The human myeloproliferative disorders: molecular pathogenesis and clonal heterogeneity

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This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

The text of this dissertation, excluding tables, figures and bibliography, does not exceed 60,000 words

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Thesis summary

The classical myeloproliferative disorders (MPD), comprising essential thrombocythaemia (ET), polycythaemia vera (PV) and idiopathic myelofibrosis (IMF), are clonal premalignant haematopoietic neoplasms associated with activating mutations in signalling pathway molecules and a variable tendency to develop acute myeloid leukaemia (AML). This thesis examined genotype-phenotype associations of *JAK2* and *MPL* mutations, the presence of clonal diversity in the MPD and the genetic events associated with progressive disease.

Mutations in *MPL* were identified in 4% of ET and 7% of IMF but not in PV. Three different acquired *MPL* mutations were identified, one of which had been reported as an inherited allele. Although *MPL* mutations did not delineate a distinct clinical or histopathological subtype of ET, molecular testing provides an important new tool in the diagnostic armamentarium. Clones homozygous for the *JAK2 V617F* mutation were identified in female but not male patients with ET, suggesting that gender differences may be important in the determination of disease phenotype. In patients with two acquired genetic alterations, a signalling pathway mutation and a cytogenetic abnormality were usually present within the same clone. By contrast, coexistence of two signalling pathway mutations indicated the presence of biclonal disease that in two patients had arisen independently and not from a shared founder clone.

RAS mutations were identified as potential cooperating events in patients with *JAK2* or *MPL* mutant IMF. In patients developing AML following a *JAK2 V617F*-positive MPD, those with *V617F*-positive leukaemia had progressed via an accelerated phase of disease and harboured acquired alterations of *RUNX1* or *EVI1*. *V617F*-negative leukaemias tended to follow directly from ET or PV, and loss of the *JAK2* mutation by reversion to wild-type due to mitotic recombination, gene deletion or gene conversion was excluded. The thesis concludes with a discussion of how clonal heterogeneity can be integrated into current models of MPD disease pathogenesis.

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Chapter 1

Introduction: The classical myeloproliferative disorders

The classical myeloproliferative disorders (MPD), comprising polycythaemia vera (PV), essential thrombocythaemia (ET) and idiopathic myelofibrosis (IMF) are clonal stem cell disorders associated with overproduction of one or more myeloid elements. PV, first described in 1892[1], is characterised by overproduction of erythroid cells, often accompanied by an increase in neutrophils and/or platelets. In ET, first described as a distinct syndrome in 1934[2], an increased platelet count is seen in the absence of significant erythrocytosis. The main clinical complication of ET and PV is thrombosis, both arterial and venous[3,4], with thrombotic complications resulting in a median survival of only 18 months in untreated PV[5]. A proportion of patients with PV or ET develop IMF, which may also present as a *de novo* disease. First described in 1879[6], IMF is characterised by bone marrow fibrosis leading to extramedullary haematopoiesis, splenomegaly and bone marrow failure. All three conditions may terminate in acute myeloid leukaemia (AML) which is generally refractory to therapy. William Dameshek, writing in 1951, was the first to suggest these three conditions may be related[7], recognising both the phenotypic overlap and tendency for phenotypic shift in the MPD.

1.1 Incidence and aetiology

The annual UK incidence of both PV and ET is in the order of 1 case per 100,000 population, with IMF being slightly less common[8]. In PV, men out number women, with the reverse seen in ET[8]. Little is known about the precise aetiology of these disorders. An increased incidence of IMF has been reported in survivors of the atomic bombs at Hiroshima and Nagasaki[9], although the incidence of PV appeared unaffected[10]. Although an increased risk of PV was reported in a cohort of nuclear weapons workers[11], the numbers affected were too small to draw firm conclusions. More recently, a cluster of PV cases was

reported around Tamaqua (Pennsylvania, USA) close to the site of a previous toxic waste dump. Although the exact nature of the chemical contamination could not be determined, the risk of PV among local residents was 4.5 times higher compared to adjacent counties[12]. Data from the comprehensive Swedish Cancer Registry has suggested a familial tendency to develop an MPD, with an increased risk of both PV and ET noted among first degree relatives of affected individuals[13,14]. Several families have been reported with multiple members affected by an MPD, with inheritance patterns suggestive of an autosomal dominant trait with incomplete penetrance[15-19]. X-chromosome inactivation studies have demonstrated clonal haematopoiesis in affected family members indicative of a genuine MPD. Of note, a proportion of apparently unaffected relatives showed erythropoietin-independent erythroid colony growth, suggesting an early phase or *forme fruste* MPD[15]. Affected families may contain individuals with different disease phenotypes, including ET, PV, IMF or chronic myeloid leukaemia (CML)[16]. Clinical phenotype and complication rates are reportedly similar in familial and sporadic cases, although some kindreds may show evidence of genetic anticipation[18].

1.2 Early studies

In 1974, *in vitro* culture studies demonstrated the ability of erythroid colonies from PV patients to grow in the absence of erythropoietin, a phenomenon not observed in normal individuals[20]. This finding of cytokine-independent differentiation became one of the hallmark features of the MPD. The clonal nature of these disorders was first elucidated in 1976, when studies of 2 female PV patients revealed expression of a single isoform of the polymorphic, X-linked *G6PD* gene in erythrocytes, granulocytes and platelets[21], a finding that was subsequently extended to patients with ET[22]. Dysregulation of several pathways has been identified in MPD patients, including up regulation of NFE2[23], PRV1[24], BCL-x_L[25] and the JAK-STAT pathway[26], and down regulation of thrombopoietin receptor (MPL) expression[27]. Many of these observations were subsequently explained by the identification of an activating mutation in the *JAK2* tyrosine kinase.

1.3 Activating mutations in Janus kinase 2 (*JAK2*) in the MPD

In 2005, four international groups described the same acquired point mutation in the *JAK2* gene in most patients with PV and around half those with ET or IMF[28-31]. Diverse approaches were used to identify this mutation, comprising dissection of signalling pathways in PV[28], high throughput sequencing of kinase genes[29,30] and sequencing of candidate genes within a region of chromosome 9 known to undergo loss of heterozygosity in PV patients[31]. JAK2, one of four JAK family cytoplasmic tyrosine kinases (comprising JAK1, JAK2, JAK3 and TYK2), is essential for signalling by the erythropoietin receptor (EpoR)[32] and thrombopoietin receptor (MPL)[33], and is also involved in signalling through the granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor[34] and interferon-γ receptors[35]. Studies of EpoR indicate that ligand binding results in a conformation change in the receptor[36], with consequent phosphorylation of JAK2 and the receptor itself resulting in activation of downstream signalling pathways[32]. JAK2 also plays a vital role in EpoR trafficking, with absence of JAK2 resulting in retention of EpoR within the endoplasmic reticulum[37]. The central role of JAK2 in haematopoiesis is highlighted by a *JAK2* knock-out mouse, which dies at embryonic day 12.5 due to a complete absence of definitive erythropoiesis[35].

