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# IDH1 and IDH2 gene mutations in South African patients with cytogenetically-normal Acute Myeloid Leukaemia.

**Walton, Ashleigh Helen**

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# *IDH1* AND *IDH2* GENE MUTATIONS IN SOUTH AFRICAN PATIENTS WITH CYTOGENETICALLY-NORMAL ACUTE MYELOID LEUKAEMIA

**Ashleigh Helen Walton**

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2013

# Declaration

I, Ashleigh Helen Walton declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

_____ day of _____ , 2013

For my husband

Home is wherever you are

# Publications and Presentations arising from this Study

Poster Presentation:

Walton A and Willem P. *IDH1* and *IDH2* mutations in Cytogenetically-Normal Acute Myeloid Leukaemia. Laboratory Medicine Congress. Johannesburg, September 2011

# Abstract

**Introduction:** Mutations in isocitrate dehydrogenase (*IDH*) 1 and 2 genes have recently been found in Acute Myeloid Leukaemia (AML) and may represent a novel class of mutations with distinct clinical and biological characteristics. They occur more frequently with normal karyotype AML and *NPM1* mutations, and may confer a mutation-specific prognostic impact. The frequency of *IDH1* and *IDH2* mutations has not yet been established in South Africa.

**Materials and Methods:** Samples from 70 cytogenetically-normal paediatric (n=9) and adult (n=61) AML patients were analyzed for $IDH1^{R132}$, $IDH2^{R140}$ and $IDH2^{R172}$ mutations with singleplex polymerase chain reaction (PCR) and direct sequencing. $IDH1^{R132}$ mutations were further investigated with a mutation-specific, multiplex PCR and agarose gel electrophoresis (AGE). The concurrence of *IDH* mutations with two common AML gene mutations, *NPM1* and *FLT3*-ITD, was also determined using a multiplex PCR and capillary electrophoresis (CE) assay for *NPM1* and *FLT3*-ITD mutations, followed by a separate singleplex PCR and AGE method for *FLT3*-ITD and a mutation-specific, multiplex real-time qualitative PCR (qPCR) for *NPM1*.

**Results:** *IDH1*$^{R132}$ mutations were detected in 5 (8.2%) adult patients and 1 (11.1%) paediatric patient. In adults, *IDH2*$^{R140}$ and *IDH2*$^{R172}$ mutations were detected in 4 (6.6%) patients and 1 (1.6%) patient, respectively. No paediatric patient had *IDH2* mutations. *IDH1* and *IDH2* mutations were heterozygous and mutually exclusive. Concurrent mutations in *NPM1* and *FLT3*-ITD were also detected in patients with mutated *IDH1*$^{R132}$ and *IDH2*$^{R140}$ but not in the patient with the *IDH2*$^{R172}$ mutation.

One sample showed discordant results between the singleplex PCR and sequencing method and the mutation-specific, multiplex *IDH1*$^{R132}$ PCR. The multiplex PCR and CE assay for *NPM1* and *FLT3*-ITD mutations was more accurate than the separate mutation-specific, multiplex real-time qPCR for *NPM1* mutations and the singleplex PCR and AGE method for *FLT3*-ITDs. For each of these, one discordant result was seen between these techniques.

**Conclusion:** Overall, 16.4% of adult and 11.1% of paediatric cytogenetically-normal AML patients had *IDH* gene mutations. Multiplex PCR and CE is the preferred method in testing for *NPM1* and *FLT3*-ITD mutations. Although the role of *IDH* mutations in AML is currently unknown, the mutation-specific, multiplex *IDH1*$^{R132}$ PCR offers a suitable and faster alternative to sequencing should these markers be used diagnostically in the future.

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# List of Abbreviations

| | |
|---|---|
| 2HG | 2-hydroxyglutarate |
| A | Adenine |
| *A* | African |
| ABI | Applied Biosystems |
| AGE | agarose gel electrophoresis |
| AML | Acute Myeloid Leukaemia |
| α-KG | alpha ketoglutarate |
| APL | Acute Promyelocytic Leukaemia |
| AR | allelic ratio |
| ASXL1 | Additional sex combs like 1 |
| BAALC | Brain and Acute Leukemia Cytoplasmic |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pairs |
| *CEBPA* | CCAAT/enhancer-binding protein alpha |
| CE | capillary electrophoresis |
| C | Cytosine |
| *C* | Caucasian |
| C | Cysteine |
| CN | cytogenetically-Normal |
| dbSNP | database of Single Nucleotide Polymorphisms |

| | |
|---|---|
| $ddH_20$ | deionized double distilled water |
| DNA | Deoxyribonucleic acid |
| DNMT3A | DNA methyltransferase 3A |
| dNTP | deoxyribonucleotide triphosphate |
| et al. | and others |
| 6-FAM | 6–carboxyfluorescein |
| *FLT3* | FMS related Tyrosine Kinase 3 |
| F | female |
| *F* | forward |
| FAB | French-American-British |
| Fig | figure |
| G | Guanine |
| G | Glycine |
| *GAPDH* | Glyceraldehyde 3-phosphate Dehydrogenase |
| H | Histidine |
| HEX | 6-carboxy-2 ,4,4 ,5 ,7,7 -hexachlorofluoresceinsuccinimidyl ester |
| HIDI | highly deionized Formamide |
| HIF1-α | hypoxia-inducible factor 1, alpha subunit |
| *IDH1* | Isocitrate Dehydrogenase |
| *IDH1/2* | Isocitrate Dehydrogenase 1/2 |
| inv | inversion |

| | |
|---|---|
| ITD | Internal tandem duplication |
| K | Lysine |
| KDM2a | lysine-specific demethylase 2A |
| KIT | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog |
| KRAS | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| L | leucine |
| M | male |
| MA | Mixed ancestry |
| MDS | Myelodysplastic syndrome |
| MLL-PTD | Mixed Leukaemia Gene Partial tandem duplication |
| MRD | minimal residual disease |
| mut | mutated |
| NCBI | National Center for Biotechnology Information |
| NED™ | 2,7',8'-benzo-5'-fluoro-2',4,7,-trichloro-5-carboxyfluorescein |
| NHLS | National Health Laboratory Service |
| *NPM1* | Nucleophosmin 1 |
| NRAS | Neuroblastoma RAS viral oncogene homolog |
| n | number |
| ND | not done |
| neg | negative |
| NS | not stated |

| | |
|---|---|
| NTC | non template control |
| OD | optical density |
| P13K | Phosphatidylinositide 3-kinases |
| p | short arm of chromosome |
| PTEN | Phosphatase and tensin homolog |
| PHF6 | plant homeodomain finger 6 |
| PCR | polymerase chain reaction |
| Q | Glutamine |
| q | long arm of chromosome |
| qPCR | qualitative PCR |
| *R* | reverse |
| R | Arginine |
| RAS/MAPK | Ras/mitogen activated protein kinase |
| ref | reference |
| RFU | relative fluorescence units |
| ROX | 6-carboxy-X-rhodamine |
| RUNX1 | runt-related transcription factor 1 |
| SNP | Single Nucleotide Polymorphism |
| STAT5 | signal transducer and activator of transcription 5 |
| T | Thymine |
| t | translocation |

| | |
|---|---|
| *TET* | Ten-Eleven-Translocation |
| TP53 | Tumor Protein 53 |
| U | unknown |
| UCT MCB | University of Cape Town Molecular and Cellular Biology Department |
| UNG | uracil *N*-glycosylase |
| UPN | unique patient number |
| *VEGF* | vascular endothelial growth factor |
| *WHO* | World Health Organization |
| *wt* | wild-type |
| *WT1* | Wilms-tumour 1 |

# List of Units and Symbols

| | |
|---|---|
| 5’ | five prime |
| 3’ | three prime |
| °C | degrees Celsius |
| % | percentage |
| kb | kilobase pairs |
| L | litre |
| ml | millilitre |
| mM | millimolar |
| mg | milligram |
| ng | nanograms |
| ng/µl | nanograms per microlitre |
| nM | nanomolar |
| µl | microliters |
| µM | micromolar |
| TM | trademark |
| ® | Registered trademark symbol |
| + | positive |
| - | negative |
| < | less than |
| > | greater than |

# 1. INTRODUCTION

## 1.1 ACUTE MYELOID LEUKAEMIA

Acute Myeloid Leukaemia (AML) is a cancer of the myeloid line of blood cells (Fig 1.1) and is characterized by the accumulation of acquired somatic molecular aberrations and the rapid, uninhibited proliferation of hematopoietic progenitor cells.[1,2] In AML, a differentiation block in early progenitor cells leads to their increased proliferation, enhanced survival and a reduced ability to undergo apoptosis.[3] These immature precursors accumulate in the bone marrow, blood and organs, obstructing the production of normal blood cells.[4] AML is a heterogeneous disease in terms of biological and clinical characteristics and treatment response.[5] Having a multi-step pathogenesis involving several molecular abnormalities, an understanding of the aetiology of AML will allow for improved diagnosis, prognosis and development of novel therapies in patients affected by this aggressive disease.[4]

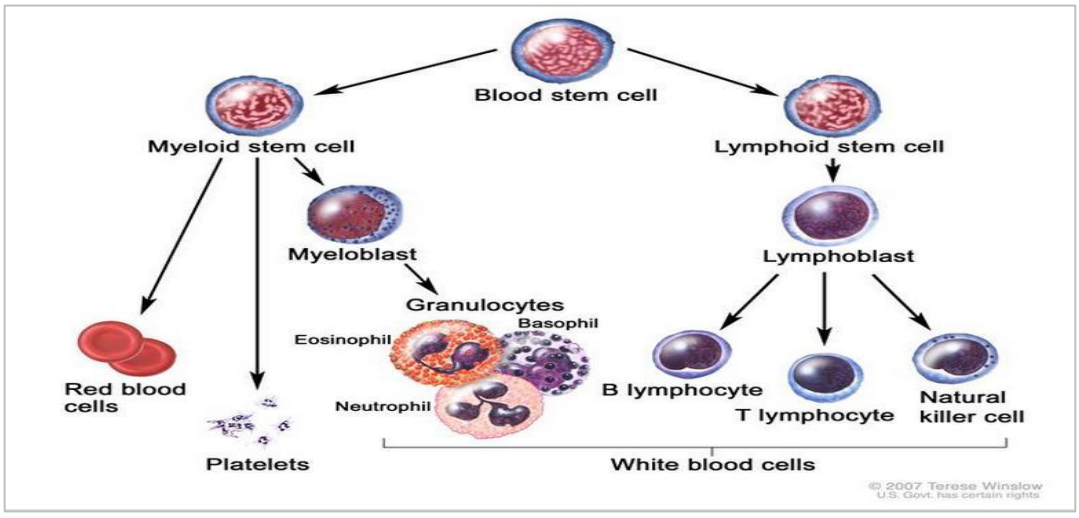



Figure 1.1 Blood cell lineage showing the Myeloid and Lymphoid pathways of Hematopoiesis. Acute Myeloid Leukaemia arises when a stem cell of the myeloid lineage is affected. Picture adapted from original.[6]

### 1.1.1 Epidemiology

In the Western population, AML has an incidence of 3.49/100 000 persons and is more prevalent in patients 65 years or older.[7] Five-year relative survival rates are 5.4% in patients ≥65 years old and 41.6% in patients <65 years old.[7] However, in the developing world there is a severe deficiency of statistics regarding the incidence and survival rates of AML. The first-world data cannot be applied to the third-world, as there are a number of environmental and genetic risk factors that can influence cancer rates in Africa. These include socio-economic status, living conditions, infectious and non-infectious diseases, extreme environments and a rural-to-urban transition.[8] Also, african populations in particular have great genetic diversity and considering AML is a complex genetic disease, this may also have an impact on leukaemia aetiology.[9]

Unfortunately, there is a lack of available data regarding the exact incidence and prevalence of AML in South Africa. The latest cancer registry available is from 2004, which includes both the chronic and acute leukaemias and combines both the lymphoblastic and myeloid lineages.[10] Other available data pertains to other African countries and is relatively old and also not specific to AML.[11,12] As such, the exact incidence and survival outcomes of AML in South Africa remains unknown.

### 1.1.2 Clinical Symptoms, Diagnosis and Treatment of AML

AML is an aggressive disease, which progresses rapidly and if left untreated, has a high fatality rate.[2,4] AML can arise *de novo* or secondary, due to history of a previous haematological disease or prior treatment with chemotherapy.[4] Common symptoms of AML are anaemia, fatigue, shortness of

breath, bruising and persistent or frequent infections.[4] A diagnosis of AML is made when testing a bone marrow sample indicates the presence of at least 20% of blasts.[4] The first-line treatment is chemotherapy with a combination of an anthracycline and cytarabine, which aims to initially reduce, and then eliminate the leukaemic cells in the patient.[13] However, despite the use of chemotherapy and even stem cell transplantation, the majority of patients will still demise from the disease.[14,15] Therefore, a better understanding of the effect of chromosomal and genetic abnormalities on the disease cells may possibly lead to the development of novel therapies thereby improving cure rates.[4]

### 1.1.3 Conventional Cytogenetics in AML

The advent of cytogenetics in the 1950's allowed for the further categorization and understanding of AML. From the initial discovery of the first cytogenetic aberrations associated with AML, over 200 different structural and numerical abnormalities have been identified to date.[16] Even today, cytogenetic findings at diagnosis are viewed as giving the single most valuable prognostic information in AML.[17] Non-random clonal aberrations e.g. insertions, deletions and translocations, are generally detectable in the leukaemic blasts in the majority of patients diagnosed with AML.[16] These not only identify biologically distinct subsets in AML, but also allow for patient risk-stratifications and adapted treatment approaches.[18] However, despite advances in cytogenetics and the introduction of molecular cytogenetic techniques e.g. fluorescent *in situ* hybridisation, a large proportion of patients still show a normal karyotype and are defined as cytogenetically-normal AML (CN-AML).[16,19]

### 1.1.4 Classification and Prognosis in AML

Two systems have been employed in the classification of AML. The French-American-British (FAB) classification system was proposed by Bennet et al.[20] in 1976 and is based on the cell morphology and degree of differentiation observed. The FAB system divides AML into 8 subgroups (M0-M7) using blast count, specific lineage and level of differentiation of the leukaemic cells as the main determining factors.[20] However, due to the heterogeneity of AML, even within the specific FAB subtypes there is a high degree of prognostic variation.[21] The second classification system, the World Health Organization (WHO) system, is a more recent classification scheme, which has more subtypes and is more inclusive.[22] However, the FAB system is still primarily used in the clinical setting.

Current prognostic indicators used in AML at diagnosis include age, cytogenetic findings, white-cell count and the occurrence of a precursor haematologic disorder.[23] Primarily, the major contributor in determining prognosis is the presence or absence of specific cytogenetic aberrations which are categorized into 3 subgroups: favourable, intermediate and poor risk (Table 1.1).[24-26] Favourable cytogenetic aberrations include inv(16), t(15;17) and t(8;21), while monosomy 5 or 7, 3q abnormalities, del(5q), t(6;9), t(6;11), t(11;19) and complex karyotypes (>3 aberrations) reflect a poor prognosis. The intermediate risk group includes t(9;11), monosomy Y, trisomy of chromosomes 8, 11, 13 or 21 and deletions of 7q, 9q, 11q or 20q.[24-26] With CN-AML patients having worse complete remission rates, relapse risk and overall survival rates than patients with favourable aberrations but better than those who have unfavourable aberrations, they are classified in the intermediate risk group.[24-26] However, a wide range of survival outcomes are displayed, with 5-year survival rates between 24% and 42%.[27,28] These different outcomes in CN-AML are most likely due to its

heterogeneous nature at the molecular level.[1] Consequently, there is a need for identifying ways to further define this group.

Table 1.1 Prognostic subgroups in AML and their cytogenetic association.

| Prognostic Subgroup | Cytogenetics |
|---|---|
| Favourable | t(15;17)(q22;q12–21)<br>t(8;21)(q22;q22)<br>inv(16)(p13q22)/t(16;16)(p13;q22) |
| Intermediate | Normal karyotype<br>t(9;11)<br>del(7q)/del(9q)/del(11q)/del(20q)<br>trisomy 8/11/13/21<br>monosomy Y |
| Unfavourable | inv(3)(q21q26)/ t(3;3)(q21;q26)<br>monosomy 5/7<br>del(5q)<br>t(6;9)/t(6;11)<br>t(11;19)<br>complex karyotype (>3 aberrations) |

### 1.1.5 CN-AML

In approximately 40-49% of adult AML patients no detectable chromosomal abnormalities can be found on standard cytogenetic analysis.[24-26] CN-AML constitute the single largest cytogenetic group in this disease (Fig 1.2). In paediatric AML patients, the frequency of normal cytogenetics is lower, with about 25% showing a normal karyotype.[16,26,27,29,30] The reasons for this discrepancy are not known, however, could be due to the differences in AML disease biology between paediatric and adult AML. As cytogenetic findings at diagnosis are the most valuable independent prognostic factor, this presents difficulties in determining outcomes in this group of AML patients.

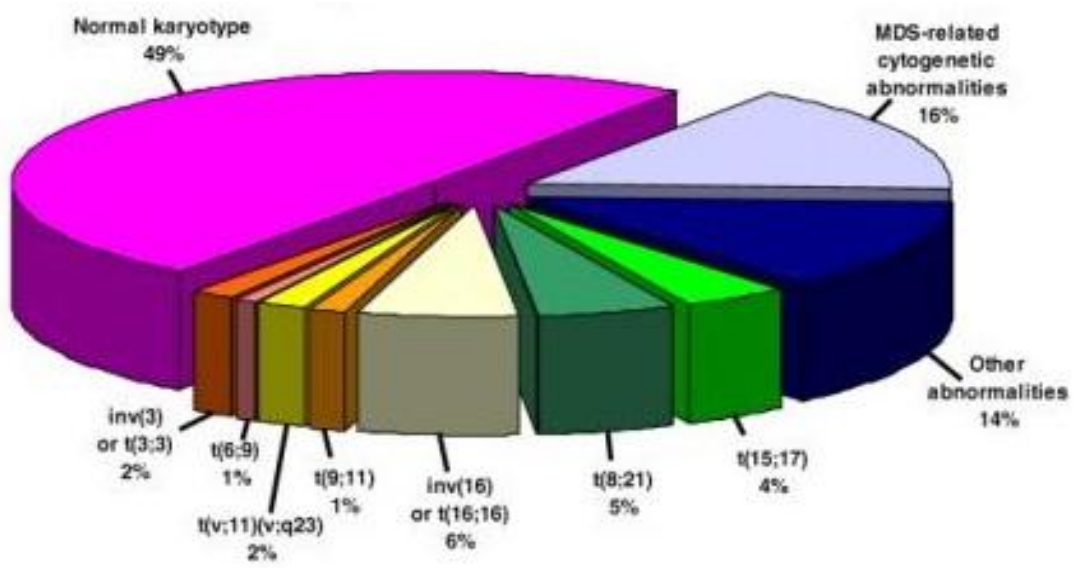



Figure 1.2 Pie-chart showing distribution of the cytogenetic aberrations and subgroups in 2363 *de novo* adult AML patients. AML with a normal karyotype comprises almost half of all AML cases in comparison to the other subgroups. t(15;17), t(8;21), inv(16)/t(16;16) represent favourable cytogenetic abnormalities. Rearrangements involving chromosomes 3 and 11 and t(6;9) are unfavourable cytogenetic abnormalities, MDS =myelodysplastic syndrome, t=translocation, inv=inversion. Picture taken from Kayser et al.[31]

### 1.1.6 Identification of Molecular Markers with Prognostic Significance

Over the past 10 years an increasing number of molecular markers have been identified that can contribute in improving individual risk assessments and determining the prognosis of AML patients, particularly those with normal cytogenetics. These molecular markers can be either a gene mutation e.g. the Nucleophosmin (*NPM1*) gene[32] and the FMS-like tyrosine kinase 3 (*FLT3*) gene[33] or they can occur as a deregulation of gene expression e.g. over-expression of the Brain and Acute Leukemia Cytoplasmic (*BAALC*) gene[34] and non-coding RNA (microRNA).[35] The presence or absence of these markers has been linked with either a favourable or unfavourable outcome and as they are not mutually exclusive, combinations of them may further define prognostic risk.[1,21]

With the development of AML thought to be a multi-step process where different genetic mutations contribute to leukaemogenesis, a 2-hit model has been hypothesized where the haematopoietic progenitor cell acquires two classes of mutations affecting cell survival, proliferation and differentiation.[36] Class 1 mutations (e.g. *FLT3*) activate signal transduction pathways affecting the proliferation and/or survival of precursor cells. These occur late and are linked with AML progression.[37] Class II mutations (e.g. *NPM1*) occur early during leukaemogenesis, interfere with transcription and cause a block in cell differentiation.[37] As the involvement of these different genetic mutations in the molecular aetiology of AML is determined, their value in patient risk stratifications and even as future targets for molecular-based therapies may be important.

One of the latest molecular markers to be identified in AML are mutations in the isocitrate dehydrogenase (*IDH*) 1 and 2 genes.[38,39] *IDH* mutations occur most frequently in CN-AML and may represent a new class of molecular mutations involved in the aetiology of AML.

## 1.2 ISOCITRATE DEHYDROGENASE 1 AND 2 GENES

Isocitrate dehydrogenases (IDHs) are enzymes that catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) and reduce nicotinamide adenine dinucleotide phosphate (NADP) to NADPH.[40] These are important steps in the regulation of cellular redox homeostasis, lipid synthesis and oxidative respiration.[40] Three classes of IDH enzymes, with different sub-cellular localizations, exist in mammals.[41] *IDH1*, located on chromosome 2q33.3, encodes a NADP-dependent isocitrate dehydrogenase located in the cytoplasm and peroxisomes.[42,43] *IDH2*, situated at 15q26.1, also

encodes an NADP-dependent IDH localized, however, to the mitochondria.[41,44] The IDH3 protein is a mitochondrial NADP-dependent enzyme consisting of alpha, beta and gamma subunits, which are encoded by genes located on chromosomes 15q25.1-q25.2, 20p13 and Xq28, respectively. No *IDH3* mutations have been detected to date.[40,45-47]

Mutations in *IDH1* were first discovered to be a common occurrence in gliomas, the most common type of primary brain tumours.[48] *IDH2* mutations have also been found in this disease but at a lower frequency.[48] All *IDH* mutations impair normal enzymatic IDH activity and are associated with a specific glioma CpG island methylator phenotype which is characterized by extensive hypermethylated loci. This phenotype is prevalent in low-grade gliomas, patients of younger age and confers a favourable prognostic outcome.[49]

### 1.2.1 *IDH* mutations in AML

In 2009, Mardis et al.[38] performed whole genome sequencing of a *de novo* AML patient with a normal karyotype, identifying a mutation in codon 132 of *IDH1* causing a change in the amino acid arginine (R). They screened a further 187 AML patients, of whom 8% had an *IDH1*$^{R132}$ mutation. Subsequently, Marcucci et al.[39] reported on two mutations in *IDH2* in codons 140 and 172 in CN-AML patients. Although *IDH2*$^{R172}$ mutations were previously found in gliomas, the *IDH2*$^{R140}$ mutation was a novel finding which had not been previously detected in tumor or normal tissue.[39,48] Since then, a number of studies have investigated *IDH* mutations in AML. To date, all reported mutations are heterozygous and single amino acid substitutions of arginine.[40,50] *IDH1* and *IDH2* mutations themselves are also largely exclusive with one another, suggesting a related biological effect.[50]

### 1.2.2 Role of *IDH* mutations in Leukaemogenesis

Proper IDH function is essential for normal cell growth and proliferation.[51] Until the discovery of *IDH* mutations in AML, no metabolic enzyme had previously been identified as being mutated in this disease.[52] All *IDH* mutations occur at residues in the active site of the enzyme and are involved with isocitrate binding.[53] In vitro enzymatic analysis showed that *IDH* mutations impair the enzyme's affinity for isocitrate and increase their affinity for NADPH and α-KG.[48,54-57] This impedes the normal oxidative carboxylation of isocitrate to α-KG and results in the gain of a neomorphic enzyme activity where α-KG is converted into 2-hydroxyglutarate (2HG).[52,54,55] 2HG is a metabolite normally present in cells at low levels.[54] In *IDH*-mutated AML patients, 2HG levels were shown to be 100-fold higher than non-IDH mutated patients.[54,56,57] 2HG is homologous to α-KG and is implicated in having an oncogenic effect by competitively inhibiting α-KG dependent enzymes such as prolyl hydroxylases (PHDs) and chromatin-modyifing enzymes e.g. histone demethylases and TET hydroxymethylases (Fig 1.3).[40,51,54,59] TET enzymes appear to be involved in the differentiation of myeloid cells.[60] They require α-KG to convert 5-methylcytosine to 5-hydroxymethylcytosine, which inhibits the re-methylation of cytosine by DNA methyltransferases during cell division.[60,61] 2HG also stabilizes hypoxia-inducible factor (HIF-1α) which has a role in angiogenesis, resulting in increased expression of vascular endothelial growth factor (VEGF), a driver of angiogenesis in cancer.[62]

*IDH1* and *IDH2* mutations display global DNA hypermethylation, disrupt *TET2* function and have a specific hypermethylation signature.[51] Although likely linked to the down-regulation of α-KG, which is required by multiple enzymes for proper functioning, the mechanism of how *IDH* mutations contribute to hypermethylation and leukaemogenesis remains to be clarified.[52] With the 2-hit model of AML leukaemogenesis involving two classes of mutations, the discovery of alterations in enzyme

encoding genes, such as *IDH1* and *IDH2,* may represent a new class of mutations involving epigenetic modifiers, adding yet another factor to the already complex pathogenesis of AML.[37,50]

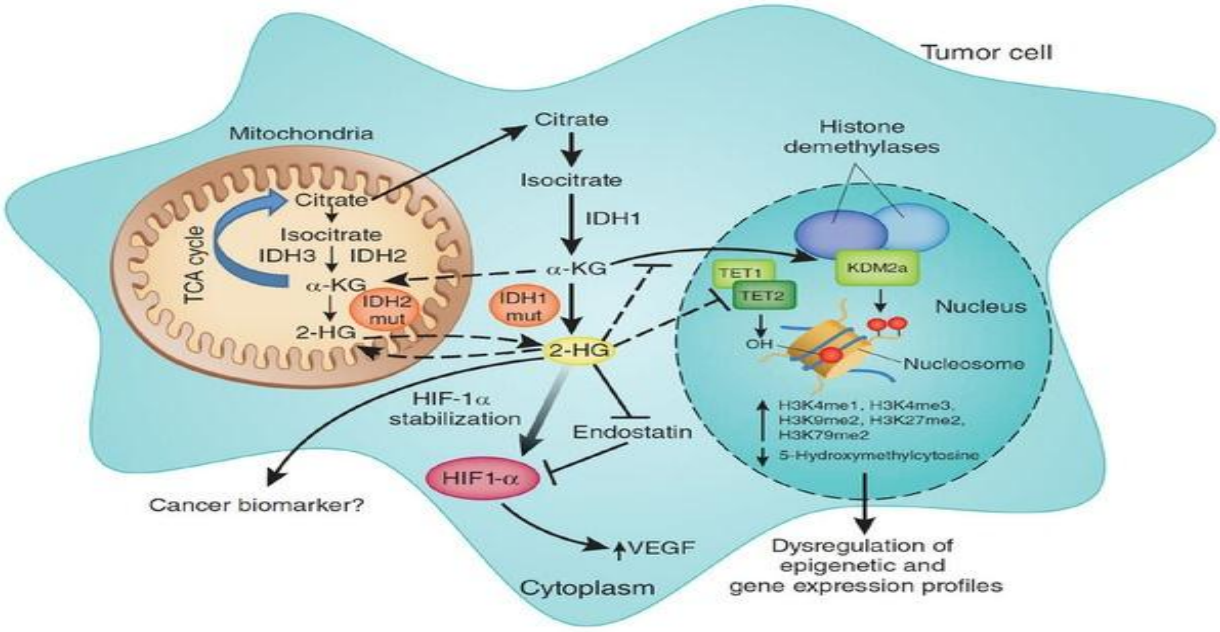



Figure 1.3 The role of mutant *IDH* activity in tumour cells. Mitochondrial tricarboxylic acid (TCA) cycle enzymes and location of the 3 isocitrate dehydrogenases are shown. Mutant *IDH1* and *IDH2* enzymes have a neomorphic activity where α-KG is converted into the oncometabolite, 2HG, affecting further pathways. 2HG inhibits α-KG binding to several histone demethylases (including KDM2a) resulting in an aberrant histone modification profile. TET1 and TET2 hydroxymethylases, which normally catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, are also inhibited. These dysregulatory epigenetic effects may cause aberrant gene expression profiles. 2HG also stabilizes HIF1-α by partly decreasing the levels of endostatin, a HIF1-α antagonist, resulting in elevated *VEGF* signaling, which is a key mediator in angiogenesis in cancer. *IDH1/2/3*=isocitrate dehydrogenase 1/2/3, 2HG=2-hydroxyglutarate, α-KG=alpha ketoglutarate, HIF1-α=hypoxia-inducible factor 1, alpha subunit, *TET1/2*= Ten-Eleven-Translocation 1/2, *VEGF*=vascular endothelial growth factor, KDM2a=lysine-specific demethylase 2A. Picture taken from Prensner and Chinnaiyan.[62]

### 1.2.3 Mutations in *IDH1*

In *IDH1*, the majority of mutations are located in exon 4 and involve codon 132 (CGT) which encodes for arginine.[40] To date, five amino acid changes involving the replacement of arginine with another

amino acid have been reported: cysteine (R132C), histidine (R132H), leucine (R132L), glycine (R132G) and serine (R132S).[39,63-65] Of these, R132C and R132H are the most common *IDH1* mutations in AML.[39,63,66]

*IDH1* has a role in cellular metabolism and is involved with lipid metabolism and glucose sensing.[40] It is one of only three cytosolic enzymes which contribute to NADPH production, essential for nucleotide and lipid synthesis.[67] *IDH1* mutations reduce the affinity for isocitrate and cause a dominant-negative inhibition of the wild-type enzyme, with gain of a neomorphic enzyme activity where α-KG is converted into 2HG.[48,54-57] The accumulation of 2HG leads to malignant progression of *IDH1*-mutated leukaemic cells.[54] Although different *IDH1*$^{R132}$ mutations exist, it is most likely the replacement of arginine itself, rather than with which specific amino acid that causes the leukaemic effect, since the impaired isocitrate binding is independent of the type of mutation present.[50]

### 1.2.4 Mutations in the *IDH2* Gene

In *IDH2,* mutations in codons 140 (CGG) and 172 (AGG) of exon 4, both of which encode for arginine, have also been identified.[39] For *IDH2*$^{R140}$ mutations, 3 amino acid substitutions are found, where the replacement of arginine with glutamine (R140Q) occurs predominantly.[39,63,68,69] Rarer substitutions of arginine with leucine (R140L) or tryptophan (R140W) have also been identified.[39] For *IDH2*$^{R172}$, the main substitution for arginine is with lysine (R172K).[39,63,66,68-71] Atypical substitutions are with methionine (R172M).[72] The *IDH2*$^{R172}$ mutation is also found in glioma patients, however, the *IDH2*$^{R140}$ mutation appears to be exclusive to AML.[39,48]

*IDH2* has an important role in regulating the tricarboxylic acid (TCA) cycle in multiple tissues.[40] Mutations in *IDH2* cause the same effect as that seen with *IDH1* mutations.[55,57] From structural modeling, the *IDH2*$^{R172}$ residue is analogous to *IDH1*$^{R132}$, which stabilizes the β-carboxyl of isocitrate (Fig 1.4).[57] Likewise, *IDH2*$^{R140}$ corresponds to residue 100 in *IDH1* and also binds directly to isocitrate. Although mutants have a reduced ability to bind isocitrate, their ability to bind and orient α-KG in the active site is still preserved.[57]

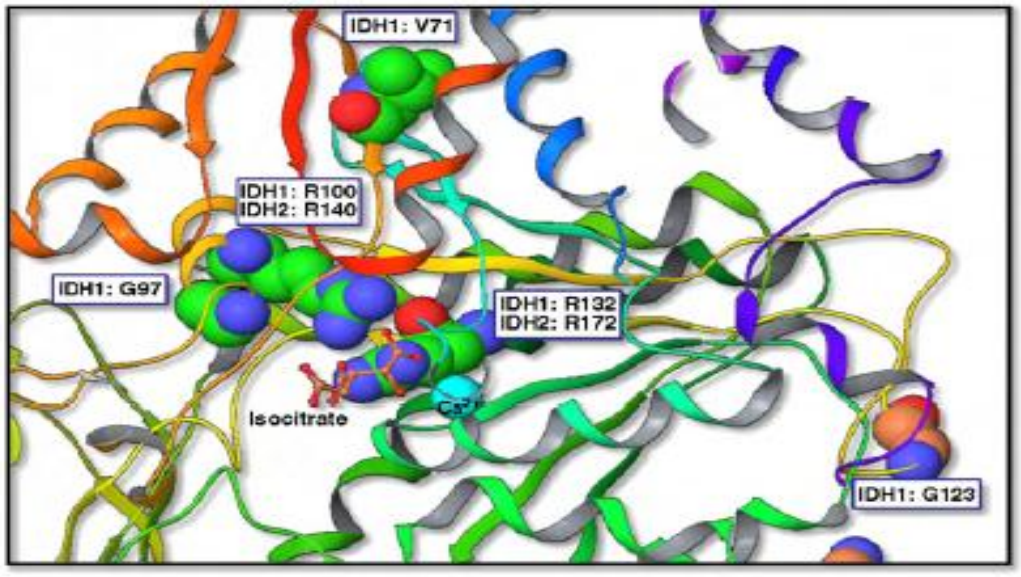



Figure 1.4 The active site of the IDH enzyme showing the locations of the *IDH1* and *IDH2* residues where mutations occur. *IDH1* R132 is analogous to *IDH2* R172 while *IDH2* R140 is analogous to *IDH1* R100. Image taken from Dang et al.[73]

### 1.2.5 Stability of *IDH* Mutations

*IDH1* and *IDH2* mutations appear to be stable. Patients without *IDH* mutations at diagnosis do not acquire these at disease relapse, while in *IDH* mutated patients, mutations disappear at complete remission and reappear at relapse.[64,74] Therefore, *IDH* mutations may be potential markers for

monitoring disease, testing for minimal residual disease (MRD) and predicting relapse. This stability also implicates *IDH* mutations as potential primary events involved in leukaemogenesis.[50]

### 1.2.6 Frequency of *IDH* mutations in CN-AML

Although *IDH* mutations have been shown to occur in AML patients with chromosomal aberrations,[74,75] like the other molecular markers identified in AML, their increased association with a normal karyotype has been firmly established.

