *et al,* 1992).

In this study an exon 9 mutation located at codon 413 was identified and was found to be within a highly conserved codon of class III RTKs and is only a short distance (5 amino acid) from the previously identified mutations at codon 419 in the c-kit receptor (Gari *et al,* 1999). The result of this study is not sufficient by itself to speculate the exact effect of this extracellular mutations of the *c-fms* gene especially with the small number of AML FAB

groups analysed in this study. Therefore, a larger group of patients should be analysed in order to clarify the occurrence of this mutation in different FAB groups of AML.

This study showed that previously identified mutations at codons 301 and 969 are not present in this group of patients. Although the number of AML patients analysed was increased (group B), none of the patients analysed had the 301 or the 969 mutation. Indeed one could argue that this might be due to the use of an insensitive technique and this was a false negative result. The sensitivity of the CSGE technique has been previously established by several studies (see chapter 3) and was also confirmed with the use of positive control (sample with known change) in each CSGE gel. This study was not the first study to report the negative finding concerning the 301 and 969 mutations in *c-fms*. Shepherd et al (1990) failed to find any mutation at codon 301 or 969 in either AML or MDS patients using direct sequencing. This findings were confirmed by another group using an allele specific restriction analysis (ASRA) protocol for the detection of these mutations (Springall *et al,* 1993). Similarly, in a study of 42 cases of AML and MDS, only one patient was identified with the 969 mutation (Jaquet *et al,* 1993). However, a normal individual has been previously reported with the 969 mutation in *c-fms* (Hirai *et al,* 1987). Recently, 70 patients with MDS were screened for *c-fms* mutations using SSCP and none of the patients analysed has a 301 or 969 mutation (Misawa *et al,* 1997). Therefore, it was recently suggested that positive results presented by some of the previous studies could be due to the use of a relatively insensitive allele specific oligonucleotide (ASO) hybridization technique (Reilly, 2002). It seems that the previously identified mutations at codon 301 and 969 are not the dominant mutations in c-fms disorders.

Chapter 5

FLT3 GENOMIC STRUCTURAL ORGANIZATION 103

FLT3 Genomic Structural Organization

5.1 Introduction

The determination of the complete sequence of genes is a significant step towards understanding the importance of these genes (Sterky and Lundeberg, 2000). In 1990 the human genome project (HGP) was launched to map and sequence the entire human genome (Sterky and Lundeberg, 2000). Automated DNA sequencing was an important breakthrough towards large-scale sequencing. Due to the large size and complexity of genomes, all genomes are sub-cloned in small pieces to suit DNA sequencing techniques (Sterky and Lundeberg, 2000).

Determination of the function of a newly sequenced gene can be achieved by experiment, although only a few genes have been studied using this approach (Andrade and Sander, 1997). Instead, earlier characterised proteins are frequently used to hypothesise a potential function of unknown protein, since proteins with similar sequence tend to posses similar functions (Sterky and Lundeberg, 2000).

5.1.1 Class III RTKs

Rosnet et al, (1991) isolated FLT3, a novel member of class III RTK. The FLT3 gene was assigned to human chromosome 13q12 and to mouse chromosome 5 (Rosnet et al, 1991b). Early data suggested that the genomic loci encoding the FLT3, c-kit and c-fms receptors share overall conservation of exon size, number, sequence and exon/intron boundary positions (Agnes et al, 1994). In addition, class III RTKs were found to occur in pairs on different chromosomes: in man, *c-kit* and *PDGFRα* gene are located as a pair on chromosome 4 (Matsui et al, 1989) and chromosome 5 in mouse (Rosnet et al, 1991a), while *c-fms* and *PDGFRβ* genes are located in tandem on human chromosome 5 (Roberts et al, 1988) and mouse chromosome 18 (Buchberg et al, 1989). Initial findings suggested that

FLT3 was the only RTK class III gene located on band q12 of chromosome 13 (Rosnet et al, 1991b). However, FLT1, a class V RTK (characterised by seven immunoglobulin-like domains), was later found to lie within the same 600-kb fragment as the *FLT3* gene on human chromosome band 13q12 and within the same 350-kb fragment on murine chromosome 5 (Rosnet et al, 1993b). These findings suggest that the RTK class III genes have arisen evolutionary as a result of a cis and trans duplication events (Andre et al, 1992; Rosnet et al, 1993b; Agnes et al, 1994; Abu-Duhier et al, 2001a).

Interest in FLT3 has been heightened by recent reports linking exonic mutations to the pathogenesis of acute myeloid leukaemia (Nakao et al, 1996; Yokota and Kiyoi, 1998; Xu et al, 1999; Abu-Duhier et al, 2000; Abu-Duhier et al, 2001b; Kottaridis et al, 2001).

5.1.2 FLT3 structure

Initial studies in 1996 of human FLT3 mRNA revealed the presence of an internal tandem duplication (ITD) mutation in a group of patients with AML (Nakao *et al,* 1996). Further studies revealed that the ITD mutations were all in-frame and were located in the sequence coding for the juxtamembrane/ tyrosine kinase 1 (JM/TK1) domains of the *FLT3* gene (Nakao *et al,* 1996; Yokota *et al,* 1997). Subsequent analyses of the PCR products from such patients demonstrated that the length of the ITD mutation varies in size (17-198 bp), with the starting and ending sites being distinct for each patient. The ITD mutations were then reported to occur in a high proportion of AML cases (20%), regardless of their FAB classification (Nakao *et al,* 1996; Rombouts *et al,* 2000). In addition, the ITD mutations were also found in a few cases of myelodysplastic syndromes (3%) and in rare cases of ALL, most of which express myeloid antigen, but not in patients with CML ,or in normal haematopoietic tissue (Yokota *et al,* 1997; Ishii *et al,* 1999; Xu *et al,* 1999).

A systematic screening of the entire human *FLT3* gene coding region will be required to determine whether additional mutations are important in the pathogenesis of AML. This

approach has been hindered, however, by the lack of knowledge of the complete genomic structure of *FLT3*. Ideally, CSGE analysis of the coding sequence of *FLT3* gene requires the PCR amplification of DNA fragments that covers at least 50bp of the intronic sequence on each side of the screened exon as the location of the mismatch within the PCR product is very important. The amplification of the 50bp intronic fragments was carried out to exclude missing any mismatch within the amplified exon. Prior to the commencement of this study, the necessary sequence information was not available to enable further mutational analysis of the *FLT3* gene.

The FLT3 protein, like all other class III RTKs, is characterized by three distinct regions. The extracellular region (541aa) is composed of five immunoglobulin like domains, a transmembrane region (21aa) contains a single domain and the cytoplasmic region (431 aa) which has a tyrosine kinase made up of an ATP-binding loop and a catalytic domain separated by a kinase insert domain (Wolf and Rohrschneider, 1999).

The organisation of the downstream part (dsp), or intracytoplasmic coding sequence; of *FLT3* was reported by Agnès et al (1993). Agnès and colleagues compared the nucleotide sequence of genomic subclones of the dsp of the human *FLT3* gene (ds-*FLT3*) with human FLT3 cDNA. The eleven exons were arbitrarily numbered according to the analogous exons of the *c-kit* gene (exons 10-21). However, the structure of the region coding for the extracellular and transmembrane domains remained unclear. Due to the chromosomal location and genomic structure of class III RTKs, a close evolutionary relationship has been suggested for this type of receptor (Andre *et al*, 1992; Rosnet *et al*, 1993b; Reilly, 2002). RTKs class III exhibit close homology, especially in the intracellular (IC) catalytic domain, in that the IC domains share overall conservation of exon size, number, sequence and exon/intron boundary sites.

5.1.3 Regulation of FLT3 transcription

It is well known that regulation of gene transcription is central both to tissue specific-gene expression and to the regulation of gene activity in response to specific stimuli (Latchman, 1997). Although transcription is performed by RNA polymerase, the enzyme needs other proteins to produce the transcript. These factors are either associated directly with RNA polymerase or aid in building the actual transcription apparatus. The general term for these associated proteins is transcription factors. Furthermore, transcription is controlled by a set of functional DNA sites realising specific functions through the interaction with the relevant proteins (Latchman, 1993). Binding to specific DNA sites is one of the essential steps for transcription factors to influence transcription either positively or negatively (Latchman, 1997).

Transcription factor binding sites are relatively short stretches of DNA, sufficiently conserved in sequence to allow specific recognition by the corresponding transcription factor. Nowadays, experimental data on DNA sequences and the functions of thousands of transcription factor binding sites are accumulated within various databases. Two of the best-known databases in this intensively developed area are EMBL Data Library and TRANSFAC. All of them provide initial sources of information for developing methods for transcription factor binding site recognition.

In this study, preliminary work was carried out to identify the possible transcription factor binding sites in the 5` region of the *FLT3* gene. This step is necessary to predict a likely effect of transcription factors on these transcription factor binding sites. Furthermore, exon size and intron/exon boundaries were identified for all exons of the *FLT3* gene by comparing the FLT3 cDNA sequence with the genomic sequence available in the NCBI database. This step was necessary to assist the screening of functionally important domains of the *FLT3* gene for mutations. In addition, amino acid homology was also analysed between the

predicted FLT3, c-kit and c-fms predicted amino acid sequence. These analyses demonstrated that the equivalent of exon 2 and exon 3 of *c-kit* are actually spliced into five separate exons in *FLT3*.

An amino acid comparison was carried out for the FLT3, c-fms and c-kit proteins. All sequences were aligned (using BLAST program: a set of similarity search programs designed to explore all of the available sequence databases (or the chosen sequence)) to allow for comparison (http://www.ncbi.nlm.nih.gov/blast).

5.2 Results

5.2.1 Amino acid sequence homology

Due to the close similarity between the FLT3, c-fms and c-kit proteins, an alignment between the predicted amino acid sequence of these 3 receptors was carried out to be able to identify their degree of homology.

```
FLT3      1 ----MPALARDGGQLPLLVVFSAMIFGTITNQDLPVIKCVLINHKNNDSSVGKSSSYPMV
c-fms     1 --------MGPGVLLLLL-VATAWHG-----QGIPVI----------------EPSVP--
c-kit     1 MRGARGAWDFLCVLLLLLRVQTGSSQ-----PSVSPG----------------EPSPPSI

FLT3     57 SESPEDLGCALRPQSSGTVYEAAAVEVDVSASITLQVLVDAPGNISCLWVFKHSSLNCQP
c-fms    29 -----ELVVKPGATVTLRCVGNGSVEWDGPASPHWTLYSDGSSSILSTNNATF-------
c-kit    40 HPGKSDLIVRVGDEIRLLCTDPGFVKWT-FEILDETNENKQNEWITEKAEAT--------

FLT3    117 HFDLQNRGVVSMVILKMTETQAGEYLLFIQSEATNYTILFTVSIRNTLLYTLRRPYFRKM
c-fms    77 ----QNTG-------TYRCTEPGDPLGGSA------AIHLYVKDPARPWNVLAQ-EVVVF
c-kit    91 -----NTG-------KYTCTN---KHGLSN------SIYVFVRDPAKLFLVDR--SLYGK

FLT3    177 ENQDALVCISESVPEPIVEWVLCDSQGE-----SCKEESPAVVKKEEKVLHELFGTDIRC
c-fms   119 EDQDALLPCLLTDPVLEAGVSLVRVRGRPLMRHTNYSFSPWHGFTIHRAKFIQS-QDYQC
c-kit   128 EDNDTLVRCPLTDPEVTN-YSLKGCQGKPLPKDLRFIPDPKAGIMIKSVKRAYHRLCLHC

FLT3    232 CARNELG---RECTRLFTIDLN-QTPQTTLPQLFLK--------VGEPLWIRCKAVHVNH
c-fms   178 SALMGGRKVMSISIRLKVQKVIPGPPALTLVPAELVRIR------GEAAQIVCSASSVDV
c-kit   187 SVDQEGKSVLSEKFILKVR------PAFKAVPVVSVSKASYLLREGEEFTVTCTIKDVSS

FLT3    280 GFGLTWELEN---KALEEGNYFEMSTYSTNRTMIRILFAFVSSVARNDTGYYTCSSSKHP
c-fms   232 NFDVFLQHNN--TKLAIPQQSDFH-----NNRYQKVLTLNLDQVDFQHAGNYSCVASNVQ
c-kit   241 SVYSTWKRENSQTKLQEKYNSWHHGDF--NYERQATLTISSARVNDS--GVFMCYANNTF

FLT3    337 ---SQSALVTIVGKGFINATNSS-EDYEIDQYEEFCFSVRFKAYP------QIRCTWTFS
c-fms   285 GKHSTSMFFRVVESAYLNLSSEQNLIQEVTVGEGLNLKVMVEAYPGLQGFNWTYLG-PFS
c-kit   297 GSANVTTTLEVVDKGFINIFPMINTTVFVNDGENVDLIVEYEAFPKPEHQQWIYMNRTFT

FLT3    387 RKSFPCEQKGLDNGYS---------ISKFCNHKHQPGEYIFHAENDDAQFTKMFTLNIRR
c-fms   344 DH-QP-EPK-LANATTKDTYRHTFTLSLPRLKPSEAGRYSFLARNPGGWRALTFELTLRY
c-kit   357 DK-WEDYPK-SENES--N-IRYVSELHLTRLKGTEGGTYTFLVSNSDVNAAIAFNVYVNT

FLT3    438 KPQVLAEASASQAS----CFSDGYPLPSWTWKKCSDKSPNCTEEITEGVWNRKANR----
c-fms   401 PPEVSVIWTFINGSGTLLCAASGYPQPNVTWLQCSGHTDRCDEAQVLQVWDDPYPEVLSQ
c-kit   412 KPEILTYDRLVNG--MLQCVAAGFPEPTIDWYFCPGTEQRCS-ASVLPV--DVQTLNSSG

FLT3    490 KVFGQWVSSSTLNMSEAIKGFLVKCCAYNSLG-TSCETILLNSPGPFPFIQDNISFYATI
c-fms   461 EPFHKVTVQSLLTVETLEHNQTYECRAHNSVGSGSWAFIPISAG---AHTHPPDEFLFTP
c-kit   467 PPFGKLVVQSSIDSSAFKHNGTVECKAYNDVGKTSAYFNFAFKGNNKEQIHP--HTLFTP
```

```
FLT3    549 GVCLLFIVVLT----------LLICHKYKKQFRYESQLQMVQVTGSSDNEYFYVDFREYE
c-fms   518 -----VVVACMSIMALLLLLLLLLYKYKQKPKYQVRWKIIESYEG--NSYTFIDPTQLP
c-kit   525 LLIGFVIVAGM-----MCIIVMILTYKYLQKPMYEVQWKVVEEING--NNYVYIDPTQLP

FLT3    599 YDLKWEFPRENLEFGKVLGSGAFGKVMNATAYGISKTGVSIQVAVKMLKEKADSSEREAL
c-fms   571 YNEKWEFPRNNLQFGKTLGAGAFGKVVEATAFGLGKEDAVLKVAVKMLKSTAHADEKEAL
c-kit   578 YDHKWEFPRNRLSFGKTLGAGAFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREAL

FLT3    659 MSELKMMTQLGSHENIVNLLGACTLSGPIYLIFEYCCYGDLLNYLRSKRE---KFHRTWT
c-fms   631 MSELKIMSHLGQHENIVNLLGACTHGGPVLVITEYCCYGDLLNFLRRKAEAMLGPSLSPG
c-kit   638 MSELKVLSYLGNHMNIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRD---SFICSKQ

FLT3    716 EIFKEHNFSFYPTFQSHPNSSMPG----------SREVQIHPD---SDQISGLHGNSFHS
c-fms   691 QDPEGGVDYKNIHLEKKYVRRDSG--------FSSQGVDTYVEMR-PVSTS-----SNDS
c-kit   695 EDHAEAALYKNLLHSKESSCSDSTNEYMDMKPGVSYVVPTKADKRRSVRIG-----SYIE

FLT3    763 EDEIEYENQKRLEEEEDLNVLTFEDLLCFAYQVAKGMEFLEFKSCVHRDLAARNVLVTHG
c-fms   737 FSEQD------LDKEDG-RPLELRDLLHFSSQVAQGMAFLASKNCIHRDVAARNVLLTNG
c-kit   750 RDVTP------AIMEDDELALDLEDLLSFSYQVAKGMAFLASKNCIHRDLAARNILLTHG

FLT3    823 KVVKICDFGLARDIMSDSNYVVRGNARLPVKWMAPESLFEGIYTIKSDVWSYGILLWEIF
c-fms   790 HVAKIGDFGLARDIMNDSNYIVKGNARLPVKWMAPESIFDCVYTVQSDVWSYGILLWEIF
c-kit   804 RITKICDFGLARDIKNDSNYVVKGNARLPVKWMAPESIFNCVYTFESDVWSYGIFLWELF

FLT3    883 SLGVNPYPGIPVDANFYKLIQNGFKMDQPFYATEEIYIIMQSCWAFDSRKRPSFPNLTSF
c-fms   850 SLGLNPYPGILVNSKFYKLVKDGYQMAQPAFAPKNIYSIMQACWALEPTHRPTFQQICSF
c-kit   864 SLGSSPYPGMPVDSKFYKMIKEGFRMLSPEHAPAEMYDIMKTCWDADPLKRPTFKQIVQL

FLT3    943 LGCQLA-DAEEAMYQNVDGRVSECPHTYQNRRPFSREMDLGLLSPQAQVEDS--------
c-fms   910 LQEQAQEDRRERDYTNLP---------SSSR------------SGGSGSSSSELEEESSS
c-kit   924 IEKQISESTN-HIYSNLAN-------CSPNRQKPVVDHSVRINSVGSTASSS--------

FLT3        ------------------------
c-fms   949 EHLTCCEQGDIAQPLLQPNNYQFC
c-kit   968 ------------QPLLVHDDV---
```

Figure 5.1. An alignment between the predicted amino acid sequence of human FLT3, c-fms and c-kit, showing the identical amino acid (Red) and the conserved (Blue) and the non-conserved (Black) amino acids. Amino acid sequences representing the immunoglobulin like domains of FLT3 are underlined.