1.4 The *JAK2 V617F* mutation: mechanisms of cellular transformation

1.4.1 Increased cellular proliferation

The *JAK2 V617F* substitution, resulting from a single base change, affects the pseudokinase (JH2) domain of the protein. This domain is required for both JAK2 activation and inhibition of basal kinase activity[38,39]. The *JAK2 V617F* mutation results in substitution of a bulky phenylalanine at a highly conserved residue, and is thought to impair autoinhibition of JAK2, leading to constitutive activation of tyrosine kinase activity[28-31]. Expression of *JAK2 V617F* leads to cytokine independent growth of various cytokine dependent cell lines, with constitutive activation of pathways implicated in the control of proliferation, differentiation and cell survival such as STAT5, PI3K/AKT and MAPK[28,30,31].

Consistent with these findings, cell line studies suggest a role for mutant JAK2 in promoting G_1/S transition[40]. Mutant JAK2 also appears resistant to inactivation by members of the suppressor of cytokine signalling (SOCS) family, particularly SOCS3[41]. Taken together, these studies indicate a role for mutant JAK2 in driving cellular proliferation by constitutive activation of signalling pathways, increased entry into cell cycle and resistance to inhibition by negative regulatory proteins.

1.4.2 Inhibition of apoptosis

The anti-apoptotic protein Bcl-x_L, a direct transcriptional target of STAT5, appears to play a central role in cellular survival in *JAK2 V617F* associated disease. Early studies demonstrated a correlation between increased Bcl-x_L expression and cytokine independent growth of erythroid cells from PV patients[25]. Subsequently over-expression of either Bcl-x_L or a constitutively active STAT5 were shown to induce cytokine independent erythroid differentiation of normal human progenitors[42]. The cellular metabolism of Bcl-x_L is also altered in the presence of mutant JAK2. In normal cells, DNA damage induced by irradiation or clastogen exposure leads to deamidation of the Bcl-x_L protein, reducing its ability to sequester pro-apoptotic proteins with consequent cell death by apoptosis[43]. This pathway is blocked in the presence of mutant JAK2 in both cells lines and patient granulocytes. Moreover, this reduction in Bcl-x_L deamidation can be overcome by chemical inhibition of JAK2[44]. A block in the Bcl-x_L deamidation pathway was also observed in *BCR-ABL1* expressing cells[44], but does not appear to be a general feature of malignancy as the pathway is intact in various cancer cell lines[43,45] and primary chronic lymphocytic leukaemia cells[46].

Mutant JAK2 also confers resistance to FAS-mediated apoptosis. Studies of erythroid progenitors from *JAK2 V617F*-positive PV patients demonstrated continued proliferation and differentiation in the presence of a FAS-activating antibody. By comparison, normal progenitors failed to proliferate in the presence of the antibody, with a significant fraction undergoing apoptosis. Biochemical

studies implicated activation of the PI3K/AKT pathway in the resistance to FAS-induced apoptosis[47].

In summary, expression of mutant JAK2 induces resistance to apoptosis via several mechanisms. Levels of functional Bcl-x_L are increased by both activation of JAK-STAT signalling and inhibition of Bcl-x_L deamidation. Mutant JAK2 expression also confers resistance to FAS-mediated apoptosis.

1.4.3 Cytokine independent differentiation

Differentiation of haematopoietic cells in the absence of cytokines is one of the hallmark features of the MPD[20]. Coexpression of mutant JAK2 with the erythropoietin or thrombopoietin receptor results in cellular transformation in the absence of cytokine (erythropoietin or thrombopoietin respectively)[48], suggesting that mutant JAK2 is able to signal via cytokine receptors in the absence of ligand binding. Consistent with this notion, studies of MPD patients demonstrated that only progenitors harbouring a *JAK2* mutation formed erythroid colonies in the absence of erythropoietin[29,49]. Moreover inhibition of JAK2 by small-interfering RNA abrogated erythropoietin independent differentiation of progenitors from PV patients[28]. *In vitro* culture of patient cells suggested that *JAK2 V617F*-positive progenitors also show an increased sensitivity to erythropoietin when compared to wild-type controls[49].

1.4.4 Mouse models of JAK2 V617F associated disease

Expression of the mouse equivalent *JAK2 V617F* in a retroviral murine bone marrow transplant system resulted in an oligoclonal MPD-like disease with many similarities to the human MPD. Transplanted mice developed marked erythrocytosis and leucocytosis followed by progression to bone marrow fibrosis and anaemia, a phenotype that was recapitulated in secondarily transplanted animals. Thrombocytosis, however, was not commonly observed, being present only in mice with low expression of mutant *JAK2*[50,51]. Other similarities to human disease observed in retroviral models include cytokine independent differentiation of erythroid and granulocytic precursors and reduced serum

erythropoietin levels[50-52]. Whereas retrovirally-induced *JAK2 V617F* expression was associated with erythrocytosis in the majority of cases, transgenic mouse lines expressing *JAK2 V617F* developed either erythrocytosis or thrombocytosis. Of note, thrombocytosis was observed in mice expressing low levels of mutant *JAK2*, whereas erythrocytosis was seen in high-expressing animals, suggesting that gene dosage may play a role in the determination of disease phenotype[53,54].

Independent studies have shown that progenitors from patients with a *JAK2 V617F*-positive MPD are able to engraft immunodeficient mice. Progenitors from MPD patients appear to be skewed towards myeloid differentiation in this model when compared to normal controls (where B-lymphoid differentiation is predominant)[55-57]. In one study, to date published only in abstract form, mice engrafted with progenitors from IMF patients developed megakaryocyte hyperplasia and bone marrow fibrosis, thus recapitulating features of human IMF[57].

1.4.5 Mechanisms of cellular transformation: summary

An identical point mutation in *JAK2* is present in the majority of MPD patients, resulting in constitutive phosphorylation of JAK2 and activation of downstream signalling pathways. Expression of mutant JAK2 is associated with increased proliferation, impaired apoptosis, cytokine independence and cytokine hypersensitivity. Retroviral and transgenic mouse models recapitulate many features of the human MPD, including erythrocytosis, thrombocytosis and bone marrow fibrosis. These findings suggest that mutant JAK2 is central to the MPD phenotype, and taken together imply a role for *JAK2 V617F* in disease initiation.