Schinittger et al.[74] had the largest AML cohort of 1414 adult patients and showed *IDH1*$^{R132}$ mutations to occur in 93 (6.6%) patients. Of the 93 *IDH1*$^{R132}$ mutated patients, 67 (72%) had a normal karyotype. Similarly, Chou et al.[75] found in a cohort of 446 *de novo* adult AML patients that 20 of the 27 (74%) *IDH1*$^{R132}$ mutated patients had a normal karyotype. A clear association was also shown by Boissel et al.[70] who investigated 520 adult *de novo* AML patients and found 34/50 (68%) *IDH1*$^{R132}$ mutated patients had a normal karyotype. Other studies have also confirmed this association of *IDH1*$^{R132}$ mutations with CN-AML.[63,66,69,72,76,77]

*IDH2*$^{R140}$ mutations are also associated with CN-AML. Green et al.[71] studied 1473 adult AML patients and found 119 (8%) to have an *IDH2*$^{R140}$ mutation. Of these mutated patients, 68 (57%) had a normal karyotype. Similarly, Nomdedeu et al.[63] studied 275 adult AML patients and found 10 (56%) of the 18 *IDH2*$^{R140}$ mutated patients to have a normal karyotype. Although few *IDH2*$^{R172}$ mutations have been identified to date in AML patients, their association with CN-AML is still evident. Boissel et al.[70] in the

same above cohort found 12/15 (80%) *IDH2*$^{R172}$ mutated patients were CN-AML. Also, Patel et al.[66] found 4 *IDH2*$^{R172}$ in in 199 adult AML patients, of which all mutations were in patients with a normal karyotype. As such, like *IDH1* mutations, *IDH2* mutations show more of an association with CN-AML than AML with abnormal karyotypes.

The overall reported frequencies for *IDH* mutations in CN-AML are 5.4-16% for *IDH1*$^{R132}$ mutations, 6-15.6% for *IDH2*$^{R140}$ mutations and 1.1-5.9% for *IDH2*$^{R172}$ mutations.[38,39,63-66,68-72,74-79] While these reported frequencies of *IDH1* and *IDH2* mutations vary between studies, this may represent true differences or alternatively may be due to different inclusion criteria between the study patients e.g. *de novo* vs. secondary AML and/or selective testing for *IDH* mutations e.g. *IDH2*$^{R140}$ vs. *IDH2*$^{R172}$ alone.[65,66,70,71] Since *IDH* mutations are more common in CN-AML, when a cohort comprising all AML subtypes is tested, the overall frequencies of *IDH* mutations are expected to be lower than when a cohort of exclusively CN-AML is tested.[39,70,72,74]

### 1.2.7 Prognostic Influence and Clinical Characteristics

In patients with gliomas, *IDH* mutations are known to confer a favourable prognosis.[48] However, in AML survival data is variable with *IDH* mutations generally shown to have either no impact,[64,66,69,79,80] an adverse impact[39,57,63,68,70,72,74] or even a favourable prognostic effect[71,75,78] on patient's treatment response and survival. Furthermore, this impact can vary when the results are stratified between cytogenetic findings, the specific *IDH* mutation and when concurrent genetic mutations are analysed simultaneously.[39,68,71,77]

Differences in the association of *IDH* mutations with age, platelet counts, FAB subtypes and gender at diagnosis when compared with *IDH* wild-type patients has been shown.[39,64,66,68,70,72,74] However, these, and prognostic differences, could also potentially be attributed to: (1) variations in the sizes of patient cohorts analyzed, (2) inclusion of *de novo* vs. secondary AML, (3) the age of patients studied - younger adults <60 years vs. older adults ≥60 years (who generally have poorer outcomes), (4) patient ethnicities (variation in different population groups exists) and (5) treatment strategies (standard vs. aggressive).[39,69]

Even the disease profiles of the two types of *IDH2* mutations varies, suggesting diverse biological contributions of the R140 and R172 mutations to leukaemogenesis.[39,71] Marcucci et al.[39] showed that in older *IDH2*-mutated AML patients, those with the R172 but not the R140 mutation, had complete remission rates lower than that of *IDH* wild-type patients. Subsequently, other studies showed *IDH2*$^{R172}$ was associated with a much worse impact than *IDH2*$^{R140}$ with the latter actually conferring a favourable outcome.[71,78] In CN-AML, *IDH2*$^{R172}$ is associated with a unique gene and microRNA expression profile.[39] This suggests a distinct biological and clinical contribution of this mutation in affected patients. As such, *IDH2* mutations should always be evaluated separately.

A recent meta-analysis of *IDH1* mutations was performed by Feng et al.[81] evaluating 15 studies covering a total of 8121 AML patients. *IDH1* mutations were shown to be associated with a poor overall survival in AML patients, but no impact on complete remission rates was seen. This poor survival may not then be due to death during induction or induction failure. Interestingly, in CN-AML,

only a lower complete remission rate was seen. These differences suggest that the clinical impact of genetic aberrations may be related to the biological context in which they are assessed. Also, other collaborating factors e.g. cytogenetic aberrations (as present in non CN-AML) may contribute to leukaemogenesis rather than simply the presence or absence of the *IDH1* mutation itself.[81]

Although the prognostic impact of *IDH* mutations in AML remains to be established, the favourable outcome seen in *IDH*-mutated glioma patients implies a disease-related effect. Like other molecular markers, their role in the leukaemogenesis of AML has not yet been fully elucidated. Nevertheless, *IDH1* and *IDH2* mutations remain valuable markers for further clarifying the molecular basis of this disease.

### 1.2.8 Impact of *IDH1* SNP rs11554137

Previously, Damm et al.[82] had shown that in the Wilms-tumour 1 (*WT1*) gene, the presence of a synonymous single nucleotide polymorphism (SNP) in the mutational region of *WT1* predicted a favourable prognosis in CN-AML patients. Wagner et al.[65] then investigated SNP rs11554137, a GGC>GGT substitution in codon 105 of the mutational exon 4 of *IDH1*, to determine the occurrence and any influence of this SNP on prognosis. They found SNP rs11554137 to occur at a prevalence of 12% in 275 CN-AML patients and in 11.7% of 120 healthy volunteers.[65] Later studies from America and Europe showed a similar incidence of SNP rs11554137 in their AML populations but did not report on frequencies in healthy volunteers.[76,80,83,84] However, Asian populations show a small frequency of SNP rs11554137 in their cohorts (in 1.5% of 68 AML patients and none in 270 healthy controls), which may be reflective of an ethnic association.[85]

Wagner et al.[65] showed a negative prognostic influence associated with the presence of SNP rs11554137 in the absence of *IDH1*$^{R132}$ mutations themselves (only 3/33 patients had both SNP rs11554137 and *IDH1*$^{R132}$ mutations. The presence of SNP rs11554137 in adult CN-AML patients was an independent prognostic factor for overall survival in univariate and multivariate analyses. In patients with SNP rs11554137, the levels of expression of *IDH1* mRNA was double that of wild-type patients, which was suggested to potentially influence *IDH1* protein levels and NADPH metabolism.[65]

In contrast, Ravandi et al.[76] showed that neither SNP rs11554137 alone or in combination with *IDH1*$^{R132}$ mutations had any influence on outcomes in adult AML. Ho et al.[86] evaluated SNP rs11554137 in paediatric and adult AML. No prognostic impact was seen in SNP rs11554137-positive paediatric patients, but in affected adult patients survival rates were significantly worse. However, these adult patients also had other adverse factors e.g. abnormal cytogenetics, older age and concurrent *FLT3*-internal tandem duplication (ITD) mutations, which may have contributed to their poorer outcomes.[74] Another study in childhood AML showed no significant differences between SNP rs11554137 and patient outcomes, which, like Ho et al.[86] may represent true findings or could be due to the variability in disease biology and treatment strategies between adult and childhood AML.[83]

As only a few studies have investigated SNP rs11554137 in AML with variable findings, its prognostic impact is unclear. Only two studies have reported SNP rs11554137 frequency in healthy controls and larger cohorts would have to be investigated for accurate comparisons with AML cohorts. A decreased survival and increased *IDH1* mRNA expression has been demonstrated in SNP rs11554137-

positive adult AML patients. However, this expression would also have to be investigated in healthy individuals to determine if it's specific for AML patients only. An ethnic association of SNP rs11554137 is apparent, and with South Africa having a diverse ethnic population, this warrants further investigations of SNP rs11554137 to determine its prevalence in AML patients within this population.

### 1.2.9 Association of *IDH* mutations with other molecular markers

Other molecular markers have also been found concurrently with *IDH1* and *IDH2* mutations (Fig 1.5).[39,72,74,79] *NPM1* mutations are the most common concurrent genetic aberration in *IDH1*$^{R132}$ and *IDH2*$^{R140}$ mutated cases.[39,63,64-66,70,74,80] Although *FLT3*-ITDs also occur with *IDH* mutations, no significant association has been reported.[39,63,70] Interestingly, the *IDH2*$^{R172}$ mutation itself is virtually mutually exclusive with all known other molecular markers.[39,63,66,70] This may implicate a novel CN-AML subgroup, which has no other identifiable genetic mutations.[50]

In CN-AML, *NPM1* mutations occur concurrently with mutated *IDH1*$^{R132}$ at a frequency of 45-83% and with *IDH2*$^{R140}$ in 25-61% of cases.[39,53,63-66,70,74,75,78,80] *FLT3*-ITDs occur with *IDH1*$^{R132}$ mutations at a frequency of 0-50% and in 17-30% of *IDH2*$^{R140}$-mutated CN-AML patients.[39,53,63-66,70,74,75,78,80] Unlike with *IDH* mutations, the clinical impacts of *NPM1* and *FLT3*-ITD mutations in AML are well characterized. In CN-AML, patients with *FLT3*-ITD mutations have worse survival rates and an inferior prognosis than patients without the ITD.[87,88] In contrast, *NPM1* mutations are a favourable prognostic factor. *NPM1*-mutated patients without concurrent *FLT3*-ITDs (referred to as the low risk genotype *NPM1*$^{mut}$/*FLT3*-ITD$^{neg}$) have significantly improved survival outcomes.[89] The prognostic influence of *IDH* mutations when stratified with *NPM1* and *FLT3*-ITD mutations has been investigated in a number

of studies. In CN-AML with *IDH1*$^{R132}$ and the *NPM1*$^{mut}$/*FLT3*-ITD$^{neg}$ low-risk genotype, a higher risk of relapse and shorter survival has been shown in these patients.[39,68,70,72]

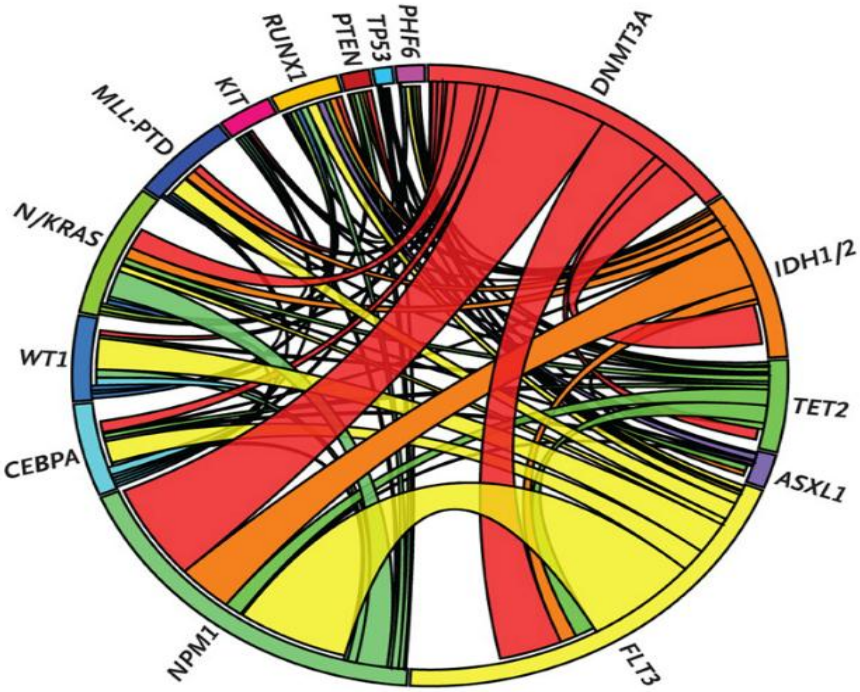



Figure 1.5 A circos diagram showing the frequency and concurrence of molecular mutations in 398 AML patients <60 years old. The arc length corresponds to the frequency of mutations in one gene, while the width of the ribbon corresponds to the percentage of patients with an additional mutation in another gene. Pairwise concurrence is only denoted once, starting with the first gene in a clockwise direction. *NPM1*, *FLT3*, *DNMT3A* (one of the most recently identified genetic markers*)* and *IDH* mutations were the most common markers seen in this study. *IDH1/2* mutations occurred most commonly with *NPM1* mutations. *IDH1*/2=Isocitrate Dehydrogenase 1/2, *FLT3*=FMS-like tyrosine kinase 3, *NPM1*=Nucleophosmin, *CEBPA*=CCAAT/enhancer-binding protein alpha, *WT1*=Wilms-tumour 1, *NRAS*=Neuroblastoma RAS viral oncogene homolog, *KRAS*=v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, *MLL*-PTD=Mixed Leukaemia Gene Partial tandem duplication, *KIT*= v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, *RUNX1*= runt-related transcription factor 1, *PTEN*=Phosphatase and tensin homolog, *TP53*=Tumor Protein 53, *PHF6*=plant homeodomain finger 6, *DNMT3A*=DNA methyltransferase 3A, *TET2*= Ten-Eleven-Translocation 2, *ASXL1*=Additional sex combs like 1. Picture taken from Patel et al. (2012)[90]

Green et al.[77] implicated the presence of a *FLT3*-ITD as a favourable prognostic factor in younger adult *IDH1*-mutated CN-AML. In contrast, the absence of a *FLT3*-ITD in *IDH1*-mutated patients was an adverse factor for relapse. They suggested these differences in prognosis may be associated with the metabolic impact of *IDH1* mutations influencing blast cells chemo-resistance depending on their context.[77] No other study has yet confirmed this favourable finding, with most also demonstrating an association between the *NPM1*$^{mut}$/*FLT3*-ITD$^{neg}$ genotype and a poorer prognosis when an *IDH1* mutation is also present.[39,68,70,72]

Further clinical trials and prospective studies will need to be performed to confirm the clinical impacts of *IDH* mutations in combination with other molecular aberrations. With *NPM1* mutations have a significant association with *IDH* mutations and with the low-risk molecular genotype, *NPM1*$^{mut}$/*FLT3*-ITD$^{neg}$ being linked to adverse outcomes in *IDH1* mutated CN-AML, these three genes should always be investigated concurrently to allow for further risk stratification in these patients.

### 1.2.10 *IDH* mutations in Adult vs. Paediatric AML

The differing molecular pathogenesis in paediatric and adult AML is important when investigating *IDH* mutations. Unlike in adult AML, the frequency of *IDH* mutations in paediatric AML is much lower (0-2.4% for *IDH1* and 0-2.2% for *IDH2* in childhood vs. 4.4-13% for *IDH1* and 10-12.1% for *IDH2* in adult AML).[63,69,71,72,75,80,83,84,91,92] In paediatric AML, *IDH* mutations tend to associate with a normal karyotype, but are also present in cases with translocations of favorable prognosis such as t(8;21) and t(15;17).[83,84,91,92] Similarly to adult AML, *IDH* mutations also occur with *NPM1* and *FLT3*-ITD mutations

in paediatric AML.[83,84] However, the low number of *IDH* mutations identified in paediatric AML patients overall precludes a reliable analysis of their co–occurrence.

Damm et al.[83] showed a favourable prognostic effect for overall survival in childhood AML patients with *IDH* mutations while Andersson et al.[84] did not. This could have been due to the presence of favourable cytogenetic aberrations.[83] Conclusive analyses of outcomes, however, are limited by the low prevalence of *IDH* mutations in this group.

### 1.2.11 Frequency of *IDH* mutations in South Africa

Since 2009, a number of studies have investigated *IDH* mutations in AML patients from Asia, Europe or America.[39,53,63-66,68-72,74-80] To date, no studies have reported on the incidence of *IDH* mutations in South Africa and the frequency of these mutations in the local population remains to be determined. Also, occurring mainly in adult AML, only a few studies have examined paediatric AML patients for *IDH* mutations. Therefore, there is a need to investigate the frequency of *IDH1* and *IDH2* mutations in AML in South Africa together with the common AML molecular markers, *NPM1* and *FLT3*-ITD, which have been shown to associate with *IDH* mutations.

## 1.3 NUCLEOPHOSMIN GENE

### 1.3.1 *NPM1* gene and Protein

The *NPM1* gene encodes for a protein that shuttles continuously between the cytoplasm and nucleolus, with a prominent location in nucleoli.[93] *NPM1* prevents protein aggregation in the

nucleolus and mediates the transport of ribosomal components through the nuclear membrane to the cytoplasm.[94] *NPM1* also controls DNA repair and centrosome duplication, while its interaction with the tumour suppressors, p53 and p14ARF (alternate reading frame), directs cell proliferation and apoptosis.[95,96]

### 1.3.2 Mutations in the *NPM1* gene

The *NPM1* gene is located on chromosome 5q35 and consists of 12 exons coding for a ~37kDa protein.[97,98] Over 40 different *NPM1* mutations have been reported to date, with almost all occurring in exon 12.[93] Mutations are generally a 4-base pair insertion at nucleotide 960 (NM_002520), with rarer insertion/deletion mutations also identified.[32] The most common *NPM1* mutation, type A - a tetranucleotide insertion of TCTG, accounts for 75-80% of all mutations. The next common, types B (tetranucleotide CATG) and D (tetranucleotide CCTG), account for 10% and 5%, respectively.[32,93] Other mutations have been reported (e.g. types C, E, F, G and H), however, these are rare.[32] *NPM1* mutations are heterozygous with the retention of a wild-type allele.[93] Patients without *NPM1* mutations at diagnosis do not acquire these at relapse. Due to their stability, they are suitable markers for MRD monitoring and in predicting disease relapse.[99]

### 1.3.3 *NPM1* mutations and Leukaemogenesis

Independent of their type, all *NPM1* mutations alter tryptophan residues and disrupt the C-terminus of the protein causing a frame-shift in the coding region.[100] This frame-shift results in a novel nuclear export signal and a longer NPM1 protein, which affects its localization to the nucleolus with its aberrant and exclusive expression in the cytoplasm of leukemic cells.[32] Mutant NPM1 proteins also form dimers with and delocalize the wild-type protein into the cytoplasm, causing the mislocalization

and destabilization of the nucleolar p14ARF with the inhibition of the tumor suppressor p53.[95,101] This altered function suggests an oncogenic role of cytoplasmic nucleophosmin critical in malignant transformation.[94] However, the exact mechanism how *NPM1* promotes leukaemogenesis remains to be established.

### 1.3.4 *NPM1* mutations in AML

*NPM1* mutations are highly associated with normal karyotype, *de novo* AML patients who have distinct clinical and biological features.[32,93] They occur in about 25-35% of all adult AML and only in 2.1-6.5% of paediatric AML.[99,102-109] In addition, the mutations seen in paediatric AML patients are typically the uncommon non-A types, in contrast to that seen in adults.[103,108] In adult CN-AML, the frequency of *NPM1* mutations is 45.7-63.8% while in paediatric CN-AML, mutations occur at a frequency of 9-26.9%.[99,102-109] To date, despite the discovery of other AML molecular markers, they remain one of the most frequently occurring genetic mutation in CN-AML (Fig 1.6).

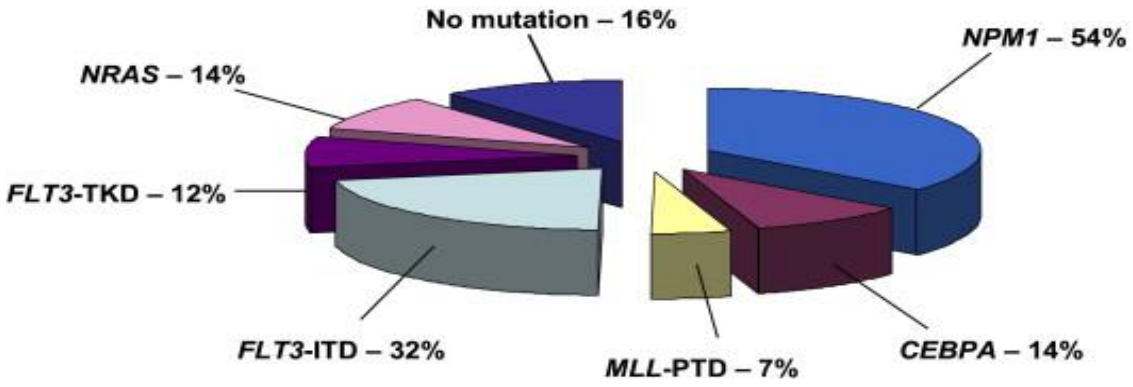



Figure 1.6 Incidence of molecular mutations in 438 CN-AML adult patients <60 years old. *NPM1* is the most common mutation followed by *FLT3*-ITD, *CEBPA*, *NRAS, FLT3*-TKD and *MLL*-PTD. Overlapping mutations are not shown, hence total percentage is >100%. *NPM1*=nucleophosmin 1, *FLT3*=FMS-like tyrosine kinase 3, ITD=internal tandem duplication, TKD=tyrosine kinase domain, *MLL*-PTD, mixed leukaemia gene partial tandem duplication, *CEBPA*=CCAAT/enhancer binding protein, *NRAS*=neuroblastoma RAS viral oncogene homolog. Picture taken from Odenike et al.[110]

Due to its favourable prognostic effect, *NPM1* is as an important molecular marker that should be investigated in AML patients. With its significant association with *IDH* and *FLT3*–ITD mutations in CN-AML,[39,70,72,105-108] these mutations should always be investigated in parallel to allow for a more comprehensive risk stratification for these patients.

## 1.4 FMS-LIKE TYROSINE KINASE 3 GENE

One of the first molecular markers to be identified in AML was an internal tandem duplication of the *FLT3* gene which inserts in the juxtamembrane domain of the receptor (Fig 1.7).[33] Another class of *FLT3* mutations occur, which are substitutions, small deletions or insertions mainly in codons 835 and 836 that affect the activation loop of the second tyrosine kinase domain.[111] Less prevalent are point mutations within the juxtamembrane domain of *FLT3* affecting different amino acid residues.[112]

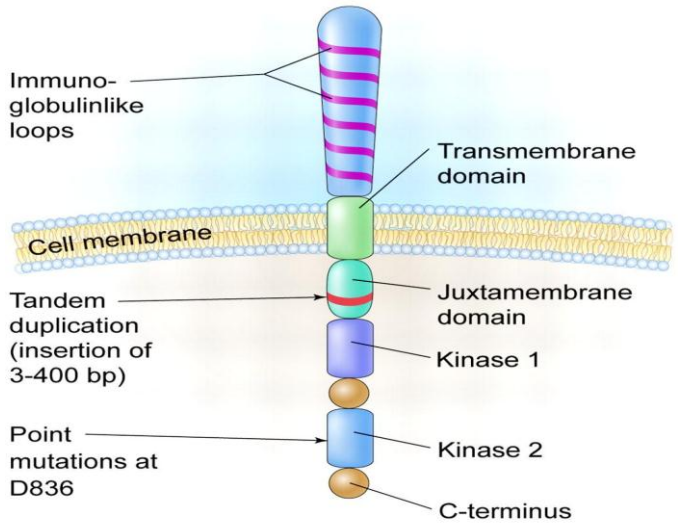



Figure 1.7 The *FLT3* tyrosine kinase receptor. The different domains and locations of the tyrosine kinase point mutations in Kinase 2 and internal tandem duplications in the Juxtamembrane domain are shown. Image by Kenneth Probst, adapted from Litzow.[113]

### 1.4.1 *FLT3* and Internal Tandem Duplications

The *FLT3* gene is located on chromosome 13q12 and consists of 24 exons encoding a membrane bound glycosylated protein.[114] ITD mutations occur between exons 14 and 15.[115] Direct duplications of this region are in a head-to-tail orientation and always maintain the reading frame.[114] The sequence length of the ITD is variable, ranging from as little as 3bp to over 400bp, with its exact location differing between cases.[115]

*FLT3*-ITD mutations are secondary events in leukaemogenesis and show instability. Patients with *FLT3*-ITDs at presentation can lose these at relapse, while patients who were wild-type can gain mutations. ITDs different to that present at diagnosis can also be acquired. As such, FLT3-ITDs have little value in MRD monitoring.[116]

### 1.4.2 Role of *FLT3*-ITD mutations in Leukemogenesis

The *FLT3* gene encodes for a member of the class III receptor tyrosine kinase family.[117] Usually expressed on the surface of early haematopoeitic progenitor cells, it aids in the proliferation, survival and differentiation of multipotent stem cells.[117] *FLT3*-ITD mutations, result in uninhibited activation of its receptor, characterized by ligand independent receptor dimerization and constitutive *FLT3* signaling. This activates the signal transducer and activator of transcription 5 (STAT5) and Ras/mitogen activated protein kinase (RAS/MAPK) and Phosphatidylinositide 3-kinases (P13K) pathways.[118] Consequently, this implicates a role for ITD mutations in the growth of leukaemic cells, further contributing to the pathogenesis of AML.

### 1.4.3 *FLT3*-ITD mutations in AML

*FLT3*-ITDs occur in about 15% of paediatric and 20-30% of adult AML patients.[115,119-122] They have a predominant occurrence in t(15;17)-positive patients as well as *de novo*, CN-AML.[119] Interestingly, in *NPM1*-mutated patients the frequency of *FLT3*-ITDs is almost double that of *NPM1* wild-type patients.[105-108] In adult CN-AML, the frequency of *FLT3*-ITDs is 28-34%, while it is present in ~30-40% of paediatric CN-AML.[104,105,115,119-124]

A significantly inferior prognosis of *FLT3*-ITDs for remission duration and survival rates in CN-AML has been well documented.[104,123,124] Specific characteristics of the ITDs have also been investigated to determine their clinical and biological impact. Investigations into the size of the ITD,[125] the number of duplications,[119] the mutant to wild-type ratio[126] and the level of expression of *FLT3*[127] have been performed to try determine any contribution to a poorer prognosis. Although the influences of these factors remains to be clarified, in CN-AML with *FLT3*-ITDs, a high mutant-to-wild-type ratio has been shown to negatively impact patient's survival.[126]

Due to the significantly worse prognostic effect in CN-AML patients with *FLT3*-ITDs, this is an important molecular marker that should be studied in this group. As they show an increased association with the favourable risk marker, *NPM1*, these two genes should always be investigated in parallel.[105-108] With the inclusion of other genetic mutations, such as *IDH1* and *IDH2*, this will allow for a better understanding of their biological and clinical impacts.

## 1.5 IN REVIEW

AML is a heterogeneous disease, particularly with regards to the varied clinical outcomes associated with cytogenetically-normal patients. Therefore, any biological and genetic insights into its pathophysiology and progression may be significant in elucidating the nature of this disease. In light of the current knowledge of *IDH* mutations and their potential epigenetic role in the leukaemogenesis of AML, they may be important molecular markers in disease development and progression.

Although molecular markers often occur singly and impart their own prognostic influence, it is also recognized that the combined role of two or more markers may exert further prognostic impacts. With the current array of already identified molecular markers, and as novel markers are found, their value in determining clinical outcomes and even serving as potential therapeutic targets may be incalculable. With a future goal of personalized, patient-specific treatment, any contributory genetic factors may be important. As such, investigations into AML patients, particularly those with normal cytogenetics who have variable clinical outcomes should be undertaken, with the aim to clarify the role of these mutations in this disease.

In South Africa, there is no knowledge of the prevalence of many of these molecular markers in AML patients, and studies investigating their frequency will be important in managing these patients when more clinical information is available in the future.

## 1.6 STUDY OBJECTIVES

Since there is currently no data available regarding the prevalence of *IDH* mutations in South African patients with AML, this study aimed to investigate the frequency of these mutations in the population, specifically in AML patients with a normal karyotype. As *IDH* mutations can be found independently as well as concurrently with other genetic mutations, influencing clinical outcomes, their occurrence with two other AML molecular markers, *NPM1* and *FLT3*-ITD, was also investigated.

Therefore, the primary objectives of this project were to:

- Assess *IDH1* and *IDH2* mutation frequency in a cohort of pediatric and adult *de novo* CN-AML presentation patients.

- Determine the incidence of two commonly occurring AML molecular markers in the same cohort and their co-occurrence with *IDH* mutations, namely :

    - *NPM1*, the most common molecular marker in AML, which confers a favourable prognosis and is associated with *IDH* mutations and a normal karyotype.
    - *FLT3*-ITD, also commonly occurring in AML, particularly with *NPM1* mutations, and associates with an unfavourable prognosis and a normal karyotype.

- Evaluate and compare the various testing methodologies used in investigating these mutations in the cohort.

# 2. MATERIALS AND METHODS

## 2.1 ETHICS

Ethics approval for the study was obtained from the institutional review board of the Steve Biko Centre for Bioethics at the University of the Witwatersrand (Ethics clearance number M091022) (Appendix A).

## 2.2 SUBJECTS

A database was compiled of all AML samples received in the Somatic Cell Genetics Unit, National Health Laboratory Service (NHLS) for routine testing over a period of 6 years (2006-2011). From this, samples from 70 CN-AML patients were identified (9 paediatric (≤16 years old) and 61 adult (>16 years old) patients). These were from patients who had no karyotypic abnormalities following routine cytogenetic analysis. Where possible, 20 metaphases had been analysed per case to exclude any clonal abnormalities.

All of the 70 samples were from bone marrow aspirates or peripheral blood specimens from *de novo* AML patients who were at the presentation stage of disease and were treatment naïve. The patient's initial diagnosis of AML was provided by the treating doctor which was based on the clinical symptoms, bone marrow morphology and flow cytometry results. Diagnostic information was obtained from the patient's test request form and the NHLS

laboratory information system. All samples for the study were available retrospectively following routine cytogenetic analysis in the Unit. For negative control samples, peripheral blood was drawn from healthy volunteers.

## 2.3 DNA ISOLATION

Following cytogenetic culture and harvesting of samples for routine karyotypic analysis, the remaining cultured cells were stored in 3:1 methanol:acetic acid fixative at -20$^{o}$C. DNA was extracted from these cultured cells using a standard manual phenol:chloroform technique (complete procedure Appendix B). This technique is superior over commercially-available, column-based extraction kits in that higher DNA yields are generally obtained. The extracted DNA was assessed for quantity (ng/µl) and purity ($OD_{260/280}$) using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA with an $OD_{260/280}$ between 1.8 and 2.0 indicated good purity with no protein contamination. The quality of the DNA was evaluated on a 2% agarose gel (Bioline, London, UK) to assess for degradation. Dilutions of 100ng/µl of DNA were prepared with dd$H_2$0 and stored at -70$^{o}$C.

## 2.4 DETECTION OF *IDH1* AND *IDH2* MUTATIONS

To detect *IDH1* and *IDH2* mutations in the cohort, samples were tested using a singleplex PCR followed by Sanger sequencing. To verify these results and evaluate the use of a more sensitive alternative technique, a mutation-specific, multiplex PCR to detect *IDH1*$^{R132}$

mutations was performed.[128] For *IDH2*, since only one type of mutation from each codon was detected with sequencing, it was unnecessary to design and optimize an *IDH2* mutation-specific multiplex PCR. The steps undertaken in testing for *IDH1* and *IDH2* mutations in the cohort are shown in the flow-diagram below (Fig 2.1).