This data, together with the previously published data by Rosnet *et al* (1993a), shows that the amino acid sequence alignment of the human FLT3 predicted amino acid sequence exhibited 18% and 19% identity with human c-fms and c-kit respectively for the extracellular domain (Rosnet *et al*, 1993a). However, the degree of homology with FLT3 is greater for the intracellular domain (47% for c-kit and 49% for c-fms) especially in the tyrosine kinase domain, which exhibits 63% and 64% homology with c-kit and c-fms, respectively. Furthermore, this strong homology between the different RTK III also holds true for the mouse homologues (see figure 5.2 for the mouse homology).

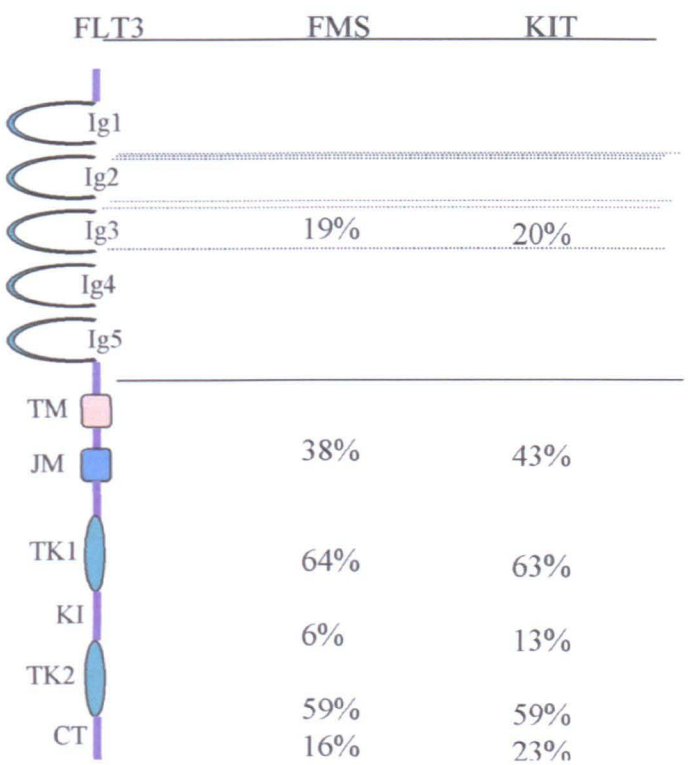


Figure 5.2 Percentage amino acid identity of mouse FLT3 receptor with other murine class III RTKs FMS and KIT.

Ig = Immunoglobulin-like domain, TM = Transmembrane,

JM =Juxtamembrene, TK = Tyrosine kinase domain, KI = Kinase Insert, CT = C-terminus.

Adapted from Rosnet and Birnbaum, 1993

5.2.2 Identification of 3 extra exons in the 5' region and determination of the exon/intron boundaries for the FLT3 gene

In order to screen the functionally important domains of the *FLT3* gene for mutations, a search was initiated for more information about the unidentified sequence of this gene. A well known molecular search tool, the basic logical alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/blast), provided by the National Centre for Biotechnology Information (NCBI) was used, to search for genomic sequences complementary to the nucleotide sequence of the human FLT3 mRNA (Accession No=18582015). A BLAST search of unfinished high throughput genomic sequences (htgs) identified two clones located on chromosome 13; namely No: 11062932 (Submitted to the NCBI; 12th November 2000), consisting of 8 unordered fragments and No: 11137776 (Submitted to the NCBI; 9th November 2000), comprising 5 unordered fragments. These clones contained twenty exons (1-20) coding for the 5' region of the *FLT3* gene. However, these two fragments did not contain the 3` sequence of the *FLT3* gene and it was not possible to identify the exact size and location for exons and introns within the most 3` region of the gene i.e. exons 21-24. Recently (7th February 2002) a new FLT3 sequence was submitted by the Sanger Centre No: 18582081. This sequence contains all the 24 exons of the *FLT3* gene.

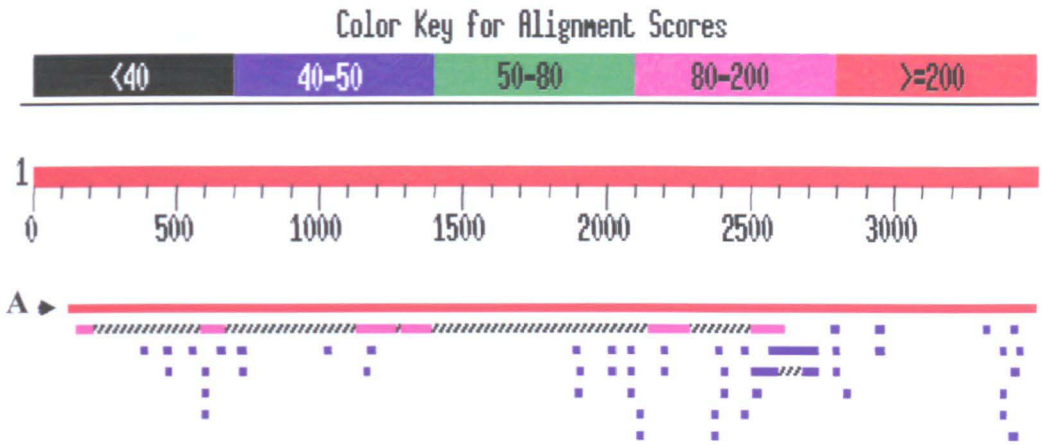


Figure 5.3. Result of the recent BLAST search using nucleotide sequence of the human mRNA corresponding to the *FLT3* gene showing genomic sequence alignment using the Unfinished High Throughput Genomic Sequence (htgs) databases. The *Homo sapiens* chromosome 13 genomic clone RP11-153M24 (labelled **A)** encompasses the whole coding sequence of the *FLT3* mRNA. All the other lines represent different clones with some homology with the *FLT3* mRNA, none had any significant homology in relation to the human *FLT3* gene. The patterned line represents sequence with an Expect (E) value that could occur by chance.

The result of the BLAST analysis showed the nucleotide sequence of the *FLT3* mRNA matched with the sequence of the identified clone (figure 5.3). The determination of the exon and intron sizes was carried out using Gene Jockey II (Biosoft). The nucleotide sequence of the *FLT3* mRNA and the identified genomic clone were put into Gene Jockey II. A search was performed to locate the sequence of each exon (based on the mRNA sequence) on the genomic sequence of the identified clone. All the predicted exon/intron boundaries agreed with the canonical acceptor and donor splice sites (Mount, 1982).

Analysis showed that the human *FLT3* gene is encoded by 24 exons, spanning approximately 100kb. Exon sizes range from 83bp to 562bp, while the characterised introns vary from 86 to 29,856bp (Table 5.1). Seven exons encode the first three immunoglobulin-like repeats, compared to four for c-kit, and include a unique 126bp exonic sequence that straddles the 3' and 5' end of exons 2 and 3 respectively, together with an intervening intron of 8434bp (figure 5.4). The first exon contains the signal sequence, the second to fourth exons encode the first Ig-like repeat, the fifth and sixth exons encode the second Ig-like repeat, while the seventh and eighth exons encode the third Ig-like repeat.

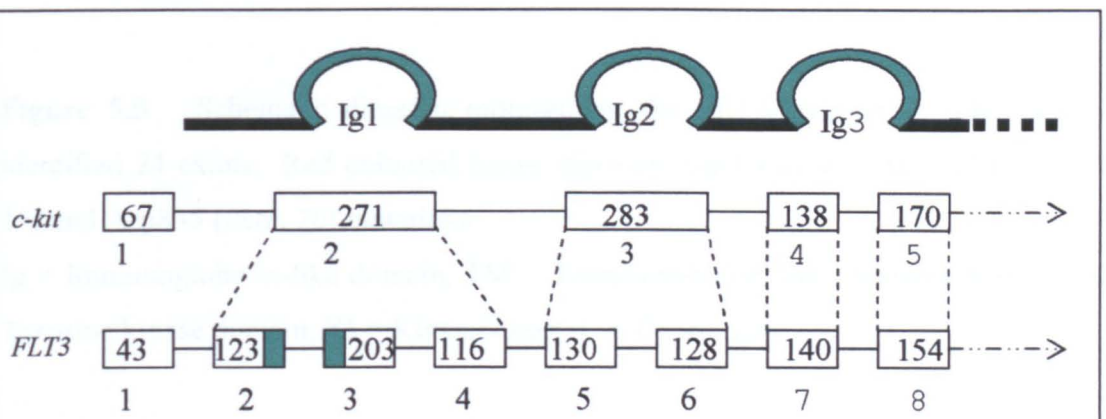


Figure 5.4. Comparison of the structural organisation of the 5' end of the human *FLT3* and *c-kit* genes in relation to the first three immunoglobulin-like (Ig) domains. Bold numbers correspond to exon numbering while exon sizes (bp) are indicated in the corresponding boxes. The shaded areas correspond to the additional 126bp of exonic sequence in exons 2 and 3.

The remainder of the *FLT3* gene is split into the same number of exons *c-kit* (16), and is highly conserved in size, sequence and exon/intron boundary positions (figure 5.5).

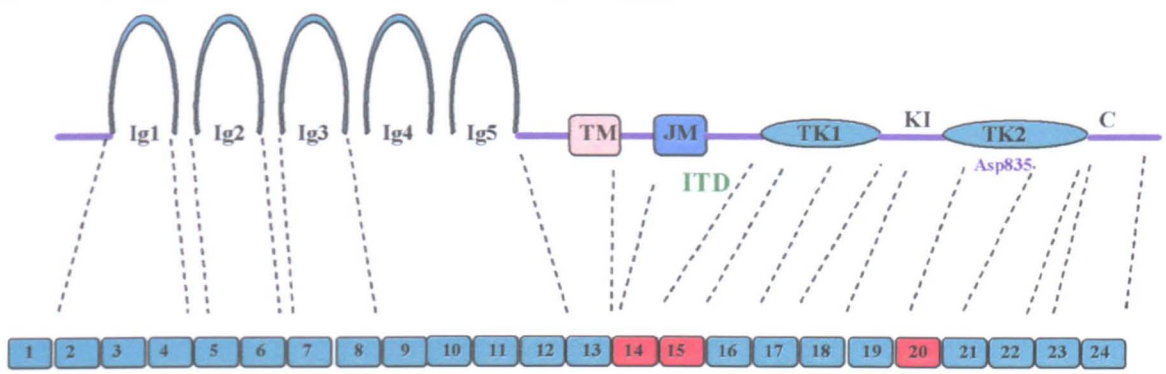


Figure 5.5 Schematic diagram representing the *FLT3* receptor in relation to the identified 24 exons. Red coloured boxes represent the location of the ITD (exons 14-15) and Asp835 (exon 20) mutations.

Ig = Immunoglobulin-like domain, TM = Transmembrane, JM =Juxtamembrene, TK = Tyrosine kinase domain, KI = Kinase Insert, C = C terminus.

Table 5.1 Exon/intron organisation of the human *FLT3* gene

Exon number	Exon length (bp)	3' Splice acceptor	5' Splice donor	Intron length (bp)
1	43	1 tccggaggccATGCCG	43 TGCTCGgtaaggcccc	29856
2	123	44 tgttttacagTTGTTT	165 CCCATGgtaaagtaac	8422
3	203	166 tgcaacgtagGTATCA	368 AAACAGgtaagtggag	4402
4	116	369 tgttttgcagAGGAGT	484 TAAGAAgtaagtccag	4673
5	130	485 gatgctttagATACCC	614 GGAAAGgtatgacaca	2323
6	128	615 ctaattgcagCTGTAA	742 CAATAGgtaacactat	320
7	140	743 catctcctagATCTAA	882 GAGGAGgtaataggac	97
8	154	883 taaaacctagGGCAAC	1036 TCGTAGgtaatgcagg	940
9	169	1037 tttcttacagAAAAGG	1205 ATACAGgtgagaccac	10986
10	104	1206 tttcaaacagCATATC	1309 TAAGAAgtaagttaaa	1142
11	109	1310 ttttctatagGGAAAC	1418 TCCCAAgtaataagga	261
12	179	1419 ttttttttcagCTGCAC	1597 CTCCAGgtacaacagt	1086
13	107	1598 tttgttgcagGCCCCT	1704 AAAAAGgtaaaagcaa	86
14	133	1705 atctctgaagCAATTT	1837 AGTTTGgtaagaatgg	90
15	105	1838 gtctttgcagGGAAGG	1942 TGAAAGgtacagtata	5598
16	111	1943 tctttgacagAAAAAG	2053 TGTCAGgtaacccact	936
17	154	2054 actatttcagGACCAA	2207 TTCCAGgtaagaggct	2144
18	83	2208 tttttaatagCATGCC	2290 CTGAAGgtaatatttt	1383
19	128	2291 tattttacagATGAAA	2418 AAGTCGgtatgctcct	4760
20	123	2419 ttcttgacagTGTGTT	2541 GGCAATgtgaggctgc	2787
21	112	2542 ttttccacagGCCCGT	2653 CACTTGgtaagttggg	335
22	100	2654 ttttcctcagGTGTGA	2753 AGAAATgtaagttcaa	600
23	106	2754 gcttttacagATACAT	2859 GAAGCGgtatgtagca	10277
24	554	2860 ccacattcagATGTAT		

Uppercase letters represent exon sequence and lowercase letters represent intron sequence. Exon sequences were numbered according to the human mRNA sequence (Rosnet *et al*, 1993a).

The revised exon numbering has resulted in previous studies using the incorrect exon numbering for the reported mutations discovered in the *FLT3* gene (figure 5.6). Therefore, the previously described location for the *FLT3* ITD i.e. exons 11- 12 is now exons 14-15. Similarly, the Asp835 that has been presumed to be located in exon 17 is actually located in exon 20 according to the revised exon numbering.

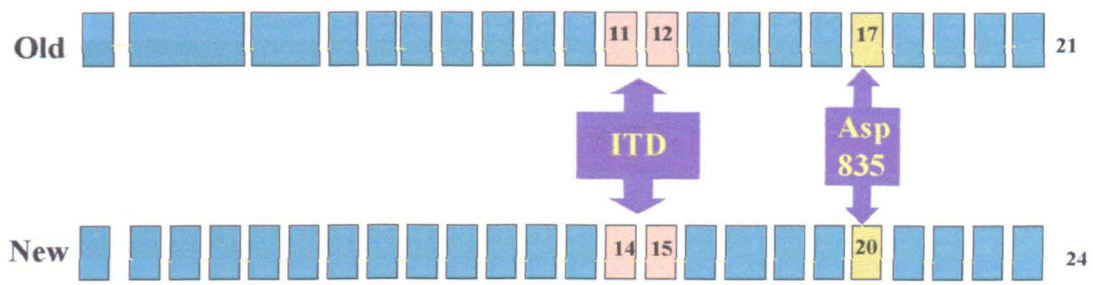


Figure 5.6. Schematic diagram showing the revised exon numbering and the resultant location of the ITD and the Asp835 mutations

The novel nucleotides and corresponding amino acid sequences of the 3` end of exon 2 and 5` end of exon 3 were used in the BLAST homology search to look for sequence homology. No significant homologous sequences, however, were found with any other previously reported protein sequence.

5.2.3 FLT3 transcription factor binding sites

To date, the 5` region of the *FLT3* gene has not been fully analysed. However, an initial study, using primer extension analysis, identified a major and minor transcription initiation site 61bp and 57 bp respectively, upstream of the translation start site (Liu *et al,* 1997). Furthermore, nucleotide sequence analysis of the 5` region revealed that the *FLT3* 5' flanking region lacks typical TATA and CAAT boxes, as do *c-kit* and *c-fms*. In this study, 200 bp of the 5' flanking sequence of the *FLT3* gene was analysed using PatSearch V1.1 (http://transfac.gbf.de) (Heinemeyer *et al,* 1998) for consensus transcription factor binding sites. No sites corresponding to TATA or CAAT sequences were identified in the 200bp fragment upstream of the reported translation start site (Small *et al,* 1994; Liu *et al,* 1997). However, seven likely transcription factor binding sites that show 100% match with the specified consensus sequence, were located within the 200 bp fragment (see table 5.2). It is noteworthy to mention that one of the seven likely transcription factor binding sites was a predicted AML1 binding site (TGCGGT) that was located 109bp upstream of the initiation codon (Figure 5.7).

