Assessment for the presence of FMS-like tyrosine kinase 3 (FLT3) and Nucleophosmin protein-1 (NPM1) mutations and their association with immunophenotypic markers in de novo Acute Myeloid Leukaemia (AML) diagnosed at a single academic centre

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Haematology.

Johannesburg, April 2012

DECLARATION

I Robyn Cara Marshall declare that this research report comprises my own work. It is being submitted for the degree of Master of Medicine in the branch of Haematology to the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree or examination at any other University.

Robyn Cara Marshall

On this day of the April 2012.

DEDICATION

To my husband, Craig, for all his patience, support and ever willing assistance in helping me complete this work.

RESEARCH OUTPUT

The work of this research report has been published or presented in the following proceedings:

Conferences:

1. Marshall RC, Tlagadi AM, Wiggill TM, Carmona SC.

   Assessment for the presence of NPM1 and FLT3 mutations in de-novo AML.

   Pathvine Congress, September 2-5th 2010, Somerset West.

   Poster Presentation.

2. Marshall RC, Tlagadi AM, Wiggill TM, Carmona SC.

   Mutational assessment in AML: experience from a South African Centre.

   XXXIII World Congress of the International Society of Hematology, October 10-13th 2010, Jerusalem, Israel.

   Poster Presentation.

3. Marshall RC, Tlagadi AM, Wiggill TM, Carmona SC.

   Mutational Assessment in AML.

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ABSTRACT

Introduction: Acute Myeloid Leukaemia (AML) is known to be a heterogeneous clonal disorder of haemopoietic progenitor cells. Recently, the molecular pathogenesis of AML has become more apparent, with the description of two commonly found mutations, namely NPM1 (Nucleophosmin protein-1) gene mutation and the FLT3-ITD (FMS-like tyrosine kinase 3 internal tandem duplication) mutation. These mutations are now routinely assessed and used in both prognostication and treatment decisions in the first world.

The primary aim of this project was to assess for the presence of NPM1 and FLT3-ITD mutations in patients diagnosed with *de novo* AML at Charlotte Maxeke Johannesburg Academic Hospital. In order to achieve this aim certain objectives were completed. This included the establishment of a detection method for both NPM1 and FLT3-ITD mutations by means of PCR as well as determining the overall number of these mutations in the study population and assessing the frequency of these mutations alone and in combination. Correlation with previous immunophenotypic analysis, cytogenetics and other routine parameters was also assessed. **Materials and Methods:** A total of 164 cases of de novo AML were collected retrospectively over time period September 2004 to December 2009. A robust method for the detection of both NPM1 and FLT3-ITD mutations was established using genomic (g) DNA, extracted from blood or bone marrow aspirate samples. Two single PCR reactions were performed, with final analysis by means of capillary electrophoresis using the ABI 3130 Genetic Analyser.

Results: Of the 164 cases analysed, NPM1 mutations were found in 26%, while FLT3-ITD was present in 21% of these cases. FLT3-ITD showed preferential targeting of the NPM1 mutation positive cases with 44% of these cases also found to be NPM1 mutation positive *(P<0.04)*. Routine parameter assessment revealed some novel findings. The median age at diagnosis for the NPM1 mutation positive only cases was 30 years; this is younger than expected. The median WCC of the FLT3-ITD only cases was $101x10^9$/l which was significantly increased compared to the NPM1 mutation only cases *(P<0.03)* and those with neither mutation present *(P<0.01)*. Immunophenotypic analysis revealed a statistically significant increase in CD14, CD11b and HLA-DR (human leucocyte antigen DR) in NPM1-mut positive samples. When assessing CD34 expression and NPM1-mut. NPM1-wt cases were significantly more likely to be CD34 positive than NPM1-mut cases. Recurrent genetic translocations were found to co-occur with NPM1 mutations. This, in particular is a rare finding which requires further investigation. **Conclusion:** This much needed test has proven to be robust in its detection of these gene mutations. It will certainly prove clinically relevant, especially as the use of molecularly targeted therapy becomes part of routine treatment.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AML	Acute myeloid leukaemia
AML M2	Acute myeloid leukaemia with maturation
AML M3/ APL	Acute promyelocytic leukaemia
AML M4	Acute myelomonocytic leukaemia
AML M5	Acute monoblastic and monocytic leukaemia
AML M5b	Acute monocytic leukaemia
ATRA	All trans retinoic acid
AUC	Area under the curve
BAALC	Brain and acute leukaemia gene, cytoplasmic
BCR3	Breakpoint cluster region 3
BMA	Bone marrow aspirate
bp	Base pair
CBF	Core binding factor
CEBPA	CCAAT enhancer binding protein a
CEBPA-mut	CEBPA mutation
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CN	Cytogenetically normal
CR	Complete remission
Crm1	Exportin 1
Del	Deletion
DFS	Disease free survival
dHPLC	Denaturing high pressure liquid chromatography

DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
EQA	External quality assurance
ERG	V-Ets erythroblastosis virus E26 oncogene like (avian)
EVI 1	Ectropic viral integration site 1 gene
FAB	French American British
FBC	Full blood count
FISH	Fluorescent in situ hybridisation
FITC	Fluorescein isothiocyanate
FLT3	FMS-like tyrosine kinase 3
FLT3-F	FLT3 forward primer
FLT3-ITD	FLT3 internal tandem duplication
FLT3-R	FLT3 reverse primer
FLT3-TKD	Tyrosine kinase domain of the FLT3 gene
FLT3-wt	FLT3 wild type
g	Genomic
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Haematoxylin and eosin stain
Hb	Haemoglobin
HIV	Human immunodeficiency virus
HLA-DR	Human leucocyte antigen DR
HMDM	Human oncoprotein murine double minute
ISCN	International System for Human Cytogenetic Nomenclature

Inv	Inversion
JAK2	Janus kinase 2
JMD	Juxta-membrane domain
KIT	C-kit receptor or CD117
KRAS	Kirsten rat sarcoma 2 viral oncogene homologue
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinase
MDS	Myelodysplastic syndrome
min	Minutes
ml	Millilitres
MLL	Mixed lineage leukaemia
MLL-PTD	Mixed lineage leukaemia partial tandem duplication
MN1	Meningioma 1
MPN	Myeloproliferative neoplasms
MRD	Minimal residual disease
NES	Nuclear export signal
NF-κB	Nuclear factor kappa beta
NHLS	National health laboratory services
nm	Nanometres
NPM1	Nucleophosmin member 1 protein
NPM1-F	NPM1 forward primer
NPM1-mut	NPM1 mutant
NPM1-R	NPM1 reverse primer
NPM1-wt	NPM1 wild type
NRAS	Neuroblastoma RAS viral oncogene homologue

OS	Overall survival
p19ARF	p19 Alternative reading frame
PBS	Phosphate buffered saline
PE	Phycoerythrin
PT	Pre treatment
PCR	Polymerase chain reaction
QPCR	Quantitative PCR
RAS	Rat sarcoma
RB	Retinoblastoma protein
RFS	Relapse free survival
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
s	Seconds
SSC	Salt, sodium citrate
SOP	Standard operating procedure
STAT5	Signal transducer and activator of transcription 5
t	Translocation
TBE	Tris-borate-EDTA
TKD	Transcription kinase domain
TP53/p53	Tumour protein 53
TSG	Tumour suppressor gene
µl	Microlitres
v	Version
WCC	White cell count

WHO	World Health Organization
WT 1	Wilms' tumour 1 gene

1 INTRODUCTION

1.1 INTRODUCTION TO ACUTE MYELOID LEUKAEMIA (AML)

Adult acute myeloid leukaemia (AML) is known to be a very heterogeneous clonal disorder of haemopoietic progenitor cells with expansion of myeloid blasts in the peripheral blood, bone marrow and other tissues (Estey and Döhner, 2006).

This is a result of the inability of the cells to regulate proliferation, together with inhibition of differentiation (Kelly and Gilliland, 2002).

The incidence of AML in North America is approximately 3.5 cases per 100 000 population per year with a median age at diagnosis of 67 years and a slight male predominance (Swerdlow et al., 2008, Jemal et al., 2009). There is currently very little published data with regards to the epidemiology and diagnosis of AML in South Africa and it was therefore set out, as part of the aims of this study, to better elucidate this.

The first classification of AML was proposed by Galton and Dacie in 1976 (Galton, 1975) and this was followed shortly thereafter by the French American British (FAB) classification, which is still used today, in conjunction with other classifications (Bennett et al., 1976). These earlier classifications were based solely on morphology and cyto-chemical staining results. Subsequently, the World Health Organization (WHO) Classification of Tumours of Haemopoietic and Lymphoid Tissues and in particular the 3rd edition (2001) was introduced which, although largely based on the FAB classification, includes both cytogenetic and immunophenotypic analysis as key components in making the diagnosis and assessing the prognosis of AML (Jaffe et al., 2001).

More recently the importance of newly identified gene abnormalities in the pathogenesis of AML has come to light. The 4th edition (2008) of the WHO classification now includes the new category of AML with gene mutations and within that, two new provisional entities: AML with mutated Nucleophosmin Member 1 Protein (NPM1) and AML with mutated CCAAT Enhancer Binding Protein α (CEBPA) (Swerdlow et al., 2008). These changes have lead to more reliable prognostic information, improved treatment stratification and a move towards targeted therapy (Swerdlow et al., 2008). By including these components into this classification, they have become part of a worldwide standard expected in diagnostic medicine. These molecular techniques should now form part of the routine diagnostics of AML.

Up to 40-50% of adult AML patients are classified as being cytogenetically normal (CN) as they display no visible chromosomal abnormality on conventional cytogenetic analysis. This heterogeneous group has a normal karyotype but widely varying epigenetics, gene expression profile (Grimwade et al., 1998, Mrózek and Bloomfield, 2006), phenotype, treatment response and prognosis (Schlenk et al., 2008). In particular these gene mutations have the potential to assist in the further sub-classification of this heterogeneous group of AMLs. A summary of the commonly found gene mutations together with their locations, common associations and outcomes are presented in Table 1.1.

The most common gene mutations in patients with a normal karyotype include those mentioned above (NPM1 and CEBPA) as well as mutation of the FMS-like tyrosine kinase 3 (FLT3). The combinations in which these gene mutations, in particular FLT3 and NPM1, occur are vital to patient outcome (Estey and Döhner, 2006, Thiede et al., 2006). The presence of the FMS-like tyrosine kinase 3 internal

tandem duplication (FLT3-ITD) mutation implies a poorer prognosis regardless of the other accompanying mutations. NPM1 mutations on their own (without the presence of a FLT3-ITD) provide a much improved prognosis (Döhner et al., 2005). The significant impact of these mutations on patient outcome as well as the relative frequency of these mutations were important determining factors in the decision to assess for both NPM1 and FLT3-ITD mutations in combination in this study.

Table 1.1 Recently identified gene mutations in AML.
A summary of their chromosomal location, frequency in CN AML, common associations and outcome including overall prognostic impact (Mrózek and Bloomfield, 2006).

Gene Symbol	Location	Frequency	Associated with	Outcome	Prognostic Impact
NPM1-mut	5q35	45-62%	FLT3-ITD and FLT3-TKD	NPM1-mut/ FLT3-wt= better CR, EFS, RFS, DFS and OS	Favourable
FLT3- ITD	13q12	28-34%	NPM1-mut Level of mutant allele important	Always inferior outcome	Adverse
FLT3-TKD	13q12	11-14%		Better OS	Favourable
MN1	22		Treatment failure Increased relapse risk	Inferior RFS, OS	Adverse
EVI 1	3q26		MLL-PTD	Overall poorer outcome	Adverse
WT1	11p13	~10%	NPM1-mut, MLL-PTD, FLT3-ITD and CEBPA	Induction failure	Adverse
MLL PTD	11q	5-10%		Shorter CR, and inferior RFS, DFS and EFS	Adverse
BAALC overexpression	8q22.3		FLT3-ITD, NPM1-wt, CEBPA-mut, MLL-PTD increased ERG	Worse CR, shorter DFS, EFS, and OS	Adverse
ERG overexpression	21		NPM1-mut, FLT3-ITD	Shorter OS	Adverse
CEBPA	19q13.1	15-20%	Less likely to carry FLT3-ITD, FLT3-TKD and MLL PTD	Better CR, EFS, DFS and OS	Favourable

Abbreviations: CN, cytogenetically normal; AML, acute myeloid leukaemia; NPM1, Nucleophosmin 1; mut, mutant; FLT3-ITD, internal tandem duplication of the FMS related tyrosine kinase 3 (FLT3) gene; FLT3-TKD, mutation of the tyrosine kinase domain of the FLT3 gene; wt, wild type; CR, complete remission; EFS, event free survival; RFS, relapse free survival; DFS, disease free survival; OS, overall survival; MN1, Meningioma 1; EVI 1, Ecotropic viral integration site 1 gene; MLL-PTD, partial tandem duplication of the myeloid/lymphoid or mixed lineage leukaemia (MLL) gene; WT 1, Wilms' tumour 1 gene; CEBPA, CCAAT/enhancer binding protein (C/EBP) alpha; BAALC, brain and acute leukaemia gene, cytoplasmic; ERG, v-ets erythroblastosis virus E26 oncogene like (avian).

1.2 AML PATHOGENESIS

It is generally accepted that AML blasts develop from normal myeloid blasts or stem cells following two forms of genetic damage, therefore requiring a double hit (Kelly and Gilliland, 2002). One insult induces an enhanced proliferative state and decreased apoptosis and the other leads to a block in differentiation by inactivating a master regulator of myeloid differentiation. Accordingly the genetic damage or mutations are classified into two classes:

Class I Mutations: Activate signal transduction pathways such as rat sarcoma (RAS) which represent small GTPases, or receptor tyrosine kinases such as FLT3 or C-kit receptor or CD117 (KIT). This constitutive activation allows for a proliferative advantage with clonal expansion of the affected haemopoietic progenitors (Kelly and Gilliland, 2002, Estey and Döhner, 2006).

Class II Mutations: Include mutations in CEBPA, Mixed Lineage Leukaemia (MLL) and NPM1 that affect transcription factors or parts of the transcription co-activation complex resulting in the impaired differentiation (Kelly and Gilliland, 2002).

These mutations rarely overlap within their class and most often at diagnosis of AML, there will be at least two of these mutations present; one from each class (Schlenk et al., 2008). A diagrammatic representation of the different class mutation in the pathogenesis of AML is presented in Figure 1.1.

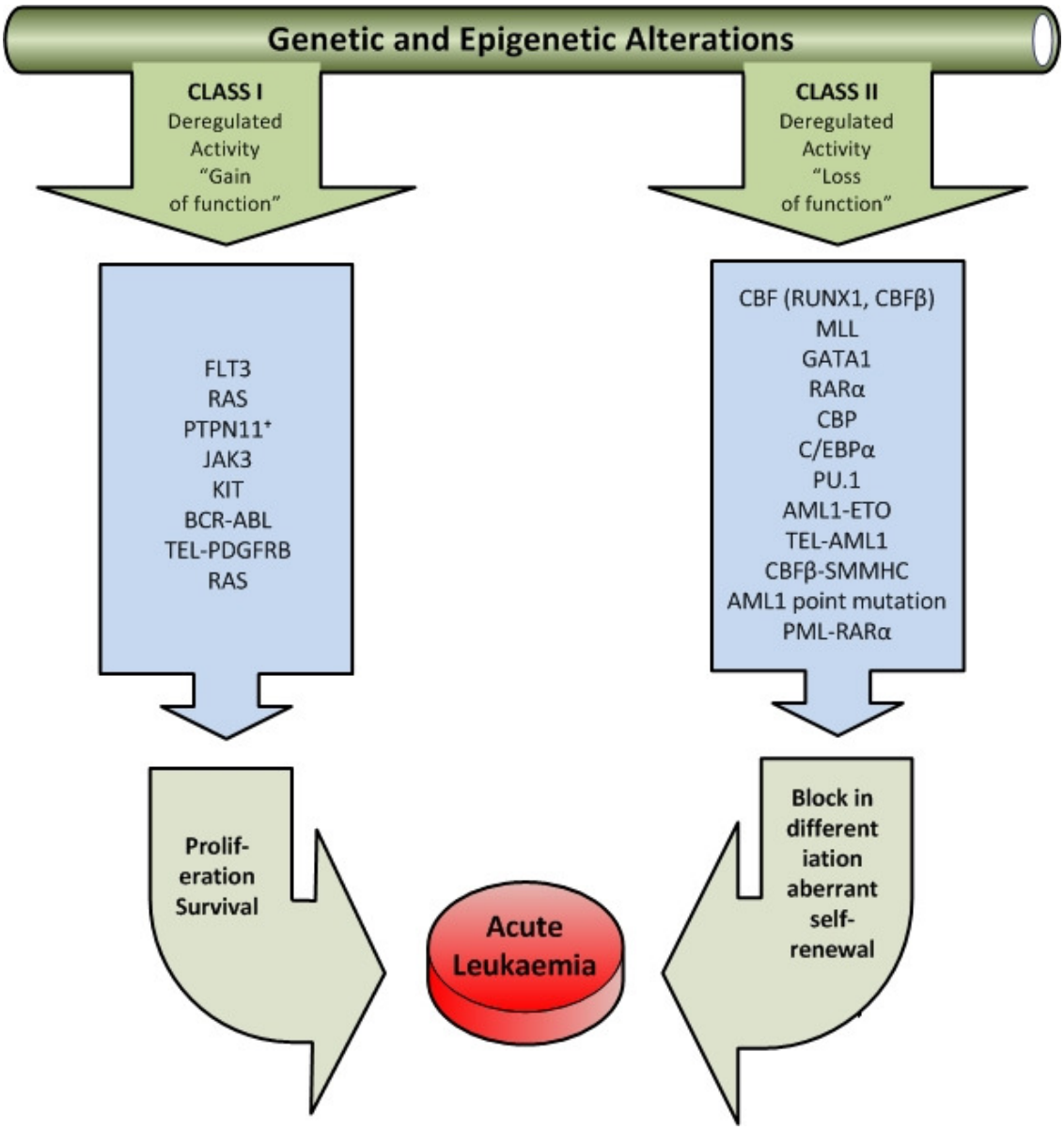


Figure 1.1 Cooperation between different class mutations in AML pathogenesis.
Mutations in Class I allow for clone proliferation and/or a survival advantage while Class II mutations allow for impaired differentiation. Often a mutation from each class may be present in a single clone. Adapted from (Schlenk et al., 2008).

1.3 NUCLEOPHOSMIN MEMBER 1 PROTEIN (NPM1)

1.3.1 OVERVIEW OF NPM1

NPM1 is ubiquitously expressed and is a highly conserved phosphoprotein (Rau and Brown, 2009). Although predominantly found in the nucleolus, it shuttles,

continuously between the nucleus and the cytoplasm (Borer et al., 1989). It has a role in various cellular processes including acting as a molecular chaperone (Szebeni and Olson, 1999), being involved in ribosome biogenesis and assisting in the regulation of centrosome duplication and therefore maintenance of genomic stability (Herrera et al., 1995, Yu et al., 2006). NPM1 is also capable of binding to several proteins such as tumour protein 53 (TP53/p53) and the proteins which interact with and regulate p53 e.g. alternative reading frame (p19ARF), Human oncoprotein murine double minute (HMDM2) and the retinoblastoma protein (Rb) (Grisendi et al., 2006, Mrozek et al., 2007).

It has recently been shown that NPM1 mutations are the most common genetic lesions detectable in AML (Noguera et al., 2005). These occur on chromosome 5q35, at exon 12 (Figure 1.2), and are somatic frame shift mutations, which are almost always heterozygous in nature.

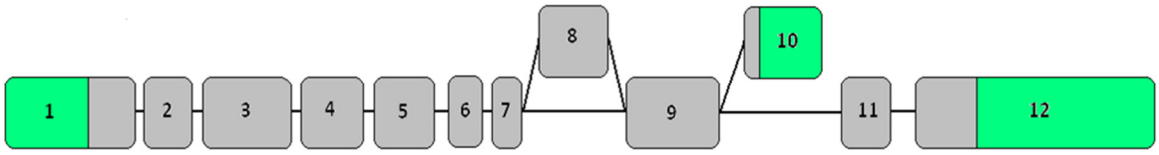


Figure 1.2 NPM1 gene structure.
This gene is found on Chromosome 5q35 and is frequently mutated in AML. The mutations (A-F) usually occur at exon 12. Green indicates the 3' and 5' and other untranslated regions. Adapted from (Falini et al., 2005).

The mutation leads to two important changes, which act together and results in the aberrant cytoplasmic expression of NPM1 as demonstrated in Figure 1.3 (Falini et al., 2005). These changes are the loss of tryptophan residues 288 and 290 (which

is required for NPM1 binding to the nucleoli) and the formation of an additional nuclear export signal (NES) motif at the C terminus of the mutant proteins. The NPM1mutant (NPM1-mut) proteins recruit the NPM1 wild type (NPM1-wt) from the nucleoli by means of dimerization and so also affect NPM1-wt functioning.

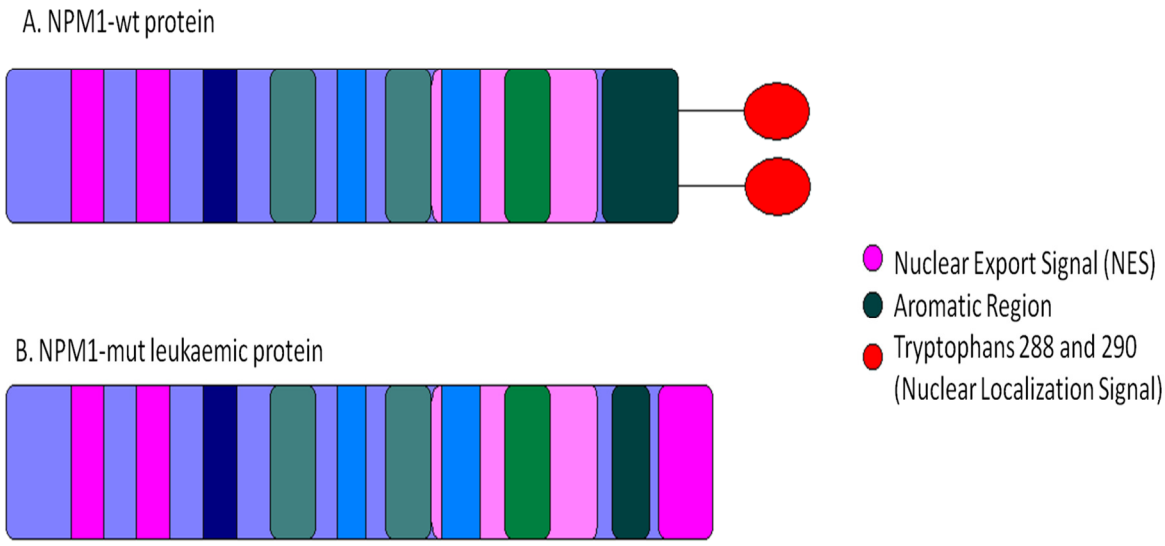


Figure 1.3 Mutation induced changes in the NPM1 protein.
NPM1-wt protein is depicted in (A) with the nuclear localization signals, tryptophan 288 and 290 present at the C-terminus. With an NPM1-mut (B) there is the creation of a new nuclear export signal and the loss of the nuclear localization signal. This results in increased nuclear export and aberrant cytoplasmic expression of the NPM1-mut. Adapted from (Falini et al., 2009).

To date a total of 55 NPM1 mutations has been described. These mutations represent either simple 4bp insertions or more complex deletion insertion mutations (Schnittger et al., 2009). Mutation A, a duplication of a TCTG tetranucleotide at position 956 to 959 of the reference sequence accounts for 75-85% of the mutations found. B and D mutations make up a further 10% and 5% respectively (Falini et al., 2005). These mutations include 4-bp insertions at position 960, which are distinct from each other and result in the same frame shift

mutation as that seen in mutation A. Mutation B has a CATG tetranucleotide insertion at position 956 to959 while Mutation D has a CCTG tetranucleotide insertion at the same position (Schnittger et al., 2009). Table 1.2 demonstrates the NPM1 wild type sequence as well as 8 of the common mutant variants. In paediatric cases, mutation A, only accounts for 11-50% of NPM1 mutation positive cases, while the other variants occur more frequently (Cazzaniga et al., 2005, Brown et al., 2007).

Table 1.2 Wild type NPM sequence aligned with 8 of the common mutant variants.
The red type indicates the nucleotide insertions seen in these mutations (Falini et al., 2005).

Type of Mutation:	**Sequence:**
Wild Type	GATCTCTG···GCAGTGGAGGAAGTCTCT TTAAGAAAATAG
Mutation A	GATCTCTGTCTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG
Mutation B	GATCTCTGCATGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG
Mutation C	GATCTCTGCGTGGCAGT···GGAGGAAGTCTCTTTAAGAAAATAG
Mutation D	GATCTCTGCCTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG
Mutation G	GATCTCTGTTTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG
Mutation H	GATCTCTGCTTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG
Mutation I	GATCTCTGTAAGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG
Mutation J	GATCTCTGTATGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG

Abbreviations: NPM1, Nucleophosmin 1; mut, mutation

1.3.2 NPM1 AND ITS ASSOCIATION WITH OTHER MUTATIONS

NPM1 mutations occur in approximately 27-35% of all adult patients with AML and more specifically in 50-60% of adult CN AML making them the most frequent genetic abnormality in this group (Falini et al., 2005). In paediatric patients with AML approximately 7.5% will have an NPM1-mut, with the incidence increasing as age increases (Cazzaniga et al., 2005, Brown et al., 2007). This mutation is also found, at a lower frequency in adult Asian populations as compared to that found in European and American studies (Koh et al., 2009). This mutation occurs in 15% of AMLs with other chromosomal aberrations, especially trisomy 8 and deletion 9q (Thiede et al., 2006). Although uncommon there have been various reports confirming that NPM1 mutations can also accompany a recurrent chromosomal translocation such as t(8;21) or t(15;17) (Suzuki et al., 2005, Verhaak et al., 2005, Thiede et al., 2006). A recent study by Haferlach *et al* concludes that any chromosomal aberrations accompanying an NPM1 mutation are secondary events. The NPM1 mutation is always the primary event (Haferlach et al., 2009) and the secondary aberrations do not appear to affect prognosis (Falini et al., 2009a, Haferlach et al., 2009). NPM1 mutations and its associations with other defined mutations are summarised in Table 1.3.

Table 1.3 Other mutations associated with NPM1-mut positivity.
Some mutations are commonly associated and others rarely associated with NPM1 mutations. Other mutations have not been found to correlate with NPM1-mut positivity (Döhner et al., 2005, Verhaak et al., 2005).

	Commonly Associated	Rarely Associated	No Correlation to Date
NPM1 Mutation Positive	CN	MLL-PTD	KIT
	Trisomy 8	Recurrent Translocations *e.g.* t(8;21), t(15;17), inv(16)	TP53
	Del 9q	CEBPA	KRAS
	FLT3-ITD		NRAS
	FLT3-TKD		

Abbreviations: NPM1, Nucleophosmin 1; mut, mutation; CN, cytogenetically normal; MLL-PTD, partial tandem duplication of the myeloid/lymphoid or mixed lineage leukaemia (MLL) gene; KIT, C-kit receptor; inv, inversion; TP53, tumour protein 53; Del, Deletion; CEBPA, CCAAT/enhancer binding protein (C/EBP) alpha; KRAS, Kirsten rat sarcoma 2 viral oncogene homologue; FLT3-ITD, internal tandem duplication of the FMS related tyrosine kinase 3 (FLT3) gene; NRAS, Neuroblastoma RAS viral oncogene homologue; FLT3-TKD, mutation of the tyrosine kinase domain of the FLT3 gene.

