Mutational analysis of the *TET2* gene in Philadelphia negative Myeloproliferative Neoplasms

By

Hanri du Plessis

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Faculty of Health Sciences

Department of Haematology and Cell Biology

University of the Free State, Bloemfontein

Supervisor: Dr. A. de Kock

Co-Supervisor: Prof. C.D. Viljoen

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DECLARATION

I certify that the dissertation hereby submitted by me for the M.Med.Sc (Human Molecular Biology) degree at the University of the Free State is my independent effort and had not previously been submitted for a degree at another university or faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

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Hanri du Plessis

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“But those who hope in the Lord will renew their strength; they will soar on wings like eagles; they will run, and not grow weary; they will walk, and not faint.”

Isaiah 40:31

CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

3’	3 prime
5’	5 prime
°C	Degree Celsius
%	Percentage
A	Adenine
ABI	Applied Biosystems
Allo-SCT	Allogenic stem cell transplantation
AML	Acute myeloid leukaemia
B (cell)	Bursa of Fabricus cell
bp	Base pairs
C	Cytosine
caC	Carboxylcytosine
CD	Cysteine-rich domain
cm	Centimetre
CML	Chronic myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
CNL	Chronic neutrophilic leukaemia
CXXC	Cysteine-rich DNA binding domain
dbSNP	Database of single nucleotide polymorphisms
del	Deletion
dL	Decilitre
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ds DNA	Double stranded deoxyribonucleic acid
DSBH	Double-stranded β-helix 2-oxyglutarate Fe (II)-dependent dioxygenase domain
EDTA	Ethylenediamine tetra acetic acid
EEC	Endogenous erythroid colony
EpoR	Erythropoietin receptor
ET	Essential thrombocythaemia
et al.	*et alia* (and others)

ETOVS	Ethics committee of the Faculty of Health Sciences of the Free State
fC	Formylcytosine
Fe	Iron
Fig.	Figure
FTA	Fast technology for analysis of nucleic acid
g	Gram
G	Guanine
G-CSFR	Granulocyte-colony stimulating factor receptor
GE	General Electric
HES	Hypereosinophilic syndrome
HG	Hydroxyglutarate
HHRH	Hereditary hypophosphatemic rickets with hypercalciuria
hmC	Hydroxymethylcytosine
HRM	High resolution melting
HSC	Haematopoietic stem cell
IDH	Isocitrate dehydrogenase
in vitro	Outside a living organism
in vivo	Inside a living organism
ins	Insertion
JAK	Janus kinase
KV	Kilovolt
L	Litre
LDH	Lactate dehydrogenase level
LOH	Loss of heterozygosity
M	Molar
mC	Methylcytosine
MDR	Multidrug resistance
MDS	Myelodysplastic syndrome
$MgCl_2$	Magnesium chloride
ml	Millilitre
MLL	Mixed lineage leukaemia
mm	Millimetre
mM	Millimolar

MPL	Myeloproliferative leukaemia virus oncogene homology
MPN	Myeloproliferative neoplasm
mRNA	Messenger ribonucleic acid
NaPi	Sodium-dependent phosphate co-transporter
NCBI	National centre for biotechnology information
NF	Neurofibromatosis
P-(glycoprotein)	Permeability glycoprotein
PCR	Polymerase chain reaction
Ph	Philadelphia
pH	Concentration of hydrogen ions in solution
PMF	Primary myelofibrosis
POP-7	Performance optimized polymer number 7
PV	Polycythaemia vera
q	Long arm of chromosome
RNA	Ribonucleic acid
rs	Reference single nucleotide polymorphism number
SLC	Solute-carrier
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
t	Translocation
T	Thymine
T (cell)	Thymus-derived cell
tRNA	Transfer ribonucleic acid
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris EDTA
TET	Ten-eleven-translocation
T_m	Melting temperature
TpoR	Thrombopoietin receptor
Tris	Tris hydroxymethyl aminomethane
TYK	Tyrosine kinase
U	Unit
UK	United Kingdom
USA	United States of America

UV	Ultraviolet
V	Volt
WHO	World health organization
WNK	With no lysine (K) kinases
www	World wide web
x g	Acceleration due to gravity
Zn	Zinc
β	Beta
µA	Micro-ampere
µl	Micro-litre
µM	Micro-molar

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CHAPTER 1

LITERATURE REVIEW

1.1 Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) are clonal haematopoietic disorders which arise in the bone marrow stem cells (Hoffbrand and Moss 2011). The outcome of these disorders is the increased proliferation of blood cells from one or more of the myeloid lineages (Jäger and Kralovics 2011) (Fig. 1.1). According to the 2008 World Health Organization (WHO) classification MPNs include polycythaemia vera (PV), essential thrombocythaemia (ET), primary myelofibrosis (PMF), chronic myeloid leukaemia (CML), chronic neutrophilic leukaemia (CNL), hypereosinophilic syndrome (HES), mast cell disease and unclassifiable MPNs (Swerdlow *et al*. 2008). Dameshek (1951) postulated that PV, ET, PMF and CML are a group of clonal myeloid disorders in which there are variable manifestations of proliferative activity of the bone marrow cells. Therefore PV, ET, PMF and CML are considered as the "classic" MPNs (Dameshek 1951). After the discovery of the Philadelphia (Ph) chromosome, which is the genetic characteristic of CML, PV, ET and PMF became recognized as the Ph-negative MPNs (Faderl *et al*. 1999; Milosevic and Kralovics 2013).

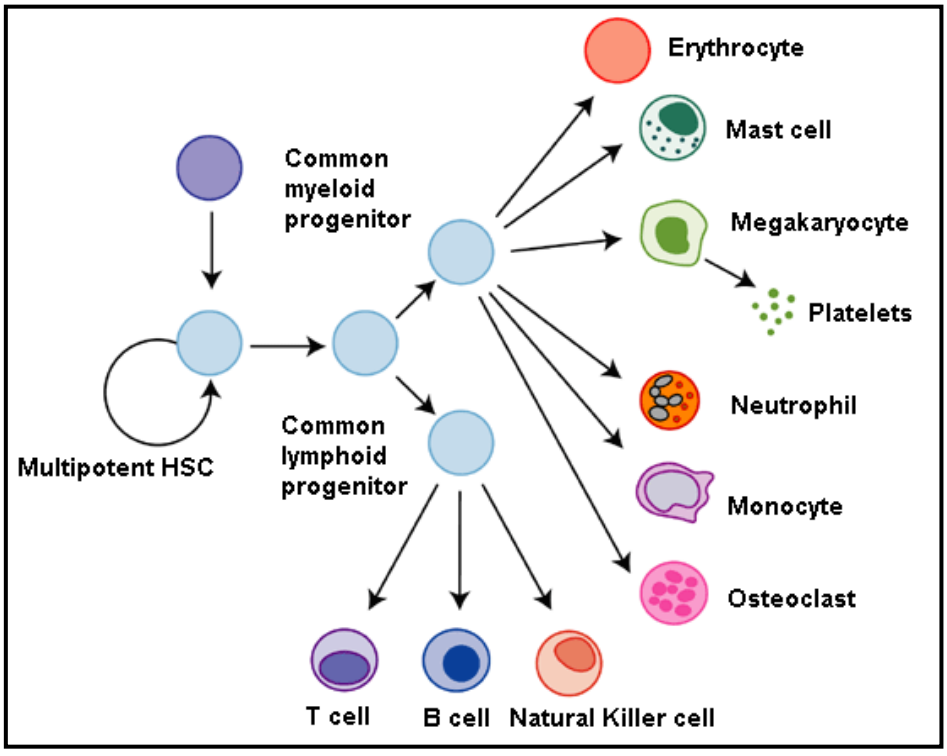

Figure 1.1: Illustration of the haematopoiesis process. Two cell lineages develop from the haematopoietic stem cells (HSCs), namely the myeloid and lymphoid cells (Copied from Qasim *et al*. 2004).

1.1.1 Polycythaemia vera

Polycythaemia vera (PV) is a clonal disorder resulting in the proliferation of haematopoietic precursors, thereby mostly progressively increasing the red cell mass (Barbui *et al*. 1995). The overproduction of erythrocytes leads to higher viscosity of the blood, which ultimately increases the patient's risk to develop thrombosis (Stuart and Viera 2004). The main symptoms of PV include fatigue, pruritus, splenomegaly and erythromelalgia (Bircher and Meier-Ruge 1988; Emanuel *et al*. 2012; Hensley *et al*. 2013). The incidence of PV is 0.7 to 2.6 per 100,000 individuals per year (Johansson 2006). Men are more prone than women to develop PV and the mean age of diagnosis is 60 years (Barbui *et al*. 1995; Marchioli *et al*. 2005).

The diagnosis of PV patients is based on the 2008 WHO diagnostic criteria (Swerdlow *et al*. 2008). The classification system consists of major and minor criteria. In order for a patient to be diagnosed with PV, two major criteria and one minor criterion have to be met. The major criteria include: (1) haemoglobin count above 18.5 g/dL for men and 16.5 g/dL for women, or other evidence of elevated red cell volume and (2) the presence of a Janus kinase *(JAK)* 2 mutation (*JAK2* V617F or *JAK2* exon 12 mutations). The minor criteria include: (1) hyperactivity of the bone marrow, (2) serum erythropoietin levels below the reference range for normal and (3) endogenous erythroid colony (EEC) development *in vitro*.

The treatment of PV is primarily based on treating the symptoms to reduce the risk of thrombosis or disease progression (Hensley *et al*. 2013). PV patients receive phlebotomy and low-dose aspirin to reduce haematocrit levels (Barbui *et al*. 2011). PV patients above 65 years of age, with a history of thrombosis and higher leukocyte levels are considered as high-risk PV patients (Barbui and Finazzi 2006; Hensley *et al*. 2013). Cytoreductive therapy, for example hydroxyurea, is recommended for high-risk PV patients (Barbui *et al*. 2011). Hydroxyurea reduces the risk of thrombosis and the progression to acute myeloid leukaemia (AML) (Prchal and Prchal 2010; Kiladjian *et al*. 2011).

1.1.2 Essential thrombocythaemia

Essential thrombocythaemia (ET) is characterized by the increased proliferation of thrombocytes with megakaryocytic hyperplasia of the bone marrow (Cervantes 2011). The predominant features of ET include haemorrhage, thrombosis and microvascular disturbances (Cervantes 2011). Microvascular disturbances may include erythromelalgia, digital ischemia, necrosis and headaches (Brière 2007). Thrombosis in ET patients could result in defects of the peripheral, neurological and cardiac systems (Brière 2007). Bleeding events occur less frequently than thrombosis and are usually present in ET patients with very high platelet levels (Van Genderen *et al*. 1996). The incidence of ET is 0.6 to 2.5 per 100,000 individuals per year (Jensen *et al*. 2000; Johansson 2006). The mean age of patients diagnosed with ET is between 50 and 70 years (Beer and Green 2010). Many ET patients present with no symptoms and are only diagnosed after a routine full blood count has been performed (Hoffbrand and Moss 2011).

The diagnosis of patients is made on the basis of the 2008 WHO diagnositic criteria for ET (Swerdlow *et al*. 2008). The WHO diagnostic criteria require that four major criteria have to be met for the diagnosis of ET patients. These criteria include: (1) platelet counts above 450 x 10^9/L, (2) proliferation action of megakaryocytes to produce high levels of large and mature megakaryocytes, (3) no correspondence to the WHO criteria for PV, PMF, CML or any other myeloid neoplasm and lastly (4) the presence of *JAK2* V617F or any other clonal marker, or in the absence of a clonal marker, no evidence of reactive thrombocytosis.

One of the major concerns for ET patients is elevated platelet counts since this may result in thrombosis or haemorrhage. Several treatment options are available to manage the platelet levels of patients. Patients younger than 40 years are considered to be low-risk (Beer *et al*. 2011) and receive low doses of aspirin (Barbui *et al*. 2011). High-risk patients who are older than 60 years and have suffered from previous thrombotic events (Brière 2007) receive cytoreductive therapy (Barbui *et al*. 2011). Hydroxyurea, a cytoreductive agent, is used as the first-line therapy to reduce the platelet levels and the risk of thrombosis in ET patients (Cortelazzo *et al*. 1995). Anagrelide, another cytoreductive agent, is recommended as the second-line therapy (Barbui *et al*. 2011). Hydroxyurea and anagrelide inhibits megakaryocyte proliferation and thus decreases the platelet levels (Solberg *et al.* 1997).

1.1.3 Primary myelofibrosis

Primary myelofibrosis (PMF) is a clonal haematopoietic stem cell disorder which is characterized by the fibrous tissue formation of the bone marrow (Campbell and Green 2006). Symptoms of PMF include extramedullary haematopoiesis with splenomegaly, anaemia and leukoerythroblastosis in the peripheral blood (Barosi 1999). The haematological characteristics of PMF may vary from leukopenia to leukocytosis, or from thrombocytopenia to thrombocytosis (Cervantes 2004). The main causes of death in PMF patients include transformation to acute leukaemia, cardiac failure, thrombosis, infection and haemorrhage (Tefferi 2000; Cervantes *et al*. 2009). The incidence of PMF is estimated to be 0.5 to 1.5 per 100,000 individuals per year (McNally *et al*. 1997; Mesa *et al*. 1999; Ridell *et al*. 2000). The mean age of presentation with PMF is 65 years (Dupriez *et al*. 1996; Cervantes *et al*. 1997; Reilly *et al*. 1997). The disorder presents as PMF or could follow on a previous disorder for example PV or ET (post-PV or post-ET myelofibrosis) (Mesa *et al*. 2007).

As defined by the 2008 WHO diagnostic criteria for PMF, three major criteria and two minor criteria have to be met in order for a patient to be diagnosed (Swerdlow *et al*. 2008). The three major criteria are: (1) megakaryocyte proliferation and atypia* usually connected with reticulin and/or collagen fibrosis and if reticulin fibrosis does not occur, the megakaryocyte changes must be accompanied by increased bone marrow cellularity, granulocyte proliferation and often decreased erythropoiesis, (2) no correspondence with the WHO criteria for CML, PV, myelodysplastic syndrome (MDS) or any other myeloid neoplasm and lastly (3) the presence of *JAK2* V617F or any other clonal marker (for example *MPL* W515K/L mutation) or no evidence of reactive bone marrow fibrosis. The minor criteria include: (1) leukoerythroblastosis, (2) increased serum lactate dehydrogenase level (LDH), (3) anaemia and (4) splenomegaly.

PMF cannot be cured and the aim of treatment is therefore to improve the quality of life for the patient. Asymptomatic PMF patients without any risk factors do not receive treatment until required (Cervantes 2004). Low-risk patients displaying splenomegaly, priurtius, symptomatic anaemia, fatigue and bone pain receive conventional therapy (Tefferi 2011). Conventional therapy may include androgens

*Megakaryocytes (small to large in size) with an abnormal nuclear and cytoplasmic percentage as well as hyperchromatic, spherical or unevenly folded nuclei and dense clustering (Swerdlow *et al*. 2008).

for anaemia and hydroxyurea for splenomegaly (Cervantes and Martinez-Trillos 2013). Treatment options for high-risk PMF patients include splenectomy, radiotherapy, experimental drugs and allogenic stem cell transplantation (allo-SCT) (Tefferi 2011). Allo-SCT is the only treatment with the potential to cure PMF (Cervantes 2004). Tefferi (2013) suggested that allo-SCT should only be considered in patients with an expected survival of less than five years and/or patients with a higher risk than 20% of progressing to AML.

1.2 Genetic abnormalities associated with Ph-negative MPNs

The *JAK2* and myeloproliferative leukaemia virus oncogene homology (*MPL*) genes are the most common genes associated with Ph-negative MPNs. The most common mutation associated with Ph-negative MPNs is the *JAK2* V617F mutation and it is found in most PV patients and more than half of ET and PMF patients (Vannucchi and Guglielmelli 2012). The *JAK2* V617F mutation is also considered the main contributor to the clinical phenotype of PV, ET and PMF (Couronné *et al*. 2010). Mutations of the *MPL* gene are less common and are only found in ET and PMF patients (Campregher *et al*. 2012). Studies of *JAK2* and *MPL* mutations have aided in the understanding of the pathogenesis of PV, ET and PMF and thus form an important part of the WHO diagnostic criteria for these three disorders (Swerdlow *et al*. 2008).

1.2.1 The *JAK2* gene

JAK2 forms part of the Janus non-receptor tyrosine kinase family which also includes JAK1, JAK3 and tyrosine kinase (TYK) 2 (McLornan *et al*. 2006). The function of JAK2 is to mediate haematopoietic signalling to ensure normal myelopoiesis (Jäger and Kralovics 2011). JAK2 mediates the signalling for cytokine receptors that lack tyrosine phosphorylation activity (Baker *et al*. 2007). These receptors include erythropoietin (EpoR), thrombopoietin (TpoR) and granulocyte-colony stimulating factor (G-CSFR) (Baker *et al*. 2007). The activation of these receptors occurs via the JAK-signal transducer and activator of transcription (STAT) signalling pathway (McLornan *et al*. 2006). The most common variant of the *JAK2* gene is the *JAK2* V617F mutation. The *JAK2* V617F mutation disrupts the autoinhibitory activity of JAK2, which ultimately leads to a constitutively active protein (Bench *et al*. 2012) (Fig. 1.2). As a result of the mutation the haematopoietic stem cells are

hypersensitive to cytokines, which provide cells with a greater survival and proliferation advantage (Kralovics *et al*. 2005). The prevalence of the *JAK2* V617F mutation is 95% in PV and 60% in ET and PMF, respectively (Vannucchi and Guglielmelli 2012). Genetic variants in exon 12 of the *JAK2* gene have also been reported to occur in PV patients who lack the *JAK2* V617F mutation (Scott *et al*. 2007). According to Scott (2011), these variants are present in approximately 1% to 3% of PV patients.

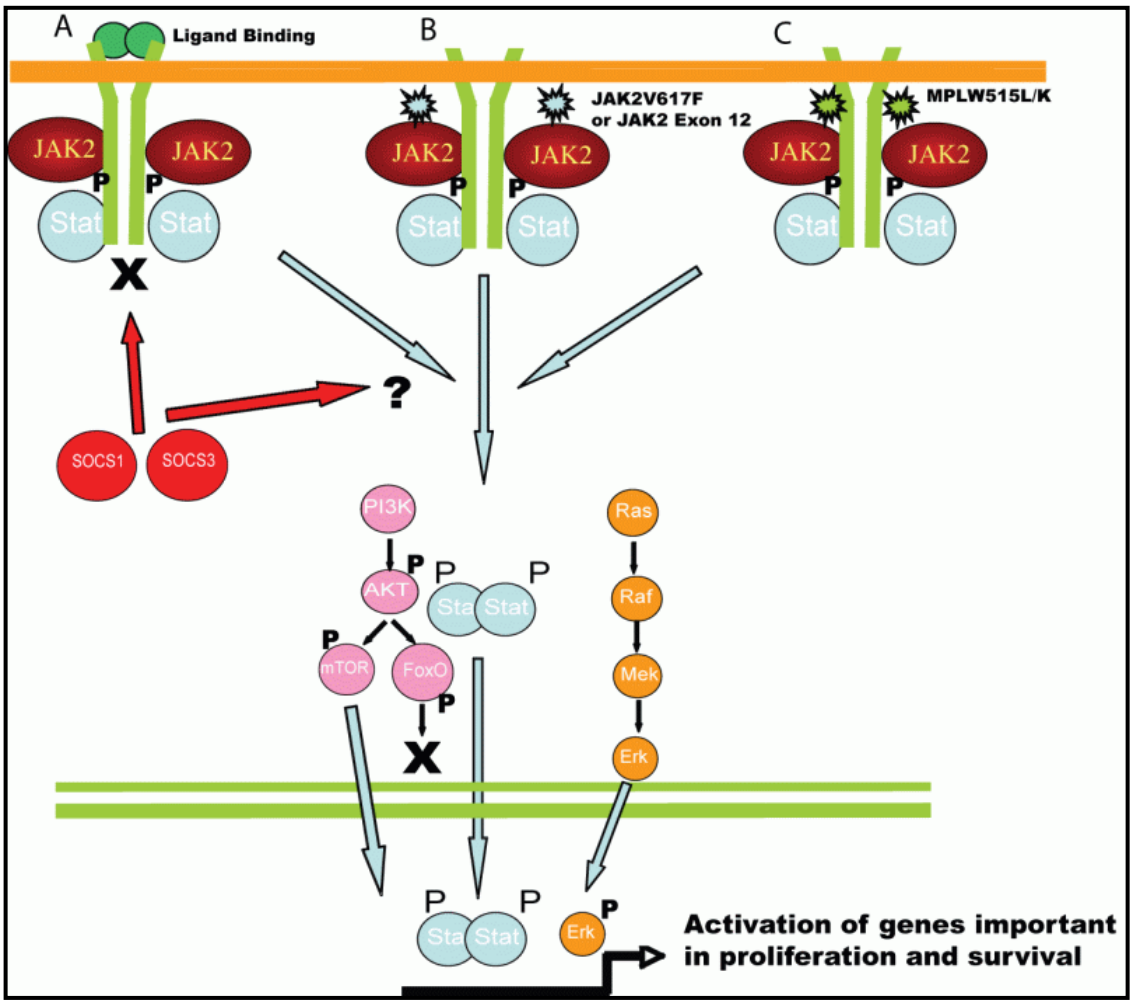

Figure 1.2: Illustration of the activation of the JAK-STAT signaling pathway. A: In the presence of a ligand the JAK2 protein is activated, STAT molecules are phosphorylated (P) and subsequently transported to the nucleus where it activates gene transcription. B and C: If *JAK2* V617F, *JAK2* exon 12 or *MPL* W515L/K mutations are present, respectively, the JAK-STAT pathway is activated without the need for ligand binding to occur (Copied from www.ommbid.mhmedical.com).