1.5 Lineage involvement in *JAK2 V617F* associated disease

In MPD patients, the *JAK2 V617F* mutation can be detected in progenitors with B-cell, T-cell, NK-cell and myeloid lineage potential, demonstrating that the mutation arises in a multipotent haematopoietic stem cell (HSC)[58]. However the mutation is detected only rarely and at low levels in peripheral blood

lymphocytes[58]. Of relevance to this observation, the transforming effects of J*AK2 V617F* are only manifest in cell lines when mutant JAK2 is co-expressed with a type I homodimeric receptor[48,59]. Type 1 homodimeric receptors, including those for erythropoietin, thrombopoietin and G-CSF, are present in myeloid but not lymphoid cells, and as such lymphocytes harbouring the *JAK2 V617F* mutation may not have a selective advantage *in vivo*. In keeping with this finding, the *JAK2 V617F* mutation appears to be exclusive to myeloid neoplasms[60,61], despite reports of *JAK2* overexpression in lymphoid malignancies[62]. Along with its common occurrence in the classical MPD, the *JAK2 V617F* mutation is found rarely in a number of other chronic myeloid malignancies including myeloproliferative-myelodysplastic syndromes and chronic neutrophilic leukaemia[63,64]. The *JAK2 V617F* mutation has also been detected in 1-8% of *de novo* AML, and shows an association with aberrations of the core binding factor complex associated with *AML1-ETO* or *CBFB-MYH11* fusion genes[60,61,65,66].

Progenitor compartment studies in PV patients have suggested an increase in the number of phenotypic HSC and common myeloid progenitors[67]. In culture experiments, however, the total number of bone marrow derived long-term culture initiating cells from PV and ET patients was no different to normal controls[49]. *In vitro* culture assays of PV HSC have suggested skewing towards erythroid differentiation when compared to normal controls, with this effect largely abrogated by the application of a chemical inhibitor of JAK2[67]. Moreover, culture of bone marrow progenitors in conditions specific for erythroid differentiation suggested increased expansion of erythroid cells from *JAK2 V617F*-positive PV and ET samples compared to normal controls. Of interest, this expansion was associated with an increase in the proportion of cells harbouring the *JAK2 V617F* mutation, suggesting mutant JAK2 drives proliferation at the later stages of erythroid differentiation[49].

In summary, the *JAK2 V617F* mutation appears specific to myeloid malignancy. Despite its presence in haematopoietic stem cells, mutant JAK2 may not provide a proliferative advantage in lymphoid cells due to the absence of a type 1 homodimeric receptor. Expression of mutant JAK2 in progenitor cells produces

a bias towards the erythroid lineage, and leads to expansion of erythroid cells at the later stages of differentiation.

1.6 JAK2 V617F: one mutation associated with three apparently distinct disease phenotypes

1.6.1 Essential thrombocythaemia and polycythaemia vera

Traditionally, PV and ET have been considered as separate but related disease entities[7]. Using sensitive detection techniques, however, the same *JAK2 V617F* mutation can be found in nearly all PV patients and around half of those with ET[29], raising questions as to how a single mutation is associated with two apparently distinct clinical phenotypes. Initial clues came from analysis of 776 ET patients enrolled into one of three prospective trials. When compared to ET patients without the *JAK2* mutation, *JAK2 V617F*-positive ET patients were found to have multiple features resembling PV, including higher haemoglobin levels and white cell counts, lower serum erythropoietin levels, increased bone marrow erythroid and granulocytic activity and higher rates of venous thrombosis. Moreover, all patients who transformed from ET to PV were positive for the *JAK2 V617F* mutation[68]. These data suggest that ET and PV form part of a biological continuum, with ET representing a *forme fruste* of PV.

Prior to the discovery of the *JAK2 V617F* mutation, loss of heterozygosity for the telomeric end of chromosome 9p had been identified in one third of PV patients[69]. This was subsequently shown to be secondary to mitotic recombination, which gives rise to a clone that is homozygous for the *JAK2 V617F* mutation with preservation of *JAK2* copy number[28,29,31]. Studies of individual progenitor colonies from MPD patients revealed that whereas a *JAK2 V617F*-homozygous clone was present in the majority of patients with PV, such clones were rare in ET[70], suggesting that gene dosage may play a role in disease phenotype. Further evidence for this hypothesis came from *JAK2 V617F* transgenic mice models, where mice with a low mutant to wild-type *JAK2* expression ratio developed an ET-like phenotype, whereas those with a high mutant to wild-type ratio manifested a PV-like disease[54]. Of note, cell line

studies have suggested that co-expression of wild-type *JAK2* may inhibit the transforming effects of the mutant allele[28]. Taken together, these studies suggest that either loss of the wild-type allele or duplication of the mutant allele may be associated with erythrocytosis and thus a PV phenotype.

A potential biological mechanism for this hypothesis was suggested by studies of normal human haematopoietic progenitors, wherein over-expression of STAT5, an important downstream target of JAK2, was associated with preferential erythroid differentiation, whereas inhibition of STAT5 favoured megakaryocyte differentiation[71]. It is possible, therefore, that homozygosity for the *JAK2 V617F* mutation results in stronger activation of STAT5 and a consequent bias towards erythroid differentiation.

The size of the chromosome 9p region involved in mitotic recombination is variable between patients, suggesting this process is not due a fragile site on 9p[31]. Of interest, rates of mitotic recombination are highly variable between normal individuals[72]. Following acquisition of the *JAK2 V617F* mutation, therefore, individuals with higher rates of mitotic recombination may be more likely to develop a *JAK2 V617F*-homozygous clone and consequently a PV phenotype. Other host genetic modifiers could also play a role in determining disease phenotype, such as inter-individual differences in erythropoietin homeostasis or JAK2 signalling. Indeed specific polymorphisms in *JAK2* and the erythropoietin receptor are over-represented in patients with either ET or PV[73].

1.6.2 Idiopathic myelofibrosis

Whereas similarities exist between the phenotype and clinical complications of ET and PV, IMF is a more distinct clinical syndrome, showing features such as bone marrow fibrosis and extramedullary haematopoiesis which are absent from both ET and PV[74]. Around half of IMF patients, however, harbour the *JAK2 V617F* mutation[29], and 15-30% of patients with ET or PV eventually develop IMF[75-77]. Acquired cytogenetic abnormalities are present in 10-15% of patients with PV or ET and up to 50% in those with IMF[78-80], suggesting a greater degree of genetic instability within an IMF clone. Consistent with this observation, IMF is

associated with features of increasing stem cell dysfunction, such as circulating myeloid and erythroid progenitors[81], increased levels of lactate dehydrogenase indicative of ineffective erythropoiesis[82], shortened survival and increased rates of progression to acute leukaemia[83,84]. Taken together, these findings suggest that IMF, presenting either *de novo* or following ET or PV, may represent an accelerated phase of disease, most likely due to the acquisition of further genetic lesions.