1.3.3 NPM1 CHARACTERISTIC FINDINGS

In general, AML with mutated NPM1 is a *de-novo* AML which has been associated with certain characteristic findings. These include a normal karyotype, association with a FLT3-ITD mutation, decreased or absent CD34 and CD133 surface expression, higher bone marrow blast counts, leucocytosis, higher platelet counts, a higher median age at presentation and a higher frequency in female patients (Thiede et al., 2006). NPM1 mutations have been associated with all FAB morphologic subtypes of AML but are particularly common in acute myelomonocytic leukaemia (AML M4) and acute monoblastic and monocytic leukaemia (AML M5). This particular association has been found both morphologically and immunophenotypically with higher expression of monocytic

differentiation associated antigens present on flow cytometric analysis (Schnittger et al., 2005). More, recently this mutation has also been associated with a distinctive gene expression as determined by gene expression microarray analysis and micro ribose nucleic acid (microRNA) profiles. A group of 42 NPM1 mutation negative cases was compared to 55 NPM1 mutation positive cases. These two AML were clearly delineated by analysis of the top 500 differentially expressed genes (Haferlach et al., 2009).

1.3.4 NPM1 AND ITS ROLE IN LEUKAEMOGENESIS AND PATIENT OUTCOME

To date, the exact role of NPM1 mutations in leukaemogenesis has remained uncertain. The NPM1-muts delocalizes p19 ARF, an important tumour suppressor gene (TSG) to the cytoplasm through its binding to the mutant NPM1 (den Besten et al., 2005). As a result p19ARF is not available to interact with HMDM2 in the nucleoplasm and thus prevents p53 initiation with a resultant compromised p53 dependent cell cycle arrest at G1/S (Colombo et al., 2006). It is also known to cause an increase in the oncoprotein cMYC which may also contribute to its leukaemogenic effect (Di Micco et al., 2006, Rau and Brown, 2009). This is demonstrated in Figure 1.4.

The presence of an NPM1 mutation in AML confers a better prognosis with a good response to induction chemotherapy (Thiede et al., 2006). This mutation is associated with improved complete remission (CR) post induction therapy as well as improved overall survival (OS), disease free survival (DFS), event free survival (EFS) and relapse free survival (RFS) provided that no concomitant FLT3-ITD

mutation is present. With FLT3-ITD absent, NPM1 mutation positive AML has a similar outcome to core binding factor (CBF) AML and CEBPA mutations *i.e.* a very favourable prognosis (Preudhomme et al., 2002).

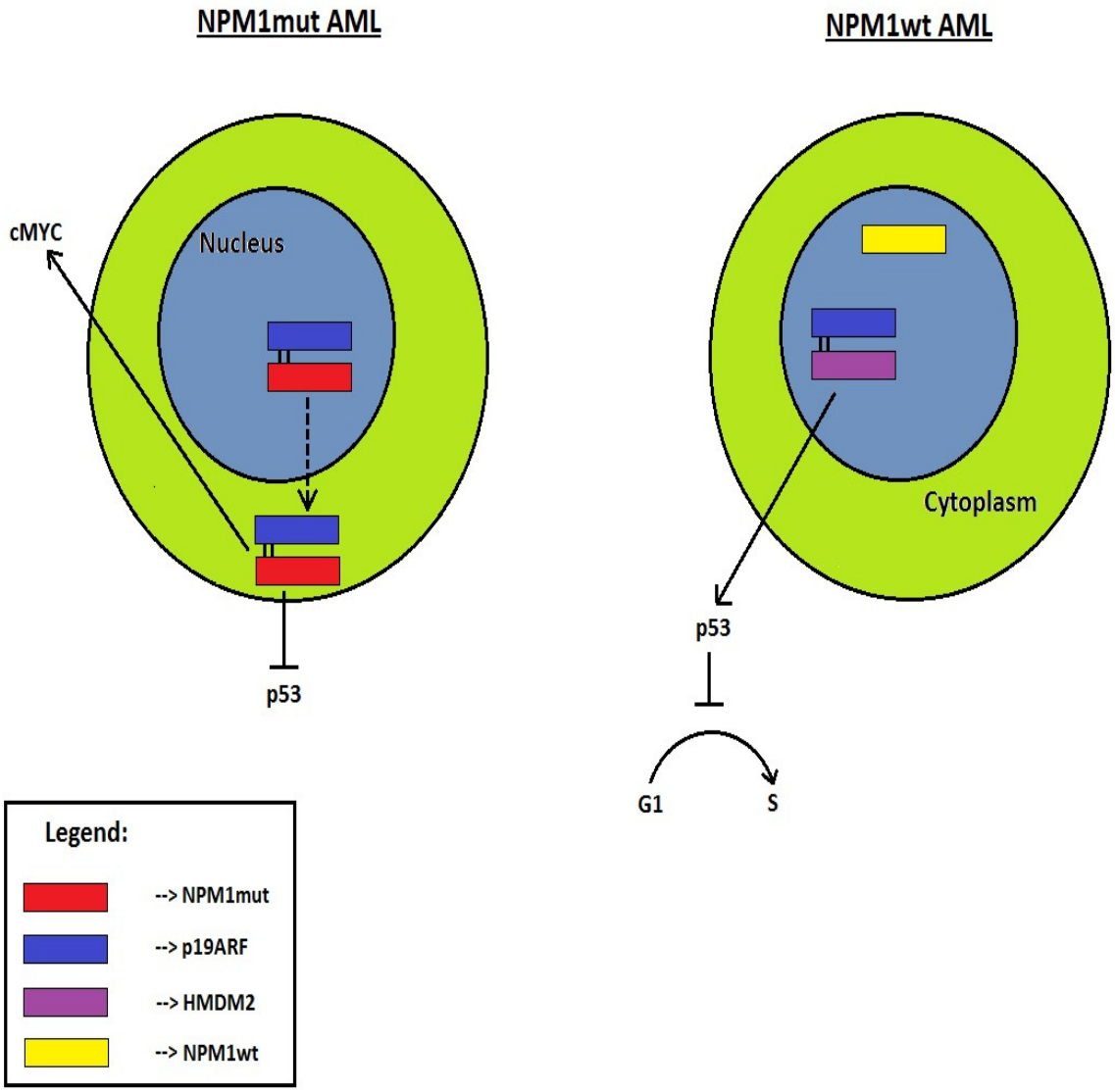


Figure 1.4 Role of NPM1mut in AML pathogenesis.
NPM1mut delocalises p19ARF to the cytoplasm and as a result is no longer available for interaction with HMDM2 in the nucleus. This then affects p53 functioning.

Abbreviations: NPM1, Nucleophosmin 1; mut, mutation; wt, wild type; p53, tumour protein 53; AML, Acute Myeloid Leukaemia; p19ARF, p19 alternative reading frame; HMDM2, human oncoprotein murine double minute.

1.3.5 NPM1 DETECTION

There are various methods to detect the presence of an NPM1 mutation. One of the most commonly used is that of immuno-histochemisty to detect the presence of aberrant cytoplasmic NPM1 and thereby the presence of a gene mutation. Immuno-histochemisty can however be difficult to interpret especially in blasts with a high nuclear to cytoplasmic ratio. Recently polymerase chain reaction (PCR) based capillary electrophoresis methods have increased in popularity as these are more sensitive than immuno-histochemisty, with diagnostic sensitivity approaching 100% (Martelli MP et al., 2008, Szankasi et al., 2008, Wertheim and Bagg, 2008). PCR and immuno-histochemical staining often make use of paraffin embedded samples as the starting material. The PCR based method can also use other materials *e.g.* peripheral blood or bone marrow aspirate (BMA) slides as the starting material. NPM1-mut detection as a tool for minimal residual disease (MRD) is a very real possibility. Not only is it a relatively frequent but also a relatively stable mutant. The use of quantitative PCR (QPCR) assays appears to be the method of choice in this regard (Gorello et al., 2006, Falini et al., 2007).

1.4 NPM1 AND FLT3 MUTATIONS

Up to 40% of patients with an NPM1 mutation also have a FLT3-ITD mutation, which is, twice as common in NPM1 mutation positive patients when compared to those with NPM1-wt (Thiede et al., 2002). The presence of a FLT3-ITD overrides the good prognosis of the NPM1 mutation positivity. Several studies, including that by Thiede *et al* confirmed that the NPM1 mutations are the primary events that preceded the acquisition of FLT3-ITD or any of the other mutations (Thiede et al.,

2002). This is supported by the observation that in some cases there are several FLT3-ITDs, while only one NPM1 mutation could be found. Furthermore, the available evidence suggests that NPM1 mutation positive AML with an abnormal karyotype has similar characteristics and prognostic outcome as NPM1 mutations in CN AML (Haferlach et al., 2009). It can then be suggested that all three of these determinants *i.e.* the presence of FLT3-ITD, NPM1 gene mutation status and cytogenetic evaluation interact in such a manner that the determination of the presence or absence of all three of these parameters are important and dependent on each other for patient outcome.

At present, within the international community, only once the diagnosis of a normal karyotype AML has been made will the assessment for NPM1 mutations and FLT3-ITD be established. With this approach, approximately 25% of AML patients will be excluded from testing due to the failure of cytogenetic analysis. This would prevent the assignment of the favourable genotype NPM1-mut/FLT3 wild type (FLT3-wt) if a chromosomal aberration or failed cytogenetic testing occurred (Mrózek et al., 2007, Reindl et al., 2006). It may therefore be more beneficial to patients not to recommend this hierarchy of testing and rather that all patients, at the time of diagnosis of AML are tested for the presence of both NPM1 and FLT3-ITD gene mutations. Once the presence or absence of these mutations has been confirmed appropriate therapeutic strategies can then be implemented.

It is currently recommended, given the favourable prognosis of AML with NPM1 mutation and no FLT3-ITD (NPM1-mut/FLT3-wt) that these patients are treated with conventional chemotherapy regimens (with or without autologous transplantation). They do not appear to gain from allogeneic transplant (Döhner et al., 2005, Schlenk et al., 2008, Rau and Brown, 2009).

1.5 FMS-LIKE TYROSINE KINASE 3 (FLT3)

1.5.1 OVERVIEW OF FLT3

The FLT3 gene is located at chromosome 13q12 and consists of 24 exons. It encodes a class 3 receptor tyrosine kinase which is important for haemopoietic progenitor cell differentiation and proliferation (Rosnet et al., 1991, Stirewalt and Radich, 2003, Döhner, 2007).

FLT3 has two domains in which mutations are found and both result in increased signal transduction (Figure 1.5). The first is the juxtamembrane domain (JMD), vital for normal kinase auto inhibition and demonstrates ITDs or rarely point mutations. The second domain is the activation loop of the tyrosine kinase domain (TKD) which demonstrates point mutations (Fröhling et al., 2002).

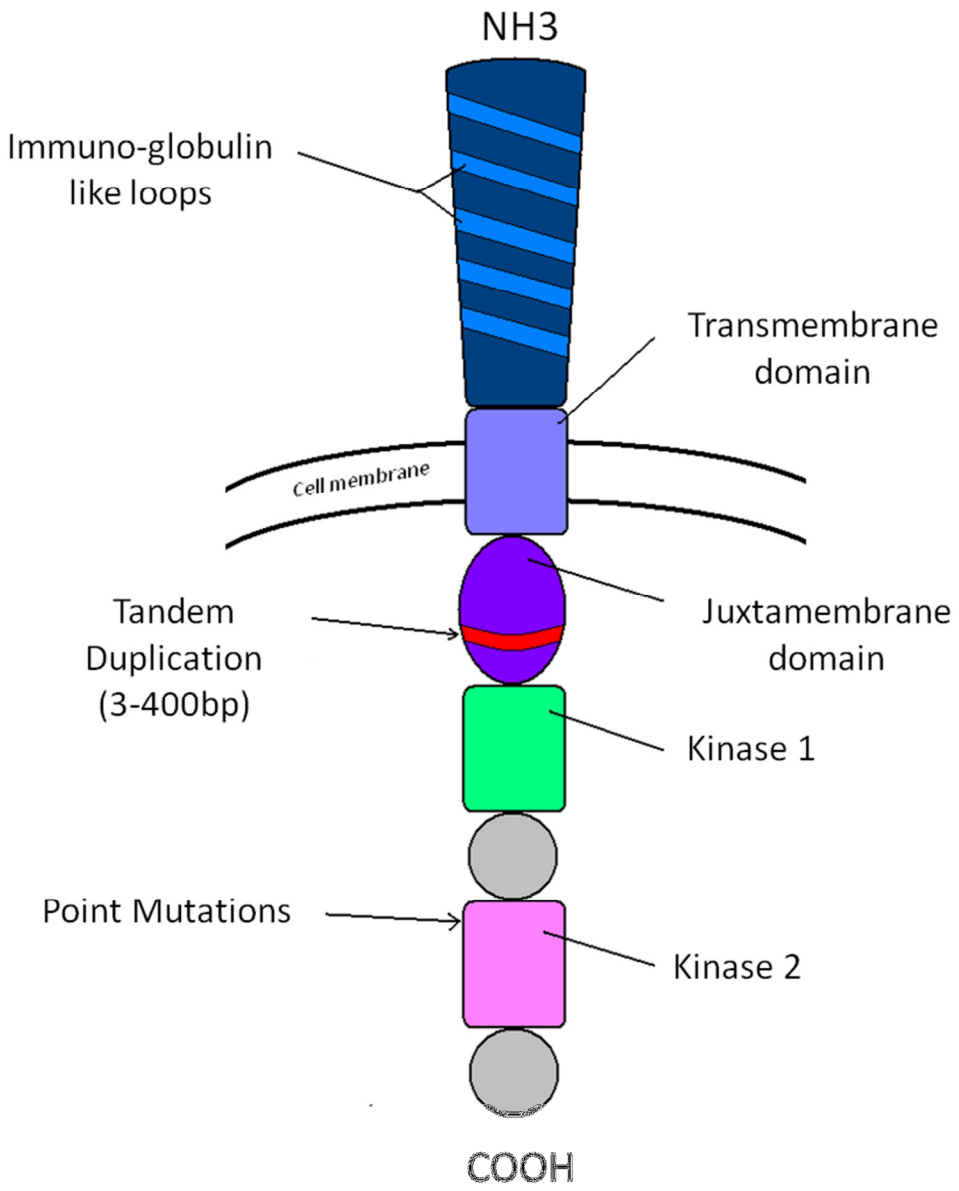


Figure 1.5 FLT3 structure.
The juxtamembrane domain is the area in which the internal tandem duplications are found. Point mutations are located in the second kinase domain. Adapted from (Kottaridis et al., 2003).

Abbreviations: FLT3, FMS related tyrosine kinase 3 gene

1.5.2 FLT3-ITD

FLT3-ITD in AML is quite diverse in both size and location. It occurs at the 5' end of the JMD in exons 14 and 15 (Nakao et al., 1996, Kottaridis et al., 2003); this mutation can vary in length from 3 to 400 nucleotides and is always in frame (Schnittger et al., 2002). FLT3-ITD produces an abnormal protein with resultant autophosphorylation, ligand-independent receptor dimerization and constitutive activation, which in turn allows for downstream signalling pathways to be activated. These include the Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5) pathway and the mitogen activated protein kinase (MAPK) pathway (Choudhary et al., 2005, Reindl et al., 2006).

FLT3-ITD is found in all forms of AML (20-40% of all cases) but, mostly in CN AML and also AML with t(6;9) and t(15;17) (Kottaridis et al., 2003, Slovak et al., 2006). With acute promyelocytic leukaemia (AML M3/APL), FLT3-ITDs presence can range from 30-39% of cases. Variant APL, however, is most often FLT3-ITD positive (up to 65-80% of cases). This increased propensity for FLT3-ITD positivity in variant AML M3, together with the common finding of leucocytosis and the shorter breakpoint cluster region 3 (BCR3) found in atypical APL suggests that these features may all have a common causative pathology (Noguera et al., 2002, Kottaridis et al., 2003).

In paediatric cases the frequency of occurrence of this mutation appears to be much lower, approximately 5-16.5% (Krstovski et al., 2009), although the clinical characteristics and outcome appear to be similar to those seen in adults (Zwaan et al., 2003).

1.5.3 FLT3-ITD CHARACTERISTIC FINDINGS

FLT3-ITDs are commonly associated with a leucocytosis, increased blast percentage in both the peripheral blood and bone marrow and with an increased lactate dehydrogenase (LDH) (Kottaridis et al., 2003, Döhner et al., 2005). Several studies have reported an association of this mutation with FAB M3 and FAB M5 and in particular acute monocytic leukaemia (AML M5b) (Schnittger et al., 2002, Thiede et al., 2002). Some patients (9-23%) will have more than one ITD mutation present. In general this has been found to range between two to five additional bands and may reflect underlying genetic instability (Kottaridis et al., 2001, Noguera et al., 2002, Kottaridis et al., 2003).

1.5.4 FLT3-ITD AND ITS ASSOCIATION WITH OTHER MUTATIONS

FLT3-ITD and its association with other mutations is summarised in Table 1.4. CEBPA does not seem to have any association with FLT3-ITD positivity (Preudhomme et al., 2002). While mixed lineage leukaemia partial tandem duplication (MLL-PTD) gene abnormalities may be associated, it is difficult to assess due to the low frequency of this in patients with AML. In general both MLL-PTD and FLT3 duplications are more commonly found in patients with a normal karyotype (Kottaridis et al., 2003). FLT3-ITD mutations has also been found in patients with t(8;21) and inv(16); these are considered in the Class II category of mutations (Kottaridis et al., 2001, Thiede et al., 2002, Schnittger et al., 2002). FLT3-ITD mutations have occasionally been found in patients with complex cytogenetics, including 11q23 and t(3;3). Neuroblastoma RAS viral oncogene homologue (NRAS) mutations and FLT3-ITD are found in combination in

approximately 0-4% of patients. This suggests that if both of these genes are mutated e in the same clone there will be no additional advantage to the clone (Kottaridis et al., 2003, Stirewalt and Radich, 2003).

Table 1.4 Other mutations associated with FLT3-ITD positivity.
Some mutations are commonly associated and others rarely associated with FLT3-ITD. Other mutations have not been found to correlate with FLT3-ITD (Kottaridis et al., 2003).

	Commonly Associated	Rarely Associated	No Correlation to date
	CN	11q23	CEBPA
	t(15;17) esp. vM3	t(3;3)	MLL-PTD
FLT3-ITD	t(6;9)	t(9;11)	
Mutation	t(8;21)	t(9;22)	
Positive	inv(16)	NRAS	
	NPM1		

Abbreviations: FLT3-ITD, internal tandem duplication of the FMS related tyrosine kinase 3 (FLT3) gene; CN, cytogenetically normal; CEBPA, CCAAT/enhancer binding protein (C/EBP) alpha; vM3, variant acute promyelocytic leukaemia; MLL-PTD, partial tandem duplication of the myeloid/lymphoid or mixed lineage leukaemia (MLL) gene; Inv, Inversion; NRAS, Neuroblastoma RAS viral oncogene homologue; NPM1, Nucleophosmin 1

1.5.5 FLT3-ITD AND PATIENT OUTCOME

Patients with the FLT3-ITD in CN AML differ from those without this gene mutation with regards to both pre-treatment characteristics and treatment outcome, which is much inferior. This has been seen for EFS, RFS and OS (Mrózek et al., 2007). Further evidence confirms that the level of the mutant allele or ratio of wild type to mutant plays an important role in determining patient outcome (Whitman et al., 2001). Furthermore, a recent report has found that FLT3-ITD status was a

prognostic marker in monocytic lineage AML and that cytogenetics was not (Koh et al., 2009).

1.5.6 FLT3-ITD AT PRESENTATION AND RELAPSE

There have been various studies to assess the presence of FLT3-ITD mutations at presentation and relapse. In a recent review, assessment of three of these studies, found that 86% of these patients maintained their original FLT3-ITD positive status at presentation and at relapse. A proportion of patients were found to either gain (10.1%) or lose (3.9%) a FLT3-ITD mutation at relapse. This not only prevents the use of FLT3-ITD mutations as a marker for assessment of MRD but also implies that the FLT3-ITD mutation is indeed a secondary event in leukaemogenesis (Kottaridis et al., 2001, Kottaridis et al., 2003). Further support for the theory of FLT3-ITD mutations as the second hit is the fact that these mutations are often only present in a subpopulation of the leukaemic cells.

All of the above is relevant for further elucidation of this mutation in its role in leukaemogenesis, patient outcome and current and future treatment strategies. It also confirms the need for this mutations detection to become part of the routine diagnostic work up in AML, just as cytogenetics has become a routine requirement.

1.5.7 FLT3-ITD DETECTION

There are various means to detect a FLT3-ITD mutation. One of the commonly used methods is standard PCR amplification with visualisation on agarose gel by

means of ethidium bromide. This technique does however have limitations, some of which include its qualitative nature, the need for increased amount of deoxyribose nucleic acid (DNA), its inability to detect both mutations simultaneously and the potential interpretative difficulty when assessing a mutant and a wild type which differ by only a few base pairs (bp) (Gleissner et al., 2001). For these reasons capillary electrophoresis is favoured. FLT3-ITD detection as part of an assessment for minimal residual disease detection has proved unsuccessful to date. FLT3-ITD is considered a second hit and does not show stability throughout the disease process (Kottaridis et al., 2003).

1.6 MOLECULAR TARGETS IN THERAPY

1.6.1 OVERVIEW OF MOLECULAR THERAPY IN AML

Traditional therapy, has improved significantly in AML in general, with new drugs such as monoclonal antibodies and all trans retinoic acid (ATRA) coming to the fore. This together with improved stem cell transplant protocols has made a significant difference to the outcome of patients with AML (Sanz et al., 2009). But, despite these advancements, there is still a need for greater improvement and it appears as if molecular targeting may be the answer.

Since the first identification of molecular changes and gene expression in AML, research and resources have been focused on finding new molecular markers; determining their interaction and understanding their pathobiology. Ideally individual targeted therapy, based on the underlying molecular abnormality is sought and currently, various trials and research are underway. Tyrosine kinase

inhibitors (TKIs) are candidate agents under investigation for the inhibition or inactivation of FLT-3 mutants (Cools et al., 2004, Sanz et al., 2009).

1.6.2 TYROSINE KINASE INHIBITORS (TKIS)

FLT3 gene mutations are characterized by aberrant tyrosine kinase activity with the constitutive activation of FLT3 in the absence of its ligand (Blum and Marcucci, 2008, Tam and Gilliland, 2008). TKIs do show some effectiveness, predominantly in the decrease of peripheral blasts. Bone marrow blasts appear to receive growth factor support from their micro-environment, thus decreasing the effectiveness of FLT3 inhibitors (Parmar et al., 2011). The overexpression of the FLT3 protein secondary to the presence of and in response to kinase inhibitors may contribute to the clinical resisitance seen (Weisberg et al., 2011). Mobilization of blasts into the peripheral blood prior to FLT3 inhibitor administration might be a viable solution to this problem (Tam and Gilliland, 2008).

Another option to improve current treatment outcomes may be to genotype all patients and thereby predict a response before deciding to initiate this form of therapy at all (Tam and Gilliland, 2008) . Alternatively new compounds may need to be developed to overcome any resistance mutations (Davies et al., 2011). In the interim, combination therapy is likely to become the standard of care, especially with recent studies showing that combination therapy decreases toxicity and increases responses (Wang et al., 2011, Wiernik, 2011).

The importance of these gene mutations with regard to patient outcome and potential management have become evident and formed the basis for the objectives of this report.

1.7 OBJECTIVES OF THIS RESEARCH REPORT

The primary aim of this research report was to assess for the presence of NPM1 and FLT3-ITD mutations in patients diagnosed with *de novo* AML at a single academic centre. In order to achieve this aim the following objectives were completed:

- The establishment of a detection method for both NPM1 and FLT3-ITD mutations by means of PCR.
- Determine the overall number of these mutations in the study population; assess the frequency of these mutations alone and in combination as well as assessing the distribution of these mutations in the various population subgroups.
- Correlate with immunophenotypic analysis of the samples and other routine parameters. These routine parameters included the haemoglobin (Hb), white cell count (WCC), platelet count and blast count at diagnosis.
- Furthermore, a comparison to the available literature was made to assess whether the patient profile found in our study compares with that found in other population groups.

The epidemiology and patient demographics of those adult patients diagnosed with *de novo* AML in South Africa has to the best of our knowledge, not been

recently assessed. This study provided all the necessary data to assess this particular patient group's characteristics and how these may differ from that seen in the developed world.

2 MATERIALS AND METHODS

2.1 SAMPLES

Patients diagnosed with de novo AML at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) were included into a database covering the period from September 2004 to December 2009. Diagnostic information and any other relevant information was extracted from the National Health Laboratory Services (NHLS) laboratory information system. Specimens were collected retrospectively from these cases. These consisted of peripheral blood smears, peripheral blood anti-coagulated with ethylenediaminetetraacetic acid (EDTA) or BMA samples. The material used was considered to be for storage and did not jeopardise routine diagnostic analysis. Negative control samples were randomly chosen from patients who were admitted to CMJAH and at the time had either a near normal full blood count (FBC) or a BMA assessment which showed no malignant infiltrate and no overt pathology. All specimens used as negative control samples were intended for the purpose of routine monitoring and/ or diagnosis and only the material remaining after routine analysis was utilised.

All cases that were diagnosed before 2008 (2004-2007) were classified as per the criteria stipulated by the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues that were published in 2001 (Jaffe et al., 2001) and those cases diagnosed from 2008 onwards were classified as per the criteria stipulated by the updated WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (Swerdlow et al., 2008). Ethical approval was obtained from the University (No.:MO90734) for the extraction of material and use of patient data.

2.2 DNA EXTRACTION

DNA was extracted from bone marrow aspirate slides, peripheral blood slides or EDTA collected peripheral blood using High Pure PCR template Preparation Kit (Roche, Mannheim, Germany) as per the manufacturer's instructions and as per the institutional Standard Operating Procedure (SOP: HAE0296v3) in the PCR laboratory. Blood wash (100-150µl) was used to remove blood and bone marrow from sample slides. Binding buffer (200 µl) and proteinase K (40µl) was then added to the sample material and incubated at 72˚C for 10 minutes (min). Isoproponol (100µl) was added and the sample then centrifuged at 8000rpm for 1min. An amount of 500µl inhibitor removal buffer was added to the sample which was again centrifuged at 8000rpm for 1min. On 2 separate occasions 500µl wash buffer was added and samples centrifuged as above. The samples were centrifuged at 14000rpm for 10 seconds to remove the remaining wash buffer from the sample. Finally pre-warmed (70˚C) elution buffer (40-50µl) was added to the filter tube and centrifuged at 8000rpm for 1min.

2.3 DNA QUALITY

Once samples were extracted the DNA quality and quantity was determined. This was performed making use of the NanoDrop® ND-1000 (NanoDrop Technologies Wilmington, Delaware USA). Both the DNA concentration and 260/280 value was recorded. This was done using ultraviolet spectrophotometry and measuring the ratio of absorbance at 260nm and 280nm. The samples were considered to be of good quality if the 260/280 ratio was between 1.8 and 2.0. If the sample yielded an undesirable result and more of the same sample was available, it was re-

extracted. If however, no further sample was available for re-extraction the already extracted samples still underwent electrophoresis and the result was interpreted provided that the internal controls were present.