1.2.2 The *MPL* gene

The *MPL* gene is responsible for encoding the thrombopoietin receptor which regulates the growth and differentiation of megakaryocytes (Milosevic and Kralovics 2013). The *MPL* W515L and *MPL* W515K mutations are the most common variants of the *MPL* gene and are situated in exon 10 (Vannucchi *et al*. 2009). The *MPL* W515L mutation, similar to *JAK2* mutations, results in the constitutive activation of the JAK2 protein, which leads to cytokine-independent growth of haematopoietic cells (Pikman *et al.* 2006) (Fig. 1.2). Until now, no *MPL* variants have been documented to occur in patients with PV (Campregher *et al*. 2012). However, the prevalence of *MPL* variants is approximately 1% to 5% in ET and 5% to 10% in PMF patients (Pardanani *et al.* 2006; Pikman *et al*. 2006).

1.3 Other genetic abnormalities associated with Ph-negative MPNs

Variants of the ten-eleven-translocation (*TET*) 2 gene have recently been found to occur in various myeloid disorders in which myeloproliferation, dysplasia and transformation to acute leukaemia are present (Mullighan 2009). Delhommeau *et al*. (2009) detected *TET2* variants in patients suffering from MPN (12%), MDS (19%), secondary AML (24%) and chronic myelomonocytic leukaemia (CMML) (22%). From this study it became clear that the *TET2* gene could contribute to the pathogenesis of several myeloid disorders, including PV, ET and PMF.

1.3.1 The *TET* genes

The *TET* oncogene family consists of *TET1*, *TET2* and *TET3*. *TET1* was the first *TET* gene to be described (Lorsbach *et al.* 2003). It was defined as the fusion partner of the mixed lineage leukaemia (*MLL*) gene in the translocation t(10;11) (q22;q23), which occurs in AML (Lorsbach *et al.* 2003). The function of TET1 is to convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (Tahiliani *et al*. 2009). According to Ito *et al*. (2010), TET1 is also important for the preservation of embryonic stem cells and inner cell mass specification. No mutations of the *TET1* gene have been found to occur in MPNs (Abdel-Wahab *et al*. 2009). *TET1*, *TET2* and *TET3* are expressed in haematopoietic cells, but the expression levels of *TET2* and *TET3* are elevated in comparison to *TET1* (Langemeijer *et al*. 2009a; Langemeijer *et al*. 2009b). Until now, no anomalies of the *TET3* gene have been associated with myeloid disorders (Langemeijer *et al*. 2009b). In addition to *JAK2*

and *MPL*, *TET2* has been found to be the only *TET* gene with a high frequency of mutations in myeloid disorders (Mohr *et al*. 2011). The TET1, TET2 and TET3 proteins share two highly conserved regions in which the catalytic domains of the proteins are present (Fig. 1.3) (Tahiliani *et al*. 2009). These regions include the cysteine-rich domain (CD) and double-stranded β-helix 2-oxyglutarate Fe (II)-dependent dioxygenase domain (DSBH). The TET1, TET2 and TET3 proteins each consists of 2138, 2002 and 1660 amino acids, respectively. The TET1 protein contains a binuclear Zn-chelating cysteine rich DNA containing (CXXC) domain that is absent in TET2 and TET3.

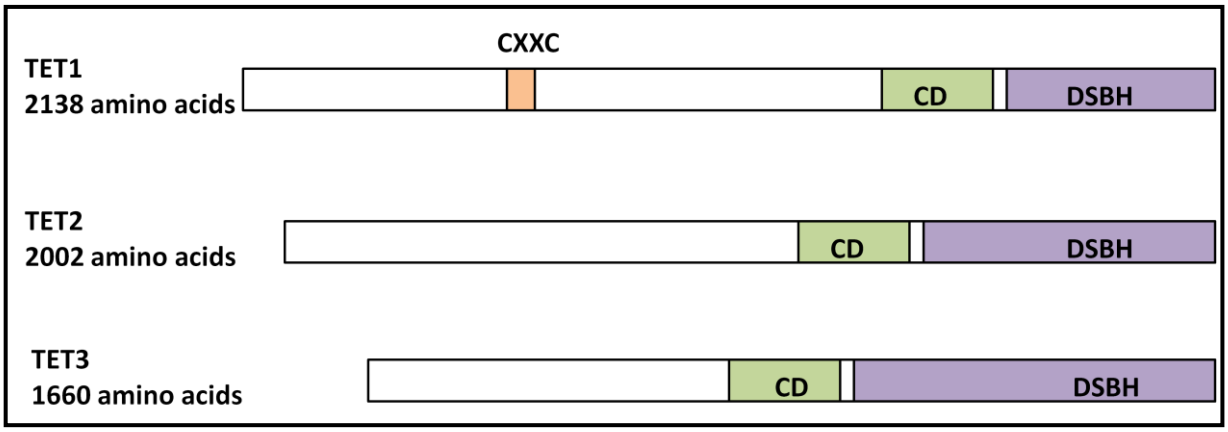

Figure 1.3: Illustration of the conserved domains shared by the three TET proteins. CXXC: Cysteine rich DNA containing domain. CD: Cysteine-rich domain. DSBH: Double stranded β-helix 2-oxyglutarate Fe (II)-dependent dioxygenase domain (Adapted from Mohr *et al*. 2011).

1.3.2 The *TET2* gene

TET2 is a tumour suppressor gene that is situated on chromosome 4q24. The *TET2* gene consists of 11 exons, of which nine are coding exons. The messenger RNA (mRNA) of *TET2* has three isoforms, which is the result of alternative splicing. The isoforms consist of 2002, 1164 and 1194 amino acids, respectively (Mohr *et al*. 2011). Variants of the *TET2* gene that occurred in the CD and DSBH domains were predicted by Mohr *et al*. (2011) to lead to abnormal protein folding, which could result in an inactive protein (Fig. 1.4). Mohr *et al*. (2011) also reported that mutations present in the DSBH domain of the TET2 protein could cause an increase in the proliferation of the mutant cell. Missense mutations are predominantly found in the conserved domains of the protein which suggests that they could interfere with the

catalytic activity of the protein and therefore result in altered protein function (Pronier and Delhommeau 2011) (Fig. 1.5). Nonsense and frameshift mutations are mainly found in the regions outside the conserved domains (Euba *et al*. 2012) (Fig. 1.5). It has been suggested that these regions acquire mutations that result in a truncated protein, which could lead to the loss of gene function (Euba *et al*. 2012).

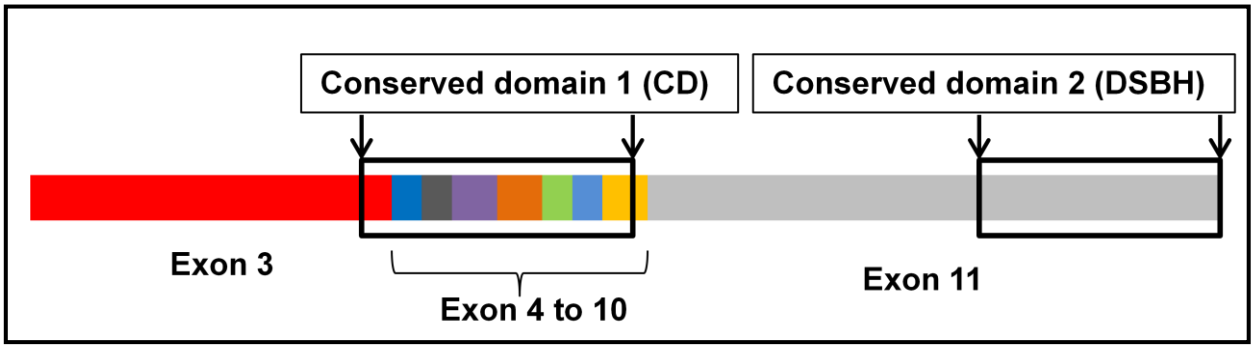

Figure 1.4: Schematic representation of the *TET2* gene structure. The CD domain of TET2 consists of amino acids 1104 to 1478 and the DSBH domain comprises amino acids 1845 to 2002 (Copied from Smith *et al*. 2010).

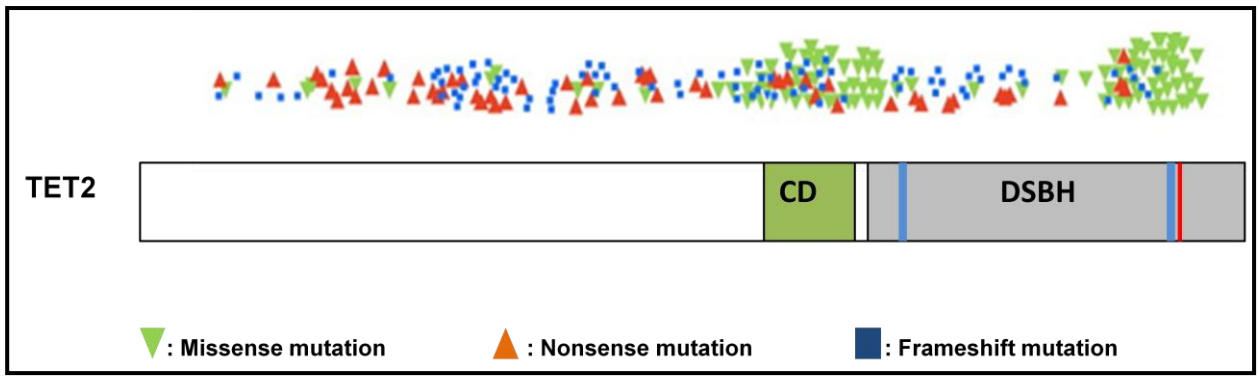

Figure 1.5: The positions of the various missense, nonsense and frameshift mutations that have been reported to occur in the *TET2* gene in myeloid disorders (Copied from Mohr *et al*. 2011).

1.3.3 The role of TET2 in haematopoiesis

TET2 has been suggested to play an important role in the regulation of haematopoiesis (Ko *et al*. 2010). TET2, similar to TET1 and TET3, acts as a catalyst for the conversion of 5-mC to 5-hmC (Ito *et al*. 2010). Ito *et al*. (2011) reported that TET2 is also able to convert 5-mC into 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). Tahiliani *et al*. (2009) proposed that the conversion of 5-mC to 5-hmC is an important transitional form involved in DNA demethylation. However, the exact function of 5-hmC and how it influences haematopoiesis is still unclear. Several authors have suggested that TET2 contributes to the regulation of the levels of 5-hmC during haematopoietic differentiation (Ko *et al*. 2010; Li *et al*. 2011; Moran-Crusio *et al*. 2011; Pronier et al. 2011a; Quivoron *et al*. 2011). Lower levels of 5-hmC have been observed in MPN patients with *TET2* variants in comparison to healthy individuals (Ko *et al*. 2010; Pronier *et al*. 2011a). Furthermore, it has been suggested that altered regulation of the conversion of 5-mC to 5-hmC could contribute to the pathogenesis of MPNs (Kunimoto *et al*. 2012). Hence, it appears that TET2 could play a role in the epigenetic regulation of haematopoietic development.

Several studies have been performed to determine the effect of defective *TET2* expression on haematopoietic development. Ko *et al*. (2010) demonstrated that decreased expression of the *TET2* gene skewed the differentiation of the haematopoietic progenitor cells towards the monocyte and macrophage cell lineages. Pronier *et al*. (2011a) reported that the knockdown of *TET2* expression increased the differentiation of haematopoietic stem cells towards monocytes at the expense of granulocytes and erythrocytes. Two other studies found that the loss of TET2 function in mice displayed characteristics similar to patients with MPNs (Li *et al*. 2011; Moran-Crusio *et al*. 2011). Deletion of the *TET2* gene resulted in splenomegaly, leukocytosis, myeloid dysplasia and expansion of the haematopoietic stem cell compartment. In another mouse model in which the deletion of *TET2* was induced, extramedullary haematopoiesis, myeloproliferation *in vivo*, monocytosis, splenomegaly and increased haematopoietic stem cell self-renewal was observed. Mice in which only one *TET2* allele was deleted also displayed extramedullary haematopoiesis and increased stem cell self-renewal. Figueroa *et al*. (2010) demonstrated that the reduced expression of *TET2* resulted in an increase in the total haematopoietic stem/progenitor cells with suppression of regular myeloid

differentiation. Altogether these studies demonstrated that TET2 is important for the regulation of normal haematopoiesis as well as myeloid differentiation later on. Therefore, abnormal function of the gene could predispose individuals to the development of MPNs.

1.3.4 *TET2* variants in MPNs

Delhommeau *et al*. (2009) was the first to describe variants in the *TET2* gene of MPN patients. Loss of heterozygosity (LOH) was discovered in both alleles of the *TET2* gene in one PV and one PMF patient, respectively. In addition, another PV patient had LOH in only one allele of the *TET2* gene. Delhommeau *et al*. (2009) discovered frameshift mutations, stop codons, deletions and amino acid substitutions in the *TET2* gene of the MPN patients. These genetic variants were observed in all of the exons of the gene and were proposed to result in the fractional or complete loss of function of TET2. Delhommeau *et al*. (2009) speculated that *TET2* mutations could be an early event in patients with MPNs.

The prognostic value of *TET2* variants in MPN patients is yet unclear (Abdel-Wahab *et al*. 2012). *TET2* mutations are somatically acquired (Langemeijer *et al*. 2009a), although one case of a germline mutation in a PV patient was reported by Schaub *et al*. (2010). Various somatic *TET2* mutations have been reported to occur in MPN patients (Fig. 1.6). It has been found that the frequency of *TET2* variants is approximately 7.0% to 16.0% in PV, 4.4% to 11.0% in ET and 7.7% to 17.0% in PMF patients (Abdel-Wahab *et al*. 2009; Delhommeau *et al*. 2009; Tefferi *et al*. 2009). A study performed by Tefferi *et al*. (2009) reported that *TET2* variants are more common in patients older than 60 years. They found that the *TET2* variants were present in 23% of patients older than 60 years compared to 4% of younger patients. Furthermore, Tefferi *et al*. (2009) found that *TET2* variants did not influence the survival, transformation to acute leukaemia, or risk for thrombosis in PV and PMF patients. However, more studies are necessary to determine the prognostic significance of *TET2* mutations in MPN patients.

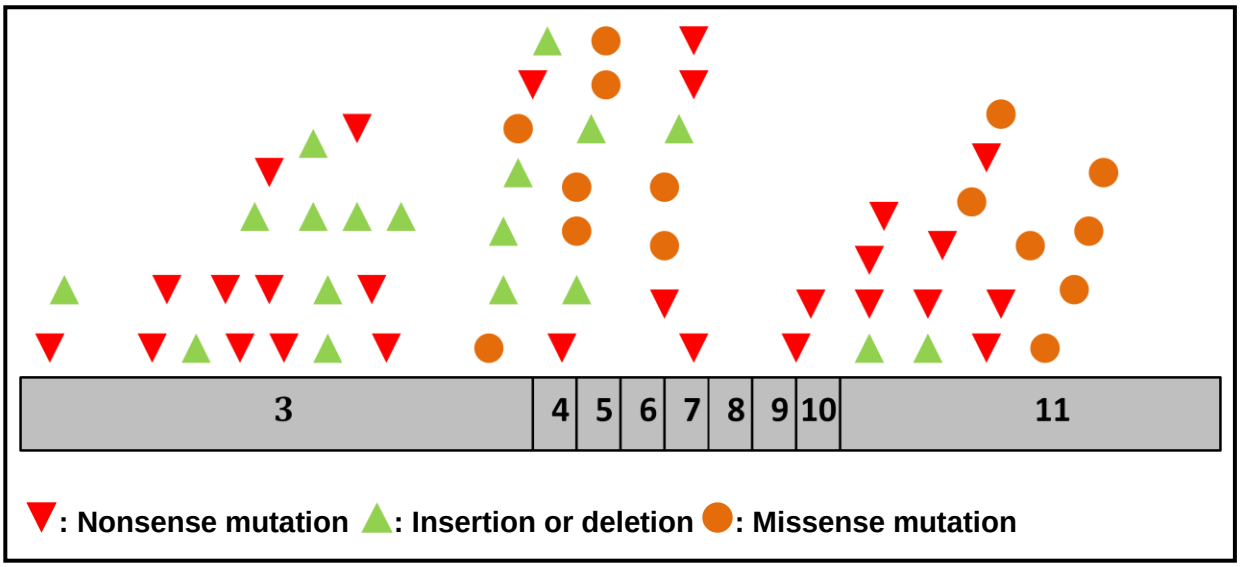

Figure 1.6: Schematic representation of the locations of the somatic missense, nonsense and frameshift mutations in the exons of the *TET2* gene that have been reported to occur in MPNs (Copied from Cimmino *et al*. 2011).

1.3.5 *TET2* variants and the *JAK2* V617F mutation

TET2 variants have been found to occur in *JAK2* V617F-positive and -negative MPN patients. Delhommeau *et al*. (2009) suggested that *TET2* variants could be present in the early stages of the disease, since these variants appear to precede the *JAK2* V617F mutation. Delhommeau *et al*. (2009) reported that *TET2* variants were present in 7% of *JAK2* V617F-negative and 14% of *JAK2* V617F-positive MPN patients. A subsequent study performed by Tefferi *et al*. (2009) reported similar results, with *TET2* variants being present in 17% and 7% of *JAK2* V617F-positive and -negative MPN patients, respectively. It was initially suspected that *TET2* variants precede the *JAK2* V617F mutation, but subsequent studies reported that it appears that *TET2* variants rather follow the *JAK2* V617F mutation (Schaub *et al*. 2010; Swierczek *et al*. 2011). Thus, it appears that the pattern of occurrence of abnormalities in these two genes do not follow in a specific order (Pronier *et al*. 2011b).

1.3.6 Mutation detection of the *TET2* gene in MPNs

TET2 is important for the conservation of normal myelopoiesis and the disturbance of its function could therefore contribute to the development of MPNs (Ko *et al*. 2010). Thus, it is important to be able to identify such variants that could lead to the abnormal function of TET2. *TET2* variants were first discovered in MPN patients

with the use of direct sequencing, comparative genomic hybridization and single nucleotide polymorphism (SNP) analysis (Delhommeau *et al*. 2009). Direct sequencing, the method of choice for *TET2* variant detection, is however expensive and time consuming (Abdel-Wahab *et al*. 2009; Saint-Martin *et al*. 2009; Tefferi *et al*. 2009; Tindall *et al*. 2009; Er and Chang 2012).

High resolution melting (HRM) analysis is a faster, easier and more cost saving method used to screen for unknown genetic variants (Wittwer *et al*. 2003; Tindall *et al*. 2009; Wittwer 2009). HRM analysis distinguishes between different genotypes by comparing the melting curves of unknown samples to the melting curve of a reference sample in which no sequence variants are present (Wittwer *et al*. 2003). HRM has been found to be more sensitive for the detection of *TET2* variants since HRM was able to detect a mutation in the initial phase of MF progression in an ET patient, while sequencing could only detect the mutation in the advanced phase of the disease progression (Martinez-Aviles *et al*. 2012).