In a murine retroviral transplantation model, an IMF-like phenotype was observed in the majority of mice by 2-4 months[28], suggesting a direct association between the *JAK2 V617F* mutation and the development of IMF. Moreover a higher *JAK2 V617F* mutant allele burden has been reported in patients who develop secondary IMF (following ET or PV) compared to patients with ET or PV, suggesting an increase in the size of the mutant clone with progressive disease[85]. Studies have indicated a role for mutant JAK2 in driving genetic instability, with increases in homologous recombination, point mutations and small deletions observed in cell lines expressing mutant compared to wild-type JAK2. Moreover foci of nuclear RAD51, a marker of DNA breakage, were more frequent in progenitors from PV patients compared to normal controls[86]. Other factors implicated in the development of IMF include reduced GATA-1 expression[87,88], increased thrombopoietin and transforming growth factor β signalling[89,90] and altered expression of molecules involved in stem cell trafficking such as CXCR4[91]. It is unclear, however, whether such changes are directly involved in the pathogenesis of IMF, or represent epiphenomena related to as yet unknown genetic lesions. As such, the specific genetic events underlying the development of IMF are yet to be fully elucidated.

1.6.3 Progression to acute myeloid leukaemia

Progression to AML is seen in 2-5% of patients with ET or PV[76,77,92] and 15-30% of patients with IMF[75,83]. Risk factors for progression to AML in patients with ET or PV include increasing age[92] and prior use of genotoxic agents such as radioactive phosphorous, alkylating agents or radiotherapy[92-94], particularly when used sequentially with hydroxycarbamide[92,95,96]. The association of single

agent hydroxycarbamide with the development of AML remains controversial[97]. Transformation to AML has been reported in a number of MPD patients who have never received cytotoxic therapy[92,98-100], suggesting that progression to acute leukaemia is part of the natural history of these disorders. Additional risk factors for AML in IMF patients include karyotypic abnormalities (other than del(20q) or del(13q)[83,101]), higher peripheral blood blast counts[84] and prior splectomy[84].

Independent studies have reported the absence of the *JAK2 V617F* mutation in leukaemic blasts from patients with a preceding *V617F*-positive MPD[102,103]. Reversion of a *JAK2 V617F*-heterozygous clone back to wild-type by mitotic recombination was excluded in 3 cases examined[102,103]. X-chromosome inactivation patterns were uninformative in a single case, with both the *V617F*-positive MPD and the *V617F*-negative AML expressing the same allele of a polymorphic X-linked gene[103]. As such, the mechanism by which a *V617F*-positive MPD transforms to a *V617F*-negative AML is yet to be elucidated. Although additional cytogenetic changes are common at the time of transformation to both *V617F*-positive and *V617F*-negative AML, these generally consist of unbalanced chromosomal gains or deletions[102,103]. AML associated aberrations including *FLT-ITD* and mutations in *NPM* have been reported in *V617F*-negative transformations[103,104], but there are as yet no reports of additional genetic events associated with progression to *V617F*-positive AML.

1.6.4 JAK2 V617F: one mutation associated with three apparently distinct disease phenotypes: summary

The same *JAK2 V617F* mutation is found in patients with ET, PV, and IMF, and in the leukaemic blasts of a proportion of patients who progress to AML. Similarities exist between ET and PV in both disease phenotype and clinical complications, suggesting that these conditions may form part of a biological continuum. Several lines of evidence suggest that the JAK2 mutant to wild-type ratio plays a role in determining the precise phenotype that is manifest, with patients harbouring *JAK2 V617F*-homozygous clones more likely to develop PV,

possibly due to increased signalling via STAT5. IMF may be best considered as an accelerated phase of disease, associated with increased genetic instability, dysregulated stem cell function and higher rates of progression to acute leukaemia, although the genetic events leading to disease evolution are currently unknown. A proportion of patients develop an acute leukaemia that may be negative for the *JAK2 V617F* mutation.

1.7 Other *JAK2* mutations in haematological disease

1.7.1 JAK2 exon 12 mutations in polycythaemia vera

Using sensitive detection techniques, up to 97% of PV patients harbour the *JAK2 V617F* mutation, located in exon 14 of the gene[29]. Subsequent sequencing of *JAK2* in *V617F*-negative PV patients identified a cluster of mutations in *JAK2* exon 12[105]. These mutations were associated with a distinct variant of PV characterised by higher haemoglobin levels and lower white cell and platelet counts compared to *JAK2 V617F*-positive PV. Expression of *JAK2* exon 12 mutations conferred cytokine independence to cytokine dependent cell lines in the presence of a type I homodimeric receptor, associated with a greater degree of downstream pathway activation compared to the *JAK2 V617F* mutation. Expression of a *JAK2* exon 12 allele (*JAK2 L539K*) in murine bone marrow resulted in marked erythrocytosis *in vivo*. The *JAK2* exon 12 mutations are predicted to lie at the interface between the kinase and pseudokinase domains, and are likely to perturb the autoinhibitory function of the pseudokinase domain. *JAK2* exon 12 mutations have not been reported in patients with ET or *de novo* IMF.

1.7.2 JAK2 kinase domain mutation in a leukaemia cell line

High-throughput screening for tyrosine-phosphorylated proteins identified a *JAK2 T875N* mutation in an acute megakaryoblastic leukaemia cell line. This mutation results in an amino acid substitution within the kinase domain at the predicted interface with the autoinhibitory pseudokinase region. As with the *JAK2 V617F* mutation, expression of *JAK2 T875N* conferred cytokine

independence to cytokine-dependent cells lines only in the presence of a homodimeric type I cytokine receptor. Expression in a murine retroviral transplant system resulted in an MPD-like phenotype characterised by erythrocytosis and variable leucocytosis. Although platelet numbers were not increased, mice expressing the *JAK2 T875N* allele showed megakaryocyte proliferation and bone marrow fibrosis[106]. To date, this mutation has not been reported in human disease.

1.7.3 JAK2 mutations in acute lymphoblastic leukaemia

A cluster of mutations centred around *JAK2 R683* within the autoinhibitory pseudokinase domain have been reported in 18% of Down's syndrome acute lymphoblastic leukaemia (ALL)[107,108]. Of interest, a mutation was also observed in a single case of non-Down's syndrome ALL in association with isochromosome 21q, suggesting cooperation between the *JAK2 R683* allele and one or more genes on chromosome 21q. Expression of these alleles conferred cytokine independence to cytokine-dependent cells lines only in the presence of a homodimeric type I cytokine receptor. It is not currently known whether a type I receptor is present in the leukaemic blasts of these patients. Expression of the *JAK2 I682-D686del* allele in murine bone marrow resulted in an MPD phenotype *in vivo* with elevation of red cell, white cell and platelet counts[108].