2.4 PCR FOR FLT3-ITD AND NPM1 MUTATIONS

PCR primer sets used to detect the presence or absence of FLT3-ITDs and the NPM1-mut have been previously described by Kottaridis *et al* (Kottaridis et al., 2001) and Falini *et al* (Falini et al., 2005) respectively. Primers for FLT3-ITD span approximately 330bp and include the common internal duplication site. The primer sets for NPM1 span approximately 170bp and cover mutations from A-F and thus cover the majority of described mutations(Huang et al., 2008). All of the primer sets were obtained from Integrated DNA Technologies and reconstituted according to instructions on the package insert. These primer sets and their sequences are presented in Table 2.1.

Table 2.1 Specific primer sequences of FLT3, NPM1 and HBG used.

Name	Primer	Sequence
FLT3-F	Forward Primer FLT3	5'FAM- AGCA ATT TAG GTA TGA AAG CCA GCTA – 3'
FLT3-R	Reverse Primer FLT3	5'-CTT TCA GCA TTT TGA CGG CAA CC – 3'
NPM1-F	Forward Primer NPM1	5'-GTT TCT TTT TTT TTT TTT CCA GGC TAT TCA AG-3'
NPM1-R	Reverse Primer NPM1	5'-HEX CAC GGT AGG GAA AGT TCT CAC TCT GC – 3'

Abbreviations: NPM1, Nucleophosmin member 1 protein; FLT3-ITD, internal tandem duplication of the FMS related tyrosine kinase 3 (FLT3) gene; F, forward; R, reverse

The PCR conditions initially used were those described by Huang *et al* (Huang et al., 2008) making use of the C1000 thermal cycler (Bio-Rad Laboratories Inc, Hercules, CA) .

The C1000 thermal cycler (Bio-Rad Laboratories Inc, Hercules, CA) was used to perform the reaction and the PCR conditions used were as follows: initial denaturing for **10**min at 95˚C, followed by **45** cycles at 95˚C for 20s, 60˚C for **60**s and 72˚C for **60s and a** final cycle of 30min at 72˚C. These conditions were used for both the NPM1 and FLT3 assays.

The PCR conditions described and the master mix as set out below in Table 2.2 was to be the standard operating procedure for the rest of the research assignment. The PCR products were then diluted to 1:10 and 1:30 and each of these electrophoresed on a 2% agarose gel (Bioline Ltd, London, UK) to assess for the presence of the appropriate PCR product and then analysed by means of capillary electrophoresis.

As presented in a multiplex method used by Huang *et al,* (Huang et al., 2008) 200ng of DNA was used per reaction. In order to obtain this amount of DNA the following calculation was used: 200ng/ 80ng/µl = 2.5µl of DNA per reaction

Table 2.2 PCR master mix protocol.
The multiplex PCR was discarded and each primer set was incorporated into its own individual master mix.

Product	Amount
Genomic DNA	2.5 µl
Primers:	
NPM1-F /NPM1-R	20pmol (0.5 µl) Forward/ 20pmol (0.5 µl) Reverse
FLT3-F /FLT3-R	20pmol (0.5 µl) Forward/ 20pmol (0.5 µl) Reverse
$MgCl_2$	25mmol/l (2.0 µl)
dNTP	2.5mmol/l (0.75 µl)
10xPCR buffer	1.35 µl
Amplitaq Gold	0.5 µl (2.5U)
Water	11.9 µl

Abbreviations: NPM1, nucleophosmin; FLT3, FMS related tyrosine kinase 3; MgCl2, magnesium chloride; dNTP, deoxynucleotide triphosphates; F, forward; R, reverse.

2.5 GEL ANALYSIS

A 50ml 2% agarose gel was prepared using 50ml 1x trisborate EDTA (TBE) buffer in a 250ml conical beaker. Once the gel had been mixed and heated and allowed to cool to 60-70˚C 2.5ul ethidium bromide (10mg/ml) was added. The gel was poured into a caste and allowed to set. The samples were then loaded together with a suitable size marker (e.g. 50bp Ladder or Low molecular weight marker). The gel was then placed in the gel tank in 1xTBE buffer with ethidium bromide at 150 volts. The gel was electrophoresed until the dye was approximately ¾ down the gel (1 hour). Following electrophoresis, the gel was visualised under UV light and a photograph taken. The presence of a band at approximately 330bp indicates the presence of FLT3-wt and at approximately 170bp NPM1-wt.

2.6 CAPILLARY ELECTROPHORESIS ANALYSIS

All samples were analysed by capillary electrophoresis on an ABI 3130 Genetic Analyser (Applied Biosciences, Inc., Foster City, CA). The labelled PCR product samples were diluted to 1:10 and 1:30 with distilled water. Of these dilutions 1µl was mixed with 8.5µl of HiDi formamide (Applied Biosystems, Inc., Foster City, CA) and 0.5µl of Genescan ROX 350 internal size standards (Applied Biosystems, Inc., Foster City, CA) and loaded in 96 well plates. This sample preparation is demonstrated in Table 2.3. These samples were then heated to 95˚C for 2min and then snap chilled on ice for 5min directly afterwards. The samples were then separated by means of the capillary electrophoresis based system on the 3130 Genetic Analyser using the POP7™ conformation analysis polymer (Applied Biosystems, Inc., Foster City, CA) and the fragment analysis default method as per the manufacturer's instructions. The raw data was then analysed using the GeneMapper version (v) 2.0 software (Applied Biosystems, Inc., Foster City, CA). All cases with a mutant peak present were included for analysis, including those with a mutant peak <100.

Table 2.3 PCR product preparation for capillary electrophoresis analysis.
Standard volumes of ROX™ Size Standard and HiDi™ Formamide are used.

Solution	Volume
ROX™ Size Standard	0.5 µl
Hi Di™ Formamide	8.5 µl
PCR product	1.0 µl
Total	**10 µl**

Reproducibility of the assay was assessed by means of repeating random (controls and mutation positive) samples as duplicated on separate days in order to determine if the expected peak size was maintained.

2.7 DILUTIONAL ASSESSMENT

Samples with known mutation positivity (NPM1-mut and FLT3-ITD respectively) were serially twofold diluted making use of extracted wild type DNA from a control sample. The NPM1 and FLT3 fragments were amplified as described above and the PCR products analysed by capillary electrophoresis. This was performed in attempt to assess the sensitivity of the assay.

Another twofold serial dilution making use of distilled H2O as the diluent was performed in a similar manner as described above. This was performed in order to assess the robustness of the assay.

2.8 FLT3 MUTANT LEVEL ASSESSMENT

The area under the curve (AUC) of both the mutant and wild type FLT3 peak generated on capillary electrophoresis is automatically recorded as part of the electropherogram data. The relative mutant level is then calculated using the AUC of the mutant and expressed as a percentage of total FLT3. The calculation is as follows: AUC FLT3-ITD x100/ AUC FLT3-wt +AUC FLT3-ITD (Gale et al., 2008). Refer to Appendix 5.1 for further details.

2.9 IMMUNOPHENOTYPIC ANALYSIS OF AML BY MEANS OF A PANEL OF ANTIBODIES

2.9.1 SAMPLE PREPARATION

Samples were prepared for immunophenotypic analysis by using accredited standard operating procedures for immunophenotypic preparation (SOP: HAE0066) as described below in the routine laboratory.

2.9.1.1 ENRICHMENT OF MONONUCLEAR CELLS

Samples were layered onto 4.5ml of Histopaque® (Sigma-Aldrich Inc., St. Louis, MO) and then centrifuged at 3500rpm for 15min. This concentrates mononuclear cells into a single layer. The enriched mononuclear cell layer was pipetted into a new tube and washed in phosphate buffered saline (PBS) (Oxoid Ltd, Basingstoke, HA).

2.9.1.2 RED CELL LYSIS

The ficoll prepared samples were incubated for 4min with 4ml, 8.9% isotonic ammonium chloride in order to lyse any remaining red cells. Debris was removed by washing the samples in 5ml of PBS. Finally the samples were centrifuged to obtain a cellular button which was then re-suspended in 1ml of PBS. Monoclonal antibodies were added to each tube containing 100µl of cells according to the manufacturer's instructions and the tube was then incubated for 15min at room temperature, in the dark, after being vortexed briefly. For each slide 0.1ml of antibody was used for staining and a total of 5 washes were performed making use of PBS.

2.10 IMMUNOPHENOTYPING ANALYSIS FOR LEUKAEMIAS

Immunophenotypic analysis using the accredited and validated standard operating procedure (SOP: HAE0042v4) was performed as part of the main laboratories routine work flow at the time of receiving the sample and the relevant request form. A number of monoclonal antibodies were added to a single tube of 100µl of cells in order to allow for multiparameter flow cytometry analysis. The monoclonal antibodies used in a particular combination were determined by the registrar or pathologist analysing the flow cytometry. Paint-A-Gate™ Pro software (BD Biosciences Inc., San Jose, CA) was used for analysis of the results on the FACS Calibur (BD Biosciences Inc., San Jose, CA). Painting allows the user to select a group of events on a dot plot and label them with an identifying colour. The events painted with a particular colour on one dot plot will be labelled by Paint-A-Gate with the same colour on all other plots generated from that data set. Paint-A-Gate can display information such as the percentage of total events or the number of events represented by the painted population. Paint-A-Gate was used also used to calculate the statistics of the painted populations. An “acute screen” was performed on either peripheral blood or a bone marrow aspirate sample when a patient is suspected of having leukaemia. This consists of a panel of monoclonal antibodies prepared in a specific combination in order to make a clear diagnosis. In some instances the samples received are not sufficient to perform an acute screen and thus a limited analysis is done. The limited analysis may still be able to distinguish a myeloid leukaemia from a lymphoid leukaemia. Monoclonal antibodies routinely performed and with particular relevance to this study are summarised in Table 2.4. The monoclonal antibodies used included CD4, CD7, CD8, CD13, CD33, CD117 and CD45 (Beckman Coulter Inc., Pasadena, CA).

CD15, CD34 and myeloperoxidase (Dako, Denmark, A/S) as well as CD3, CD5, CD14 and anti-HLA (BD Biosciences Inc., San Jose, CA). The diagnosis of an AML was only made once the various criteria as stipulated in the WHO 2008 classification had been met.

Table 2.4 Monoclonal antibodies routinely used in diagnosing an AML.
Indication of their positivity in the different French-American-British (FAB) groups (Bordessoule et al., 1993).

Antigen	FAB classification							
	M0	M1	M2	M3	M4	M5	M6	M7
CD7 (PE)	-/+	-/+	-/+	-	-/+	-/+	-	-
CD11b (FITC)	-	-	-/+	-	+/-	+	-	-
CD13 (PE)	+	+	+	+	+	+	-/+	-/+
CD14 (FITC)	-	-	-	-	+/-	+	-	-
CD15 (FITC)	+/-	+/-	+	+/-	+	+	-	-
CD33 (PE)	+	+	+	+	+	+	-/+	-/+
CD34 (FITC)	+	+	+/-	-	-	-	-	-
CD45 (PE)	+	+	+	+	+	+	+/-	+/-
Glycophorin A (FITC)	-	-	-	-	-	-	+/-	-
MPO	-	+	+	+	+/-	-	-	-
HLA-DR (FITC)	+/-	+/-	+/-	-	++/+++	++/+++	+/-	+/-
CD117 (PE)	+	+	+	-	+/-	-	+/-	+/-
CD4 dim (FITC)	-	-	-	-	+/-	+/-	-	-

Abbreviations: FAB, French American British; CD, clusters of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; MPO, myeloperoxidase; HLA-DR, human leukocyte antigen- DR.

2.11 SEQUENCING REACTION AND ANALYSIS

In order to sequence the PCR products obtained from 3 of the FLT3-ITD mutated and 2 of the NPM1 mutated samples the ABI PRISM BigDye™ Terminator version 3.1 ready reaction cycle sequencing kit was used by both the NHLS and Inqaba

Biotec. The initial PCR product was quantified by measuring absorption at 260nm using the Nanodrop Spectrophotometer. This was then followed by a DNA clean up step. A total of 3µl of PCR product was added to the 17µl reaction volume used. As demonstrated in Table 2.5, the following was prepared on ice:

Table 2.5 Cycle Sequencing PCR Master Mix.

Solution	Volume
Terminator ready reaction mix	2 µl
5x buffer	4 µl
PCR product	5.0 µl
Primer 3.2pmol/0.1µl	6.4µl
Deionised water	4.6 µl
Total	**10 µl**

The template was then mixed and spun and placed in the thermal cycler. The following cycling parameters were used: 96˚C for 1min, 96˚C for 10sec, 50˚C for 5sec, 60˚C for 4min for 25 cycles and 4˚C for infinity.

Following this, a cleanup of the cycle sequencing product takes place. This is done using the DyeEx® 2.0 Spin Kit (Qiagen Inc., CA, USA). This is done in order to remove the unincorporated dye terminators from the sequencing reaction.

The DyeEx® 2.0 Spin Kit columns are vortexed in order to resuspend them. The columns are then centrifuged for 3 min at 750 xg in an Eppendorf centrifuge to elute the sample. The DyeEx® 2.0 Spin column is placed in a newly labelled microcentrifuge tube and the sample applied to the centre of the column. It is then centrifuged again as described above. The eluted samples are then dried for

35min in the miVac DNA concentrator. These dried samples are then resuspended in 10µl of HighDye Formamide.

All of the PCR products obtained were then prepared for capillary electrophoresis on the ABI 3130 Genetic Analyser (Applied Biosciences, Inc., Foster City, CA) using the POP7™ conformation analysis polymer (Applied Biosystems, Inc., Foster City, CA) and the Sequence Analysis default method as per the manufacturer's instructions. The samples were then analysed using the Sequence Scanner Software v1.0 (Applied Biosystems, Inc., Foster City, CA) and the alignment to the reference sequences was made using Geneious Pro 5.0 (Biomatters Ltd., Auckland, New Zealand). The samples being assessed for FLT3-ITD mutations were aligned to the Gene Bank accession number NG_00706 and the samples being assessed for an NPM1 mutation were aligned to the accession number NM_002520.

2.12 CYTOGENETIC ANALYSIS

Cytogenetic analysis was performed on bone marrow aspirate smears submitted by the treating doctor. The samples were prepared, in the routine laboratory, for analysis and interpreted by using accredited standard operating procedures for cytogenetic analysis and preparation (SOP: HAE0220) as described below.

Fresh bone marrow of 1.0-2.0ml was obtained from the initial aspirate in a lithium heparin tube and kept at room temperature. Cultures were then initiated within 24 hours of collection.

2.12.1 CULTURE INITIATION

The specimen was centrifuged at 1500rmp for 10min at room temperature. The majority of the supernatant was removed with a sterile Pasteur pipette, leaving a small amount above the pellet. The pellet was re-suspended in the remaining supernatant. For the 24-48 hour culture 0.25-0.5ml of the suspension was added to a sterile 50ml culture flask which contains 3-5ml of complete supplemented medium. This was then incubated at 37℃ overnight.

2.12.2 HARVESTING PROCEDURE AND SLIDE MAKING

The culture was centrifuged at 1500rpm for 8-10min. The supernatant was then removed and 0.1ml of colemid and 6ml of warm potassium chloride (37˚C) was added. This was then mixed and incubated for 25min at 37˚C. A total of 3 drops of cold 3:1 methanol acetic acid was added, mixed and the solution centrifuged at 1500rpm for 8-10min. The supernatant was removed and the pellet re-suspended again. Another 5ml of cold 3:1 methanol acetic acid was added and the solution thoroughly mixed. The solution was again centrifuged at 1500rpm for 8-10min, the supernatant is removed and the pellet re-suspended and another 5ml of cold 3:1 methanol acetic acid was added and the solution thoroughly mixed. Slides were then made according to the slide preparation protocol.

2.12.3 REPORTING OF RESULTS

The results were signed off by the head of the unit or laboratory manager and included a tabulation of cells analysed, their chromosome counts, karyotypes description by the International System for Human Cytogenetic Nomenclature (ISCN) (2005) system. A total of 20 metaphases were analysed.

2.13 FISH ANALYSIS

FISH analysis was performed on bone marrow aspirate smears submitted by the treating doctor. The samples were prepared for analysis and interpreted by using accredited standard operating procedures for FISH analysis and interpretation (SOP: HAE0234 and HAE0244) as described below.

2.13.1 SAMPLE PREPARATION

The unstained bone marrow aspirate slides were pre-treated with methanol for 30min at a temperature of -20˚C and then dehydrated in a series of graduated ethanol solutions of 70%, 90% and a 100% for 5min each at room temperature.

The slides were then air dried at 37˚C. The slides are then warmed at 60˚C for 5min and put into a denaturing solution for another 5min. Afterwards they are immediately put in ice cold ethanol solutions of 70%, 90% and a 100% for 5min each. The slides were then air dried in an up right position.

2.13.2 FISH PROBES

The probes used included the following: LSI BCR/ABL ES dual colour translocation probe (Vysis, Abbott, Illinois, USA), LSI PML/RARA dual colour translocation probe, LSI TEL/AML ES dual colour translocation probe, LSI MLL dual colour probe, LSI ETO spectrum orange/ LSI AML1 spectrum green, LSI CBF B, inv (16) dual colour probe

The probes used were defrosted and kept in the dark and vortexed briefly before 1µl of the probe, was mixed together in an Eppendorf tube together with 2ul of Sabax water and 7µl of the hybridization buffer and then spun for 5sec at 13000rpm and vortexed.

The probes were then denatured at 76˚C by floating the Eppendorf tube in a water bath for 5min. The probe tube was put on flaked ice, immediately for 5min, then centrifuged again for 5sec at 13000 rpm and then put back on ice.

The probe mixture (10 µl) was dispensed onto the slides and covered with a cover slip. The slides were put in a slide carrier and placed in a beaker which was then incubated at 37˚C overnight to allow for hybridization to take place.

Slides were removed from the incubator and briefly dipped into 50% formamide and the coverslip shaken off from the slide. The slides were then washed in 50% formamide for 10min. This was repeated twice.

The slides were washed in 2x salt, sodium citrate (SSC) for 10 min in a shaking waterbath and then washed in 2xSSC and Tween® for 5min at 42-46˚C. An amount of 50µl of DAPI was added to 50ml of 2xSSC at room temperature and the slides were put into this solution and placed on the shaker for 15min. The coplin jar is covered to protect it from light. The slides were then washed in Tween solution at room temperature for 2min. One drop of vectashield mounting solution was dispensed onto each slide. A large cover slip was then placed onto each slide. The slides were kept in the dark for analysis under fluorescent microscope.

2.13.3 REPORTING RESULTS

There were between 100-200 cells analysed and the findings reported as a percentage. The presence of a specific fusion gene was reported as a positive result and the absence of the fusion gene as a negative.

2.14 NPM1 CYTOPLASMIC STAINING

FLEX Monoclonal Mouse Anti-Human Nucleophosmin, Clone 376, ready-to-use (Dako, Denmark, A/S) was used as per manufacturer's instructions to stain paraffin embedded trephine samples for cytoplasmic NPM1. Trephine tissue was cut into sections of approximately 4 µm and pre-treated with heat induced epitope retrieval, using EnVision FLEX Target Retrieval Solution, High pH (Dako, Denmark, A/S). The pre-treatment (PT) Link Autostainer (Dako, Denmark, A/S) was used for this pre-treatment procedure. The pre-heat temperature was 65˚C which was followed by increasing the temperature to 97˚C for 20min (epitope retrieval temperature) and then cooling back down to 65˚C. The samples were then removed from the Autostainer rack and immediately dipped into the PT Link rinse station, which contained Envision FLEX Wash Buffer. The samples were then left in the wash buffer for 1-5min. The staining steps using the FLEX Monoclonal Mouse Anti-Human Nucleophosmin, Clone 376, ready-to-use was pre-programmed into the Autostainer Link software (Dako, Denmark, A/S). For each slide 0.1ml of antibody was used for staining with an incubation time of 20min. A total of 5 washes were performed making use of PBS.

2.15 STATISTICAL ANALYSIS

All of the clinical features at presentation were compared according to the mutation status *i.e.* NPM1 and FLT3-ITD mutation positivity. Categorical variables such as sex, FAB subtype and immunophenotypic markers were compared using the Fischer's exact test. The continuous variables such as age, platelet count, Hb and WCC were compared using the Kruskal-Wallis test and the Mann-Whitney U

test. These tests were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Differences between the results of comparative tests were considered statistically significant if the 2-sided *P* value was less than 0.05.

3 RESULTS

3.1 INTRODUCTION

A number of experiments were performed in order to optimise the assay. This included assessing DNA purity and integrity as well as PCR optimisation. Various alternative methods were used in an attempt to prove NPM1-mut and FLT3-ITD positivity. This included NPM1 cytoplasmic staining and sequencing; these results are reported below. Once these findings had been evaluated and reported the patient sample results were assessed and analysed accordingly.

Figure 3.1 represents a summary of the samples initially identified as being AML positive and the various exclusion criteria which were applied.

Of the 236 samples, 1 of these were excluded based on age at diagnosis (<17 years), another 29 were excluded based on their initial WHO diagnosis *i.e.* transformation from myelodysplastic syndrome (MDS) or myeloproliferative neoplasms (MPN) or a diagnosis of biphenotypic, ambiguous or undifferentiated lineage leukaemia. A further 5 samples were excluded based on only having a disease population of <10% and lastly another 5 samples were excluded as o result was obtained on the assay. Of the 197 samples which were processed, 32 were either controls or formed part of the longitudinal assessment of the 3 cases described in section 3.14. After the above exclusions were taken into consideration a total of 164 samples were available for further analysis. Of these 164 cases were assessed immunophenotypically, 164 cases had FBC parameters available for comparison, 102 samples had cytogenetic or FISH results available, 5 samples were sequenced and 32 samples with corresponding trephine samples available were assessed for cytoplasmic NPM1 staining.

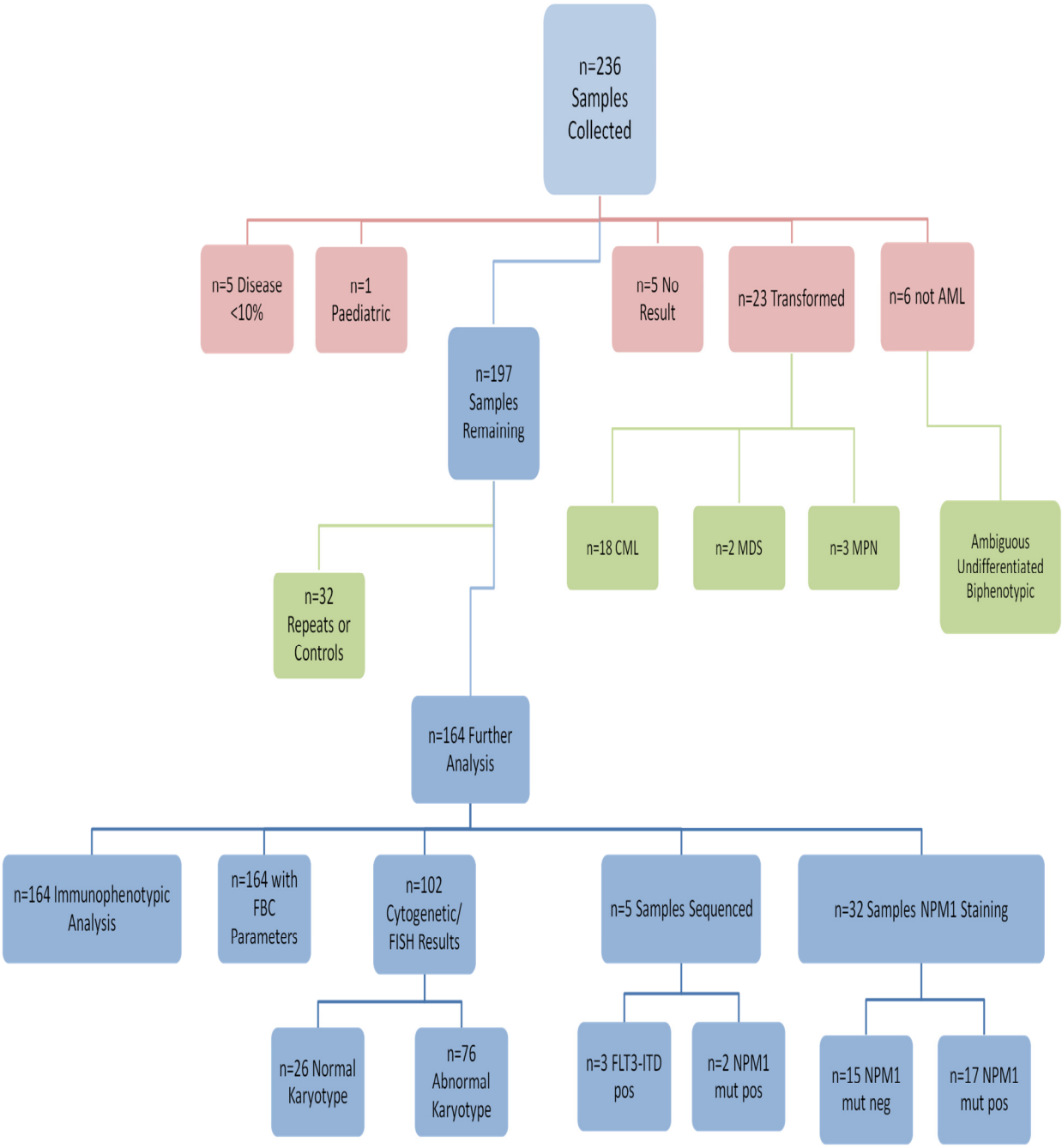


Figure 3.1 Flow diagram indicating the number of samples collected, reason for exclusion and number of cases analysed.
A number of these were patients with t(9;22) that had transformed to an AML. A total of 164 de novo adult AML samples remained for further analysis.

Abbreviations: AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; CE, capillary electrophoresis; FBC, full blood count; FISH, fluorescent in situ hybridisation; FLT3-ITD, internal tandem duplication of the FMS related tyrosine kinase 3 (FLT3) gene; NPM1, nucleophosmin 1 gene; Pos, positive; Neg, negative; Mut, mutation

3.2 DNA PURITY

Prior to the assessment for NPM1 and FLT3-ITD, the DNA quality with regards to protein contamination was assessed. Of the 152 samples which were assessed for DNA quality 38% (58) were found to have a 260/280 ratio between 1.8-2.0 which is considered to be pure DNA. A ratio below 1.8 indicates protein contamination while a value <1.5 may still be considered to be acceptable in certain circumstances. A total of 27.7% (42) samples were found to have a 260/280 ratio of <1.5. If more sample was available on these poor quality samples it was re-extracted, if not the original sample was still used.

3.3 DNA INTEGRITY

The DNA integrity of 32 random samples from the study cohort was assessed by means of confirming the presence of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. In Figure 3.2 a 2% agarose gel (Bioline Ltd, London, UK) using 1xTBE buffer (Merk, Darmstadt, Germany) illustrates representative samples with adequate DNA integrity. A DNA ladder was used (O'GeneRuler™ 50bp DNA ladder ready-to-use) to confirm the expected molecular weight for the GAPDH product.