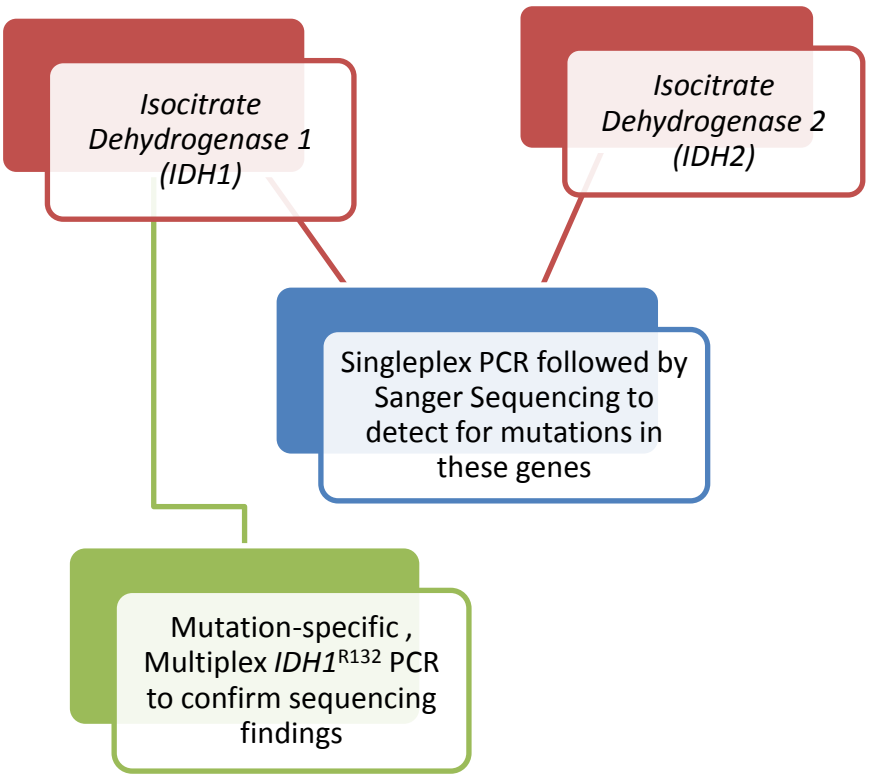



Figure 2.1 Flow-Diagram showing the steps followed in testing for *IDH1* and *IDH2* Mutations in the 70 CN-AML samples

### 2.4.1 Singleplex PCR and Sanger Sequencing of *IDH1* and *IDH2*

A singleplex PCR was used to amplify the exons where point mutations were expected to occur in the *IDH1* and *IDH2* genes. Exon 4 of *IDH1* (which included mutation codon R132 and SNP rs11554137) was amplified with intronic primers incorporating the entire exon.[39] For

*IDH2*, as codon R172 is situated in close proximity to the 3' end of the exon which could be problematic to sequence using published primers, novel primers were designed. With these, the amplified region included the whole of exon 4 which had the R140 and R172 mutation codons. Primer3 software[129] was used to design the primers and primer-BLAST[130] confirmed that they were specific for the *IDH2* gene. *IDH1* and *IDH2* primer sequences are given in Table 2.1 and primer binding locations and regions of interest shown in Figure 2.2.

Table 2.1 Primer sequences and sizes of PCR products for the *IDH1* and *IDH2* singleplex PCR.

| Gene | Direction | Sequence | Size of PCR product (bp) | Manufacturer |
|---|---|---|---|---|
| *IDH1* | Forward | 5'-AGCTCTATATGCCATCACTGC-3' | 410 | ABI |
| *IDH1* | Reverse | 5-AACATGCAAAATCACATTATTGCC-3' | - | ABI |
| *IDH2* | Forward | 5'-GCCCACACATTTGCACTCTA-'3 | 609 | ABI |
| *IDH2* | Reverse | 5'-GAAAGGAAAGCCACGAGACA-3' | - | ABI |

*IDH1* primers taken from Marcucci et al.[39] Novel primers were designed for *IDH2*. ABI=Applied Biosystems, Carlsbad, CA, bp=base pairs.

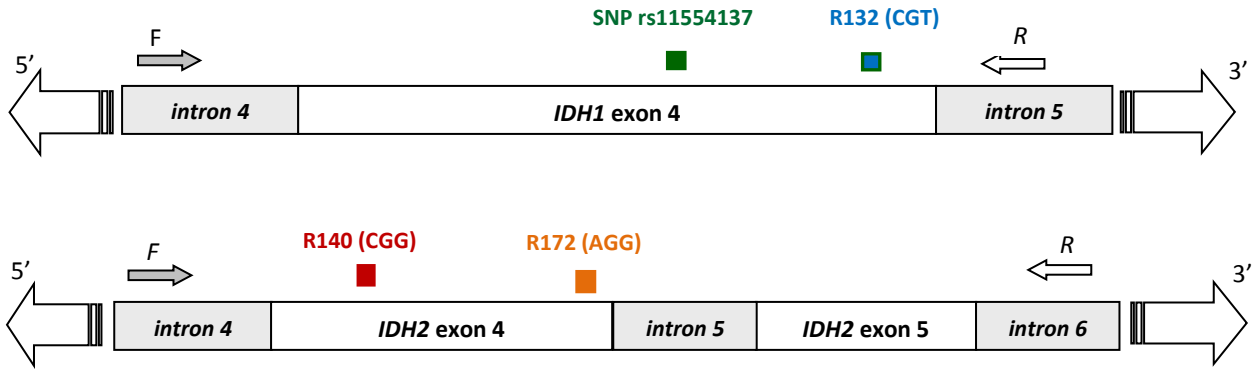



Figure 2.2 Primer-binding locations and regions of interest in *IDH1* and *IDH2*. The locations of the regions of interest are indicated by the coloured blocks and labels. The location of the primers in the introns and their orientation on the DNA are shown and labeled as "*F*" for forward or "*R*" for reverse. The novel *IDH2* primers were designed to bind in the intronic regions to ensure that only DNA was amplified. The exon/intron boundaries and 5'-3' orientation of the DNA are shown.

For both *IDH1* and *IDH2*, 100ng of DNA was added to a PCR reaction consisting of final concentrations of 0.25U BioTaq DNA Polymerase (Bioline), 1x buffer (Bioline), 0.8mM dNTPs (Promega, Wisconsin, USA), 1.6mM $MgCl_2$ (Bioline) and 0.8µM each primer (Applied Biosystems, Carlsbad, CA), made to a final volume of 25µl with $ddH_20$. PCR cycling was performed at an initial denaturation at 95$^oC$ for 5 minutes, 35 cycles of 95$^oC$ for 1 minute, 65$^oC$ for 1 minute and 72$^oC$ for 1 minute with a final extension of 72$^oC$ for 7 minutes. PCR cycling was performed in a G-Storm GS482 Thermal Cycler (Labtech, East Sussex, UK). A positive control (a DNA sample that was known to successfully amplify both the *IDH1* and *IDH2* genes) and a non-template control (with no DNA added) were included with each reaction to demonstrate successful functioning of the PCR and to ensure that no contamination was present in the reagents, respectively. Following PCR amplification, 3µl of PCR product was combined with 1µl of 6x Orange DNA loading dye (Thermo Scientific) and resolved on a 2% agarose gel (Bioline) stained with 1000x GelRed™ (Biotium, Hayward, CA). A GeneRuler 100bp DNA ladder (Thermo Scientific) was run concurrently to determine band size.

Following successful amplification of the specific exons of the *IDH1* and *IDH2* genes, sequencing was performed to determine whether mutations were present. The remaining PCR products were purified using the Biospin PCR Purification Kit (Bioer, Hangzhou, China) according to the manufacturer's instructions. This removed all excess dNTPs, $MgCl_2$ and Taq Polymerase, which may have inhibited the cycle-sequencing reaction. The purified PCR

product was assessed for quantity (ng/µl) and purity ($OD_{260/280}$) using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Thirty nanograms of purified product was then cycle-sequenced using the BigDye Terminator v3.1 Cycle-Sequencing Kit (Applied Biosystems) following the manufacturer's guidelines. The *IDH1* and *IDH2* forward and reverse PCR primers were used to sequence in both directions (Table 2.1). The cycle-sequencing reaction was performed in an Eppendorf Mastercycler (Hamburg, Germany). Following cycle-sequencing, the products were purified with 75% Isopropanol (Sigma-Aldrich, St. Louis, MO) to remove any unincorporated BigDye Terminator contaminants (complete procedure Appendix B). The purified products were loaded on a 96-well plate, which was then separated and analysed on the 3730 Genetic Analyser (Applied Biosystems). Samples were injected in a 50cm 48-capillary array using POP-7 Polymer (Applied Biosystems) at 15kV.

Following sequencing, the sequences obtained for each sample were compared to known reference sequences obtained from Genbank.[131] For *IDH1*, the published reference sequence was NG_0233119 and for *IDH2* was NG_023302. Samples were analysed individually for mutations using Sequence Scanner (v1.0) software and the reference, forward and reverse sequences aligned and analysed using Geneious (v5.6.5) software to determine whether any mutations were present in these exons.

### 2.4.2 Mutation-specific, multiplex PCR for *IDH1*$^{R132}$ mutations

Following sequencing, a mutation-specific, multiplex PCR was performed and products separated on an agarose gel to confirm the presence or absence of *IDH1*$^{R132}$ mutations.[128] This was to determine whether this technique would be a suitable alternative to singleplex PCR and sequencing to test for these mutations. Multiplex PCR allows for the simultaneous amplification of multiple loci in a single reaction with the use of more than one primer pair.[132] Furthermore, mutation-specific multiplex PCR, where primers specific for each mismatch in a target allele are combined in a single reaction mix, allow for the simultaneous amplification of specific mutations using one set of PCR conditions.[128]

In 2010, Chou et al.[128] described a mutation-specific, multiplex PCR for detecting all known *IDH1*$^{R132}$ mutations. Mutation-specific primers were designed for all possible *IDH1*$^{R132}$ types, including an additional mutation (R132P, CGT to CCT) that has not been reported to date. The principle of the primer-template match-mismatch is demonstrated in Figure 2.3.

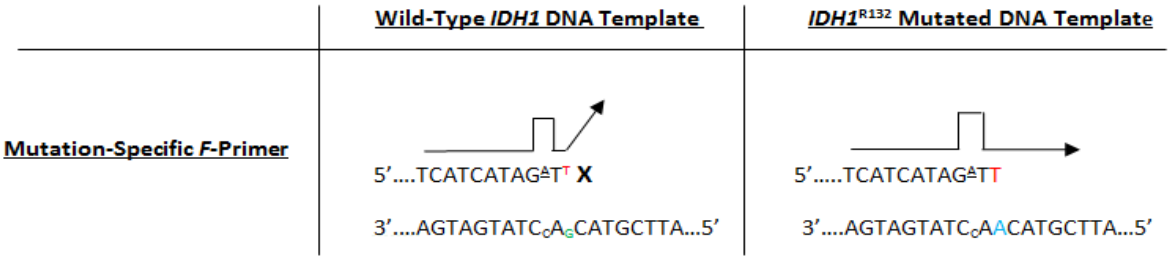



Figure 2.3 Schematic diagram illustrating the effects of primer/template base-pair mismatch. The wild-type *IDH1* template and the forward (*F*) primer for the *IDH1*$^{R132C}$ mutation are shown. The primer's third last nucleotide (underlined) has been intentionally mutated (to avoid background signals) while the last nucleotide (red) matches the mutant sequence (blue), but not the wild-type sequence (green). Primer extension will only occur when the specific mutation is present. Image adapted from Little.[133]

The *IDH1*$^{R132}$ mutation-specific, multiplex PCR was performed on the 70 CN-AML samples. Six forward *IDH1*$^{R132}$ mutation-specific primers, a common reverse primer and an upstream common forward primer, which served as an internal control, were included (Table 2.2 and Fig 2.4). The combined use of these primers would amplify a larger 393bp wild-type product, while in cases where an *IDH1*$^{R132}$ mutation was present, a smaller 264bp or 263bp product (depending on the specific R132 mutation) would also be co-amplified.

Table 2.2 Primer sequences, mutations and PCR product sizes for the *IDH1*$^{R132}$ mutation-specific, multiplex PCR.

| Direction | *IDH1* Mutation | Sequence | Size of PCR product (bp) | Manufacturer |
|---|---|---|---|---|
| *F* | R132S | 5'-TGGATGGGTAAAACCTATCATCATAGATA-3' | 264 | ABI |
| *F* | R132C | 5'-TGGATGGGTAAAACCTATCATCATAGATT-3' | 264 | ABI |
| *F* | R132G | 5'-TGGATGGGTAAAACCTATCATCATAGATG-3' | 264 | ABI |
| *F* | R132P | 5'-GGATGGGTAAAACCTATCATCATAGGACC-3' | 263 | ABI |
| *F* | R132H | 5'-GGATGGGTAAAACCTATCATCATAGGACA-3' | 263 | ABI |
| *F* | R132L | 5'-GGATGGGTAAAACCTATCATCATAGGACT-3' | 263 | ABI |
| Common-*F* | - | 5'-TGAGAAGAGGGTTGAGGAGTTCAAGT-'3 | 393 | ABI |
| Common-*R* | - | 5'-AATGTGTTGAGATGGACGCCTATTTGT-3' | - | ABI |

The last nucleotide matches only the mutant sequence, while the third last nucleotide (underlined) was intentionally mutated to prevent background signals. Primers taken from Chou et al.[128] *F*-forward, *R*=reverse, ABI=Applied Biosystems, R=Arginine, S=Serine, C=Cysteine, G=Glycine, P=Proline, H=Histidine, L=Leucine, ABI=Applied Biosystems, bp=base pairs.

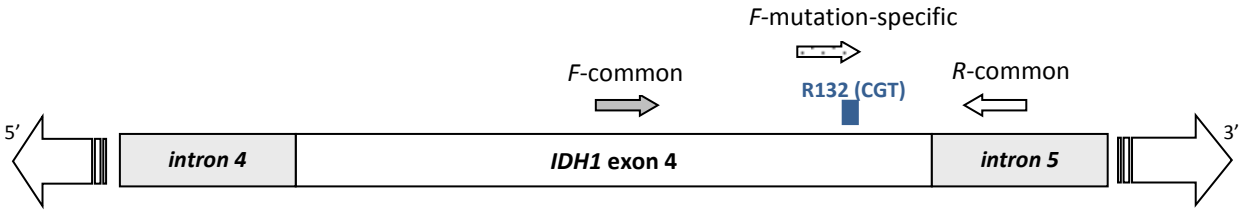



Figure 2.4 Location of codon R132 and primer-binding sites of the *IDH*$^{R132}$ mutation-specific, multiplex PCR. The location of codon R132 is indicated by the blue block. The binding-sites of the primers and their orientation on the DNA is shown and labeled as either "*F*-common" or "*F* mutation-specific" for the forward primers and "R-common" for the reverse primer. Exon/intron boundaries and the 5'-3' orientation of the DNA are shown.

One hundred nanograms of DNA was added to a PCR reaction consisting of final concentrations of 0.1µM common forward primer, 0.5µM each of the other 7 primers and 1x AmpliTaq® Gold 360 MasterMix (Applied Biosytems), made to a final volume of 25µl with $ddH_20$. Amplification was performed at an initial denaturation at 95$^oC$ for 7 minutes, 40 cycles of 95$^oC$ for 30 seconds, 62$^oC$ for 30 seconds and 72$^oC$ for 45 seconds with a final extension of 72$^oC$ for 7 minutes. PCR cycling was performed in a Veriti™ 96 Well Fast Thermal Cycler (Applied Biosystems). A positive control (a sample with an *IDH1*$^{R132}$ mutation detected by sequencing), a negative wild-type control and a non-template control were included. Following PCR amplification, 3µl of PCR product was combined with 1µl of 6x Orange DNA loading dye (Thermo Scientific) and resolved on a 2% agarose gel (Bioline) stained with 1000x GelRed™ (Biotium, Hayward, CA). A GeneRuler 100bp DNA ladder (Thermo Scientific) was run concurrently to determine band size.

To confirm the specificity of the primers, a wild-type *IDH1* sample was directly sequenced as previously described (refer to 2.4.1) using the common forward and reverse primers and the sequence obtained aligned to the *IDH1* Genbank reference sequence NG_0233119.[131]

The results obtained for testing for *IDH1*$^{R132}$ mutations using the mutation specific, multiplex PCR were compared to those obtained from sequencing to evaluate the different testing methodologies and confirm whether any discrepant results were seen between these two techniques.

## 2.5 DETECTION OF *NPM1* AND *FLT3*-ITD MUTATIONS

In the cohort, mutations in the *NPM1* and *FLT3* genes were initially investigated concurrently using a multiplex PCR followed with fragment analysis by capillary electrophoresis (CE).[134] These results were then verified separately for each gene using a multiplex, mutation-specific, real-time qualitative PCR (qPCR) for *NPM1*,[135] while internal tandem duplications in *FLT3* were tested for using a singleplex PCR and agarose gel electrophoresis (AGE).[136] The steps undertaken in testing for *NPM1* and *FLT3*-ITD mutations in the cohort are shown in the flow-diagram below (Fig 2.5).

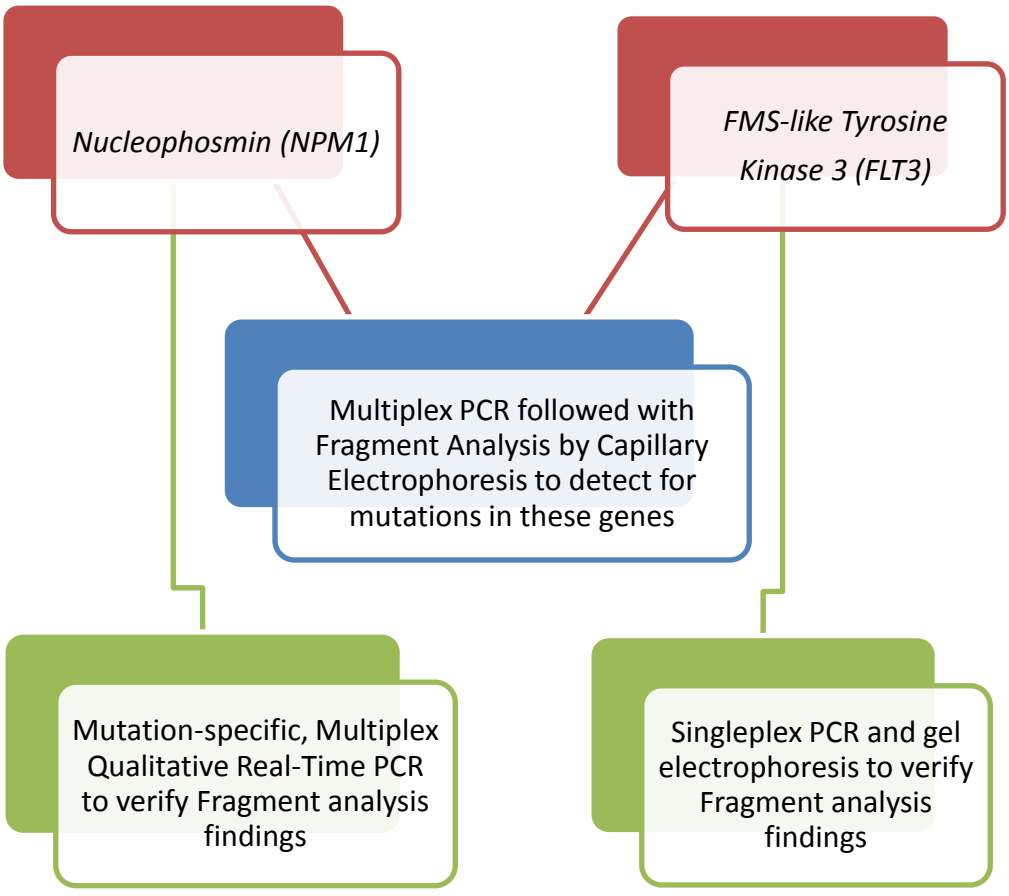



Figure 2.5 Flow-Diagram showing the steps followed in testing for *NPM1* mutations and *FLT3-ITDs* in the 70 CN-AML samples.

### 2.5.1 Multiplex PCR and Fragment Analysis by CE for the detection of *NPM1* and *FLT3*-ITD mutations

Fragment analysis is when a size estimate of a fluorescent DNA fragment is made relative to a size standard of DNA fragments of known lengths. One primer is labeled with a specific fluorescent dye, which is incorporated in the DNA fragment during amplification. During CE, the fragments are separated by size following injection into the capillary. As they pass the laser beam, the dyes are fluoresced, light of different wavelengths is emitted and the fluorescent signal converted into digital data for analysis. In fragment analysis, the use of multiple different fluorescent dyes enables different DNA fragments to be detected in one sample. A relative size can then be determined for each fragment.[137]

In 2008, Huang et al.[134] described a single-tube multiplex PCR and CE assay for the simultaneous amplification of the *NPM1*, *FLT3* and *HBG* genes. In this assay, the primer set for *NPM1* amplifies a 172bp fragment that includes the clustered multiple insertion sites (for the common mutations A, B and D), as well as the insertion/deletion sites (for the rare mutations C, E, G and H) (Fig 2.6a). This will detect virtually all reported *NPM1* mutations to date. Another primer set, specific for *FLT3*, spans a 330bp fragment that includes the common ITD insertions sites (exons 14-15) (Fig 2.6b). The *HBG* gene, amplified concurrently by a third primer set, generates a larger 474bp fragment which serves as a control for DNA integrity and PCR efficiency.

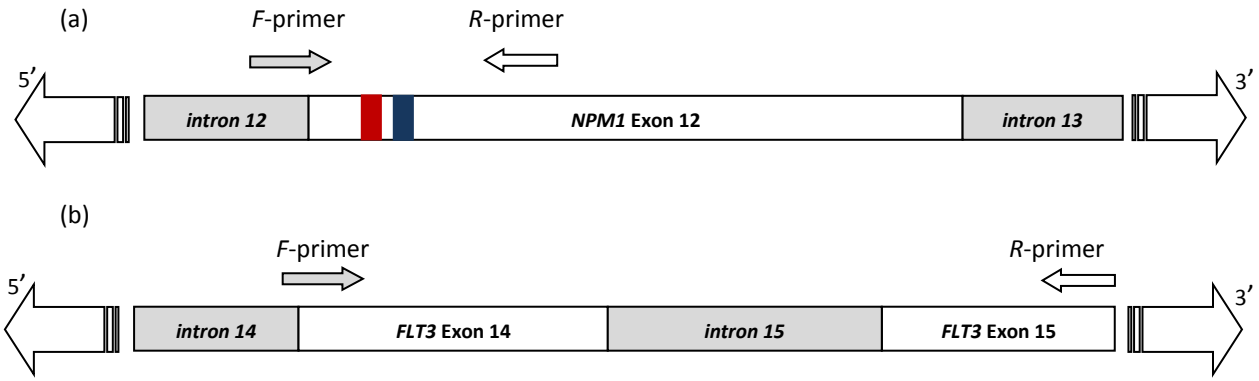



Figure 2.6 Primer-binding locations of the *NPM1* and *FLT3* primers in the multiplex PCR. The exon/intron boundaries and 5'-3' orientation of the genomic DNA are shown. (a) Exon 12 of the *NPM1* gene showing the primer-binding locations. The red block indicates the site of the 4bp insertion mutations (types A, B, C and D) while the blue block indicates the insertion/deletion mutation site (types E, G and H). (b) *FLT3* gene showing the primer-binding locations. ITDs vary in size and can occur at different locations in exons 14 and 15. *F*=forward, *R*=reverse.

#### 2.5.1.1 Multiplex PCR for *NPM1*, *FLT3* and *HBG*

For the multiplex PCR the reaction solution consisted of 100ng of gDNA added to a master-mix of 1x AmpliTaqGold® 360 MasterMix, 0.3µM *NPM1* primers, 0.2µM *FLT3* primers and 0.32µM *HBG* primers, made to a final volume of 25µl with $ddH_20$. PCR cycling was performed in a Veriti™ 96 Well Fast Thermocycler (Applied Biosystems). The following PCR conditions were used: Activation of AmpliTaq Gold® at 95°C for 5 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 1 minute at 72°C, and a final extension at 72°C for 7 minutes. A wild-type control, a non-template control and a positive control for each gene (once detected) were included in every run. Primer sequences and choice of fluorophore are given in Table 2.3.

Table 2.3 Primer sequences and expected PCR product sizes for the multiplex PCR and CE assay for *NPM1*, *FLT3* and *HBG*.

| Direction | Mutation | Sequence | Size of wild-type PCR product (bp)* | Manufacturer |
|---|---|---|---|---|
| *F* | *NPM1* | 5'-NED-ATT TCT TTT TTTTTTTTT CCA GGC TAT TCA AG-3' | 172 | ABI |
| *R* | *NPM1* | 5'-CAC GGT AGG GAA AGT TCT CAC TCT GC-3' | - | ABI |
| *F* | *FLT3* | 5'-6-FAM-AGCA ATT TAG GTA TGA AAG CCA GCTA-3' | 330 | ABI |
| *R* | *FLT3* | 5'-CTT TCA GCA TTT TGA CGG CAA CC -3' | - | ABI |
| *F* | *HBG* | 5'-HEX– CCA GGA GAG CCA AGG ACA GGT ACG-3' | 474 | ABI |
| *R* | *HBG* | 5'-AGA TCC CCA AAG GAC TCA AAG AAC C-3' | - | ABI |

To simplify analysis following CE, each forward primer was labeled with a different fluorophore (the colour of each is indicated). Primers taken from Huang et al.[134] *As the sizes obtained from CE are relative and may vary depending on the specific Genetic Analyser used, the actual sizes obtained may not match these sizes exactly. *F*=forward, *R*=reverse, ABI=Applied Biosystems, bp=base pairs.

### 2.5.1.2 CE for *NPM1*, *FLT3* and *HBG*

Following PCR amplification, the fluorescently-labeled PCR products were diluted 1:15 with HIDI-formamide (Applied Biosystems). For the reaction, 0.5µl of diluted product was combined with 9.3µl of HIDI-formamide and 0.2µl of GS500 ROX-size standard (Applied Biosystems) and loaded on a 96-well plate. The GS500 ROX size-standard consists of 16 fragments that measure from 35 to 500bp which covers the expected fragment sizes for the 3 genes. After denaturation at 95$^{o}$C for 2 minutes in a GeneAmp® PCR system 9700 (Applied Biosystems), products were immediately placed on ice for 2 minutes before separated and analysed on the 3500 Genetic Analyzer (Applied Biosystems). Samples were injected in a 50-cm-long, 8-capillary array using POP-7 polymer at 15 kV at 60$^{o}$C. The resulting data was analyzed using GeneMapper Software v4.1 (Applied Biosystems). The recommended

minimum peak height for the 3500 Genetic analyzer (Applied Biosystems) was 175 relative fluorescence units (RFU) for all genes.[137] For *NPM1* and *FLT3*-ITD mutations, for a 10% limit of detection of mutant cells in heterozygotes and 5% sensitivity of detecting heterozygote mutant peaks, a wild-type peak of ≥3500 RFU was required for a negative result.[138] Samples that did not reach this value were repeated using a 1:10 dilution.

### 2.5.1.3 Considerations and Optimization Procedures

The 3500 Genetic analyzer (Applied Biosystems) used had been calibrated for dye sets DS30 and DS33, therefore, with these dye sets, the allowable fluorophores for labeling the primers were 6-FAM™, VIC®, NED™, HEX™ and ROX™ (Table 2.4).

Table 2.4 The available dye sets and corresponding fluorophores for use with the 3500 ABI Genetic Analyzer.

**Applied Biosystems Standard Dye Sets for Genotyping Applications**

| Dye Set | DS-02 | DS-20[1] | DS-30 | DS-31 | DS-32 | DS-33 | DS-34 | DS-40 |
|---|---|---|---|---|---|---|---|---|
| Filter Set | E5 | A | D | D | F | G5 | C | S |
| Blue Dyes | dR110 | 5-FAM™ | 6-FAM™ | 6-FAM | 5-FAM | 6-FAM | 6-FAM | 6-FAM |
| Green Dyes | dR6G | JOE™ | HEX™ | VIC® | JOE | VIC | TET™ | dR6G |
| Yellow Dyes | dTAMRA™ | TAMRA™ | NED™ | NED | NED | NED | HEX | |
| Red Dyes | dROX™ | ROX™ | ROX | ROX | ROX | PET® | TAMRA | |
| Orange Dyes | LIZ® | | | | | LIZ | | LIZ |

[1]Dye primer matrix standards.

The colours of each dye and label are shown. The DS-30 dye set was used in the *NPM1* and *FLT3*-ITD CE assay. Table taken from Applied Biosystems dye set card.[139]

As the intensity of the fluorescence obtained depends on the dye used, the sample concentration and/or primer concentration needs to be optimized to obtain the optimal dye signal strength.[137] The final choice of fluorophore for *NPM1*, *FLT3* and *HBG* was NED, 6-FAM and HEX, respectively, which all emit different signal intensities (Table 2.5). Also, these have different emission spectrums, which simplifies analysis of CE results. As the PCR was multiplexed and the amount of DNA added to the PCR consistent, the concentration of fluorescently-labeled primers added to the PCR reaction master-mix was varied, according to the intensity of each fluorophore, until optimal results were obtained.

Table 2.5 Maximum Dye absorption, Emission, and Intensities of the Fluorophores used for CE.

| Dye | Absorption Max | Emission Max | Relative Intensity | Intensity (not to scale) |
|---|---|---|---|---|
| 6-FAM™ | 494 nm | 522 nm | 100 RFU | |
| HEX™ | 535 nm | 553 nm | 50 RFU | |
| NED™ | 546 nm | 575 nm | 40 RFU | |

Each forward primer for *NPM1*, *FLT3* and *HBG* was labeled with NED™, 6-FAM™ and HEX™, respectively. The 6-FAM™ dye has the highest intensity of the three dyes, thereby requiring less *FLT3* forward primer to be added in the PCR reaction. Table adapted from DNA Fragment Analysis User Guide.[138] RFU=relative fluorescent units, 6-FAM™=6-carboxyfluorescein, HEX™= 6-Hexachlorofluorescein, NED™= 2,7,8 benzo-5-fluoro-2,4,7-trichloro-5-carboxyfluorescein.

For the 3500 Genetic Analyzer (Applied Biosystems), recommended conditions for signal intensity and the size-standard to sample peak heights should be met for achieving optimal

results. As the analyzer can convert a limited fluorescence signal into a digital value, signal intensities should be within the range of 175-10 000 RFU with a maximum saturation of 30 000 RFU. To balance the signal intensities, the peak heights for the size standard should be 30-100% of that for the sample.[137] This was achieved by trying various primer concentrations for each gene, with a standard amount of the ROX marker for CE, until the intensities were balanced.

### 2.5.2 Singleplex PCR and AGE to detect *FLT3*-ITDs

To verify the *FLT3*-ITD results from the multiplex PCR and CE assay, all samples were tested with a separate singleplex PCR and analyzed with AGE.[136] As ITD insertions can vary in size and position between exon 14 and 15, the juxtamembrane domain of *FLT3*, which includes these exons, and the intervening intron were amplified (primer binding locations are given in Figure 2.6 and primer sequences are given in Table 2.6).

Table 2.6 Primer sequences and PCR product sizes for the *FLT3* singleplex PCR.

| Direction | Primer Sequence | Size of wild-type PCR product (bp) | Manufacturer |
|---|---|---|---|
| Forward | 5'-GCAATTTAGGTATGAAAGCCAGC-3' | 329 | ABI |
| Reverse | 5'-CTTTCAGCATTTTGACGGCAACC-3' | - | ABI |

Primers taken from Kiyoi et al.[136] ABI = Applied Biosystems, bp=base pairs

One hundred nanograms of DNA was added to a reaction mix consisting of 1x AmpliTaq Gold® 360 Master Mix and 0.8µM each primer, made to a final volume of 25µl with $ddH_20$.

PCR cycling was performed in a Veriti™ 96 Well Fast Thermocycler (Applied Biosystems). The PCR conditions were as follows: Activation of AmpliTaq Gold® at 95$^{O}$C for 5 minutes followed by 35 cycles of 30 seconds at 95$^{O}$C, 30 seconds at 57$^{O}$C and 1 minute at 72$^{O}$C, and a final extension at 72$^{O}$C for 7 minutes. A positive control (a sample with a *FLT3*-ITD mutation from CE), a negative wild-type control and a non-template control were included. Three microliters of PCR product was resolved on a 4% agarose gel (Bioline). As ITDs can be relatively small (≥3bp), the higher concentration agarose gel allowed for greater band discrimination. For all samples, a wild-type *FLT3* band of 329bp was expected, while in cases with ITDs, a band (or bands) larger than the wild-type band would also be seen.