TGCCCAACCT CTCCGCTCCC GCCTCGGTCC CTGCCTCTGG GGAGAGGGTT
CCTCCCCCCT TCCACTTTGC ACCAGTCCGA GGGAATTTGC GGTCGGTGAC
GCGCATCCTT AAGAGAGCCA CCTGCAGCGC GAGGCGCGCC GCTCCAGGCG
GCATCGCAGG GCTGGGCCGG CGCGGCCTGG GGACCCCGGG CTCCGGAGGC
C ATG GCG CGG C

Figure 5.7. Nucleotide sequence of 5' flanking sequence of the *FLT3* gene showing predicted transcription factor binding sites (Blue) and a translation start site (Red). Sequences in pink represent nucleotide sequence shared by two likely transcription factor binding sites.

ATG = Translation start site

Note: The minimum match percentage was set to 100% match to discard any match that might occur by chance.

Table 5.2 Possible transcription factor binding sites within the 200bp fragment 5` to the *FLT3* gene initiation site.

SITE	Pattern	Site at position*
AP2	CCCMNSSS |||||||| CCCCGGGC	-13
GCF	SCGSSSC ||||||| GCGCGCC	-62
GCF	SCGSSSC ||||||| GCGCCGC	-60
GCF	SCGSSSC ||||||| CCGGCGC	-29
GCF	SCGSSSC ||||||| GCGCGGC	-26
CREB	GNTGACGY |||||||| GGTGACGC	-100
AML1	TGCGGT |||||| TGCGGT	-109

The minimum matching window was set to a value of 6 to exclude sequence generated by chance.

AP2 = The mammalian transcription factor AP-2 is a sequence-specific DNA-binding protein expressed in neural crest lineages and regulated by retinoic acid

GCF = A factor that interacts with GC-rich sequences and positively regulates both housekeeping genes and cellular oncogenes.

CREB = cAMP response element binding protein.

AML1 = The leukaemia-associated transcription factor, acute myeloid leukaemia 1.

M = A or C , S = C or G

N = A, C, G, or T

Y = C or T

* Numbering from the A of the ATG translation start site.

5.3 Discussion

Data has been presented of the genomic organisation of the extracellular and transmembrane coding domains of the human *FLT3* receptor. This work, together with the data of Agnes et al, (1994), demonstrates that the entire *FLT3* gene comprises 24, rather than the previously assumed 21 exons. The ligand binding and receptor dimerization sites are encoded by the first 8 exons of *FLT3*, which have the least degree of homology with other RTK class III receptors. Thus, it appears that the five extracellular immunoglobulin-like repeats are encoded by the first 12 exons of the *FLT3* gene. Exon 13 encodes the C' terminus of the extracellular part and the central hydrophobic TM domain, while the JM region is encoded by exon 14. Exons 15-22 encodes the TK1 and TK2 and the KI domains while the last two exons (23-24) encode the protein's C-terminus.

5.3.1 FLT3 identity with other RTK III

As presented in section 5.2.1, the identity between *FLT3* and other RTK class III (*c-kit, c-fms*) decreases in the extracellular part of the receptor, an observation that was previously reported by a number of researchers (Rosnet and Birnbaum, 1993; Rosnet *et al*, 1993a; Agnes *et al*, 1994). However, it is well known that RTKs class III ligands are peptide regulatory factors that bind to the receptor as associated dimers (Pandit *et al*, 1992; Rosnet and Birnbaum, 1993). Furthermore, RTK class III ligand dependent activation takes place through binding of a dimerized ligand to their cognate receptor (Ullrich and Schlessinger, 1990; Rosnet and Birnbaum, 1993); a fact that may explain the decreased identity between RTK III extracellular domains as a result of cognate ligand specificity.

However, this ligand -receptor specificity is not applicable in the intracellular part, as there is no direct interaction between the ligand and its receptor. The greater homology for the intracellular domain is the result of conservation of the kinase activity as the result of the

evolution from a common ancestral gene by cis and trans duplication (Rosnet *et al,* 1991b; Andre *et al,* 1992)(chapter1).

5.3.2 Structural analysis of the *FLT3* gene

In relation to the position of mutations identified in the *FLT3* gene, the data indicates that the prognostically important *FLT3* ITD mutations (see chapter 6) are located in exons 14 and 15 rather than exons 11 and 12 as previously assumed. Furthermore, the recently identified point mutations affecting Asp835 (see chapter 6) are located in exon 20 rather than exon 17. The complete genomic structure of the *FLT3* gene will be of great interest for groups considering carrying out a full mutational analysis of the *FLT3* gene. Such a step is important, especially after the finding of Fenski and colleagues (2000) who demonstrated a lack of correlation between constitutive activation of *FLT3* and ITD mutations (Fenski *et al,* 2000). Fenski et al suggested that other mechanisms of activation must be operational; a hypothesis supported by the recent finding of the *FLT3* Asp835 mutation in AML patients (see chapter 6) (Abu-Duhier *et al,* 2001b; Yamamoto *et al,* 2001). Therefore, for the first time the exact location, size and sequence of *FLT3* gene is available to carry out more investigation and mutational analysis for the gene most commonly mutated in AML.

5.3.3 Transcription binding sites in the 5` *FLT3* gene

Prediction of transcription factor binding sites is a basic step for the analysis of gene regulatory networks. It is generally agreed that proteins other than RNA polymerase are required for transcription. These are called transcription factors. In this study, a 200bp fragment in the 5' region of the *FLT3* gene was analysed to identify predicted transcription factor binding sites. Analysis of the 5' region revealed the presence of 7 possible transcription factor binding sites (*AML1*, *CREB*, *AP-2*, *GC* (4 sites)). Some of these transcription factor binding sites appear to be important, for example the cAMP response

element binding (CREB) protein has been identified as a crucial factor mediating a transcriptional response to elevated levels of cAMP and Ca2+ (Latchman, 1997). Furthermore, it was suggested that CREB may play a role in causing alterations of gene expression important to angiogenesis in the *KDR/Flk1* gene (Mayo *et al*, 2001). However, the role of CREB protein need to be examined in *FLT3*. AP-2 is a sequence-specific DNA-binding protein expressed in neural crest lineage and regulated by retinoic acid. It was demonstrated that the expression of *c-kit* is regulated by the AP-2 transcription factor (Bar-Eli, 1999). It is therefore suggested to investigate the mechanisms of *FLT3* transcriptional regulation starting by isolation the 5' flanking region of the human *FLT3* gene and characterizing its promoter activity in haematopoietic cells.

In haematopoiesis, a functional analysis of wild-type *AML1* and its fusion proteins is yielding important information on the mechanisms of transcription (Zhang *et al,* 2001). In human leukaemia, the *AML1* gene is the most frequent target of chromosome translocations e.g. t(8;21), t(12;21) and t(3;21) (Cherry *et al,* 2001). The *AML1* gene encodes a transcription factor that regulates a number of target genes that are essential for normal haematopoiesis (Downing, 2001). Initial examination of human *c-fms* expression revealed that it is upregulated upon myeloid commitment during haematopoiesis. This suggests an important role for the transcription factor during myeloid cell differentiation (Shapiro and Look, 1995). Further analysis of the *c-fms* promoter region revealed that *AML1* and other transcription factors i.e. *C/EBP* and *PU.1*, bind directly to the *c-fms* promoter region, indicating that the human *c-fms* gene is a direct target of *AML1* (Zhang *et al,* 2001). Furthermore, transient transfection analysis of the functional promoter demonstrates that *AML1*, in conjunction with its heterodimer partner *CBFβ,* can activate this promoter. *C/EBP* and *AML1* are important factors for regulating a critical haematopoietic growth factor receptor, the *c-fms* receptor, suggesting a mechanism for how the *AML1* fusion protein could

contribute to acute myeloid leukaemia (Zhang *et al,* 1994; Zhang *et al,* 1996). These previous studies shows that *AML1* plays an important role in regulating the promoter activity of the *c-fms* gene (Zhang *et al,* 2001). An initial study was performed showing that *AML1* did actually stimulate *FLT3* expression (Qian and Small, 1996). Consequently, a similar role for *AML1* could be assumed within the 5' region of the human *FLT3* gene due to the presence of a binding site for *AML1* and the close relationship between RTK class III. In addition, *AML1* alteration could contribute to impaired differentiation of haematopoietic cells (Yergeau *et al*, 1997), which seems to be important in the "two-hit" model proposed for the development of AML (see chapter 7) (Gilliland and Griffin, 2002). This speculation, however, requires further mutational and functional analysis to be carried out in the 5` region of the *FLT3* gene to uncover this valuable information.

Chapter 6

Mutation analysis of the *FLT3* receptor

6.1 Introduction

6.1.1 Normal function of FLT3 receptor

It is established that a number of cell growth factors, acting through specific receptors strictly regulate the proliferation and differentiation of normal haematopoietic cells (Nicola, 1989). It is also confirmed that proteins with tyrosine kinase activity, not only play an important role in the transduction pathways for cell growth and differentiation of normal cells but also carry potential transforming activity.

FLT3 is thought to play a crucial role in haematopoiesis, for example, it is preferentially expressed on primitive $CD34^+$ haematopoietic stem cells, while it's ligand (FLT ligand, FL) is synthesised by bone marrow stromal cells (Lyman *et al,* 1993). Furthermore, FL is known to stimulate primitive haematopoietic cells by binding to *FLT3* receptor and causing receptor dimerization, leading to the activation of the receptor tyrosine kinase and receptor autophosphorylation (Hannum *et al,* 1994; Graddis *et al,* 1998). Activated signals are transduced by the phosphorylated *FLT3* through association with various cytoplasmic proteins, including ras GTPase-activation protein, phospholipase C, and Src family tyrosine kinases (Lyman *et al,* 1993). In addition, stimulation with an agonist antibody against *FLT3* gives rise to an expansion of both myeloid and lymphoid cells (Zeigler *et al,* 1994), while antisense oligonucleotides against *FLT3* block the formation of mature myeloid progenitor cells in long term bone marrow cultures (Small *et al,* 1994). *FLT3* plays a regulatory effect due to the interaction of *FLT3*/Flk2 with its ligand on pluripotent stem cells, immature lymphocytes and early progenitor (Matthews *et al,* 1991a).

6.1.2 FLT3 Internal tandem duplication

Recently, an internal tandem duplication (ITD) in the juxtamembrane (JM) domain of the *FLT3* gene was identified in a small group of AML patients (Nakao *et al,* 1996). The ITD, located within the JM region, was demonstrated to be a somatic mutation of the *FLT3* gene occurring in 17% of AML patients (Nakao *et al,* 1996). In a subsequent study involving a large number of haematological malignancies, ITD mutations were restricted to AML and myelodysplastic syndrome (MDS) (Yokota *et al,* 1997). *FLT3* ITD has now been shown to be present in a high percentage of AML cases (20%), regardless of the FAB classification (Yokota *et al,* 1997; Xu *et al,* 1999; Rombouts *et al,* 2000) and in MDS (5%) (Kiyoi *et al,* 1998) but never in CML, or normal haematopoietic tissue (Yokota *et al,* 1997; Ishii *et al,* 1999). *FLT3* ITD has been rarely reported in patients with acute lymphoblastic leukaemia, where interestingly aberrant myeloid antigen expression may be present (Xu *et al,* 1999; Nakao *et al,* 2000; Xu *et al,* 2000).

A relationship between the *FLT3* ITD and peripheral white blood cell count and high lactate dehydrogenease level was noticed in acute promyelocytic leukeamia (M3) (Kiyoi *et al,* 1998), while the appearance of *FLT3* ITD mutations during transformation of MDS, or at relapse in AML, suggest that ITD mutations could have a role in promoting leukaemic progression (Horiike *et al,* 1997; Nakano *et al,* 1999).

The patient-specific duplicated sequence insertions are always in open reading frame and result in mutant *FLT3* with extended JM domains (Nakao *et al,* 1996). Although there is a variation of length and location between the ITD in different patients, the altered *FLT3* mutation is always transcribed in-frame and encodes mutant *FLT3* with an extended JM domain (Nakao *et al,* 1996). The *FLT3* ITD appears to result in constitutive dimerization of

the receptor and autophosphorylation of specific tyrosine residues irrespectively of the type of mutation (Kiyoi *et al,* 1998; Hayakawa *et al,* 2000).

6.1.3 Pathogenic role of FLT3 ITD

The previously discussed data suggests a pathogenic role for *FLT3* in acute myeloid leukaemia (Yokota *et al,* 1997; Fenski *et al,* 2000; Rombouts *et al,* 2000). The ITD mutation is in open reading frame and results in a protein that acts in a dominant fashion, stimulating the growth of the leukemic cells (Yokota *et al,* 1997). In another study, *FLT3* receptor was demonstrated to cause proliferation of AML cells *in vitro*, by stimulating the proliferation and inhibiting apoptosis of AML cells (Lisovsky *et al,* 1996), thus blocking the cellular apoptotic response to conventional chemotherapy (Meshinchi *et al,* 2001a).

Finally, functional FLT3 expression in most cases of AML, as well as in the majority of immortalised human myeloid and monocytic cell lines (Birg *et al*, 1992; Rosnet *et al*, 1993a; Meierhoff *et al*, 1995; Drexler, 1996), emphasises the pathological role for FLT3 in acute myeloid leukaemia.

6.1.4 FLT3 Asp835 mutation

A number of previous studies have documented mutations in tyrosine kinase receptors in the kinase domain, such as those affecting codons 814 and 816 in mouse and human c-kit respectively (Furitsu *et al,* 1993). Such *c-kit* mutations are present in human and mouse mast cell lines, as well as being associated with human systemic mastocytosis and its associated leukaemia (Furitsu *et al,* 1993; Longley *et al,* 1999; Sotlar *et al,* 2000).

Recently it was reported that expression of the *FLT3* gene containing an ITD in COS-7 cells resulted in constitutive autophosphorylation of the receptor (Kiyoi *et al,* 1998), however,

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6.2 Results

6.2.1 Molecular analysis of the *FLT3* gene

6.2.1.1 Exon 9 and exon 11

Due to the great similarity between RTK class III, screening of the homologus regions of the mutation hot spots previously reported in the *c-fms* gene at codon 301 (exon 7) and at codon 413 (exon 9) was carried out for the *FLT3* gene i. e. codon 350 in exon 9 and codon 450 in exon 11.

As described in chapter 2, exon 9 was amplified by PCR of genomic DNA from all groups of AML (n=123) (A, B, C1, C2) except group D (n=185) and all cases of IMF patients (n=40) (see chapter 2 for details). None of the patients analysed showed abnormal CSGE patterns (Figure 6.1).

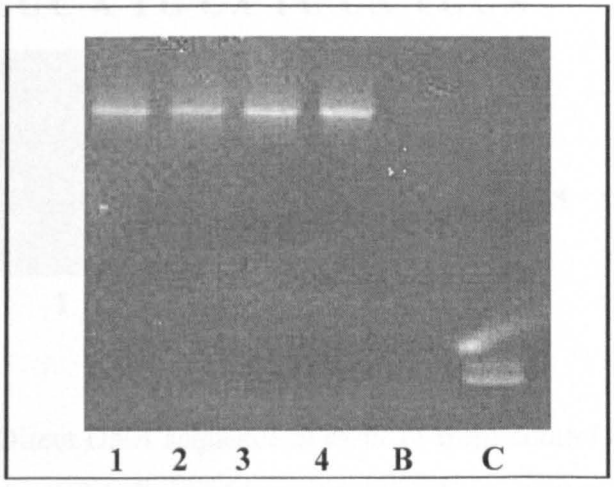

Figure 6.1 CSGE analysis of *FLT3* exon 9 demonstrating the profiles obtained for four AML patients (lanes 1-4) and a blank (B) and a gel control (C).

Similarly, PCR of genomic DNA from 123 (group A, B, C1 and C2) cases of AML and 40 cases of IMF were amplified using primers for exon 11of the *FLT3* gene. 42 out of 123 AML cases and 10 out of forty cases of IMF and 27 out of 70 normal individuals had abnormal patterns on CSGE (Figure 6.2). This pattern was the same in all individuals. Sequence analysis identified the presence of a C→T substitution a position 1300-3 of the *FLT3* gene (Figure 6.3).

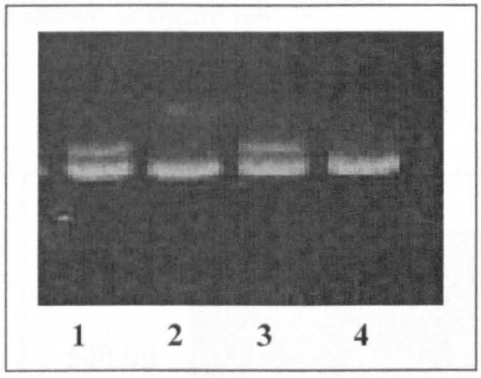

Figure 6.2 CSGE analysis of exon 11 shows CSGE variants. Lanes 1 and 3 show abnormal CSGE profiles in 2 AML patients. Lanes 2 and 4 show normal CSGE patterns in AML patients.