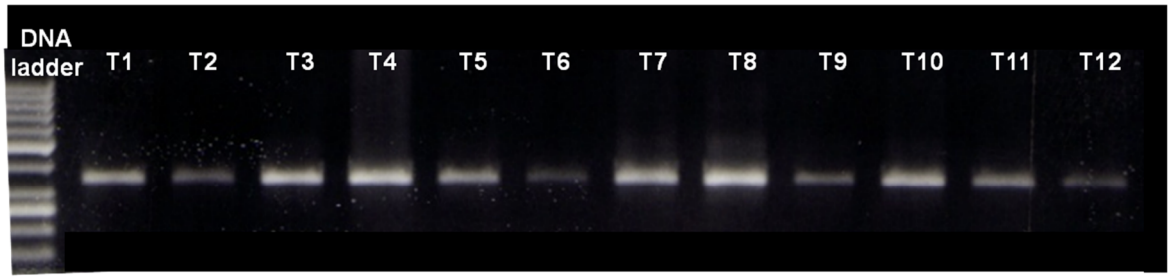


Figure 3.2 PCR for GAPDH.
The PCR products were separated on 2% agarose gel using 1x TBE buffer to confirm DNA integrity Samples T1-T12 are all cases diagnosed with de novo AML from which DNA was extracted for NPM1 and FLT3-ITD assessment.

Abbreviation: GAPDH, Housekeeping gene found in human cells.

Initially PCR products for NPM1 and FLT3 were analysed by gel electrophoresis. Figure 3.2 demonstrates results from 2% agarose gels (Bioline Ltd, London, UK) using 1x TBE buffer to assess the individual NPM1 and FLT3 PCR reactions. A 2% agarose gel (Bioline Ltd, London, UK) was used for the electrophoresis of the PCR products. This was able to discriminate the FLT3 band region from the NPM1 band region by assessing for the expected molecular weights (O'GeneRuler™ 50bp DNA ladder ready-to-use). Results from samples N5-N8, Q1, Q2 F5, F8 and T26-T28 are displayed in Figure 3.3. The gel assessments were done as part of the initial troubleshooting to confirm that products were being obtained in the expected regions before further analysis by means of capillary electrophoresis took place.

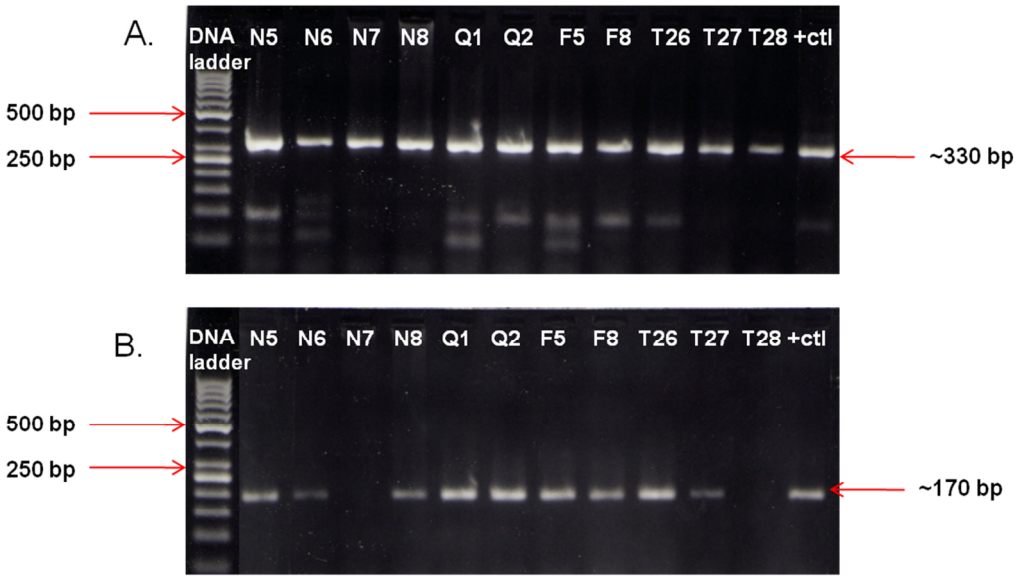


Figure 3.3 Agarose gels demonstrating gene products for FLT3 and NPM1.
(A) FLT3 ~330bp (B) NPM1 ~170bp. Both genes have different molecular weights and have been assessed relative to a 50bp DNA ladder. All the cases (N5-N8, Q1-Q2, F5, F8 and T26-T28) were de novo AML cases. Sample N7 and T28 did not yield a product for NPM1.

Abbreviations: FLT3, FMS-like tyrosine kinase receptor gene; NPM1, nucleophosmin1 gene; +ctl, positive control.

3.4 PCR, INITIAL GEL AND CAPILLARY ELECTROPHORESIS ASSESSMENTS

The PCR master mix and conditions for performing the reactions were optimised. Initially the AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Inc., Foster City, CA) volume was increased in order to obtain a product. Another of the optimisation steps was to determine which polymerase enzymes were the most suitable for use in this project. The results (Figure 3.4) suggest that AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Inc., Foster City, CA) was better suited compared to AmpliTaq ® DNA Polymerase (Applied Biosystems, Inc.,

Foster City, CA) for this particular PCR reaction. A 2% agarose gel (Bioline Ltd, London, UK) was used to electrophorese the same samples, but different DNA polymerase enzymes were used. All the other variables *e.g.* the thermal cycler used, the buffer, the cycling temperatures and the cycling times were kept constant.

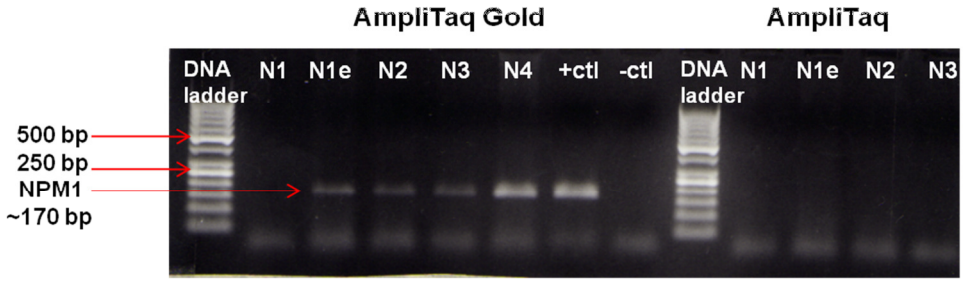


Figure 3.4 Comparison of commercially available DNA polymerases.
AmpliTaq Gold® and AmpliTaq® DNA Polymerase. The PCR product being assessed is NPM1. The PCR conditions were the same for both DNA polymerases. AmpliTaq Gold appears to have a better PCR product yield with 5 of the 6 expected products expressed. A 2% agarose gel was used.

All the samples (N1-N4) were extracted from cases diagnosed with AML.

Abbreviations: NPM1, nucleophosmin 1 gene; N1e, N1 re-extracted.

Other changes made in order to optimise the results included increasing the number of cycles from 35 to 45, increasing the denaturing melting temperature from 10min to 15min and increasing both the annealing time and the extension time from 40 sec respectively to 60 sec each. Initially both 2% and 2.5% agarose gels were used before it was determined that 2% gels yielded a clearer PCR product band. All of these optimisation steps were part of an initial attempt to

obtain PCR product on the gel and then to improve the brightness of the bands present.

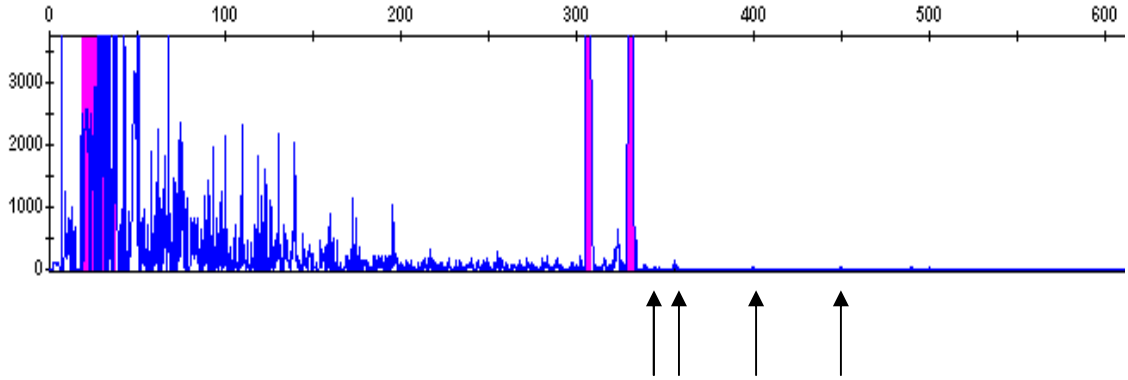
PCR product for capillary electrophoresis was assessed as neat, 1:10 and 1:30 dilutions. The 1:30 dilution resulted in the highest peak intensities.

3.5 CONTROLS

The controls consisted of 18 EDTA blood samples and 4 bone marrow aspirate slides, stored, post-analysis of the requested tests. The blood and slides used to assess for FLT3 and NPM1 by means of capillary electrophoresis. The samples were obtained from non-oncology wards and clinics and there was no suspicion or evidence of an acute leukaemia. As they were all expected to be negative for both mutations, these samples were used as negative controls to determine the position and intensity level of background noise. Of the 18 blood samples and the 4 bone marrow aspirate slides all were found to be NPM1-wt (as expected) with almost no background noise. There were no peaks found in the NPM1-mut region (*i.e.* 4bp larger than NPM1-wt). All the samples were also all FLT3-wt and accordingly had a peak in the expected region of 330bp. There was also a peak in the region of 333bp in 11 of these samples (peripheral blood samples). This was also relatively common in the study samples and was excluded as background noise. Furthermore, 4 of the samples had peaks in the region of 338bp (peripheral blood samples); this was also commonly noted in the study samples and was also considered to be part of the background noise. Figure 3.5 demonstrates electropherogram images with low background noise intensity seen in C2 and absent background noise in C6. All samples have noise prior to the peaks of

interest. Table 3.1 demonstrates all the control samples together with the NPM1-wt and FLT3-wt peak position and intensity as recorded in each control. This includes the 18 blood samples (C1-C18) and the 4 bone marrow aspirate slide samples (C19-C22).

A.



B.

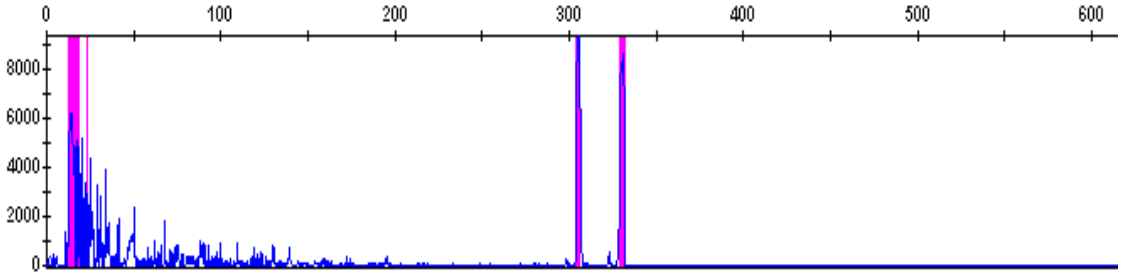


Figure3.5 Examples of electropherograms produced in control samples.
Sample C2 (A) has low peak intensities of background noise as indicated by the arrows. Sample C6 (B) has no background noise recorded.

Table 3.1 NPM1-wt and FLT3-wt size and intensity in control samples.
Any background noise peaks was also assessed in these 18 controls.

SAMPLE	NPM1-wt SIZE	NPM1-wt INTENSITY	FLT3-wt SIZE	FLT3-wt INTENSITY
C1	169.34 171.04	8247 8741	329.65 331.11 333.4	9143 9156 58
C2	169.1 170.9 187.36	7740 7396 54	329.76 333.61 338.36 355.48	8049 470 66 140
C3	169.27 170.87	8295 8469	330.13 333.48	8241 555
C4	169.41 171.0	8213 8744	330.32 333.64	8989 326
C5	169.27 170.97	8277 8377	330.94 333.64	9036 120
C6	169.31 170.9	8050 8507	329.62 330.74	8073 8644
C7	170.91	8963	330.84	9146
C8	169.43 171.01	8646 8590	331.19	4839
C9	169.22 170.07	7681 4473	329.62 333.44	8617 529
C10	170.1 187.95	7669 64	329.59 333.53	7590 704
C11	170.08 171.88	7789 8329	329.72 333.44	8839 436
C12	170.23	7920	330.42 338.36	9023 50
C13	169.9	8758	330.94	10240
C14	169.4	7756	331.07 333.49 337.21	10173 50 241
C15	169.28	8485	331.1	2852
C16	169.43	8493	330.94	10240
C17	169.36	7366	331.27	1927
C18	170.98	9082	330.96 333.56 337.34	10365 83 66
C19	169.95	23244	329.82	2625
C20	169.92	10360	329.16	34
C21	169.87	15134	329.81	69
C22	169.93	227	329.86	268

Abbreviations: NPM1-wt, nucleophosmin 1 gene wild type; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

3.6 SEQUENCING RESULTS

In order to attempt to validate the NPM1 and FLT3-ITD mutation results, 3 of the FLT3-ITD mutated and 2 NPM1 mutated samples (on capillary electrophoresis) were amplified by means of standard procedures with the FLT3 primer pairs and NPM1 primer pairs described. The PCR products were purified by the standard methods and were sequenced directly using FLT3-reverse (FLT3-R) and NPM1-forward (NPM1-F) primers. These primers were used as they were not fluoro-labelled. In total of 2 of the FLT3-ITD positive samples and 1 of the NPM1 mutation positive samples were sequenced by Inqaba Biotec™ while the other 1 FLT3-ITD positive and 1 NPM1 mutation positive sample were sequenced by our laboratory (NHLS). Samples sequenced to assess for FLT3-ITD mutations were aligned to the Gene Bank accession number NG_00706 and a sequence trace was obtained for each (Figure 3.6). Samples sequenced to assess for an NPM1 mutation were aligned to the accession number NM_002520. Unfortunately, although sequencing obtained results, only the wild type was amplified and the mutants were not identified.

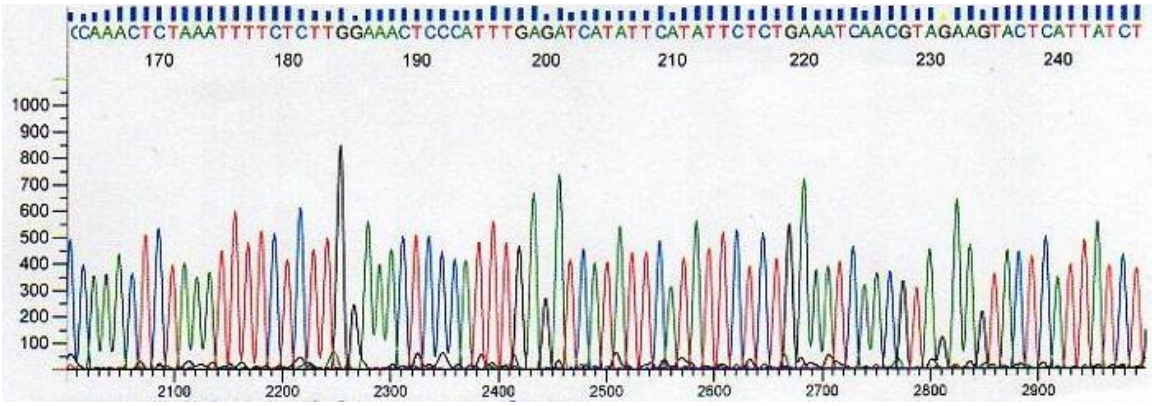


Figure 3.6 DNA sequence analysis of Sample N44.
The FLT3-R PCR primer was used as a sequencing primer. No internal tandem duplications were identified in this section or any other.

Abbreviations: FLT3, FMS related tyrosine kinase 3; R, reverse.

3.7 NPM1 CYTOPLASMIC STAINING

FLEX Monoclonal Mouse Anti-Human Nucleophosmin, Clone 376, (Dako, Denmark, A/S) was used to assess for cytoplasmic NPM1 positivity. The presence of cytoplasmic NPM1 is known to concur, in most cases, with the presence of an NPM1 mutation (Falini et al., 2006). A total of 32 samples were assessed. These 32 samples were chosen as they were found to have corresponding, adequate quality, stored trephine biopsy samples which could be used for staining. Of these, 18 were NPM1-mut positive on molecular testing and 14 were NPM1-wt. These samples were assessed by 2 pathologists blinded to the molecular results. Pathologist 1 had correctly assessed the cytoplasmic or nuclear positivity of NPM1 in 59% (19/32) and pathologist 2 in 53% (17/32) of samples. The mutant peak intensity did not appear to affect the ability to determine cytoplasmic NPM1 positivity. These details are depicted in Table 3.2. Figure 3.7 demonstrates the

presence of NPM1 nuclear and cytoplasmic staining (A and B) as seen in NPM1 mutation positive cases as well as the presence of nuclear NPM1 staining only (C and D) as expected in NPM1 wild type cases.

Table 3.2 Assessment of NPM1 cytochemical staining in NPM1 mut positive samples.
The diagnosis of NPM1-mut positivity was made by means of CE and mutant peak intensity documented. The cytoplasmic and nuclear staining of these samples were then assessed by 2 pathologists blinded to the CE results

SAMPLE	NPM1-mut INTENSITY On CE	Pathologist 1	Pathologist 2
R 52	8857	Cytoplasmic	Cytoplasmic
R 57	7000	Cytoplasmic	Nuclear
F19	8392	Cytoplasmic	Cytoplasmic
R 35	6077	Nuclear	Nuclear
N14	8686	Cytoplasmic	Cytoplasmic
N49	523	Cytoplasmic	Cytoplasmic
N55	8556	Cytoplasmic	Nuclear
N64	611	Cytoplasmic	Cytoplasmic
T9_R9	8862	Cytoplasmic	Cytoplasmic
R 11	109	Nuclear	Cytoplasmic
R 17	407	Cytoplasmic	Cytoplasmic
R 21	208	Cytoplasmic	Nuclear
N56	386	Cytoplasmic	Nuclear
R30_T7	243	Nuclear	Cytoplasmic
T14_F11	198	Cytoplasmic	Cytoplasmic
T8	3793	Nuclear	Nuclear
R 8	314	Cytoplasmic	Cytoplasmic
N24	209	Cytoplasmic	Cytoplasmic

Abbreviations: ; NPM1-mut, nucleophosmin 1 gene mutant; CE, capillary electrophoresis .

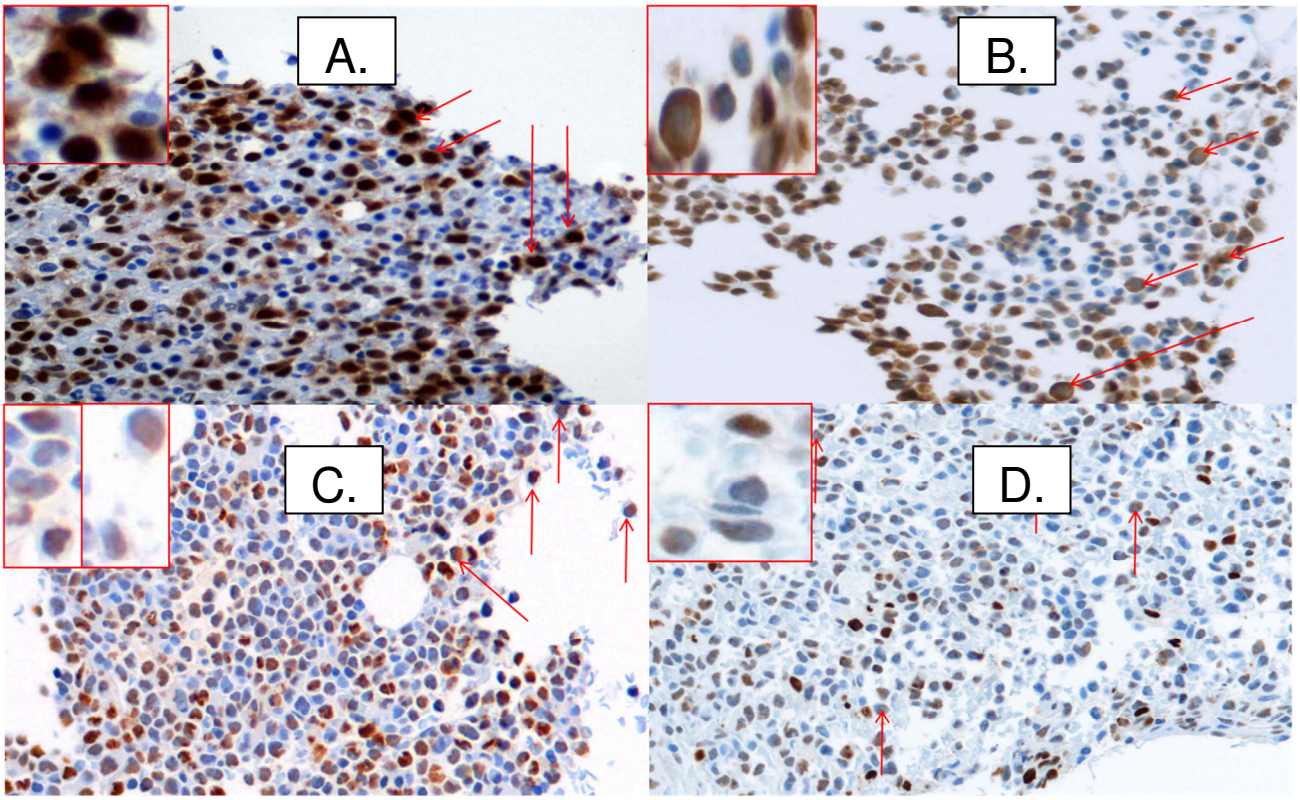


Figure 3.7 NPM1 cytoplasmic staining.
(A) and (B) 40x magnification demonstrates NPM1 cytoplasmic positivity while (C) and (D) 40x magnification demonstrates NPM1 cytoplasmic negativity and nuclear positivity. Cytoplasmic positivity is in keeping with NPM1-mut positivity and cytoplasmic negativity with NPM1-wt on molecular testing. The arrows indicate individual cells with cytoplasmic NPM1 positivity in A and B and nuclear positivity in C and D respectively. The insets are enlargements of the stained tissue blocks in order to see the individual cells more clearly.

Nikon ECLIPSE E400 microscope (Nikon, Tokyo, Japan) Altra 20 soft imaging system Olympic digital camera (Olympic, Hamburg, Germany)

3.8 CAPILLARY ELECTROPHORESIS ASSAY

Utilising capillary electrophoresis, NPM1-wt displayed consistent bands at approximately 170bp and NPM1-mut bands were displayed at approximately 174bp (Figure 3.8A). FLT3-wt was displayed at approximately 330bp while the FLT3-ITD varied in length from 9bp to 163bp (Figure 3.8B) as expected by the predicted size that the amplification primers spanned.

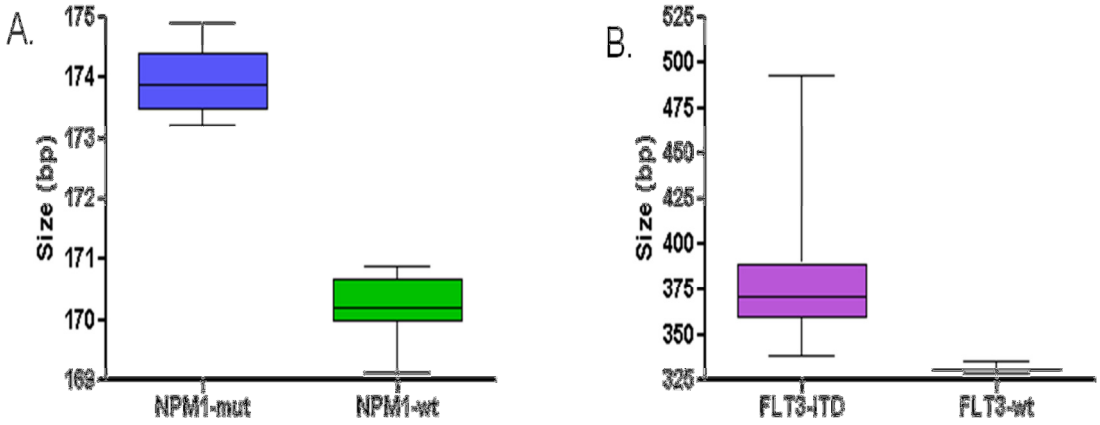


Figure 3.8 Capillary electrophoresis for NPM1 and FLT3.
(A) NPM1-mut and NPM1-wt and (B) FLT3-ITD and FLT3-wt sizes. All of the 164 samples analysed by capillary electrophoresis were included. Both the NPM1 and FLT3-ITD mutations are significantly different ($P<0.05$) in size compared to their respective gene wild type.

Abbreviations: bp, base pairs; NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

Reproducibility of the assay was partially assessed by means of retrospective analysis of samples. A limited set of randomly selected samples (15) were re-assessed using the same methods as previously described. Of these samples 6 were controls and the other 11 samples were positive for either FLT3-ITD or NPM1-mut or both. All of these samples confirmed both the presence of the wild type and the mutants previously described in each sample. All of the wild type and mutant peaks reported appeared to be of a similar bp size to those reported on initial electrophoresis.

Figure 3.9 demonstrates NPM1 and FLT3 gene wild type on capillary electrophoresis, while Figures 3.10 and 3.11 demonstrates NPM1 and FLT3-ITD mutations respectively. Sample R63 and sample R38 were both positive for an

NPM1-mut as well as FLT3-ITD and were thus used as examples. Both samples had more than one FLT3-ITD present, but only the mutant with the highest peak intensity was labelled. The NPM1-wt fragment lengths can range from 169 to 171bp with what appears to be the presence of a fragment length polymorphism.