HRM is a post-PCR analysis method that is used to detect sequence variants (Taylor 2009; Tindall *et al*. 2009). HRM follows conventional PCR, which is performed in the presence of an intercalating double-stranded DNA (dsDNA) binding dye (Krypuy *et al*. 2007; Taylor 2009) (Fig. 1.7 A). Following PCR, the PCR products are denatured in temperature increments (Reed *et al*. 2007). The increasing temperature results in decreased fluorescence as the double stranded DNA becomes single stranded and the dsDNA binding dye is released (Taylor 2009; Er and Chang 2012). A characteristic melting profile is created from the denaturing DNA and released fluorescence (Ririe *et al*. 1997).

The raw melting curve data is normalized in order to remove background as a result of the fluorescence variance between samples (Montgomery *et al*. 2007; Taylor 2009) (Fig. 1.7 B). The pre- and post-melt regions are selected to normalize the fluorescence of the melting region, which enables the differences between the various melting curves to become more distinctive and allows similar sequence variants to cluster together (Wittwer *et al*. 2003) (Fig. 1.7 C). The different sequence variants are visualized on a difference plot, which magnifies the differences between samples by subtracting the normalized fluorescence data of a wild type sample from the fluorescence data of an unknown sample (Wittwer *et al*. 2003; Montgomery *et al*. 2007) (Fig. 1.7 D). Each sample has a characteristic melting temperature (T_m),

which is defined as the temperature at which 50% of the double stranded DNA is single stranded (Erali and Wittwer 2010). Differences in the T_m and melting curve profile of samples is used to distinguish between different sequence variants (Reed *et al*. 2007). Homozygous samples are, however, more difficult to detect since the differences in T_m is not always prominent (Liew *et al*. 2004). Heterozygous samples are easier to detect due to the presence of a sequence variant, which alters the melting curve shape (Graham *et al*. 2005). HRM has been found to be more sensitive than sequencing for the detection of genetic variants (Nomoto *et al*. 2006). Sequencing is, however, still required to be performed after HRM in order to characterize the genetic variant (Do and Dobrovic 2009).

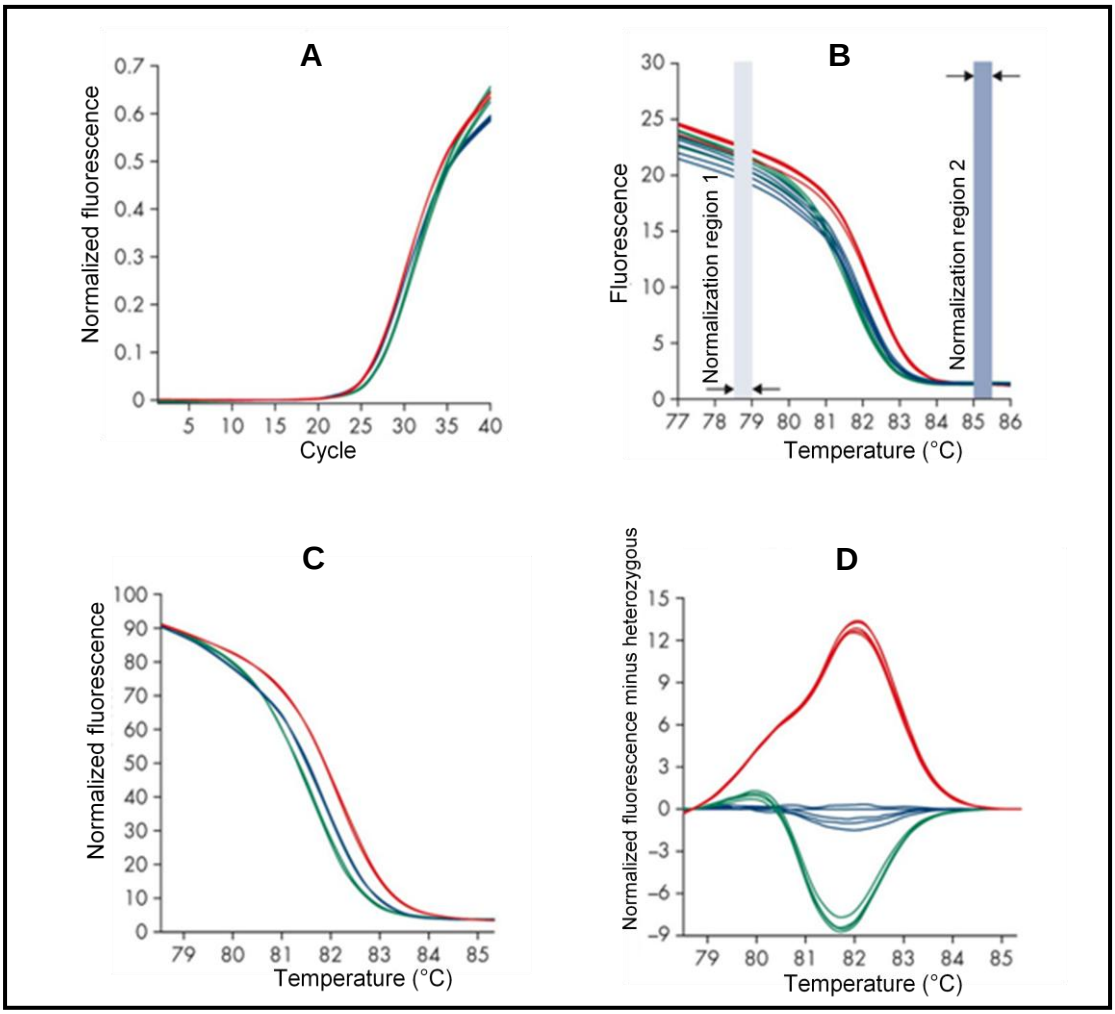

Figure 1.7: The process of HRM analysis. A: The amplification of the PCR is firstly reviewed to establish whether it is sufficient for HRM. B: After HRM, the melting curve data is normalized by selecting pre- and post-melting regions that excludes the melting region. C: Example of the normalized melting curves. D: The different variants are compared to a wild type sample that was selected as reference and the differences between the samples are displayed on a difference plot. The blue curves represent the wild type (or reference) samples whereas the green and red curves represent samples that deviated from the reference samples due to the presence of sequence variants (Copied from www.Qiagen.com).

1.4 Conclusion

MPNs are clonal blood disorders in which the levels of myeloid cells are elevated. The genetic basis of Ph-negative MPNs still remains complex, since several genes have been found to contribute to the pathogenesis of these blood disorders. The discovery of the *JAK2* V617F mutation has contributed greatly to the understanding of the pathogenesis of Ph-negative MPNs. However, the *JAK2* V617F mutation is absent in approximately 5% of PV and 40% of ET and PMF patients, respectively (Vannucchi and Guglielmelli 2012). Therefore, other genes have to be investigated as possible explanations for the development of Ph-negative MPNs. Variants of the *TET2* gene have been found in PV, ET and PMF patients. The *TET2* gene is thought to contribute to the homeostasis of haematopoiesis. Disturbance of the function of the *TET2* gene has revealed to influence myeloid differentiation. Thus, variants within the *TET2* gene could result in abnormal or increased proliferation of myeloid cells. Variants of the *TET2* gene have been found to occur in all of the exons of the gene (Bacher *et al*. 2010). Therefore, Euba *et al*. (2012) suggested that mutational studies of the *TET2* gene should include the entire coding region of the gene. The aim of this study was therefore to screen Ph-negative MPN patients for possible variants in the nine coding exons of the *TET2* gene.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Rationale

The *JAK2* and *MPL* genes form an important part of the 2008 WHO diagnostic criteria for the Ph-negative MPNs. However, variants of the *JAK2* and *MPL* genes are not always present in patients suffering from PV, ET and PMF. Previous studies have indicated that variants of the *TET2* gene could contribute to the pathogenesis of PV, ET and PMF (Delhommeau *et al*. 2009; Pronier *et al*. 2011a). Therefore, the *TET2* gene was investigated for possible genetic variants in PV, ET and PMF patients.

2.2 Aim and objectives of the study

The aim of the study was to perform mutational analysis of the *TET2* gene in PV, ET and PMF patients using sequencing and HRM analysis.

2.3 Study design

The study was an observational descriptive study. HRM analysis and sequencing was used to detect variants in the *TET2* gene of PV, ET and PMF patients.

2.4 Study group

The study group consisted of ten PV, five ET and ten PMF patients (Table 2.1). The control group consisted of ten healthy individuals from the staff of the Department of Haematology and Cell Biology, University of the Free State, Bloemfontein. The PV, ET and PMF patients included in the study visit the Haematology clinic at the Universitas Hospital situated in Bloemfontein, South Africa, on a regular basis. A clinician ensured that the patients included in the study were correctly diagnosed according to the 2008 WHO diagnostic criteria for PV, ET and PMF (Swerdlow *et al*. 2008). Approximately 4 ml of peripheral blood was collected in ethylenediamine tetra acetic acid (EDTA) tubes from each patient and healthy individual. To determine whether *TET2* variants could be acquired at a later stage in the progression of Ph-negative MPNs, two and three blood samples were collected from one ET (E1) and one PMF (M2) patient, respectively, and were included in the

mutational screening of *TET2*. The *JAK2* V617F status of each PV, ET and PMF patient was determined prior to the study as part of routine diagnostic testing (Table 2.1). A full blood count was performed on the control samples to confirm that the individuals were healthy (results not shown). Informed consent was obtained from each patient and healthy individual before collection of the blood samples. A unique number was assigned to each patient to identify their blood sample (Table 2.1). This study was an amendment to an existing protocol for which ethics approval has been granted from the ethics committee of the Faculty of Health Sciences, University of the Free State (ETOVS 15/08).

Table 2.1: Summary of the PV, ET and PMF patients included in the study.

Patient number	Diagnosis	*JAK2* V617F *status*
P1	Polycythaemia Vera	Positive
P2	Polycythaemia Vera	Positive
P3	Polycythaemia Vera	Positive
P4	Polycythaemia Vera	Negative
P5	Polycythaemia Vera	Positive
P6	Polycythaemia Vera	Positive
P7	Polycythaemia Vera	Positive
P8	Polycythaemia Vera	Positive
P9	Polycythaemia Vera	Positive
P10	Polycythaemia Vera	Positive
E1*	Essential Thrombocythaemia	Negative
E2	Essential Thrombocythaemia	Positive
E3	Essential Thrombocythaemia	Positive
E4	Essential Thrombocythaemia	Positive
E5	Essential Thrombocythaemia	Negative
M1	Primary Myelofibrosis	Positive
M2**	Primary Myelofibrosis	Positive
M3	Primary Myelofibrosis	Positive
M4	Primary Myelofibrosis	Positive
M5	Primary Myelofibrosis	Positive
M6	Primary Myelofibrosis	Negative
M7	Primary Myelofibrosis	Negative
M8	Primary Myelofibrosis	Negative
M9	Primary Myelofibrosis	Negative
M10	Primary Myelofibrosis	Positive

*A second blood sample (taken 8 months after collection of first sample) was obtained from patient E1. The two samples were referred to as samples E1.1 and E1.2, respectively, throughout the study.

**Two additional blood samples were collected from patient M2 (taken 6 and 11 months, respectively, after collection of the first sample). The three samples were referred to as samples M2.1, M2.2 and M2.3, respectively, throughout the study.

2.4.1 Inclusion criteria

PV, ET and PMF patients diagnosed by a clinician according to the 2008 WHO diagnostic criteria (Swerdlow *et al*. 2008).

2.4.2 Exclusion criteria

Patients not diagnosed with PV, ET and PMF.

2.5 Methods

2.5.1 Preparation of patient DNA on fast technology for analysis of nucleic acid paper

For each patient sample, approximately 125 µl of the peripheral blood collected in the EDTA tubes was blotted onto fast technology for analysis of nucleid acid (FTA™) paper (Whatman™, Buckinghamshire, UK). Once the blood makes contact with the FTA™ paper, the DNA is captured in the fibres of the paper and remains immobilized (GE Healthcare 2010). The DNA captured in the paper remains stabilized and are protected from external factors such as UV light, nucleases and oxidation (Qiagen 2010). The DNA on the FTA™ paper can be stored for several years at room temperature between 15°C and 25°C (Qiagen 2010). After the blood was blotted onto the FTA™ paper, the paper was allowed to dry for approximately one hour. Subsequently, discs were punched from the FTA™ paper and washed to remove the haemoglobin which has the potential to inhibit the PCR process (Al-Soud and Rödstrom 2001). The washing of the FTA™ paper discs was performed according to the protocol of Whatman™ FTA™ for blood DNA (GE Healthcare 2010).

Discs were punched from the FTA™ paper using a 1.2 mm punch. The discs were placed into a 1.5 ml Eppendorf tube after which 200 µl of FTA Purification Reagent (Whatman™, Buckinghamshire, UK) was added. The discs were incubated in the FTA Purification Reagent for 5 minutes. The FTA Purification Reagent was removed and the wash step repeated twice. This was followed by two washes with 0.1 x TE buffer (10 mM Tris and 0.1 mM EDTA pH 8.0). The 0.1 x TE buffer was discarded after each wash. Finally, the FTA discs were dried in a speed vacuum (Speedvac® SC110, Savant) at 165 x g for approximately one hour. Finally, the dried FTA™

discs were used as template for the PCR. The unused FTA™ paper discs were stored at room temperature for later use.

2.5.2 PCR amplification

The PCR reactions were performed using 24 primer sets specific for the nine coding exons of the *TET2* gene. The primer sequences for exons 3, 4 and 5 were obtained from Olcaydu *et al*. (2011) and Saint-Martin *et al*. (2009) (Table 2.2). Additional primers were designed for exons 6, 7, 8, 9, 10 and 11 using the online primer design program Primer3Plus (Untergasser and Nijveen 2007) using the NC_000004.11 reference sequence obtained from the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) (Table 2.2).

Each PCR reaction contained 5 µl of 5 x GoTaq® Flexi Buffer (Promega, Madison, USA), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.2 µl of 5 U/µl GoTaq® DNA Polymerase (Promega, Madison, USA), 0.2 µl of 100 µM forward and reverse primer (Table 2.2), respectively, and nuclease free distilled water to a final volume of 25 µl. For primer sets 10-2 and 11-2, a volume of 1 µl of 10 µM forward and reverse primer, respectively, was used in the PCR reaction mixture. A Hot Start PCR procedure was used on the ThermoHybaid®PX2 thermal cycler (Thermo Scientific). The cycling conditions were 5 minutes at 95˚C for one cycle, followed by 15 seconds at 95˚C, 15 seconds at the specific annealing temperature of each primer set, 45 seconds at 72˚C for 32 cycles, followed by 7 minutes at 72˚C for one cycle.

Table 2.2: Primers used for the PCR amplification of the *TET2* gene.

Exon	Primer	Sequence (5'-3')	Reference
3	Primer 3-1F	CAGTTTGCTATGTCTAGGTATTCCG	Olcaydu *et al*. (2011)
	Primer 3-1R	AGGCCCACTGCAGTTATGTG	
	Primer 3-2F	TGAACCTTCTCTCTCTGGGC	Olcaydu *et al*. (2011)
	Primer 3-2R	GTCTGTGCGGAATTGATCTG	
	Primer 3-3F	CACACATGGTGAACTCCTGG	Olcaydu *et al*. (2011)
	Primer 3-3R	AAGCAATTGTGATGGTGGTG	
	Primer 3-4F	TCTGTTCAGGTTCCAGCAG	Olcaydu *et al*. (2011)
	Primer 3-4R	TGCTGGCAGTTGTCCTGTAG	
	Primer 3-5F	GCCTCAGAATAATTGTGTGAACAG	Olcaydu *et al*. (2011)
	Primer 3-5R	TTTTGGAACTGGAGATGTTGG	
	Primer 3-6F	AAATTCCAACATGCCTGGG	Olcaydu *et al*. (2011)
	Primer 3-6R	TTCACCATGAAAACATTCTTCC	
	Primer 3-7F	TCCCAGAGTTCACATCTCCC	Olcaydu *et al*. (2011)
	Primer 3-7R	AGTTGCGCAGCTTGTTGAC	
	Primer 3-8F	TTTTGCAGGAAACAAGACCC	Olcaydu *et al*. (2011)
	Primer 3-8R	AAACTGCTTCAGATGCTGCTC	
	Primer 3-9F	TTAAGGTGGAACCTGGATGC	Olcaudy *et al*. (2011)
	Primer 3-9R	AGCCTTTACAAATTGCTCCG	
4	Primer 4F	CCTTAATGTGTAGTTGGGGGTTA	Saint-Martin *et al*. (2009)
	Primer 4R	CTTTGTGTGTGAAGGCTGGA	
5	Primer 5F	ATCCAGTTTGCTTGGCGTAG	Saint-Martin *et al*. (2009)
	Primer 5R	GGCATGAGTCTTTGATCTGG	
6	Primer 6F	TGCAAGTGACCCTTGTTTTG	du Plessis 2012*
	Primer 6R	ACCAAAGATTGGGCTTTCCT	
7	Primer 7F	GCACAGCCTATATAATGCTATCCA	du Plessis 2012*
	Primer 7R	TGTCATATTGTTCACTTCATCTAAGC	
8	Primer 8F	AAGGGGAATAATCTAACTGATAGTCTC	du Plessis 2012*
	Primer 8R	AAATATTTTTGGACATAGGTCATTAGT	
9	Primer 9F	AAAACTAACTACTTTCGCATTCACA	du Plessis 2012*
	Primer 9R	GCAGTGTGAGAACAGACTCAACA	
10	Primer 10-1F	CACGTTTTCTTTGGGACCTG	du Plessis 2012*
	Primer 10-1R	CTGCAGCTTTCTTGGCTTCT	
	Primer 10-2F	CAGGATGTTAGCAGAGCCAGT	du Plessis 2012*
	Primer 10-2R	TTCATTTTTAATATACCACACAACACA	
11	Primer 11-1F	CATTTAAGTATCCTCACTAGCCTTCA	du Plessis 2012*
	Primer 11-1R	TGGATAAGGACTAACTGGATTGG	
	Primer 11-2F	TGTCAACTCTTATTCTGCTTCTGG	du Plessis 2012*
	Primer 11-2R	GGCTGAGACTGGGGAGAATA	
	Primer 11-3F	TGGAAACCTATCAGTGGACAA	du Plessis 2012*
	Primer 11-3R	GAAGTGGCCATCCATCTCAT	
	Primer 11-4F	ATGCAGGGAGATGGTTTCAG	du Plessis 2012*
	Primer 11-4R	CCCATTAGCTGTGTGGGAAA	
	Primer 11-5F	CTCTTCATGCCCTGCATCTC	du Plessis 2012*
	Primer 11-5R	GAGAATTGACCCATGAGTTGG	

	Primer 11-6F Primer 11-6R	AGACAGCGAGCAGAGCTTTC TTTGCCATGGGATTTCTGA	du Plessis 2012*
	Primer 11-7F Primer 11-7R	CGTGAGAAAGAGGAAGAGTGTG GAACTATACTACTGACAGGTTGGTTG	du Plessis 2012*

*Primer sets designed using Primer3Plus with reference sequence NC_000004.11.

F: Forward primer. R: Reverse primer.

2.5.3 Gel electrophoresis

After completion of the PCR reaction, gel electrophoresis was performed to confirm the presence of the correct fragment size. The PCR product was resolved on an ethidium bromide (3 µl) stained 2.5% agarose gel in 1 x TAE buffer (2 M Tris acetate and 0.05 M EDTA pH 8). The gel was run at 120 V for approximately 45 minutes. A molecular weight marker (Promega, Madison, USA) was also resolved on the gel to confirm the fragment size of the PCR products. Afterwards, the gel was visualized under UV light with the 3 UV™ Transilluminator (Syngene). A gel image was obtained using a Nikon S8100 digital camera, after which it was documented.

2.5.4 HRM PCR

The HRM PCR reactions were performed on the ABI 7500 Fast (Applied Biosystems) with the MeltDoctor™ HRM master mix (Applied Biosystems, Foster City, USA). Each PCR reaction consisted of 10 µl of MeltDoctor™ HRM master mix, 1 µl of the 1:100,000 diluted PCR product and nuclease free distilled water to a final volume of 20 µl. The primer concentration in the HRM PCR reaction mixture for exons 3 (primers 3-1 to 3-9), 4, 5, 6, 7, 8, 10 (primer 10-1) and 11 (primer 11-3) was 1000 nM for the forward and reverse primer, respectively. For primers 9, 10-2, 11-1, 11-2, 11-4, 11-5, 11-6 and 11-7 a concentration of 500 nM forward and reverse primer, respectively, was added to the reaction mixture. The cycling conditions for the amplification were 10 minutes at 95˚C for one cycle, followed by 15 seconds at 95˚C, 15 seconds at the specific annealing temperature for each primer set and 45 seconds at 72˚C for 40 cycles. The cycling conditions for the HRM melt curve was 10 seconds at 95˚C, 1 minute at 60˚C followed by 30 seconds at 95˚C with a transition of 0.03˚C per second and finally 15 seconds at 60˚C.