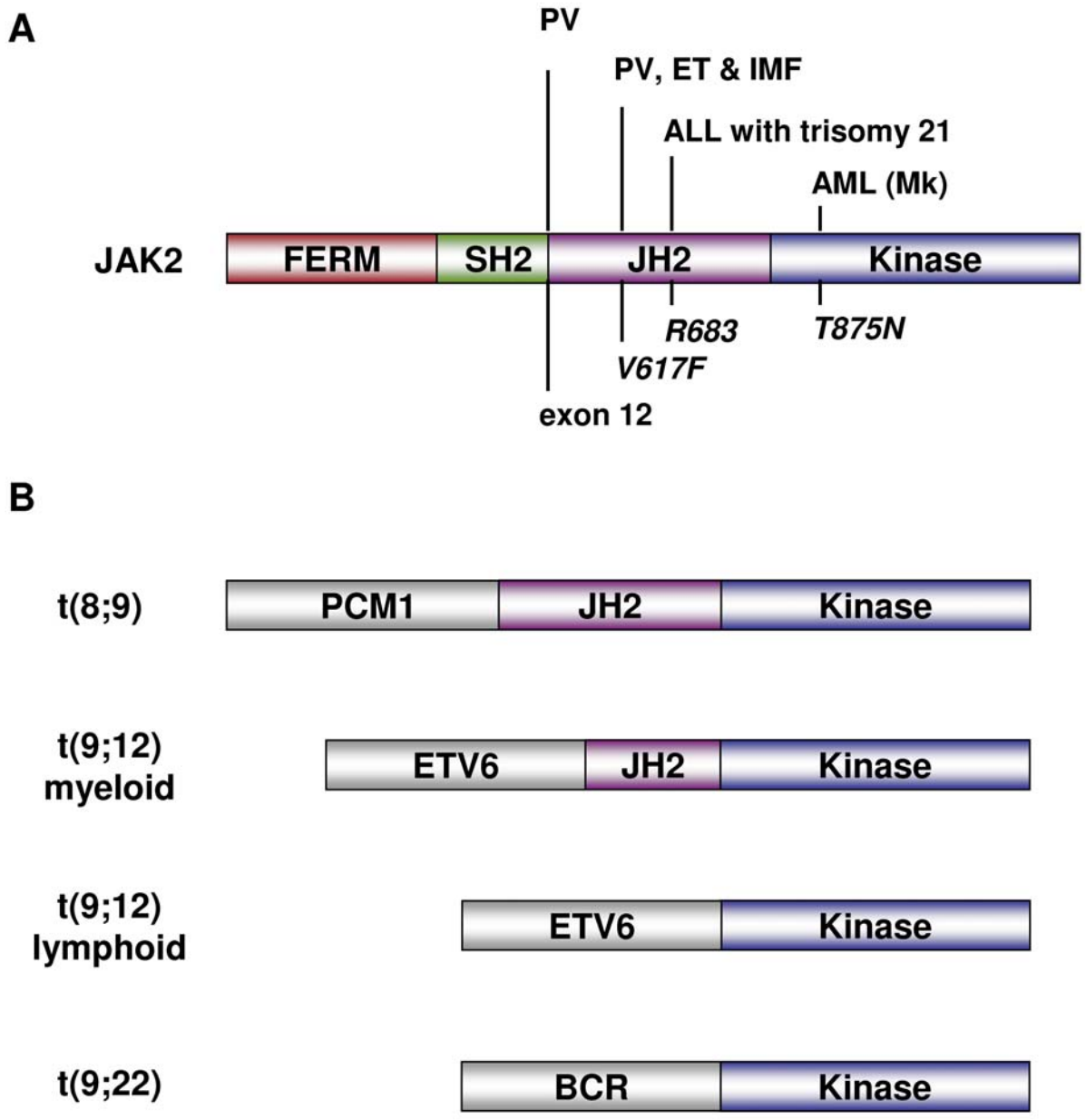

Figure 1.1. Oncogenic mutations and rearrangements of *JAK2*. (A) *JAK2* mutations and their associated haematopoietic malignancies. (B) Chromosomal translocations leading to rearrangement of *JAK2*. PV: polycythaemia vera; ET: essential thrombocythaemia; IMF: idiopathic myelofibrosis; ALL: acute lymphoblastic leukaemia; AML: acute myeloid leukaemia; Mk: megakaryocytic.

1.7.4 JAK2 rearrangements in myeloid and lymphoid neoplasia

Translocations resulting in a fusion gene in which *JAK2* is the 3' partner have been reported in myeloid and lymphoid leukaemias (Figure 1.1). In all cases, the 5' partner (*ETV6*[109,110], *BCR*[111] or *PCM1*[112]) contains a protein-protein interaction domain, suggesting that forced dimerisation leads to constitutive JAK2 kinase activation. Of note, expression of the *ETV6-JAK2* fusion conferred cytokine independent growth to a cytokine dependent cell line in the absence of a homodimeric type I receptor[113]. Transplantation of *ETV6-JAK2* expressing human cord blood progenitors into immunodeficient mice resulted in an IMF-like phenotype with anaemia and bone marrow fibrosis[114].

Table 1.1. Activating mutations and rearrangements of *JAK2* in human haematopoietic malignancy.

Mutation	Cell line transformation	Mouse bone marrow transplantation	Human disease
JAK2 V617F	Only with type I homodimeric receptor	Erythrocytosis +/- leucocytosis Bone marrow fibrosis	PV (97%) ET (50%) IMF (50%) AML (1-8%)
***JAK2* exon 12**	Only with type I homodimeric receptor	Erythrocytosis Leucocytosis	PV (3%)
JAK2 T875N	Only with type I homodimeric receptor	Erythrocytosis +/- leucocytosis Bone marrow fibrosis	AML cell line
JAK2 R683	Only with type I homodimeric receptor	Erythrocytosis Leucocytosis Thrombocytosis	Down's syndrome ALL (18%)
ETV6-JAK2	Without type I homodimeric receptor	Anaemia Bone marrow fibrosis	CML-like B-ALL T-ALL
BCR-JAK2	Unknown	Unknown	CML-like AML
PCM1-JAK2	Unknown	Unknown	CML-like B-ALL T-ALL

PV: polycythaemia vera; ET: essential thrombocythaemia; IMF: idiopathic myelofibrosis; AML: acute myeloid leukaemia; ALL: acute lymphoblastic leukaemia; CML: chronic myeloid leukaemia

1.7.5 Other JAK2 mutations in haematological disease: summary

Oncogenic alterations in *JAK2* are associated with both myeloid and lymphoid malignancy (Table 1.1; Figure 1.1). Point mutations are associated with either loss of autoinhibitory function or activation of the kinase domain, and these alleles are only able to transform cell lines in the presence of a type 1 homodimeric receptor. By contrast, translocations involving *JAK2* appear to result in constitutive dimerisation and consequent activation of JAK2 without the need for a type 1 receptor.