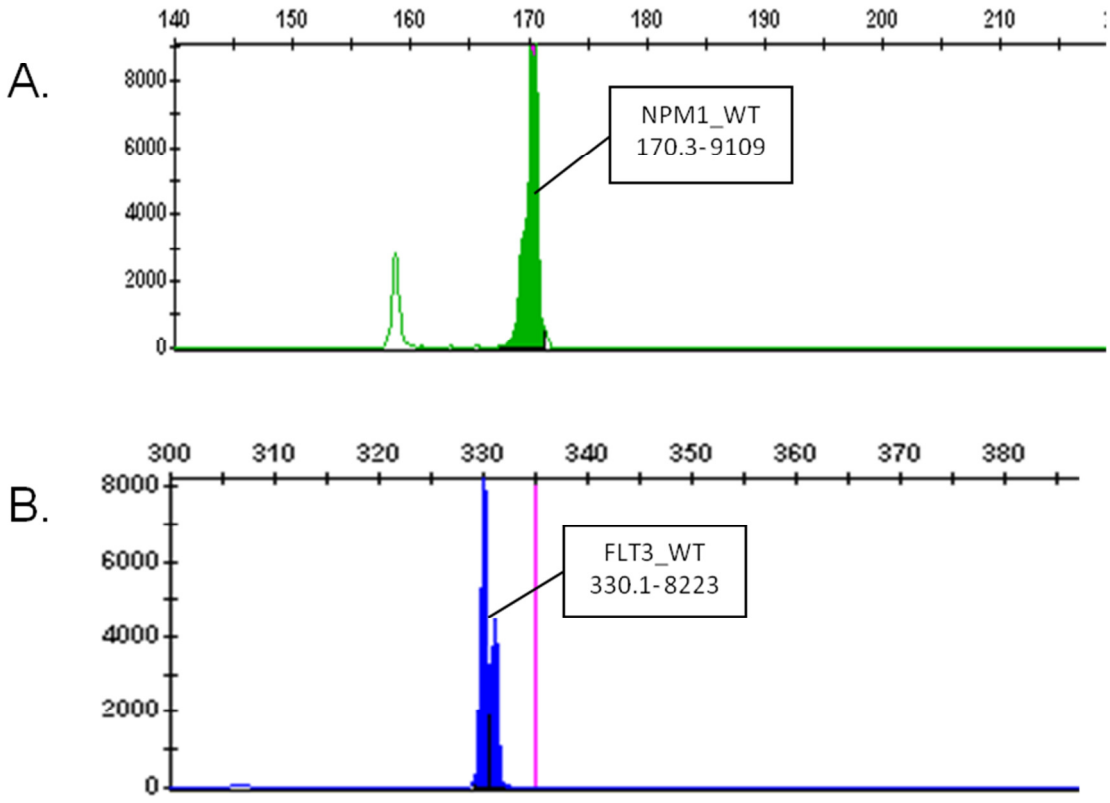


Figure 3.9 Capillary electrophoresis pattern of NPM1 and FLT3 wild type peaks.
Demonstration of (A) NPM1 and (B) FLT3 wild type peaks. Sample R64 (A) demonstrates an NPM1 wild type 'single peak' at 170.30bp with a peak intensity of 9109, while Sample T18 (B) demonstrates a FLT3 gene wild type peak at 330.1 bp with a peak intensity of 8223.

Abbreviations: bp, base pairs; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type

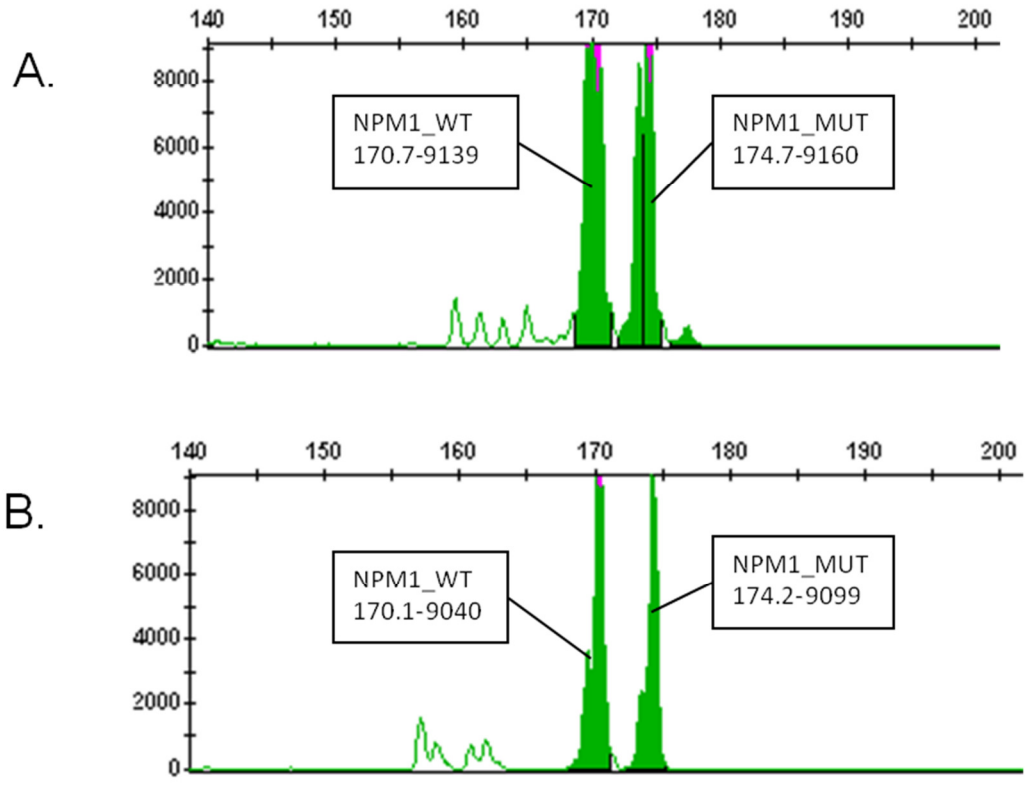


Figure 3.10 Capillary electrophoresis patterns showing heterozygosity for NPM1-wt and mutant peaks.
Sample (A)R63 and (B)R38 are heterozygous for NPM1. The bp position and peak intensities are displayed for both the NPM1-mut and NPM1-wt peaks.

Abbreviations: bp, base pairs; NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type.

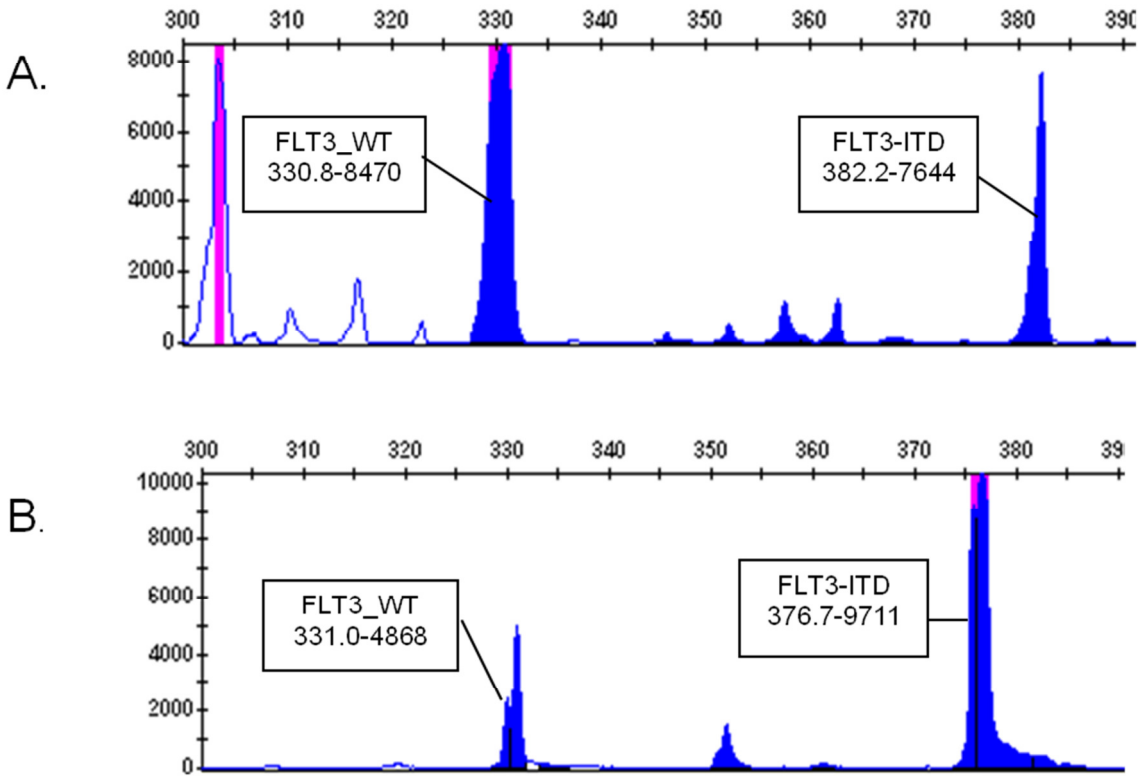


Figure 3.11 Capillary electrophoresis patterns showing heterozygosity for FLT3-wt and FLT3-ITD peaks.
Sample (A) R63 and (B)R38 are positive for FLT3-wt and FLT3-ITD. The bp position and peak intensities are displayed as part of the labelling above.

Abbreviations: bp, base pairs; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

More detailed information of each sample which was found to be either NPM1 mutation positive, FLT3-ITD mutation positive or which was found to have dual positivity can be found in Appendix 5.2. Figure 3.12 demonstrates a common phenomenon where two NPM1 wild type fragments, with a 1bp difference was noted.

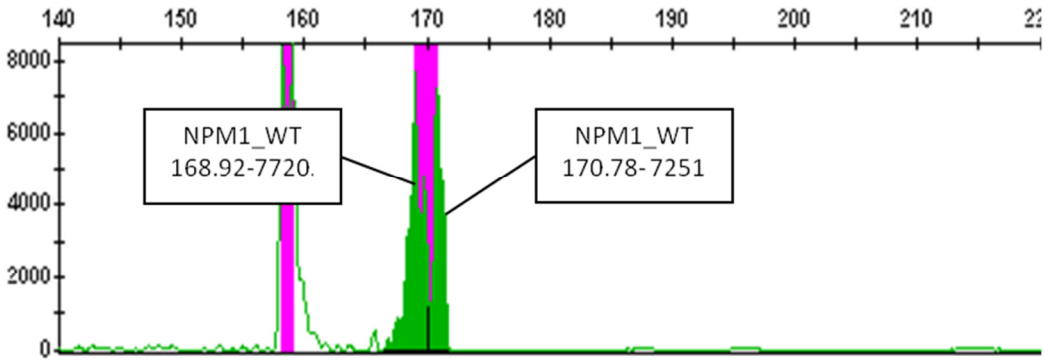


Figure 3.12 NPM1-wt 'double peak' at 169bp and 170.78 bp.
This suggests the presence of an intronic fragment length polymorphism.

Abbreviations: bp, base pairs; NPM1-wt, nucleophosmin 1 gene wild type.

3.9 CRITERIA FOR NPM1 AND FLT3-ITD MUTATION POSITIVITY

All of the cases in our cohort found to have a peak corresponding to the expected mutation peak position were included for analysis. This included those with a low level of mutation peak fluorescent intensity (arbitrary units). As part of this study a more comprehensive analysis of all the NPM1-mut and FLT3-ITD positive cases was done to try and determine a clear limit of "noise to signal ratio" and consequently confirm the true presence of these mutations with a low level peak fluorescent intensity. In order to establish the appropriate value to consider as a "cut-off" between positive and indeterminate, all the fluorescent intensities of the respective mutant peaks (NPM1 or FLT3) were determined. Figure 3.13 demonstrates all of these mutation positive cases ranked by fluorescent intensity for NPM1-mut and FLT3-ITD respectively. This area of research is part of ongoing work in the laboratory.

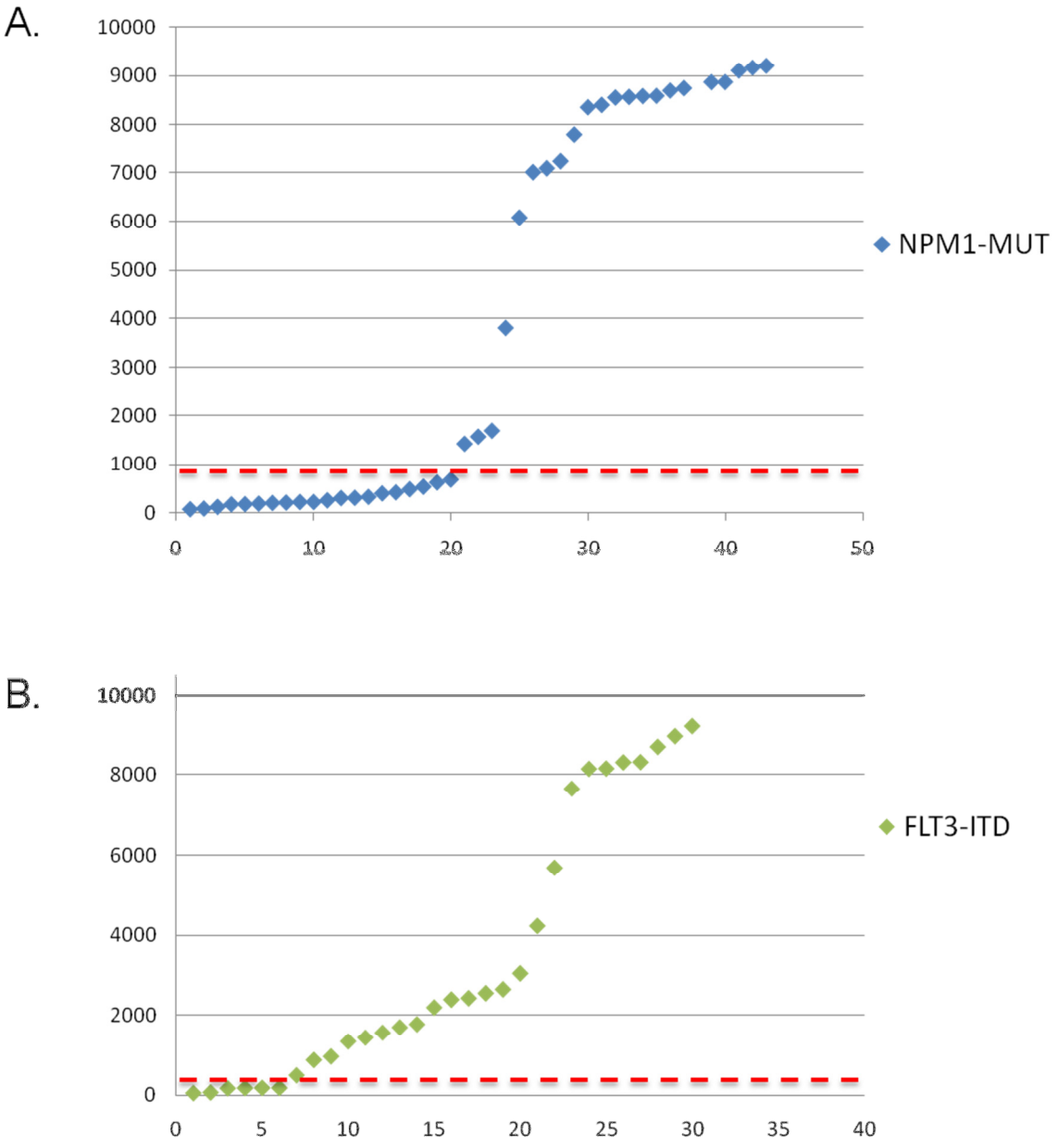


Figure 3.13 Determination of "cut-offs" for indeterminate peak intensities.
(A) Analysis of NPM1-mut peak intensity. A peak intensity of >1000 may be an appropriate value at which to consider the sample as mutation positive. (B) Analysis of FLT3-ITD peak intensity. A peak intensity of >500 may be an appropriate value at which to consider the sample as mutation positive.

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication.

The use of a “cut-off” value of >100 was also considered after correspondence with two authors who had recently published information in this regard (Szankasi et al., 2008, Wertheim and Bagg, 2008, Buban et al., 2011). The first correspondent includes all patient samples with a mutation peak of any intensity into the positive group including those with an intensity of <100. The second correspondent uses a peak intensity of >100 as a determinant to call a result positive. For illustrative purposes only, if the criterion, of a peak intensity of >100, was used, a slight variation in the frequency of both NPM1-mut and FLT3-ITD was found, with the frequency for both decreasing (Figure3.14). All peak intensity levels, including those with values of <100 were used in the analysis of our study cohort.

Also, when analysing the control samples any background noise was recorded for both intensity and position. This noise did not peak at intensities >70 in the NPM1 assay and did not occur in the expected size range for this mutation. The background noise did not peak at intensities >150 in the FLT3 assay.

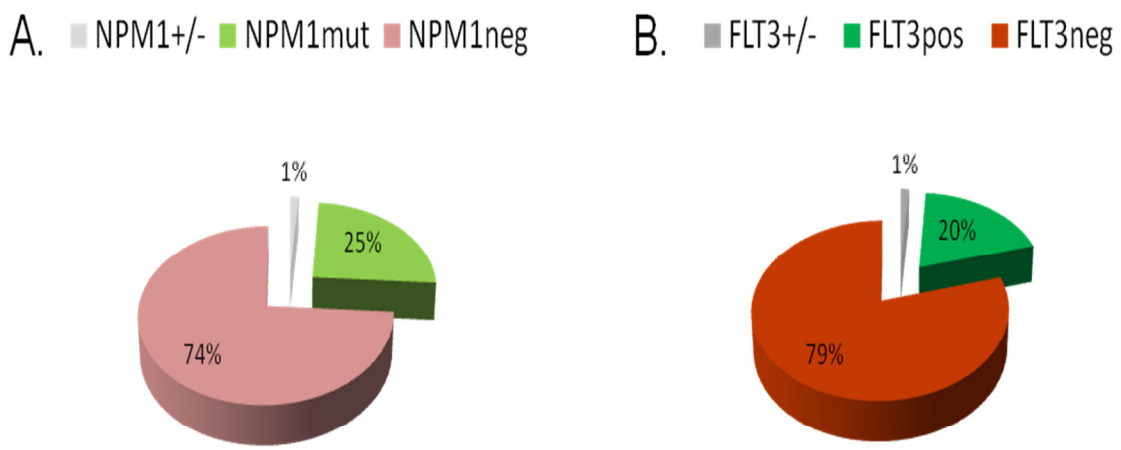


Figure 3.14 NPM1-mut and FLT3-ITD using peak intensities of >100 as a "cut-off".
Assessment of (A) NPM1-mut and (B) FLT3-ITD in AML cases. Samples with a peak intensity of <100 were considered to be within an indeterminate range.

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

3.10 DILUTIONAL STUDY

It has been reported that PCR and capillary electrophoresis has a diagnostic sensitivity of 100% and an analytical sensitivity of 5%. This means that all the known NPM1 mutations can be detected provided that the leukaemic cells represent 5% of the cells present.

Sample N55 which was found to be NPM1-mut positive with a sample blast count of approximately 85% blasts was used in a serial dilution study to attempt to determine the assay sensitivity. A serial dilution using Sample N55 as the mutant DNA and a control sample as the wild type DNA was assessed by means of capillary electrophoresis. The dilution series included a 1:64 dilution which

remained positive for the mutation. This finding suggested that the assay was indeed sensitive for the detection of the NPM1 mutation.

Sample T2 which was found to be FLT3-ITD positive with a sample blast count of approximately 96% blasts was used in a serial dilution study to attempt to determine the assay sensitivity. A serial dilution using Sample T2 as the mutant DNA and a control sample as the wild type DNA was assessed by means of capillary electrophoresis. The dilution series included a 1:64 dilution which remained postitive for the mutation. This finding suggested that the assay was indeed sensitive for the detection of the NPM1 mutation.

A sample that was confirmed NPM1 mutation positive and FLT3-ITD positive with a blast count of 30% was serially diluted with distilled H_2O and assessed by means of capillary electrophoresis. The sample was electrophoresed neat up to and including 1:128 dilution. Both the wild type and mutant remained present in all the dilutions with a minimal drop in peak values with increased dilution. This suggests that the capillary electrophoresis method of detection is robust.

3.11 FLT3 MUTANT LEVEL

The FLT3 mutant level was determined to further assist in prognostication. The cohort of FLT3-ITD positive samples, seen in Table 3.3 and Figure 3.15 was divided into the three risk group categories as suggested by Gale *et al.* (Gale et al., 2008) which found the three groups to have different outcomes. The three risk groups were determined by assessing the relative mutant level. This was calculated by using the area under the mutant peak curve and expressing it as a

percentage of the total FLT3 (Further described in Appendix 5.1). The risk categories are low risk (<25% mutant), intermediate risk (25% to 50% mutant) and high risk (>50% mutant). This risk assessment appears to affect both RR and OS with those with a high total mutant level doing significantly worse than those in the intermediate group. Those in the low risk group had a significantly worse RR compared to those with FLT3-wt (Gale et al., 2008).

Table 3.3 Analysis of the FLT3-ITD/ FLT3-wt ratio.
The percentage FLT3-ITD mutant in the sample impacts on clinical outcome.

	Low Risk (<25%)	**Intermediate Risk (25-50%)**	**High Risk (>50%)**
Number of Patients (%)	15 (50%)	12(40%)	3(10%)
Median Mutant Level	6%	40%	64%

Abbreviations: FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

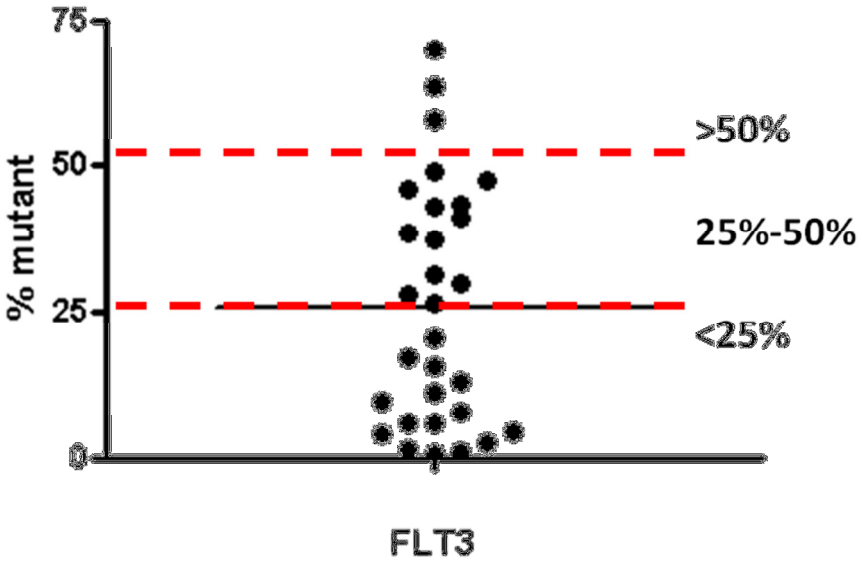


Figure 3.15 Percentage FLT3-ITD relative to total FLT3 in that sample.
These have then been subdivided into three different risk categories.

Abbreviations: FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3, FMS-like tyrosine kinase receptor gene.

3.12 PATIENT CHARACTERISTICS

Of the 164 cases analysed 37% (61/164) were found to have one of the specified mutations *i.e.* NPM1-mut, FLT3-ITD or both.

In total, 21% (34/164) were found to have FLT3-ITD and 26% (42/164) were NPM1-mut positive. A total of 9% (15/164) of the cases were found to have both these mutations occurring in combination (Figure 3.16). FLT3-ITD mutations were found in 36% (15/42) NPM1-mut cases, which is statistically significantly higher when compared with 16% (19/122) of the NPM1-wt cases (*P<0.04)*. This data confirms that NPM1-mut and FLT3-ITD characterise major, partially overlapping subgroups in AML. For this reason all further analyses were performed according

to 4 groups. These groups consisted of the two mutations in combination, each individually and a wild type group which was negative for both these mutations.

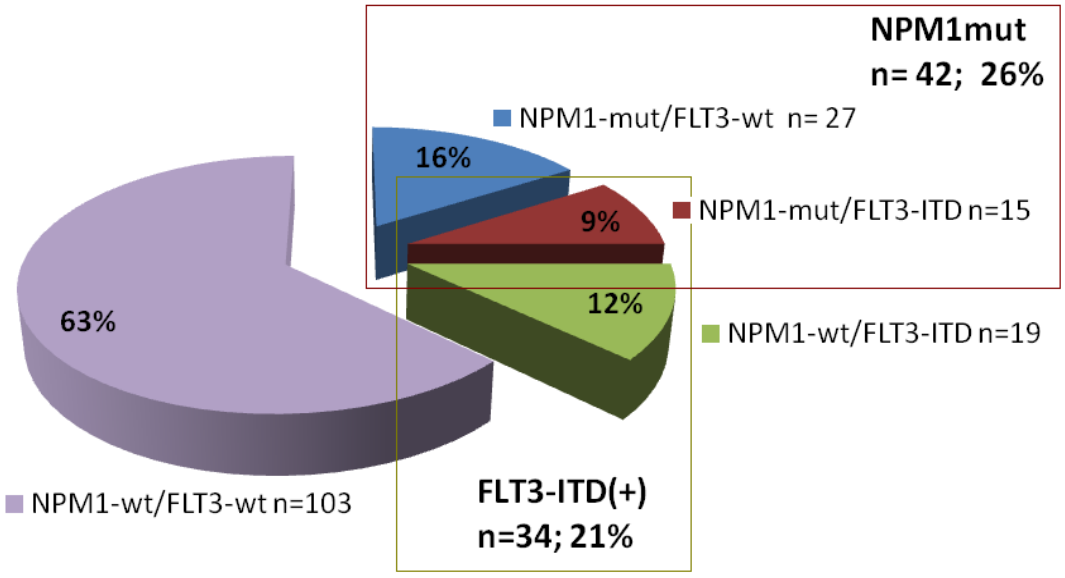


Figure 3.16 Frequency of NPM1 and FLT3-ITD mutations.
Assessment of the frequency of NPM1 and FLT3-ITD mutations in relation to each other as well as those patients diagnosed with AML but negative for these specific gene mutations.

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

This cohort of 164 cases had a minor female to male predominance with 51% (84/164) female and 49% (80/164) male patients. It has been reported that NPM1-mut tends to occur with a higher frequency in female patients with a male: female ratio of 1:1.5 (Thiede et al., 2006). In this patient population, 60% (25/42) of the patients who were NPM1-mut positive were also female (*i.e.* male to female ratio of 1:1.5) (Figure 3.17).

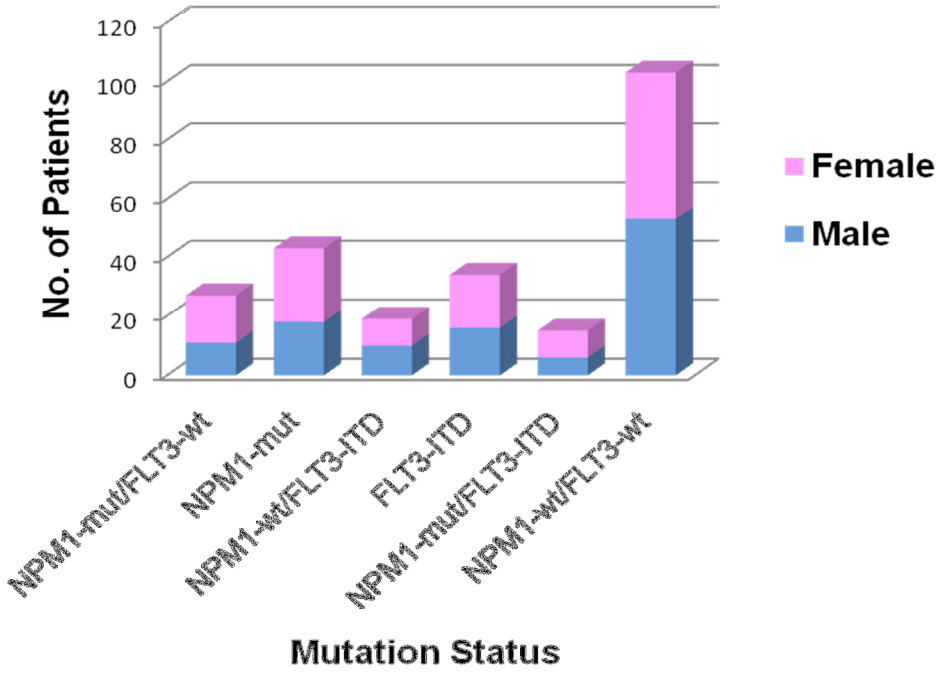


Figure 3.17 Gender relative to the various mutation combinations.

No statistical difference was found between the different mutation groups.

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type; no; number.