2.5.5 Purification of the PCR product for the sequencing reactions

The PCR product was purified prior to sequencing using Illustra™ ExoStar™ 1-Step (GE Healthcare, Buckinghamshire, UK). Purification of the PCR product was performed to remove excess primers and nucleotides that could interfere with the sequencing. The purification was done according to the protocol of the Illustra™ ExoStar™ 1-Step product. A volume of 2 µl of ExoStar™ 1-Step reagent was added to 5 µl of the PCR product. The mixture was incubated for 15 minutes at 37˚C, followed by 15 minutes at 80˚C. The purified PCR product was used as template for the subsequent sequencing reactions.

2.5.6 DNA sequencing

The sequencing reactions were performed using the BigDye® Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Two sequencing reactions were set up with the forward and reverse primer, respectively. Each sequencing reaction consisted of 2 µl of the BigDye Terminator mix, 1 µl of the sequencing buffer, 1.6 µl of 1 µM primer, 5 µl of the purified PCR product and nuclease free distilled water to a final volume of 10 µl. The cycling conditions were 1 minute at 96˚C for one cycle, followed by 10 seconds at 95˚C, 5 seconds at the specific annealing temperature for each primer set and 4 minutes at 60˚C for 25 cycles.

The purification of the sequencing product was performed using the ethanol/sodium acetate precipitation method according to the ABI PRISM BigDye® Terminator v3.1 Ready Reaction Cycle Sequencing kit protocol (Applied Biosystems, Foster City, USA). A solution containing 3 µl of 3 M sodium acetate (pH 5), 63 µl of 95% ethanol and 14.5 µl nuclease free distilled water was made. A total volume of 10 µl of the sequencing product was added, the solution vortexed for 30 seconds, centrifuged at 12,700 x g for 15 seconds, followed by incubation in the dark for 30 minutes. The mixture was centrifuged for a further 30 minutes at 12,700 x g. The supernatant was removed and 250 µl of 70% ethanol added to wash the pellet by vortexing for 2 minutes, followed by centrifugation at 12,700 x g for 10 minutes. Thereafter, the supernatant was discarded and the pellet was dried at 90˚C for 1 minute. A volume of 25 µl Hi-Di™ Formamide (Applied Biosystems, Foster City, USA) was added to the pellet, followed by vortexing for 1 minute and centrifugation at 12,700 x g for 30

seconds. The sequencing samples were denatured at 95˚C for 2 minutes and cooled on ice for 5 minutes. Finally, the sequencing product was vortexed for 10 seconds and centrifuged for 30 seconds at 12,700 x g. The final product was stored in the dark at 4˚C until capillary electrophoresis was performed.

The sequencing samples were run on the ABI Prism 3130 Genetic Analyser (Applied Biosystems). The DNA fragments were separated in a coated capillary filled with performance optimized polymer 7 (POP-7). The samples were electro-kinetically injected for 12.5 seconds at 1.2 KV and separated for 47 minutes at 8.5 V/cm, 5 µA at 60˚C. The Sequencing Analysis Software (v5.3.1) (Applied Biosystems) was used to analyse the sequencing results. The data obtained from the analysis was visualized using an online program, Chromas Lite v2.3.1 (www.technelysium.com.au/chromas_lite.html). The data was compared to the NC_000004.11 reference sequence obtained from NCBI (http://www.ncbi.nlm.nih.gov/) using the online program Lalign (Huang and Muller 1991).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Primer design

A total of 24 primer sets were used for the PCR, sequencing and HRM reactions to analyze the nine coding exons of the *TET2* gene. The primers were designed to cover the entire coding region of *TET2*. Multiple primer sets were used for exons 3 (3,454 bp), 10 (354 bp) and 11 (1,454 bp) to ensure that the fragment sizes were suitable for sequencing and HRM analysis. The amplicon size for the different primer sets ranged from 233 bp to 698 bp (Table 3.1).

3.2 Annealing temperature optimization of the primers for *TET2*

In order to achieve optimal PCR results the annealing temperatures of each of the 24 primer sets was optimized. The optimization of the primer annealing temperature is important to ensure maximum product yield without the presence of non-specific amplification product (Rychlik *et al*. 1990). The annealing temperature of the primers was optimized by using a temperature gradient ranging from 50°C to 69°C. FTA discs from one control sample were used to optimize the annealing temperatures of the 24 primer sets. The optimized annealing temperatures for the 24 primer sets of *TET2* varied between 57°C and 62°C (Table 3.1). Non-specific amplification products were not visible at the optimized annealing temperatures for the 24 primer sets (data not shown). The optimized annealing temperature of each primer set was also used for subsequent HRM and sequencing reactions.

Table 3.1: The optimized annealing temperatures for the 24 primer sets for the PCR amplification of *TET2*.

Exon	Primer	Fragment size (bp)	Optimized annealing temperature (°C)
3	Primer 3-1	698	62
	Primer 3-2	540	61
	Primer 3-3	558	62
	Primer 3-4	538	61
	Primer 3-5	528	62
	Primer 3-6	544	61
	Primer 3-7	545	62
	Primer 3-8	547	62
	Primer 3-9	588	62
4	Primer 4	267	58
5	Primer 5	356	62
6	Primer 6	340	62
7	Primer 7	285	62
8	Primer 8	248	62
9	Primer 9	233	62
10	Primer 10-1	298	62
	Primer 10-2	234	57
11	Primer 11-1	287	62
	Primer 11-2	297	62
	Primer 11-3	298	62
	Primer 11-4	288	62
	Primer 11-5	290	62
	Primer 11-6	298	60
	Primer 11-7	300	62

3.3 HRM analysis of the *TET2* gene

Of the 24 primer sets tested, HRM analysis only proved successful for amplicon from two primer sets. This conclusion was based on the comparison of the HRM analysis to the sequencing results. The two primer sets successfully used for HRM analysis were primer pairs 11-4 and 11-5 in exon 11, respectively.

3.3.1 Successful HRM analysis results of primer pairs 11-4 and 11-5 in exon 11

The difference plot obtained from the HRM analysis for primer pair 11-4 in exon 11 identified three variant groups in addition to the wild type group (Fig. 3.1). Sequencing of patient samples from the variant groups confirmed the presence of four previously published single nucleotide polymorphisms (SNPs). The variants detected included I1762V (Gerhard *et al*. 2004; Langemeijer *et al*. 2009a), H1778R (Langemeijer *et al*. 2009a), L1721W (Langemeijer *et al*. 2009a) and E1786E (Schuster *et al*. 2010). The first variant group indicated on the difference plot consisted of patients that were heterozygous for three different SNPs which included I1762V (adenine to guanine base change), H1778R (adenine to guanine base change) and L1721W (thymine to guanine base change) (Fig. 3.2 and 3.3). The second variant group included patients homozygous for the H1778R and I1762V SNPs, while the third variant group consisted of samples heterozygous for E1786E (base change of a guanine to an adenine). Sequencing of the samples, as well as the wild type group, confirmed the presence or absence of any sequence variants.

The difference plot for the HRM analysis of primer pair 11-5 in exon 11 identified one variant group in the patient samples. Sequencing of samples from the variant group confirmed the presence of the heterozygous E1786E SNP (Schuster *et al*. 2010), the result of a base change of a guanine to an adenine (Fig. 3.4). Sequencing of samples from the wild type group confirmed the absence of any sequence variants.

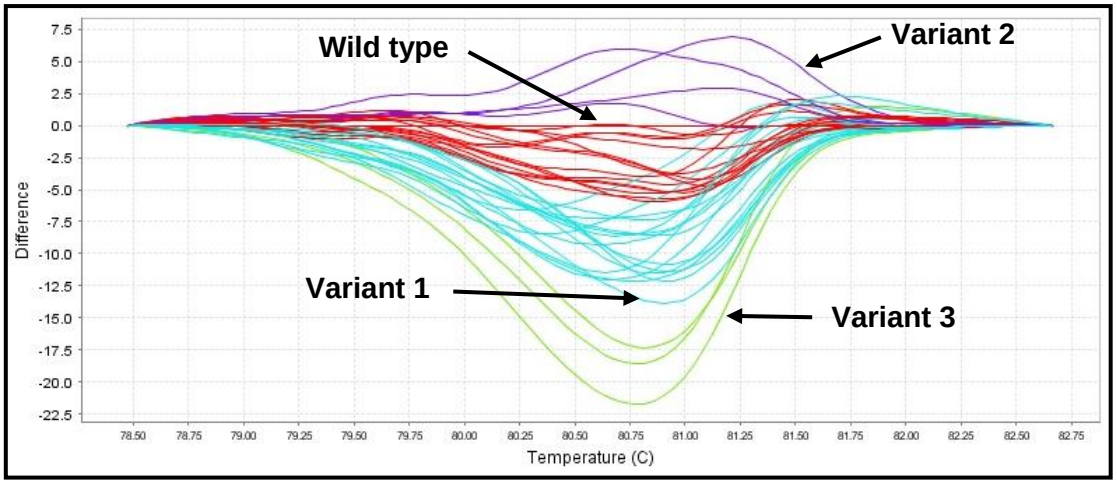

Figure 3.1: Difference plot obtained from the HRM analysis of primer 11-4 in exon 11. Three variant groups were detected in the patient samples. Blue curves: Variant one, purple curves: variant two, green curves: variant three and red curves: wild type.

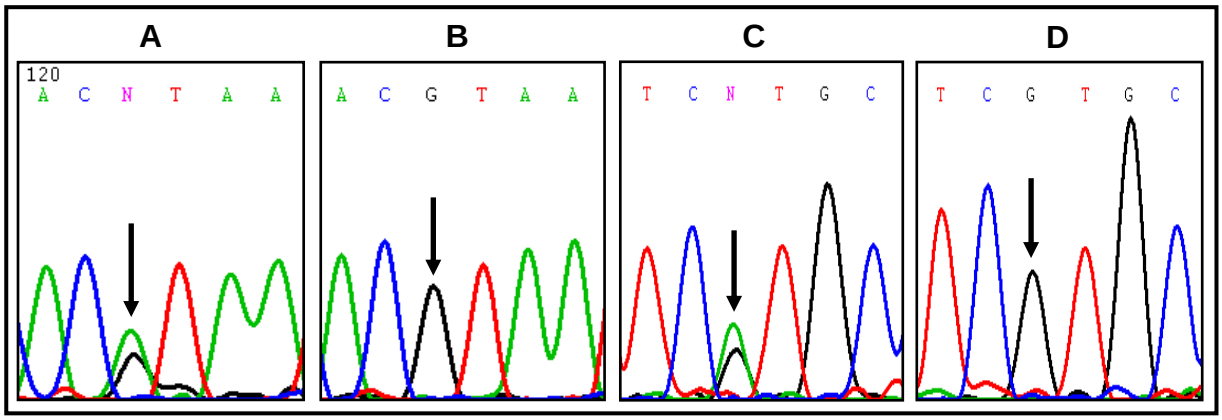

Figure 3.2: Sequence chromatographs of the I1762V and H1778R SNPs detected with primer 11-4 in exon 11. A: Chromatograph of the heterozygous I1762V SNP in patient P1. B: Chromatograph of the homozygous I1762V SNP in patient M7. C: Chromatograph of the heterozygous H1778R SNP in patient M1. D: Chromatograph of the homozygous H1778R SNP in patient P5. The arrows indicate the positions of the respective SNPs.

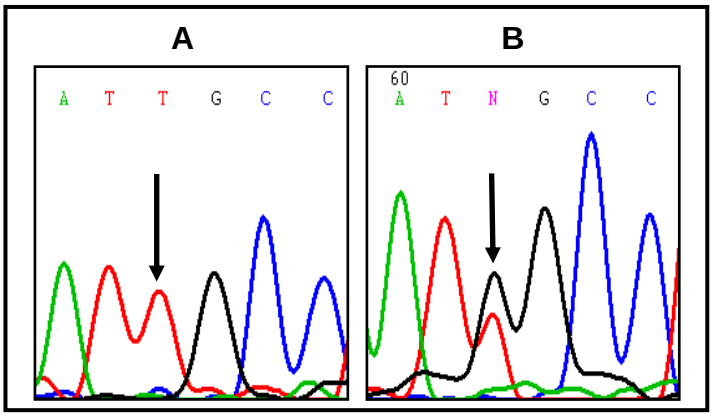

Figure 3.3: Sequence chromatographs of the L1721W SNP detected with primer 11-4 in exon 11. A: Chromatograph of the wild type sequence. B: Chromatograph of the heterozygous L1721W SNP. The arrows indicate the position of the L1721W SNP.

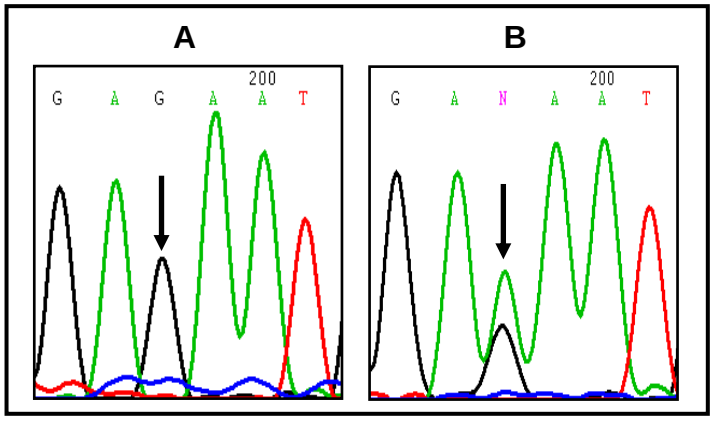

Figure 3.4: Sequence chromatographs of the E1786E SNP detected with primer 11-5 in exon 11 in patient E5. A: Chromatograph of the wild type sequence. B: Chromatograph of the heterozygous E1786E SNP. The arrows indicate the position of the E1786E SNP.

3.3.2 Unsuccessful HRM analysis of exons 3 to 11 of *TET2*

Unfortunately, there was no correlation between the HRM analysis results and the presence of *TET2* variants detected with sequencing in exons 3, 4, 5, 6, 7, 8, 9, 10 and 11 (with the exception of primer sets 11-4 and 11-5). Sequencing of samples where HRM analysis failed, revealed that either the variants identified with HRM analysis did not contain SNPs, or that the presence of SNPs was not in concordance with variants versus wild type grouping. The presence of non-specific amplification products, primer dimer, multiple melting domains and the occurrence of high melting background on the melting curves was suspected to be the cause of the discrepancy between the HRM analysis results and sequencing results.

3.3.2.1 Variant groups identified with HRM not in concordance with sequencing results

Sequencing of exons 4, 6, 7, 10 (primer pairs 10-1 and 10-2) and 11 (primer pairs 11-1, 11-2, 11-6 and 11-7) did not detect any SNPs in any patient samples while HRM analysis identified variant groups.

- Exons 4, 6, 10 (primer set 10-1) and 11 (primer sets 11-1 and 11-6): The cause of the discrepancy between the sequencing results and HRM analysis results of exons 4, 6, 10 (primer set 10-1) and 11 (primer sets 11-1 and 11-6) was suspected to be the presence of non-specific amplification products and primer dimer. For example, the difference plot obtained from the HRM analysis of exon 4 identified two variant groups in the patient samples. Sequencing of samples from the two variant groups, however, confirmed the absence of any *TET2* variants in exon 4 of the patient samples. Gel electrophoresis of the HRM products of exon 4 confirmed the presence of non-specific amplification products in a number of patient samples (Fig. 3.5). The melting domain observed on the derivative melting curve of amplicon for exon 4 had a similar melting profile to the theoretical derivative melting curve of exon 4, which had two melting domains (Fig. 3.6 and 3.7). The theoretical melt curve of exon 4 was determined using the online melting curve prediction Umelt software program (v2.02) (www.dna.utah.edu/umelt/umphp) (Dwight *et al*. 2011).

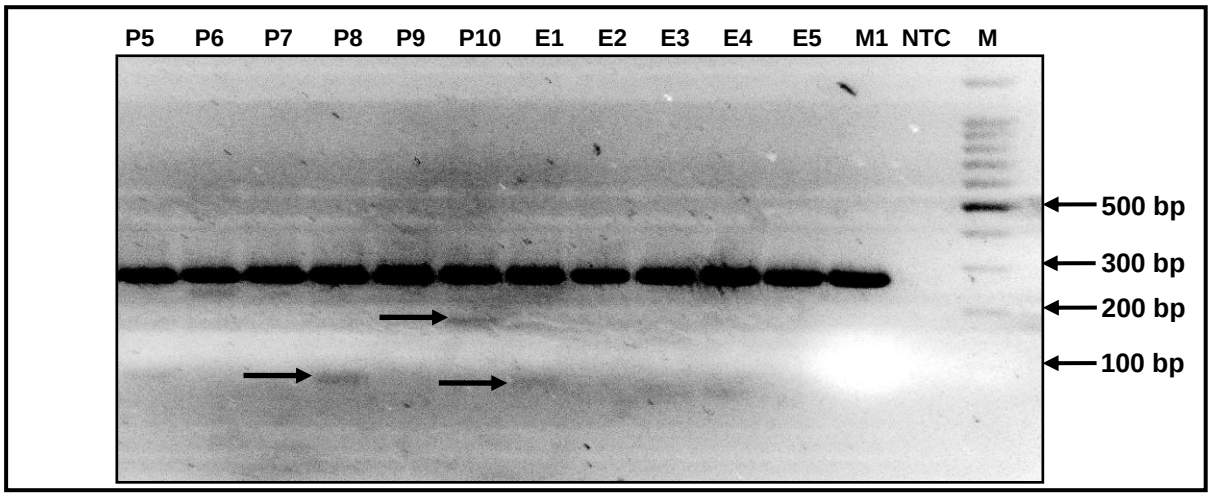

Figure 3.5: Gel image of the HRM PCR products of exon 4. The correct amplicon size of 267 bp was obtained with all the patient samples (displaying samples P5 to M1). Non-specific amplification products were observed at various patient samples (indicated with arrows). Lane NTC: No template control and lane M: 100 bp molecular weight marker.

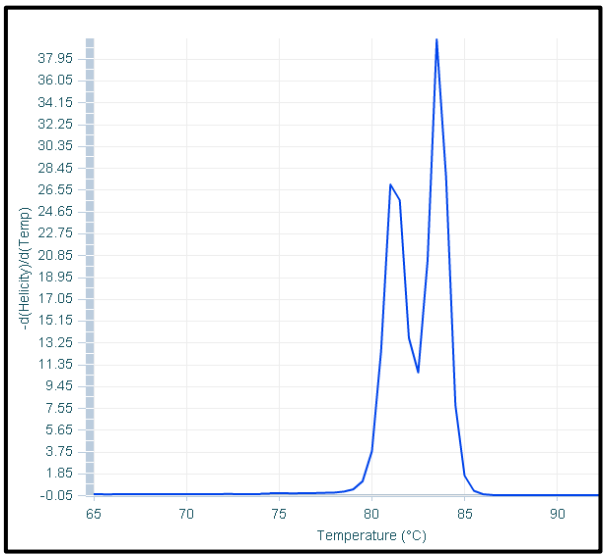

Figure 3.6: The predicted derivative melting curve of exon 4 as determined using the online Umelt software (www.dna.utah.edu/umelt/umphp) (Dwight *et al*. 2011).

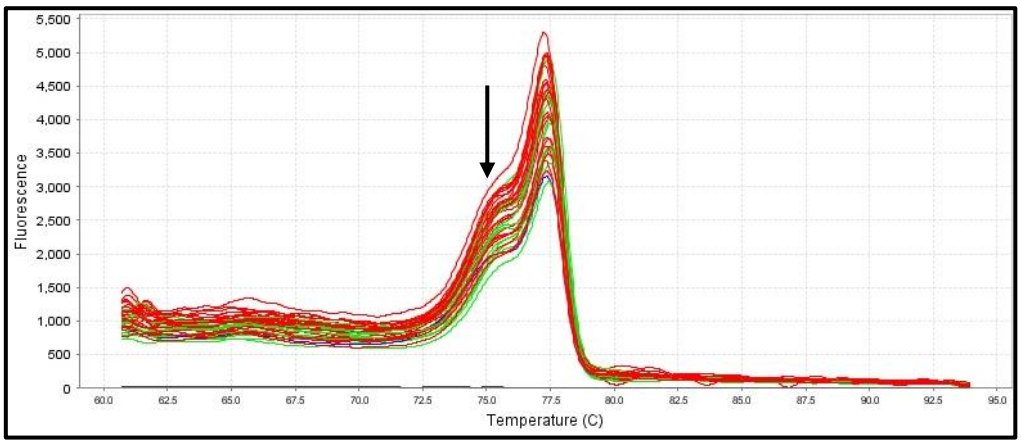

Figure 3.7: Derivative melting curves of exon 4. The arrow indicates the slight elevation of the first melting domain.