To confirm the specificity of the primers for the *FLT3* gene, the PCR product of a wild-type sample was directly sequenced as previously described (refer to 2.4.1) using the forward and reverse PCR primers, and the sequence obtained aligned to the *FLT3* published reference sequence NC_000013 obtained from Genbank.[131]

Following testing of all CN-AML samples for *FLT3*-ITD mutations, the results obtained from the singleplex PCR and AGE were compared to those from the multiplex PCR and CE method to confirm whether any discrepant results were seen between these two techniques.

### 2.5.3 Mutation-specific, Multiplex real-Time qPCR to detect *NPM1* mutations

To verify the results obtained from CE for the *NPM1* gene, the samples were tested with a mutation-specific, multiplex real-time qPCR using TaqMan® methodology. TaqMan®-based chemistry is highly specific for the target of interest. A fluorogenic probe is used, which is labeled with a 5' fluorescent reporter dye and a 3' quencher. When the probe is intact, the quencher reduces the fluorescence of the reporter dye by fluorescence resonance energy transfer (FRET). During PCR, if the target is present, the fluorogenic probe binds to the complementary region on the DNA between the primers. As the target extends during amplification, the fluorescent reporter dye is cleaved from the probe by the DNA polymerase. The probe dissociates from the strand and extension continues. With the quencher in further proximity, the emission from the fluorescer dye can then be detected, which is increased in subsequent amplification cycles (Fig 2.7).[140]

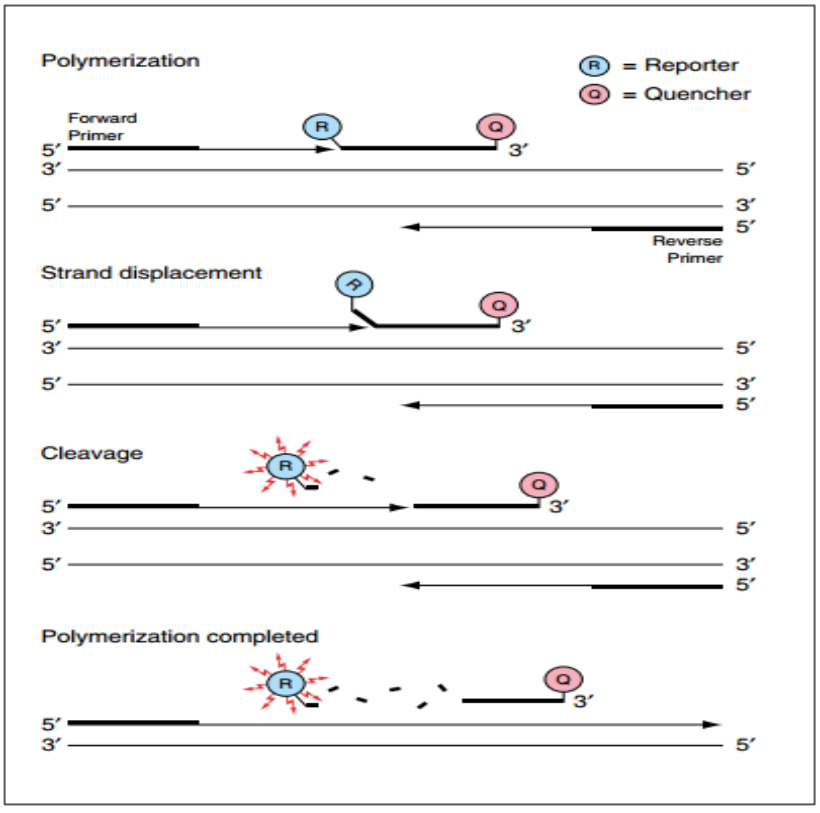



Figure 2.7 Principle of the TaqMan® probe-based chemistry. The probe is labeled with a 5' reporter dye (fluorescer) and 3' quencher. As the forward primer extends, the reporter dye is cleaved from the probe and fluorescence emitted. As further amplification cycles occur, the amount of fluorescence is directly proportional to the amount of PCR product produced. Picture taken from Taqman® Universal Master Mix Protocol, Applied Biosystems.[141]

The choice of quencher also contributes to the specificity of the assay; therefore, it was decided to label the *NPM1* probe with minor groove binder (MGB) as a quencher. DNA probes with MGB form very stable duplexes with single-stranded DNA targets and have a higher melting temperature (Tm). The Tm of the probe has to be at least 10$^{o}$C higher than that of the primers, and as MGB increases the probe's Tm (in contrast to other quenchers), this was the optimal choice.[142,143] Together, the use of a probe and MGB quencher greatly increases the test specificity and significantly reduces background signals and false-positives. As such, this assay was a highly efficient detection method to identify *NPM1* mutations.

In this multiplex, mutation specific real-time qPCR, described by Barakat et al.,[135] 6 *NPM1* mutation-specific primers (for mutations A, B, D, E, G and H) were used with a common Taqman® 6-carboxyfluoresein-labeled MGB probe and a common reverse primer (Fig 2.8). As the forward primers are mutation-specific, only those samples that have *NPM1* mutations should produce an amplification curve, while no curve should be seen in wild-type cases.




Figure 2.8 The sequences and binding locations of the primers and probe used in the mutation-specific, multiplex real-time *NPM1* qPCR. The 6 mutation-specific forward primers are indicated in green whilst the reverse primer and probe are in red and yellow, respectively. Picture taken from Barakat et al.[135]

To assess for the quality of the DNA and verify any negative results, the Glyceraldehyde 3-phosphate Dehydrogenase (*GAPDH*) gene was also amplified for each sample. Primer and probe sequences for *GAPDH* are given in Table 2.7.

Table 2.7 Primer and probe sequences and PCR product sizes for *GAPDH* for the multiplex qPCR.

| Direction | Primer Sequence | Size of PCR product (bp) | Manufacturer |
|---|---|---|---|
| Forward | 5'-CCCCACACACATGCACTTACC-3 | 97 | ABI |
| Reverse | 5'-CCTAGTCCCAGGGCTTTGATT-3' | - | ABI |
| Probe | 5'-FAM-AAAGAGCTAGGAAGGACAGGCAACTTGGC-TAMRA-3 | - | ABI |

Primers and probe taken from Zhong et al.[144] ABI = Applied Biosystems, bp=base pairs

For *NPM1*, 100ng of gDNA was added to a reaction mix consisting of 1x TaqMan® Universal Master Mix (Applied Biosystems), 400nM primers and 200nM probe. For *GAPDH,* the reaction mix was the same, except 300nM of each primer was used. The TaqMan® Universal Master Mix contained Amperase uracil *N*-glycosylase (UNG) which removed any uracil incorporated into single or double-stranded DNA preventing the re-amplification of carryover-PCR products.[145] This was an extra available precautionary step to exclude any possible contamination in the samples e.g. from previous PCRs, aerosols etc. Real-time analysis was performed on the 7500 PRISM Sequence Detection System (Applied Biosystems) using the following PCR conditions: an initial 2 minutes at 50$^{o}$C (to activate UNG), 10 minutes at 95$^{o}$C (for denaturation of DNA and activation of AmpliTaq® Gold enzyme) then 40 cycles of 15 seconds at 95$^{o}$C and 1 minute at 61$^{o}$C. Samples were tested in duplicate. A positive control (a sample with an *NPM1* mutation on CE), a negative wild-type control and a non-template control were included in each run. Results were analyzed with the Sequence

Detection Software v1.3.1 (Applied Biosystems). The post-amplification threshold was set to 0.1 with a start and end baseline of 3 and 15 cycles, respectively. The Ct value for *GAPDH* amplification for the samples had to be <30. This showed that the DNA in the sample was of a suitable quality and that a valid result had been obtained.

Following the initial qPCR run, the PCR products were resolved on a 2% agarose gel to confirm that the expected amplicon sizes were obtained for *NPM1* and *GAPDH*.

Those samples positive for an *NPM1* mutation were re-tested in a singleplex reaction for the common mutations A, B and D to determine the exact mutation present.

### 2.5.4 Sequencing to determine the type of *NPM1* mutations

As one sample showed an *NPM1* mutation on the CE results, but not with the qPCR, it was sequenced to determine the reason for this discrepancy. A novel forward primer was designed to bind downstream of a 13 Thymine-base mononucleotide region in intron 11 of *NPM1* which may have caused polymerase slippage during PCR. Hence, the binding location was very close to the *NPM1* mutation site. The primers were initially checked with primer-BLAST[106] and confirmed to be specific for the *NPM1* gene. Primer sequences are given in Table 2.8 and binding locations and mutation hot-spots in Figure 2.9.

Table 2.8 Primer sequences and expected PCR product sizes for PCR and sequencing of *NPM1*.

| Direction | Primer Sequence | Size of PCR product (bp) | Manufacturer |
|---|---|---|---|
| Forward | 5'-TTTTTTCCATATTCAAGATC-3' | 450 | UCT MCB |
| Reverse | 5'-CAAGACTATTTGCCATTCCTAAC-3' | - | ABI |

Reverse primer taken from Falini et al.[32] The forward primer was a novel design. UCT MCB = University of Cape Town Molecular and Cellular Biology Department, ABI = Applied Biosystems, bp=base pairs

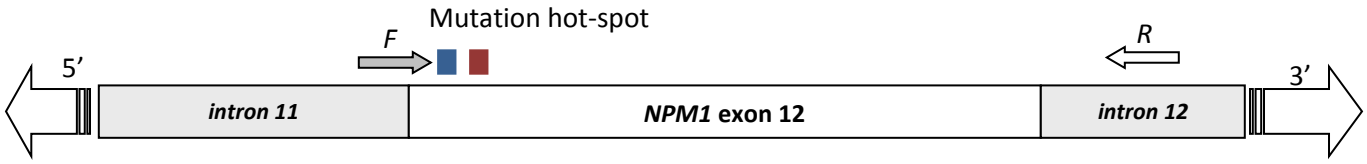



Figure 2.9 Primer-binding locations and *NPM1* mutation hot-spots. The location of the primers in the introns and their orientation on the DNA are indicated and labeled as "*F*" for forward or "*R*" for reverse. The blue block indicates the locations of *NPM1* mutations A, B and D, while the red block, mutations C, E, G and H. Other mutation types would also have been amplified with this primer set. The exon/intron boundaries and 5'-3' orientation of the genomic DNA are shown.

100ng of DNA was added to a PCR master-mix consisting of final concentrations of 1x AmpliTaq® Gold 360 MasterMix Kit (Applied Biosytems) and 0.4uM each primer (Applied Biosystems), made to a final volume of 25µl with $ddH_20$. Amplification was performed at an initial step of 95°C for 5 minutes to activate the AmpliTaq® Gold, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute with a final extension of 72°C for 7 minutes. Reactions were run in a Veriti™ 96 Well Fast Thermocycler (Applied Biosystems). A negative control (a DNA sample known not to have an *NPM1* mutation) and a non-template control (with no DNA added) were included with each PCR reaction.

PCR product purification and cycle sequencing was performed as previously described (refer to 2.4.1). Due to the close proximity of the forward primer to the mutation hot-spot, only the reverse primer was used for sequencing. After separation and analyzing on the 3500 Genetic Analyser (Applied Biosystems), the resulting *NPM1* sequence was compared to exon 12 of *NPM1* reference sequence NG_016018.[131]

## 2.6 DATA ANALYSIS

The results obtained for the *IDH1*, *IDH2*, *NPM1* and *FLT3*-ITD mutations in the CN-AML cohort were analyzed to determine the frequency and concurrence of these mutations. A comparison of the different testing methodologies used in the study was also performed to review the advantages and disadvantages of each technique.

# 3. RESULTS

## 3.1 COHORT DEMOGRAPHICS

Seventy CN-AML patients were available for this study, comprising 9 paediatric (≤16 years) and 61 (>16 years) adult patients. The paediatric cohort had a mean age of 10.1 years (median 11 years) and a male to female ratio of 1:2. The adult cohort had a mean age of 50 years (median 51 years) and a male to female ratio of 1:1.2. The ethnicities of the paediatric patients were 4 African, 2 Caucasian, 1 Indian, 1 of Mixed Ancestry and 1 where ethnicity could not be determined with certainty. In the adult patients, the ethnicities were 30 African, 23 Caucasian, 3 Indian and 5 where ethnicity could not be determined with certainty.

## 3.2 RESULTS FROM SINGLEPLEX PCR AND SEQUENCING FOR *IDH1* AND *IDH2* MUTATIONS

Of the 70 CN-AML patients analysed by PCR and sequencing, 11 (15.7%) patients had mutations in either *IDH1*$^{R132}$, *IDH2*$^{R140}$ or *IDH2*$^{R172}$ (Table 3.1). None of the patients had both *IDH1* and *IDH2* mutations concurrently.

### 3.2.1 *IDH1*

Six (8.6%) patients, 5 adults and 1 child, had mutations in *IDH1* codon R132, which were all heterozygous point mutations. Three patients had a conversion of CGT>TGT resulting in an

arginine to cysteine substitution (R132C) (Fig 3.1a) and 3 other patients had a conversion of CGT>CAT resulting in an arginine to histidine (R132H) substitution (Fig 3.1b). The mean age of the adult patients with *IDH1* mutations was 46.4 years (median 53 years). The paediatric patient was 15 years old. Other reported *IDH1* mutations, R132S, R132G and R123L were not found.

Eight (11.4%) patients, all adults, had SNP rs11554137 (codon 105, GGC>GGT). These were all heterozygous C/T mutations (Fig 3.1c). Five patients only had SNP rs11554137, 1 patient had $IDH1^{R132}$ and SNP rs11554137, 1 patient had *FLT3*-ITD and SNP rs11554137 and 1 patient had $IDH1^{R132}$, $NPM1^{mut}$ and SNP rs11554137.

One paediatric patient had a silent mutation in codon 131 of *IDH1*, just upstream of the reported mutation codon R132 (Fig 3.1d). This was a heterozygous point mutation at position 3 in the codon causing a T>A (GGT>GGA) substitution which did not change the amino acid glycine (393T>A, p.G131G). Patel et al.[146] had also identified this same mutation in 1 out of 146 AML patients.

### 3.2.2 *IDH2*

Five (7.1%) patients had *IDH2* gene mutations; 4 had the R140 mutation with a heterozygous conversion of CGG>CAG leading to an arginine to glutamine (R140Q) substitution (Fig 3.1e).

One patient had a R172 mutation with a heterozygous conversion of AGG>AAG resulting in an arginine to lysine (R172K) substitution (Fig 3.1f). Other reported *IDH2* mutations, R140L, R140W and R172M were not found. No paediatric patient had *IDH2* mutations. The mean age of the patients with *IDH2*$^{R140}$ mutations was 53 years (median 48 years). The *IDH2*$^{R172}$ mutated patient was 66 years old.

Two adult patients had silent mutations. Both were located at position 3 in codon 143 of *IDH2* exon 4, with a heterozygous substitution of G>C (CTG>CTC) which did not change the amino acid leucine (L143L, c.429G>C) (Fig 3.1g). This synonymous mutation had been previously reported in dbSNP (SNP rs144712130).[147] One of these patients also had an *IDH2*$^{R140}$ mutation (Fig 3.1h).

Table 3.1 Clinical information and Sequencing results from the CN-AML patients with *IDH1* or *IDH2* mutations.

| | | | ***IDH1* mutation** | | | ***IDH2* mutation** | |
|---|---|---|---|---|---|---|---|
| **UPN** | **Age/Gender** | **Ethnicity** | **nucleotide change** | **amino acid change** | **SNP rs11554137** | **nucleotide change** | **amino acid change** |
| 1 | 17/F | *A* | wt | wt | C/T | wt | wt |
| 17 | 52/M | *C* | wt | wt | wt | CGG>CAG | R140Q |
| 20 | 66/F | *A* | wt | wt | wt | AGG>AAG | R172K |
| 21 | 54/M | *C* | CGT>TGT | R132C | wt | wt | wt |
| 24 | 41/F | *A* | wt | wt | C/T | wt | wt |
| 27 | 75/F | MA | wt | wt | C/T | wt | wt |
| 30 | 60/M | *C* | wt | wt | C/T | wt | wt |
| 33 | 53/F | *A* | CGT>CAT | R132H | wt | wt | wt |
| 34 | 19/M | *A* | CGT>TGT | R132C | wt | wt | wt |
| 40 | 42/F | *C* | CGT>CAT | R132H | C/T | wt | wt |
| 44 | 60/F | *A* | wt | wt | C/T | wt | wt |
| 45 | 27/F | *A* | wt | wt | C/T | wt | wt |
| 46 | 10/F | *C* | GGT>GGA | none (G131G) | wt | wt | wt |
| 48 | 15/F | *A* | CGT>CAT | R132H | wt | wt | wt |
| 51 | 74/F | *A* | wt | wt | wt | CGG>CAG | R140Q |
| 55 | 41/M | U | wt | wt | wt | CGG>CAG | R140Q |
| 57 | 44/M | U | wt | wt | wt | CGG>CAG<br>CTG/CTC | R140Q<br>none (L143L) |
| 60 | 64/F | *C* | CGT>TGT | R132C | C/T | wt | wt |
| 64 | 28/F | *A* | wt | wt | wt | CTG/CTC | none (L143L) |

UPN=unique patient number; SNP=single nucleotide polymorphism, M=male; F=female; *A*=African; *C*=Caucasian; MA=mixed ancestry; U=unknown, wt=wild type, Nucleotide abbreviations: A=Adenine, C=Cytosine, G=Guanine, T=Thymine; Amino acid abbreviations: C=Cysteine, G=Glycine, H=Histidine, K=Lysine, L=Leucine, Q=Glutamine, R=Arginine.

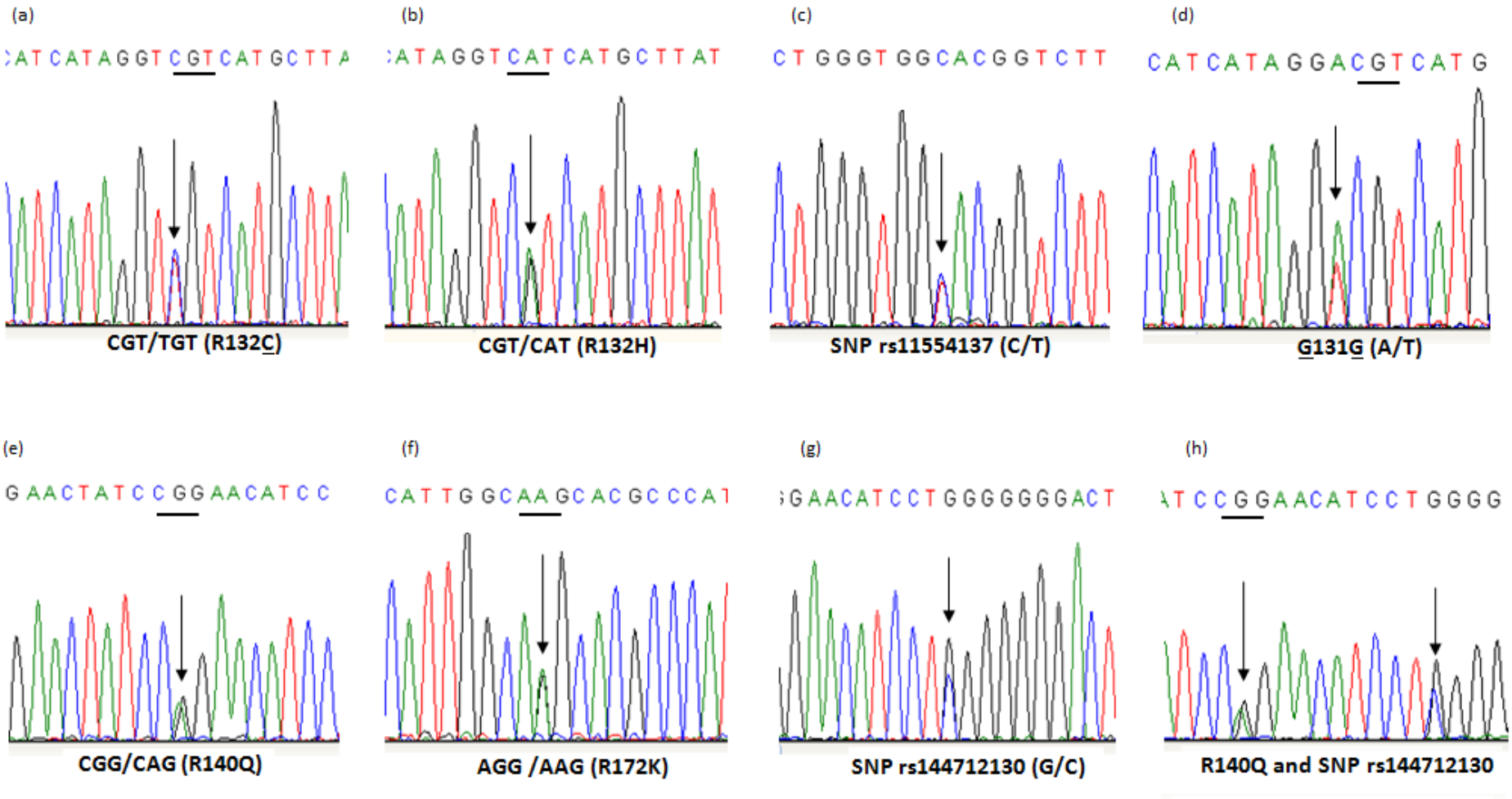



Figure 3.1 Sequence electropherograms of the various *IDH* mutations detected in the CN-AML cohort. Mutation codons are underlined and mutations are indicated with an arrow. The nucleotide sequence obtained for the sample is given above each image (a) The *IDH1* R132C mutation, CGT to TGT (b) The *IDH1* R132H mutation, CGT to CAT (c) *IDH1* SNPrs11554137 (d) The *IDH1* G131G silent mutation seen in one patient directly before mutation codon R132 (e) The *IDH2* R140Q mutation, CGG to CAG (f) The *IDH2* R172K mutation, AGG to AAG (g) *IDH2* SNP rs144712130 (h) The *IDH2* R140Q mutation with SNP rs144712130. SNP=single nucleotide polymorphism, Nucleotide abbreviations: A=Adenine, C=Cytosine, G=Guanine, T=Thymine; Amino acid abbreviations: C=Cysteine, G=Glycine, H=Histidine, K=Lysine, L=Leucine, Q=Glutamine, R=Arginine.

## 3.3 RESULTS FROM THE *IDH1*$^{R132}$ MUTATION-SPECIFIC, MULTIPLEX PCR AND AGE

An *IDH1*$^{R132}$ mutation-specific, multiplex PCR and AGE was used to verify the *IDH1*$^{R132}$ results from Sanger sequencing. All the samples (6/70) that had *IDH1*$^{R132}$ mutations by singleplex PCR and sequencing showed concordant results with the multiplex PCR and AGE. In these cases, a second smaller band in addition to the wild-type *IDH1* band was seen on the gel, indicating the presence of a mutation (Fig 3.2).

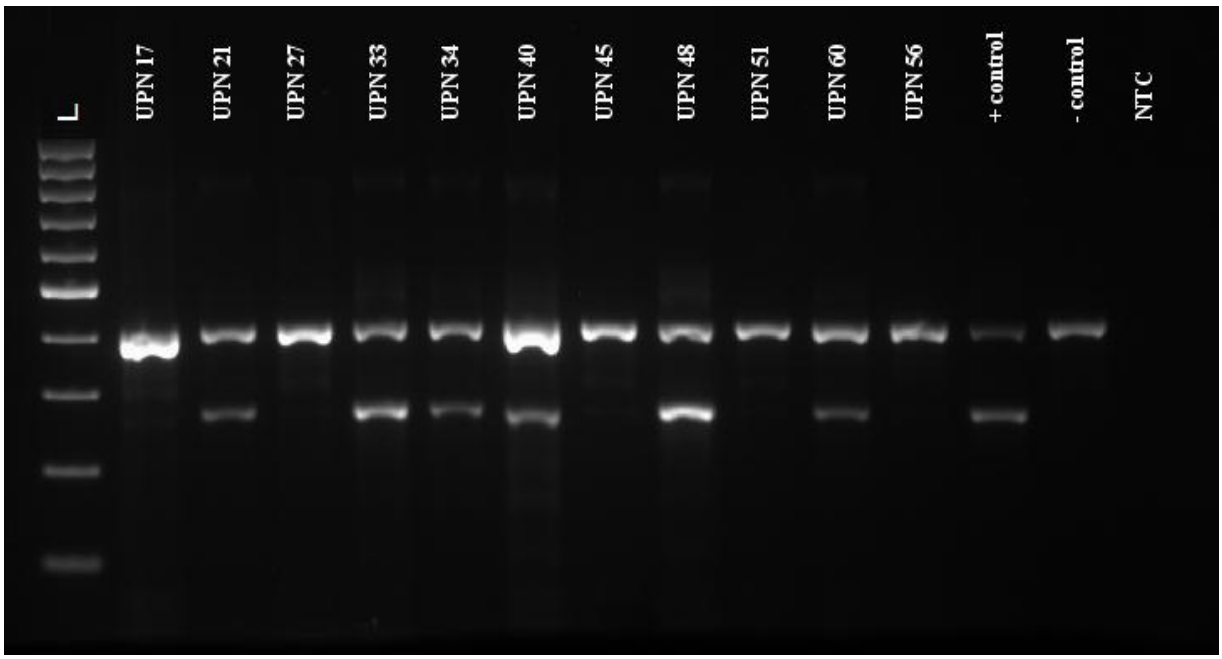



Figure 3.2 Gel image of a selection of *IDH1*$^{R132}$ positive and wild-type PCR products following amplification with the mutation-specific, multiplex PCR. PCR products were resolved at 110V for 120 minutes on a 2% agarose gel. The bright band of the 100bp ladder (L) is 500bp. A wild-type band of 393bp is seen in all samples, with *IDH1*$^{R132}$ mutated samples (UPN21,33,34,40,48,60) also having an additional smaller band of 263/264bp. UPN=unique patient number, NTC=Non template control, bp=base pairs, +=positive, -=negative,

The intensity of the PCR products on the gel was indicative of the level of the *IDH1* wild-type allele compared to the *IDH1*$^{R132}$ mutated allele. This could be verified with the sequencing results i.e. for sample UPN40, the intensity of the *IDH1*$^{R132}$ mutated PCR product on the gel was less than that for the wild-type one. Similarly the height of the mutated peak on the sequencing electropherogram was approximately half that of the wild-type allele (Fig 3.3a). For sample UPN48, the intensity of the PCR product on the gel for the mutated allele was slightly brighter than the wild-type allele. Similarly, on the sequencing electropherogram, the mutant peak was slightly higher than the wild-type peak (Fig 3.3b).

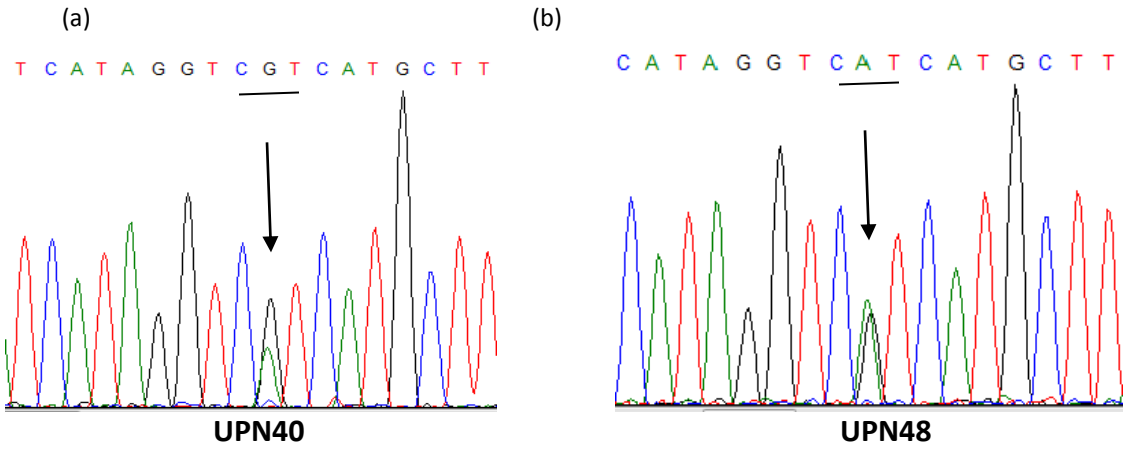



Fig 3.3 Sequence electropherograms for samples UPN40 and UPN48 showing *IDH1* mutation codon R132 and the peak heights of the mutant and wild-type alleles. *IDH1* R132 mutation codons are underlined and mutations are indicated with an arrow. The sequence obtained for the sample is given above (a) In sample UPN40 with *IDH1* R132H (CGT to CAT) mutation, the height of the mutated nucleotide "A" is approximately half of the wild-type nucleotide "G" indicating the level of the mutated allele is half that of the wild-type allele in the sample. (b) In sample UPN48 with *IDH1* R132H (CGT to CAT) mutation, the mutant allele is slightly higher than the wild-type allele indicating a slightly higher level of mutant allele than wild-type allele is present in the sample. UPN=unique patient number, A=Adenine, C=Cytosine, G=Guanine, T=Thymine

### 3.3.1 Discordant findings for sample UPN46

All samples in the cohort, except one, who had no *IDH1*$^{R132}$ mutations detected with the singleplex PCR and sequencing only showed the wild-type *IDH1* band on the gel after amplification with the mutation-specific, multiplex PCR.

For sample UPN46, however, in addition to the wild-type *IDH1* band, another faint band was seen on the agarose gel, corresponding to the size expected for mutated *IDH1*$^{R132}$ (Fig 3.4a). Although this was initially thought to be due to the presence of low levels of mutated *IDH1*$^{R132}$ in the sample, comparison with the sequencing findings showed that this sample was from the paediatric patient who had the silent G131G, c.393T>A point mutation in *IDH1* directly upstream of codon R132 (Fig 3.4b). As such, the band on the gel was most likely a false-positive finding occurring as a result of the point mutation decreasing the specificity of one or more of the mutation-specific primers.

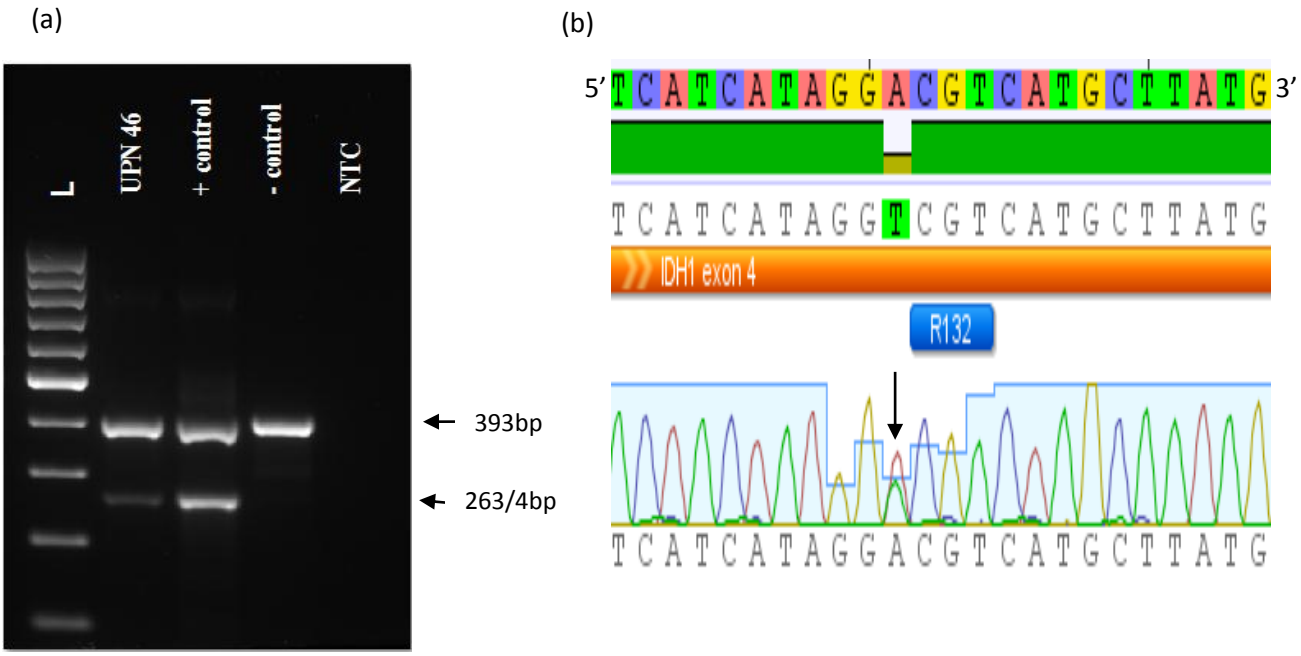



Figure 3.4 Gel image of the *IDH1*$^{R132}$ mutation specific, multiplex PCR products and sequencing results for sample UPN46 showing the G131G T>A point mutation. (a) PCR products were resolved at 110V for 120 minutes on a 2% agarose gel. The bright band of the 100bp ladder (L) is 500bp. Sample UPN46 is shown to have the wild-type band (393bp) as well as a fainter 263/264bp *IDH1*$^{R132}$ mutated band (b) Sequencing results showing the *IDH1* mutation codon R132 (blue block) and the G131G T>A point mutation (indicated by the arrow) in codon 131 directly preceding it. The sequence obtained for the sample is shown above (in colour) with the reference sequence (plain) given in 5' to 3' orientation below. UPN=unique patient number, NTC=Non template control, bp=base pairs, +=positive, -=negative,

The sample was re-tested in a singleplex PCR for each of the mutation-specific forward primers and the common reverse primer to determine which forward primer was causing this effect. The primer for the R132H mutation was shown to amplify this region and the primer for the R132L mutation to a lesser extent. Figure 3.5 demonstrates the primer-sequence base pair matching of these two primers with the wild-type and mutated sequence, however, a reason for this occurrence could not be determined. The 3' terminal base does not match in

either primer, which should result in failed amplification. No similar results were seen in the other samples.