The median age of diagnosis of AML was found to be 40 years in this cohort of cases. Within all the patient groups the age range was found to be similar with the exception of those who were NPM1-mut only (NPM1-mut/FLT3-wt) and those who had both mutations (NPM1-mut/FLT3-ITD). This data set showed that those who were NPM1-mut positive were younger than expected with a median age of 30 years, while those who were mutation positive for both (NPM1-mut/FLT3-ITD) had a median age of 60 years (*P>0.05*) (Figure 3.18).

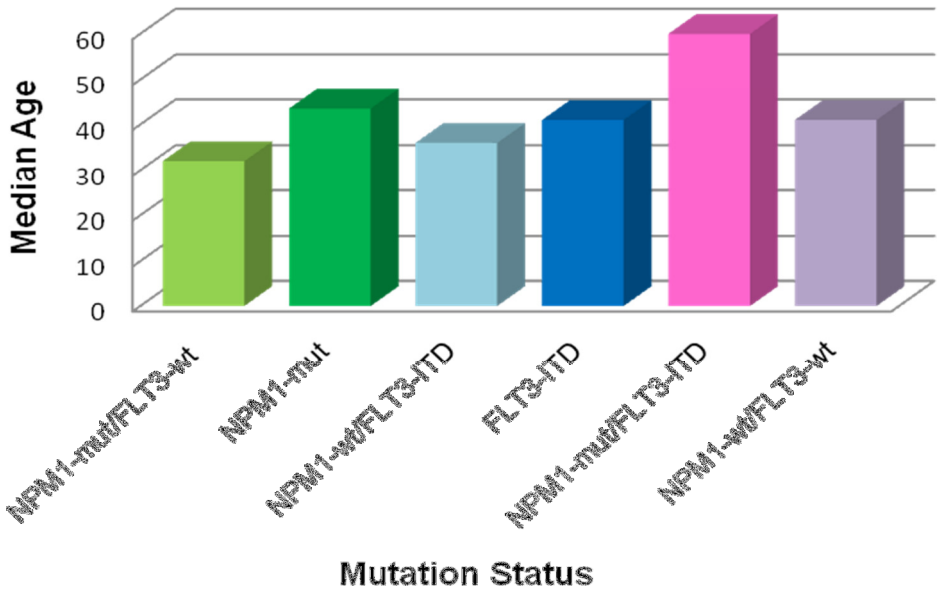


Figure 3.18 Age relative to NPM1 and FLT3-ITD mutation status.

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

Of the NPM1-mut only (NPM1-mut/FLT3-wt) cases, with a FAB diagnosis documented, 55% (11/20) were either FAB M4 or M5. In those patients who were NPM1-wt 34% (26/76) patients were classified as FAB M4 or M5. Furthermore, 38% (6/16) of the samples that were FLT3-ITD only and had been classified by means of FAB were found to be FAB M4 or M5. FAB M5 was found to be the most common classification group, in both the NPM1-mut only (NPM1-mut/FLT3-wt) and the FLT3-ITD only (NPM1-wt/FLT3-ITD) group. In the group negative for both (NPM1-mut/FLT3-ITD) these mutations, FAB M2 and M3 were the most common FAB classification. Although not statistically significant these trends concur with

those reported in the literature (Swerdlow et al., 2008). Patient characteristics for age, gender and FAB classification are summarised in Table 3.4.

The anaemia and thrombocytopenia at diagnosis as previously described (Swerdlow et al., 2008) was confirmed in patients who were NPM1-mut positive. A leucocytosis and high blast count was also confirmed (Swerdlow et al., 2008) but was not found to be statistically significantly higher than in the other sub-groups investigated (*P>0.05*).

The expected leucocytosis and high blast count in those with FLT3-ITD positivity was also found to be present in this cohort of FLT3-ITD positive samples (Thiede et al., 2006, Suzuki et al., 2007). These clinical characteristics are summarized in Table 3.5. Compared to AML patients without NPM1 or FLT3-ITD mutations patients showing both of these mutations simultaneously (NPM1-mut/FLT3-ITD) had statistically significantly higher median Hb (7.4 g/dl versus 9.5 g/dl; $P< 0.05$). Furthermore, patients with both mutations (NPM1-mut/FLT3-ITD) were also found to have a statistically significantly higher median Hb (9.5 g/dl versus 6.8 g/dl; $P< 0.03$) when compared to those with NPM1-mut only (NPM1-mut/FLT3-wt) as well as when compared to those with FLT3-ITD only (NPM1-wt/FLT3-ITD) (9.5 g/dl versus 6.9 g/dl; $P< 0.04$).

Patients with the NPM1-mut only (NPM1-mut/FLT3-wt) were found to have a statistically significantly reduced median platelet count (24.5 $x10^9$/l versus 53.5 $x10^9$/l; $P< 0.05$) when compared to those with only an FLT3-ITD (NPM1-wt/FLT3-ITD) and this appears to be a novel finding.

Table 3.4 Assessment of age, gender and AML FAB classification.
This is assessed relative to the presence of NPM1 and FLT3-ITD mutations.

	FLT3-ITD negative		FLT3-ITD positive	
CHARACTERISITCS	**NPM1-mut n=27**	**NPM1-wt n=103**	**NPM1-mut n=15**	**NPM1-wt n=19**
Gender:	n=27	n=103	n=15	n=19
Male (%)	11 (40%)	53 (52%)	6 (40%)	10 (53%)
Age (years):	n=26	n=101	n=15	n=19
Median	30	41	60	36
Range	18-79	17-80	19-67	17-80
FAB:	n=27	n=103	n=15	n=19
MO (%)	1 (4%)	6 (6%)	0 (0%)	1 (5%)
M1 (%)	0 (0%)	0 (0%)	3 (20%)	0 (0%)
M2 (%)	6 (22%)	20 (19%)	2 (13%)	4 (21%)
M3 (%)	2 (7%)	21 (20%)	0 (0%)	3 (16%)
M4 (%)	3 (11%)	8 (8%)	1 (7%)	1 (5%)
M5 (%)	8 (30%)	18 (17%)	4 (27%)	5 (26%)
M6 (%)	0(0%)	1 (1%)	0 (0%)	0 (0%)
M7 (%)	0 (0%)	2 (2%)	0 (0%)	2 (11%)
NO RESULT For FAB (%)	7 (25%)	27 (26%)	5 (33%)	3 (16%)

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type; FAB, French American British

The WCC count of those patients who were only FLT3-ITD positive (NPM1-wt/FLT3-ITD) demonstrated a marked leucocytosis with a median WCC of 101 $x10^9$/l. This was found to be statistically significantly increased when compared to the median WCC of those with an NPM1-mut only (NPM1-mut/FLT3-wt) (101 $x10^9$/l versus 15.9 $x10^9$/l; $P< 0.03$) and those with neither an NPM1-mut nor FLT3-ITD (101 $x10^9$/l versus 12.2 $x10^9$/l; $P< 0.02$).

Table 3.5 Clinical characteristics at diagnosis.
These have been subdivided according to the presence of NPM1 and FLT3-ITD mutations.

	FLT3-wt, n=130		**FLT3-ITD, n=34**	
CHARACTERISITCS	**NPM1-mut n=27**	**NPM1-wt n=103**	**NPM1-mut n=15**	**NPM1-wt n=19**
Median Hb level, g/dl (range)	6.8 (2.5-12)	7.4 (2.5-12)	9.5 (7.0-13.6)	6.9 (3.0-13.1)
Missing	12	32	3	3
Median Plt count, $x10^9$/L (range)	24.5 (9-151)	32 (6-1149)	43 (9-157)	53.5 (11-398)
Missing	11	32	4	3
Median WCC, $x10^9$/l (range)	15.9 (1.1-78.4)	12.15 (0.69-459.4)	10.23 (2.2-376)	101 (1.6-582)
Missing	11	32	3	3
Median blasts at Dx, % (range)	52 (6-85)	57.5 (8-95)	72.5 (14-95)	65.0 (13-96)
Missing	2	1	1	0

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutation; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type; Hb, haemoglobin; Plt, platelet; WCC, white cell count; Dx, diagnosis.

3.13 NPM1 AND FLT3-ITD MUTATIONS AND IMMUNOPHENOTYPING

All the immunophenotypic markers commonly used as part of routine flow cytometric assessment were investigated to determine if there was any significant association between marker expression and NPM1 and FLT3-ITD mutational status.

More specifically CD14, CD11b and human leucocyte antigen DR (HLA-DR) were statistically significantly increased in all the samples which were NPM1-mut positive (FLT3-ITD or FLT3-wt) *(P<0.05)*. When assessing those samples which had both mutations (NPM1-mut/FLT3-ITD), it was noted that CD56 was negative in 100% (8/8) of the samples compared to the wild type samples of which 35% (22/63) of the samples assessed were CD56 positive (*P<0.05*).

When assessing CD34 expression and NPM1-mut, 48.7% (19/39)) of the cases that were NPM1-mut positive were also reported to be CD34 positive. The CD34 analysis of two representative cases are shown in Figure 3.19. The CD34 dot plots demonstrate a CD34 positive case (A) and another with virtual absence of CD34, that was considered negative (B).

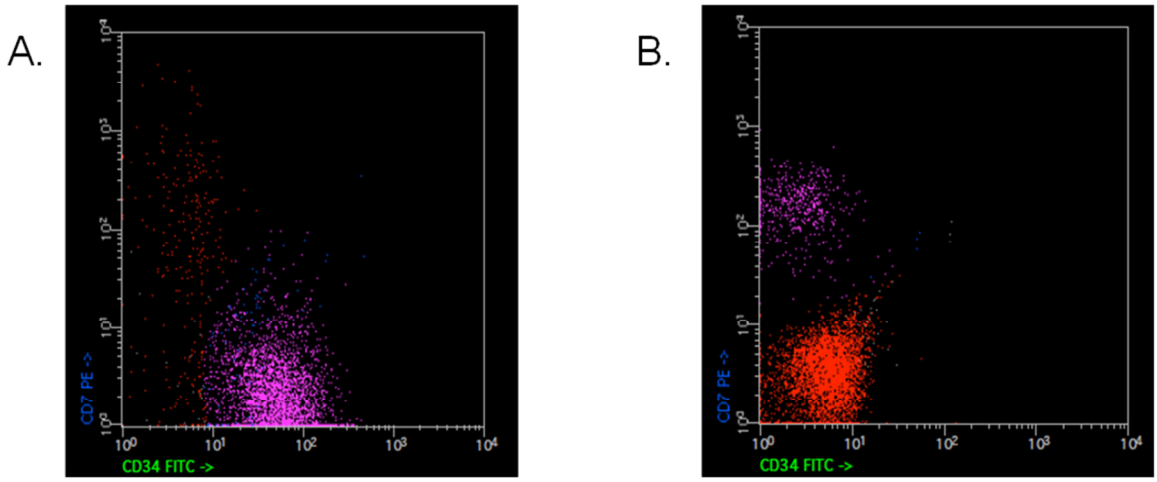


Figure 3.19 Dot plot of CD34 (FITC) expression on the X axis vs. CD7 (PE) on the Y axis in NPM1 mutant cases.
(A) CD34 positive population of cells is represented by the purple events and (B) CD34 negative population is represented by the red events. The purple population represents mature T-cells. These assessments were made in conjunction with an isotypic negative control (not in this figure).

Those samples that were found to be NPM1-wt were statistically significantly more likely to be CD34 positive than NPM1-mut cases as shown in Figure 3.20 (*P<0.001*). When assessing NPM1-mut only (NPM1-mut/FLT3-wt), 44% (11/25) of the samples were found to be CD34 negative. This is a higher percentage than described in the international literature.

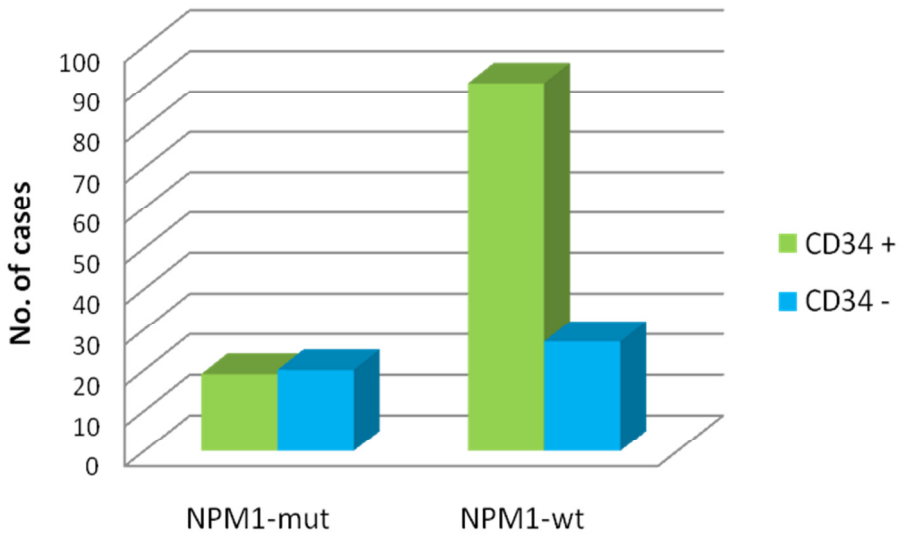


Figure 3.20 CD34 expression in NPM1-mut and NPM1-wt cases.
NPM1-wt cases are statistically significantly more likely to be CD34 positive than NPM1-mut cases (P<0.001).

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type.

As expected CD 13, a myeloid marker, was positive in 90% (35/38) NPM1-mut positive samples assessed. A similar result with another myeloid marker, CD33, was observed with 97% (38/39) NPM1-mut positive samples assessed.

3.14 NPM1 AND FLT3-ITD MUTATIONS AND CYTOGENETIC EVALUATION

Of the 42 patients who were diagnosed as NPM1-mut positive 29% (12/42) had an abnormal cytogenetic result and 19% (8/42) were found to be cytogenetically normal. The remaining 52% (22/42) had no cytogenetic result available (*i.e.*no fluorescent in situ hybridisation (FISH) or karyotype results). Figure 3.21 gives an

indication of the frequency of cytogenetic abnormalities seen in NPM1 mutation positive cases.

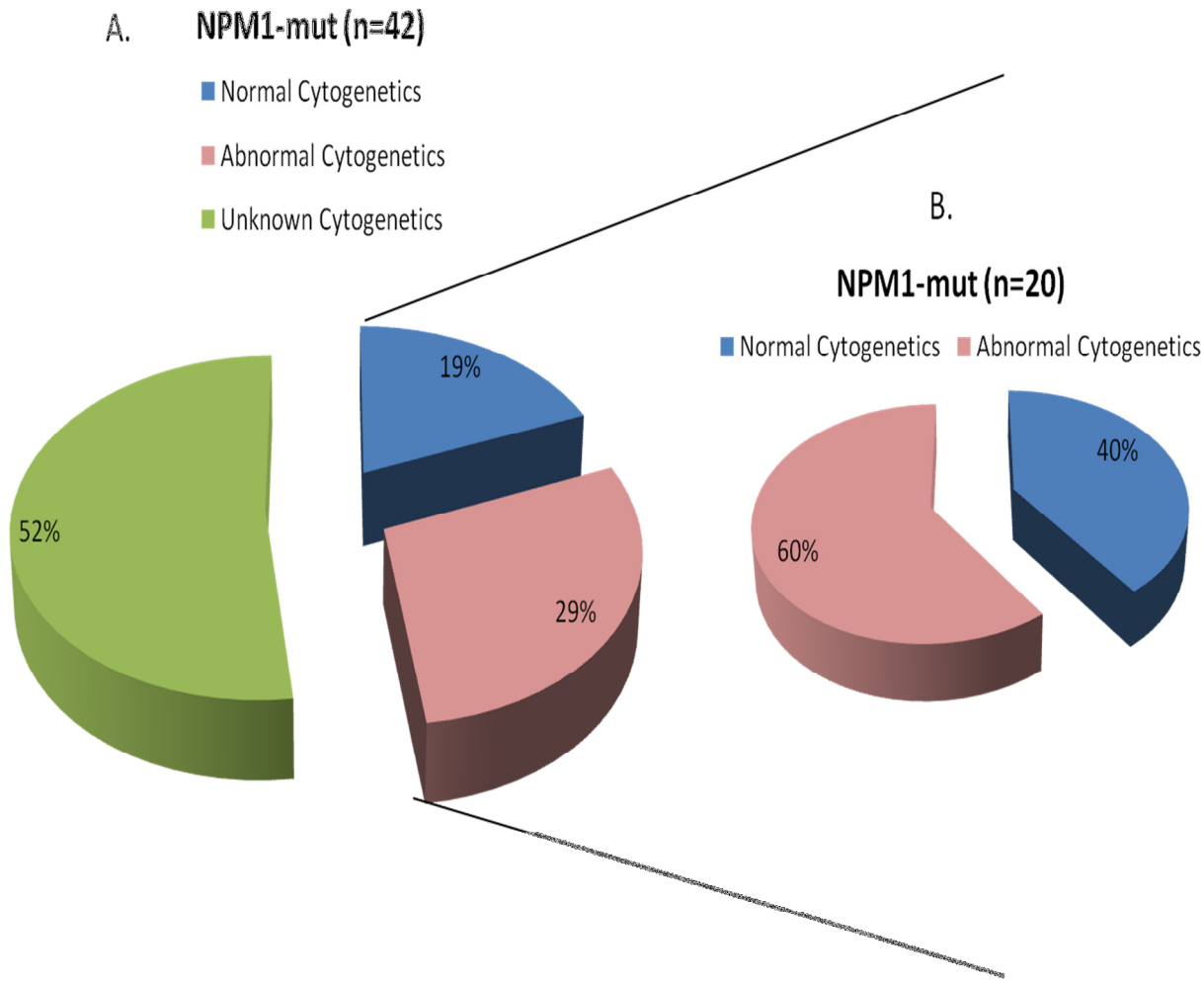


Figure 3.21 NPM1-mut cases and cytogenetic results.

(A) NPM1-mut positive cases and the cytogenetic findings for the group as a whole and (B) closer assessment of the NPM1-mut positive cases that have a cytogenetic result recorded.

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant.

Those samples that were NPM1-mut positive with an abnormal karyotype are presented in Table 3.6. Both the mutant position and peak intensity have been included. The intensity of NPM1-mut peaks was determined in relation to the

presence of the above mentioned genetic mutations. The majority of the samples and in particular those with a recurrent chromosomal translocation as classified by WHO 2008, have mutation peak intensities of <550.

Table 3.6 Confirmed abnormal karyotype in NPM1-mut positive samples.
The NPM1-mut position and intensity has also been included.

SAMPLE	CYTOGENETICS	MUT POSITION	MUT INTENSITY
R8	Inv(16)	173.76	314
R30	5% cells positive for inv16	173.48	243
N49	t(15;17)	173.58 174.42	523 400
R11	+11	174.22	109
R51	del(13)(q14)	174.27	7223
N93_N23	t(3;5)(q25;q34)	173.24	294
R35	inv(9)	174.61	6077
R14_F18	del(7)(q?33),t(8;21)(q22;q22)	173.61	61
N24	t(8;21)	173.67	209
R36_F23	t(8;21)	173.32	161
R3	t(15;17)	173.56	165
R53	11q23	173.7	290

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; inv, inversion; t, translocation; del, deletion.

FLT3-ITD mutations were found in 36% (15/42) of NPM1-mut cases compared with 16% (19/122) of the NPM1-wt cases (*P<0.04)*. Figure 3.22 illustrates how FLT3-ITD preferentially targets those patients who are NPM1-mut positive. This association has also been reported by others (Thiede et al., 2006).

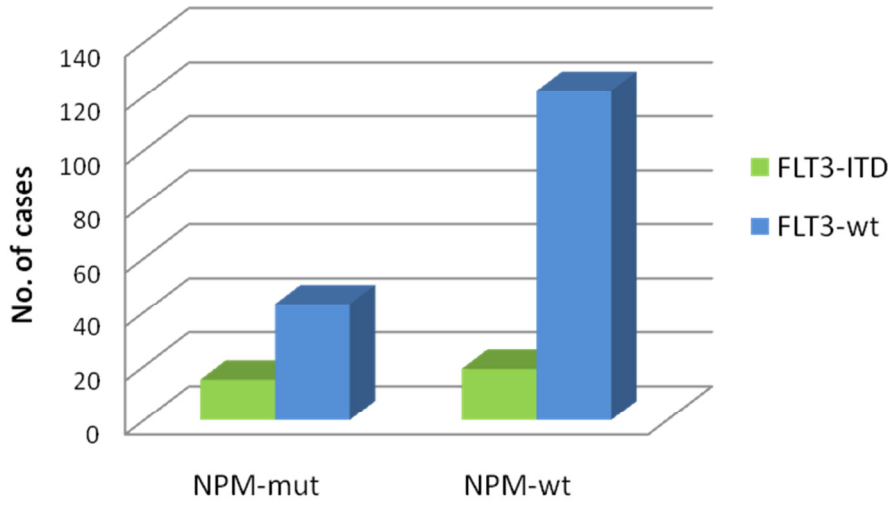


Figure 3.22 FLT3-ITD preferentially target NPM1-mut positive samples.
44% (15/34) of the FLT3-ITD positive samples are also NPM1-mut positive.

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

Of the FLT3-ITD cases, 58% (11/19) had cytogenetic abnormalities detected while 42% (8/19) were found to be CN. Those with confirmed abnormal FISH analysis results were included in the cytogenetically abnormal group even though not all of them had a karyotype result available. The other 15 either had failed cytogenetic results, or no genetic analysis was requested at all. Of those who were FLT3-ITD only (NPM1-wt/FLT3-ITD), 13 had a karyotype result available of which 38% (5/13) had a normal karyotype (*P>0.05*). Only 3 of the 34 samples that were FLT-ITD positive carried a t(15;17) (*P>0.05*). All FLT3-ITD positive cases with abnormal cytogenetics are summarised in Table 3.7. Some of these abnormal karyotypes are commonly associated with FLT3-ITD, while others are less frequently associated and others very rarely associated. The mutation peak position and

intensity is included in order to better assess whether some of these FLT3-ITD mutations and rare cytogenetic associations may form part of the described indeterminate range. A summary of both the FLT3-ITD and NPM1-mut results relative to the karyotype findings is represented in Table 3.8.

Table 3.7 Confirmed abnormal karyotype in FLT3-ITD positive samples.
The FLT3-ITD mutation position and intensity has also been included.

SAMPLE	CYTOGENETICS	MUT POSITION	MUT:WT RATIO
R27	t(15;17)	371.13	0.75
T2	t(15;17)	371.22	0.15
F3	t(15;17)	344.04 352.34	0.77
F6	t(8;21)	355.41	0.01
T15/N31/T27	t(8;21)	385.0	0.04
F4	t(8;21)	391.02	0.03
R11	47XX,+11	360.07	0.19
R51	del (13)	378.98 408.85	0.39
F24	46,XX,del(12)(p?13) [8]/45,XX,der(8;12)(q10;q10) [2]/46,XX [2]	392.32 415.72 424.43	0.63
N93	46,XY,t(3;5)(q25;q34)	372.91	0.60
N94	46,XY,t(2;17)(p?23;q25)	339.28	0.03

Abbreviations: FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; t, translocation; del, deletion.

Table 3.8 Cytogenetic results relative to NPM1 and FLT3 mutations.
The cytogenetic results (FISH and/or karyotype) were documented at diagnosis. In some of these cases there were no karyotype results but FISH analysis was able to confirm an abnormal result. Assessment for single or multiple cytogenetic abnormalities was therefore not possible in those cases with FISH analysis only.

	NPM1-mut/ FLT3-wt	FLT3-ITD/ NPM1-wt	NPM1-mut/ FLT3-ITD	NPM1-wt/ FLT3-wt
Karyotype:	n=14	n=13	n=6	n=69
Normal Cytogenetics (%):	5 (36%)	5(38%)	3 (50%)	13(19%)
Abnormal Cytogenetics (%):	9(64%)	8(62%)	3(50%)	56(81%)
No. with Abnormalities:	n=5	n=7	n=3	n=34
Single Abnormality (%):	4(80%)	2(29%)	3(100%)	14(41%)
Multiple Abnormalities (%):	1(20%)	5(71%)	0	20(59%)

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type; FISH, fluorescent in situ hybridisation.

3.15 LONGITUDINAL CASE STUDY ASSESSMENT

An assessment of 10 cases, which had samples available from different time periods in the course of their disease, was made. A longitudinal analysis was performed to determine whether these two gene mutations remain stable throughout the disease course. In particular, it has been reported that NPM1 mutations are more stable than FLT3-ITD mutations and may be used for the detection of MRD (Falini et al., 2009). Occasionally both these mutations may be gained or lost at relapse (Suzuki et al., 2005). Analysis of this study cohort cases demonstrated no gains of either of the mutations, however, loss of NPM1-mut in a single sample was noted.

Case 1 (Samples N71 and N28):

At diagnosis the patient was found to be NPM1-mut positive only (NPM1-mut/FLT3-wt). At relapse a similar analysis profile was found (Table 3.9).

Table 3.9 NPM1-mut positivity confirmed at diagnosis and relapse.

	Diagnosis Position	Diagnosis Intensity	Relapse Position	Relapse Intensity
NPM1-wt	170.62	8812	169.96	8446
NPM1-mut	174.51	8542	174.03	8484
FLT3-wt	330.1	7320	330.34	8686

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

Case 2 (Sample T15, N31 and T27):

At diagnosis (T15), the patient was found to have a FLT3-ITD but was NPM1-mut negative (NPM1-wt/FLT3-ITD). At relapse 1 (N31) and at relapse 2 (T27) FLT3-ITD mutation was found at the same position as at diagnosis and the NPM1-mut was absent (Table 3.10).

Table 3.10 FLT3-ITD positivity at diagnosis and at both relapses.

	Diagnosis Position	Diagnosis Intensity	Relapse 1 Position	Relapse 1 Intensity	Relapse 2 Position	Relapse 2 Intensity
NPM1-wt	170.41	9440	170.77	7586	170.18	5205
FLT3-wt	330.0	9488	331.14	9901	329.98	9812
FLT3-ITD	385.28	8309	385.34	477	385.32	560

Abbreviations: NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

Case 3: (Sample T1 and T28):

As seen in Table 3.11 both diagnosis and relapse samples are positive for a FLT3-ITD found at approximately the same position. The ratio of intensity for wild type: mutant was much less on follow up. An NPM1-mut was found at diagnosis but was not present on follow up.

Table 3.11 FLT3-ITD at presentation and relapse.
The NPM1-mut present at presentation was not found at relapse.

	Diagnosis Position	Diagnosis Intensity	Relapse Position	Relapse Intensity
NPM1-wt	169.33	8018	169.96	8446
NPM1-mut	173.35	669	Not present	8484
FLT3-wt	330.81	9680	330.34	8686
FLT3-ITD	418.69	3030	417.0	211

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

Another 2 cases which were assessed longitudinally were found to be NPM1-mut and FLT3-ITD positive (NPM1-mut/FLT3-ITD) at diagnosis and at relapse.

A further 5 longitudinal assessments of cases who were initially both NPM1-wt and FLT3-wt were made. At relapse both these mutations were assessed again and no gains of either of these mutations were found at relapse.

The findings of those cases assessed longitudinally were as expected. Case study 3 demonstrated a loss of the NPM1-mut; this is a less common finding with the presence of NPM1-mut likely to be used as a measure for minimal residual disease in the future (Palmisano et al., 2007).