In another example, the difference plot from the HRM analysis of exon 6 identified one sequence variant in only patient sample P9 (Fig. 3.8). A second melting domain was observed on the derivative melting curves of exon 6 (Fig. 3.9). Gel electrophoresis of the HRM products of exon 6 revealed the presence of primer dimer smaller than 100 bp in all patient samples (Fig. 3.10). Erali and Wittwer (2010) suggested that primer dimers can be identified as smaller melting peaks with a lower melting temperature on the derivative melting curves obtained from HRM analysis. The presence of the second melting domain in the derivative melting curves of exon 6 could therefore be attributed to the presence of primer dimer, since the theoretical derivative melting curve of exon 6 only had one melting domain (Fig. 3.11). Sequencing of exon 6 in all the patient samples, including patient sample P9, detected no *TET2* variants. The HRM product of patient sample P9 had an additional fragment of approximately 250 bp and it is speculated that the melting behaviour of patient sample P9 deviated from the other wild type samples due to the presence of the non-specific amplification product of 250 bp (Fig. 3.10).

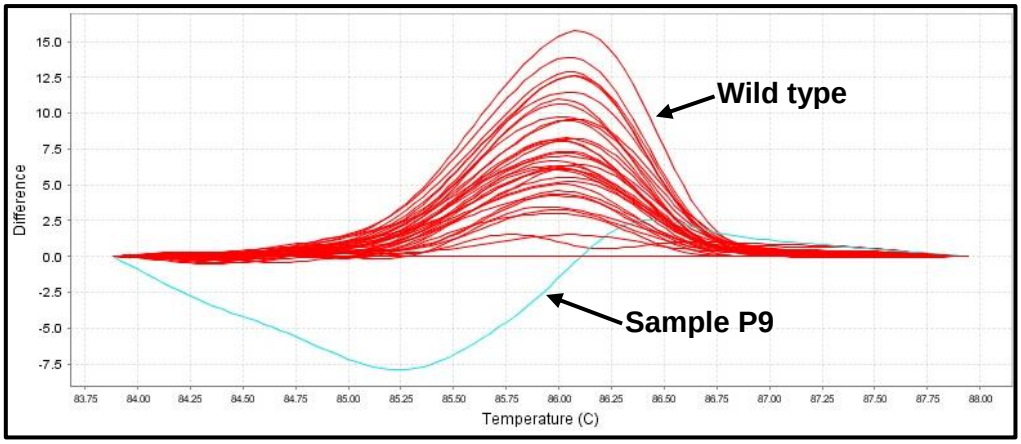

Figure 3.8: Difference plot obtained from the HRM analysis of exon 6. Only patient sample P9 deviated from the wild type group.

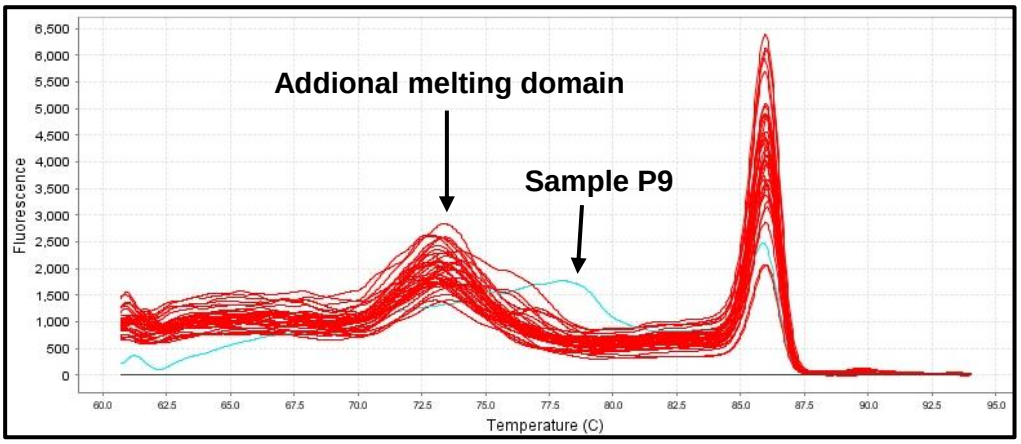

Figure 3.9: Derivative melting curves of exon 6. An additional melting domain was observed on the derivative melting curves of exon 6. The melting curve of patient sample P9 deviated from the other patient samples.

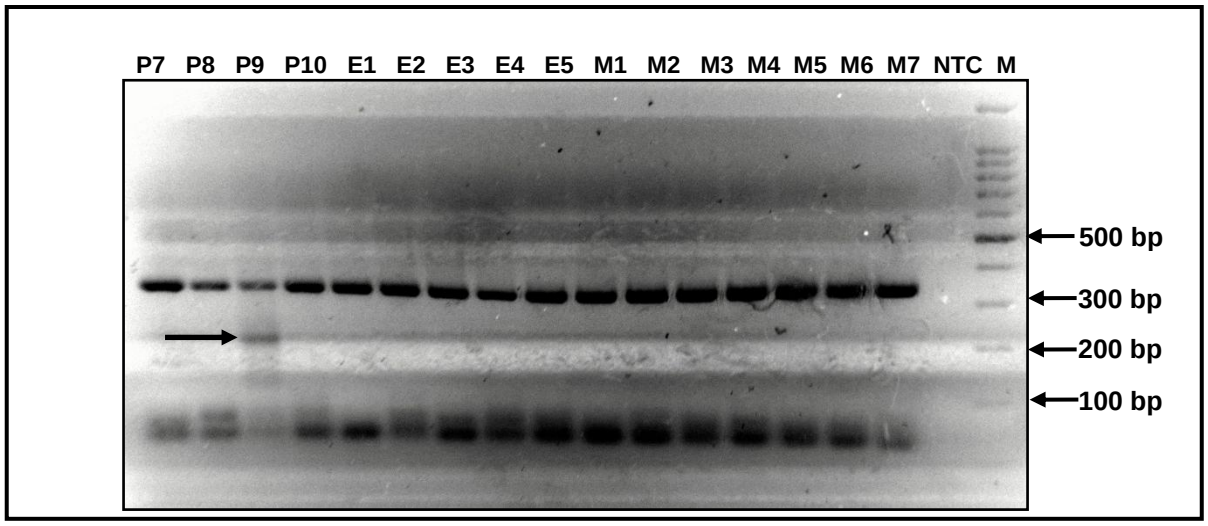

Figure 3.10: Gel image of the HRM PCR products of exon 6. The correct amplicon size of 356 bp was observed with all the patient samples (displaying samples P7 to M7). Primer dimer smaller than 100 bp were observed with all the samples while patient sample P9 had an additional non-specific amplification product of approximately 250 bp (indicated with arrow on gel image). Lane NTC: No template control and lane M: 100 bp molecular weight marker.

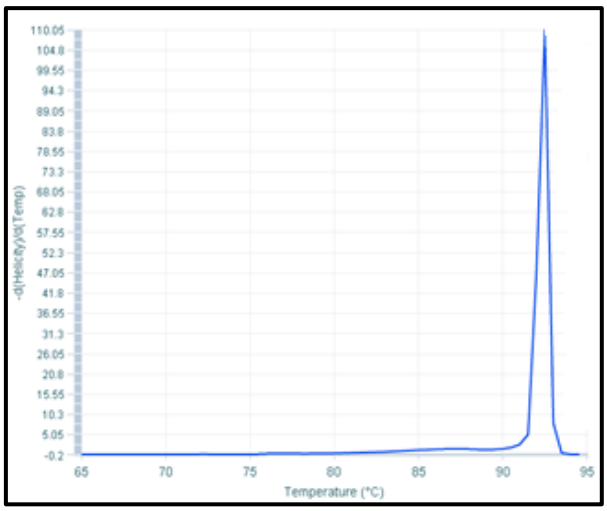

Figure 3.11: The predicted derivative melting curve of exon 6 as determined using the online Umelt software (www.dna.utah.edu/umelt/umphp) (Dwight *et al*. 2011).

- Exons 7 and 11 (primer sets 11-2 and 11-7): The presence of multiple melting domains in the melting curves of exons 7 and 11 (primer sets 11-2 and 11-7) was suspected to cause the HRM analysis results and sequencing results to not correspond. For example, the HRM analysis of exon 7 identified two sequence variants in the patient samples. Sequencing of the patient samples, however, did not detect any *TET2* sequence variants. The melting curves of exon 7 had four melting domains while the theoretical melting curves only had two melting domains (Fig. 3.12 and 3.13). Gel electrophoresis of the HRM products of exon 7 confirmed the presence of primer dimer in all patient samples (Fig. 3.14). The primer dimers were suspected to cause the multiple melting domains, which eventually resulted in the sequencing and HRM results to not correspond. A previous study performed by Reed and Wittwer (2004) reported that multiple melting domains are easier to analyze than those with a single melting domain. In contrast, Krypuy *et al*. (2007) suggested that multiple melting domains should be avoided for HRM analysis, since multiple domains mask the presence of sequence variants. In the current study it was found that multiple melting domains reduced the sensitivity of sequence variant detection and should be avoided for accurate HRM analysis.

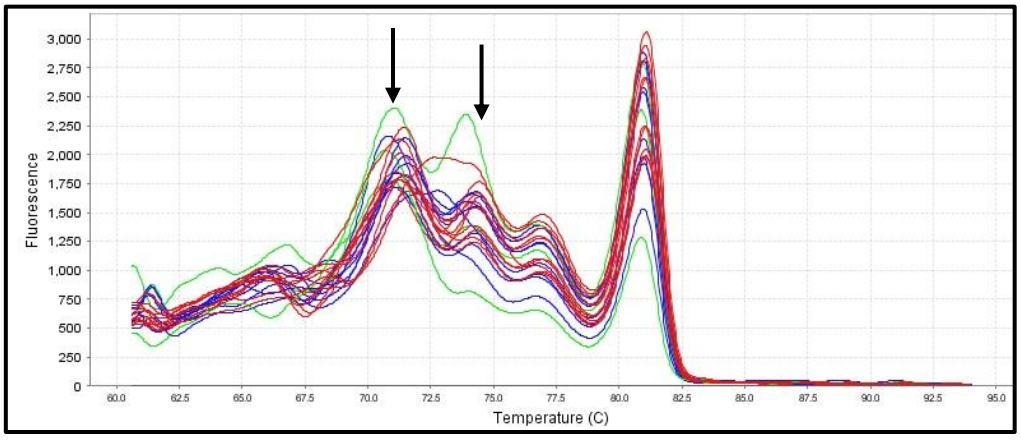

Figure 3.12: Derivative melting curves of exon 7. The arrows indicate the positions of the additional melting domains.

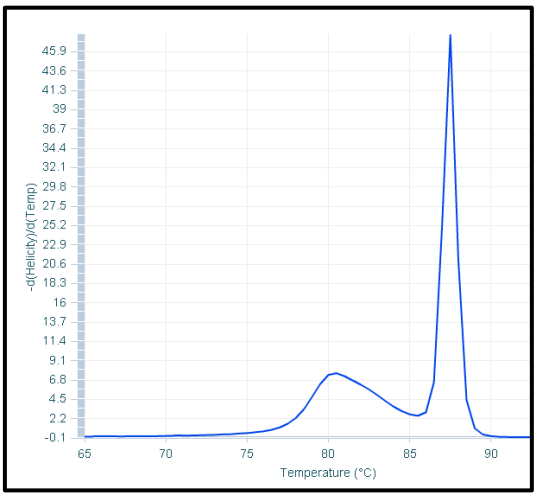

Figure 3.13: The predicted derivative melting curve of exon 7 as determined using the online Umelt software (www.dna.utah.edu/umelt/umphp) (Dwight *et al*. 2011).

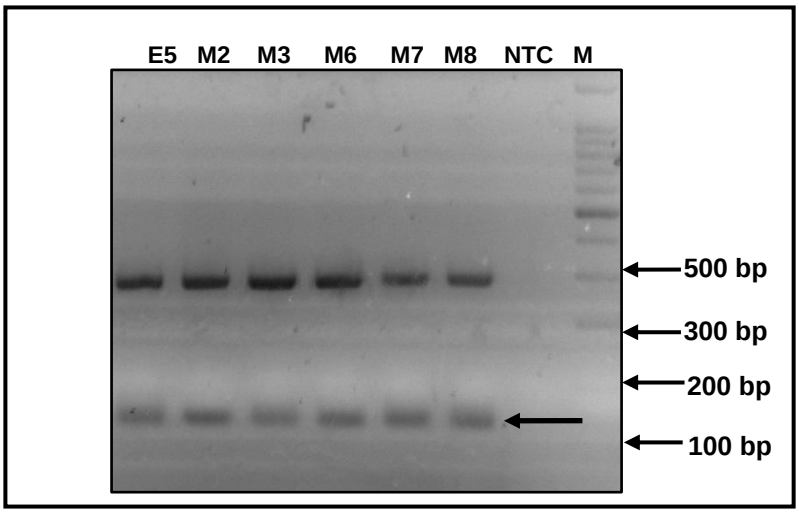

Figure 3.14: Gel image of the HRM PCR products of exon 7. The correct amplicon size of 293 bp and primer dimer smaller than 100 bp was observed with all the patient samples (displaying patient samples E5, M2, M3, M6, M7 and M8). The arrow on the gel image indicates the position of the primer dimers. Lane NTC: No template control and lane M: 100 bp molecular weight marker.

- Primer set 10-2 in exon 10: High melting background observed in the derivative melting curve of primer set 10-2 was suspected to be the cause of the discrepancy between the sequencing results and HRM analysis results (Fig. 3.15). The HRM analysis of primer set 10-2 identified two variant groups in the patient samples. Sequencing of samples with primer set 10-2, however, did not detect any sequence variants. Gel electrophoresis of the HRM products revealed the presence of primer dimer in all patient samples. The primer dimers, as well as the background PCR amplification, were speculated to cause the incorrect HRM analysis results of primer set 10-2.

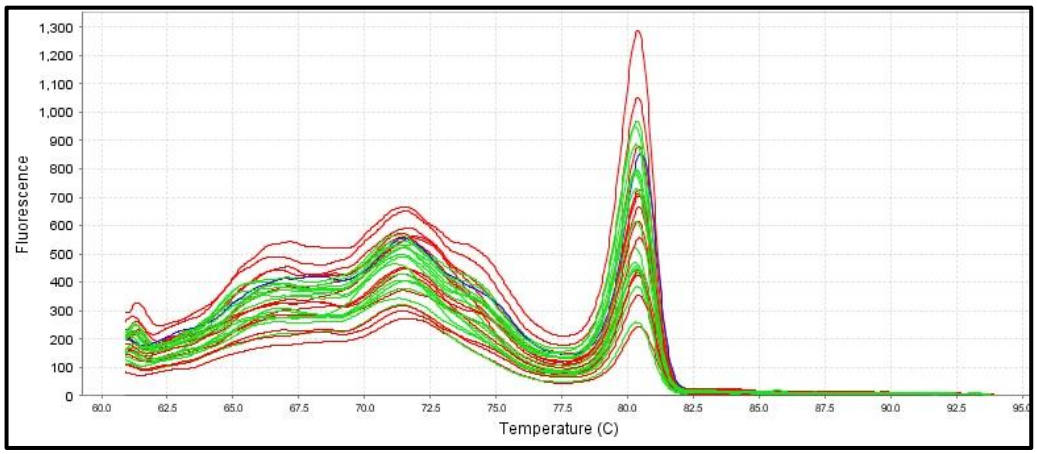

Figure 3.15: Derivative melting curves of primer 10-2 in exon 10.

3.3.2.2 SNPs detected with sequencing not identified with HRM analysis

Sequencing of exons 3, 5, 8, 9 and 11 (primer pair 11-3) detected SNPs in a number of patients, but there was no variant versus wild type grouping concordance with the HRM analysis.

- Exons 3, 5, 8 and 11 (primer set 11-3): Multiple melting domains and/or primer dimer observed with exons 3, 5, 8 and 11 (primer set 11-3) were suspected to result in incorrect HRM analysis results. Although the HRM analysis of exon 8 identified four variant groups in the patient samples, sequencing only confirmed the presence of one variant, the c.3955-3C>T SNP, (Schuster *et al*. 2010) in patient sample P3. Three melting domains were present in the melting curves of exon 8, while the theoretical melting curves only had two melting domains (Fig. 3.16 and 3.17). Gel electrophoresis of the HRM products of exon 8 confirmed the presence of primer dimer in all the patient samples. The additional melting domains in exon 8 were suspected to be the result of the primer dimer.

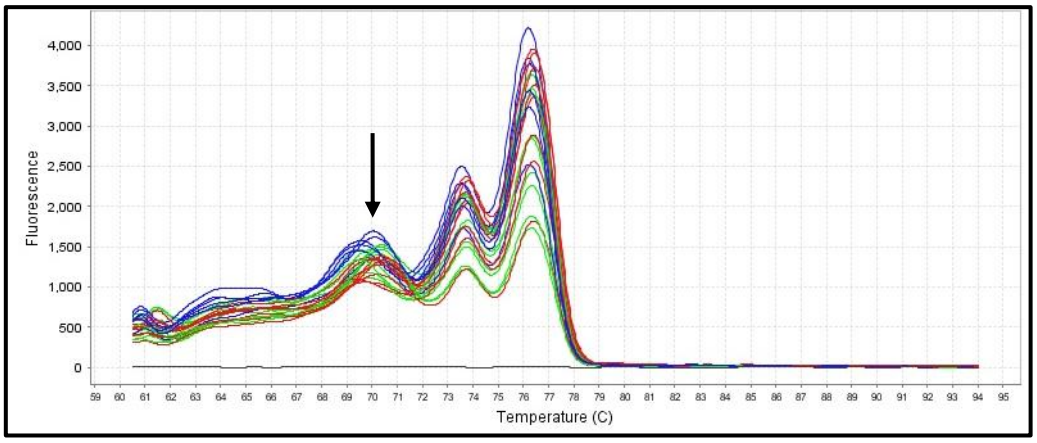

Figure 3.16: Derivative melting curves of exon 8. The arrow indicates the position of the additional melting domain.

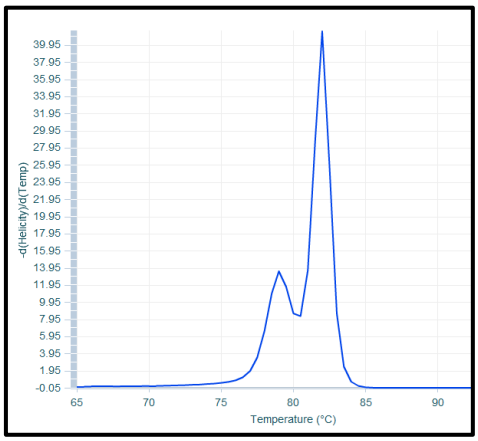

Figure 3.17: The predicted derivative melting curve of exon 8 as determined using the online Umelt software (www.dna.utah.edu/umelt/umphp) (Dwight *et al*. 2011).

- Exon 9: High melting background was observed in the melting curves of exon 9. It was suspected that the high melting background interfered with the HRM analysis of exon 9. The difference plot obtained from the HRM analysis of exon 9 identified three variant groups in the patient samples. Sequencing confirmed that patient samples from the first variant group were heterozygous for the H1380H SNP (Haga *et al*. 2002), while patient samples from the second variant group were homozygous for the H1380H SNP. The only patient sample that was identified as variant 3 was confirmed with sequencing to be heterozygous for the R1359H mutation (Kosmider *et al*. 2009). The sequencing and HRM analysis results of four patient samples, however, did not correspond. Since high melting background was observed on the melting curves of exon 9 (Fig. 3.18), it was decided to perform gel electrophoresis with the HRM products. Gel electrophoresis confirmed the presence of primer dimer in the HRM products of all the patient samples. Thus, the presence of primer dimer and background PCR amplification appears to have contributed to the incorrect HRM analysis results of exon 9.