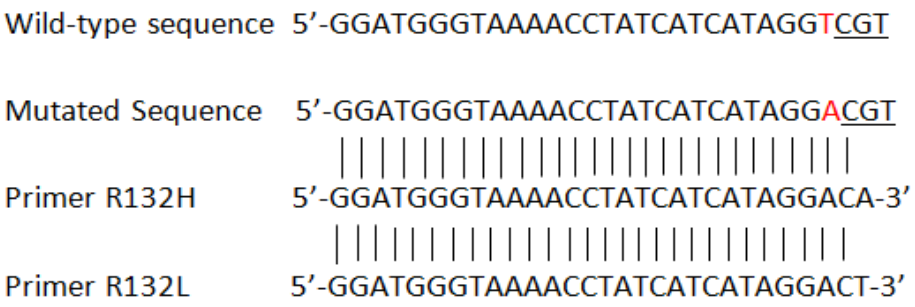



Figure 3.5 Wild-type and mutated sequences for sample UPN46 showing base pair matching of *IDH1*$^{R132}$ multiplex primers R132H and R132L. Sample UPN46 had a T>A mutation directly preceding *IDH1* codon 132 which caused amplification with primer R132H and to a lesser extent with primer R132L. Although the sequences of the primers and sample were compared, no reason for this occurrence could be established. The wild-type base "T" and mutated base "A" are indicated in red. *IDH1* R132 codon is underlined. The vertical lines indicate a 100% matching of the primer to the sequence. The most 3' nucleotide in both cases did not match either primer, which theoretically should have prevented amplification. Nucleotide abbreviations: A=Adenine, C=Cytosine, G=Guanine, T=Thymine, Amino Acid abbreviations: H=Histidine, L=Leucine, R=Arginine.

### 3.3.2 PCR Artifact due to DNA polymerase slippage during multiplex PCR amplification of a mononucleotide repeat region in intron 5 of *IDH1*

Following PCR amplification and sequencing of an *IDH1*$^{R132}$ wild-type sample to confirm the specificity of the primers in the multiplex PCR, an N-1 stutter product was seen in the electropherogram (Fig 3.6). This artifact occurred during the replication of a region of 10 consecutive Thymine bases in intron 5 of *IDH1* which caused the growing strand to dissociate from and re-anneal incorrectly with the template strand during PCR. When the forward primer was used for sequencing, as the mononucleotide region occurred after *IDH1* exon 4

which incorporated the regions of interest, SNP rs11554137 and codon R132, a clear sequence read could be obtained prior to the repeat region. However, as the mononucleotide region occurred in intron 5, sequencing using the reverse primer resulted in the stutter product encompassing the whole of exon 4 of *IDH1*, interfering with mutational analysis of this region. Re-designing the reverse primer to bind after this mononucleotide repeat region would prevent this artifact from occurring.

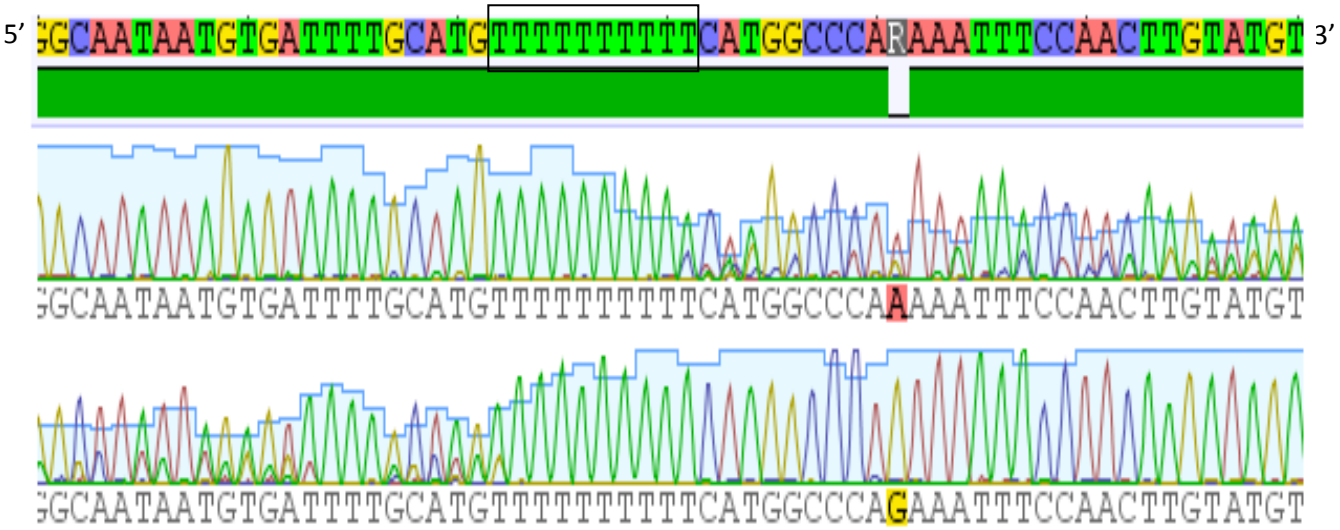



Figure 3.6 Sequencing results showing a 10-base pair region of repeat Thymine bases in intron 5 of *IDH1* resulting in DNA polymerase slippage during PCR. The electopherograms obtained from the forward and reverse primers are seen at the top and bottom, respectively. The repeat region of T-bases has been blocked. An N-1 stutter product is seen in both directions following the mononucleotide region. The *IDH1* consensus sequence is shown in the 5’-3’ direction above. Codon R132 occurs 5’ to the displayed region. Further downstream a base “G” (yellow) has been called erroneously as an “A” (red) in the forward sequence, also as a result of the polymerase slippage. A=Adenine, C=Cytosine, G=Guanine, T=Thymine

### 3.3.3 Singleplex PCR and. Sanger Sequencing vs. Mutation-specific, Multiplex *IDH1*$^{R132}$ PCR and AGE

Although one discordant result was seen between the two techniques, this was due to the presence of a point mutation in the codon preceding *IDH1* mutational codon R132 affecting the mutation-specific primers in the multiplex PCR. However, due to its occurrence in only 1 of 70 CN-AML samples in this cohort and as it has not been previously reported before by other studies, this point mutation is most likely rare. Should it be necessary to determine the specific *IDH1*$^{R132}$ mutation present in affected samples, despite the DNA polymerase slippage that occurs during the replication of the mononucleotide region in intron 5 of *IDH1* with the multiplex PCR, a clear sequence read is still obtainable using the common forward primer. However, determining the specific type of mutation present is unlikely to add any clinically valuable information. As such, with the mutation-specific, multiplex PCR and AGE method being more time efficient, less laborious and having a much higher reported sensitivity than the singleplex PCR and sequencing method,[129] it is a simple alternative method in testing for *IDH1*$^{R132}$ mutations.

## 3.4 RESULTS FROM MULTIPLEX PCR AND CE FOR *NPM1* AND *FLT3*-ITD MUTATIONS

The *NPM1* and *FLT3* genes were analysed to determine the frequency of any mutations in the CN-AML cohort and their co-occurrence with *IDH1* and *IDH2* mutations.

The multiplex PCR and CE results showed that 42/70 (60%) patients had wild-type forms of *NPM1* and *FLT3*. All samples amplified the control gene *HBG* (Fig 3.7a). Nineteen (27.1%) patients had *NPM1* mutations (Table 3.2). These were all 4bp insertions. The wild-type peak of 171bp was seen in all samples, with an additional peak at 175bp in *NPM1* mutated samples (Fig 3.7b). Of the 19 patients with *NPM1* mutations, 4 were paediatric patients (mean and median age 12.5 years) and 15 were adult patients (mean age 51.7 years, median age 51 years).

Twelve (17.1%) patients had a *FLT3*-ITD (Table 3.2). All samples had a 329bp wild-type peak and *FLT3*-ITD mutated samples also had 1-4 additional peaks of varied sizes and heights (Fig 3.8a-c). The smallest ITD was 3bp and the largest was 210bp. The majority of the ITD peaks were higher than the recommended minimum limit of 175 RFU, however, 5 peaks were lower. These samples were re-tested using a 1:10 dilution and the peaks were confirmed to be true FLT3-ITDs. Of the 12 patients with *FLT3*-ITDs, 11 were adult patients (mean age 49.7 years, median age 52 years). The paediatric patient was 10 years old.

Four (21.1%) patients, 3 adults and a paediatric patient had concurrent *NPM1* and *FLT3*-ITD mutations (Fig 3.8c and Table 3.2).

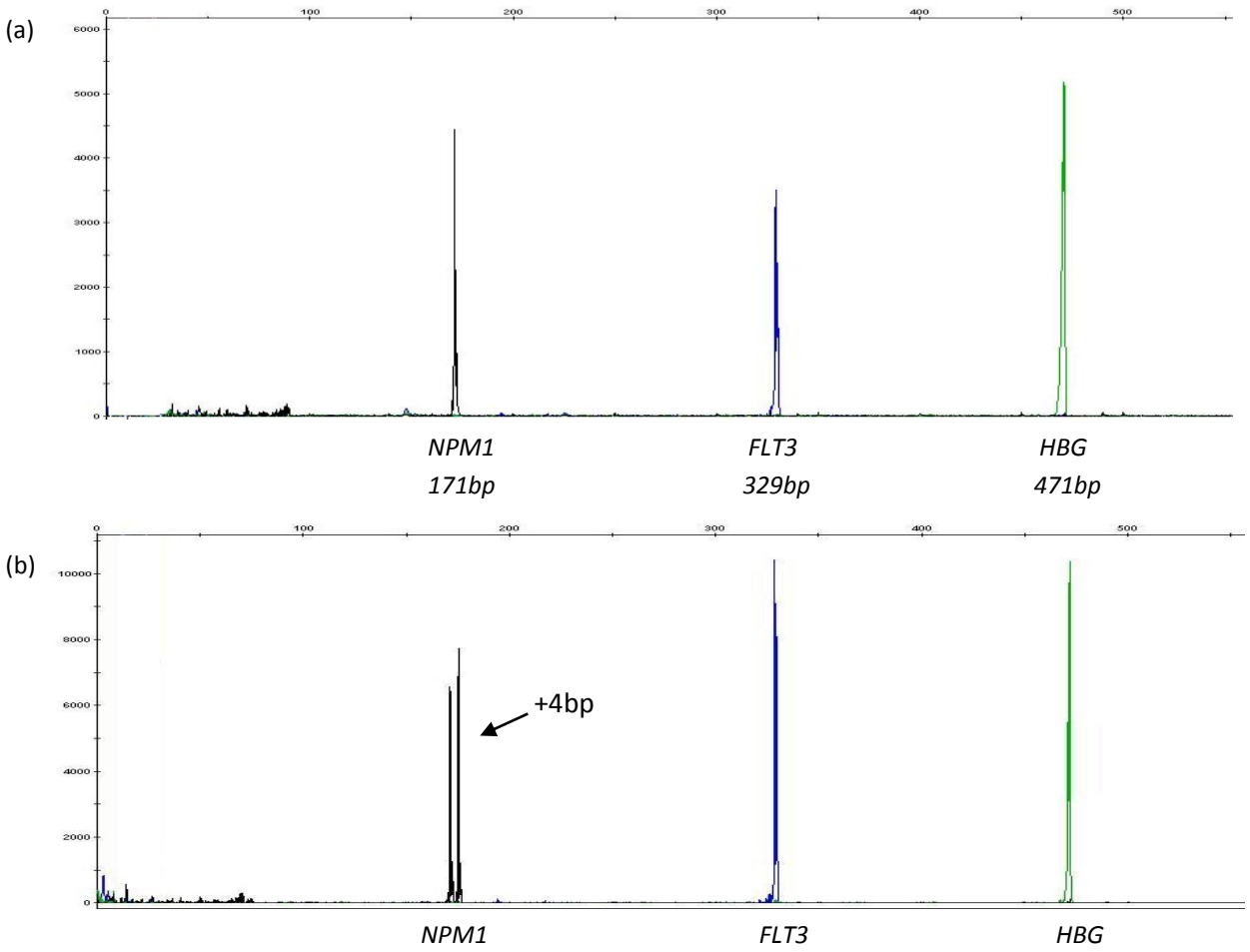



Figure 3.7 CE results of the multiplex PCR for *NPM1*, *FLT3* and *HBG*. The X-axis shows the sizes in base pairs while the Y-axis is labeled with the RFU value. (a) an *NPM1* and *FLT3*-ITD wild-type sample (UPN24) showing single peaks for each gene (b) an *NPM1* mutated sample (UPN66) showing the wild-type peak and a 4bp insertion. The wild-type sizes expected for the 3 PCR products are shown below. bp=base pairs, RFU=relative fluorescence units

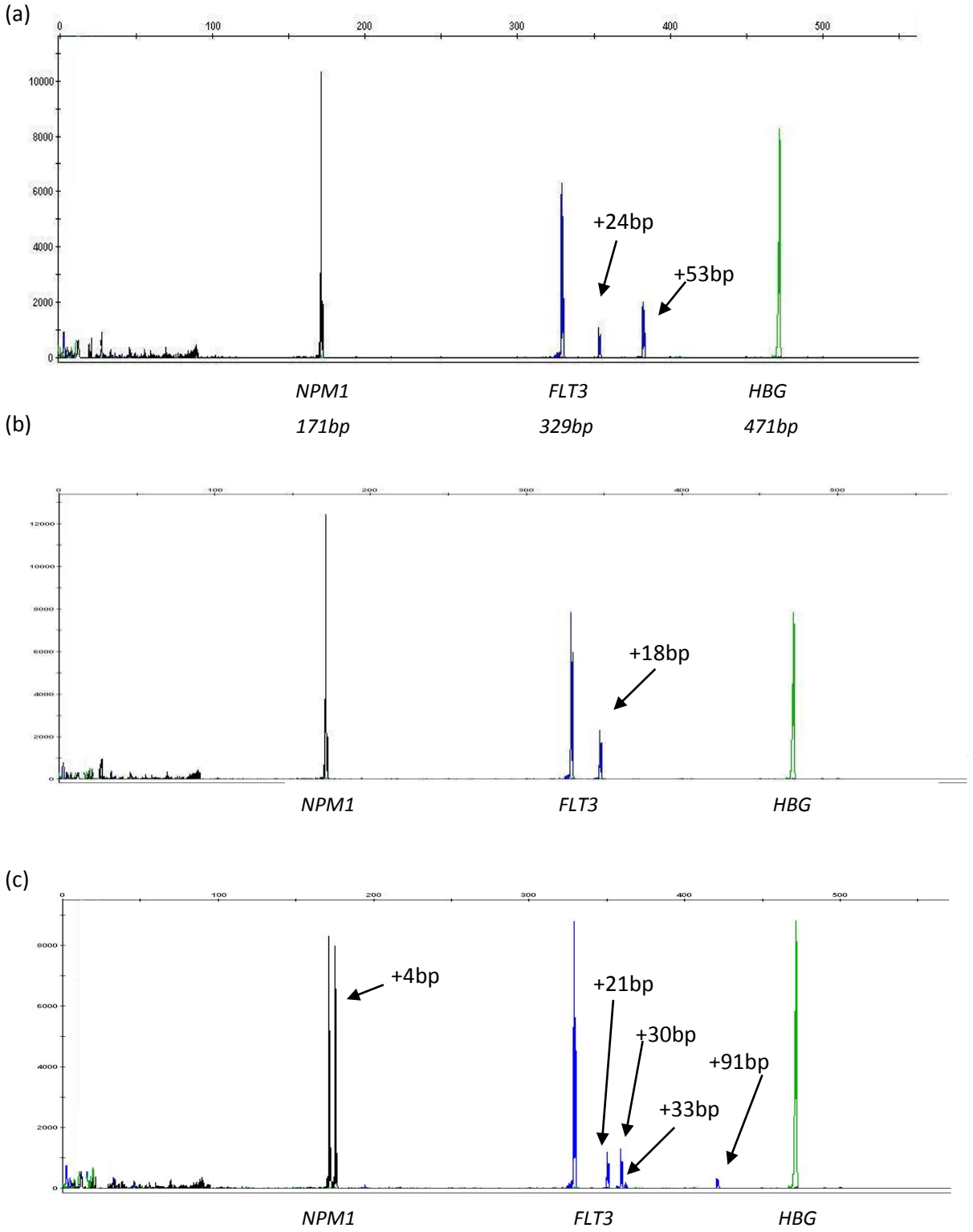



Figure 3.8 CE results of the multiplex PCR for *NPM1*, *FLT3* and *HBG*. The X-axis shows the sizes in base pairs and the Y-axis the RFU value. (a) a *FLT3*-ITD mutated sample (UPN42) with the wild-type peak and 2 larger ITD peaks (b) a *FLT3-ITD* mutated sample (UPN51) with the wild-type peak and 1 ITD peak (c) an *NPM1* and *FLT3*-ITD mutated sample (UPN17). As *FLT3*-ITDs are always in frame and hence divisible by 3bp, the sizes allocated to the various ITDs are most likely due to the relative sizing of the insertions during CE. The wild-type sizes expected for the 3 PCR products are shown below. bp=base pairs, RFU=relative fluorescence units

Table 3.2 Results of the multiplex PCR for the CN-AML patients who had *NPM1* and *FLT3-ITD* gene mutations.

| UPN | Age/Gender | Ethnicity | *NPM1* | *FLT3*-ITD | RESULT |
|---|---|---|---|---|---|
| 4 | 48/M | I | 171/175bp | wt | *NPM1 +* |
| 8 | 51/M | *C* | 171/175bp | wt | *NPM1+* |
| 9 | 68/M | *A* | 171/175bp | wt | *NPM1+* |
| 10 | 21/F | *A* | 171/175bp | wt | *NPM1+* |
| 25 | 14/F | *A* | 171/175bp | wt | *NPM1 +* |
| 31 | 51/F | *A* | 171/175bp | wt | *NPM1+* |
| 40 | 42/F | *C* | 171/175bp | wt | *NPM1+* |
| 48 | 15/F | *A* | 171/175bp | wt | *NPM1+* |
| 49 | 66/M | *A* | 171/175bp | wt | *NPM1+* |
| 52 | 39/M | *C* | 171/175bp | wt | *NPM1+* |
| 62 | 39/F | *A* | 171/175bp | wt | *NPM1+* |
| 63 | 11/M | U | 171/175bp | wt | *NPM1+* |
| 65 | 84/F | *A* | 171/175bp | wt | *NPM1+* |
| 67 | 40/M | *A* | 171/175bp | wt | *NPM1+* |
| 70 | 69/F | *C* | 171/175bp | wt | *NPM1+* |
| 6 | 24/M | *A* | wt | 329/353bp | *FLT3*-ITD+ |
| 23 | 20/F | *A* | wt | 329/353bp | *FLT3*-ITD+ |
| 27 | 75/F | *C* | wt | 329/361bp | *FLT3*-ITD+ |
| 35 | 63/M | *A* | wt | 329/335bp | *FLT3*-ITD+ |
| 42 | 59/F | *C* | wt | 329/353/382bp | *FLT3*-ITD+ |
| 51 | 74/F | *A* | wt | 329/347bp | *FLT3*-ITD+ |
| 54 | 46/F | *C* | wt | 329/332bp | *FLT3*-ITD+ |
| 66 | 29/M | I | wt | 329/354bp | *FLT3*-ITD+ |
| 17 | 52/M | *C* | 171/175bp | 329/350/359/362/420bp | *NPM1/FLT3*-ITD+ |
| 34 | 19/M | *A* | 171/175bp | 329/350/367*/379*bp | *NPM1/FLT3*-ITD+ |
| 46 | 10/F | *C* | 171/175bp | 329/376/471*bp | *NPM1/FLT3*-ITD+ |
| 69 | 86/F | *C* | 171/175bp | 329/353/382*/540*bp | *NPM1/FLT3*-ITD+ |

The wild-type *NPM1* peak is 171bp, with all mutated samples having an additional peak corresponding to a 4bp insertion. The wild-type *FLT3* peak is 329bp. *FLT3*-ITDs varied in size with the relative sizes indicated. More than 1 ITD occurred in 5/12 (41.7%) *FLT3* mutated patients. The sizes of the mutant peaks were calculated by subtracting the size of the wild-type allele from the mutant allele. F=female, M=male, I=Indian, *C*=Caucasian, *A*=African, U=unknown, bp=base pairs, wt=wild-type,.*=peak heights ≤175RFU (confirmed in a repeat assay using a 1:10 dilution)

## 3.5 RESULTS FROM SINGLEPLEX PCR AND AGE FOR *FLT3*-ITD MUTATIONS

Singleplex PCR and AGE were used to confirm the *FLT3*-ITD results obtained from the multiplex PCR and CE method. Sanger sequencing, used initially to test for the specificity of the *FLT3* primers, confirmed that they were specific for the region of interest.

In all cases, the wild-type *FLT3* gene, seen as a 329bp band on the agarose gel, was amplified showing suitable DNA quality. All samples, except one, that had *FLT3*-ITD mutations by the multiplex PCR and CE method had mutations with the singleplex PCR and gel electrophoresis method (Fig 3.9). In these cases, one or more bands larger than the wild-type band were seen on the gel.

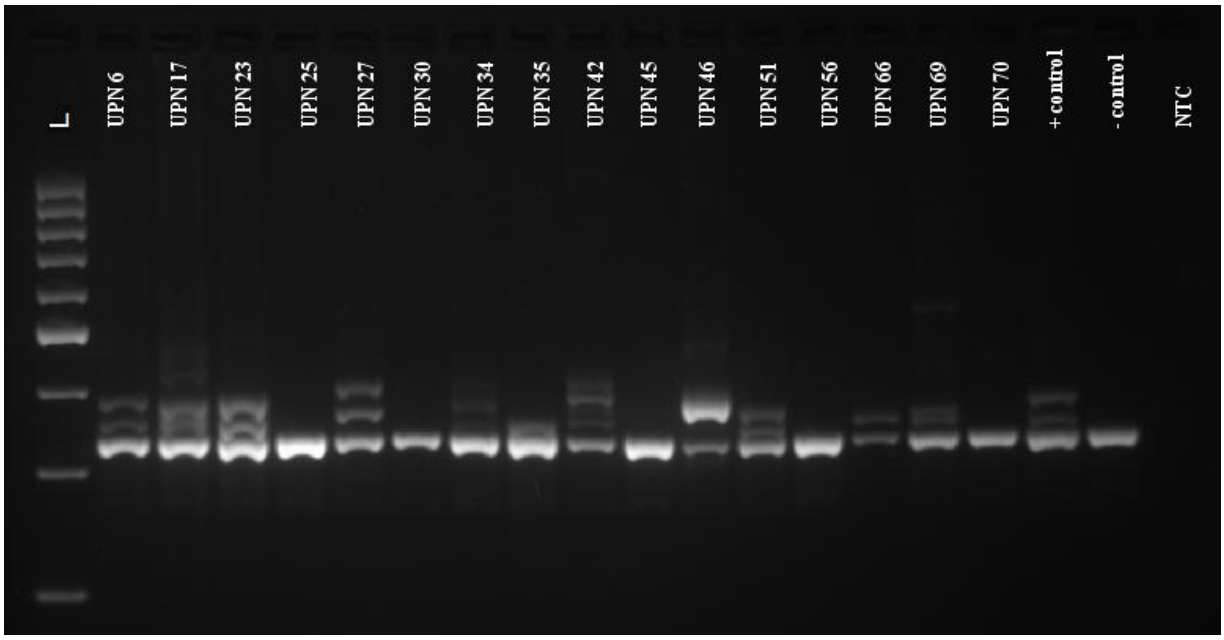



Figure 3.9 Gel image of a selection of *FLT3*-ITD mutated and wild-type PCR products following amplification with the singleplex PCR. PCR products were resolved at 80V for 300 minutes on a 4% agarose gel. The bright band of the 100bp ladder (L) is 500bp. A wild-type *FLT3* band of 329bp is seen for all samples and those with a *FLT3*-ITD also have one or more larger bands of different sizes (UPN6,17,23,27,34,35,42,46,51,66 and 69). UPN=unique patient number, NTC=non template control, bp=base pairs, +=positive, -=negative

### 3.5.1 Discordant findings for sample UPN54

One discordant result was seen for sample UPN54 which showed a *FLT3*-ITD with the multiplex PCR and CE method but not with singleplex PCR and AGE. Evaluating the CE results showed that this patient had a single ITD 3bp's in size. Due to this small size, it was unlikely for the ITD band to be differentiated from the wild-type band on the 4% agarose gel (Fig 3.10a), and as such could only be detected with CE (Fig 3.10b). Similarly, for sample UPN17 who showed multiple ITDs with CE (Fig 3.8c), 2 of these ITDs differed by 3bp and also could also not be distinguished individually on the gel (Fig 3.9). Sample UPN35 with the second smallest ITD, a 6bp insertion on CE, did show both the wild-type and ITD band on the gel (Fig 3.9). This indicates that any ITDs of 3bp's in size would not be discernible using AGE.

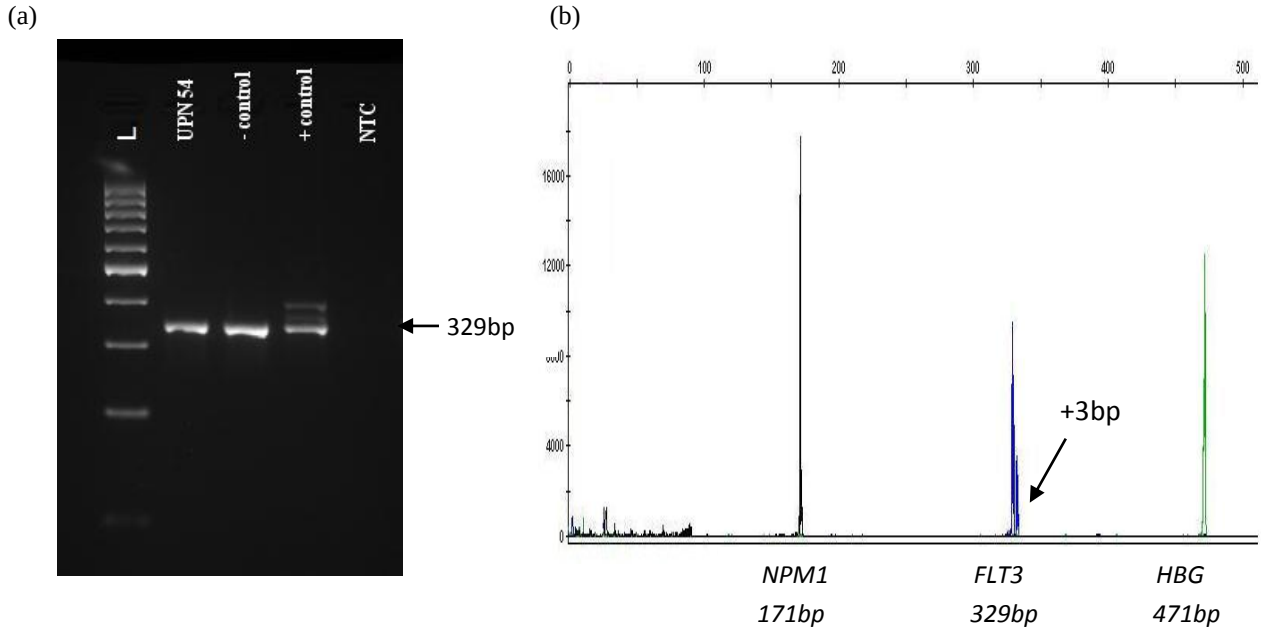



Figure 3.10 Gel image of the *FLT3* singleplex PCR and AGE results and of the CE results of the multiplex PCR of *NPM1*, *FLT3* and *HBG* for sample UPN54. (a) PCR products were resolved at 80V for 240 minutes on a 4% agarose gel. The bright band of the 100bp ladder (L) is 500bp. Only the wild-type *FLT3* band of 329bp could be seen but not the 3bp ITD. (b) The CE results of the multiplex PCR for *NPM1*, *FLT3* and *HBG* showing a 3bp *FLT3*-ITD. The X-axis shows the sizes in base pairs while the Y-axis is labeled with the RFU value. The wild-type sizes expected for the 3 PCR products are shown below. UPN=unique patient number, NTC=non template control, bp=base pairs, +=positive, -=negative, RFU=relative fluorescence units

### 3.5.2 Artifact due to heteroduplex formation between *FLT3* wild-type and ITD PCR products

*FLT3*-ITD positive samples had one or more larger bands on the agarose gel, which varied according to the size of the ITD/s. The approximate sizes of the bands correlated with the size of the ITD as determined by CE. However, extra ITDs that had not been detected by the multiplex PCR and CE (Table 3.2 and Fig 3.11a) were seen in some *FLT3*-ITD mutated samples (UPN6, 23, 27, 42 and 51) on the agarose gel (Fig 3.9).

This finding represents an artifact and can be explained by heteroduplex formation between the wild-type and ITD PCR products. This results in an additional, larger band on the gel, which does not represent an independent ITD (Fig 3.11b). Therefore, although *FLT3*-ITD mutated samples appeared on average to have two or more ITDs with AGE, the presence of multiple ITDs was misrepresented by the formation of these heteroduplexes.

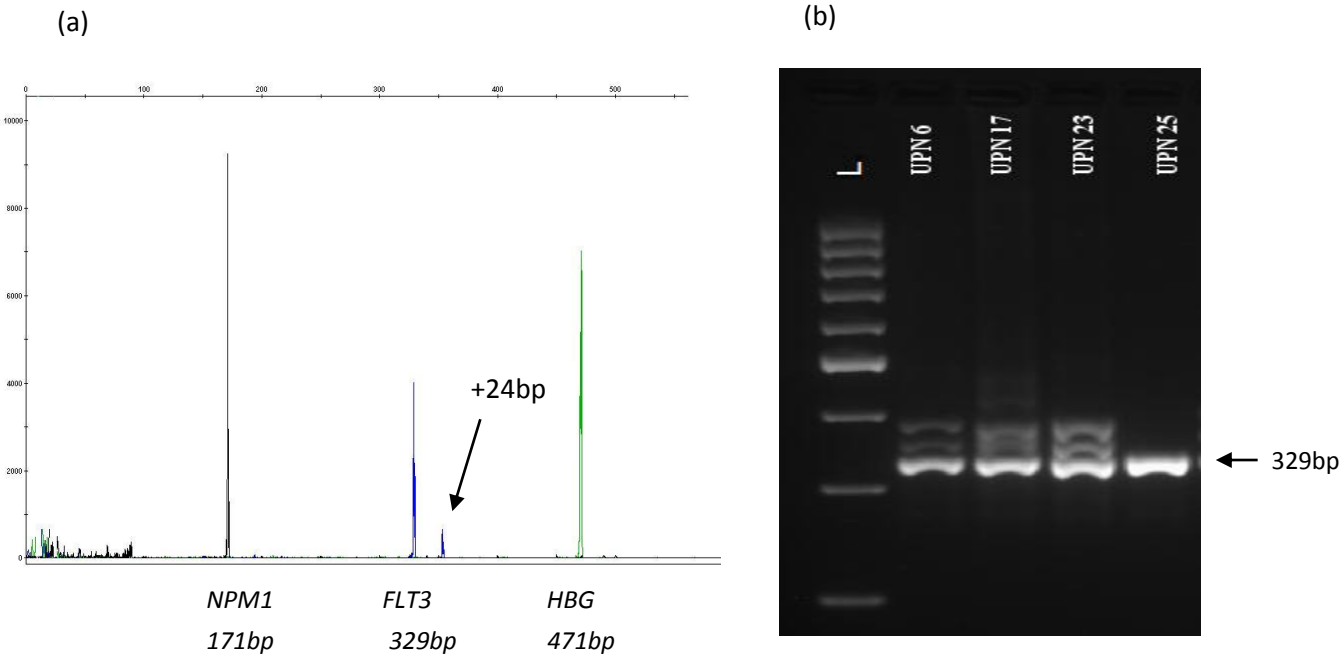



Figure 3.11 CE result of UPN6 for the multiplex PCR of *NPM1*, *FLT3* and *HBG* and gel image of the singlexplex *FLT3* PCR product. (a) With CE, only the *FLT3* wild-type peak and 1 ITD were seen. As CE is denaturing it will only detect single-stranded PCR products. The wild-type sizes expected for the 3 PCR products are shown below. (b) Image modified from Fig 3.9. Sample UPN6 showed 2 ITDs on the gel. However, this is an artifact due to the formation of heteroduplexes between the *FLT3*-ITD and wild-type PCR products. The bright band of the 100bp ladder (L) is 500bp. UPN=unique patient number, bp=base pairs, RFU=relative fluorescence units

### 3.5.3 Singleplex PCR and AGE vs. Multiplex PCR and CE to detect *FLT3*-ITD mutations

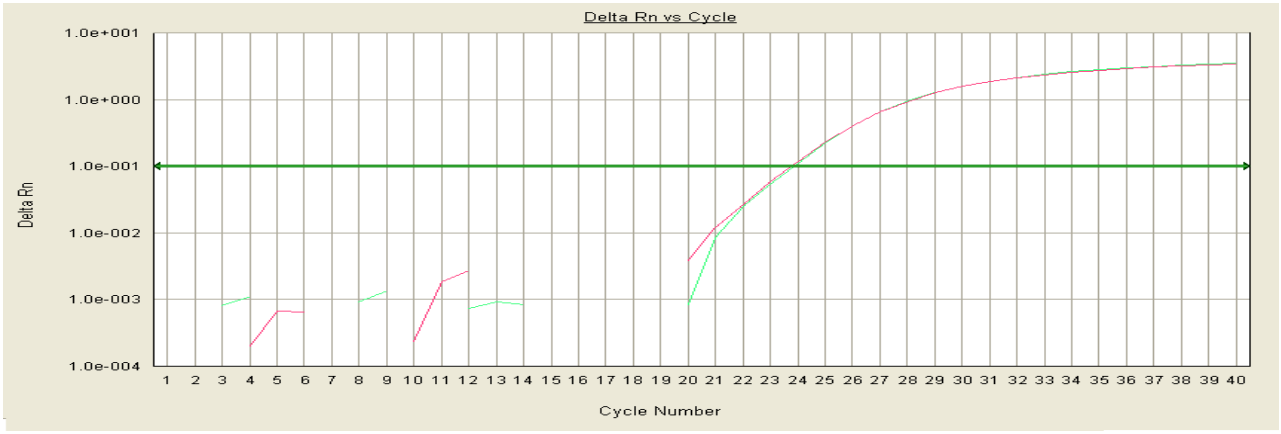
Overall in the CN-AML cohort, one sample showed discordant results between the two techniques. Since singleplex PCR and AGE could not distinguish ITDs 3bp's in size and with heteroduplex formation occurring between wild-type and mutant alleles, this method may miss ITDs or over-estimate the frequency of multiple ITDs. In contrast, the multiplex PCR and CE method could detect *FLT3*-ITDs 3bp's or larger in size, and since a denaturing reagent is used which prevents heteroduplex formation, only single-stranded PCR products are

detected. As such, this method is a much more accurate technique than singleplex PCR and AGE in detecting *FLT3*-ITD mutations.