1.8 Therapeutic considerations in the myeloproliferative disorders

Thrombotic complications are the leading cause of morbidity and mortality in patients with ET or PV[3,4], and reducing the frequency of thrombosis is the main aim of therapy in these patients. Established risk factors for thrombotic events include past history of thrombosis[76,115-118], age over 60 years[76,116-118], leucocytosis[76,119-121], increased haematocrit (PV patients)[122] and presence of a *JAK2 V617F* mutation (ET patients)[68,119,123]. A clear association between degree of thrombocytosis and thrombotic risk in ET or PV has not been established[115,117,119]. Whereas the vast majority of patients with PV will require treatment to lower the haematocrit, rates of thrombosis in ET are more variable, such that ET patients can be stratified and treated according to thrombotic risk[115,124-126]. In IMF, survival is significantly reduced due to bone marrow failure and transformation to acute leukaemia[82,127]. At present, IMF therapy is largely supportive, aimed at ameliorating symptoms due to bone marrow failure and extramedullary haematopoiesis.

1.8.1 Anti-platelet therapy

A large, randomised controlled trial in PV demonstrated a reduction in thrombotic events in those taking aspirin[3]. Retrospective studies also suggest that aspirin may prevent thrombotic complications in ET patients[128,129]. An association between aspirin use and haemorrhage has been reported, particularly in patients with very high platelet counts[128], although this remains to

be proven in a prospective study. Based on current evidence, aspirin is recommended for all ET and PV patients unless otherwise contraindicated. Although there is little data concerning the use of newer anti-platelet agents such as clopidogrel, their proven track record in the prevention of atherosclerotic vascular disease suggests they may be appropriate for patients unable to tolerate aspirin.

1.8.2 Venesection

Reduction of the haematocrit to <0.45 has been associated with a decreased risk of thrombotic complications in patients with PV[122], and is suitable therapy for patients with an isolated erythrocytosis. However given the association of leucocytosis with thrombotic complications[121], many patients will require therapy with cytoreductive agents.

1.8.3 Hydroxycarbamide (previously known as hydroxyurea)

Hydroxycarbamide, a ribonucleotide reductase inhibitor, is the only cytoreductive agent proven to reduce thrombotic events in a randomised controlled trial[124]. Major complications of this drug include reversible myelosuppression and ulceration of the buccal mucosa or lower leg. Although hydroxycarbamide appears non-carcinogenic when used to treat sickle cell disease, an inherited non-clonal disorder of erythrocyte production[130], controversy remains concerning leukaemogenicity when used in the MPD[97]. Although several retrospective studies have suggested an increased risk of leukaemia in hydroxycarbamide treated patients[131-133], others have not observed this association[96,100,134]. Problems with these studies include small patient numbers, retrospective data collection and relatively short follow-up. Overall, any increased risk of leukaemia is likely to be small, and should be balanced against the likely reduction in thrombotic risk.

1.8.4 Interferon

Recombinant interferon alpha is effective at controlling blood counts in MPD patients, although there is little evidence regarding efficacy in the prevention of thrombotic complications[135]. Treatment is often associated with significant side-effects, including flu-like symptoms and psychiatric disturbance that may mandate cessation of therapy. Of note, interferon therapy in *JAK2 V617F*-positive PV patients may lead to a significant reduction in peripheral blood mutant allele burden, with occasional patients achieving a molecular remission[136].

1.8.5 Anagrelide

Anagrelide, a quinazoline derivative, reduces the platelet count by inhibition of megakaryocyte differentiation[137]. Although the white cell count is unaffected, anaemia is common and may be delayed in onset and progressive over the first years of treatment[68,138]. Up to a third of patients cannot tolerate anagrelide because of side effects, many of which result from its vasodilatory and positive inotropic effects, including fluid retention, palpitations and arrhythmias, heart failure and headaches[4,138]. Although anagrelide is not cytotoxic, and therefore free from leukaemogenic risk, the PT-1 randomised controlled trial demonstrated inferiority to hydroxycarbamide in the prevention of thrombotic complications in ET. In this study, anagrelide treated patients experienced higher rates of arterial thrombosis, major haemorrhage and progression to myelofibrosis, despite equivalent control of the platelet count[4]. Comparison of ET patients in the PT-1 and Italian prospective studies, however, revealed actuarial rates of first thrombosis by 2 years of 4% (hydroxycarbamide +/- aspirin, both studies), 8% (anagrelide plus aspirin, PT-1 study) and 26% (no cytoreductive therapy, Italian study)[139], suggesting that anagrelide provides at least partial protection from thrombotic complications.

1.8.6 Alkylating and radiomimetic agents

Radioactive phosphorous and alkylating agents such as busulphan and chlorambucil are effective at controlling blood counts, but are associated with an increased risk of acute leukaemia, particularly when used sequentially with hydroxycarbamide[92,95,96,133]. As such, these agents should be reserved for older patients unable to tolerate safer therapies. Pipobroman, a piperazine derivative, is effective at reducing blood counts in MPD patients, although there is little direct evidence for efficacy in thrombosis prevention[95,140]. Despite structural similarities to other alkylating agents, pipobroman does not appear to be associated with high rates of leukaemic transformation[95,140,141], but should none-the-less be avoided in the treatment of younger patients.

1.8.7 Therapeutic strategies for IMF

Therapy for IMF, occurring *de novo* or secondary to ET or PV, is essentially supportive, with transfusion of blood products for symptomatic anaemia or bleeding providing the mainstay of treatment. Cytoreductive therapy such as hydroxycarbamide may be useful in patients with thrombocytosis or progressive splenomegaly. Danazol, a synthetic testosterone derivative, alleviates anaemia in around one third of patients[142]. Thalidomide has also been reported to improve haemoglobin and occasionally platelet counts[143]. The mechanism of action of thalidomide is not fully understood but probably includes immunomodulation, antiangiogenic affects and modulation of cytokines including tumour necrosis factor-alpha. Early studies have suggested that thalidomide analogues such as lenalidomide may also be useful in reducing splenomegaly and alleviating cytopenias[144]. Splenectomy may be considered for those with significant splenic discomfort and may also reduce or obviate the need for red cell transfusion[145]. Splenectomy in patients with IMF carries a significant risk of adverse outcome, including bleeding, infection and thrombocytosis leading to thrombosis or haemorrhage, and is associated with operative mortality rates of 5-10%[145]. Splenic irradiation has occasionally been used for patients with symptomatic splenomegaly who are unfit for surgery, although this procedure

may be complicated by severe and occasionally prolonged cytopenias[146]. Allogeneic bone marrow transplantation has been used with some success in younger patients with IMF, with reduced intensity conditioning extending the age range of the procedure[147]. The clinical course of IMF is highly variable, however, with some patients surviving for long periods with stable disease. Bone marrow transplantation should therefore be reserved for those with clear evidence of progressive disease.