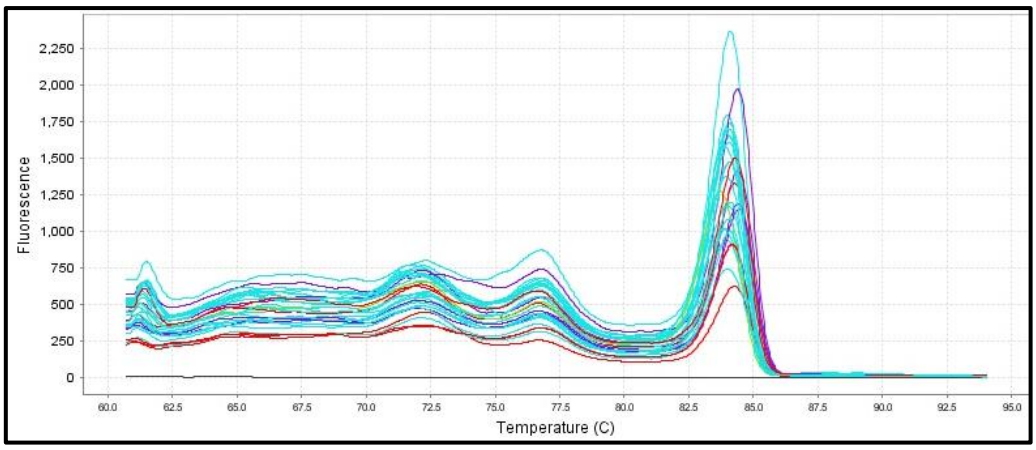

Figure 3.18: Derivative melting curves of exon 9.

3.3.3 Optimization of the HRM analysis by reducing the primer concentration

Since primer dimers were a concern with the successful application of HRM analysis in the current study, further optimization was performed to prevent primer dimer formation. Reducing the primer concentration in the initial PCR and subsequent HRM PCR reactions was used to eliminate the presence of primer dimer. The reduction in primer concentration, however, did not eliminate the occurrence of primer dimer. The primer concentration used for the initial PCR was reduced from 0.8 µM to 0.4 µM for both the forward and reverse primer. After gel electrophoresis was performed it appeared that primer dimer was not present in the initial PCR products (Fig. 3.19 A). HRM analysis was subsequently performed on the PCR products. The primer concentration for the HRM PCR was reduced from 1.0 µM to 0.5 µM for the forward and reverse primer, respectively. Gel electrophoresis of the HRM PCR products revealed the presence of primer dimer of approximately 60 bp in size (Fig. 3.19 B). With a higher primer concentration the derivative melting curves from the HRM analysis had two melting domains (Fig 3.20). This was expected to change with a different primer concentration. However, when a lower primer concentration was used in the initial PCR and subsequent HRM PCR, the derivative melting curves were similar to when a higher primer concentration was used (Fig. 3.21). Thus, it appeared that reducing the primer concentration did not prevent the formation of primer dimer and therefore the presence of multiple melting domains in the derivative melting curves.

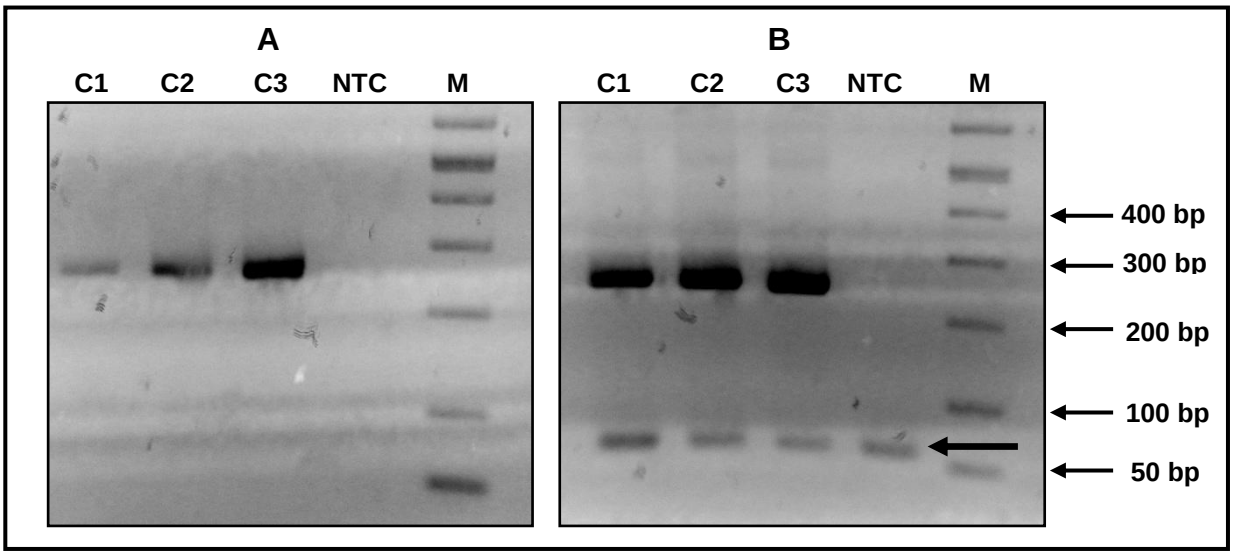

Figure 3.19: Gel images of the initial PCR and subsequent HRM PCR products with a decreased primer concentration of exon 8. A: PCR products of three control samples (C1 to C3) with a primer concentration of 0.4 µM. The expected amplicon of 248 bp was obtained with all the samples without any non-specific amplification products. B: HRM PCR products of the three control samples (C1 to C3) with a primer concentration of 0.5 µM. Primer dimers of approximately 60 bp were observed with all the samples (indicated with an arrow on the gel image). Lane NTC: No template control and lane M: 50 to 2500 bp molecular weight marker.

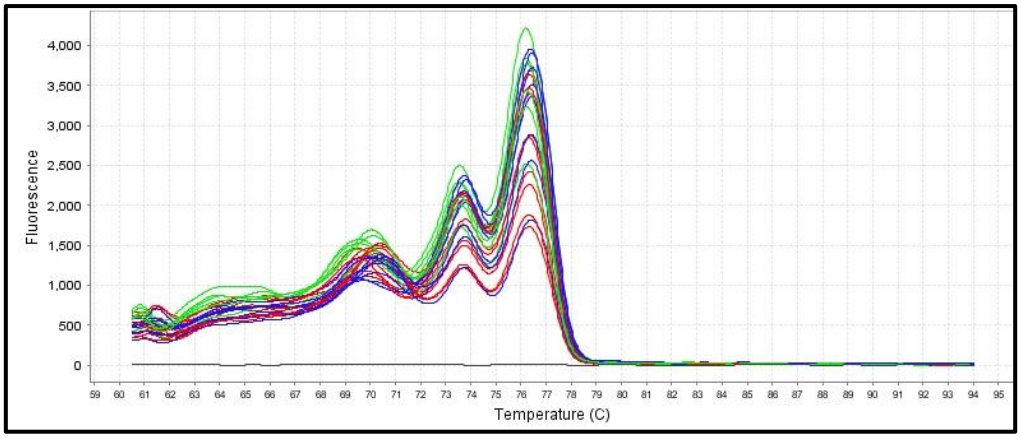

Figure 3.20: Derivative melting curves of exon 8 with a higher primer concentration in the initial PCR and subsequent HRM PCR.

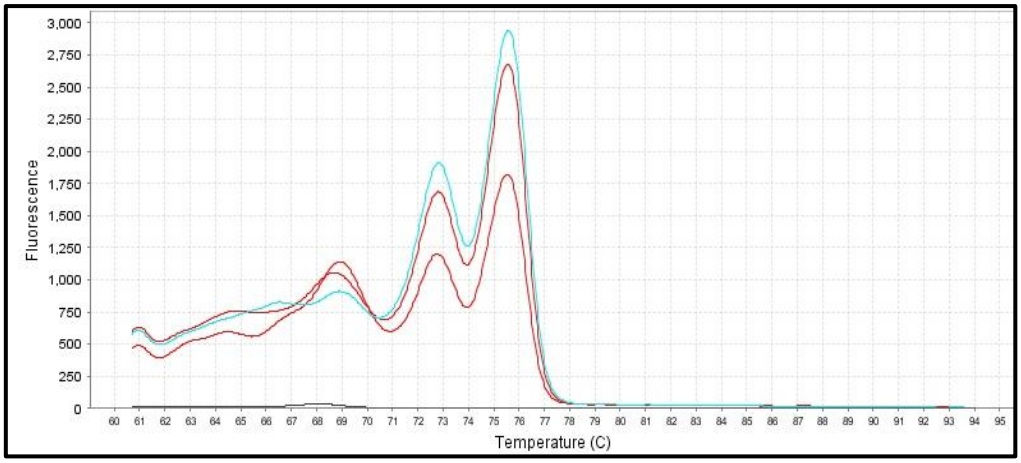

Figure 3.21: Derivative melting curves of exon 8 with a decreased primer concentration in the initial PCR and subsequent HRM PCR.

3.3.4 Conclusion of the HRM analysis of *TET2*

HRM analysis could not be used successfully to detect sequence variants in the nine coding exons of the *TET2* gene. With the exception of primer sets 11-4 and 11-5 in exon 11, there was no concordance between the HRM analysis results and sequencing results. Primer dimer, non-specific amplification, multiple melting domains and high melting background appeared to decrease the sensitivity of the HRM analysis in the current study. A possible reason for why the HRM failed in the current study is that the primers were designed for PCR and not specifically for HRM. Amplicon size, which is an important consideration for HRM analysis, was considered during primer design, but it appeared that non-specific binding of the

primers occurred. It is therefore suggested that new primers have to be designed for successful HRM analysis of *TET2*. While it is possible that using primers specifically designed for HRM could result in non-specific amplification products and primer dimer formation, these issues will have to be addressed in future for the successful application of HRM analysis for *TET2*.

3.4 Sequencing analysis of the *TET2* gene

3.4.1 *TET2* variants detected in both *JAK2* V617F-positive and -negative MPN patients

Previous studies have found that *TET2* variants are more common in *JAK2* V617F-positive MPN patients than in *JAK2* V617F-negative MPN patients (Delhommeau *et al*. 2009; Tefferi *et al*. 2009). In the current study, six of the seven *JAK2* V617F-negative MPN patients had *TET2* variants as opposed to 12 of the 18 *JAK2* V617F-positive MPN patients. Thus, *TET2* variants appeared to be more common than expected in *JAK2* V617F-negative MPN patients in this study. The discrepancy between the results of the current study with previous literature could be attributed to the difference in the study cohort sizes, since Delhommeau *et al*. (2009) and Tefferi *et al*. (2009) included 206 and 203 MPN patients, respectively, in comparison to the 25 MPN patients in the current study. It is also possible that *TET2* variants only occurred during the advanced stages of the PV, ET and PMF diseases, which could not be detected in the current study.

A number of *TET2* variants were found in both *JAK2* V617F-positive and *JAK2* V617F-negative MPN patients in the current study, including one missense SNP (V218M) and four synonymous SNPs (E628E, S795S, Q1030Q and S1039S) found in exon 3 in 17 out of 25 patient samples (Table 3.2). Synonymous SNPs H1380H and E1786E were detected in four and two patient samples in exons 9 and 11, respectively. Two insertion variants (g.92960_92967insATAGATAG and g.92969_92972insTAGA) were found in intron 2 of *TET2* in 10 out of 24 patient samples. One deletion variant (g.101930_101931delTG) was detected in intron 4 of the *TET2* gene in four of the 25 patient samples.

Table 3.2: Summary of *TET2* variants detected in both *JAK2* V617F-positive and -negative MPN patients.

SNP	Type	Result		Number of patients	Type of MPN
		Nucleotide change	Amino acid change		
V218M	Missense	G to A	Valine to Methionine	17	PV, ET, PMF
E628E	Synonymous	G to A	None	2	PMF
S795S	Synonymous	C to T	None	4	ET, PMF
Q1030Q	Synonymous	G to A	None	3	PV, ET, PMF
S1039S	Synonymous	G to A	None	13	PV, ET, PMF
H1380H	Synonymous	T to C	None	4	PV, ET, PMF
E1786E	Synonymous	G to A	None	2	ET, PMF
g.92960_92967ins ATAGATAG	Intronic insertion	Insertion of ATAGATAG	None	4	PV, PMF
g.92969_92972ins TAGA	Intronic insertion	Insertion of TAGA	None	6	ET, PMF
g.101930_101931del TG	Intronic deletion	Deletion of TG	None	4	ET, PMF

* A – adenine; C – cytosine; G – guanine; T – thymine.

- V218M missense SNP: The V218M SNP (Bechtel *et al*. 2007; Langemeijer *et al*. 2009a) causes an amino acid change from a valine to a methionine (Fig. 3.22). This SNP has previously been described in patients with different myeloid related disorders as well as control subjects and genome studies (Bechtel *et al*. 2007; Langemejier *et al*. 2009a; Chou *et al*. 2011; Kohlmann *et al*. 2011; Li *et al*. 2011; Martinez-Aviles *et al*. 2012; Lin *et al*. 2014). In the current study the V218M variant was present in Black, Caucasian and Coloured individuals. There does not appear to be studies on the effect of this SNP on protein function and its occurrence in normal individuals suggests that it is a natural variant of *TET2*.

- E628E synonymous SNP: The synonymous E628E SNP has been reported in normal individuals from European and African populations in a genome study performed by Bustamante *et al*. (2005). In the current study SNP E628E was found in Black and Coloured individuals. The occurrence of this SNP in normal individuals suggests that it is a natural variant of the *TET2* gene.

- S795S synonymous SNP: The synonymous S795S SNP has been described in a genome study that consisted of individuals from the Khoisan and Bantu populations in Southern Africa (Schuster *et al*. 2010). The S795S SNP was present in Black individuals in the current study. Since this SNP has previously been found in normal individuals, it is suspected that S795S is a natural variant of *TET2*.

- Q1030Q synonymous SNP: The Q1030Q variant has previously been found in normal individuals from European and African descent (Bustamante *et al*. 2005). SNP Q1030Q was detected in Black individuals in the current study. The presence of SNP Q1030Q in normal individuals suggests that it is a natural variant of *TET2*.

- S1039S synonymous SNP: SNP S1039S (Haga *et al*. 2002) has previously been described in MPN patients, control subjects as well as in genome studies (Ismael *et al*. 2012; Martinez-Aviles *et al*. 2012) (Fig. 3.23). In the current study the S1039S variant was found in Black and Coloured individuals. Previous studies did not observe any clinical differences between MPN patients with or without this SNP (Ismael *et al*. 2012). Since SNP S1039S has previously been found in normal individuals, it is speculated that this SNP is a natural variant of *TET2*.

- H1380H synonymous SNP: The H1380H SNP (Haga *et al*. 2002) has previously been found in MPN patients as well as in control subjects and genome studies (Ismael *et al*. 2012). SNP H1380H SNP was present in Black individuals in the current study. The occurrence of H1380H in normal individuals suggests that this SNP is a natural variant of the *TET2* gene.

- E1786E synonymous SNP: The E1786E SNP has previously been described in the Khoisan and Bantu populations from Southern Africa (Schuster *et al*. 2010). In the present study variant E1786E occurred in Black individuals. The presence of this variant in normal individuals suggest that it is a natural variant of *TET2*.

There appears to be no studies on the impact of the aforementioned synonymous *TET2* variants on protein function. Although synonymous mutations have no effect on the amino acid sequence, recent literature suggests that these SNPs could slow the process of translation and result in altered protein folding and subsequent protein

function (Kimchi-Sarfaty *et al*. 2007; Bartoszewski *et al*. 2010). When synonymous SNPs result in the occurrence of irregular codons in the messenger RNA (mRNA) the rate of translation could be slower in comparison to when frequent codons are present (Komar 2007). The slower rate of translation is due to the pause of the ribosome, which is influenced by the availability of transfer RNAs (tRNAs) that supply the amino acids during translation (Komar 2007). Slower translation may affect the folding and the final conformation of the protein (Kimchi-Sarfaty *et al*. 2007). Kimchi-Sarfaty *et al*. (2007) found that a synonymous SNP in the multidrug resistance 1 (*MDR1*) gene altered the activity of the P-glycoprotein, which changed its substrate specificity.

- Insertion variant g.92960_92967insATAGATAG: The g.92960_92967insATAGA TAG insertion variant was originally described in different population groups in a genome study performed by Montgomery *et al*. (2013). Individuals with this insertion variant in the current study included Black, Caucasian and Coloured individuals (Fig. 3.24). There does not appear to be studies on the effect of this intronic insertion on protein function, but since it was previously found in normal individuals, it is speculated that it occurs as a natural variant in *TET2*.

- Insertion variant g.92969_92972insTAGA: The g.92969_92972insTAGA insertion variant has previously been described in diverse population groups during a genome study performed by Mills *et al*. (2006). This insertion variant was found in Coloured and Black individuals in the current study. There does not appear to be studies on the impact of this insertion variant on protein function. Since this intronic insertion was previously found in normal individuals, it is possible that this insertion is a natural variant of the *TET2* gene.

- Deletion variant g.101930_101931delTG: The g.101930_101931delTG deletion variant was initially found in a mixed South African population group (rs 376067407) (Sherry *et al*. 2001). In the current study this deletion variant of two bases occurred in Black individuals (Fig. 3.25). It appears that the impact of this variant on protein function has not yet been investigated. The occurrence of this deletion variant in normal individuals suggests that it is a natural variant of *TET2*.

Although it appears that there are no studies on the effect of the deletion and insertion variants detected in the current study, previous studies have reported that

intronic deletions and insertions could interfere with gene splicing. Ganguly *et al*. (2003) detected an intronic insertion of 13 bases in the Factor VIII gene that resulted in the skipping of an exon during gene splicing. The subsequent loss of 39 amino acids resulted in a non-functional A3 domain of the Factor VIII protein, which resulted in Haemophilia A. Another intronic insertion of 320 bp in the neurofibromatosis type 1 (*NF1*) gene lead to the skipping of an exon during gene splicing and resulted in the loss of 77 amino acids of the NF1 protein (Wallace *et al*. 1991). A previously found intronic deletion of 101 bases in the *SLC*34A3 gene that was associated with Hereditary Hypophosphatemic Rickets with Hypercalciuria (HHRH) resulted in a truncated intron, which caused abnormal RNA splicing (Ichikawa *et al*. 2006). The truncated intron was not eliminated during splicing and resulted in the insertion of 22 amino acids in the encoded sodium-dependent phosphate co transporter (NaPi-IIc) protein. A larger intronic deletion consisting of 41,241 bp detected in the *WNK1* gene (associated with hypertension) was found to cause a significant increase in gene expression (Wilson *et al*. 2001).

The SNPs detected in both *JAK2* V617F-positive and -negative MPN patients in the current study appear to be due to natural variation in the population. These SNPs were, however, not found in the control samples, which could be due to the small size of the control group. No novel SNPs were found in both *JAK2* V617F-positive and -negative MPN patients in the current study.

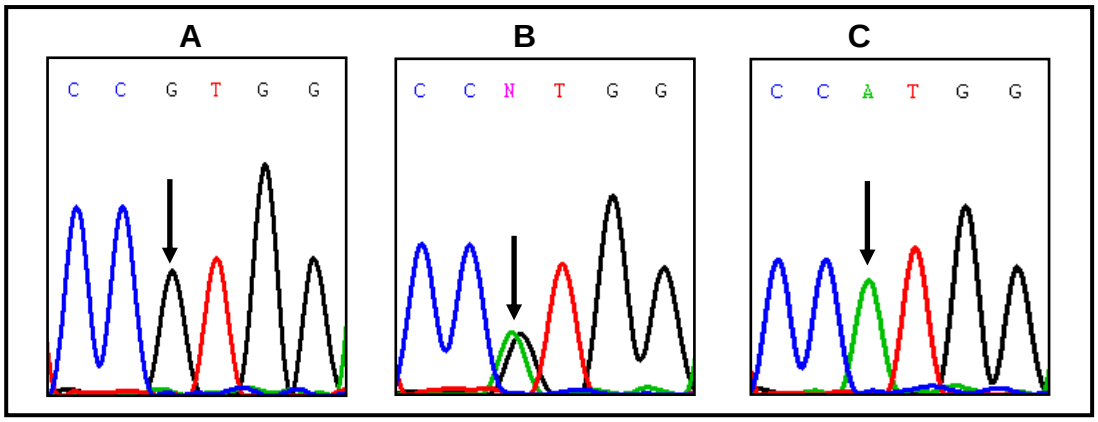

Figure 3.22: Sequence chromatographs of the V218M SNP detected in exon 3 of *TET2*. A: Wild type sequence with a guanine at base 652 in exon 3. B: Heterozygous V218M SNP in patient P5. C: Homozygous V218M SNP in patient P3 with an adenine at base 652. The arrows indicate the position of the SNP in the respective chromatographs.