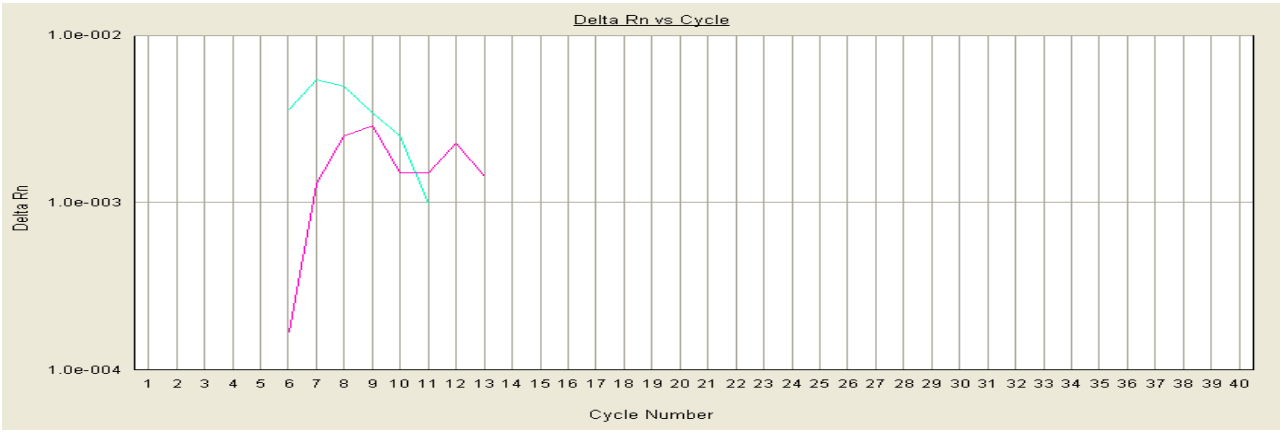
## 3.6 RESULTS FROM MUTATION-SPECIFIC, MULTIPLEX REAL-TIME qPCR FOR *NPM1* MUTATIONS

A mutation-specific, multiplex real-time qPCR was used to verify the findings of the multiplex PCR and CE method for *NPM1*. In all cases, the control gene, *GAPDH* was amplified (Fig 3.12a) indicating the suitable quality of DNA and the absence of PCR inhibitors. Samples that had wild-type *NPM1* on the multiplex PCR and CE showed no amplification product with qPCR i.e. an "undetected" result was obtained (Fig 3.12b). All samples, except one, that had *NPM1* mutations on multiplex PCR and CE showed *NPM1* amplification curves with qPCR. This was indicated by a positive Ct value (this value is determined from the point at which the amplification curve crosses the threshold line) following PCR amplification (Fig 3.12c).

(a) *GAPDH* (control gene)




*(b)* *NPM1* wild-type




(c) *NPM1* mutation positive

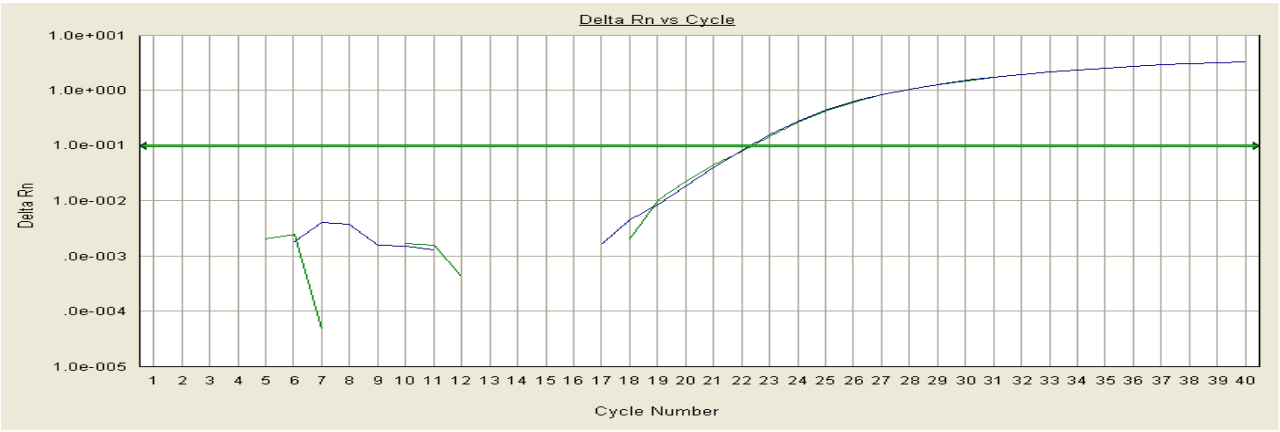


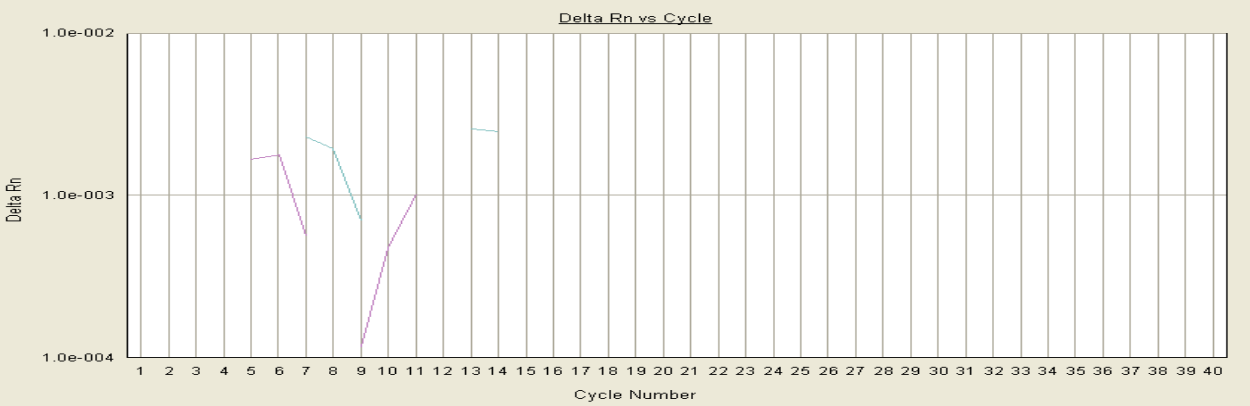

Figure 3.12 Amplification plots of *GAPDH,* wild-type *NPM1* and mutated *NPM1* for the mutation-specific, multiplex *NPM1* qPCR. The X-axis and Y-axis represent the Cycle number and Delta Rn (Rn minus baseline), respectively. Rn=normalized reporter where the fluorescence emission intensity of the reporter dye is divided by the fluorescence emission intensity of the passive reference dye. The baseline is represented by the green line (set to 0.1) (a) Sample UPN5 showing amplification of the control gene, *GAPDH,* which was seen for all samples. (b) *NPM1* wild-type sample UPN5 showing no amplification curve. The coloured lines represent the normal background fluorescence (c) *NPM1* mutated sample UPN63 with an amplification curve for each duplicate.

### 3.6.1 Discordant findings for sample UPN10

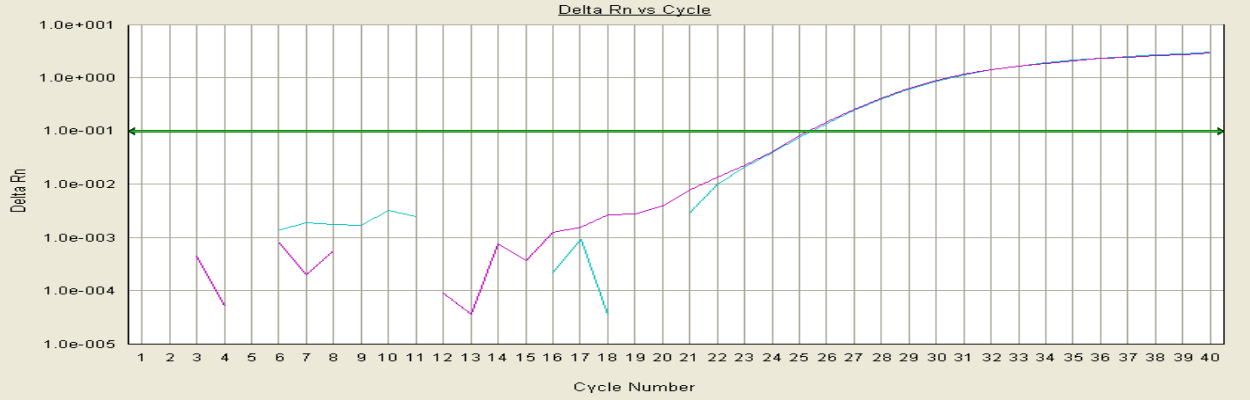
One discordant result was seen between the mutation-specific, multiplex qPCR and the multiplex PCR and CE findings. On CE, sample UPN10 showed both the wild-type *NPM1* peak and a +4bp mutated peak. However, with the qPCR there was no *NPM1* amplification, which gave an "undetected" result (Fig 3.13a,b). This same result was obtained when the sample was re-tested and also when 50 amplification cycles, rather than 40 cycles, were used.

The sample was also re-tested with multiplex PCR and CE and still showed the patient to have an *NPM1* mutation (Fig 3.13c). Based on these findings, this patient was thought to have an *NPM1* mutation, which was a 4bp insertion (as determined by CE), however, was probably one of the rarer mutations and not types A, B, or D, since these mutation-specific primers did not yield any amplification product with the qPCR. Attempts at sequencing this sample, despite a number of optimizations, only yielded noisy sequence reads which precluded accurate analysis (Fig 3.14).

*(a) NPM1*




*(b) GAPDH*




(c) CE

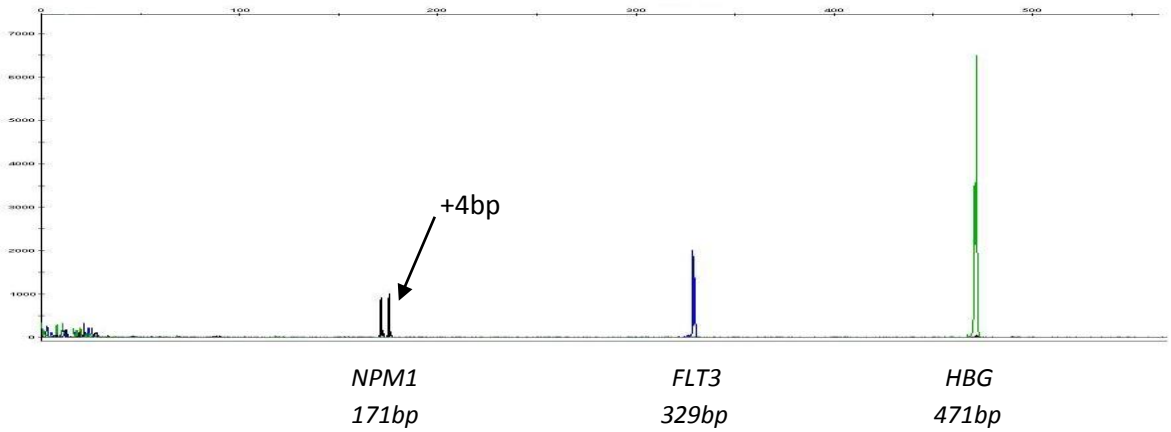



Figure 3.13 Amplification plots for *NPM1* and *GAPDH* for the mutation-specific, multiplex *NPM1* qPCR and CE results from the multiplex PCR for *NPM1*, *FLT3* and *HBG* for sample UPN10. (a) No amplification for *NPM1* was obtained using the multiplex qPCR (b) *GAPDH* amplified indicating adequate DNA quality. The X-axis and Y-axis represent the Cycle number and Delta Rn (Rn minus baseline), respectively. The baseline is represented by the green line (set to 0.1) (c) CE results showing the presence of a +4bp *NPM1* mutation. The X-axis shows the sizes in base pairs while the Y-axis is labeled with the RFU value. The wild-type sizes expected for the 3 PCR products are shown below. bp=base pairs, RFU=relative fluorescence units

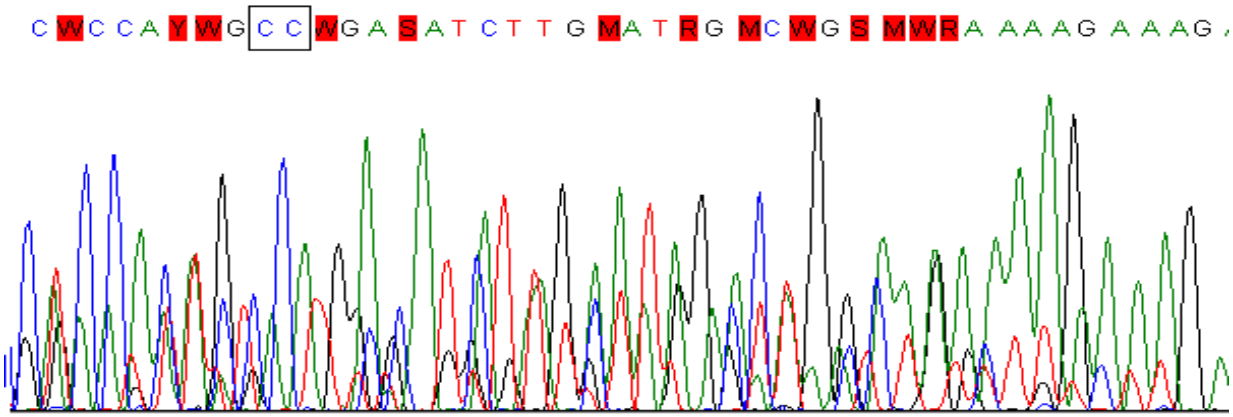


Figure 3.14 Sequencing electropherogram of *NPM1* for sample UPN10 showing a noisy sequence read. The reverse sequence is displayed. The noisy sequence read precluded accurate analysis. The boxed region indicates where the 4bp insertion mutation occurs between these 2 nucleotides.

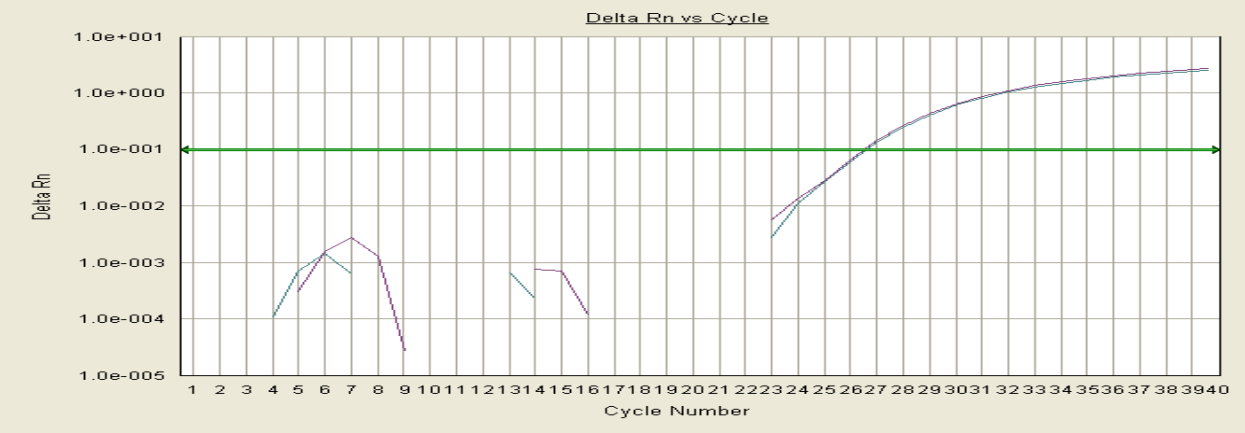
### 3.6.2 Singleplex qPCR to determine the specific *NPM1* mutation present in mutated samples

After confirmation of the mutation status with the multiplex, mutation-specific qPCR, a singleplex qPCR using each primer separately for the common mutations A, B and D was performed to determine the exact type present in mutated samples (since CE only identified 4bp insertions, this excluded types E, G and H, which would have yielded mutations larger than 4bp in size and were, therefore, not tested by singleplex qPCR). However, even with the singleplex PCR, amplification for all three mutation types was observed for each sample (Fig 3.15a-c). This showed that the specificity of the mutation-specific primers was not exclusive to any particular type. Since only one base pair differed between primers A and D, if an *NPM1* mutation A (which accounts for 95% of all *NPM1* mutations) was present, primer D could still potentially bind and amplify that region. Similarly, although two base pairs differed between primers A and B, if mutation A was present, primer B could still bind. In this case,

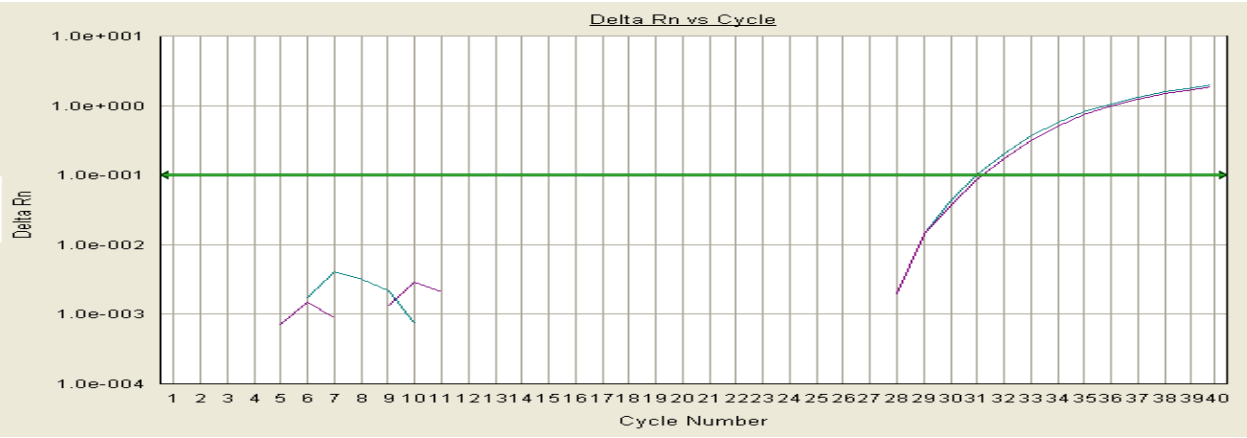
the Ct values were higher than that obtained for types A and D (Fig 3.15b) indicating that the two base pair difference may have caused a lower specificity to the target sequence.

Increasing the annealing temperature of the qPCR to 63$^{o}$C to make the primers more specific did not improve the results. Therefore, it was not possible to determine the exact *NPM1* mutation present in mutated cases using the qPCR. This would require an improved and more specific primer design, however, would be unlikely to add any valuable clinical information. Despite these observations regarding the reduced primer specificity of the qPCR, this will only impact on cases which have *NPM1* mutations, since when the qPCR was performed as a multiplex PCR, no amplification curves were seen for any wild-type *NPM1* samples, but only with those who had *NPM1* mutations.

(a) *NPM1* Mutation type A




(b) *NPM1* Mutation type B




(c) *NPM1* Mutation type D

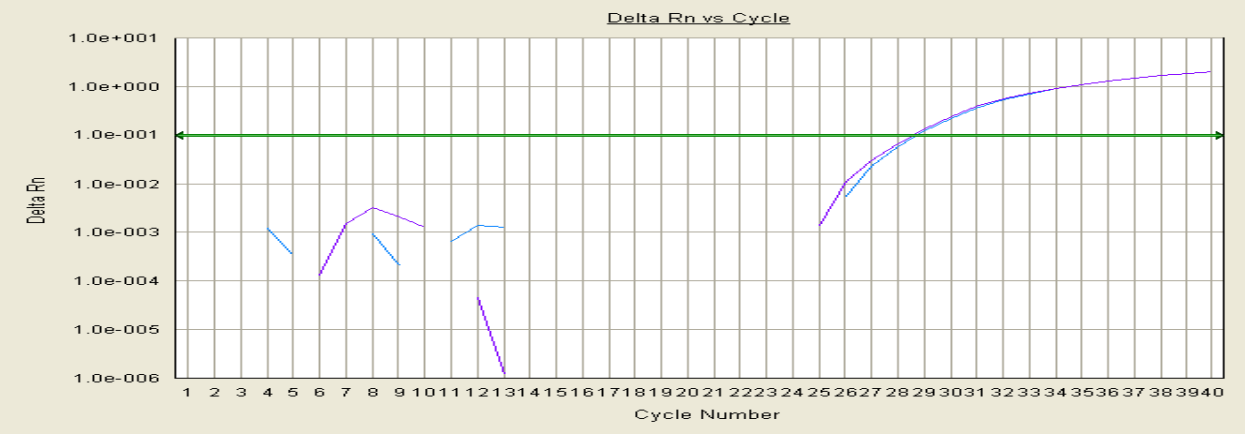



Figure 3.15 Amplification plots for *NPM1* mutated sample UPN9 from the mutation-specific, singleplex *NPM1* qPCR for mutation types A, B and D. (a) Mutation type A amplification curve (Ct=26.46) (b) Mutation type B amplification curve with the highest Ct value (Ct=31.07) indicating the least efficient amplification of this type during PCR and (c) Mutation type D amplification curve (Ct=28.58). The X-axis and Y-axis represent the Cycle number and Delta Rn (Rn minus baseline), respectively. The baseline is represented by the green line (set to 0.1)

### 3.6.3 Multiplex PCR and CE vs. Multiplex, mutation-specific Real-Time qPCR to detect *NPM1* mutations

Overall, one sample in the cohort had discrepant results between the two techniques. Although the qPCR has a much higher reported sensitivity than that for the multiplex PCR and CE method (0.1% vs. 5%, respectively)[135,136] it is disadvantaged by a non-specific primer design and can also give false-negative results in cases where rarer mutations occur. As such, the multiplex PCR and CE method, which can detect all *NPM1* exon 12 mutations, is a more suitable technique in testing for mutations in this gene.

## 3.7 *IDH1*, *IDH2*, *NPM1* AND *FLT3*-ITD MUTATIONS - PAEDIATRICS VS. ADULTS

### 3.7.1 Paediatric CN-AML

Nine paediatric patients were available for the study. No *IDH2* mutations were observed in any paediatric patients. Overall, 1 (11.1%) paediatric patient had an $IDH1^{R132}$ mutation, 4 (44.4%) had an *NPM1* mutation and 1 (11.1%) had a *FLT3*-ITD.

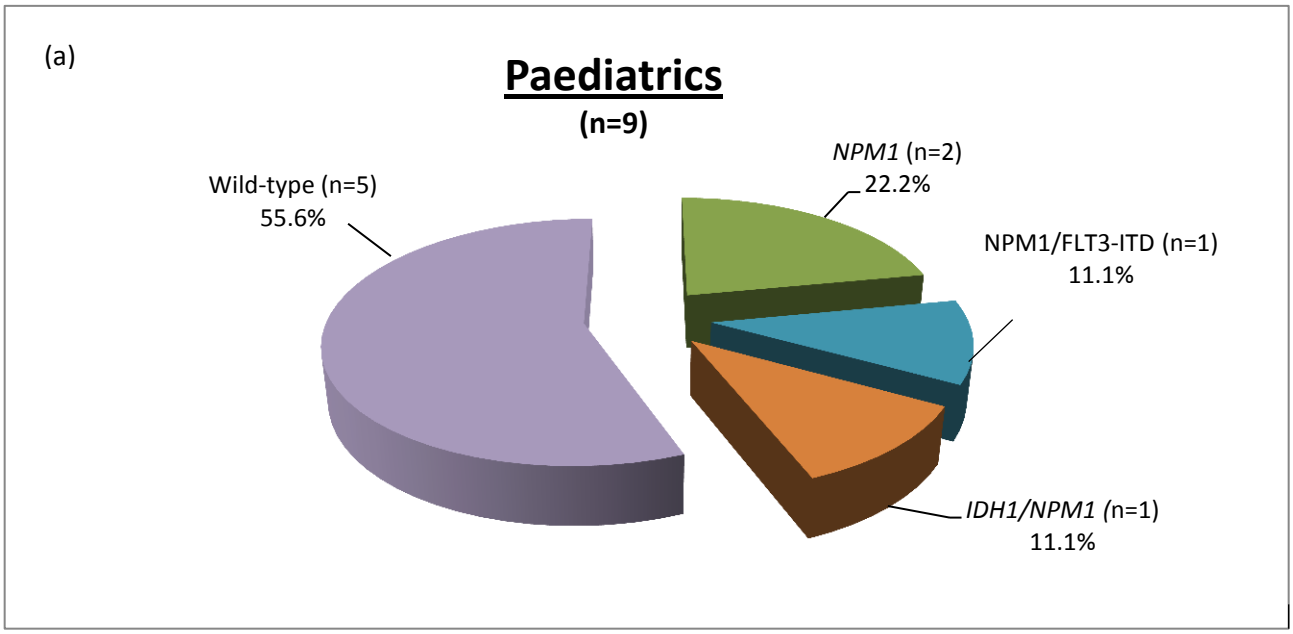
Of these, 1 (11.1%) paediatric patient had an $IDH1^{R132}$/$NPM1^{mut}$ and 1 (11.1%) had an $NPM1^{mut}$/*FLT3*-ITD mutation concurrently (Fig 3.16a). Two (22.2%) paediatric patients only had an *NPM1* mutation while the remaining 5 (55.6%) patients were wild-type for all mutations.

### 3.7.2 Adult CN-AML

From the 61 adult CN-AML patients, 5 (8.2%) had an *IDH1*$^{R132}$, 4 (6.6%) had an *IDH2*$^{R140}$, 1 (1.6%) had an *IDH2*$^{R172}$, 15 (24.6%) had an *NPM1* mutation and 11 (18%) had a *FLT3*-ITD.

Of these, 1 (1.6%) adult patient each had an *IDH1*$^{R132}$/*NPM1*$^{mut}$, *IDH2*$^{R140}$/*FLT3*-ITD, *IDH1*$^{R132}$/*NPM1*$^{mut}$/*FLT3*-ITD, *IDH2*$^{R140}$/*NPM1*$^{mut}$/*FLT3-ITD* and *NPM1*$^{mut}$/*FLT3*-ITD mutation concurrently (Fig 3.16b). Three (4.9%) patients each only had an *IDH1* or *IDH2* mutation, while 11 (18%) patients had an *NPM1* mutation and 7 (11.5%) patients had a *FLT3*-ITD only. Overall, 32/61 (52.5%) patients were wild-type for all mutations.

Patient demographics of the paediatric and adult CN-AML cohorts are given in Table 3.3 and Table 3.4, respectively.



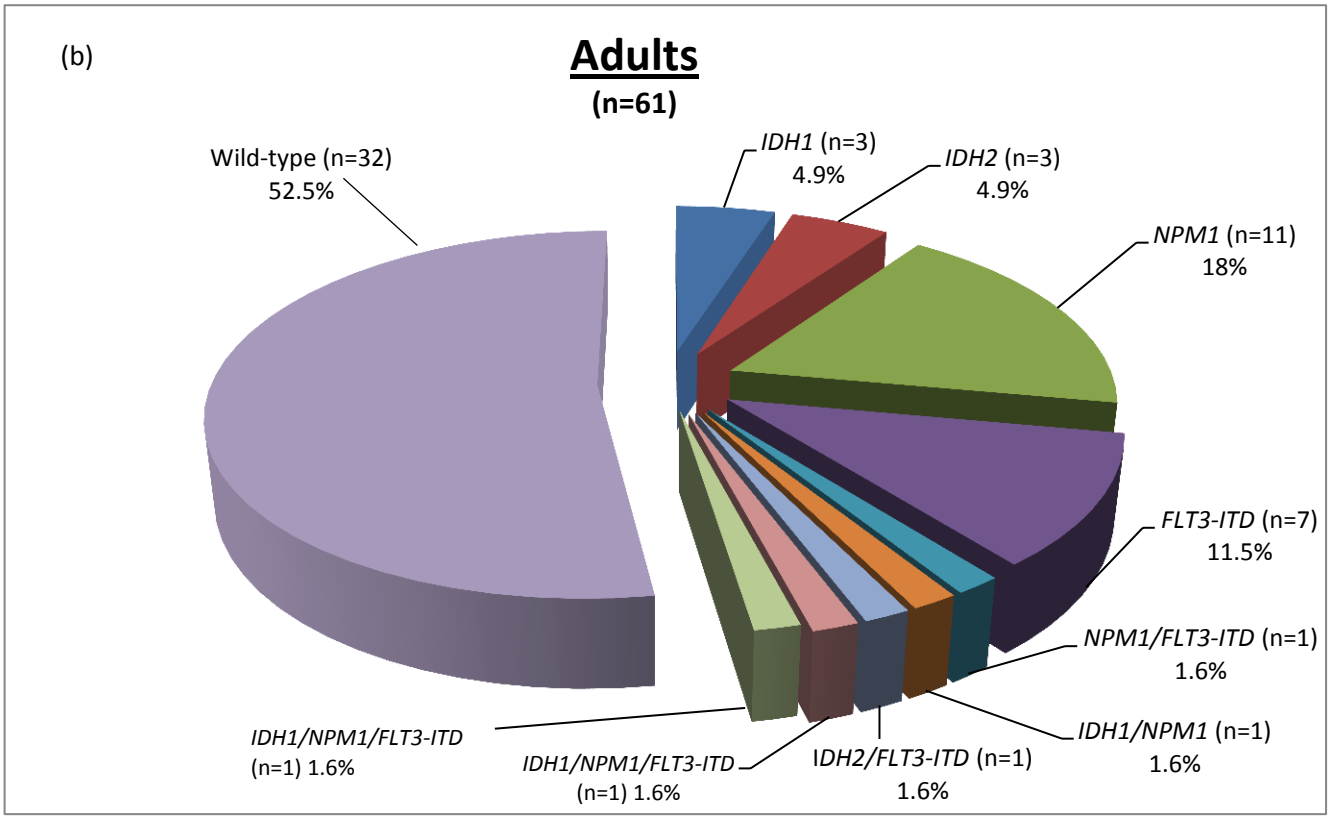




Figure 3.16 Pie charts showing the distribution of *IDH1*, *IDH2*, *NPM1* and *FLT3*-ITD mutations in the (a) Paediatric and (b) Adult CN-AML cohorts. (a) The distribution and concurrence of mutations in the paediatric cohort (n=9). No *IDH2* mutations were observed in any paediatric patients. (b) The distribution and concurrence of mutations in the adult cohort (n=61). All mutations except *IDH1* and *IDH2* occurred concurrently.

Table 3.3 Patient Demographics of the Paediatric CN-AML cohort.