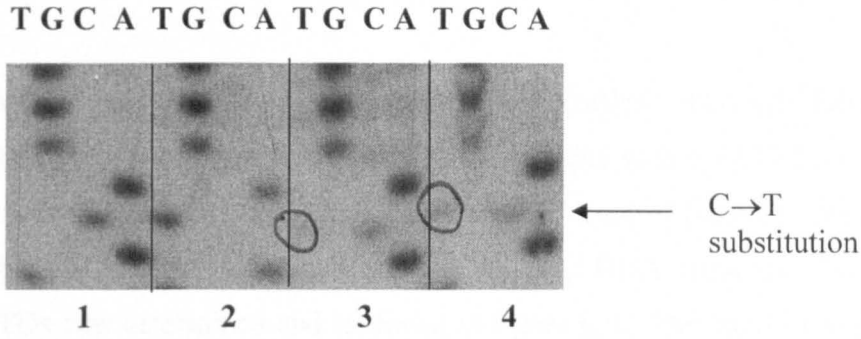

Figure 6.3 Direct DNA sequence of exon 11 from control and patient samples showing the control individual homozygous CC (sequence 1) homozygous TT (sequence 2) and heterozygous C→T substitution at position 1300-3 in two patients (sequences 3 and 4)

6.2.1.2 *Exon 14 and 15 ITD*

The genomic DNA of 151 patients (111 AML from groups (A, B, C1), and 40 IMF) with myeloproliferative disorders was screened for mutations, in exons 14 and 15 of the *FLT3* gene using specific primers (see section 2.3.4.1). 14 AML but no IMF patients showed an abnormal profile following PCR amplification and polyacrylamide gel electrophoresis of exon 14 and 15. The abnormal profile represents the previously reported internal tandem duplication. PCR products of four patients representing different sizes of ITD (27-111 bp) are shown in figure 6.4.

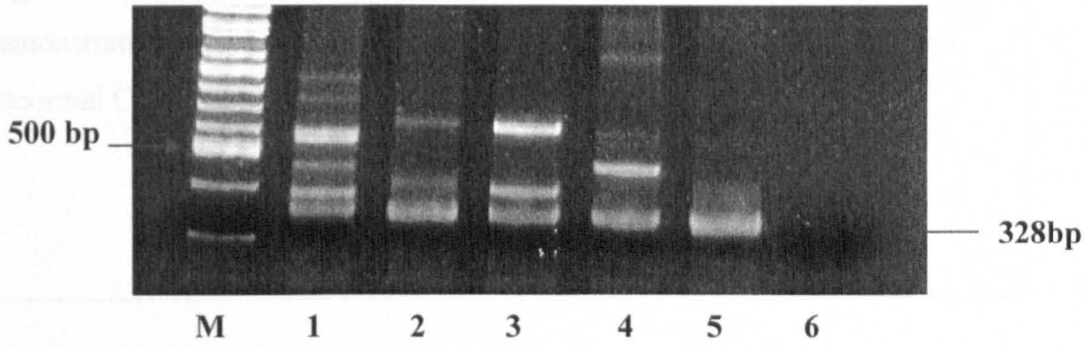

Figure 6.4. Polyacrylamide gel illustrating profiles obtained following amplification from genomic DNA of four AML patients with a *FLT3* ITD (lanes 1-4), a normal individual (lane 5) and a 'no template' control (lane 6). An arrow indicates the 500bp band in the marker lane (M). The DNA sequence of each of the four ITDs was determined and is shown in figure 6.6. The band of 328bp in each individual (lanes 1-5) represents wild-type *FLT3* sequence. Additional larger bands represent the ITD in each of the four AML patients, plus heteroduplexes formed between wild-type and ITD DNA strands in each patient.

CSGE was performed to confirm the presence or absence of any other mutations in exon 14 and 15. CSGE analysis of ten AML patients with an ITD is shown in figure 6.5.

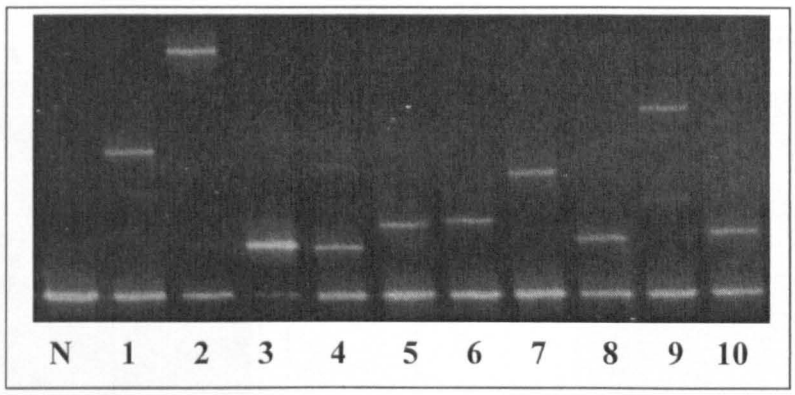

Figure 6.5 CSGE analysis of exons 14-15 of the *FLT3* gene demonstrating the profiles obtained for a normal individual (N) and abnormal CSGE profiles (lanes 1-10) in AML patients.

After sequencing four selected AML patients, three patients showed a simple ITD and one showed an ITD plus insertion. Sequencing gels representing a normal individual and an AML patient with an ITD are shown in figure 6.6. All four patients shown in figure 6.4 were sequenced and the ITD sequence was identified for each of them (Figure 6.7). None of the forty patients with IMF showed abnormal profiles on the polyacrylamide gel or CSGE for exon 14 and 15. Furthermore, none of the 70 normal individuals had an abnormal profile on CSGE.

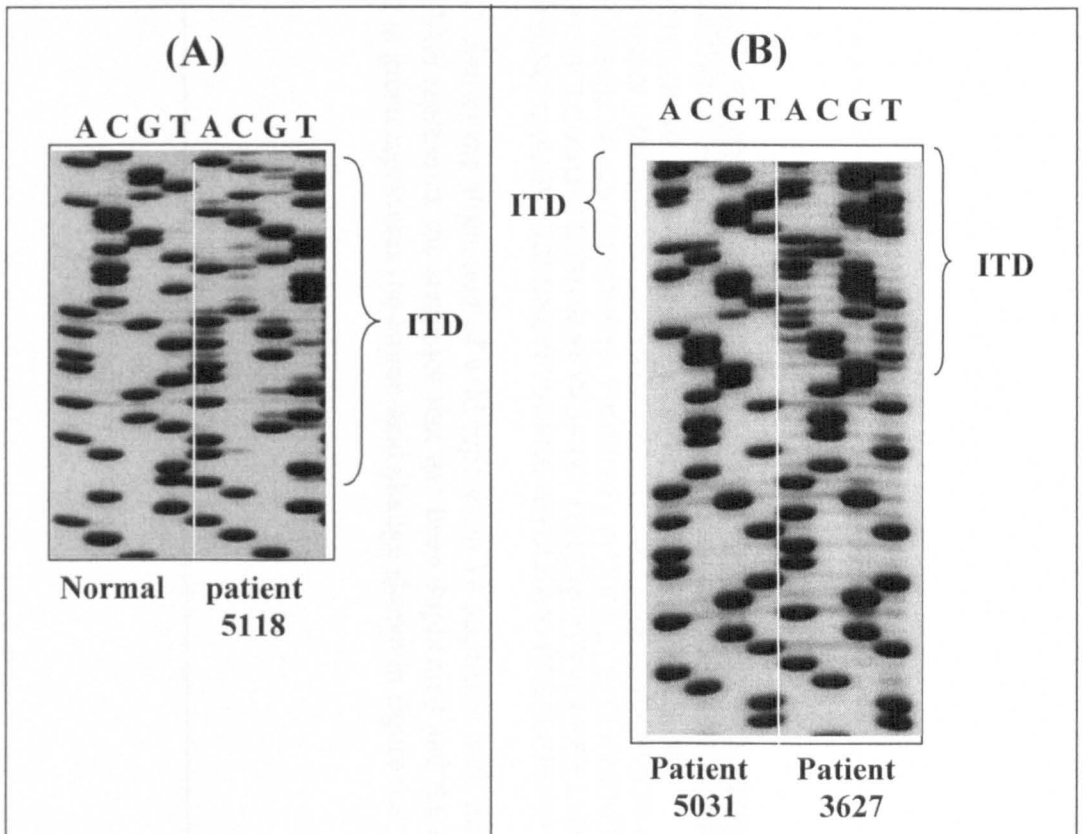

Figure 6.6. (**A**) DNA sequence analysis gel of the ITD in exon 14-15 demonstrating normal sequence and an ITD sequence in an AML patient (5118). (**B**) DNA sequence analysis gel of the ITD in two patients with an ITD. See fig 6.7 for ITD sequence of each patient.

W QFRYESQLQMVQVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF
5117 QFRYESQLQMVQVTGSSDNEYFYV DFREYEYDLDFREYEYDLKWEFPRENLEF
5118 QFRYESQLQMVQVTGSSDNEYF YVDFREYEYYVDFREYEYDLKWEFPRENLEF
5031 QFRYESQLQMVQVTGSSDNEYFYVDFREY GSSDNEYFYVDFREY EYDLKWEFPRENLEF
3627 QFRYESQLQMVQVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF*GKNV* QVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF

Figure 6.7 Shows the alignment of wild type exon 14 sequence with the sequence of four AML patients with ITD mutations. Bold letters in blue represents the sequence that has been duplicated and the underlined letters represent the actual duplicated sequence. Italic text in green represents the amino acid change shown in figure 6.8

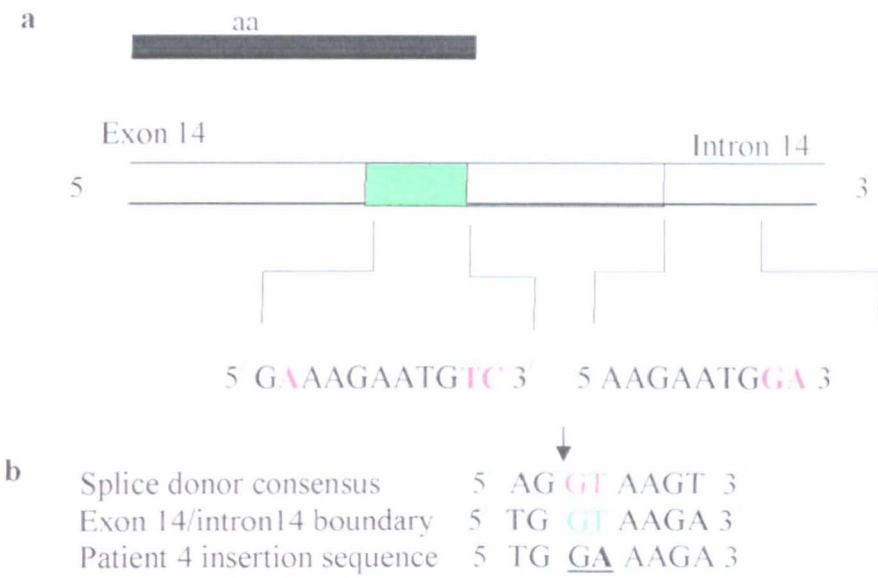

Figure 6.8. Schematic representation of the exon 14-intron 14 boundary in patient 3627. **A)** The extent and position of the 111-bp duplicated nucleotide sequence encoding 37 amino acids within exon 14 and the corresponding original region of wild-type sequence across the exon 14-intron 14 boundary from which it is derived are shown by the black and blue bars respectively. The green bar indicates the 11-bp sequence derived form the intronic duplicated sequence encoding the novel four amino acid (aa) motif. The sequence of this and the original wild-type intron 14 sequence are shown: the single base pair mismatch between these two sequences is highlighted in bold red text. **B)** Aligned nucleotide sequence of the splice donor site consensus, the exon 14 donor splice site of wild-type *FLT3* and the duplicated sequence present in exon 14 of patient 3627. Underlined nucleotides indicate the invariant GT dinucleotide present in the splice donor site at the 5′ end of intron 14. The intron-exon boundary is shown by an arrow. The mismatched nucleotides present in this region of duplicated sequence within exon 14 of patient 3627 are shown in bold.

6.2.1.3 *Exon 20*

In this stage of patient analysis, due to the fact that some AML patients were not suitable to be statistically analysed (no PCR product in previous tests (ITD), no PCR product in the current test (Asp835) or poor risk criteria) another 12 patients were added. Therefore, this group i.e. group ABC20 (n=97) patients with AML, were analysed for sequence alterations in exon 20 of the *FLT3* gene. Due to the lack of sufficient information about the intronic sequence, a 194bp fragment comprising 101bp of the 3' end of exon 20 and 93bp of the 5' end of intron 20 was amplified. This was subjected to analysis by CSGE.

Initial observation highlighted the presence of an abnormal band in some AML samples. Although it was possible to distinguish diffuse bands from the normal band, a reliable result demonstrating the presence of either one or two band was not possible (Figure 6.9). Further tests were needed to identify the type and numbers of changes present in exon 20.

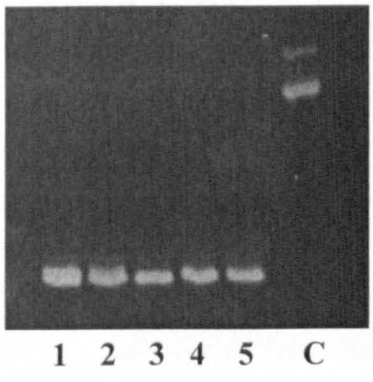

Figure 6.9. CSGE analysis of exon 20 of the *FLT3* gene demonstrating diffuse bands (lanes 1 and 2) and normal bands (lanes 3, 4, 5) and a gel control sample (C).

Initial sequencing of DNA from three samples, representing two AML patients with diffuse bands and one individual with a normal CSGE pattern, revealed the presence of a GAT→CAT change at nucleotide 2503 predicted to result in an Asp835His mutation in one AML sample (5104). This nucleotide change was not present in the other AML sample

(5018) or in the normal individual (Figure 6.10). However, an A→G intronic change was identified 57bp from the 3' end of exon 20. This intronic change was identified in AML patient 5018 (Figure 6.11).

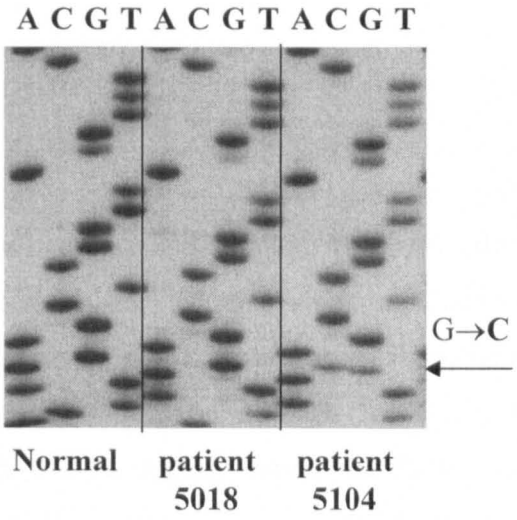

Figure 6.10. DNA sequencing gel showing wild type sequence in a normal individual, AML patient without any mutation (5018) and abnormal sequence with a single-base substitution GAT→CAT at codon 835, predicted to result in an amino acid change Asp835His in patient 5104.

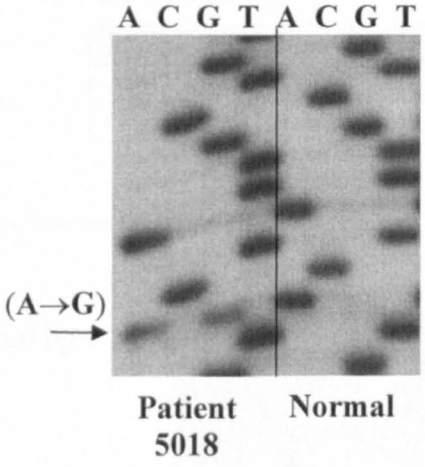

Figure 6.11. Sequencing gel showing the sequence of exon 20 of the *FLT3* gene with a single-base substitution A→G (57bp from the 3' end of exon 20) in patient 5018 and a wild-type sequence (normal).

Analysis of the sequences involved in the nucleotide alterations affecting codon 835 demonstrated that any sequence alteration affecting the codon would destroy the restriction enzyme site for EcoRV. In addition, the nucleotide 2541+57 A→G change destroyed a site for NlaIII. Therefore, in order to screen the 97 samples for all possible codon 835 mutations, the 194bp fragment was digested with EcoRV (Figure 6.12), while NlaIII was used to screen for the intronic A to G change (Table 6.1 and Figure 6.13.)

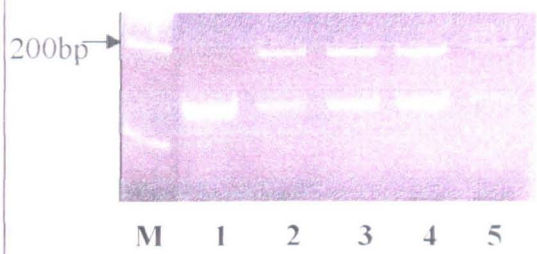

Figure 6.12. Polyacrylamide gel demonstrating the product obtained after restriction enzyme analysis using EcoRV enzyme. Lane 1 shows an AML patient without an Asp835 mutation, lanes 2, 3, 4 and 5 show Asp835 mutations (lane 5 is an AML patient with an Asp835 deletion).

Table 6.1. Fragments, frequency and sizes for PCR products using NaIII.