3.16 SUMMARY OF RESULTS

A retrospective analysis was performed of samples from adult patients diagnosed with *de novo* AML at CMJAH from September 2004 until December 2009. A total of 164 AML patient samples were assessed for the presence of NPM1 and FLT3-ITD mutations occurring alone and in combination.

FLT3-ITD was found in 21% (34/164) and NPM1-mut in 26% (42/164) of these cases. A total of 9% (15/164) were found to have both these mutations occurring in combination (NPM1-mut/FLT3-ITD). FLT3-ITD was found in 36% (15/42) NPM1-mut cases.

Of the cases analysed, 49% (80/164) were male and 51% (84/164) female. The median age at diagnosis was found to be 40 years (range: 17-80 years). Of the male patients, 20% (16/80) were 60 years or older at diagnosis and 23% (19/84) of female patients were 60 years or older at diagnosis. FBC results at diagnosis were

assessed; with WCC results available for 115 cases and Haemoglobin (Hb) and platelet count results available for 114 cases. The median WCC was 12.6 $x10^9$/l (0.69-582), the median Hb was 7.6 g/dl (range: 2.5-13.6) and the median platelet count was 32.5 $x10^9$/l (range: 6-1149). Furthermore, blast count data was collected on 160 of these cases with a median of 58% (range: 6-97) at diagnosis.

Of the total number of cases analysed 26% (42/164) had no FAB classification assigned, 21% (35/164) cases were diagnosed as FAB M5, 20% (32/164) as FAB AML with differentiation (AML M2) and 16%(26/164) as FAB M3.

Assessment of cytogenetics revealed that only 46% (75/164) of cases had documented karyotype results available. Of these, 35% (26/75) were found to have a normal karyotype and the other 65% (49/75) had abnormal cytogenetics recorded. Of the 49 with abnormal cytogenetics 49% (24/49) had single mutations and 51% (25/49) presented with multiple mutations (including complex karyotypes).

Table 3.12 Summary of patient characteristics at diagnosis.

Characteristics at Diagnosis	% (n)
Male, %	49%
Female, %	51%
Age > 60 yrs, %	21.5%
Median age, yrs	40 yrs
Median WCC, x10^9/L	12.6
Median Hb, g/dl	7.6
Median plt, x10^9/L	32.5
FAB Classification	
No FAB % (n)	26% (42/164)
M2 % (n)	20% (32/164)
M3 % (n)	16% (26/164)
M5 % (n)	21% (32/164)
Karyotype	
Present % (n)	46% (75/164)
Normal % (n)	35% 26/75)

Abbreviations: Yrs, years; WCC, white cell count; Hb, haemoglobin; plt, platelet; FAB, French American British

4 DISCUSSION

4.1 INTRODUCTION

An assay which involves PCR amplification of genomic (g) DNA and fragment analysis by capillary electrophoresis has been optimised and used to assess for the presence of NPM1 and FLT3-ITD mutations in a *de novo* adult AML population. This is an assay which as yet, to the best of our knowledge, has not been routinely performed in a South African centre. It allows for the simultaneous detection of both NPM1 and FLT3-ITD mutations. These recently discovered key mutations in AML should always be assessed in conjunction with each other, not only initially but throughout follow up (Suzuki et al., 2007, Schlenk et al., 2008, Gale et al., 2008). PCR and subsequent capillary electrophoresis is the only available assay to detect both mutations together and to assess for all or almost all NPM1 mutation subtypes. As an alternative FLT3-ITD is being detected by means of a PCR based gel assay and NPM1 by means of Q-PCR, PCR and sequencing or PCR and denaturing high performance liquid chromatography (dHPLC) amongst others (Wertheim and Bagg, 2008). Each of the tests has both advantages and disadvantages with cost and diagnostic sensitivity being the major determinants for their use in a routine molecular diagnostic laboratory. This newly instituted PCR and capillary electrophoresis testing is highly sensitive and relatively low cost (Wertheim and Bagg, 2008). The detection limit (analytical sensitivity) of this form of testing has been determined by a least two separate investigators and it appears that the assay can detect mutations present in concentrations as low as 5% (Noguera et al., 2005, Szankasi et al., 2008, Wertheim and Bagg, 2008). Introduction of this testing into routine use will allow

clinicians to gain an improved understanding of the pathobiology of each individual patient's disease: to provide a more accurate prognostic assessment and better stratify treatment decisions (such as when to consider bone marrow transplantation or more experimental drug regimens and when to include patients in clinical trials). This is of particular relevance in those who are assessed to have a normal karyotype or indeed those whose cytogenetic assessment fails. The robustness of this assay has been confirmed and has provided results despite, at times, being presented with suboptimal quality samples. Although assay specificity and accuracy was not determined in this research project further experimentation is ongoing in this regard. Furthermore, peripheral blood can be used as an alternative to bone marrow which makes obtaining a sample for diagnosis much simpler.

4.2 LIMITATIONS OF THE STUDY

The current study shows certain limitations which are acknowledged in this section.

The first limitation is the number of cases included in the study. Although a total of 164 cases were included in this study, when these are analysed in subgroups the patient numbers in each subgroup are small.

The second limitation is the retrospective nature of the study together with the lack of clinical correlation for the cases included in this study. In particular, patients who are NPM1 mutation positive are often noted to have extra-medullary involvement and this could not be assessed. An assessment of survival in this cohort was also

not possible due to limited ethical clearance on this study which stipulated that only NHLS available patient data could be used. This limitation could be overcome in several ways, including seeking consent from patients at the time of diagnosis and this is planned for subsequent prospective studies.

The third limitation relates to the nature of the samples obtained. Many of them were collected in a heparin tube which is likely to result in inhibition of the PCR reaction and some samples were excluded on this basis. Furthermore, samples (especially those of single slide extracts) yielded limited quantities of DNA thus, more extensive testing, such as sequencing of these samples was not possible. This limitation could be overcome by obtaining a sample of bone marrow in an EDTA tube at the time of doing the diagnostic bone marrow aspirate in a prospective study. The sample could be stored and DNA extracted at a later stage once the diagnosis of AML has been confirmed (the diagnosis is routinely made within less than 24 hours of the sample being collected).

The fourth limitation was the lack of a reliable positive internal control. An NPM1 cell line has recently been obtained, which in future work will be incorporated into routine diagnostics as a positive control. Alternative positive controls could be derived by cloning well characterised PCR reaction products of positive cases.

4.3 ASSAY PROBLEMS

4.3.1 PCR TROUBLESHOOTING

The decision to use the fragment analysis method was partly based on the readily available instruments and staff expertise in PCR diagnostics and this method has become one of the most commonly used for the detection of NPM1 and FLT3-ITD

mutations, as it is the only one that can potentially provide both of these mutation results simultaneously (Noguera et al., 2005, Wertheim and Bagg, 2008). The importance of the detection of these mutations in combination has been confirmed in various studies as their impact on prognosis alone and in combination is significantly different (Mrózek et al., 2007, Haferlach et al., 2009).

As presented in section 2.5 revisions to the master mix (Table 2.2) and PCR conditions were initially made before a final protocol was adopted. Individual PCR reactions for each of the two sets of primers (NPM1 and FLT3-ITD) were instituted and became the standard operating procedure for the rest of the research assignment. However, in the future the laboratory will attempt to optimise the multiplex reaction in order to reduce the cost of this assay. Different polymerase enzymes were tested for the PCR conditions set out for this experiment leading to a switch from AmliTaq® DNA Polymerase to AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Inc. Foster City, CA).

Possible reasons for the initial poor PCR results included poor DNA concentration of the samples extracted secondary to DNA degradation over time as fresh blood samples and BMA slides were not used. In addition, there may have been inhibition of the PCR reaction by a contaminant (such as heparin used in samples for immunophenotyping).

The poor DNA integrity was confirmed by assessing for PCR products for GAPDH on gels on a random selection of the extracted patient samples. Of the 31 samples analysed only 48% (15/31) showed product to be present on the gel. This confirms that the DNA integrity of the samples available for testing plays an important role in the ability to produce a result.

Randomly chosen fresh blood samples and previously extracted BMA samples (rather than stored slides) were then used to assess for any differences in DNA integrity. The former samples were much more consistent in providing a result, in particular provided much higher peak intensities on capillary electrophoresis. Therefore, it was concluded that blood or BMA samples taken in an EDTA tube (rather than made into a slide and stored) should be the preferred source material for routine analysis going forward.

The role of the extraction method used was also assessed. Some samples were extracted in duplicate using the QIAquick gel extraction kit (Qiagen, GmbH, Germany) as per the manufacturer's instructions as well as the High Pure PCR template Preparation Kit (Roche Mannheim, Germany). The DNA concentrations obtained with the QIAquick gel extraction kit (Qiagen, GmbH, Germany) were lower than that of the High Pure PCR Kit. Thus, the decision to continue using the High Pure PCR template Preparation Kit in the rest of our experiments was made.

Testing of samples after all the optimisation steps yielded good results on both the gels and the capillary electrophoresis.

4.3.2 SAMPLE QUALITY

Most of the samples used were assessed by means of NanoDrop® ND-1000 (NanoDrop Technologies Wilmington, Delaware USA) in order to determine both DNA concentration and quality.

Some of the samples assessed were found to be of suboptimal quality. The reasons for this included: age of specimen with DNA degradation likely, storage of

samples in suboptimal conditions and many of the samples available for extraction were poorly spread, single slides with little DNA present.

Despite the above mentioned limitations of the samples available, 38% (58/152) of the samples assessed for DNA quality and then included in the study were considered to be of good quality. Those samples that were suboptimal in nature, for the various reasons mentioned above, and still yielded a result with high peak intensities on fragment analysis were included in the study sample. The ability to obtain results with high peak intensities despite sub-optimal samples suggests that this is a robust assay. Furthermore, a total of 232 samples were collected, many of which were extracted and the test performed using this assay. Only 5 of the extracted samples did not yield a result and were therefore excluded from the study. The rest of the samples that underwent electrophoresis, were either included in the study, formed part of the longitudinal assessment of patients or formed part of the control group. Refer to Figure 2.1 for further details.

Other investigators have made use of a similar approach for mutation diagnosis with capillary electrophoresis, but have used RNA and reverse transcriptase PCR (RT-PCR) instead (Noguera et al., 2005). RNA instability is, however, a major limiting factor to this method, especially in a retrospective study. This is particularly relevant in our setting where many of the samples are referred from peripheral centres with the interval between obtaining the specimen and analysis is often more than 24 hours. The clear advantages of using DNA include a stable and reliable starting test material and that the test can be performed on paraffin embedded samples as well as fresh samples.

A means to determine adequate DNA integrity, successful DNA extraction and successful PCR amplification is found in the NPM1 PCR reaction. To date all the

NPM1 mutant cases reported in the literature have been reported as being heterozygous (Swerdlow et al., 2008, Falini et al., 2007). Thus, the presence of a wild type peak or fragment can be considered as a positive internal control.

4.4 OPTIMISATION OF INTERPRETATION OF RESULTS

4.4.1 CAPILLARY ELECTROPHORESIS PEAK SIZE

The primers for this assay have been designed in such a way that the FLT3 primer spans approximately 330bp and the NPM1 primer spans approximately 170bp (Huang et al., 2008). This then allows for the inclusion of the common internal tandem duplication sites and the sites for NPM1 mutations A-F, respectively. The study by Huang *et al.* (Huang et al., 2008) showed that amplified NPM1-wt product was found at approximately 167bp and that the amplified FLT3-wt product was found at approximately 328bp. The NPM1-mut was found to be a single peak at approximately 171bp and FLT3-ITD produced large peaks from 349bp upwards with the largest ITD peak being 424bp in length. The ITD thus varied significantly from the FLT3-wt with a size ranging from 18bp to 94bp. Other studies have shown that the FLT3-ITD mutation size varies from 12bp to as many as 204bp (Schnittger et al., 2002, Kottaridis et al., 2003). Some reports have even identified ITDs as small as 3bp (Schnittger et al., 2002).

Our results differed from that described above with NPM1-wt approximately 170bp in size. The NPM1-mut, did however, remain 4bp larger at 174bp. The results for FLT3-wt were also shifted, at approximately 330bp with the mutant peaks ranging in size from 15bp- 163bp larger than the wild type. The peaks appeared to be

shifted, with an approximate 2bp difference, from that published by another laboratory (Huang et al., 2008) using the same primers. This is not an unexpected finding as capillary electrophoresis fragment analysis does not measure an absolute size but it is rather an assessment of relative size. Various factors may affect the end result, which include a different instrument, a different polymer, the use of a different capillary and the use of a different size standard (Wätzig et al., 1998). Numerous sample types were assessed including stored BMA samples, stored blood slides and fresh blood samples (control samples). All of these yielded peaks with a bp size as described above. A single sample that was found to be mutation positive for both NPM1 and FLT3-ITD, together with a negative control (FLT3-wt/NPM1-wt) was used throughout the rest of the study to confirm consistency.

Up to 23% of patients will have more than one FLT3-ITD mutation present at one time. This can include up to five additional bands (Kottaridis et al., 2001, Noguera et al., 2002, Kottaridis et al., 2003) and may reflect an underlying genetic instability. Additional FLT3-ITD mutation bands were also found in 44% (15/34) of the samples assessed in this study. The significance of more than one peak has not been confirmed to affect prognosis or treatment response and to date the significance of this is unknown (Gale et al., 2008).

The NPM1-wt fragment lengths range from 169 to 171bp with some samples yielding two wild type peaks, a ‘double peak’, which is approximately 1 bp apart with or without the presence of a mutant peak. Length polymorphisms in the poly-T track in the intronic portion of the amplified fragment have recently been described (Szankasi et al., 2008), thus providing an explanation for this finding. Based on this the NPM1-wt fragments will occur within this 3bp range and by further

extrapolation the NPM1-mut fragments will also range by the same 3bp with an expected bp length, from 173 to 175bp. Furthermore, it was found in the literature that by using a high fidelity polymerase this wild type ‘double peak’ could almost be eliminated and that its presence did not interfere with the identification of any of the mutant peaks (Schlenk et al., 2008). These wild type ‘double peaks’, were also consistently found in this study, but did not affect the ability to detect mutant peaks and for this reason such a high fidelity polymerase was not incorporated into the methods of this study.

4.4.2 CAPILLARY ELECTROPHORESIS PEAK INTENSITY

The assessment of fragment analysis results revealed that the peak intensities of both the NPM1 and the FLT3-ITD mutations had a very wide range. Some samples had intensities of less than 100 while others were more than 10 000. Correspondence with two individuals specialising in the field of myeloid leukaemias was initiated (Adam Bagg, personal communication on April 30; 2010 and Phillippe Szankasi personal communication on May 3; 2010). Both of these correspondents had recently published information with regards to the testing strategies available for these gene mutations (Szankasi et al., 2008, Wertheim and Bagg, 2008, Buban et al., 2011). The presence of low mutant peaks in their fragment analysis results was confirmed. Both of these correspondents make use of similar methods, both to each other and to the methods described in this study’s materials and methods section. The first correspondent (Adam Bagg) reports any mutation peak as positive, including those with peak intensities below 100. The sensitivity of the assay in use by his laboratory has been confirmed and they are

confident that these findings are significant. The second correspondent (Phillippe Szankasi) considers mutant peak intensities greater than 100 to be positive.

The incorporation of an indeterminate group as part of the interpretation of the results is worth considering, although this was not performed in the final analysis of the results of this study. This will allow for the sample in question to be re-extracted for PCR, for the original PCR product to be prepared again and electrophoresis on the gene analyser to take place to assess for fragment analysis or alternatively to receive a fresh sample from the patient. These samples will either be bone marrow or peripheral blood depending on the clinical scenario e.g. blast count in the blood, or whether treatment has been started.

As previously mentioned, it is known that FLT3-ITD positivity in an AML patient is a poor prognostic feature. The impact of FLT3-ITD positivity, mutant level, size and number of mutations in these patients remains uncertain. Recently, it was found that the most significant marker for poorer prognosis was mutant level rather than the presence of the mutant itself (Gale et al., 2008).

4.4.3 FLT3 RATIO RESULTS

The reporting of the mutant levels, as recorded on the electropherogram has become routine practice. It was recently noted that those with a low mutant to wild type level had a better long term outcome while those with a high FLT3-ITD mutant ratio were at a higher risk of early relapse (Gale et al., 2008). A previous study had suggested that patients with very low FLT3-ITD mutant level ratios had a similar prognosis to those who were wild type (Cloos et al., 2006). All of this is

available for reporting when making use of the capillary electrophoresis method and the allelic burden has been assessed in more detail in the results section. If this approach is adopted then the presence of a low mutant peak and the decision to call it positive or alternatively indeterminate should have no real impact on patient prognosis or on physician treatment decisions compared to those who are diagnosed as wild type. This does not, however, mean that these peaks and mutant ratios should not be reported. It will remain important for follow up of these patients with a definite need to be particularly vigilant.

The importance of allelic burden is a finding which is also relevant in the assessment of other mutation states. JAK2 V617F mutation allelic burden is an important prognostic marker in MPN (Theocharides et al., 2008, Hussein et al., 2009).

FLT3-ITD mutant level, however, is affected by a number of factors which include the number of blasts present in the sample as well as the number of cells that have the mutant clone present (sub-clone). As mentioned, the study by Gale *et al.* allowed for the division of their patient cohort into three risk group categories. RR and OS were affected by the FLT3-ITD allelic burden and was the basis for this risk classification (Gale et al., 2008). Of the cases that were FLT3-ITD positive in our study and had a mutation level recorded, 50% (15/30) were found to be in the low risk category with a mutant to wild type peak of less than 25%.

Other methods of assessing FLT3-ITD and NPM1 mutants are valuable in order to confirm the molecular findings described.

4.5 SEQUENCE ANALYSIS

The “direct visualisation” of this method would have been particularly useful for confirmation of the molecular findings. However, none of the samples that were sequenced (n=5) revealed the expected mutation. Although DNA sequencing is generally a robust technique, problems do occur. This method has limited sensitivity due to the background noise in the generated chromatograms and a positive result is often dependent on a high mutant/wild type ratio, *i.e.* requiring at least 20% mutant in the sample (Izmailov et al., 2002, Steensma, 2006). This is particularly relevant in NPM1 cases in which homozygosity of the mutant has never been recorded and in FLT3-ITD where often it is a secondary mutation which may only form part of a small clone (Izmailov et al., 2002, Steensma, 2006, Falini et al., 2007). Furthermore, the same primers that were used for the PCR reaction were also used for the sequencing reaction. This could have resulted in the area of interest being too close to the primer and therefore falling into the initial area of noise. Some of the traces had short read lengths: a delayed start to the trace signal may have resulted in the area of mutation being missed and primer dimers may have formed in the sequencing reaction. Methods to improve the sensitivity of sequencing include cloning the mutant DNA prior to sequencing or blast cell enrichment of the specimen (Hyodo et al., 2003, Kiyoi and Naoe, 2006). These methods will be employed as part of the ongoing research.

4.6 CYTOPLASMIC NPM1 ASSESSMENT

In general aberrant expression of cytoplasmic nucleophosmin can be used as a surrogate for NPM1-mut positivity (Falini et al., 2006, Swerdlow et al., 2008). This

method of detection of NPM1 mutations was included in the study not only as a potential means to confirm the molecular findings but also to assess its potential usefulness as an adjunct to capillary electrophoresis.

A number of limitations are present with this form of detection. This includes that, immuno-staining with anti-NPM1 is not used as a single marker but rather anti-C23 (nucleolin) is used in conjunction with this stain. C23 is used as control as it is always nuclear in NPM1 mutation positive cases (Falini et al., 2005). Furthermore, this form of NPM1 detection has been optimised for histology only, accurate detection of cytoplasmic positivity can be difficult in high nuclear to cytoplasmic ratio blasts and its use in detection of MRD is not reported and is unlikely to be useful (Falini et al., 2005, Wertheim and Bagg, 2008). Also, the detection of NPM1 mutations without knowledge of the FLT3 status is unhelpful in the further management stratification of these patients. These limitations were also evident in the use of this stain in this study cohort.

The corresponding trephine samples of the bone marrow aspirate slides were not always available and as a result only 32 samples were assessed for NPM1 cytoplasmic positivity (NPM1-mut status). A 73% concordance was found between the immuno-histochemical staining and the molecular results. Although this was useful in confirming some of the findings from the molecular assessment, some of the samples were found to have equivocal staining and it did not prove to be of any value in those cases which have a low NPM1 mutant peak intensity.

Another alternative would be to use flow cytometric analysis to detect for the presence of cytoplasmic nucleophosmin and thereby the presence of the NPM1 gene mutation (Oelschlaegel et al., 2010). This method may be incorporated into further research in this area.

4.7 PATIENT CHARACTERISTICS

An overall assessment of the frequency and general patient characteristics of *de novo* AML in our population had not been recently reported. For this reason all *de novo* AML cases were assessed generally and then for the more specific subgroups of NPM1 and FLT3-ITD mutation positivity.

4.7.1 DEMOGRAPHICS

There is often a delay in accessing health care in developing countries and this results in up to 95% of cancer patients in general being diagnosed late with a consequent poor outcome (Andela, 2006) . During this delay nearly half of these patients would have sought advice from a traditional healer or private medical practioner (Mesfin et al., 2009). The reasons stated for this delay in seeking care include hoping for spontaneous remission, a way to defer cost, negative perception of health institutions, illiteracy and to avoid the possible diagnosis of HIV/AIDS (Yomi and Gonsu, 1995, Mavhu et al., 2010). These are important points to consider when assessing patient demographics and comparing results from a developing country to that of the developed world.

Taking this into account, it is known that NPM1 mutations are generally associated with *de novo* AML with no prior history of MDS, MPN or other prior malignancies for which treatment was received. The male to female ratio in all subtypes of AML is 3:2 but NPM1 mutation positive subgroups have a male to female ratio of 1:1.5 (Thiede et al., 2006). An unexpected slight female predominance was found in the total study cohort of 164 AML cases. In the NPM1 mutation positive cases there was a male to female ratio of 1:1.5 which was not statistically significant *(P>0.05)*. The reason for the slight female predominance in the AML group as a whole is not

certain and would be beyond the scope of this project. However, a recent report has indicated that there is often a delay by men in seeking medical assistance with regard to certain chronic illnesses (Mavhu et al., 2010). It is possible that this may also occur in acute onset illnesses.

The median age of diagnosis of AML was found to be 40 years in this study cohort which is significantly lower than the reported figure of 63 years (Jemal et al., 2005) as reported in the cancer statistics of the United States of America. Sub-Saharan Africa is known to have vastly different population demographics compared to first world western countries, with a much younger population in general: This could contribute to the younger age at presentation seen in our study There is also a much lower life expectancy mostly due to the HIV pandemic and other infectious diseases (Kahn et al., 2007).

NPM1mutation positivity is commonly found in adults (30%) diagnosed with AML with a median age at diagnosis for those who are NPM1-mut only is approximately 60 years (Thiede et al., 2006). Our data set showed that NPM1-mut only (NPM1-mut/FLT3-wt) cases were found to be younger than expected with a median age of 30 years as represented in Figure 3.17. It was, however, noted that patients' ≥60 years formed 32% (13/41) of the NPM1-mut positive subgroup while in the NPM1-wt subgroup those ≥60 years only accounted for 18% (22 /120). An increase in the number of older patients with both mutations occurring simultaneously was noted, with those ≥60 years accounting for 53% (8/15). There was a statistically significant difference noted when assessing those aged ≥60 years in the NPM1-mut/FLT3-ITD group compared to the NPM1-wt/FLT3-wt group *(P<0.002)*. This seems to indicate a gain in mutations with increasing age.

There has been no significant association between FLT3-ITD positivity and age or gender in the literature (Kottaridis et al., 2001, Thiede et al., 2002) . No significant association was found in this study cohort either.

4.7.2 FBC AND BLAST COUNT FINDINGS

AML with mutated NPM1 is a *de-novo* AML which has been associated with certain characteristic findings. These patients are often anaemic and have a thrombocytopenia, but present with a higher WCC, platelet count and blast count at diagnosis when compared to other AML types (Döhner et al., 2005). The anaemia and thrombocytopenia at diagnosis was confirmed in the NPM1-mut positive cases as represented in Figure 3.4.

Those with NPM1-mut only (NPM1-mut/FLT3-wt) were found to have a significantly reduced median platelet count when compared to those with FLT3-ITD only (NPM1-wt/FLT3-ITD) and this appears to be a novel finding as this has, to my knowledge, not been documented in any of the international literature.

In addition, a leucocytosis and high blast cell count was present in the NPM1-mut as well as the NPM1-mut only group (NPM1-mut/FLT3-wt), but was not statistically different than in the other AML sub-groups assessed in this project. The international literature found similar findings as that described above.

The expected leucocytosis and high blast count in those with FLT3-ITD positivity was also found to be present (Suzuki et al., 2007). The WCC count of those cases that were FLT3-ITD positive only (NPM1-wt/FLT3-ITD) was significantly increased when compared to those with an NPM1-mut only (NPM1-mut/FLT3-wt) *(P<0.03)*, and those without either of the mutations (NPM1-wt/FLT3-wt) *(P<0.01)*. This finding has not been documented in the international literature. As a further

novel finding, those cases with both mutations in combination (NPM1-mut/FLT3-ITD) had a significantly *(P< 0.04)* higher median Hb (9.5 g/dl) when compared to the other three groups (NPM1-mut only, FLT3-ITD only and wild type for both gene mutations). As the group numbers analysed are small, all of these novel findings need to be assessed in greater detail and would form part of future work.

4.7.3 FAB CLASSIFICATION

NPM1 mutations occur most commonly in those patients diagnosed as FAB M4 and M5 and there have been reports that 80-90% of monocytic leukaemias are NPM1-mut positive (Falini et al., 2005, Swerdlow et al., 2008). In this study cohort, FAB M5 was also found to be the most common subgroup, comprising 40% (8 of 20) of those who were NPM1-mut positive only (NPM1-mut/FLT3-wt). FAB M2 was found to be the second most common group in those who are NPM1-mut positive.

Several studies have reported an association of the FLT3-ITD mutation with FAB M3 and FAB M5 (in particular M5b). Conversely, it is not commonly associated with M6 and M7 (Schnittger et al., 2002, Thiede et al., 2002). A total of 31% (5 of 16) of the FLT3-ITD positive only samples were classified as FAB M5 according to the FAB classification system.

FAB M5 was found to be the most common classification group, in both the NPM1-mut only (NPM1-mut/FLT3-wt) and the FLT3-ITD only (NPM1-wt/FLT3-ITD) group. In the group negative for both (NPM1-wt/FLT3-wt) these gene mutations FAB M2 and M3 were the most common FAB classification. Although, not statistically significant these findings concur with that found in the literature(Falini et al., 2005, Swerdlow et al., 2008).

4.8 IMMUNOPHENOTYPIC ANALYSIS

Blasts in the NPM1 wt group were found to be significantly more commonly CD34 positive (*(P< 0.05)* than in the NPM1 mut group. It was, however, expected that most of the NPM1-mut positive samples would be CD34 negative (Falini et al., 2005), but only 51% (20 of 39) of NPM1-mut positive samples were CD34 negative . The reporting of CD34 positivity may be affected by various factors and needs to be assessed in more detail. Most studies assess the presence or absence of CD34 expression by means of immuno-histochemistry staining, this is not as sensitive as flow cytometric assessment (Falini et al., 2005). Also, of those studies that do assess CD34 presence by means of flow cytometric analysis, anti-CD34 FITC is used. In our centre it is possible that for some of these cases the panel of HLA-DR/CD33/CD34 is used in making the diagnosis of AML. This means that a PERCP anti-CD34 monoclonal antibody is used, which will be brighter than that seen when using FITC anti-CD34. This may result in the higher estimation of CD34 positive cells. Furthermore, Paint-A-Gate PRO Software (BD Biosciences Inc., San Jose, CA) is used for flow cytometric analysis, this may also result in an over estimation of the positivity of CD34.