1.8.8 JAK2 inhibitors

Since the identification of the *JAK2 V617F* mutation, several small molecule inhibitors of the JAK2 protein have been tested in animal models or early clinical trials. JAK2 inhibitors are able to attenuate the disease phenotype in mouse models[51,148-150], and *in vitro* studies of human cells have suggested a selective effect of JAK2 inhibitors on *JAK2 V617F*-positive haematopoiesis[151,152]. Early clinical trials in humans, however, have suggested limited efficacy of JAK2 inhibitors in IMF patients. As JAK2 is the sole tyrosine kinase associated with the erythropoietin and thropmbopoietin receptors, JAK2 inhibition is associated with a predicatble, dose dependent anaemia and thrombocytopenia, limiting the therapeutic utility of this agent in many patients. Reduction in spleen size associated with freedom from blood transfusion has been reported in rare IMF patients (although similar responses are also seen in occassional hydroxyurea-treated patients). A proportion of patients report improvement in constitutional symptoms, an effect that may be related to inhibition of JAK1 with consequent reduction in cytokine secretion[153,154].

1.9 The thrombopoietin receptor, MPL, and the myeloproliferative disorders

The thrombopoietin receptor, MPL, was first identified through the study of transforming retroviruses in murine bone marrow. Transduction of murine haematopoietic progenitors with murine friend virus along with the replication incompetent myeloproliferative leukaemia virus (MPLV) was shown to induce proliferation and terminal differentiation of granulocyte, erythroid and

megakaryocyte lineages in the absence of growth factors[155]. Subsequent cloning of the MPLV virus pin-pointed the *v-mpl* gene as responsible for this activity, and identified a murine homologue, *c-mpl*, with structural homology to previously identified haematopoietic growth factor receptors[156]. Following identification of the human homologue (MPL)[157], studies suggested a specific role for this receptor in megakaryopoiesis, with transfection of human progenitor cells with *MPL* antisense RNA inhibiting megakaryocyte but not erythroid or granulocytic colony formation[158]. In 1994, the MPL ligand, thrombopoietin (Tpo), was identified using diverse approaches: capture and protein sequencing of Tpo on immobilised MPL[159,160], chemical mutagenesis to produce a thrombopoietin secreting cell line[161] and conventional protein purification and sequencing[162,163]. Subsequent studies using Tpo-neutralising antibodies confirmed that Tpo/MPL signalling is required for terminal megakaryocyte differentiation[164].

Studies of *MPL* knockout mice have suggested a role for Tpo/MPL signalling not only in megakaryopoiesis, but also in the maintenance of the HSC compartment[165]. Mice lacking the MPL receptor are born at the expected Mendelian ratio with platelet counts around 6% of normal. *In vitro* progenitor assays, however, demonstrated a significant reduction in erythroid and granulocyte as well as megakaryocyte colony formation[165]. Moreover in competitive reconstitution assays into normal mice, *MPL* knockout bone marrow showed only 10-15% of normal repopulating activity[166], suggesting either a numeric or functional HSC defect. MPL expression segregates with stem cell activity in both human and murine haematopoietic progenitors[167], and Tpo alone is able to maintain murine HSC in an undifferentiated state in liquid culture[168]. Recent work has suggested that Tpo/MPL signalling acts to maintain HSC in a quiescent state[169,170]. *TPO* knockout mice were shown to have normal numbers of HSC in the mid-gestation foetal liver, with HSC numbers subsequently falling through the life of the animal[169]. Taken together, these studies indicate a role for Tpo/MPL signalling in maintaining the quiescent state of HSC, with loss of this pathway leading to stem cell exhaustion.

Loss of function mutations in *MPL* are the cause of congenital amegakaryocytic thrombocytopenia (CAMT) in humans. This autosomal recessive disorder is

manifest by severe thrombocytopenia from birth and the development of aplastic anaemia between 1-3 years of age. A number of different mutations have been reported, with some correlation between genotype and phenotype[171]. The development of aplastic anaemia in these patients is consistent with mouse models, with loss of Tpo/MPL signalling leading to a gradual depletion of the HSC compartment.

The first *MPL* gain of function mutation identified in humans was the *MPL S505N* allele reported in a Japanese pedigree with dominantly inherited familial thrombocytosis[172]. The mutation was shown to segregate with affected family members across three generations, and was absent from unaffected relatives. Expression of *MPL S505N* resulted in cytokine independent proliferation of a cytokine dependent cell line, associated with constitutive activation of downstream targets such as MEK and STAT5. The amino acid change is within the transmembrane region of MPL, although the precise mechanisms leading to cellular transformation are not known.

Comparison of MPL and EpoR, which share around one third homology at the protein level, identified a five amino acid juxtamembrane motif in MPL that was absent from EpoR. Deletion or alteration of this motif resulted in constitutive activation of the MPL receptor, suggesting an autoinhibitory role for this region[173]. Subsequent analysis of patient samples identified acquired mutations at *MPL W515*, within the juxtamembrane region, in 9% of patients with *JAK2 V617F*-negative IMF and 1% of patients with ET[174,175]. Expression of the *MPL W515L* allele resulted in cytokine-independent growth of several cytokine dependent cell lines, associated with constitutive phosphorylation of downstream targets including JAK2, STAT3, STAT5, AKT and ERK[174]. Transplantation of mice with bone marrow expressing the *MPL W515L* allele resulted in an MPD-like phenotype characterised by marked thrombocytosis, splenomegaly, splenic infarction and reduced life expectancy[174]. More recently, an acquired *MPL T487A* mutation was reported in a child with acute megakaryoblastic leukaemia. Of note, expression of this allele in a mouse model resulted in a similar phenotype to expression of the *MPL W515L* allele.

In summary, Tpo/MPL signalling is required for both terminal differentiation of megakaryocytes and maintenance of the HSC compartment. Loss of MPL function in humans is associated with profound thrombocytopenia and progression to aplastic anaemia. A germline gain of function mutation has been associated with dominantly inherited familial thrombocytosis. Acquired mutations in *MPL* have been reported in IMF, occasional patients with ET and a single case of acute megakaryoblastic leukaemia.

1.10 Genetic complexity in the MPD: are mutations in *JAK2* disease initiating events?

JAK2 mutations are present in most patients with PV and half of those with ET or IMF[29,105,176]. *JAK2 V617F* or exon 12 mutations result in dysregulated JAK2 kinase activity, induce growth factor independence in factor-dependent cell lines[28,30,31,48,105] and are sufficient to produce an MPD phenotype in retroviral transplant models[28,105]. Taken together these results demonstrate the centrality of *JAK2* mutations to their associated MPD and imply a role for such mutations in disease initiation. However several observations have suggested a degree of genetic complexity in the human MPD that may be interpreted as evidence for clonal haematopoiesis prior to the acquisition of a *JAK2* mutation.

An inherited predisposition to develop an MPD is supported by both kindred studies[15-19] and registry data[14]. Affected family members manifest a genuine clonal disorder associated with acquired mutations in *JAK2* and clinical features indistinguishable from sporadic MPD[15,16,19]. Although the nature of the inherited allele is currently unknown, these observations have led to speculation that mutations in *JAK2* may be insufficient alone to cause a clinical MPD, with an additional genetic event being inherited in familial cases and acquired in sporadic cases.