Sample (frequency)	Fragment size (bp)
A allele (0.76)	38, 71, 85
G allele (0.24)	71, 123

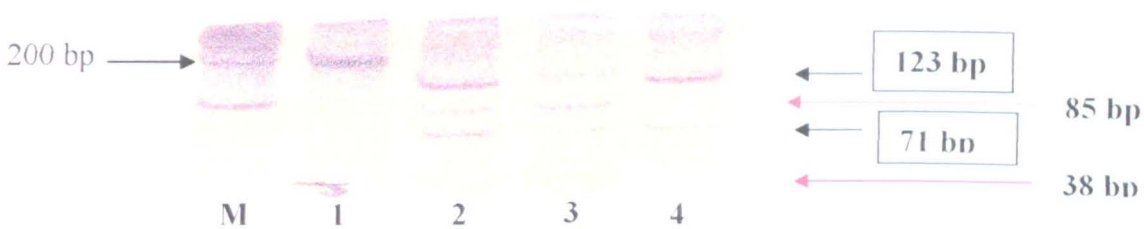

Figure 6.13. Polyacrylamide gel stained with silver stain demonstrating the product obtained after restriction enzyme analysis using NlaIII. Undigested PCR product is shown in lane 1, heterozygous polymorphism A/G in lane 2, homozygous A/A in lane 3 and homozygous G/G in lane 4.

Changes detected with EcoRV were confirmed by direct DNA sequencing (Figure 6.14). Seven AML samples (7.2%) possessed a codon 835 mutation; Asp835Tyr (n=5), Asp835His (n=1) and Asp835 del (n=1). Five of these cases were classified as AML M4, of which three demonstrated inv(16) (see Table 6.2). FLT3 ITD mutations had been identified in 15 patients, however no case possessed both an ITD and Asp835 mutation. Codon 835 mutations were not detected in the 70 control samples using EcoRV analysis.

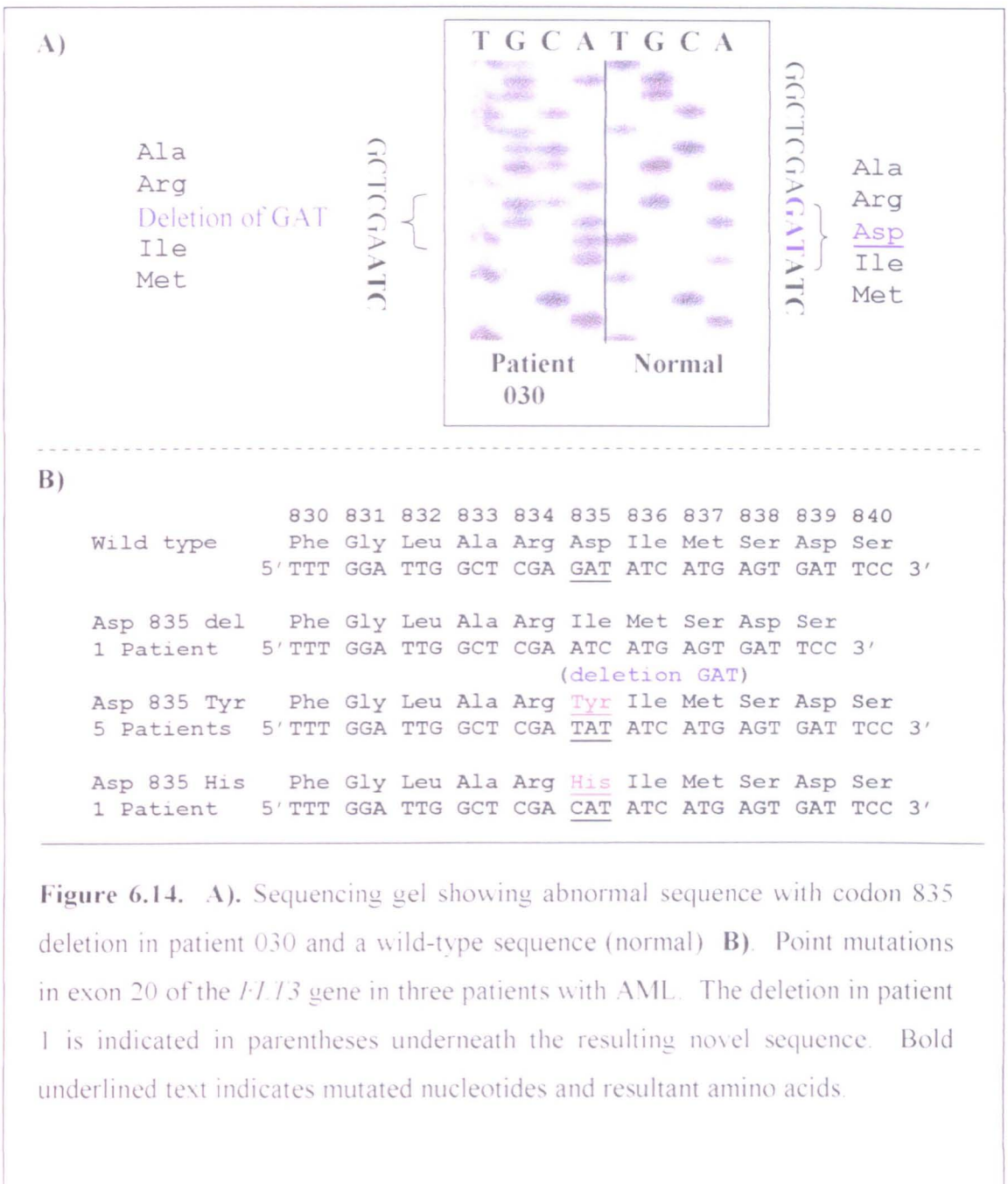

Figure 6.14. **A).** Sequencing gel showing abnormal sequence with codon 835 deletion in patient 030 and a wild-type sequence (normal). **B).** Point mutations in exon 20 of the *FLT3* gene in three patients with AML. The deletion in patient 1 is indicated in parentheses underneath the resulting novel sequence. Bold underlined text indicates mutated nucleotides and resultant amino acids.

Table 6.2. Patients characteristics of seven AML cases possessing an Asp835

Patient	Sex	Age	FAB	Cytogentics	Mutation	Survival (months)
030	F	73	M4	46,XX[30]	Asp835del	0 +
1920	M	32	M4	47,XY,inv(16)(p13q22),+22/46,XY(*)	Asp835Tyr	1 +
5041	F	48	M4	46,XX[30]	Asp835Tyr	3 +
5269	M	51	M4	46,X,-Y,+8,inv(16)(p13q22)[20]	Asp835Tyr	50
5370	M	24	M6	46,XY,add(5)(q31),del(5)(q22q34)[14]	Asp835Tyr	48
040	M	62	M4	46,XY,inv(16)[3]/47,XY,inv(16),+22[21]/46,XY[3]	Asp835Tyr	3 +
5104	M	32	M3	46,XY,t(15;17)(q24;q21)[5]	Asp835His	1 +

Analysis date January 2001

The nucleotide 2541+57A>G change was shown to be a polymorphic change, with an allelic frequency of 0.24 for the G and 0.76 for the A allele in the 70 control samples. The Asp835 residue was noted to be highly conserved amongst the class III RTKs (Figure 6.15).

```
          807                                   835
FLT3 (H)  CVHRD LAARN VLVTH GKVVK ICDFG LAR D I MSDSN (Abu-Duhier et al 2001)
          810                                   838
FLT3 (M)  CVHRD LAARN VLVTH GKVVK ICDFG LAR D I LSDSS (Fenski et al, 2000)
          788                                   816
c-kit (H) CIHRD LAARN ILLTH GRITK ICDFG LAR D I KNDSN (Longley et al, 1999)
          786                                   814
c-kit (M) CIHRD LAARN ILLTH GRITK ICDFG LAR D I RNDSN (Tsujimura et al, 1994)
          789                                   817
c-kit (R) CIHRD LAARN ILLTH GRITK ICDFG LAR D I RNDSN (Tsujimura et al, 1995)
          774                                   802
c-fms (H) CIHRD VAARN VLLTN GHVAK IGDFG LAR D I MNDSN (Glover et al, 1995)
          772                                   800
c-fms (M) CIHRD VAARN VLLTS GHVAK IGDFG LAR D I MNDSN YVVKG N (NI)
```

Figure 6.15. Cross-species comparison of amino acid sequence of RTK class III receptors demonstrating the location of the highly conserved aspartic acid resides. References indicate known activating point mutations.
H= Human, M=Mouse, R=Rate, NI=Not identified.

185 extra AML samples (group D) were added to the study to increase the number of the AML samples to determine if there was any significant difference in survival for a larger group of patients. Seven patients were confirmed to have Asp835 mutations using EcoRV restriction enzyme digest and by automated DNA sequencing.

6.2.2 Survival analysis

6.2.2.1 ITD mutation

The Kaplan-Meier (KM) method (Kaplan and Meier, 1958), was used to calculate the survival differences between the two groups of AML patients (patients with and without mutations), and were compared by the Mantel-Cox test. Stat-view software (Abacus Concepts, Inc), available in the Division of Genomic Medicine was used to measure survival. The AML patients were separated into two groups according to the cytogenetic data: a good risk group (n=62) defined as those with an inv(16) (n=37), t(8;21) (n=15) or t(15;17) (n=10) and a standard risk group (n=44) which included patients who lacked good and/or poor risk karyotypes. Five patients were excluded from the analysis because they were poor risk patients, defined as monosomy 7 and/or 5, 5q-, anomalies of 3q and complex re-arrangements. The analysis shows that the *FLT3* ITD mutation is a strong prognostic factor. The mean survival for patients without the mutation was 29.1 months compared to 12.8 months for patients with an ITD mutation. Therefore, patients lacking the ITD mutation survived longer than patients with the mutation. In addition, the data shows that the incidence of *FLT3* mutations is lower in the good risk group (6.5%) compared to (22.7%) in the standard risk group. The *FLT3* ITD was of prognostic significance for patients in both the good and standard risk groups (Figure 6.16).

6.2.2.2 Codon 835 mutations

Similarly, survival analysis was carried out for the 97 AML patients (group ABC20) screened for both the ITD and Asp835 mutation. 15 AML patients with ITD were excluded from the analysis because it had already been shown that ITD was of adverse prognostic significance. Kaplan-Meier cumulative survival analysis was carried out for 75 AML patients lacking a *FLT3* ITD and compared to seven AML patients possessing a *FLT3* codon 835 mutation. The Asp835 mutations alone were not shown to be of prognostic significance (Figure 6.17), although a larger number of patients need to be studied. However, the lack of prognostic significance for Asp835 mutations has been confirmed in 429 cases of AML (Yamamoto *et al,* 2001), as well as by two other groups (Inami *et al,* 2001; Frohling *et al,* 2002), who examined, respectively 170 and 224 AML patients.

Criteria	Patients with ITD	Patients without ITD
Median age	37.7	41.8
t(8;21)	1	15
inv(16)	2	35
t(15;17)	1	9
5q- 7q-	0	5
Median survival	10	23
FAB type (total)	14	97
M0	0	7
M1	5	7
M2	0	29
M3	1	9
M4	5	30
M5	3	9
M6	0	6
Good prognosis	4	58
Standard prognosis	10	34
Poor prognosis	0	5
Male	7	59
Female	7	38

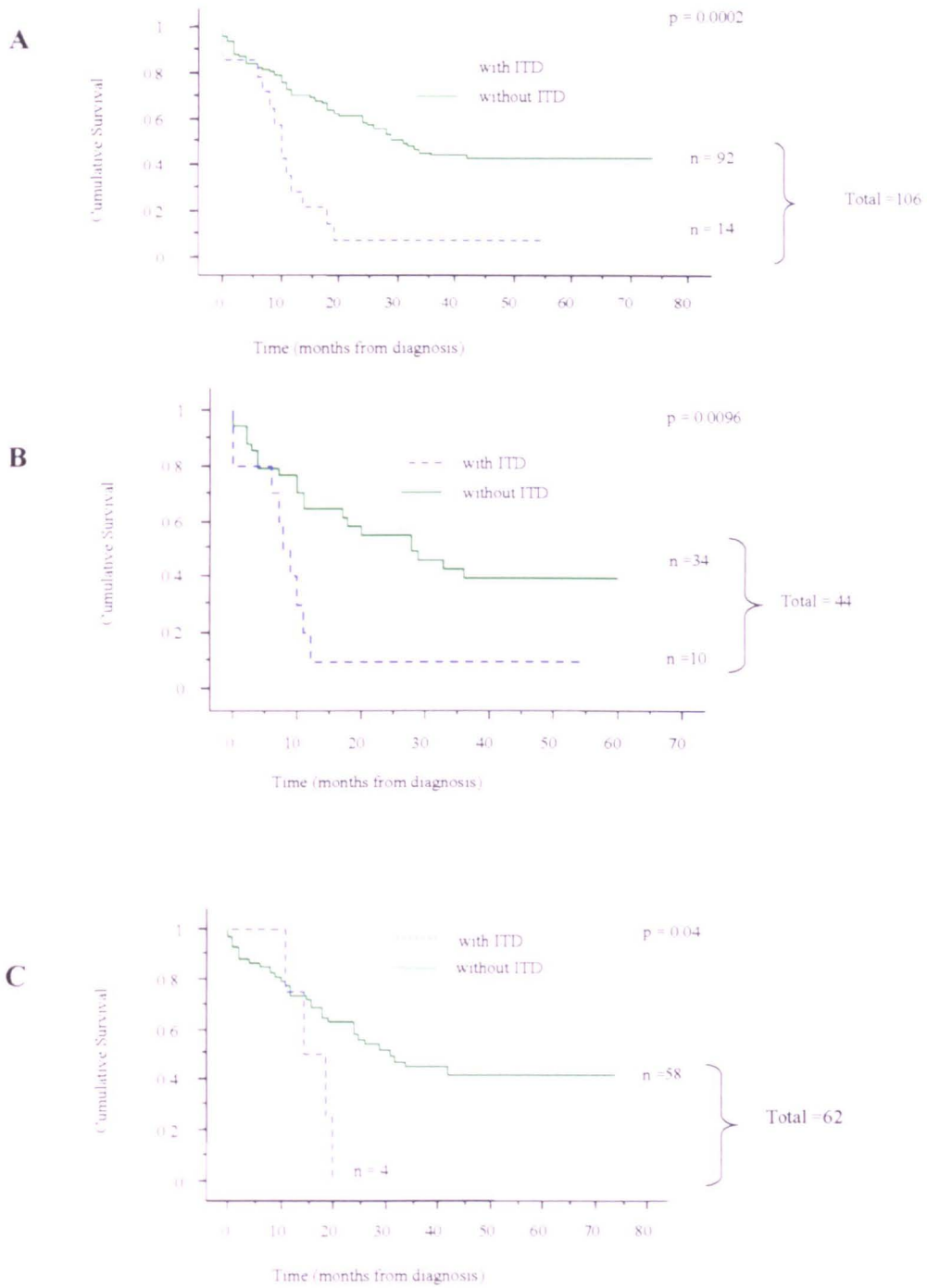

Figure 6.16 Kaplan-Meier survival plots for patients with (blue broken line) and without (solid green line) a *FLT3* ITD in three groups (according to cytogenetic criteria) of AML patients. (A) All patients, (B) Standard risk patients, (C) Good risk patients.

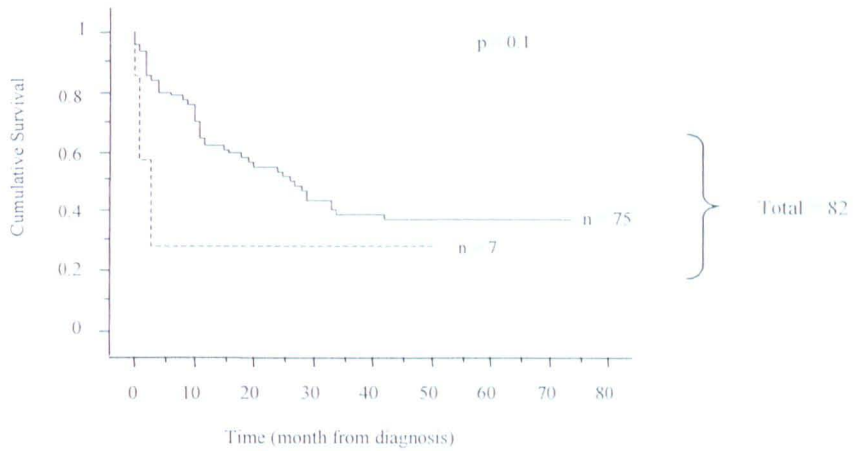

Figure 6.17 Kaplan-Meier cumulative survival plot of 82 AML patients. 75 AML patients lacking either a *FLT3* ITD or codon 835 mutation (solid line) and the seven AML patients possessing a *FLT3* codon 835 mutation (broken line). p-values were derived from the log-rank test (Mantel-Cox).

6.3 Discussion

6.3.1 Exon 9 and exon 11

FLT3 is a tyrosine kinase receptor with strong sequence similarity to *c-fms* and *c-kit* and other members of the RTK type III subfamily with a similar overall structure (Yarden and Ulrich, 1988). Interestingly, a number of *c-kit* mutations have been reported in the extracellular, juxtamembrane and tyrosine kinase domains of the receptor. An earlier study from our laboratory reported the presence of exon 8 in frame deletion plus insertion mutations in AML with inv(16), involving the highly conserved Asp419 codon (Gari *et al,* 1999). In the human mast cell leukaemia cell line HMC-1; Val560Gly and Asp816Val mutations have been reported in the tyrosine kinase domain (Furitsu *et al,* 1993). Furthermore Nagata and Longley, in two different studies reported the presence of Asp816Val mutation in cells from patients with aggressive mast cell disease (Nagata *et al,* 1995; Longley *et al,* 1996). Additionally Asp816Tyr substitution was reported recently in *c-kit* (Beghini et al, 1998). Importantly, the identification of this substitution provided the first direct evidence for such mutations leading to the development of human acute leukaemia (Beghini *et al*, 1998; Beghini *et al*, 2000b).