4.9 CYTOGENETIC ASSESSMENT

Of the 164 cases in this study cohort, 21% (34/164) were found to be FLT3-ITD positive and 26% (42/164) were NPM1-mut positive while 9% (15/164) were found to have dual positivity (NPM1-mut/FLT3-ITD). All of these findings together with the propensity for FLT3-ITD to target those with an NPM1-mut are in keeping with findings in the literature (Thiede et al., 2006, Swerdlow et al., 2008).

Recently research has focused only on those patients who were diagnosed with CN AML (Fröhling et al., 2002, Döhner et al., 2005, Falini et al., 2005). Many of the adult AML patients in our records did not have their karyotype assessed at diagnosis. Some of the pre-analytical reasons included that the test had not been requested by the treating doctor, samples had not been sent in the correct tubes and in some cases inadequate samples were received. Analytical reasons included the relatively common growth failure of the cell culture. As a result, a reliable assessment of those who were indeed normal karyotype was not possible and the decision to only look at those patients with a confirmed normal karyotype was not a feasible option.

The presence of an aberrant karyotype, including recurrent translocations, occurring in conjunction with NPM1 and FLT3-ITD mutations has been reported (Suzuki et al., 2005, Verhaak et al., 2005, Thiede et al., 2006, Palmisano et al., 2007)). As little or no data is currently available in South Africa the decision not to limit our assessment to those with either no karyotype or normal karyotype results only was made. Both known associations and any unexpected, novel findings could be missed if the sample group was too limited.

In most laboratories, approximately 25% of AML patients are currently excluded from NPM1 and FLT3-ITD testing due to the failure of cytogenetic analysis and are also possibly excluded from the assignment of the prognostically favourable genotype (NPM1-mut/FLT3-wt) if a chromosomal aberration is present (Mrózek et al., 2007). In view of the above assessment and our findings in this study it may indeed be valuable to test all AML patients for both FLT3-ITD and NPM1 mutations.

Palmisano *et al* found that, from 11 patients who were confirmed NPM1-mut positive on sequencing, two were positive for t(15;17) and two were positive for inv(16) (Palmisano et al., 2007). Another two patients were found to have complex karyotypes. There has also been a case report documented, where a patient was diagnosed as having an NPM1 mutation (low mutation peak intensity) and was found to have not only one recurrent translocation but two (Falini et al., 2008). This patient had both a t(8;21) and an inv(16) mutation. When assessing the mutation status of our patient cohort with a recurrent chromosomal translocation and an NPM1mutation present, a common feature was the presence of a low NPM1mutation peak intensity and by association a low NPM1-mut /NPM1-wt ratio. The recurrent translocations, as mentioned in the 2008 WHO classification, were present only in NPM1 mutation positive samples with mutant peak intensities of less than 550. Two other samples (R51 and R35) with a mutation peak intensity >550 also had another cytogenetic abnormality noted. Further details are reported in Appendix 5.2.

In view of the limited numbers of patients with dual recurrent translocations and NPM1 mutations some of these results reported in the literature have been reassessed on a retrospective basis by Falini *et al* (Falini et al., 2008). Some of these samples were found to be negative for NPM1mutation on repeat testing, while in others cases, sample registration errors were found for the PCR and/ or the karyotyping results (Falini et al., 2008). Other samples, however, could not be disregarded as having an error and were found to be correct results. It will, certainly be valuable to reassess those patients in our cohort who were found to have both recurrent translocations and an NPM1 mutation. Fresh samples at

relapse for both FISH and capillary electrophoresis analysis would be particularly useful.

The possibility of using the peak intensity value of >550 for NPM1 mutation positivity may also be a realistic consideration as all the recurrent translocations which occurred simultaneously with an NPM1 mutation were found in cases with NPM1 mutation peak intensities of <550. Reporting of the NPM1-mut/NPM1-wt ratio is another assessment tool which is likely to be valuable in the future and is readily available from data produced in the capillary electrophoresis assessment. This ratio would then be able to correct for the low mutant peak intensities.

Laboratory error as well as the sensitivities and specificities of the diagnostic test may not be the only factors contributing to these findings. Ethnic differences have been confirmed to affect disease pathogenesis. Even in a recent study assessing NPM1 mutations in Asian populations, it was found that the incidence of this mutation was significantly lower than in European populations (Koh et al., 2009).

The possibility remains that the occurrence of two or more specific genetic markers (in particular NPM1 mutations in conjunction with another) may in fact be co-incidental or alternatively a true association, which is as yet not understood. The assessment of these recurrent translocations together with NPM1 mutations should form part of an ongoing investigation in all future patients diagnosed with *de novo* AML.

Of the 34 patient samples found to be FLT3-ITD positive, 44% (15/34) of them were also NPM1 mutation positive. This was found to be a statistically significant association ($P<0.05$) and is a common finding in most other studies (Thiede et al., 2006). This is suggestive of the NPM1 mutation being the first of the 2 mutations to occur. FLT3-ITD is not as stable and often develops later in the disease process

as a secondary event (Cloos et al., 2006, Palmisano et al., 2007, Schnittger et al., 2009).

FLT3-ITD is found in all forms of AML, but is commonly associated with CN AML and with t(6;9) and t(15;17) (Kottaridis et al., 2003, Slovak et al., 2006). Of the 34 patients who were found to have a FLT3-ITD mutation, 58% (11/34) had cytogenetic abnormalities detected (those with confirmed abnormal FISH analysis results were included in this group even though not all of them had a karyotype result available), while 42% (8/34) were found to be cytogenetically normal. Of those who were FLT3-ITD positive only (NPM1-wt/FLT3-ITD) 13 had a karyotype result available of which 38% (5/13) had a normal karyotype (*P>0.05*). Only 3 of the 34 samples that were FLT-ITD positive were also t(15;17) positive (*P>0.05*). In this study the expected significant association between FLT3-ITD positivity and t(15;17) and CN AML was not found. The total number of those with a cytogenetic or FISH result was small and this may have limited accurate assessment.

4.10 ASSESSMENT OF MINIMAL RESIDUAL DISEASE (MRD)

NPM1 mutations are the most common genetic aberration in CN AML and are one of the more stable mutations over the course of the disease (Falini et al., 2009). In assessing for MRD, it has also been shown that mutant copy numbers coincide well with response to chemotherapy and are predictive of haematologic relapse (Gorello et al., 2006). For these reasons there is merit in attempting to use this mutation as a potential marker for MRD follow up. Various techniques are available for assessing for the presence of NPM1 mutations. Immuno-histochemistry and capillary electrophoresis have a diagnostic sensitivity of

approximately 100%, with the ability to detect almost all of the NPM1 mutation types (A-F). Capillary electrophoresis has an analytical sensitivity of approximately 5% and thus limits its potential as a test for assessing MRD (Szankasi et al., 2008, Wertheim and Bagg, 2008). QPCR has an analytic sensitivity of 0.01-0.1% but is limited, in most assays, to only diagnosing NPM1 mutation A (Ottone et al., 2008, Wertheim and Bagg, 2008). For most of the other mutations there may be a need to develop patient specific primers, which has cost implications. The answer to assessing for MRD in our laboratory, as a future undertaking, may lie in using a combination of these methods.

Although using NPM1 as a marker for MRD appears promising, there have been two recent reports confirming the loss of this mutation at relapse (Suzuki et al., 2005, Papadaki et al., 2009) with Papadaki *et al.* finding this in 9.5% and Suzuki *et al.* finding this in 11.7% of their patient cohorts. They suggest that this may in fact be a limitation to its use as a marker for assessing MRD. This particular finding, of a loss of NPM1 mutation at relapse, was also noted in a single patient that formed part of the longitudinal follow up and was reported as Case 3 in the results section. With the reported lower limit analytical sensitivity of approximately 5% and the reported evidence of a distinct correlation of NPM1 mutation ratio with blast percentage (Papadaki et al., 2009); the relapse blast percentage of 80% in this particular case, Case 3, was adequate for NPM1 mutation assessment by means of capillary electrophoresis.

The presence of FLT3-ITD is not considered useful for assessing MRD. At relapse some patients have shown a loss, a gain, a change in the length as well as a change in the number of FLT3-ITD mutations. A gain can occur in up to 23% of patients and may suggest the presence of an oligo-clone which dominates in

relapse (Cloos et al., 2006). Patients may gain a new mutation and lose the old mutation during disease progression. It is, however, important to continue to monitor for this mutation as it has been found that if the FLT3-ITD remained present at relapse there had then been a significantly shorter time to relapse and if the FLT3-ITD had been lost at relapse the time to relapse was found to be significantly longer (Kelly and Gilliland, 2002).

4.11 IMPACT ON CINICAL OUTCOME

It is has been well established in the recent literature that the presence of an NPM1 mutation improves overall survival and confers a better prognosis for CN AML patients, while the presence of FLT3-ITD positivity results in much poorer prognosis and the combination of the two mutations appears to provide an intermediate prognosis (Döhner et al., 2005, Gale et al., 2008). Those patients with an NPM1 mutation usually respond well to induction chemotherapy (Arber, 2001). This sensitivity to chemotherapy may be secondary to the mutated NPM1 binding to nuclear factor kappa beta (NF-κB) which confers resistance to chemotherapy by sequestering it in the cytoplasm and therefore inactivating it (Cilloni et al., 2008). When NPM1 is mutated, a NES motif is created which then reinforces the Crm-1 (exportin1) dependent movement of NPM1 into the cytoplasm (Bolli et al., 2007). Targeting abnormal Nucleophosmin trafficking to the cytoplasm by means of Crm-1 inhibitors is a potential target (Dong et al., 2009).

NPM1 mutations are always heterozygous in AML and it has been suggested that some normal NPM1 is required for leukaemic cell survival (Falini et al., 2007).

Another possibility for therapy might then be to target the residual NPM1-wt which the leukaemic cell still requires to survive. (Falini et al., 2009)

It was also recently found that the addition of an already well known drug, ATRA to a conventional chemotherapy regimen improved survival in those patients who had an NPM1 mutation only (NPM1-mut/FLT3-wt) (Schlenk et al., 2009).

FLT3-ITD positivity is well known to have an adverse effect on patient outcome and is relatively common in AML patients; this makes it an attractive target for the design of specific inhibitory therapies such as FLT3-inhbitors, a specific form of TKIs (Sanz et al., 2009). However, the effectiveness of FLT3-inhibitors as single agents has been disappointing overall and clinical testing in combination with standard chemotherapeutic regimens have shown better results (Levis et al., 2004). Some of these FLT3 inhibitors, as part of combination chemotherapy, are currently in phase III clinical trials (Sanz et al., 2009).

4.12 RECOMMENDATIONS

Further improvements on testing methodology can be made via a prospective study making use of fresh DNA samples. Cases included at diagnosis need to be assessed on a longitudinal basis and monitored for survival, time to relapse and mutation status at relapse in collaboration with the treating haematologists or oncologists.

Those patients who have been found to be NPM1 mutation positive in conjunction with a recurrent translocation should be assessed in more detail. It may be of value to obtain a fresh sample from these particular cases if relapse occurs for

reassessment of mutation status. This will enable the further elucidation of the uncommon finding of an NPM1 mutation and a recurrent cytogenetic translocation and allow confirmation of differences in our setting. All patients diagnosed with AML should be assessed for NPM1 and FLT3-ITD gene mutations, not only those who are assessed to be CN. This decision may need to be reviewed at a later stage once sufficient data for our patient population has been obtained and the cost implications of testing have been fully evaluated.

Immunophenotypic interpretation of prospective studies should be assessed in more detail. Anti-CD34 FITC only, should be used to determine CD34 positivity on these samples and a comparison made to the immuno-histochemical anti-CD34 stain. It is also suggested that a single person review these results, at least initially, in order to exclude inter-observer variation.

This testing should be expanded to include paediatric patients as well. Despite the lower frequency of these mutations in paediatric patients with AML, approximately 7.5% will have an NPM1 mutation (Brown et al., 2007) and 5-16.5% (Krstovski et al., 2009) a FLT3-ITD mutation. The outcome and treatment strategies for paediatric patients are also affected by the presence of these mutations (Zwaan et al., 2003, Cazzaniga et al., 2005, Brown et al., 2007).

Sequence analysis of both mutations but in particular, for NPM1 mutations will be useful. This will determine if the commonly reported mutations are indeed also the most common in our population and whether it will be financially viable to implement the use of Q-PCR in combination with capillary electrophoresis in order to better assess for MRD. The primers used for sequencing needs to be re-evaluated along with the various other possible reasons for the initial failure of this technique.

Use of a known mutation positive cell line to include as part of the routine assay would confirm the reproducibility of our results.

Consideration could be given to the establishment of a laboratory external quality assurance (EQA) programme.

Other well known gene mutations and various combinations of these such as CEBPA, WT1, NRAS, KRAS and MLL-PTD (Kelly and Gilliland, 2002, Mrózek and Bloomfield, 2006) should ideally also be assessed as they are also known to affect the outcome of patients. As a further project, multiplex mutational testing making use of Luminex based systems may be a consideration. Currently, a commercial Luminex assay which detects four (A, B, D and J) of the NPM1 gene mutations (RNA based) is being validated. This will allow detection of 85-95% of the known NPM1 exon 12 mutations (Hafez et al., 2010). The potential exists to assess for both the common recurrent translocations (normally determined by karyotyping or FISH analysis) as well as these gene mutations and others in one assay (Wertheim and Bagg, 2008).This would mean that all the clinically relevant mutations for AML could be detected in a single assay. In order for this to be feasible all of the PCR and hybridisation conditions for the karyotypic abnormalities and gene mutations would need to be similar.

4.13 CONCLUSION

The presence of both NPM1 and FLT3-ITD mutations were assessed by means of a PCR based method. Analysis by Genescanning capillary electrophoresis yielded results in which the intensity and exact position of the mutation as well as the

mutant level were easily determined. The PCR method for the detection of these gene mutations is relatively simple and visualization is clear.

The combined assessment of NPM1 and FLT3-ITD allows for identification of subgroups of patients with markedly different prognosis. NPM1-mut only (NPM1-mut/FLT3-wt) confers a favourable outcome, while FLT3-ITD positivity is generally unfavourable, with FLT3-ITD overriding the good prognosis of NPM1 mutation positivity when they occur in conjunction. FLT3-ITD peak intensity relative to the FLT3-wt peak intensity is also known to affect prognosis with a high FLT3-ITD/FLT3-wt ratio predicting a poorer OS and RR (Gale et al., 2008).

It is well known that the diagnosis, assessment of severity and prognostication of most diseases requires not only multiple special investigations but a detailed history and clinical examination as well. This is especially true for a complicated multi-dimensional disease such as acute leukaemia. The complexity of this particular neoplasm is easily evidenced in the WHO classification where numerous factors are incorporated into diagnostic algorithms to make the final diagnosis. This includes morphology, cyto-chemical stains, immunophenotyping, clinical findings and most recently genetic information. Some of these parameters are used to make a diagnosis, whereas others are more specifically used for prognostication. When assessing so many parameters at so many different levels it becomes evident that not all individuals will always comply with a definitive category but indeed groups of patients may differ from the norm. In particular this needs to be taken into account when assessing minority groups which are often not included in the studies used to establish these international guidelines.

Currently there is limited data available describing AML in South Africa. This study not only provides information about these newly described gene mutations but

also gives insight into the epidemiology and patient characteristics of those adults diagnosed with *de novo* AML. The frequency of these mutations in newly diagnosed AML patients concurs with reported studies.

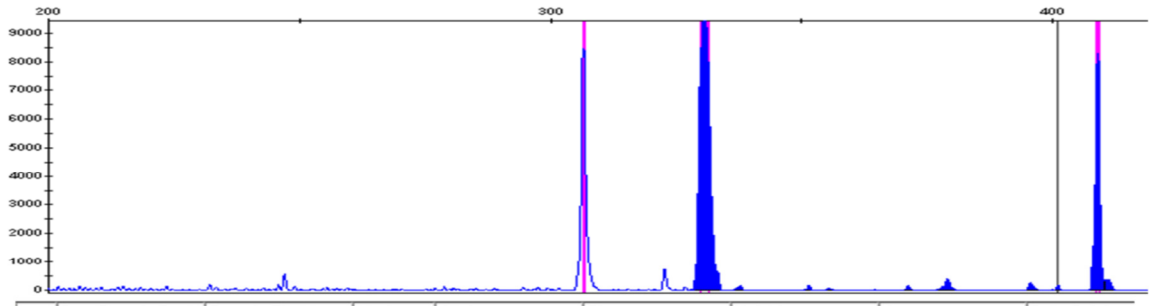
An assessment of these mutations relative to some of the special investigations commonly used in our setting was made. This included comparison to routine investigations (FBC) and those more specific to the diagnosis of acute leukaemia (immunophenotyping and cytogenetics). Some of the findings presented appear to be novel. In particular, the occasional occurrence of a recurrent translocation together with an NPM1 mutation is an unexpected yet interesting result.

The determination of the presence of these gene mutations will now form part of the standard testing in any patient diagnosed with *de novo* AML at any of the Gauteng provincial hospitals. As part of an ongoing assessment and follow up the results obtained from this routine implementation of testing will continue to be compared to those parameters described in this study. Furthermore, patient outcome will be prospectively assessed in collaboration with the treating haematologist.

5 APPENDIX

5.1 FLT3 MUTATION LEVEL

The AUC of both the mutant and wild type FLT3 peak generated on capillary electrophoresis is automatically recorded as part of the electropherogram data and is displayed in Figure 5.1. The relative mutant level or allelic burden can then be calculated using the following: AUC FLT3-ITD x100/ AUC FLT3-wt +AUC FLT3-ITD (Gale et al., 2008).



	Dye/Sample Peak	Sample File Name	Marker	Allele	Size	Height	Area	Data Point
	B,96	FLT3_R38_C08_(			211.1	123	632	2839
	B,97	FLT3_R38_C08_(			319.17	146	2068	3886
	B,98	FLT3_R38_C08_(			329.94	2476	17557	3988
	B,99	FLT3_R38_C08_(			330.98	4976	37525	3998
	B,100	FLT3_R38_C08_(			333.48	145	2719	4022
	B,101	FLT3_R38_C08_(			351.52	1474	19862	4205
	B,102	FLT3_R38_C08_(			361.09	183	2361	4305
	B,103	FLT3_R38_C08_(			375.81	9177	81861	4456
	B,104	FLT3_R38_C08_(			376.7	10313	162192	4465
	B,105	FLT3_R38_C08_(			382.05	437	8459	4519
	B,106	FLT3_R38_C08_(			384.74	120	2619	4546

Figure 5.1 Electropherogram and data table (GeneMapper software).
This demonstrates the wt and the mutant peak sizes, intensities and area under the curve for Sample R38.

5.2 NPM1 AND FLT3-ITD MUTATION POSITIVE SAMPLES

Table 5.1 includes all the samples which were found to be NPM1 mutation positive. Included in this table are the wild type and the mutant size and peak intensities.

Table 5.1 NPM1-mut positive samples.
All the wild type and mutation peak intensities and sizes are included.

SAMPLE	NPM1-wt SIZE	NPM1-wt INTENSITY	NPM1-mut SIZE	NPM1-mut INTENSITY
R 18	170.02	8603	173.64	1562
R 38	170.68	9139	174.69	9160
R 51	170.34	8585	174.27	7223
R 52	170.04	8836	174.09	8857
R 57	170.65	8433	174.14	7000
R 63	170.14	9040	174.2	9099
B3	170.38	8809	174.82	9198
F19	169.28	8692	173.45	8392
N12	170.7	7616	173.59 174.44	8595 8573
N17	169.12 170.73	7517 7506	174.7	7785
N44	169.19	7442	173.24 174.82	7330 7078
N99	170.68	9092	174.41	8578
T1_T28	169.33 170.85	8018 7516	173.35	669
R 35	170.49	9397	174.61	6077
N10	170.76 169.03	7344 7214	173.44	1415
N14	169.23	8443	173.95	8686
N40	169.07	7886	173.92	1684
N49	169.16 170.74	8765 8465	173.58 174.42	523 400
N55	169.32	8507	174.32	8556
N59	169.96	8907	173.8	8860
N61	169.29 170.77	7724 7607	174.77	8343
N64	170.25	2320	174.25	611
N71_N28	170.62	8812	174.51	8542
T9_R9	170.69	8990	174.9	8862

R 11	169.98	9126	174.22	109
R 17	170.54	1046	174.54	407
R 21	169.3	8475	173.64	208
R 31	169.3	8325	173.33	75
R 3	170.03	9249	173.56	165
R 8	169.98	8938	173.76	314
R 10	170.9	9228	173.78	471
R 53	170.72	8883	173.7	290
B5	169.43 171.02	8495 8181	173.88	176
N24	170.07	8541	173.67	209
N56	170.71	8870	173.35	386
R14_F18	169.30 170.80	8005 8101	173.61	61
R30_T7	170.8	9059	173.48	243
R36_F23	170.76	9233	173.32	161
T14_F11	170.46	451	174.44	198
T17	169.33 170.96	8661 8574	173.24	189
R73	169.96	8628	174.63	8736
T8	169.26 170.88	6913 6848	173.47	3793

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type.

Sample R51 had a mutation peak intensity of 7223 and was found to have a *del(13q)* while sample R35 had a peak mutation intensity of 6077 and was found to have an *inv(9)* on cytogenetic analysis. The electropherogram plot of Sample R51 is displayed in Figure 5.2.

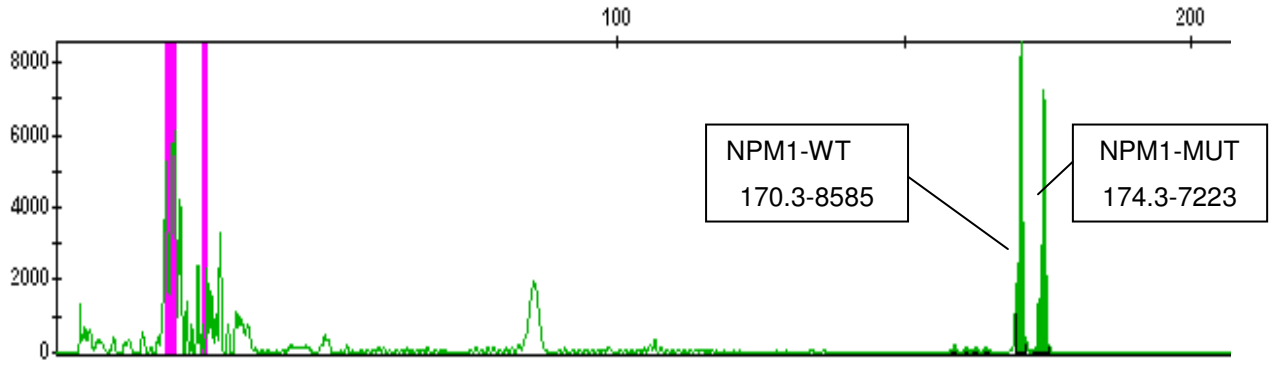


Figure 5.2 High NPM1-mut peak intensity in sample positive for del(13q).

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; NPM1-wt, nucleophosmin 1 gene wild type.

Table 5.2 and 5.3 includes all the samples which were found to be FLT3-ITD positive (NPM1-wt/FLT3-ITD) and positive for both mutations simultaneously (NPM1-mut/FLT3-ITD). Included in these tables are the wild type and the mutant size and peak intensities.

Table 5.2 FLT3-ITD positive samples.
All the wild type and mutation peak intensities and sizes are included.

	SAMPLE	FLT3-wt SIZE	FLT3-wt INTENSITY	FLT3-ITD SIZE	FLT-ITD INTENSITY
1.	R 38	330.98	4868	351.52 375.81 376.70	1465 8575 9711
2.	R 51	330.75	9435	378.98 408.85	400 8302
3.	R 57	330.93	9324	345.74 355.34	184 163
4.	R 63	330.75	8470	382.23	7644
5.	B3	330.67	9851	368.48	1439
6.	F19	329.43	8794	346.52 356.53 370.68	2077 2916 9430
7.	N12	330.94	8933	388.02 396.55	2383 1790
8.	N17	331.08	9947	348.61	972
9.	N44	331.04	7809	379.8 406.00	1432 5681
10.	N99	329.87 330.92	4421 8143	351.44 359.93 362.85 421.51	679 1699 196 572
11.	T1_T28	330.81	9680	418.69	3030
12.	R 11	330.81	9238	360.07	2539
13.	R 17	331.0	6348	374.1	1565
14.	R 21	330.88	8485	359.54	1347
15.	R 27	330.44	8730	371.13	8955
16.	R 64	330.87	8770	365.51	2418
17.	R 65	330.55	9755	365.19 378.15 393.5	1111 1340 8139
18.	F24	330.69	9783	392.32 415.72 424.43	8149 296 8149
19.	F3	330.81	10032	344.04 352.34	2187 327
20.	F4	330.89	8148	391.02	184
21.	F6	330.07	9034	355.41	81
22.	N11	330.59	10049	361.59 374.98	1005 1227

				387.76	10350
23.	**N30**	330.63	9929	356.77 376.8	1776 741
24.	**N32**	331.19	10234	493.29	881
25.	**N33**	331.17	9774	493.33	70
26.	**N37**	331.29	9509	355.39	174
27.	**N60**	330.68	9044	359.63	9204
28.	**N62**	330.89	9379	340.67 348.5 365.04	9483 2497 9483
29.	**N75**	329.78	8694	367.23	499
30.	**N94**	330.86	9646	339.28	56
31.	**T15_N31_T27**	330.0	9488	385	8309
32.	**T2**	330.79	10026	371.22	8688
33.	**T20**	330.72	10012	353.98 382.77	2637 3144

Abbreviations: FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication; FLT3-wt, FMS-like tyrosine kinase receptor gene wild type.

Table 5.3 Samples with dual positivity for NPM1-mut and FLT3-ITD.
Mutation size and intensity for both are demonstrated.

SAMPLE	NPM1-mut SIZE	NPM1-mut INTENSITY	FLT3-ITD SIZE	FLT-ITD INTENSITY
R 38	174.69	9160	351.52 376.70	1465 9711
R 51	174.27	7223	378.98 408.85	400 8302
R 57	174.14	7000	345.74 355.34	184 163
R 63	174.2	9099	382.23	7644
B3	174.82	9198	368.48	1439
F19	173.45	8392	346.52 356.53 370.68	2077 2916 9430
N12	173.59 174.44	8595 8573	388.02 396.55	2383 1790
N17	174.7	7785	348.61	972
N44	173.24 174.82	7330 7078	379.8 406.00	1432 5681
N99	174.41	8578	351.44 359.93 362.85 421.51	679 1699 196 572
T1_T28	173.35	669	418.69	3030
R 11	174.22	109	360.07	2539
R 17	174.54	407	374.1	1565
R 21	173.64	208	359.54	1347

Abbreviations: NPM1-mut, nucleophosmin 1 gene mutant; FLT3-ITD, FMS-like tyrosine kinase receptor gene internal tandem duplication.

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