**PAEDIATRIC**

| **≤16 years** | | **Whole cohort (n=9)** | | ***IDH1*$^{wt}$ (n=8)** | | ***IDH1-R132*$^{mut}$ (n=1)** | | ***IDH2*-R140$^{mut}$ (n=0)** | | ***IDH2*-R172$^{mut}$ (n=0)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | n | % | n | % | n | % | n | % | n | % |
| **Gender** | | | | | | | | | | | |
| | Male | 3 | 33.3 | 3 | 37.5 | 0 | 0 | - | | - | |
| | Female | 6 | 66.7 | 5 | 62.5 | 1 | 100 | - | | - | |
| **Age, median (range)** | | 11 (1-15) | | 10.5 (1-15) | | 15 (15) | | - | | - | |
| **Types of *IDH1* mutations** | | 1 | 11.1 | | | | | | | | |
| | R132H | - | | - | | 1 | 100 | - | | - | |
| | R132C | - | | - | | 0 | 0 | - | | - | |
| | R132S | - | | - | | 0 | 0 | - | | - | |
| | R132G | - | | - | | 0 | 0 | - | | - | |
| | R132L | - | | - | | 0 | 0 | - | | - | |
| **Types of *IDH2* mutations** | | 0 | 0 | - | | - | | 0 | 0 | 0 | 0 |
| ***NPM1*** | | | | | | | | | | | |
| | Wild-type | 5 | 55.6 | 5 | 62.5 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Mutated | 4 | 44.4 | 3 | 37.5 | 1 | 100 | 0 | 0 | 0 | 0 |
| ***FLT3*-ITD** | | | | | | | | | | | |
| | Wild-type | 8 | 88.9 | 7 | 87.5 | 1 | 100 | 0 | 0 | 0 | 0 |
| | Mutated | 1 | 11.1 | 1 | 12.5 | 0 | 0 | 0 | 0 | 0 | 0 |

Mut=mutated, wt=wild-type, n=number, Amino acid abbreviations: C=Cysteine, G=Glycine, H=Histidine, K=Lysine, L=Leucine, S=Serine,

Table 3.4 Patient Demographics of the Adult CN-AML cohort.

| **ADULT** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **>16 years** | | **Whole cohort (n=61)** | | ***IDH*$^{wt}$ (n=51)** | | ***IDH1-R132*$^{mut}$ (n=5)** | | ***IDH2*-R140$^{mut}$ (n=4)** | | ***IDH2*-R172$^{mut}$ (n=1)** | |
| | | n | % | n | % | n | % | n | % | n | % |
| **Gender** | | | | | | | | | | | |
| | Male | 28 | 45.9 | 23 | 45 | 2 | 40 | 3 | 75 | 0 | 0 |
| | Female | 33 | 54.1 | 28 | 55 | 3 | 60 | 1 | 25 | 1 | 100 |
| **Age, median (range)** | | 51 (17-93) | | 51 (17-93) | | 53 (19-64) | | 48 (41-74) | | 66 (66) | |
| **Types of *IDH1* mutations** | | 5 | 8.2 | | | | | | | | |
| | R132H | | - | | - | 2 | 40 | | - | | - |
| | R132C | | - | | - | 3 | 60 | | - | | - |
| | R132S | | - | | - | 0 | 0 | | - | | - |
| | R132G | | - | | - | 0 | 0 | | - | | - |
| | R132L | | - | | - | 0 | 0 | | - | | - |
| **Types of *IDH2* mutations** | | 5 | 8.2 | | | | | | | | |
| | R140Q | | - | | - | | - | 4 | 100 | | - |
| | R140L | | - | | - | | - | 0 | 0 | | - |
| | R140W | | - | | - | | - | 0 | 0 | | - |
| | R172K | | - | | - | | - | | - | 1 | 100 |
| | R172M | | - | | - | | - | | - | 0 | 0 |
| | R172S | | - | | - | | - | | - | 0 | 0 |
| ***NPM1*** | | | | | | | | | | | |
| | Wild-type | 46 | 75.4 | 43 | 76.8 | 3 | 60 | 3 | 75 | 1 | 100 |
| | Mutated | 15 | 24.6 | 13 | 23.2 | 2 | 40 | 1 | 25 | 0 | 0 |
| ***FLT3*-ITD** | | | | | | | | | | | |
| | Wild-type | 50 | 82 | 46 | 82.1 | 4 | 80 | 2 | 50 | 1 | 100 |
| | Mutated | 11 | 18 | 10 | 17.9 | 1 | 20 | 2 | 50 | 0 | 0 |

Mut=mutated, wt=wild-type, n=number, Amino acid abbreviations: C=Cysteine, G=Glycine, H=Histidine, K=Lysine, L=Leucine, S=Serine, Q=Glutamine, R=Arginine, W=Tryptophan, M=Methionine

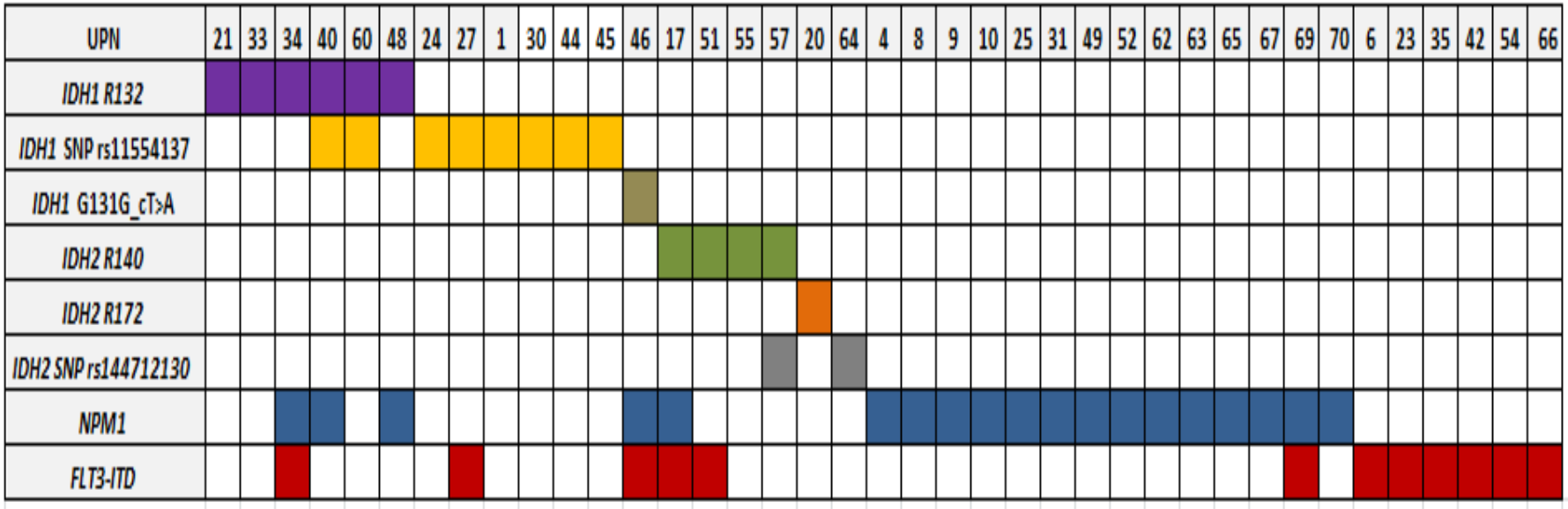
## 3.8 RESULT SUMMARY FOR *IDH*, *NPM1* AND *FLT3-ITD* MUTATIONS

From the CN-AML cohort, 6 (8.6%) patients had *IDH1*$^{R132}$ mutations, 4 patients (5.7%) had *IDH2*$^{R140}$ mutations, 1 (1.4%) patient had the *IDH2*$^{R172}$ mutation, 19 patients (27.1%) had *NPM1* mutations and 12 (17.1%) patients had the *FLT3*-ITD. The mutation pattern and their concurrence are shown in Table 3.5.

*IDH1* and *IDH2* mutations were mutually exclusive with each other. The low-risk favourable genotype, *NPM1*$^{mut}$/*FLT3*-ITD$^{neg}$ was seen in 15 (21.4%) patients of which 2 of these also had concurrent *IDH1*$^{R132}$ mutations. *IDH1* SNP rs11554137 was found in 8 (11.4%) patients and two additional types of silent mutations, SNP rs144712130 in *IDH2* and G131G_cT>A in *IDH1*, were seen in 3 other patients.

The complete results from the entire CN-AML cohort are presented in Table 3.6 with all the mutation-positive patients in Table 3.7.

Table 3.5 Mutation Patterns of the CN-AML patients who had one or more mutated genes.




The coloured blocks indicate the presence of the mutation while the clear blocks show the absence of the mutation. *IDH1* and *IDH2* mutations are mutually exclusive with each other. Unlike as seen with *IDH1*$^{R132}$ and *IDH2*$^{R140}$, *IDH2*$^{R172}$ does not have a concurrent *NPM1* or *FLT3*-ITD mutation. Patient samples not shown (n=31) indicated they were wild-type for these genes. Samples are organized according to *IDH1* mutation status, then by *IDH2*, *NPM1* and *FLT3*-ITD. UPN=unique patient number, AML=Acute Myeloid Leukaemia

Table 3.6 Complete patient results from the entire CN-AML cohort.

| UPN | Age/Gender | Ethnicity | *IDH1* | | *IDH2* | | *NPM1* | *FLT3* | Mutation Summary |
|---|---|---|---|---|---|---|---|---|---|
| | | | SNP rs11554137 | R132 | 140 | 172 | | | |
| 1 | 17/F | *A* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 2 | 35/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 3 | 49/F | *C* | wt | wt | wt | wt | wt | wt | - |
| 4 | 48/M | I | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 5 | 93/M | C | wt | wt | wt | wt | wt | wt | - |
| 6 | 24/M | *A* | wt | wt | wt | wt | wt | **ITD** | *FLT3-ITD* |
| 7 | 58/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 8 | 51/M | *C* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 9 | 68/M | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 10 | 21/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 11 | 64/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 12 | 1/F | U | wt | wt | wt | wt | wt | wt | - |
| 13 | 18/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 14 | 51/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 15 | 35/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 16 | 59/F | U | wt | wt | wt | wt | wt | wt | - |
| 17 | 52/M | *C* | wt | wt | **R140Q** | wt | **+4bp** | **ITD** | *IDH2* R140Q, *NPM1, FLT3-ITD* |
| 18 | 20/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 19 | 60/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 20 | 66/F | *A* | wt | wt | wt | **R172K** | wt | wt | *IDH2* R172K |
| 21 | 54/M | *C* | wt | **R132C** | wt | wt | wt | wt | *IDH1* R132C |
| 22 | 62/M | *A* | wt | wt | wt | wt | wt | wt | - |
| 23 | 20/F | *A* | wt | wt | wt | wt | wt | **ITD** | *FLT3-ITD* |
| 24 | 41/F | *A* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 25 | 14/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 26 | 57/F | U | wt | wt | wt | wt | wt | wt | - |
| 27 | 75/F | *C* | **C/T** | wt | wt | wt | wt | **ITD** | *IDH1* SNP rs11554137, *FLT3-ITD* |
| 28 | 59/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 29 | 29/F | *C* | wt | wt | wt | wt | wt | wt | - |
| 30 | 60/M | *C* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 31 | 51/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 32 | 15/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 33 | 53/F | *A* | wt | **R132H** | wt | wt | wt | wt | *IDH1* R132H |
| 34 | 19/M | *A* | wt | **R132C** | wt | wt | **+4bp** | **ITD** | *IDH1* R132C, *NPM1, FLT3*-ITD |

| UPN | Age/Gender | Ethnicity | *IDH1* | | *IDH2* | | *NPM1* | *FLT3* | Mutation Summary |
|---|---|---|---|---|---|---|---|---|---|
| | | | SNP rs11554137 | R 132 | 140 | 172 | | | |
| 35 | 63/M | *A* | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| 36 | 63/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 37 | 28/M | I | wt | wt | wt | wt | wt | wt | - |
| 38 | 6/M | I | wt | wt | wt | wt | wt | wt | - |
| 39 | 22/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 40 | 42/F | *C* | **C/T** | **R132H** | wt | wt | **+4bp** | wt | *IDH1* SNP rs11554137,*IDH1* R132H, *NPM1* |
| 41 | 71/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 42 | 59/F | *C* | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| 43 | 5/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 44 | 60/F | *A* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 45 | 27/F | *A* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 46 | 10/F | *C* | wt | **G131G_cT-A** | wt | wt | **+4bp** | **ITD** | *IDH1* SNP[#], *NPM1,FLT3*-ITD |
| 47 | 14/M | *A* | wt | wt | wt | wt | wt | wt | - |
| 48 | 15/F | *A* | wt | **R132H** | wt | wt | **+4bp** | wt | *IDH1* R132H, *NPM1* |
| 49 | 66/M | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 50 | 42/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 51 | 74/F | *A* | wt | wt | **R140Q** | wt | wt | **ITD** | *IDH2* R140Q, *FLT3*-ITD |
| 52 | 39/M | *C* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 53 | 55/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 54 | 46/F | *C* | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| 55 | 41/M | U | wt | wt | **R140Q** | wt | wt | wt | *IDH2* R140Q |
| 56 | 68/M | *A* | wt | wt | wt | wt | wt | wt | - |
| 57 | 44/M | U | wt | wt | **R140Q and L143L_cG>C** | wt | wt | wt | *IDH2* R140Q, *IDH2* SNP* |
| 58 | 37/F | MA | wt | wt | wt | wt | wt | wt | - |
| 59 | 74/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 60 | 64/F | *C* | **C/T** | **R132C** | wt | wt | wt | wt | *IDH1* SNP rs11554137, *IDH1* R132C |
| 61 | 46/F | *A* | wt | wt | wt | wt | wt | wt | - |
| 62 | 39/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 63 | 11/M | U | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 64 | 28/F | *A* | wt | wt | **L143L_cG>C** | wt | wt | wt | *IDH2* SNP* |
| 65 | 84/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 66 | 29/M | I | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| *67* | 40/M | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| *68* | 75/M | *C* | wt | wt | wt | wt | wt | wt | - |
| 69 | 86/F | *C* | wt | wt | wt | wt | **+4bp** | **ITD** | *NPM1, FLT3-ITD* |
| 70 | 69/F | *C* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |

UPN=unique patient number, F=female, M=male, *A*=African, *C*=Caucasian, MA=Mixed ancestry, I=Indian, U=unknown, wt=wild-type, bp=base pair, SNP*= SNP rs144712130 in *IDH2*, SNP[#]=G131G in *IDH1.* Nucleotide abbreviations: A=Adenine, C=Cytosine, G=Guanine, T=Thymine; Amino acid abbreviations: C=Cysteine, G=Glycine, H=Histidine, K=Lysine, L=Leucine, S=Serine, Q=Glutamine, R=Arginine, W=Tryptophan, M=Methionine

Table 3.7 Results from the CN-AML patients who had one or more mutations in *IDH1, IDH2, NPM1 and/or FLT3*.

| UPN | Age/Gender | Ethnicity | SNP rs11554137 | *IDH1* R 132 | *IDH2* 140 | *IDH2* 172 | *NPM1* | *FLT3* | Mutation Summary |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 17/F | *A* | **C/T** | wt | wt | wt | wt | **ITD** | *IDH1* SNP rs11554137 |
| 4 | 48/M | I | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 6 | 24/M | *A* | wt | wt | wt | wt | wt | **ITD** | *FLT3-ITD* |
| 8 | 51/M | *C* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 9 | 68/M | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 10 | 21/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 17 | 52/M | *C* | wt | wt | **R140Q** | wt | **+4bp** | **ITD** | *IDH2* R140Q, *NPM1, FLT3-ITD* |
| 20 | 66/F | *A* | wt | wt | wt | **R172K** | wt | wt | *IDH2* R172K |
| 21 | 54/M | *A* | wt | **R132C** | wt | wt | wt | wt | *IDH1* R132C |
| 23 | 20/F | *A* | wt | wt | wt | wt | wt | **ITD** | *FLT3-ITD* |
| 24 | 41/F | *A* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 25 | 14/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 27 | 75/F | *C* | **C/T** | wt | wt | wt | wt | **ITD** | *IDH1* SNP rs11554137, *FLT3-ITD* |
| 30 | 60/M | *C* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 31 | 51/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 33 | 53/F | *A* | wt | **R132H** | wt | wt | wt | wt | *IDH1* R132H |
| 34 | 19/M | *A* | wt | **R132C** | wt | wt | **+4bp** | **ITD** | *IDH1* R132C, *NPM1,FLT3*-ITD |
| 35 | 63/M | *A* | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| 40 | 42/F | *C* | **C/T** | **R132H** | wt | wt | **+4bp** | wt | *IDH1* SNP rs11554137,*IDH1* R132H, *NPM1* |
| 42 | 59/F | *C* | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| 44 | 60/F | *A* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 45 | 27/F | *A* | **C/T** | wt | wt | wt | wt | wt | *IDH1* SNP rs11554137 |
| 46 | 10/F | *C* | wt | **G131G** | wt | wt | **+4bp** | **ITD** | *IDH1* SNP[#], *NPM1,FLT3*-ITD |
| 48 | 15/F | *A* | wt | **R132H** | wt | wt | **+4bp** | wt | *IDH1* R132H, *NPM1* |
| 49 | 66/M | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 51 | 74/F | *A* | wt | wt | **R140Q** | wt | wt | **ITD** | *IDH2* R140Q, *FLT3*-ITD |
| 52 | 39/M | *C* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 54 | 46/F | *C* | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| 55 | 41/M | U | wt | wt | **R140Q** | wt | wt | wt | *IDH2* R140Q |
| 57 | 44/M | U | wt | wt | **R140Q** | wt | wt | wt | *IDH2* R140Q, IDH2 SNP* |
| 60 | 64/F | *C* | **C/T** | **R132C** | wt | wt | wt | wt | *IDH1* SNP rs11554137, *IDH1* R132C |
| 62 | 39/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 63 | 11/M | U | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 64 | 28/F | *A* | wt | wt | **wt** | wt | wt | wt | *IDH2* SNP* |
| 65 | 84/F | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 66 | 29/M | I | wt | wt | wt | wt | wt | **ITD** | *FLT3*-ITD |
| *67* | 40/M | *A* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |
| 69 | 86/F | *C* | wt | wt | wt | wt | **+4bp** | **ITD** | *NPM1, FLT3-ITD* |
| 70 | 69/F | *C* | wt | wt | wt | wt | **+4bp** | wt | *NPM1* |

UPN=unique patient number, F=female, M=male, *A*=African, *C*=Caucasian, MA=Mixed ancestry, I=Indian, U=unknown, wt=wild-type, bp=base pair, SNP*= SNP rs144712130 in *IDH2*, SNP[#]=G131G in *IDH1*. Nucleotide abbreviations: A=Adenine, C=Cytosine, G=Guanine, T=Thymine; Amino acid abbreviations: C=Cysteine, G=Glycine, H=Histidine, K=Lysine, L=Leucine, S=Serine, Q=Glutamine, R=Arginine, W=Tryptophan, M=Methionine

# 4. DISCUSSION

Mutations in *IDH1* and *IDH2* genes have recently been added to the increasing repertoire of molecular mutations identified in AML. Until now, no study has reported on the frequency of *IDH1* and *IDH2* mutations in the South African population. In our CN-AML cohort, the incidence of *IDH1*$^{R132}$, *IDH2*$^{R140}$ and *IDH2*$^{R172}$ mutations and their occurrence with two other molecular markers, *NPM1* and *FLT3*-ITDs, was determined using a combination of different molecular techniques.

## 4.1 *IDH1* AND *IDH2* MUTATIONS IN SOUTH AFRICAN CN-AML

With different frequencies of genetic mutations, normal karyotypes and clinical outcomes between paediatric and adult AML patients, these groups should be analysed separately.[1,31,70] In this study, *IDH1*, *NPM1* and *FLT3*-ITD mutations were detected in both adult and paediatric patients, however, *IDH2* mutations were only identified in adult patients.

### 4.1.1 Frequency of *IDH* mutations in Adult CN-AML

#### 4.1.1.1 *IDH1* mutations

In our CN-AML cohort, *IDH1*$^{R132}$ mutations were found in 8.2% of adult patients. This correlated with the reported frequencies of 5.4-16% in other adult CN-AML studies.[39,63-65,70,74,80] Table 4.1 shows a comparison of our cohort *IDH1*$^{R132}$ mutation findings with other published studies.

The *IDH1* R132C and R132H mutations are the two most commonly reported *IDH1* R132 mutations in both unselected AML and CN-AML[39,63,65,66,73] and were the only mutations detected in the current study (Table 3.4). Only two studies did not find R132C mutations in their CN-AML cohorts, however, they also had the lowest reported frequencies of *IDH1* R132 mutations in CN-AML of all the studies (Table 4.1).[53,80] These studies reported R132C mutations in one patient each, both of which had cytogenetic abnormalities. Overall, the frequency and type of *IDH1* mutations found in the SA population are similar to those found in international cohorts.

Table 4.1 List of studies and patient details of *IDH1*$^{R132}$ mutations in CN-AML.

| Author (ref) | Publication Year | Region | Types | n | Age | *IDH1*$^{R132}$ in CN-AML | *IDH1*$^{R132}$ / *NPM1*$^{mut}$ | *IDH1*$^{R132}$ / *FLT3-ITD*$^{mut}$ |
|---|---|---|---|---|---|---|---|---|
| Current study | 2013 (thesis) | South Africa | *de novo* CN-AML | 61 | 17-93 (51) | 5/61 (8.2%) | 2/5 (40%) | 1/5 (20%) |
| Nomdedeu (63) | 2012 | Spain | AML | 275 | 17-73 | 15/120 (12.5%) | 7/15 (46.7%) | 4/15 (26.7%) |
| Patel (90) | 2011 | America | all AML (no APL) | 199 | 17-89 (55) | 11/127 (8.7) | 5/11 (45%) | 3/11 (27%) |
| Zhang (53) | 2011 | China | *de novo* AML | 365 | 15-74 (39) | 6/111 (5.4%) | 3/6 (50%) | 0/6 (0%) |
| Ho (80) | 2010 | America | *de novo* AML (no APL) | 274 | 18-88 (63) | 6/85 (7.1%) | 5/6 (83%) | 3/6 (50%) |
| Chou (64) | 2010 | Taiwan | *de novo* AML (no APL) | 493 | 18-90 (53) | 20/227 (8.8%) | 11/20 (55%) | 6/20 (30%) |
| Schnittger (74) | 2010 | Germany | all AML | 1414 | 17-93 (66) | 67/673 (10%) | 37/65 (57%) | 14/64 (22%) |
| Wagner (65) | 2010 | Germany | de novo or secondary CN-AML | 275 | 17-60 (47) | 30/275 (10.9%) | 17/30 (57%) | 4/30 (13%) |
| Marcucci (39) | 2010 | America | *de novo* CN-AML | 358 | 19-83 (61) | 47/358 (13.1%) | 34/48 (71%) | 10/49 (20%) |
| Boissel (70) | 2010 | France | *de novo* AML (no APL) | 520 | 17-70 (48) | 34/213 (16%) | 21/34 (62%) | 6/33 (18%) |

Age ranges and medians are shown. These lists are not all-inclusive as other *IDH1* studies have been published; however, these were excluded based on a lack of data regarding CN-AML being available. Ref=reference, APL=Acute Promyelocytic Leukaemia, ref=reference, n=number, CN=cytogenetically normal, ND=not done, NS=not stated

#### 4.1.1.2 *IDH2* mutations

*IDH2* mutations were found in 8.2% of our adult CN-AML patients (6.6% with *IDH2*$^{R140}$ and 1.6% with *IDH2*$^{R172}$). Reported frequencies in adult CN-AML for *IDH2*$^{R140}$ mutations are from 8.3-15.6%[39,63,78] and for *IDH2*$^{R172}$ mutations from 2-5.9%.[39,63,66,70] Therefore, a lower incidence of both types of *IDH2* mutations was observed in our cohort. This could, however, be due to our lower number of CN-AML patients tested in comparison with other studies. Table 4.2 shows a comparison of our cohort's *IDH2* mutation findings with those from other published studies.

The *IDH2*$^{R140}$ mutations were all R140Q substitutions. This correlated with published data where R140Q mutations are predominantly found.[39,63,68,69] For the *IDH2*$^{R172}$ mutation, the R172K substitution is most common, representing virtually all reported types to date.[39,63,66,68,70] Only one *IDH2*$^{R172}$ mutation was found in our cohort, which was the R172K substitution as expected.

Table 4.2 List of studies and patient details of *IDH2*$^{R140}$ and *IDH2*$^{R172}$ mutations in CN-AML

| Author (ref) | Publication Year | Region | Types | n | Age | *IDH2*$^{R140}$ freq in CN-AML | *IDH2*$^{R140}$ / *NPM1*$^{mut}$ | *IDH2*$^{R140}$ / *FLT3*-ITD$^{mut}$ | *IDH2*$^{R172}$ freq in CN-AML | *IDH2*$^{R172}$ / *NPM1*$^{mut}$ | *IDH2*$^{R172}$ / *FLT3*-ITD$^{mut}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Current study | 2013 (thesis) | South Africa | *de novo* CN-AML | 61 | 17-93 (51) | 4/61 (6.6%) | 1/4 (25%) | 2/4 (50%) | 1/61 (1.6%) | 0 | 0 |
| Nomdedeu (63) | 2012 | Spain | AML | 275 | 17-73 | 10/120 (8.3%) | 6/10 (60%) | 3/10 (30%) | 3/120 (2.5%) | 0 | 0 |
| Patel (66) | 2011 | America | all AML (no APL) | 199 | 17-89 (55) | ND | - | - | 4/196 (2%) | 0 | 0 |
| Boissel (78) | 2011 | France | CN-AML | 205 | NS | 18/205 (8.8%) | 11/18 (61%) | 3/18 (17%) | ND | - | - |
| Boissel (70) | 2010 | France | *de novo* AML (no APL) | 520 | 17-70 (48) | ND | - | - | 12/205 (5.9%) | 0 | 0 |
| Marcucci (39) | 2010 | America | *de novo* CN-AML | 358 | 19-83 (61) | 56/358 (15.6%) | 31/56 (57%) | 15/56 (27%) | 13/358 (3.6%) | 0 | 0 |

Age ranges and medians are shown. These lists are not all-inclusive as other *IDH2* studies have been published; however, these were excluded based on a lack of data regarding CN-AML being available. Ref=reference, APL=Acute Promyelocytic Leukaemia, ref=reference, n=number, CN=cytogenetically normal, ND=not done, NS=not stated

### 4.1.2 Association of *IDH1* and *IDH2* mutations with clinical characteristics

*IDH1* and *IDH2* mutations were detected in African and Caucasian CN-AML patients (and two patients of unknown ethnicity) in our cohort. No mutations were found in the Indian and Mixed ancestry groups, however, they did constitute a small proportion of the cohort overall so this finding was not unexpected. A more representative cohort incorporating a higher proportion of Indian and Mixed ancestry patients would be required for any racial comparisons.

No major differences in ages and gender between *IDH1* and *IDH2* mutated and wild-type patients were seen (Table 3.3 and 3.4). However, this analysis is limited by the small number of mutations detected in the cohort overall. Further work in a larger series of AML and CN-AML South African patients will have to be performed to confirm our findings.

### 4.1.3 Association of *IDH* mutations with *NPM1* and *FLT3*-ITD mutations in adult CN-AML

In our adult CN-AML cohort, 16% (10/61) of patients had *IDH* mutations, 25% (15/61) had *NPM1* mutations and 18% (11/61) had *FLT3*-ITD insertions (Table 3.4). From published data, *NPM1* occurs concurrently with $IDH1^{R132}$ mutations in at least half or more (45-83%) of adult CN-AML patients (Table 4.1).[39,63-66,70,74,80] In the present study, 40% of $IDH1^{R132}$ mutated adult

patients had a concurrent *NPM1* mutation, which was slightly lower than the reported frequencies. *FLT3*-ITD occurs in none to half of *IDH1*$^{R132}$ mutated adult CN-AML patients (Table 4.1).[53,63-66,70,74,80] With 20% of adult patients in our cohort having *IDH1*$^{R132}$ and *FLT3*-ITD mutations concurrently, this correlated with the published frequencies.

In CN-AML with *IDH1*$^{R132}$ mutations, the low-risk genotype, *NPM1*$^{mut}$/*FLT3*-ITD$^{neg}$ has been associated with a high risk of relapse and shorter survival.[39,68,70,72] From published data, the frequency of this genotype ranges from 15-55% of *IDH1*$^{R132}$ mutated adult CN-AML.[39,63,64,65,66,80] In our cohort it was seen in 20% of *IDH1*$^{R132}$ mutated adult patients, comparable to published studies.

For *IDH2* mutations, about two-thirds of adult CN-AML patients have *IDH2*$^{R140}$ and *NPM1* mutations (Table 4.2).[39,63,78] With 25% of the adult patients in our cohort having *IDH2*$^{R140}$ and *NPM1* mutations concurrently, this was almost half than expected. Although our overall frequency of *IDH2*$^{R140}$ mutations was lower, a low concurrence with *NPM1* mutations was also observed. The reported frequencies of *IDH2*$^{R140}$ mutations with *FLT3*-ITDs are 17% to 30% (Table 4.2).[39,63,78] In this study, the 50% observed was higher. These differences could be due to the low number of patients in our cohort as compared with other studies or they could represent true findings which warrant further investigation. No concurrent *NPM1* or *FLT3*-ITD mutations were seen with the *IDH2*$^{R172}$ patient. However, this was expected as studies have

shown virtually no association of *IDH2*$^{R172}$ mutations with other molecular markers in either unselected AML or CN-AML.[39,63,66,70,71]

It was thought that the lower frequency of *IDH1* and *IDH2* mutations in combination with *NPM1* mutations in our cohort could be due to a lower prevalence of *NPM1* mutations themselves as compared to other studies. Indeed, *NPM1* mutations occurred in 25% of adult patients in the present cohort (Table 3.4). With about 46-64% of adult CN-AML reported to have *NPM1* mutations,[99,102-109] their frequency in our cohort was much lower than expected. Even the frequency of *FLT3*-ITDs was lower, with only 18% of adult patients harbouring these mutations (Table 3.4), not the expected third.[104,105,116,120-125]

Unfortunately, no published data on *NPM1* and *FLT3*-ITD mutation frequencies in South Africa is currently available. A recent MMed study in South Africa investigated the frequency of *NPM1* and *FLT3*-ITD mutations in 164 adult AML patients.[148] Of these, 26% of patients had *NPM1* mutations and 21% had *FLT3*-ITDs, which both correlated with the frequencies reported by other studies. From the *FLT3*-ITD cases, 42% had a normal karyotype. This is higher than in other studies where about a third of CN-AML patients have *FLT3* insertions. Although 19% of the *NPM1* mutated patients had a normal karyotype, this is not an accurate indication as half of these patients had no cytogenetic result available. This was due to either the culture failing to grow or the test not being requested by the oncologist. As such, the overall proportion of *NPM1* mutated patients with normal cytogenetics in the cohort could not be determined.[148]

Studies on larger South African cohorts, including AML and CN-AML, would have to be performed in the future to confirm these findings.

### 4.1.4 Frequency of *IDH1* and *IDH2* Mutations in Paediatric CN-AML

Nine paediatric patients were available from the cohort for analysis. One *IDH1*$^{R132}$ mutation was found in this group (Table 3.3). From childhood AML studies, the reported frequencies of *IDH1*$^{R132}$ mutations range from 0-2.4% in AML and from 0-4.9% in CN-AML.[80,83,84,91,92] Table 4.3 shows our cohort findings for *IDH1* and *IDH2* mutations in comparison with other published studies.

No *IDH2* mutations were found in our paediatric cohort. This was not unexpected given the low frequency of these mutations in childhood AML. In AML, only 1.8% of paediatric patients have *IDH2*$^{R140}$ mutations.[83,84,92,149] To date, only one *IDH2*$^{R172}$ mutation has been reported in paediatric AML (Table 4.3).[149] This implies an age-association of this mutation with adult patients predominantly. The *IDH1*$^{R132}$ mutation in our cohort was found in a 15-year old female patient. Both types of *IDH* mutations have not been described in children younger than 3 years, reinforcing the idea that these mutations are somewhat age related.[50]

A meta-analysis was performed of all the childhood AML studies. From a total of 1321 paediatric patients from America, Asia and Europe, 16 (1.2%) *IDH1* and 19 (1.4%) *IDH2* mutations have been reported in total.[80,83,84,91,92,149] However, *IDH* mutations in childhood AML are not significantly associated with a normal karyotype. They also occur in patients with favourable cytogenetic abnormalities such as t(8;21) and t(15;17) or with other abnormal cytogenetic aberrations, which are generally more common in childhood AML.[50] Since only paediatric patients with a normal karyotype were included in this study, this design omitted patients with abnormal cytogenetics who may have had *IDH* mutations.

In paediatric AML, studies, are limited by the low number of *IDH* mutations in this group. As such, diagnostic testing for *IDH* mutations in childhood AML does not have any current value in risk-stratifications for the disease.