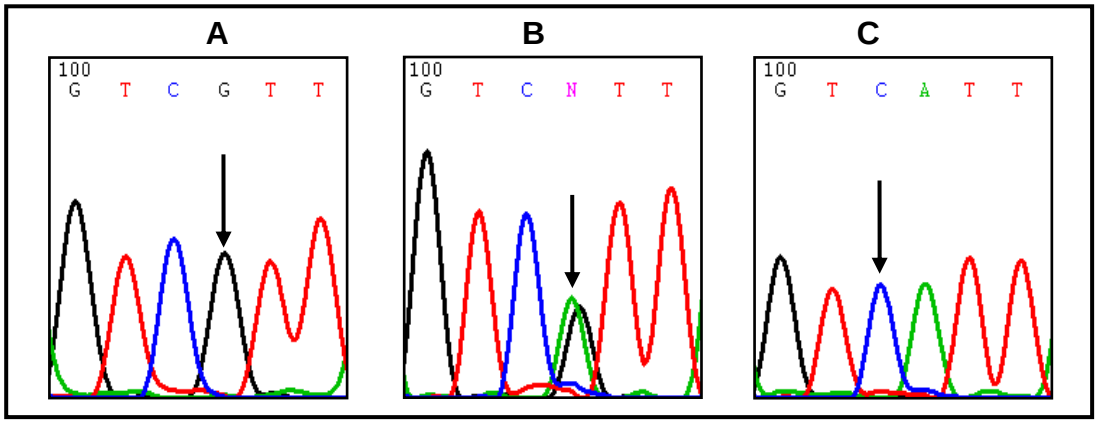

Figure 3.23: Sequence chromatographs of the S1039S SNP detected in exon 3 of *TET2*. A: Wild type sequence with a guanine at base 3117 in exon 3. B: Heterozygous S1039S SNP in patient E5. C: Homozygous S1039S SNP with an adenine at base 3117 in exon 3 in patient P10. The arrows indicate the position of the SNP in the respective chromatographs.

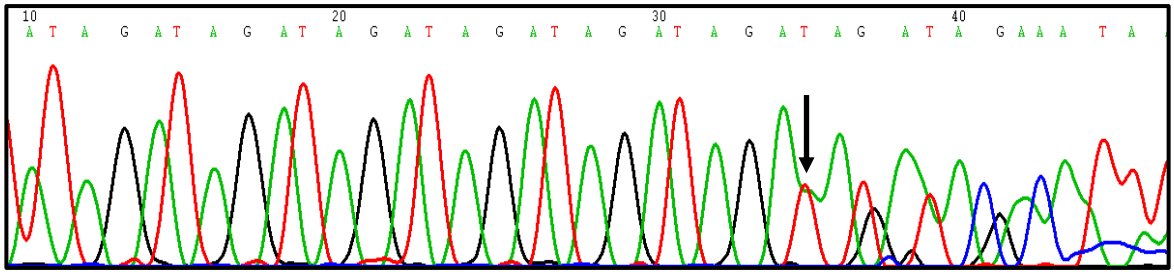

Figure 3.24: Sequence chromatograph of the g.92960_92967insATAGATAG insertion variant in intron 2 of *TET2* in patient M8. The arrow indicates the start of the double sequence, which is the result of the insertion of eight bases (ATAGATAG). The double sequence started at base 35 in the chromatograph and continued throughout the sequence.

Figure 3.25: Alignment of the deletion variant (g.101930_101931delTG) in intron four in patient E4 with the reference sequence (NC_000004.11) from NCBI (www.ncbi.nlm.nih.gov/). The position of the deletion is approximately 22 bases before the start of exon 5. The sequence of exon 5 is indicated in the blue text.

3.4.2 *TET2* variants detected in *JAK2* V617F-positive MPN patients

A number of *TET2* variants were only found in *JAK2* V617F-positive MPN patients in the current study (Table 3.3). These variants were found in exons 3 (Y1149S), 9 (R1359H), 11 (H1778R) and intron 7 (c.3955-3C>T). No novel *TET2* variants were detected in the *JAK2* V617F-positive MPN patients in the current study.

Table 3.3: Summary of *TET2* variants detected only in *JAK2* V617F-positive MPN patients.

SNP	Type	Result		Number of patients	Type of MPN
		Nucleotide change	Amino acid change		
Y1149S	Missense	A to C	Tyrosine to Serine	4	PV, ET, PMF
R1359H	Missense	G to A	Arginine to Histindine	1	PV
H1778R	Missense	A to G	Histidine to Arginine	2	PV, PMF
c.3955-3C>T	Intronic SNP	C to T	None	1	PV

* A – adenine; C – cytosine; G – guanine; T – thymine.

- Y1149S missense SNP: The Y1149S missense SNP (Bustamante *et al*. 2005) results in an amino acid substitution of tyrosine with serine. This missense SNP has previously been described in different population groups (Bustamante *et al*. 2005). In the current study SNP Y1149S was found in Black individuals. SNP Y1149S is present in the cysteine-rich domain of the protein, which suggests that it could interfere with the catalytic activity of the protein. However, it appears that no studies have been done previously to determine its influence on protein function. Since Y1149S was previously found in normal individuals, it is possible that this SNP is a natural variant of the *TET2* gene.

- R1359H missense mutation: The R1359H mutation (Kosmider *et al*. 2009) causes an amino acid change from arginine to histidine (Fig. 3.26). The R1359H missense mutation has previously been described in myeloid related disorders (Kosmider *et al*. 2009; Chou *et al*. 2011). This missense mutation has been associated with increased white blood cell counts and decreased platelet levels (Kosmider *et al*. 2009; Chou *et al*. 2011). In the current study, the Caucasian individual with the R1359H mutation, had increased neutrophil and increased platelet levels. Although this mutation is present in the cysteine-rich domain of the TET2 protein and could interfere with the catalytic activity, it appears that no studies have been done to determine the impact of this mutation on protein function.

- H1778R missense SNP: The H1778R SNP (Langemeijer *et al*. 2009a) results in an amino acid substitution of histidine with arginine. This missense SNP has been described in myeloid related disorders, as well as control subjects (Langemeijer *et al*. 2009a; Nibourel *et al*. 2010; Chou *et al*. 2011; Kohlmann *et al*. 2011; Martinez-Aviles *et al*. 2012; Weissmann *et al*. 2012). In the current study SNP H1778R was detected in Caucasian and Black individuals. Since the H1778R SNP has previously been found in normal individuals, this variant is suspected to be a natural variant of *TET2*. Furthermore, it appears that no studies have been done to establish the impact of this missense variant on protein function.

- Intronic SNP c.3955-3C>T: The c.3955-C>T intronic SNP (Schuster *et al*. 2010) is located in the splice region of *TET2* in intron 7 (Fig. 3.27). This intronic SNP was initially described in the Khoisan and Bantu populations from Southern Africa (Schuster *et al*. 2010). In the current study this intronic SNP was present in one Coloured individual. It appears that no studies have been done on the effect of this SNP on the splicing of *TET2*. The occurrence of this SNP in normal individuals, however, suggests that it is a natural variant of *TET2*. Variants present in the splicing regions of genes have been reported to affect protein splicing and either expose individuals to the development of disorders, or change the severity of a disease phenotype (Wang and Cooper 2007). In a previous study an intronic SNP, also located in the splice region in intron 7 of *TET2*, was associated with abnormal gene splicing and decreased protein expression (Swierczek *et al*. 2011).

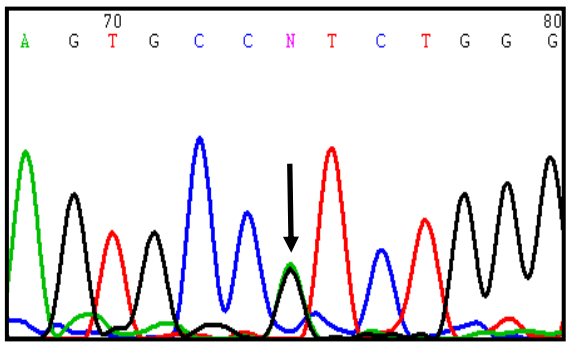

Figure 3.26: Sequence chromatograph of the R1359H mutation detected in exon 9 in patient P5. The arrow indicates the position of the heterozygous R1359H mutation with an adenine and guanine at base 4076 in exon 9.

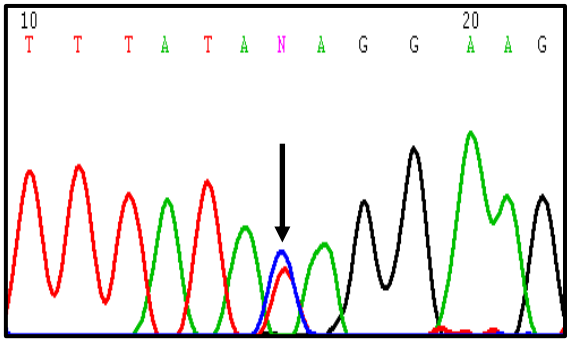

Figure 3.27: Sequence chromatograph of the c.3955-3C>T intronic SNP detected in patient P3. The arrow indicates the position of the SNP.

3.4.3 *TET2* variants detected in *JAK2* V617F-negative MPN patients

A total of five *TET2* variants were only detected in *JAK2* V617F-negative MPN patients in the current study (Table 3.4). These *TET2* variants were present in exon 3 (A347A, N767D and Q810R) and intron 2 (g.92961_92968delTAGATAGA and g.92963_92970delGATAGATA). Since the *JAK2* V617F mutation, which is the main contributor to the pathogenesis of MPNs, was not present in these patients *TET2* could have an impact on the development of the respective disorders. It is, however, unknown whether *JAK2* exon 12 or *MPL* mutations that form part of the WHO diagnostic criteria for PV, ET and PMF and also contributes significantly to the development of Ph-negative MPNs, occurred in these patients. No novel *TET2* variants were found in the *JAK2* V617F-negative MPN patients in this study.

Table 3.4: Summary of *TET2* variants detected only in *JAK2* V617F-negative MPN patients.

SNP	Type	Result		Number of patients	Type of MPN
		Nucleotide change	Amino acid change		
A347A	Synonymous	G to A	None	1	PMF
N767D	Missense	A to G	Asparagine to Aspartic acid	1	ET
Q810R	Missense	A to G	Glutamine to Arginine	1	ET
g.92961_92968 delTAGATAGA	Intronic deletion	Deletion of TAGATAGA	None	1	PMF
g.92963_92970 delGATAGATA	Intronic deletion	Deletion of GATAGATA	None	1	PMF

* A – adenine; C – cytosine; G – guanine; T – thymine.

- A347A synonymous SNP: The synonymous A347A SNP has previously been described in a genome study that included individuals from the Khoisan and Bantu populations from Southern Africa (Schuster *et al*. 2010). In the current study the A347A SNP was found in one Black individual (Fig. 3.28 A). Since A347A has previously been found in normal individuals, it is possible that this SNP is a natural variant of *TET2*.

- N767D missense SNP: The missense N767D SNP (Metzeler *et al*. 2011) results in an amino acid substitution of asparagine with aspartic acid. This SNP was previously described in acute myeloid leukaemia (AML) patients (Metzeler *et al*. 2011). Metzeler *et al*. (2011) reported that it is not yet clear whether N767D is a natural variant of *TET2* or a disease causing mutation. In the current study this variant was present in one Black individual (Fig. 3.28 B). There appears to be no studies on the influence of this missense variant on protein function.

- Q810R missense SNP: The Q810R missense SNP (Bentley *et al*. 2008) causes an amino acid change from glutamine to arginine. This SNP has previously been described in MPN patients, control subjects and genome studies (Bentley *et al*. 2008; Martinez-Aviles *et al*. 2012). In the current study this variant was detected in one Black individual (Fig. 3.28 C). There appears to be no studies investigating the effect of this missense SNP on protein function. The occurrence

of SNP Q810R in normal individuals, however, suggests that it is a natural variant of *TET2*.

- Deletion variant g.92961_92968delTAGATAGA: The g.92961_92968delTAGATAGA deletion variant has previously been described in a genome study that included a diverse population group (Mills *et al*. 2006). This deletion variant was detected in one Black individual in the current study. Furthermore, it appears that the g.92961_92968delTAGATAGA deletion variant is a natural variant of *TET2* due to its previous occurrence in normal individuals.

- Deletion variant g.92963_92970delGATAGATA: The g.92963_92970delGATAGATA deletion variant has been recorded in the database of SNPs (dbSNP) in NCBI (rs 10569647) (Sherry *et al*. 2001) after it was found in an unidentified population group. In the current study this deletion variant was present in one Black individual. It appears as if the impact of this deletion variant on protein function has not been investigated previously.

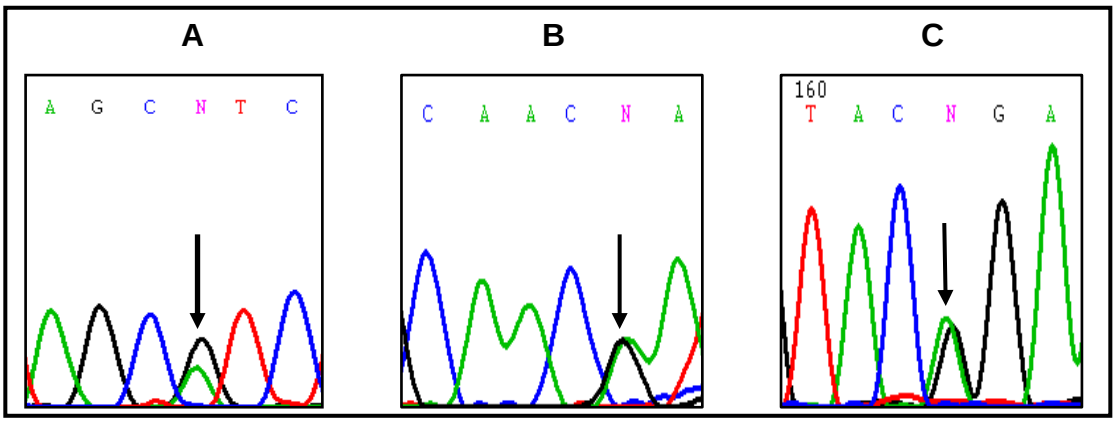

Figure 3.28: Sequence chromatographs of the A347A, N767D and Q810R SNPs detected in exon 3 in *TET2*. A: Heterozygous A347A SNP in patient M6. B: Heterozygous N767D SNP in patient E1. C: Heterozygous Q810R SNP in patient E5. The arrows indicate the positions of the SNPs in the respective sequences.

3.4.4 *TET2* variants detected in control samples

Five *TET2* variants were detected in patient as well as control samples in the current study (Table 3.5). These variants were therefore speculated to be natural variants of the *TET2* coding sequence and not implicated in MPN development. These variants were found in exon 3 (P363L), 11 (L1721W and I1762V) and intron 2 (g.92960_92967delATAGATAG and g.92962_92969insAGATAGAT).

Table 3.5: Summary of *TET2* variants detected in control samples.

SNP	Type	Result		Number of patients	Number of controls	Type of MPN
		Nucleotide change	Amino acid change			
P363L	Missense	C to T	Proline to Leucine	1	2	PV
L1721W	Missense	T to G	Leucine to Tryptophan	2	3	PV, ET
I1762V	Missense	A to G	Isoleucine to Valine	2	3	PV, PMF
g.92960_92967 delATAGATAG	Intronic deletion	Deletion of ATAGATAG	None	9	8	PV, PMF
g.92962_92969 insAGATAGAT	Intronic insertion	Insertion of AGATAGAT	None	3	1	PMF

* A – adenine; C – cytosine; G – guanine; T – thymine.

- Missense P363L SNP: The missense P363L SNP (Langemeijer *et al*. 2009a) results in an amino acid substitution of proline with leucine. This SNP has been reported in different myeloid related disorders, including control subjects (Langemeijer *et al*. 2009a; Nibourel *et al*. 2010; Chou *et al*. 2011; Kohlmann *et al*. 2011; Langemeijer *et al*. 2011; Martinez-Aviles *et al*. 2012; Weissmann *et al*. 2012). In the current study SNP P363L was found in Caucasian individuals (Fig. 3.29). This missense SNP does not appear to have any clinical implications.

- Missense L1721W SNP: The missense L1721W SNP (Langemeijer *et al*. 2009a) causes an amino acid change from leucine to tryptophan. SNP L1721W has been associated with different myeloid related disorders as well as control subjects (Langemejier *et al*. 2009a; Nibourel *et al*. 2010; Chou *et al*. 2011; Kohlmann *et al*. 2011; Langemeijer *et al*. 2011; Martinez-Aviles *et al*. 2012;

Weissmann *et al*. 2012). The L1721W SNP was detected in Black and Caucasian individuals in the present study. It appears that no studies have been done to establish the effect of this missense variant on protein function.

- Missense I1762V SNP: The missense I1762V SNP (Gerhard *et al*. 2004; Langemeijer *et al*. 2009a) results in an amino acid substitution of isoleucine with valine. This SNP has been reported to occur in various myeloid related disorders as well as control subjects and genome studies (Gerhard *et al*. 2004; Langemejier *et al*. 2009a; Nibourel *et al*. 2010; Kohlmann *et al*. 2011; Langemeijer *et al*. 2011; Weissmann *et al*. 2012). SNP I1762V was detected in Black, Caucasian and Coloured individuals in the current study. The I1762V SNP has previously been associated with a better chance of survival in AML patients due to lower treatment mortality (Kutney *et al*. 2010). There was no clinical difference between AML patients with or without this missense variant (Kutney *et al*. 2010). There appears to be no studies investigating the effect of this missense variant on the survival of MPN patients.

- Deletion variant g.92960_92967delATAGATAG: The g.92960_92967delATAGATAG deletion variant was initially detected in a genome study that included individuals from African, European, Chinese and Japanese descent (Montgomery *et al*. 2013). This intronic deletion was present in Black, Caucasian and Coloured individuals in the current study (Fig. 3.30). It appears that no studies have been done to establish the effect of this intronic deletion on protein function.

- Insertion variant g. 92962_92969delAGATAGAT: The g.92962_92969delAGATAGAT insertion variant has been recorded in the database of SNPs (dbSNP) in NCBI (rs 72224084) (Sherry *et al*. 2001) after it was found in an unidentified population group. In the present study this intronic insertion was detected in Black and Caucasian individuals and it appears as if the impact of this insertion on protein function has not previously been investigated.

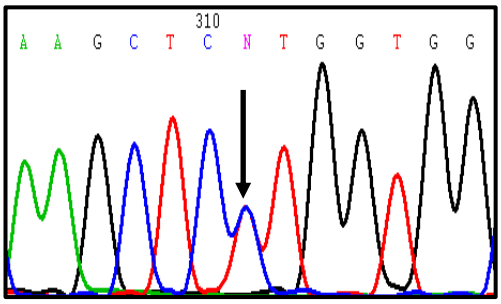

Figure 3.29: Sequence chromatograph of the P363L SNP detected in exon 3 in *TET2*. The arrow indicates the position of the heterozygous P363L SNP in patient P2.

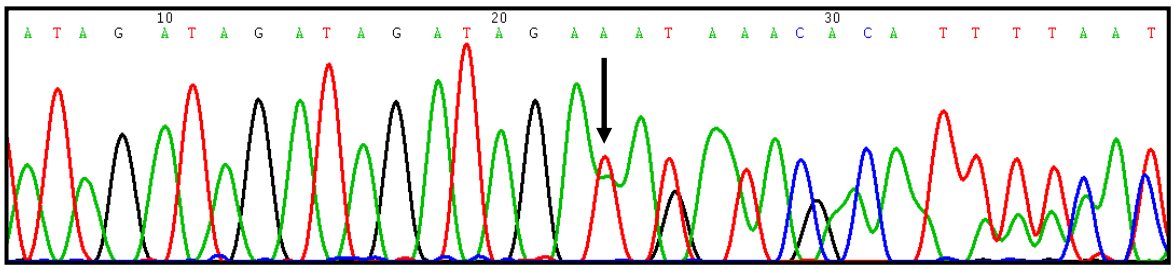

Figure 3.30: Sequence chromatograph of the g.92960_92967delATAGATAG deletion variant in intron 2 of *TET2* in patient P1. The arrow indicates the start of the double sequence, resulting from the eight base deletion (ATAGATAG). The double sequence was observed until the end of the sequence chromatograph.