Similarly, mutations in codon 301 in the *c-fms* gene are believed to lead to a conformational change that mimics ligand binding, resulting in constitutive tyrosine activity (Ridge *et al,* 1990). Therefore, the homologous location in the *FLT3* gene, namely codon 350 in exon 9, was screened in our study to identify possible pathologically important mutations in this location. None of the AML or the IMF patients showed any mutations in exon 9, a result that excludes the presence of similar mutations to the codon 301 changes found in *c-fms* in both group of patients. Furthermore, in the homologous location to the codon 413 mutation found in the *c-fms* gene i.e codon 450 in exon 11 of the *FLT3* gene, a C→T polymorphism

was identified in the *FLT3* gene in the two patients groups (AML and IMF) as well as in the normal individuals at position 1300-3, a result that excludes the likelihood of an abnormal effect of this nucleotide change in the AML or the IMF patients.

6.3.2 ITD in AML and IMF patients

Little is known so far about the pathogenic consequences of the *FLT3* ITD, however, expression of mutant *FLT3* (containing ITD) in COS-7 cells was reported to induce constitutive autophosphorylation of the receptor (Kiyoi *et al,* 1998). Surprisingly, in a study carried out by Fenski et al (2000), it was not possible to correlate the constitutive autophosphorylation of the receptor with the ITD in some AML samples. Therefore, it was suggested that other mechanisms of activation might be operational, resulting from either mutation elsewhere in the gene or from autocrine mechanisms.

The presence of the *FLT3* ITD mutations in exon 14 and 15 have been reported previously by other groups (Nakao *et al,* 1996; Kiyoi *et al,* 1997). In the study performed by Nakao et al, (1996) further analysis of DNA samples obtained at complete remission shows that three patients, initially harbouring a *FLT3* ITD mutation, had no ITD mutations, a fact that confirms the somatic origin of this mutation. In the present study, it was shown that in these AML patients (Number: 5117, 5118 and 5031) the nucleotide sequence analysis revealed an in-frame insertion and amino acid duplication of almost perfect copies of flanking sequence derived completely from within exon 14. Furthermore, a close inspection of the ITD in a further AML patient (Number: 3627) indicated that the ITD spanned the exon 14-intron 14 boundary and that the duplicated sequence was similar to the template sequence from which it originated, differing by just three bases, including a T→A transversion 8 bp from the 3` end of the inserted sequence. It would appear that the T→A transversion is in a sequence derived from the original exon 14-intron 14 splice site consensus sequence. In fact, this

transversion interrupts an important GT dinucleotide consensus sequence, present in normal donor splice sites. Interestingly, if this important regulatory sequence was not disrupted, it would be expected that any similar event would result in a 'mutated' *FLT3* gene sequence capable of utilizing this duplicated splice site in preference to the normal site, and in such a case an entirely normal protein would be produced. Alternatively, it might be possible that a previously benign duplication event may undergo a secondary mutation (within the duplicated splice site) that, due to the growth advantage present as an outcome of both mutations, causes proliferation of this clone. On the other hand, it is possible that an inserted copy of the disrupted splice site will produce an altered allele that will cause the production of a mutated protein with the potential to cause uncontrollable growth of the clone in which it originates.

Data from the current study shows that *FLT3* ITD occurs in 12.6% of adults with AML at diagnosis, a frequency somewhat lower than the 20-25% published by other groups (Nakao *et al,* 1996; Yokota *et al,* 1997; Kiyoi *et al,* 1999; Kottaridis *et al,* 2001). However, it was noticed that patients in the good risk group (according to the cytogentic definition) had a lower incidence of the ITD than standard and high risk groups. This low incidence of the ITD mutations in the good risk group has been recently confirmed (Frohling *et al,* 2002; Thiede *et al,* 2002). Our interpretation of this low percentage of positive ITD in our AML patients is due to the presence of a high proportion of patients within the good risk group, when compared to other reports with high percentages of the *FLT3* ITD mutation.

6.3.3 Prognostic significance of the ITD

It is clear that *FLT3* ITD mutations are associated with an adverse prognosis and that this is independent of standard karyotype findings (Abu-Duhier *et al,* 2000). Out of the 106 AML cases analysed, fourteen cases were identified having *FLT3* ITD and possessed a

significantly lower survival when compared with patients without a *FLT3* ITD. Thirteen out of the 14 patients with a *FLT3* ITD died within 18 month of diagnosis. A similarly significant difference in survival has been also noticed by other groups (Kiyoi *et al*, 1999; Kottaridis *et al*, 2001; Gilliland and Griffin, 2002) especially in patients under the age of 60 (Kottaridis *et al*, 2001; Whitman *et al*, 2001; Frohling *et al*, 2002; Thiede *et al*, 2002). For example, Kottaridis *et al*, (2001) noted 231/854 (27%) patients to be positive for ITD. *FLT3* ITD mutations correlated with high leukocyte and blast cell counts, decreased remission induction rates ($p=0.005$), and decreased disease free survival (DFS), event free survival (EFS) and overall survival (OS) ($p< 0.001$ for each). Furthermore, *FLT3* ITD has been shown to be an adverse prognostic factor in two paediatric studies (Iwai *et al*, 1999; Meshinchi *et al*, 2001b). Overall, it would appear that AML patients possessing a *FLT3* ITD should be regards as having high risk disease, irrespective of cytogenetic and alternative therapy should be considered.

An interesting finding in the recent study by Kottaridis *et al*, (2001), is the marked variation in the incidence of *FLT3* ITD among FAB groups. The frequency was the highest in AML M3 i.e. t(15;17) at 36% and only 9% and 7% in t(8;21) and inv(16) respectively. A similar trend was noted in the present study but the lower percentage in M3 may reflect the small number studied for example, 10% of the t(15;17) and 6.3% and 5.4% of t(8;21) and inv(16) respectively. Importantly, the *FLT3* gene appears to be the most commonly mutated gene identified to date in AML. This findings gives impetus to the search for specific kinase inhibitors for the treatment of AML (Abu-Duhier *et al*, 2001b; Kottaridis *et al*, 2001).

6.3.4 Asp835 screening in AML and IMF patients

Previous studies have demonstrated that several *c-kit* mutations are associated with mast cell leukaemia and AML. Theses are located in two distinct regions, the JM domain and at Asp816, within the activation loop (A-loop) (Yamamoto *et al,* 2001). As mentioned earlier, Asp835 of the *FLT3* gene corresponds to Asp816 of c-kit. Furthermore the presence of an activating Asp816 mutation within the A-loop of c-kit has been reported in AML as well as in human mast cell leukaemia cell line HMC-1 (Furitsu *et al*, 1993; Beghini *et al*, 2000a).

To identify additional *FLT3* gene mutations, exon 20 was screened. The result indicates that Asp835 mutations occur in approximately 7% of AML patients. Recently the frequency of this mutation has been confirmed by two different groups (Inami *et al,* 2001; Yamamoto *et al,* 2001). It is noteworthy that no patient possessed both a *FLT3* ITD and an Asp835 mutation, that is they were mutually exclusive. Therefore, in the total of 97 AML cases screened for both mutations, 23% possessed *FLT3* mutation. However, in the study carried out by Yamamoto *et al*, (2001) one AML patient had both an Asp835 and an ITD. Further investigation revealed that these mutations occurred on different alleles. Similarly, although two different Asp835 mutations were identified in a further AML patient, each occurred on different alleles (Yamamoto *et al,* 2001).

Asp835 is highly conserved in RTKs and is thought to play an important role in receptor activation. It is hypothesised that Asp835 mutations can cause constitutive activation by causing an active conformation in the A-loop, similar to the constitutive activation caused by the substitution of Asp to Tyr or Val in *c-fms* (Asp802) and murine *FLT3* (Asp838). It is likely, therefore, that the aspartate within the A-loop is a key regulatory amino acid within the RTKs (Morley *et al,* 1999; Fenski *et al,* 2000). None of the IMF patients, or normal individuals screened had an Asp835 mutation. This finding agrees with that of Yamamoto *et*

al, (2001) which also showed that in one patient with Asp835 at initial diagnosis, the mutation was lost at CR. Therefore, these results confirmed that Asp835 mutation of *FLT3* are somatic mutations associated with leukaemia.

6.3.5 Survival analysis

6.3.5.1 ITD

Kaplan-Meier analysis showed that the *FLT3* ITD mutation was a strong adverse prognostic factor. In fact, the effect of the mutation were so significant that all patients, except one, died within 18 month of diagnosis. Patients lacking the mutation survived significantly longer (29.1 months) than those with an ITD (mean 12.9 months; p=0.0002).

Earlier studies on AML patients showed that cytogenetic data is the most important prognostic factor in AML (Grimwade *et al,* 1998). In the current study, patients were separated into two groups; a good risk group (n=62) and a standard risk group (n =44). The percentage of the *FLT3* ITD mutations were lower in the good risk group (6.5%) compared to the standard risk group (22.7%) and was of prognostic significance for the two cytogenetic groups. As mentioned earlier, this variation in the incidence of the *FLT3* ITD mutation was also noticed in different cytogenetic groups (Kottaridis *et al,* 2001).

6.3.5.2 Asp835

An Asp835 mutation was present in approximately 7% of AML patients (7/97), an incidence significantly lower than that of the ITD in 13.2 % (14/106). The Asp835 mutation alone was not found to be of prognostic significance in the initial cohort of 97 AML patients. Therefore, 185 further AML samples selected randomly have been screened to clarify if this low frequency of Asp835 mutation among the patients was due to our selection bias for good risk patients. In fact the percentage of Asp835 was lower (3.8%; 7 of 185) in this new group

of AML patients that was randomly selected. Therefore, it is expected that there is no prognostically significant effect of the Asp835 change in AML, a fact confirmed later by the finding of Yamamoto *et al,* (2001).

Chapter 7

General Discussion

Different groups of AML patients (A, B, C, and D) and a group of local IMF patients were investigated for the presence of genetic alteration in *c-fms* and selected exons of the *FLT3* gene. The establishment of CSGE analysis for the *c-fms* gene was carried out in this study. Furthermore, novel and previously identified polymorphic nucleotide alterations in the *c-fms* gene were investigated. In total, twelve different apparently polymorphic alterations were identified, five alterations that resulted in silent amino acid changes and 7 intronic nucleotide alterations. Three of these changes have been previously reported as polymorphisms. Furthermore, important mutations that have been previously identified in the *c-fms* gene (i.e 301 and 969 mutation) were also investigated. In addition, three novel nucleotide changes resulting in amino acid substitution were identified in exons 6, 8 and 9. In exon 6, a G→T substitution that predicts an amino acid change at codon 245 (Ala245Ser) was identified in two AML patients. None of the IMF or the normal individuals screened had this change. Exon 8 showed an A→G substitution in 13 patients (5 AML and 8 IMF) at position 1385, which predicts an amino acid change at codon 362 (His362Arg). However, normal individuals were also identified with the same change in exon 8 of the *c-fms* gene. In exon 9, a novel G→A substitution at codon 413 (Gly413Ser), was identified in four patients (2 AML and 2 IMF) out of the 143 patients (103 AML and 40 IMF) screened for exon 9 (2.8%). None of the normal individual screened had this change. Furthermore, none of the patients and normal individuals analysed had the 301 or the 969 mutation in *c-fms*.

In the other part of the study, which concerns the *FLT3* gene, it was shown, together with the data of Agnes et al, (1994) that the entire *FLT3* gene comprises 24, rather than the previously assumed 21 exons. In addition, the homologous location for codon 301 of *the c-fms* gene, namely codon 350 in exon 9 of the *FLT3* gene, was screened to identify possible

pathologically important mutations in this location. None of the AML or the IMF patients showed any mutations in exon 9, a result that excludes the presence of similar mutations to the codon 301 changes found in *c-fms* in both group of patients. Furthermore, the homologous location to the codon 413 mutation found in the *c-fms* gene i.e codon 450 in exon 11 of the *FLT3* gene was also investigated, similarly no mutation was identified in the *FLT3* gene. The homologous for codon 969 of the *c-fms* was not located in the *FLT3* coding sequence, therefore, no mutation analysis was performed.

Furthermore, the *FLT3* ITD was investigated in the AML and IMF patients. The data from this study showed that *FLT3* ITD occurs in 12.6% of adults with AML at diagnosis and that AML patients possessing a *FLT3* ITD should be regarded as having high risk disease, irrespective of cytogenetics. This study also identified the presence of an Asp835 mutation that occurs in approximately 7% of AML patients. None of the IMF patients, or normal individuals, screened had the Asp835 mutation. However, the Asp835 mutation was not found to be of prognostic significance in this group of AML patients, a result that was confirmed by others afterwards.

In general, this study has provided further evidences that class III RTKs are linked to the pathogenesis of a number of haematological malignancies. Indeed, *FLT3* is believed to be the target gene most commonly mutated in AML. Furthermore, the presence of the *FLT3* ITD appears to be the strongest independent prognostic factor in AML. Mutations of other RTKs of class III have also been linked to leukaemia, including *c-kit*, *PDGFRβ* and *c-fms*.

It is likely that the identification of additional pathogenically important RTKs will increase in the future. In addition, it is promising that targeting RTKs with specific inhibitors can lead to clinical improvement (Druker, 1999) and this approach may eventually lead to the

development of disease-specific therapy customized to the pattern of RTK expression of any given leukaemic clone (Gupta *et al,* 2002).

An association between the presence of *FLT3* ITD and poor prognosis has been reported in AML (Kiyoi *et al,* 1999; Rombouts *et al,* 2000). For example, in a survival study in patients under the age of 60 years, the *FLT3* ITD mutation was the strongest prognostic factor (Kiyoi *et al,* 1999; Kondo *et al,* 1999; Kottaridis *et al,* 2001). In childhood AML, a similar result was also reported (Kondo *et al,* 1999) while a significant reduction in the remission rate in the *FLT3* ITD population has been documented (Rombouts *et al,* 2000). Furthermore, a recent study reported that *FLT3* ITD was the most important factor predicting relapse following complete remission (CR) (Kottaridis *et al,* 2001). A similar paediatric AML study reported that no child with a *FLT3* mutation achieved complete remission (Meshinchi *et al,* 2001). *FLT3* ITD is thought to be the most common mutation described in AML (Abu-Duhier *et al,* 2000). However, the most common cause for treatment failure in AML is relapse (Kottaridis *et al,* 2001), therefore, identifying patients with a high risk of relapse would be very helpful, and could lead to the introduction of alternative therapy for such patients (Kottaridis *et al,* 2001).

Recently, ideas have been built up with regard to the role of *FLT3* mutations in leukaemia, although the mechanism of *FLT3* activation has not been, so far, fully elucidated. However, insights into the role of *FLT3* mutation can be gleamed from the study of other non-receptor and receptor tyrosine kinases, such as Eph (Ephrins) (Stapleton *et al,* 1999; Binns *et al,* 2000; Hubbard, 2001; Wybenga-Groot *et al,* 2001), Epidermal growth factor receptor 2 (Burke *et al,* 1997; Burke and Stern, 1998), insulin-like growth factor-1 receptor (IGF1R) (Favelyukis *et al,* 2001), fibroblast growth factor receptor 1 (Mohammadi *et al,* 1996; Mohammadi *et al,* 1997) and EGF receptors (Weiss and Schlessinger, 1998). As discussed in chapter 1, it appears that the kinase domain is maintained in an inactive conformation by the effect of the

domain within the receptor tyrosine kinase. The A-loop folds into the active sites preventing access of ATP and substrates. However, it is believed that the activated A-loop folds out of the active site as the result of phosphorylation of critical amino acids thereby allowing access to ATP and substrate. Favelyukis *et al,* (2001) have shown that intermolecular autophosphorylation of specific residues in the A-loop of IGF1R stabilizes the loop in a conformation that facilitates catalysis. Furthermore, prevention of autoinhibition has been shown, by substitution mutation, to reside at a highly conserved aspartic acid residue which increases the ability of the unphosphorylated kinase to bind to the ATP (Till *et al,* 2001). In addition, the JM domain is another autoinhibitory domain in a subset of RTKs. In the Eph receptor for example, it has been shown that, in addition to the autoinhibitory activity of the A-loop, the JM domain contains a second autoinhibitory domain regulated by tyrosine phosphorylation (Binns *et al,* 2000). The mechanisms of autoinhibtion were determined by structural analysis of the JM and kinase domain of an autoinhibited unphosphorylated form of EphB2 (Wybenga-Groot *et al,* 2001). It was shown that the A-loop in the receptor was blocked from achieving an activated conformation due to the presence of a helical conformation in the JM domain. Phosphorylation of the conserved JM tyrosine residues, however relieved the association of the JM segment with the kinase domain and liberated phosphotyrosine sites for binding with the SH2 domain of target proteins (Wybenga-Groot *et al,* 2001).

It has been suggested, therefore, that activation in some class III RTKs (i.e. *c-kit* and *FLT3*) could result from mutations in the JM region which may cause the JM domain to fall away from the kinase, permitting kinase activation, transphosphorylation, and initiation of signalling (Gilliland and Griffin, 2002). Furthermore, this model of activation could explain how different types of mutation within the JM domains of different RTKs leads to receptor activation (Gilliland and Griffin, 2002).