Table 4.3 *IDH* mutations in Paediatric AML and CN-AML.

| | | | | | | Whole Cohort | | | CN-AML | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Author (ref)** | **Publication Year** | **Region** | **Types** | **n** | **Age** | ***IDH1*$^{R132}$** | ***IDH2*$^{R140}$** | ***IDH2*$^{R172}$** | ***IDH1*$^{R132}$** | ***IDH2*$^{R140}$** | ***IDH2*$^{R172}$** |
| Current study | 2013 (thesis) | South Africa | *de novo* AML | 9 | 1-15 (11) | - | - | - | 1/9 (11.1%) | 0% | 0% |
| Ho (80) | 2011 | America | *de novo* AML (no APL) | 180 | 0.2-20.8 (9.5) | - | 3/180 (1.7%) | 1/180 (0.6%) | - | 0% | 0% |
| Oki (92) | 2011 | Japan | *de novo* AML | 115 | NS | 1/115 (0.9%) | 0% | 0% | 0% | 0% | 0% |
| Pigazzi (76) | 2011 | Italy | AML (no APL) | 165 | 1.4-17.7 (11.3) | 4/165 (2.4%) | - | - | 3/139 (2.2%) | - | - |
| Damm (83) | 2011 | Netherlands/Germany | AML | 459 | <18 | 8/459 (1.7%) | 10/459 (2.2%) | 0% | NS | NS | 0% |
| Andersson (71) | 2011 | America | AML | 227 | NS | 3/227 (1.3%) | 5/227 (2.2%) | 0% | 2/41 (4.9%) | 2/41 (4.9%) | 0% |
| Ho (149) | 2010 | America | *de novo* AML | 257 | 0-21 (9.8) | 0% | - | - | 0% | - | - |

*IDH* mutation frequencies in the whole AML cohort and the CN-cohort are shown. Age ranges and medians are shown. No cytogenetic result of the one patient with the *IDH2*$^{R172}$ mutation was available. *These studies included patients from the same cohorts. ¥165 patients were initially investigated then an additional 97 cytogenetically-normal paediatric AML patients. Ref=reference, APL=Acute Promyelocytic Leukaemia, n=number, CN=cytogenetically normal, ND=not done, NS-not stated.

### 4.1.5 Association of *IDH* mutations with *NPM1* and *FLT3*-ITD mutations in paediatric CN-AML

Interestingly, 4/9 (44%) paediatric patients in the cohort had *NPM1* mutations, which is almost double the expected frequency.[99,102-109] In contrast, at 11%, the frequency of FLT3-ITD mutated paediatric patients was much lower than the expected 30-40%.[115,119-124] However, these findings are most likely due to the very low cohort size. The one paediatric patient in our cohort with an *IDH1*$^{R132}$ mutation had a concurrent *NPM1* mutation. The occurrence of *NPM1* with *IDH1*$^{R132}$ and *IDH2*$^{R140}$ mutations was suggested in studies of childhood AML.[83,84] Also, a concurrence with *FLT3*-ITD mutations has been observed.[83,91] The only reported paediatric patient to date who had an *IDH2*$^{R172}$ mutation also had a *NPM1* mutation.[150] However, due to the very low frequency of *IDH* mutations in paediatric AML patients, no association with these genetic markers can be made.

### 4.1.6 Single Nucleotide Polymorphisms in *IDH1* and *IDH2*

In this CN-AML cohort, *IDH1* SNP rs11554137 and two other silent mutations were identified.

#### 4.1.6.1 *IDH1* SNP rs11554137

Eight studies have reported on *IDH1* SNP rs11554137 with frequencies varying from 1.3-15.4% in AML and from 0-12% in CN-AML.[65,69,76,83,84,86] In our CN-AML cohort, we found 11.4% of CN-AML patients had SNP rs11554137, which correlated with the published frequencies (Table 4.4).

Table 4.4 *IDH1* SNP rs11554137 frequencies and concurrence with *IDH* mutations in Adult and Paediatric AML and CN-AML

| | | | | | | | | Whole Cohort | |
|---|---|---|---|---|---|---|---|---|---|
| **Author (ref)** | **Publication Year** | **Region** | **Types** | **n** | **Cohort Age** | **Frequency in Cohort** | **Frequency in CN-AML** | **SNP+*IDH1*$^{mut}$** | **SNP+*IDH2*$^{mut}$** |
| **Adult** | | | | | | | | | |
| Current study | 2013 (thesis) | South Africa | *de novo* AML | 61 | 17-93 (51) | - | 8/61 (13.1%) | 2/8 (25%) | 0 (0%) |
| Chotirat (69) | 2012 | Thailand | AML | 230 | 13-86 (44) | 3/230 (1.3%) | NS | NS | NS |
| Ho (86) | 2011 | America | *de novo* AML (no APL) | 274 | 18-88 (63) | 30/274 (11%) | 11/274 (4%) | 0/30 (0%) | ND |
| Ravandi (76) | 2011 | America | AML | 170 | 17-73 (53) | 24/170 (14%) | 17/170 (10%) | 2/24 (8.3%) | 4/24 (16.7%) |
| Wagner (65) | 2010 | Germany | CN-AML | 275 | 17-60 (47) | - | 33/275 (12%) | 3/33 (9.1%) | ND |
| Zou (85) | 2010 | China | AML (+other) | 68 | NS | 1/68 (1.5%) | NS | NS | NS |
| **Paediatric** | | | | | | | | | |
| Current study | 2013 (thesis) | South Africa | *de novo* AML | 9 | 1-15 (11) | - | 0/9 (0%) | 0 | 0 |
| Ho (86) | 2011 | America | AML (no APL) | 253 | 0.07-21.6 (9.8) | 27/253 (11%) | 3/253 (1.2%) | NS | ND |
| Damm (83) | 2011 | Netherlands/Germany | AML | 459 | <18 | 47/460 (10%) | NS | NS | NS |
| Andersson (84) | 2011 | America | AML | 227 | paediatric | 35/227 (15.4%) | NS | NS | NS |

Frequencies of *IDH1* SNP rs11554137 in the entire AML cohort and in the CN-AML cohort are shown. Age ranges and medians are shown. The frequencies of the SNP with *IDH* mutations (where available) could only be determined for the whole cohort and not for CN-AML. Ref=reference, SNP=SNP rs11554137 APL=Acute Promyelocytic Leukaemia, n=number, mut=mutated, CN=cytogenetically normal, ND=not done, NS-not stated.

All substitutions were heterozygous as expected. In our cohort, although 25% of SNP rs11554137-positive patients also had an *IDH1*$^{R132}$, this high proportion is unreliable given the low number of patients analysed and since previously reported frequencies of concurrent *IDH1*$^{R132}$ mutations with SNP rs11554137 are low (0-9.1%).[65,76,80] While all the SNP rs11554137-positive patients in our cohort were adults, similar frequencies are reported in paediatric and adult AML.[76, 83,84,86]

Ravandi et al.[76] reported a strong association between SNP rs11554137 and a normal karyotype – in 17 of 24 (71%) AML patients, however, this is the only paper to comment on this finding. In contrast, Wagner et al.[65] found SNP rs11554137 at nearly equal frequencies in non CN-AML (12.9%) and CN-AML (12%). Ho et al.[80] found a higher prevalence of SNP rs11554137 in AML patients with an abnormal karyotype. Therefore, an association of SNP rs11554137 with CN-AML is unlikely.

Studies of AML patients from Asian, European and American cohorts have indicated a potential ethnic association of SNP rs11554137, with Asian populations showing a minimal prevalence compared to Europeans and Americans. Zou et al.[85] found SNP rs11554137 in only 2 out of 456 Chinese patients with various haematological diseases (including 68 AML patients) and none in 270 Chinese healthy controls. Similarly, Chotirat et al.[69] found SNP rs11554137 in only 3 out of 230 AML patients from Thailand. When only non-Asian cohorts are included (i.e. from America and Germany), SNP rs11554137 frequencies are similar (10-

15.4%) between these populations.[76,83,84,86] A germline genetic diversity between Asian and non-Asian people may explain this difference in frequencies, since *IDH* mutation frequencies in Asian AML cohorts are comparable to their western counterparts.[69,75]

In further support of an ethnic association, the SNP is more prevalent in individuals of African descent. Ho et al.[95] found a number of black adult AML patients (30%) in their cohort were positive for SNP rs11554137. In the present study, half (4/8) of the SNP rs11554137-positive patients were African and the remainder Caucasian. Although this polymorphism was not found in the Indian and Mixed ancestry groups, they did represent a small proportion of this cohort overall. A larger South African AML cohort would have to be tested for any comparisons.

Given similar SNP rs11554137 frequencies between AML patients and healthy volunteers, and since no studies have conclusively confirmed any clinical impact of SNP rs11554137 in these patients, it probably has no prognostic significance in AML. Although this polymorphism may be associated with other currently unidentified mechanisms in leukaemogenesis, particularly in protein translation and function due to its alteration to the coding sequence, the role it plays in AML, if any, remains to be established. Until then, routine analysis for SNP rs11554137 should not be included in the diagnostic repertoire of testing.

#### 4.1.6.2 *IDH2* SNP rs144712130 and an *IDH1* G131G silent mutation

Two patients in our cohort had a silent mutation in codon 143 of the *IDH2* gene. This was SNP rs144712130, which was previously reported in two studies.[84,90] In adult AML, Patel et al.[90] found 2 out of 146 patients (1.4%) had SNP rs144712130, while in paediatric AML, Andersson et al.[84] found 2 out of 227 (0.9%) patients had SNP rs144712130. With 2.9% of our CN-AML patients having SNP rs144712130, its overall frequency is very low.

One patient in our cohort had a silent mutation in codon 131 of the *IDH1* gene. This mutation had also been previously reported by Patel et al.[90] who found it in 1 out of 146 (0.7%) adult AML patients. Other papers have not reported this finding in their AML cohorts, and with a frequency of 1.4% in our cohort it most likely occurs in a small proportion of patients overall.

### 4.1.7 PROGNOSTIC SIGNIFICANCE OF *IDH* MUTATIONS IN AML

Unfortunately, due to lack of available clinical data for the patients in this study cohort, no analysis of outcomes could be performed. As such, although the frequencies of *IDH1* and *IDH2* mutations could be determined, it was not possible to establish their prognostic impact whether alone, or in combination with *NPM1* and *FLT3*-ITD mutations

With current *IDH* prognostic impacts having been based on observational studies and retrospective analysis, future prospective studies or randomized trials are needed.[68] However, the studies to date demonstrate that *IDH* mutations have a likely unfavourable (or possibly favourable in the case of *IDH2*$^{R140}$ mutations) prognostic impact, which warrants further investigations and implementation in a routine diagnostic set up.

## 4.2 ASSESSMENT OF EXPERIMENTAL TECHNIQUES

In the current cohort, different testing methodologies were used to test for *IDH*, *NPM1* and *FLT3*-ITD mutations. For each test, its limitations, time to obtain a result and ease of use must be considered when deciding which technique to use.

### 4.2.1 Singleplex PCR and Sanger Sequencing vs. Mutation-specific, Multiplex PCR to detect *IDH1* mutations

From published studies, screening for *IDH* mutations is commonly performed by PCR of exon 4 followed by direct sequencing.[39,70,75,76] However, a more sensitive, less labourious and cost-efficient method would be preferable.

Chou et al.[129] developed the mutation-specific, multiplex PCR using an AML cohort which had been previously tested for *IDH1*$^{R132}$ mutations with a singleplex PCR followed by sequencing.

They determined their sensitivity of this method to be 0.5% (i.e. 1:200). This was much higher than that for sequencing where they could only reliably detect *IDH1*$^{R132}$ mutations in up to 1:10 (10%) of wild-type alleles.[128] When our cohort was tested using both techniques, near-perfect concordance between the two methods was seen. The one discordant result (from sample UPN46) was due to a silent mutation directly upstream of codon R132 that affected the binding of some of the mutation-specific, multiplex PCR primers. From the other *IDH1* studies published to date, this silent mutation was previously reported in only one other patient,[90] therefore, is most likely very rare and as such, this false-positive finding with the *IDH1*$^{R132}$ multiplex PCR is unlikely to be a common occurrence.

Unlike with sequencing, the multiplex PCR and gel electrophoresis method cannot distinguish between the different types of *IDH1*$^{R132}$ mutations. However, it is thought that it is the replacement of arginine, rather than with which amino acid that has a clinical impact.[50] Therefore, determining the specific *IDH1*$^{R132}$ mutation present would be unlikely to add any clinical value.

Since mutation-specific, multiplex *IDH1*$^{R132}$ PCR and gel electrophoresis is a much more sensitive, faster and time efficient method than the singleplex PCR and sequencing, it is the preferred method in testing for these mutations (Table 4.5).

Table 4.5 Comparison of the Mutation-specific, multiplex *IDH1*$^{R132}$ PCR and Gel electrophoresis method with the Singleplex PCR and Sequencing to detect *IDH1*$^{R132}$ mutations

| ***IDH1***$^{R132}$ | **Mutation-specific, Multiplex PCR/AGE** | **Singleplex PCR/ Sequencing** |
|---|---|---|
| Analytical sensitivity$^{\dagger}$ | 1:200 | 1:10 |
| Time to result$^{\beta}$ | ±4 hours | ±11 hours |
| Use for MRD | Very sensitive | Not recommended |
| *IDH1*$^{R132}$ mutations detected | All types | All types |
| Nature of result | Qualitative | Qualitative |

$^{\dagger}$as determined by Chou et al.[129] MRD=minimal residual disease, $^{\beta}$=for 1 sample.

### 4.2.2 Multiplex PCR and CE vs. Mutation-specific, Multiplex real-time qPCR to detect *NPM1* mutations

A number of molecular tests are available to test for *NPM1* mutations in genomic DNA. One such method is PCR of the mutational region followed by CE.[104] Although quantitative methods are used to assess *NPM1* mutation burden and for MRD, the clinical value of this has yet to be determined.[135]

Although CE requires no complex analysis, when mutant allele levels are low and/or non-specific background peaks occur, this may interfere with accurate analysis of the results. For the present study, by increasing the dilution of the PCR product to 1:15, very little background noise was seen. All samples from the cohort amplified *NPM1* wild-type peaks higher than the minimum recommended limit of 175 RFU (this limit is based on the specific genetic analyzer

used).[138] Also, the peak heights of the *NPM1* wild-type and mutant alleles were comparable, giving a mean ratio of 1.2 (range 0.85-2.13). This showed that the level of mutant cells in the *NPM1* mutated samples were relatively high. This could, however, be due to the inclusion of only presentation patients in our cohort who are expected to have higher levels of disease.

Huang et al.[134] determined their sensitivity of this multiplex PCR and CE assay to be 1:20 (i.e. 5%). They also used the same primers in a singleplex PCR with CE for *NPM1* and *FLT3* separately and found no differences in the individual sensitivities when compared with multiplexing. Barakat et al.,[135] who designed the mutation-specific, multiplex *NPM1* qPCR assay, determined its sensitivity to be at least 1:1000 (0.1%). Although this is much higher than that for the multiplex PCR and CE assay, all samples, apart from one, in our cohort that were positive for *NPM1* mutations on CE were also positive with the qPCR.

An unexpected finding of the qPCR was that the A, B and D mutation-specific primers had reduced specificity for their particular mutation type. Fortunately, this did not affect the ability of the assay to detect *NPM1* mutations, which was its primary aim. Designed to be preferentially used as a multiplex PCR, when all mutation-specific primers were incorporated in a single reaction mix, the expected amplification curve was seen in *NPM1* mutated cases. However, in the singleplex PCR, reduced primer specificity was seen where all *NPM1* mutated cases amplified types A, B and D when these primers were used individually. As the 3' ends of the primers for types E, G and H are dissimilar (Fig 2.8), they would have much higher

specificity for their mutation type. As such, rarer 4bp mutations may actually be present in *NPM1*-positive samples, as the primers for the A, B and D mutations may potentially still bind to and amplify these rare mutations e.g. the tetranucleotide insertion sequence for types I, K, L, N, R varies from that for types A, B and D by one nucleotide each.[93] Therefore, although only 6 types of mutations are specifically tested for with the qPCR, rarer 4bp types may also be detected. However, since the analytical sensitivity of the qPCR was 100% (i.e. no wild-type *NPM1* sequences were co-amplified), this assay should still be a suitable for determining *NPM1* mutation status.

The one discordant result (from sample UPN10) that caused a false-negative result with the qPCR was most likely due to it being one of the rarer *NPM1* mutation types that could not be amplified even with the reduced specificity of the mutation-specific primers. Unfortunately, attempts to sequence this sample resulted in noisy sequence reads; hence the exact mutation could not be determined. Sequencing of other *NPM1* mutated samples also yielded noisy, unanalysable results, which was not improved after several optimization attempts. Since the same samples were used for sequencing other genes, such as *IDH1* and *IDH2*, which yielded good electropherograms with little noise, this phenomenon appears specific to *NPM1*. In *NPM1*-mutated samples, a wild-type sequence with a mutated sequence showing a 4 base-pair shift was expected. However, even *NPM1* wild-type sequences yielded noisy sequence data. In the reference sequence (NG_016018),[131] no repeat mono-nucleotide regions were identifiable which could have cause DNA polymerase slippage. Therefore, no reason could be established for this occurrence.

With a much higher reported sensitivity than the multiplex PCR and CE, the main value of the qPCR is in MRD monitoring. It also has a faster turn-around-time than the multiplex PCR and CE, however, TaqMan® probes are expensive. The one false-negative result obtained with the qPCR is concerning. Without an alternative test being performed, this would have been missed. With the multiplex PCR and CE, all *NPM1* exon 12 mutations can be detected, and it is also relatively time saving. Also, since several different genes can be tested for simultaneously, this further reduces time to obtain a result. Therefore, the multiplex PCR and CE remains the preferred method to use in testing for *NPM1* mutations (Table 4.6).

Table 4.6 Comparison of the Multiplex PCR and CE method with the Mutation-specific, multiplex real-time qPCR to detect *NPM1* mutations

| **NPM1** | **Multiplex PCR/ CE** | **Mutation-specific, Multiplex qPCR** |
|---|---|---|
| Analytical sensitivity[†] | 1:20 | >1:1000 |
| Time to result[β] | ±5 hours | ±3 hours |
| Use for MRD | Less sensitive | Very sensitive |
| Mutations detected | All in exon 12 | 6 types (A,B,D,E,G,H)[£] |
| Nature of result | Semi-quantitative | Quantitative[#] |

[†]as determined by Huang et al.[134] and Barakat et al.[135] , [β]=for 1 sample, £=including other rare +4bp mutations, [#]=with the use of a standard curve for *NPM1* and the control gene. MRD=minimal residual disease.

### 4.2.3 Multiplex PCR and CE vs. AGE to detect *FLT3-ITD* mutations

The sensitivity of the multiplex PCR and CE assay to detect *FLT3*-ITD mutations was reported to be 1:20 (i.e. 5%).[134] The lower detection limit identifies 10% of disease cells heterozygous for the ITD i.e. 5% of the total *FLT3* alleles. For the recommended minimum detection limit of 175 RFU, a wild-type peak height of ≥3500 RFU for a valid negative result would be required.[138]

Three samples, UPN34, UPN46 and UPN69, showed additional ITDs below the minimum peak limit of 175 RFU with CE. Due to the absence of background noise these were thought to represent true low-level ITD insertions. Indeed, on the agarose gel, all of these ITD bands in each corresponding sample could be seen, although with faint intensities. Each case should, therefore, be assessed on an individual basis when using the multiplex PCR and CE assay, as excluding peaks below the minimum limit of 175 RFU may exclude valid ITD insertions.

As *FLT3*-ITDs vary in size, when using CE, non-specific pull-up peaks and background noise should be eliminated or reduced. This will prevent false-positive results and ensure any non-wild-type peak is indicative of an ITD. In cases where the wild-type *FLT3* peak did not reach the minimum level of 3500 RFUs, these samples were re-tested using a dilution of 1:10 rather than the 1:15. Although the same findings were obtained, increased background noise and over-saturation of the peaks complicated analysis. A better option for increased sensitivity and specificity would be to label both the forward and reverse *FLT3*-ITD primers with two

different fluorophores. Peaks incorporating both colours would represent true ITDs and not background noise.[138] In this cohort, since CE showed none, to very low, levels of background noise, the labeling of only one primer was deemed adequate.

*FLT3*-ITDs are always in-frame and in multiples of 3bp.[115] However, the ITD size determination with CE did not always reflect this. Buban et al.[150] showed the ITD size given by CE to be 7% lower than that determined by sequencing. They explained with CE, variation of ±0.5bp for the wild-type and mutant peaks would cause a ±1bp change in the size of the ITD. For exact sizing, sequencing would have to be performed. Although ITD size has been associated with worse clinical outcome, this has not been conclusively established and, therefore, does not offer any current clinical value.[120,125]

Shires et al.[151] could reliably detect 1:50 (i.e. 2%) ITD mutant to wild-type *FLT3* alleles with their singleplex PCR and AGE, which is higher than that reported for the multiplex PCR and CE method (5%).[134] Although singleplex PCR and AGE would appear superior in amplifying low level ITDs, the multiplex PCR and CE method was still capable of detecting all the ITDs that AGE had identified.

With the singleplex PCR and AGE, the presence of multiple ITDs was over-estimated. This was due to the formation of heteroduplexes between the *FLT3* wild-type and ITD PCR products

resulting in a larger band on the agarose gel.[150] As seen in 5/12 (42%) cases in our cohort, although these larger bands were generally the same intensity as those for the ITD, they did not show a peak with CE. For CE, as the PCR products are first denatured with formamide and heat prior to being loaded on the genetic analyser, this eliminates heteroduplex formation. As such, CE gives a more reliable result than AGE for detection of *FLT3*-ITDs.

A serious disadvantage of AGE is that small duplications may be missed, as evident by a false-negative result from one *FLT3*-ITD mutated sample. Sample UPN54 had only one 3bp ITD, which would have been missed had only AGE been used. Due to the poor outcomes associated with *FLT3*-ITD, mutated patients may undergo more aggressive treatment regimens e.g. bone marrow transplantations.[138] This may not have been made available to this patient in the clinical setting should this test have been performed diagnostically. Similarly, as was seen with sample UPN17, AGE was also unable to distinguish between two ITDs which differed by 3bp in size. In this case, other ITDs were present, and this patient would not have been misdiagnosed. This finding was also seen by Buban et al.[150] where AGE was unable to separate two ITDs in one sample that differed in size by 3bp. However, CE could detect both of these ITDs. In our cohort, a 6bp ITD in sample UPN35 could be detected by both AGE and CE. Therefore, AGE cannot distinguish between ITDs that differ by 3bp and will also give false-negative results in cases where a single 3bp ITD insertion is present.

Although the singleplex PCR and AGE method is relatively simple and economical to perform, it is limited by its inability to detect small insertions and its over-estimation of multiple ITDs due to heteroduplex formation. Since the clinical importance of ITD size and presence of multiple ITDs is not yet clarified and not tested routinely, AGE would anyway be unsuitable for this. With the multiplex PCR and CE method having a high reported analytical sensitivity and more accurate than the singleplex PCR and AGE, this is the most suitable technique in analyzing for *FLT3*-ITD mutations (Table 4.7).

Table 4.7 Comparison of the Multiplex PCR and CE method with the Singleplex PCR and AGE method to detect *FLT3*-ITD mutations

| **FLT3-ITD** | **Multiplex PCR/CE** | **Singleplex PCR/AGE** |
|---|---|---|
| Analytical sensitivity[†] | 1:20 | 1:50 |
| Time to result[β] | ±5 hours | ±4 hours |
| Sizes of ITDs detected | ≥3bp | ≥6bp |
| Mutations detected | All *FLT3*-ITDs | Almost all *FLT3*-ITDs[#] |
| Nature of result | Semi-quantitative | Qualitative |

[†]as determined by Huang et al.[134] and Shires et al.[151] [β]=for 1 sample, [#]where ITDs differ by 3bp in size.

## 4.3 TESTING FOR *IDH*, *NPM1* AND *FLT3*-ITD MUTATIONS

Should testing for *IDH1* and *IDH2* mutations be offered in the clinical setting, cheaper and more sensitive alternatives to sequencing should be used where possible, particularly in settings with limited resources. The *IDH1*$^{R132}$ multiplex PCR offers such an alternative. A similar test could also be developed for *IDH2*$^{R140}$ and *IDH2*$^{R172}$ mutations. With the WHO

recommending testing for *NPM1* and *FLT3*-ITD in AML patients, and since the clinical effect of these mutations should always be considered in combination, simultaneous testing of these two genes is preferential over separate testing. Multiplex PCR and CE, with its ease of use, high analytical sensitivity and ability to detect virtually all *NPM1* and *FLT3*-ITD mutations concurrently, remains the recommended choice of method in testing for these mutations in AML patients.

# 5. CONCLUSION

The leukaemogenesis of AML is a complex and multi-step process with a broad molecular component. An understanding of the biological pathway of AML and the contribution of genetic alterations is vital in elucidating the nature of this aggressive disease. Complete prognostic assessment of AML patients has advanced not only with cytogenetic analysis, but also with the mutational profiling of specific genetic mutations. With the current repertoire of already identified molecular markers, and as novel markers are found, their value in determining clinical outcomes and even serving as potential therapeutic targets may be incalculable. Mutations in *IDH1* and *IDH2*, enzyme-encoding genes which influence cytosine methylation pathways with aberrant DNA methylation, are two such molecular markers. Although their prognostic influence in AML remains to be established, they are important in routine diagnosis, improving risk stratification and prediction of disease outcomes, particularly in patients with normal cytogenetics. Due to their stability they are also useful markers of minimal residual disease.

*IDH* mutations, like other international studies, have now been shown to occur in South African CN-AML patients. Further work clarifying the role of *IDH* mutations in the molecular biology of AML and their prognostic impact should be undertaken to determine the contribution of these mutations in this disease.

# 6. APPENDICES

## Appendix A

### Ethics Clearance Certificate

**UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG**
Division of the Deputy Registrar (Research)

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)**
R14/49 Ms Ashleigh Walton

| | |
|---|---|
| **CLEARANCE CERTIFICATE** | **M091022** |
| **PROJECT** | Investigation of Molecular Markers with Prognostic Significance in Acute Myeloid Leukemia Patients in the South African Population |
| **INVESTIGATORS** | Ms Ashleigh Walton. |
| **DEPARTMENT** | Molecular Medicine and Haematology |
| **DATE CONSIDERED** | 2009/10/30 |
| **DECISION OF THE COMMITTEE*** | Approved unconditionally |

**Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.**

**DATE** 2009/11/15 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr P Willem

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**DECLARATION OF INVESTIGATOR(S)**

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

# Appendix B

## Reagent Recipes

DNA EXTRACTIONS

**10X Phosphate Buffered Saline (PBS) (pH 7.4)**

Dissolve the following in 800ml $dH_20$:

- 80g NaCl
- 2g KCl
- 14.4g $Na_2HPO_4$
- 2.4g $KH_2PO_4$

Adjust pH to 7.4 with HCl

Add $dH_20$ to make up to 1L

Sterilize by autoclaving

Store at room temperature

Dilute 1:10 for 1xPBS

**1M Tris-HCL (pH 8.0)**

Dissolve the following in 70ml $dH_20$:

- 12.1g Tris base (Mw = 121.10g/mol)

Adjust to pH 8.0 with concentrated HCL

Add $dH_20$ up to 100ml

Sterilize by autoclaving

Store at room temperature (indefinitely)

Note: the pH of Tris solutions are temperature-dependent, make pH measurements at room temperature

**10M NaOH**

Dissolve the following in 200ml $dH_20$:

- 80g NaOH

The solution will heat up quickly

Cool and store at room temperature in a plastic bottle

**0.5M EDTA (pH 8.0)**

Dissolve the following in 800ml $dH_20$:

- 186.1 g EDTA disodium salt ($Na_2EDTA\text{-}2H_2O$)

Add ~50 mL 10MNaOH to pH to 8.0

Add $dH_20$ to 1L

Sterilize by autoclaving

Store at room temperature

Note: EDTA will only dissolve when the pH is adjusted to 8.0 or higher

**Lysis Buffer**

50mM Tris-HCL

1mM EDTA

0.5% Tween-20

Sterilize by autoclaving

Store at room temperature

**3M Sodium Acetate (pH 5.2)**

Dissolve the following in 200ml $ddH_20$:

- 204.15g sodium acetate(trihydrate - MW 136.08)

Adjust to pH 5.2 (about 90ml glacial acetic acid)

Add $dH_20$ up to 500ml

Sterilize by autoclaving

Store at room temperature

AGAROSE GEL ELECTROPHORESIS

**5x TBE (Tris-Borate-EDTA) buffer**

Dissolve the following in $dH_20$ to make 1L:

- 5x concentrate TBE powder blend (Sigma-Aldrich)

Sterilize by autoclaving

Store at room temperature

Can precipitate during storage, if this occurs, place the bottle in hot water until fully dissolved

Dilute 1:4 before use

**2% Agarose gel**

Add to 100ml 1xTBE buffer :

- 2g Agarose powder (Bioline, London, UK)

Boil until clear

Add 10µl Gel-Red (Biotium, Hayward)

Pour into tray and allow to set

Makes 3 gels

## DNA Extraction from Cultured Cells

Note: large cell pellets were halved and DNA extracted from the one half.

1. The cells were centrifuged at 3824xg in an 5810R Centrifuge (Eppendorf, Hamburg, Germany) for 4 minutes, fixative removed and 200 µl 1xPBS added.

2. The cells were centrifuged at 3824xg for 4 minutes, 1xPBS removed and discarded.
3. Five hundred microlitres of Lysis Buffer (which lysed the cells) and 40µl of 10mg/ml Proteinase K (which digested the protein) were added to the cells which were incubated at 55$^{o}$C while shaking in an Thermomixer Compact (Eppendorf, Hamburg, Germany) at 1400rpm.
4. Once the cells were homogenized (10 minutes to 2 hours), the Proteinase K was deactivated at 80$^{o}$C for 10 minutes to prevent further degradation of the DNA.
5. One volume of Phenol (Sigma-Aldrich, St. Louis, MO)(to remove protein impurities from the DNA) was added to the cells which were vortexed and centrifuged at 17949xg for 3 minutes.
6. The upper aqueous phase (containing the DNA) was transferred to a new tube while the lower phase which contained the protein was not disturbed.
7. Half volumes each of Phenol (Sigma-Aldrich) and Chloroform (Sigma-Aldrich)(removes residual phenol) were added, the sample vortexed and centrifuged at 17949xg for 3 minutes.
8. The supernatant was transferred to a new tube and one volume of Chloroform (Sigma-Aldrich) added, sample vortexed and centrifuged at 17949xg for 3 minutes.
9. After the supernatant was transferred to a new tube, a 1/10$^{th}$ volume of 3M Sodium Acetate (which neutralized the charge on the sugar phosphate backbone so the DNA precipitated out) and 2.5 volumes of ice-cold 100% Ethanol (Merck)(which made the DNA less hydrophilic causing it to be precipitated) were added and the sample stored at -70$^{o}$C overnight.

10. The following day, the DNA was centrifuged at 17949xg at 4$^{o}$C for 30 minutes and ethanol removed.
11. Two hundred microlitres of ice-cold 70% ethanol was added (which removed any residual salt from the DNA).
12. The DNA was then centrifuged at 17949xg at 4$^{o}$C for 10 minutes, ethanol removed and the DNA air-dried.
13. Once dried, 50-100µl (depending on the pellet size) of TE buffer (Promega, WI, USA) was added and the DNA re-suspended on a shaking platform overnight.
14. The following day the extracted DNA was assessed for quantity (ng/µl) and purity ($OD_{260/280}$) using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).
15. A 100ng dilution was made of the DNA with $dH_20$ and run on a 2% agarose gel (Bioline, London, UK) to assess for degradation.

## Isopropanol Purification of Cycle-Sequencing Products for Sanger Sequencing

1. Following cycle-sequencing, 80µl of 75% Isopropanol (Sigma-Aldrich, St. Louis, MO) was added to each sample and vortexed briefly.
2. The samples were then transferred into a 96-well plate (Applied Biosystems) according to a designated layout (for the ABI 3730 Genetic analyser with a 48-capillary array, samples were loaded in alternate rows and for the ABI 3500 Genetic analyser with an 8-capillary, samples were loaded in a single row).
3. The plate was sealed with a rubber 96-well plate cover (Applied Biosystems) and

incubated in the dark at room temperature for 30 minutes.

4. The plate was then centrifuged at 2000xg in a 5810R Centrifuge (Eppendorf, Hamburg, Germany) for 45 minutes, rubber plate cover removed and inverted onto paper towel.
5. The Isopropanol was removed by centrifuging the plate for 1 minute at 100xg.
6. The plate was air-dried for 5 minutes and samples re-suspended in 10µl Hi-Di Formamide (Applied Biosystems) and covered with a septa lid (Applied Biosystems). (For the 3730 DNA analyser all of the alternate rows contained Hi-Di Formamideand for the ABI 3500 with an 8-capillary, each row used had Hi-Di Formamide).
7. The plate was centrifuged at 50xg for 30 seconds to ensure all the samples were at the bottom of the well and loaded onto the Genetic analyzer.

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