3.4.5 Accumulation of *TET2* variants in MPN patients

It has been suggested in literature that *TET2* variants are more common in MPN patients older than 60 years as opposed to younger patients (Tefferi *et al*. 2009). *TET2* variants detected in the current study were not significantly more prevalent in MPN patients older than 60 years as suggested in literature. However, the size of the current study cohort (25 MPN patients) has to be considered and is significantly smaller compared to the study of Tefferi *et al*. (2009) (239 patients). Furthermore, it is possible that *TET2* mutations accumulate in genomes that are less stable, which would not exclude younger individuals but would more likely include older individuals.

A question that has arisen in literature regarding the involvement of *TET2* in MPN development is whether the accumulation of *TET2* mutations in MPN patients plays

a role in disease progression. Saint-Martin *et al*. (2009) and Brecqueville *et al*. (2012) found that *TET2* variants accumulate in MPN patients over time. Saint-Martin *et al*. (2009) found a second *TET2* mutation (p.Asn857fs) seven years after the detection of the first *TET2* mutation (p.Arg550X) in a 38 year old PV patient. The PV patient was diagnosed with myelofibrosis five months after detection of the second *TET2* mutation. The authors concluded that the mutational burden of *TET2* increase during progression of the disease. Brecqueville *et al*. (2012) detected a second *TET2* mutation (p.Arg550X) 48 months after the discovery of an initial *TET2* mutation (p.Ala1355HisfsX8) in a 56 year old PMF patient. It appears that *TET2* variants could occur early on in the disease, during progression of the disease or when transformation to AML occurs (Delhommeau *et al*. 2009; Abdel-Wahab *et al*. 2010; Nguyen-Khac *et al*. 2010). In the current study two patient samples were tested at different time intervals over 11 months (data not shown). Compared to previous studies there was no further accumulation of *TET2* variants in these patients. However, this might be as a result of the shorter duration of observation in the current study (11 months compared to 24 months or 7 years).

3.4.6 Location of detected variants in the *TET2* gene

According to literature *TET2* variants are more common in the largest exons of the gene: exons 3 and 11 (Bacher *et al*. 2010). In the current study, many of the *TET2* variants (nine of the 19 variants) were detected in exon 3, while two *TET2* variants were found in exon 11. No variants were found in exons 4, 5, 6, 7, 8 and 10, while two variants were detected in exon 9. A total of six variants were detected in introns 2, 4 and 7.

3.4.7 Conclusions of the sequencing analysis of *TET2*

The role of *TET2* variants in the development of MPNs is currently unclear. It has been suggested that there is a greater accumulation of *TET2* variants in MPN patients with the *JAK2* V617F mutation. Contrary to this, in the current study more of the *JAK2* V617F-negative MPN patients had *TET2* variants in comparison to patients with the *JAK2* V617F mutation. However, this discrepancy in results could be due to the difference in study cohort size. The relationship between *TET2* and the *JAK2* gene is yet unclear. Since *TET2* variants occur in *JAK2* V617F-negative MPN

patients, it is possible that these two genes function separate from each other and affect MPN development independently.

It has been found that the isocitrate dehydrogenase (*IDH*) 1 and 2 genes could influence the catalytic activity of TET2. Figueroa *et al*. (2010) reported that 2-hydroxyglutarate (HG) produced by mutant *IDH1* and *IDH2* inhibits the conversion of 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC), which is catalyzed by TET2 (Figueroa *et al*. 2010). The hydroxylation of 5-mC is speculated to be involved in DNA demethylation and inhibition of this process by *IDH* mutations could result in DNA hypermethylation (Figueroa *et al*. 2010). While the role of 5-hmC in haematopoiesis is currently unknown, it has been found that *IDH* mutations and decreased expression of *TET2* altered haematopoietic differentiation and caused an increase in the production of haematopoietic stem or progenitor cells (Figueroa *et al*. 2010). *IDH* mutations have been found to occur in the presence or absence of *JAK2* and *TET2* mutations in Ph-negative MPNs, with a higher frequency in PMF patients (Pardanani *et al*. 2010; Tefferi *et al*. 2010). However, *IDH* mutations appear to be most common in AML patients who were initially diagnosed with MPNs and are therefore, similar to *TET2*, speculated to be involved in leukemic transformation (Abdel-Wahab *et al*. 2010; Pardanani *et al*. 2010; Tefferi *et al*. 2010). Therefore, it appears that the function of TET2 is influenced by other genetic abnormalities, which are perhaps necessary to occur in order for TET2 to have an impact on the haematopoietic development in MPN and post-MPN AML patients.

In the current study more than one *TET2* variant was found in individual patients. The number of *TET2* variants varied from two to seven per patient (Tables 3.6, 3.7 and 3.8). Previous studies have reported up to four *TET2* variants in MPN patients (Delhommeau *et al*. 2009; Saint-Martin *et al*. 2009; Brecqueville *et al*. 2012). The later studies did not suggest any possible implications of this on the development of MPNs. Therefore, it is unknown whether more than one *TET2* variant could predispose individuals to the development of MPNs, or have any prognostic value in these patients.

It has also been shown in the current study that SNPs in *TET2* may occur as natural variants in a population. Since most of the SNPs detected in the current study were initially found during reported genome studies, it is possible that these *TET2* variants

are not specific to MPNs. Given that the clinical implications of most of the *TET2* variants detected in the current study is unknown, we suggest that expression studies should be done in future to determine the effect of these SNPs, deletions and insertions on TET2. Based on the current literature it doesn't appear as if variants in the *TET2* gene play a direct role or have prognostic value in MPNs. However, the body of literature on *TET2* variants is quite small and it is possible that specific variants are risk factors for the development of MPNs and the potential role of *TET2* in MPNs therefore needs to be investigated.

Table 3.6: Summary of *TET2* variants detected in the PV patients.

Patient	Variant	Variant type	Location	*JAK2* V617F status	Age	Gender
P1	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	74	Female
	I1762V*	Missense	Exon 11			
P2	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	35	Male
	P363L*	Missense	Exon 3			
	L1721W*	Missense	Exon 11			
P3	g.92960_92967insATAGATAG	Intronic insertion	Intron 2	Positive	65	Female
	V218M	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
	c.3955-3C>T	Intronic SNP	Intron 7			
P4	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Negative	47	Female
P5	g.92960_92967insATAGATAG	Intronic insertion	Intron 2	Positive	64	Female
	V218M	Missense	Exon 3			
	R1359H	Missense	Exon 9			
	H1778R	Missense	Exon 11			
P6	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	60	Female
P7	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	58	Male
	V218M	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
	Q1030Q	Synonymous	Exon 3			
	H1380H	Synonymous	Exon 9			
P8	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	30	Male
	V218M	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
P9	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	69	Male
P10	g.92960_92967insATAGATAG	Intronic insertion	Intron 2	Positive	71	Female
	V218M	Missense	Exon 3			
	Y1149S	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			

* Variants also detected in control samples.

Table 3.7: Summary of *TET2* variants detected in the ET patients.

Patient	Variant	Variant type	Location	*JAK2* V617F status	Age	Gender
E1	g.92969_92972insTAGA	Intronic insertion	Intron 2	Negative	60	Female
	V218M	Missense	Exon 3			
	N767D	Missense	Exon 3			
	Q1030Q	Synonymous	Exon 3			
	H1380H	Synonymous	Exon 3			
E2	g.92969_92972insTAGA	Intronic insertion	Intron 2	Positive	74	Female
	V218M	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
	Y1149S	Missense	Exon 3			
E3	L1721W*	Missense	Exon 11	Positive	67	Female
E4	g.92969_92972insTAGA	Intronic insertion	Intron 2	Positive	63	Female
	V218M	Missense	Exon 3			
	S795S	Synonymous	Exon 3			
	S1039S	Synonymous	Exon 3			
	Y1149S	Missense	Exon 3			
	g.101930_101931delTG	Intronic deletion	Intron 4			
E5	g.92969_92972insTAGA	Intronic insertion	Intron 2	Negative	82	Male
	V218M	Missense	Exon 3			
	Q810R	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
	S795S	Synonymous	Exon 3			
	g.101930_101931delTG	Intronic deletion	Intron 4			
	E1786E	Synonymous	Exon 11			

* Variants also detected in control samples.

Table 3.8: Summary of *TET2* variants detected in the PMF patients.

Patient	Variant	Variant type	Location	*JAK2* V617F status	Age	Gender
M1	g.92962_92969insAGATAGAT*	Intronic insertion	Intron 2	Positive	63	Male
	V218M	Missense	Exon 3			
	S795S	Synonymous	Exon 3			
	g.101930_101931delTG	Intronic deletion	Intron 4			
	H1778R	Missense	Exon 11			
	E1786E	Synonymous	Exon 11			
M2	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	58	Male
	V218M	Missense	Exon 3			
	Q1030Q	Synonymous	Exon 3			
	S1039S	Synonymous	Exon 3			
	H1380H	Synonymous	Exon 9			
M3	g.92962_92969insAGATAGAT*	Intronic insertion	Intron 2	Positive	62	Male
	V218M	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
M4	g.92969_92972insTAGA	Intronic insertion	Intron 2	Positive	59	Female
	V218M	Missense	Exon 3			
	E628E	Synonymous	Exon 3			
	S1039S	Synonymous	Exon 3			
M5	g.92960_92967delATAGATAG*	Intronic deletion	Intron 2	Positive	84	Male
M6	g.92961_92968delTAGATAGA	Intronic deletion	Intron 2	Negative	54	Male
	V218M	Missense	Exon 3			
	A347A	Synonymous	Exon 3			
	S795S	Synonymous	Exon 3			
	g.101930_101931delTG	Intronic deletion	Intron 4			
M7	g.92963_92970delGATAGATA*	Intronic deletion	Intron 2	Negative	67	Male
	I1762V*	Missense	Exon 11			

M8	g.92960_92967insATAGATAG	Intronic insertion	Intron 2	Negative	71	Female
	V218M	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
M9	g.92962_92969insAGATAGAT*	Intronic insertion	Intron 2	Negative	56	Female
	V218M	Missense	Exon 3			
	E628E	Synonymous	Exon 3			
	S1039S	Synonymous	Exon 3			
M10	g.92969_92972insTAGA	Intronic insertion	Intron 2	Positive	59	Female
	V218M	Missense	Exon 3			
	S1039S	Synonymous	Exon 3			
	Y1149S	Missense	Exon 3			
	H1380H	Synonymous	Exon 9			

* Variants also detected in control samples.

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SUMMARY

Myeloproliferative neoplasms (MPNs) are clonal haematopoietic stem cell disorders which are characterized by the excessive proliferation of one or more of the myeloid cell lineages. Mutations in several genes have been found to contribute to the pathogenesis of the Philadelphia (Ph) -negative MPNs. These MPNs include polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF). The most common genes associated with Ph-negative MPNs are the *JAK2* and *MPL* genes, which also form an integral part of the 2008 World Health Organization (WHO) diagnostic criteria for PV, ET and PMF. Mutations in the *JAK2* and *MPL* genes are, however, not always detected in PV, ET and PMF patients. The ten-eleven-translocation (*TET*) 2 tumor suppressor gene has been identified to be a potential contributor to the normal development of haematopoietic cells, particularly the myeloid blood cells. While the exact role of TET2 in haematopoiesis is not yet completely understood, mutations in the *TET2* gene have recently been found to occur in PV, ET and PMF patients.

It has been suggested that mutations occurring in the conserved domains of *TET2* could interfere with the catalytic activity of the protein, which ultimately has the potential to result in an inactive tumor suppressor gene. *TET2* mutations situated outside of the conserved domains also have the potential to alter protein function. Defective expression of *TET2* has previously been shown to alter haematopoietic development, resulting in the increased production of various myeloid cells. Previous studies in which mice models were used illustrated that the loss of TET2 function leads to characteristics similar to that of MPN patients. The aim of our study was to screen 25 PV, ET and PMF patients for mutations in the *TET2* gene using high resolution melting (HRM) analysis and DNA sequencing analysis.

In this study only two of the 24 primer sets could successfully be used to detect mutations using HRM analysis. The presence of non-specific amplification products, primer dimer, multiple melting domains and high melting background were suspected to be the causes of the unsuccessful HRM analysis. The DNA sequencing analysis of the *TET2* gene was, however, successful. No novel variants were found in the current study. A total of 24 previously published *TET2* variants were detected in the 25 Ph-negative MPN patients. The *TET2* variants included missense single

nucleotide polymorphisms (SNPs), synonymous SNPs, intronic SNPs, intronic deletions and intronic insertions. Unlike in previous studies, *TET2* variants appeared to be more common in *JAK2* V617F-negative MPN patients than in *JAK2* V617F-positive MPN patients. It is speculated that most of the *TET2* variants detected in the current study occur naturally in the *TET2* gene and not exclusively in MPN patients.

The prognostic value of *TET2* variants in MPNs is as yet unknown. Since most of the SNPs in the current study appeared to be natural variants of the *TET2* gene, it is possible that *TET2* does not play a direct role in the development of MPNs. However, limited literature regarding the function of *TET2* and how it contributes to abnormal haematopoiesis is available. Further investigation is therefore needed to establish the exact role of *TET2* in the pathogenesis of MPNs and whether variants of this gene could predispose individuals to the development of MPNs.

Key words: Myeloproliferative neoplasm (MPN), polycythaemia vera (PV), essential thrombocythaemia (ET), primary myelofibrosis (PMF), *TET2*, tumor suppressor gene, high resolution melting (HRM), DNA sequencing, single nucleotide polymorphisms (SNP)

OPSOMMING

Miëloproliferatiewe neoplasmas (MPNs) is klonale hematologiese stamselsiektes wat deur die oormatige produksie van een of meer van die miëloïede bloedsellyne gekenmerk word. Mutasies in verskeie gene word met die patogenese van die Philadelphia (Ph) -negatiewe MPNs geassosieer. Die Ph-negatiewe MPNs bestaan uit polisitemie vera (PV), essensiële trombositemie (ET) en primêre miëlofibrose (PMF). Mutasies in die *JAK2* en *MPL* gene is die algemeenste genetiese afwykings in Ph-negatiewe MPNs en vorm dus ‘n belangrike deel van die 2008 Wêreld Gesondheids Organisasie (WGO) diagnostiese kriteria vir PV, ET en PMF. *JAK2* en *MPL* mutasies word egter nie altyd in PV, ET en PMF pasiënte aangetref nie. Die tien-elf-translokasie (*TET*) 2 tumor onderdrukker geen is onlangs met Ph-negatiewe MPNs geassosieer en word gespekuleer om tot die normale ontwikkeling van bloedselle by te dra, veral van die miëloïede sellyn.

Mutasies wat in die gekonserveerde domeine van *TET2* geleë is, word gespekuleer om in te meng met die katalitiese aktiwiteit van die proteïen wat ‘n onaktiewe tumor suppressor geen tot gevolg kan hê. Mutasies wat buite die gekonserveerde domeine van *TET2* geleë is, het die potensiaal om die funksie van die proteïen te verander. Vorige studies het getoon dat die gebrekkige uitdrukking van *TET2* abnormale hematopoïese, insluitende verhoogde produksie van sekere miëloïede selle kan veroorsaak. Studies waarin muismodelle gebruik is, het getoon dat die verlies van die *TET2* geen simptome soortgelyk aan wat in MPN pasiënte waargeneem word, kan veroorsaak. Die doel van hierdie studie was om hoë-resolusie-smeltkromme (HRS) analise en deoksiribonukleïensuur (DNS) volgordebepaling te gebruik om vas te stel of mutasies in die *TET2* geen van 25 PV, ET en PMF pasiënte voorkom.

Slegs twee van die 24 priemstukke in die huidige studie kon suksesvol gebruik word met die HRS analise vir die identifisering van variante in die *TET2* geen. Die oorsake vir die onsuksesvolle HRS analise was die teenwoordigheid van nie-spesifieke amplifiseringsprodukte, priemstuk dimere, veelvoudige smeltdomeine en hoë smeltingsagtergrond. Die DNS volgordebepaling van die *TET2* geen was egter suksesvol. Geen nuut beskryfde *TET2* variante is in die huidige studie geïdentifiseer nie. ‘n Totaal van 24 *TET2* variante wat van te vore beskryf is, is in die PV, ET en

PMF pasiënte gevind. Die *TET2* variante sluit aminosuur veranderende enkelnukleotiedpolimorfismes (ENPs), sinonieme ENPs, introniese ENPs, introniese delesies en introniese invoegings in. Die geïdentifiseerde *TET2* variante in die huidige studie was meer algemeen in *JAK2* V617F-negatiewe MPN pasiënte in vergelyking met *JAK2* V617F-positiewe MPN pasiënte. Vorige studies het egter getoon dat *TET2* mutasies meer algemeen is in *JAK2* V617F-positiewe MPN pasiënte. Dit blyk of meeste van die geïdentifiseerde variante in die huidige studie natuurlike variante van die *TET2* geen is.

Die prognostiese waarde van *TET2* variante in MPN pasiënte is tans onbekend. Terwyl dit blyk of meeste van die geïdentifiseerde variante natuurlik voorkom in die *TET2* geen, is dit moontlik dat *TET2* nie ‘n direkte rol in die ontwikkeling van MPNs speel nie. Daar is tans beperkte wetenskaplike literatuur rakende die funksie van *TET2* en die impak van mutasies in hierdie geen op hematopoiëse beskikbaar. Toekomstige studies moet dus uitgevoer word om te bepaal of *TET2* variante moontlike risikofaktore kan wees vir die ontwikkeling van MPNs om sodoende die rol van *TET2* in die patogenese van MPNs vas te stel.

Sleutel terme: Miëloproliferatiewe neoplasmas (MPN), polisitemie vera (PV), essensiële trombositemie (ET), primêre miëlofibrose (PMF), *TET2*, tumor onderdrukker geen, hoë-resolusie-smeltkromme (HRS) analise, DNS volgordebepaling, enkelnukleotiedpolimorfismes (ENPs)

APPENDIX A

CONSENT FORM

A) Consent to participate in the study

You have been asked to participate in a research study.

You have been informed about the study by Dr André de Kock.

You may contact Dr André de Kock at (051) 405 3283 at any time if you have questions about the research or if you are injured as a result of the research.

You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, University of the Free State (UFS) at telephone number (051) 405 2812 if you have any questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation.

If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

_____	_____
Signature of Participant	Date
_____	_____
Signature of Study Leader *(Where applicable)*	Date
_____	_____
Signature of Witness *(Where applicable)*	Date

B) Information document

Title of the research project: Mutational analysis of the *TET2* gene in Philadelphia negative myeloproliferative neoplasms.

Introduction:

I, Dr André de Kock, am doing research on mutations found in myeloproliferative neoplasms (MPNs). Research is just the process to learn the answer to a question. In this study we want to learn more about the mutations that occur in MPNs and if we have novel mutations that occur in South African individuals. This study involves research and will not influence your treatment. This study could have future spin-offs in the diagnosis and treatment of MPNs.

Invitation to participate:

We are asking/inviting you to participate in a research study *(or asking for your permission to include your child in a research study)*.

What is involved in the study?

We will collect 4-10ml of your blood. The genetic material will be taken from your blood cells for further study. The study involves searching for changes that may have occurred in your genetic material. These changes may be involved in the development and progression of your disease. After the study, the material obtained from your blood will be destroyed. About 40 individuals from South Africa will be invited to take part in this study.

Risks of being involved in the study:

There are no risks involved for you in the study; only the discomfort of having a blood sample taken from you by a professional nurse or doctor.

Benefits of being in the study:

The information we will gather from the analysis of your blood sample will provide us with genetic information on MPNs as well as information of the genetic make-up of the local populations.

Alternative procedures or courses of treatment that might benefit the subject:

The information gathered in this study may influence future diagnosis and treatment of patients with MPNs.

The subject will be given pertinent information on the study while involved in the project and after the results are available.

Participation is voluntary:

Refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

Reimbursements:

There is no reimbursement for this study.

Confidentiality:

Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Ethics Committee for Medical Research and the Medicines Control Council.

If results are published, this may lead to individual/cohort identification.

Contact details of researcher(s):

(For further information/reporting of study-related adverse events)

Dr André de Kock

(051) 405 3283.

Contact details of REC Secretariat and Chair:

(For reporting of complaints/problems)

Ms H Strauss, Research Division

Internal Post Box G40.

E-mail address: gndkhs.md@mail.uovs.ac.za