As discussed earlier, it has been established that either length mutations in the juxtamembrane domain (i.e. ITD) or activating mutations within the A-loop (Asp835), result in constitutive activation of the *FLT3* kinase. Furthermore, STAT5 and RAS/MAPK pathway have been shown to be activated by *FLT3* ITD mutation (Hayakawa *et al,* 2000; Mizuki *et al,* 2000). Recently, Kelly *et al,* (2002) using a bone marrow transplant (BMT) assay have shown that retroviral transduction of *FLT3* ITD mutation into primary murine bone marrow cells results in a myeloproliferative phenotype (Kelly *et al*, 2002b). Furthermore, a leukaemic phenotype occurred in syngenic recipient mice following the injection of 32D or Ba/F3 cells stably transfected with constitutively activated *FLT3* (Kelly *et al*, 2002b). Similarly Asp835 mutations in *FLT3* result in constitutive kinase activation (Yamamoto *et al*, 2001).

7.1 Is there a cure?

Due to the high frequency and poor prognosis of *FLT3* mutations in AML, there is intense study to develop suitable inhibitors that can safely be used in a leukaemic patients. The recent reports of STI571 (Signal transduction inhibitor 571) as an bcr-abl kinase inhibitor in CML have encouraged a similar approach towards the inhibition of *FLT3* (Reilly, 2002). Initial work has been carried out using cell cultures and murine models of leukaemia mediated by *FLT3* ITD (Levis *et al,* 2001; Naoe *et al,* 2001; Tse *et al,* 2001). Two tyrophostin drugs have been shown to possess *FLT3* inhibitory activity, namely AG1296 and AG1295 (Levis *et al,* 2001; Tse *et al,* 2001). AG1296, a selective inhibitor of *FLT3*, *KIT* and *PDGFRβ*, inhibits the growth of Ba/F3 transformed by *FLT3* ITD (Tse *et al,* 2001). It has also been shown that STAT5A/B activation and other downstream molecules, can be inhibited, together with *FLT3* ITD autophosphorylation, by AG1296 (Tse *et al,* 2001). In

addition, a related compound AG1295 can also inhibit *FLT3*, and has been shown to have activity against primary AML blasts harbouring *FLT3* ITD (Tse *et al*, 2001).

Therefore, tyrophostin, although too toxic to be considered for clinical use in humans, provides clear evidence that *FLT3* inhibition may be an effective approach for treatment of a subset of leukaemic patients (Gilliland and Griffin, 2002). Currently, there is intensive activity to find a suitable inhibitor for mutated *FLT3* in humans. Recently, a few promising reports have emerged. For example, CEP-701 (Cephalon compound), an orally bioavailable inhibitor of both *FLT3* ITD and the A-loop mutants, has been shown to have activity in a murine model that involved injection of *FLT3* ITD transfected Ba/F3 cells (Allebach *et al*, 2001). More importantly, primary AML cells containing *FLT3* ITD, also appear to respond (Levis *et al*, 2002). Furthermore, CT53518, a known *PDGFRβ* and *c-kit* inhibitor, has also been shown to inhibit *FLT3* ITD, although it has no activity against the Asp835 A-loop mutants (Yu *et al*, 2001).

SU5614, developed by SUGEN Inc, has also been also been shown to posses inhibitory activity for *FLT3* in that it selectively induces growth arrest, apoptosis and cell cycle arrest in Ba/F3 and *FLT3* mutated AML cell lines. In addition, SU5614 reverses the anti-apoptotic and proliferative activity of *FLT3* ligand (FL) in FL-dependant cells. Interestingly, SU5614 has not shown cytotoxic activity in leukaemic cell lines which express either a non-activated *FLT3* or a total lack of *FLT3* protein. STAT3, STAT5 and MAPK are also downregulated by SU5614 (Spiekermann *et al*, 2002b). Two other SUGEN Inc, inhibitors (SU11248 and SU5416) have been reported to have activity in inhibition of *FLT3* ITD transformed cells *in vitro* and *in vivo* (O'Farrell *et al*, 2001; Yee *et al*, 2002). PKC412, from Novartis Pharmaceuticals, is a benzoylstaurosporine that was originally developed as a VEGFR inhibitor, and was tested with minimal toxicity in a phase I trial in solid tumour patients. In addition to its effect on VEGER, PKC412 is an inhibitor of several kinases including protein

kinase C (PKC) and spleen tyrosine kinase (SYK), and is an effective submicromolar inhibitor of *FLT3* ITD in cell culture and murine model of leukaemia (Weisberg *et al,* 2002).

Overall, *FLT3* mutations occur in about 30% of AML cases. N-RAS and K-RAS mutations account for another 20% of AML cases (Gilliland and Griffin, 2002), while A-loop mutations have been reported at codon 816 in *c-kit* in 5% of cases (Beghini *et al,* 2000), and *c-kit* exon 8 insertion/deletions mutation can also occur (Gari *et al,* 1999). However, there are several lines of evidence indicating that additional mutations are required for the development of an AML phenotype. For instance, in a murine bone marrow transplant assay, mutant *FLT3* expression was not sufficient by itself to cause AML. However, mutant *FLT3* shows a transforming activity similar to other constitutively activated tyrosine kinase such as BCR/ABL, TEL/ABL, TEL/PDGFR and TEL/JAK2 (Gilliland and Griffin, 2002). It has been noticed previously that these fusions are associated with chronic myeloproliferative phenotypes in humans and result in a myeloproliferative disorder when expressed in animal models. Although the constitutively activated tyrosine kinase is required for the myeloproliferative phenotype, it does not, by itself, cause AML, which is characterized by impaired differentiation of haematopoietic progenitors. Therefore, it has been suggested that additional mutations are required, in addition to activating mutations in *FLT3* for the development of AML (Gilliland and Griffin, 2002).

This hypothesis is supported by the finding that *FLT3* mutations are frequently accompanied by other gene re-arrangements and point mutations that result in maturation block. For example, *FLT3* ITD has been reported in patients with t(8;21), inv(16), t(15;17), 11q23 gene re-arrangements involving MLL, and MLL internal tandem repeat mutations (Kiyoi *et al,* 1997; Jamal *et al,* 2001; Kottaridis *et al,* 2001; Nomdedeu *et al,* 2001; Thiede *et al,* 2002). These translocations result in expression of fusion genes, including the *AML1/ETO*, *CBFβ/SMMHC* and *PML/RARα*, which although not sufficient to cause leukaemia by

themselves (Castilla *et al,* 1999), contribute to impaired differentiation of haematopoietic cells (Castilla *et al,* 1996; Yergeau *et al,* 1997; Okuda *et al,* 1998).

The above data support the concept of "two-hit" model for AML in which the *FLT3* ITD enhances proliferation and/or survival through activation of the STAT, RAS/MAPK and PI3K/AKT pathways, while the expression of fusion proteins such as *AML1/ETO* result in impaired differentiation and produce the AML phenotype (Dash and Gilliland, 2001).

It, therefore, thought that the use of drug combinations that inhibit class I (mutations that result in constitutively activated tyrosine kinase, and do not affect differentiation) and class II (mutations that result in impaired haematopoietic differentiation, but are not sufficient to cause leukaemia) mutations may be very successful in the treatment of AML. For example, drugs that inhibit *FLT3* may be highly effective in combination with ATRA for treating APL associated with the PML/RARα gene rearrangement (Gilliland and Griffin, 2002).

These recent finding of new, and important, RTK class III mutations are providing a greater understanding of the pathogenesis of AML. Furthermore, combining this data with our growing knowledge of the different signalling components and the various pathways activated by oncogenic tyrosine kinases may provide us with a better understanding of the variety of biological processes that are controlled by these kinases.

Although there are more mutations being discovered within the *FLT3* gene such as the one recently reported in the A-loop at codon 840 (Spiekermann *et al*, 2002a), a relevant question is what are the class I mutations in AML patient that lack a *FLT3* or *c-kit* mutation. This is being investigated in our laboratory for patients with inv(16). A total of 110 cases of AML, exhibiting either inv(16) or t(8;21), were screened for *c-kit* (exon 8 insertion/deletion and Asp816) and *FLT3* (ITD and Asp835) gene mutations. Approximately 40% of AML and inv(16) possessed a class I mutation involving either the *c-kit* or the *FLT3* gene, a finding

that supports the two-hit pathogenic model for CBF AML. The nature of the class I mutation in the remaining 60% of inv(16) cases, to enable the development of full-blown leukaemia is presently unknown. Interestingly, however the RAS gene mutations, have been reported in 48% patients with inv(16) (Schnittger *et al*, 2001).

It is noteworthy that a large number of intracellular signalling proteins bind the phosphotyrosine on the activated RTKs, including the GTPase-activated protein (GAP), phospholipase C-γ (PLC-γ), phosphotidylinositol 3`-kinase (PI3K), growth factor receptor binding protein 2(Grb2) and Src-like non-receptor tyrosine-kinases (Alberts *et al*, 1994; Porter and Vaillancourt, 1998; McCubrey *et al*, 2000). In addition, the activation of these proteins is known to initiate the serine/threonine phosphorylation cascades, resulting in activation of the transcription factors and modulation of cellular processes by gene transcription (McCubrey *et al*, 2000). For instance, Ras signalling cascades are known to be involved the activation of serine/threonine kinases (McCubrey *et al*, 2000). There are three human Ras genes (H-ras, N-ras and K-ras) that localize to the inner surface of the plasma membrane and function as GDP/GTP-regulated switches (Campbell *et al*, 1998). Furthermore, Ras proteins are known to be crucial to a number of signalling pathways including those mediated by RTKs. Autophosphorylation of specific tyrosine residues could be initiated by ligand binding causing the existence of phosphotyrosyl binding sites for the SH2 domain of adaptor proteins such as Shc and Grb2 which, through their interaction with other proteins, then activate Ras (Prendergast and Gibbs, 1994; Campbell *et al*, 1998). Therefore Ras activation will transfer the signal to Raf serine/threonine kinases and then down a cascade of cytoplasmic proteins (Campbell *et al*, 1998). The activity of an antiapoptotic protein of the Bcl-2 family was found to be influenced by the serine/threonine kinases (Blagosklonny *et al*, 1999; McCubrey *et al*, 2000). It was demonstrated that in

normal cell cycle Bcl-2 becomes serine/threonine phosphorylated at several sites during the G2 to M phase transition (Haldar *et al*, 1997; McCubrey *et al*, 2000).

In general activation or over expression of RTKs were seen in several cancers including leukaemia (Drexler, 1996; Porter and Vaillancourt, 1998). The involvement of serine/threonine signalling pathways in leukaemogenesis is a complex process that is not yet fully understood (McCubrey *et al*, 2000). However, mutations and uncontrolled activation of Ras have been documented in different type of human leukaemia (Janssen *et al*, 1987; Ahuja *et al*, 1990; Hirsch-Ginsberg *et al*, 1990; Imamura *et al*, 1993; Sawyers *et al*, 1995; Beaupre and Kurzrock, 1999). It was thought therefore, that termination of the pathways enhancement that lead to cellular proliferation and the dysfunctional suppression of the pathways leading to apoptosis and cell cycle arrest play an important role in the pathogenesis of leukaemia (Porter and Vaillancourt, 1998; McCubrey *et al*, 2000). Tyrosine kinase inhibitors were introduced for leukaemia therapy due to their activity in different cellular pathways in the cellular signalling cascade. There are two classes of tyrosine kinase inhibitors that are being developed: inhibitors of the tyrosine kinase ATP binding site and inhibitors of the substrate binding site (Levitzki and Gazit, 1995; Klohs *et al*, 1997; Levitt and Koty, 1999). Furthermore, other methods, although less specific, were also introduced for kinase inhibition. These include down regulation of kinase expression using antisense molecules and inhibition of ligand binding using monoclonal or polyclonal antibodies (figure 7.1) (Ravandi *et al*, 2002).

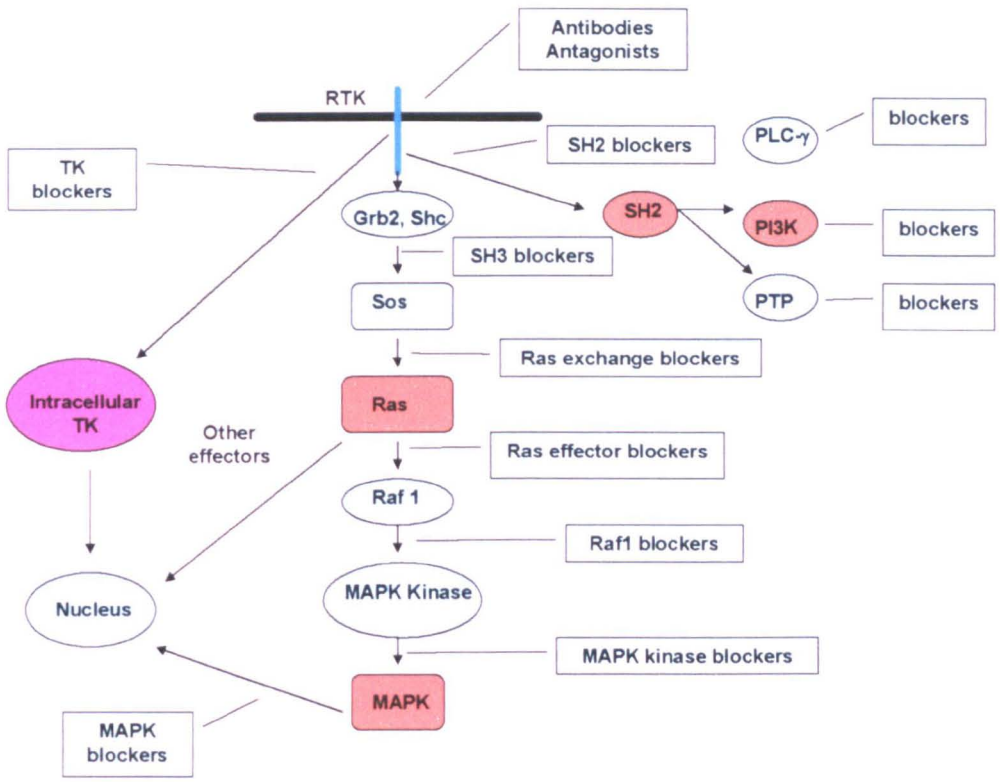

Figure 7.1. Targets for modulation of RTK signalling. Possible targets of the tyrosine kinase receptor pathways that can be interrupted by a number of agents under development.

Adapted from (Ravandi et al, 2002).

7.2 Future work

In relation to the *c-fms* gene analysis, large-scale epidemiological studies might be needed to assess the importance and influence of each polymorphism (Schork et al, 2000). In addition, further analysis at the RNA level would be required for the TT→AG change at position 1189+67-68 in intron 6 due to its potential functional relevance (discussed in chapter 3). A larger group of patients should be analysed in order to clarify the occurrence of the identified alteration in different FAB groups of AML. While, functional analysis for the effect of some of these alterations should be performed in order to gather a clear idea concerning its effect in AML and IMF.

Recently, an important question was raised regarding the presence of activating mutations in FLT3 in other myeloproliferative disorder such as IMF (Kelly et al, 2002b). This study has provided the result of *FLT3* analysis in a group of IMF patients and showed that no *FLT3* ITD or codon 835 mutations were present in this group of patients (Abu-Duhier *et al*, 2002). However, it would be interesting to carry out mutational analysis for the entire *FLT3* gene to identify any other mutations that might cause receptor activation.

In addition, prediction of transcription factor binding sites was an essential step for the analysis of gene regulatory networks. Seven possible transcription factor binding sites were predicted for FLT3 in this study, however, it is tremendously important to perform an *in vitro* transcription studies to confirm the role of these transcription factor binding sites. Furthermore, it is also important to screen the promoter region of the FLT3 to identify any variation that could change the transcriptional activity of the FLT3.

In general, there are many interesting questions that still need an answer, for instance, what other tyrosine kinases that are activated in AML patients that do not have *FLT3* or *c-kit*

mutation, similarly this question might be valid for other myeloproliferative disorders. Furthermore, another question was brought up recently in regards to the identification of signalling pathways used by tyrosine kinase oncogene that are either unique or shared, and required for transformation (Scheijen and Griffin, 2002).

Furthermore, more *in vitro* and clinical studies for signalling proteins would expand our knowledge of the complex cellular communication pathways and lead into the identification of more specific and possibly less toxic therapy for leukaemia.

It is possible that in the future, all leukaemia patients will have detailed genotypic analysis to identify the full spectrum of tyrosine kinase mutations, rearrangements, single nucleotide polymorphisms in genes that will influence the outcome or response to therapy. Eventually, these data may collectively help us to produce a "finger print" for each leukaemic patient, which ultimately will determine treatment and prognosis (Kelly *et al,* 2002a).

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Appendices

Appendix A. Details of AML patients in group A representing all FAB classification.

Number	Age	Survival	Sex	FAB	Mutation	Cytogenetics
5003	19	10	F	M1	ITD	OCA
5004	48	61	M	M3		t(15;17)
5010	39	1	M	M3		t(15;17)
5012	45	1	F	M5		OCA
5013	23	29	F	M2		inv(16)
5017	52	60	M	M3		t(15;17)
5018	42	60	F	M1		OCA
5021	55	2	M	M1		46;XY
5022	18	29	M	M5		OCA
5023	43	9	M	M4		inv(16)
5024	16	16	F	M4		OCA
5025	52	24	M	M4	c-kit	inv(16)
5026	56	2	M	M1		inv(16)
5027	51	24	M	M3		t(15;17)
5030	42	5	M	M1		OCA
5031	49	8	F	M4	ITD	46;XX
5032	49	60	F	M1		46;XX
5033	23	60	F	M5		46;XX
5034	54	2	F	M2		t(8;21)
5035	17	28	M	M6		46;XY
5040	37	10	F	M4		OCA
5041	48	3	F	M4	835	46;XX
5047	34	58	F	M2	c-kit	t(8;21)
5050	51	58	F	M4		46;XX
5051	19	18	F	M3	ITD	t(15;17)
5052	47	2	F	M2		46;XX
5054	57	4	M	M4		46;XY
5057	45	42	F	M3		t(15;17)
5059	58	57	M	M2		46;XY
5061	36	11	M	M5	ITD	46;XY
5063	40	57	M	M2		46;XY
5072	56	56	F	M2		OCA
5073	33	56	F	M4		inv(16)
5074	48	10	F	M2		OCA
5077	56	0	F	M4	ITD	46;XX
5079	42	7	M	M4	ITD	46;XY
5081	42	56	M	M2		inv(16)
5086	54	56	F	M3		t(15;17)
5093	49	10	F	M3		t(15;17)
5095	49	56	M	M2		46;XY
5097	58	56	M	M4	c-kit	inv(16)
5101	52	6	F	M1	ITD	46;XX
5104	32	1	M	M3	835	t(15;17)
5105	48	0	F	M5		OCA
5117	35	0	F	M5	ITD	46;XX
5118	29	9	M	M1	ITD	OCA
5124	15	55	F	M3		t(15;17)
5132	34	55	M	M5	ITD	OCA
5158	59	11	M	M0		OCA
5160	24	7	F	M5		OCA

Appendix A. Details of AML patients in group A representing all FAB classification

Number	Age	Survival	Sex	FAB	Mutation	Cytogenetics
5174	42	18	M	M0		t(9;11)
5191	54	0	M	M5		OCA
5195	33	53	M	M5		OCA
5200	32	52	M	M6		46;XY
5228	53	1	F	M0		OCA
5308	49	33	M	M6		46;XY
5318	23	4	M	M0		46;XY
5370	24	48	M	M6	835	OCA
5528	58	14	M	M6		OCA
5532	52	20	M	M6		OCA

See appendix D for abbreviations details

Appendix B. Details of AML patients in group B (patients with inv(16) or t(8;21) (cytogenetic abnormality).

Number	Age	Survival	Sex	FAB	Mutation	Cytogenetics
1468	51	18	M	M4		
1511	45	74	F	M4		inv(16)
1514	53	74	M	M4		t(8;21)
1521	41	74	F	M2		t(8;21)
1592	42	19	M	M2	c-kit	inv(16)
1622	26	72	M	M4		inv(16)
1645	44	34	M	M2		t(8;21)
1695	26	12	F	M4		inv(16)
1704	47	69	F	M2		t(8;21)
1735	40	14	M	M4	ITD	inv(16)
1799	31	8	M	M2		t(8;21)
1920	32	1	M	M4	835	inv(16)
1947	29	32	M	M4		inv(16)
3570	56	15	F	M4	c-kit	inv(16)
3627	74	10	F	M1	ITD	inv(16)
3710	56	0	M	M1		inv(16)
5134	53	55	F	M2	c-kit	inv(16)
5153	46	11	F	M4		inv(16)
5224	29	51	M	M2		t(8;21)
5269	51	50	M	M4	835	inv(16)
5341	41	0	M	M4	c-kit	inv(16)
5369	48	11	M	M2		t(8;21)
5400	62	4	M	M4		inv(16)
5449	16	19	M	M1	ITD	t(8;21)
5607	20	40	M	M2		t(8;21)
5615	30	16	M	M2		t(8;21)
5620	57	26	M	M4		inv(16)
5722	49	37	F	M4		inv(16)
5771	37	36	M	M2		t(8;21)
5859	40	6	F	M4	c-kit	inv(16)
5970	38	25	M	M4		inv(16)
6162	35	18	F	M4	c-kit	inv(16)
6185	23	37	F	M2		t(8;21)
6202	38	37	M	M4		inv(16)
6262	58	2	M	M4		inv(16)
6286	31	23	F	M2		inv(16)
6294	34	23	M	M4		inv(16)
6357	32	21	F	M4	c-kit	inv(16)
6428	49	19	F	M5		inv(16)
6442	51	20	M	M4		inv(16)
6552	49	12	M	M1	c-kit	inv(16)
6581	39	15	M	M2		inv(16)
6621	21	13	M	M		inv(16)

Appendix C. Details of AML group C1 and C2

AML group C1 (patients from RHH)

Number	Age	Survival	Sex	FAB	Mutation	Cytogenetics
GB310798	30	18	M	M2		46;XY
TB050899	56	6	M	M0		46;XY
MG040397	30	36	M	M5		46;XY
TH050697	59	33	M	M2		OCA
SJ170898	49	17	M	M2		t(8;21)
DL030297	23	31	F	M2		t(8;21)
LP051197	28	12	M	M4	ITD	OCA
JR071197	44	28	F	M2		46;XX

AML group C2 (patients from RHH)

Number	Age	Survival	Sex	FAB	Mutation	Cytogenetics
WA050598	79	2	M	M0		46;XY
PC080997	49	27	M	M4		46;XY
VG070598	73	0	F	M4	835	46;XX
PG131197	26	3	M	M4	835	inv(16)
NG270898	70	3	M	M1		46;XY
JH130598	65	17	M	M0		46;XY
JJ060499	74	9	M	M1		46;XY
AK110199	84	11	M	M1		46;XY
EL080997	85	1	F	M1		OCA
AW011097	28	0	M	M5	ITD	OCA
SW240899	69	4	F	M1	ITD	46;XX
DW110898	71	2	M	M2		t(8;21)

Appendix D. Details of all AML patients (group A, B, C1 and C2) included in the study

Number	Age	Survival	Sex	FAB	FLT3 mutations		c-fms	c-kit	Cytogenetics
					ITD	Asp835			
1468	51	18	M	M4	NPCR	ND	NPCR	Neg	
1511	45	74	F	M4	NPCR	ND	NPCR	Neg	inv(16)
1514	53	74	M	M4	Neg	Neg	Neg	Neg	t(8;21)
1521	41	74	F	M2	Neg	NPCR NIS	Neg	Neg	t(8;21)
1592	42	19	M	M2	Neg	Neg	Neg	Exon 8	inv(16)
1622	26	72	M	M4	Neg	Neg	Neg	Neg	inv(16)
1645	44	34	M	M2	Neg	Neg	Neg	Neg	t(8;21)
1695	26	12	F	M4	Neg	Neg	Neg	Neg	inv(16)
1704	47	69	F	M2	Neg	Neg	Neg	Neg	t(8;21)
1735	40	14	M	M4	ITD	Neg	Neg	Neg	inv(16)
1799	31	8	M	M2	Neg	Neg	Neg	Neg	t(8;21)
1920	32	1	M	M4	Neg	835	NPCR	Neg	inv(16)
1947	29	32	M	M4	Neg	NPCR NIS	Neg	Neg	inv(16)
3570	56	15	F	M4	Neg	Neg	Neg	Exon 8	inv(16)
3627	74	10	F	M1	ITD	Neg	Neg	Neg	inv(16)
3710	56	0	M	M1	Neg	Neg	Neg	Neg	inv(16)
5003	19	10	F	M1	ITD	Neg	Neg	Neg	OCA
5004	48	61	M	M3	Neg	Neg	Neg	Neg	t(15;17)
5010	39	1	M	M3	Neg	Neg	Neg	Neg	t(15;17)
5012*	45	1	F	M5	Neg NIS	ND	Neg	Neg	OCA
5013	23	29	F	M2	Neg	Neg	Neg	Neg	inv(16)
5017	52	60	M	M3	Neg	Neg	Neg	Neg	t(15;17)
5018	42	60	F	M1	Neg	Neg	Neg	Neg	OCA
5021	55	2	M	M1	Neg	Neg	Neg	Neg	46;XY
5022	18	29	M	M5	Neg	Neg	Neg	Neg	OCA
5023	43	9	M	M4	Neg	Neg	Neg	Neg	inv(16)
5024*	16	16	F	M4	Neg NIS	ND	NPCR	Neg	OCA
5025	52	24	M	M4	Neg	Neg	Neg	Exon 8	inv(16)
5026	56	2	M	M1	Neg	Neg	245 (E6)	Neg	inv(16)
5027	51	24	M	M3	Neg	NPCR NIS	Neg	Neg	t(15;17)
5030*	42	5	M	M1	Neg NIS	ND	NPCR	Neg	OCA
5031	49	8	F	M4	ITD	NPCR NIS	Neg	Neg	46;XX
5032	49	60	F	M1	Neg	NPCR NIS	Neg	Neg	46;XX
5033	23	60	F	M5	Neg	Neg	Neg	Neg	46;XX
5034	54	2	F	M2	Neg	ND	Neg	Neg	t(8;21)
5035	17	28	M	M6	Neg	Neg	NPCR	Neg	46;XY
5040	37	10	F	M4	Neg	Neg	Neg	Neg	OCA
5041	48	3	F	M4	Neg	835	Neg	Neg	46;XX
5047	34	58	F	M2	Neg	Neg	Neg	Exon 8	t(8;21)
5050	51	58	F	M4	Neg	Neg	Neg	Neg	46;XX
5051	19	18	F	M3	ITD	Neg	Neg	Neg	t(15;17)
5052	47	2	F	M2	Neg	Neg	Neg	Neg	46;XX
5054	57	4	M	M4	Neg	Neg	Neg	Neg	46;XY
5057	45	42	F	M3	Neg	Neg	Neg	Neg	t(15;17)
5059	58	57	M	M2	Neg	Neg	Neg	Neg	46;XY
5061	36	11	M	M5	ITD	Neg	Neg	Neg	46;XY
5063	40	57	M	M2	Neg	Neg	Neg	Neg	46;XY
5072	56	56	F	M2	Neg	Neg	NPCR	Neg	OCA
5073	33	56	F	M4	Neg	Neg	Neg	Neg	inv(16)
5074	48	10	F	M2	Neg	Neg	Neg	Neg	OCA

Appendix D. Details of all AML patients (group A, B, C1 and C2) included in the study

Number	Age	Survival	Sex	FAB	FLT3 mutations		c-fms	c-kit	Cytogenetics
					ITD	Asp835			
5077	56	0	F	M4	ITD	Neg	Neg	Neg	46;XX
5079	42	7	M	M4	ITD	Neg	Neg	Neg	46;XY
5081	42	56	M	M2	Neg	Neg	Neg	Neg	inv(16)
5086	54	56	F	M3	Neg	Neg	Neg	Neg	t(15;17)
5093	49	10	F	M3	Neg	Neg	Neg	Neg	t(15;17)
5095	49	56	M	M2	Neg	Neg	NPCR	Neg	46;XY
5097	58	56	M	M4	Neg	Neg	NPCR	Exon 8	inv(16)
5101	52	6	F	M1	ITD	Neg	NPCR	Neg	46;XX
5104	32	1	M	M3	Neg	835	413	Neg	t(15;17)
5105	48	0	F	M5	NPCR	Neg	Neg	Neg	OCA
5117	35	0	F	M5	ITD	Neg	Neg	Neg	46;XX
5118	29	9	M	M1	ITD	Neg	Neg	Neg	OCA
5124	15	55	F	M3	NPCR	ND	NPCR	Neg	t(15;17)
5132	34	55	M	M5	ITD	Neg	Neg	Neg	OCA
5134	53	55	F	M2	Neg	Neg	Neg	Exon 8	inv(16)
5153	46	11	F	M4	Neg	Neg	Neg	Neg	inv(16)
5158	59	11	M	M0	Neg	Neg	Neg	Neg	OCA
5160	24	7	F	M5	Neg	NPCR NIS	Neg	Neg	OCA
5174	42	18	M	M0	Neg	Neg	Neg	Neg	t(9;11)
5191	54	0	M	M5	NPCR	ND	NPCR	Neg	OCA
5195	33	53	M	M5	Neg	Neg	NPCR	Neg	OCA
5200	32	52	M	M6	Neg	Neg	NPCR	Neg	46;XY
5224	29	51	M	M2	Neg	Neg	Neg	Neg	t(8;21)
5228*	53	1	F	M0	Neg NIS	ND	Neg	Neg	OCA
5269	51	50	M	M4	Neg	835	Neg	Neg	inv(16)
5308	49	33	M	M6	Neg	Neg	245(E6)	Neg	46;XY
5318	23	4	M	M0	Neg	Neg	Neg	Neg	46;XY
5341	41	0	M	M4	Neg	Neg	413	Exon 8	inv(16)
5369	48	11	M	M2	Neg	Neg	Neg	Neg	t(8;21)
5370	24	48	M	M6	Neg	835	NPCR	Neg	OCA
5400	62	4	M	M4	Neg	Neg	Neg	Neg	inv(16)
5449	16	19	M	M1	ITD	Neg	Neg	Neg	t(8;21)
5528*	58	14	M	M6	Neg NIS	ND	Neg	Neg	OCA
5532	52	20	M	M6	NPCR	Neg	Neg	Neg	OCA
5607	20	40	M	M2	Neg	Neg	Neg	Neg	t(8;21)
5615	30	16	M	M2	Neg	Neg	Neg	Neg	t(8;21)
5620	57	26	M	M4	Neg	Neg	Neg	Neg	inv(16)
5722	49	37	F	M4	NPCR	ND	NPCR	Neg	inv(16)
5771	37	36	M	M2	Neg	Neg	Neg	Neg	t(8;21)
5859	40	6	F	M4	Neg	Neg	Neg	Exon 8	inv(16)
5970	38	25	M	M4	Neg	Neg	Neg	Neg	inv(16)
6162	35	18	F	M4	Neg	NPCR NIS	Neg	Exon 8	inv(16)
6185	23	37	F	M2	Neg	Neg	Neg	Neg	t(8;21)
6202	38	37	M	M4	Neg	NPCR NIS	Neg	Neg	inv(16)
6262	58	2	M	M4	Neg	Neg	NPCR	Neg	inv(16)
6286	31	23	F	M2	Neg	NPCR NIS	NPCR	Neg	inv(16)
6294	34	23	M	M4	Neg	NPCR NIS	Neg	Neg	inv(16)
6357	32	21	F	M4	Neg	NPCR NIS	Neg	Exon 8	inv(16)
6428	49	19	F	M5	NPCR	NPCR NIS	Neg	Neg	inv(16)
6442	51	20	M	M4	Neg	NPCR NIS	Neg	Neg	inv(16)

Appendix D. Details of all AML patients (group A, B, C1 and C2) included in the study

Number	Age	Survival	Sex	FAB	FLT3 mutations		c-fms	c-kit	Cytogenetics
					ITD	Asp835			
6552	49	12	M	M1	Neg	Neg	Neg	Exon 8	inv(16)
6581	39	15	M	M2	Neg	Neg	Neg	Neg	inv(16)
6621	21	13	M	M	Neg	ND	Neg	Neg	inv(16)
GB310798	30	18	M	M2	Neg	Neg	ND	ND	46;XY
TB050899	56	6	M	M0	Neg	Neg	ND	ND	46;XY
MG040397	30	36	M	M5	Neg	Neg	ND	ND	46;XY
TH050697	59	33	M	M2	Neg	Neg	ND	Neg	OCA
SJ170898	49	17	M	M2	Neg	Neg	ND	ND	t(8;21)
DL030797	23	31	F	M2	Neg	ND	ND	Neg	t(8;21)
LP051197	28	12	M	M4	ITD	Neg	ND	ND	OCA
JR071197	44	28	F	M2	Neg	Neg	ND	ND	46;XX
WA050598	79	5	M	M0	NIS	Neg	ND	ND	46;XY
PC080997	49	27	M	M4	NIS	Neg	ND	ND	46;XY
VG070598	73	0	F	M4	NIS	835	ND	ND	46;XX
PG131197	26	3	M	M4	NIS	835	ND	ND	inv(16)
NG270898	70	3	M	M1	NIS	Neg	ND	ND	46;XY
JH130598	65	17	M	M0	NPCR NIS	Neg	ND	ND	46;XY
JJ060499	74	9	M	M1	NIS	Neg	ND	ND	46;XY
AK110199	84	11	M	M1	NIS	Neg	ND	ND	46;XY
EL080997	85	1	F	M1	NIS	Neg	ND	ND	OCA
AW011097	28	0	M	M5	ITD NIS	Neg	ND	ND	OCA
SW240899	69	4	F	M1	ITD NIS	Neg	ND	ND	46;XX
DW110898	71	2	M	M2	NIS	Neg	ND	ND	t(8;21)

ITD= Patient with internal tandem duplication
Neg =Negative
NPCR= No PCR product
ND= Sample was not don for a specific mutation
NIS = Not included in the statistical analysis
OCR= other cytogenetic abnormalities
* = Patients that were excluded from the statistical analysis because they were patients with poor risk criteria
Note: all samples marked with ND were not included in the statistical analysis for that mutation.