Studies have described female MPD patients with a *JAK2 V617F*-positive MPD in whom clonality of peripheral blood granulocytes, as assessed by X-inactivation patterns, was in excess of *JAK2 V617F* mutant allele burden[177,178], leading to the hypothesis that the *JAK2* mutation had arisen within a pre-existing

clonal expansion. The results of these studies, however, are complicated by the phenomenon of age related skewing within the haematopoietic compartment, present in up to a half of normal older females[179,180]. Skewing of X-chromosome usage may represent a stochastic occurrence due to an age related decrease in HSC numbers resulting in reliance on a smaller number of stem cells to maintain haematopoiesis. Such skewing has been observed in felines undergoing autologous transplantation with small numbers of cells[181]. Alternatively, polymorphisms in X-chromosome genes may impart a subtle survival benefit, such that either the maternal or paternal allele is selected for over the lifetime of the individual. Evidence for this model again comes from the study of cats, where breeding experiments demonstrated a consistent selection of the same parental X-chromosome as the cats aged[182]. Moreover, in a study of skewed X-chromosome usage in human monozygotic twins, the over-represented X-allele was concordant between twin pairs[183]. Skewing has also been observed in T-cells from older females, albeit to a lesser degree than in granulocytes[180]. In feline studies, increasing X-chromosome skewing in granulocytes was not mirrored by skewing in T-cells. Following autologous bone marrow transplantation, however, reconstituted cats showed skewing of both granulocytes and T-cells[182]. This result is consistent with the notion that skewing occurs at the HSC level, with the long survival of T-cells maintaining a non-skewed pattern in some individuals[102].

Discordant clonality has also been reported in patients harbouring a *JAK2* mutation and a deletion of chromosome 20q, where granulocyte clonality measured by *JAK2* mutant allele burden was less than clonality assessed by the size of the 20q deleted clone[177]. The clonal relationship of the two genetic lesions, however, was not known in these cases.

Patients with a *JAK2 V617F*-positive MPD may progress to acute myeloid leukaemia, and in a proportion of such cases the leukaemia is negative for the *JAK2* mutation[102,103]. Similarly erythropoietin independent erythroid colonies (endogenous erythroid colonies: EEC) cultured from patients with a *JAK2 V617F*-positive MPD may be negative for the *JAK2* mutation[184,185]. It has been hypothesised in such cases that both the *JAK2* mutant MPD and *JAK2* wild-type

disease (AML or EEC) arise from a shared clonal expansion. In a single case of *JAK2* wild-type AML, fluorescent *in-situ* hybridisation studies suggested the presence of the same clonal cytogenetic abnormality in both the *JAK2*-mutant MPD and *JAK2* wild-type AML[103]. However analysis of single progenitor colonies was not performed, and as such this finding cannot be taken as definitive proof that the two stages of disease arose from a shared clonal expansion.

Of interest, similar lines of evidence have been cited in support of a pre-*BCR-ABL1* phase of clonal haematopoiesis in CML. In a proportion of females with CML, a skewed X-chromosome inactivation pattern was detected in *BCR-ABL1* negative immortalised B cells, with the over-represented allele being the same as the allele expressed by the *BCR-ABL1* clone[186,187]. Moreover in one patient chromosomal abnormalities were detected in immortalised B-cell lines expressing the same X-linked allele as the *BCR-ABL1* clone, but not in lines expressing the alternative allele, suggesting the presence of a genetically unstable clone prior to the acquisition of *BCR-ABL1*. CML patients may develop *BCR-ABL1* negative clones harbouring acquired chromosomal abnormalities, with a proportion of such patients developing *BCR-ABL1* negative myelodysplasia or acute leukaemia. Such clones were observed more frequently in patients who had received prior cytotoxic therapy but have also been reported in patients treated solely with the tyrosine kinase inhibitor imatinib[188-190]. The *BCR-ABL1* fusion gene has also been detected in individuals without an overt MPD[191], leading to the suggestion that *BCR-ABL1* alone may be insufficient to cause disease in humans.

In summary, biochemical and mouse model data suggests that *JAK2* mutations are central to the phenotype of their associated disease. However studies have suggested a degree of genetic complexity in human disease which may be interpreted as evidence for the establishment of clonal haematopoiesis prior to the acquisition of these lesions. Thus far, however, evidence for such a pre-*JAK2* phase of clonal haematopoiesis remains circumstantial and direct evidence for or against this hypothesis is lacking.

Chapter 2

Materials and Methods

2.1 Preparation of human blood cells

Blood samples were obtained from MPD patients and normal controls after obtaining informed consent. The acquisition, storage and analysis of these samples was covered under the project entitled 'The causes of clonal blood cell disorders' which was approved by the Regional Ethics Committee (MREC 03/5/22 & 07/MRE05/44). Venous blood (10-50mls) was obtained by venepuncture from the antecubital fossa after application of tourniquet pressure. Blood was collected into tubes containing either ethylenediaminetetraacetic acid (EDTA), preservative free heparin or sodium citrate as an anticoagulant. All samples were processed within 24 hours of venepuncture.

2.1.1 Separation of platelets from peripheral blood

The following protocol was developed by Dr George Vassiliou as part of his PhD project. The protocol has been optimised to give a platelet recovery of 38% ± 6.2%, with an average of 0.32 white blood cells per 10^6 platelets. Given that 10^9 platelets contain approximately 1µg RNA, with 10^7 T-cells or granulocytes containing approximately 14.4µg or 5.9µg RNA respectively, RNA isolated using this protocol is >99% platelet derived.

Whole blood taken into EDTA was placed into a 15mL polypropylenetube (BD Bioscience, CA, USA) and centrifuged at 150g for 20 minutes at room temperature. The upper phase of platelet-rich plasma (PRP) was removed down to 1.5mL from the cell pellet, placed into a clean 15mL tube and centrifuged at 150g for 20 minutes. The PRP was again removed down to 0.5mL from the cell pellet, placed in a clean 15mL tube and centrifuged for a third time at 150g for 20 minutes. The PRP was removed down to 0.5mL from the cell pellet and

aliquoted into 2mL eppendorf tubes, each containing 35µL of prewashed anti-CD45 magnetic beads (Dynabeads, Dynal AS, Norway). The tubes were incubated for 30 minutes at room temperature with mixing. The tubes were then applied to a magnet and inverted 10 times to allow binding of white cells. The PRP was carefully removed whilst the tubes were still applied to the magnet, and placed into clean 2mL eppendorf tubes. These tubes were then reapplied to the magnet and inverted 10 times, following which the PRP was removed and pooled in a clean 15mL tube. The PRP was centrifuged at 1,600g for 10 minutes to pellet the platelets. The platelets were washed once in phosphate-buffered saline (PBS), re-pelleted, lysed in Tri-reagent (Sigma, MO, USA) at 10^8 -10^9 platelets/mL and stored at -80^{o}C.

2.1.2 Separation of mononuclear cells and granulocytes from peripheral blood

20mls of sodium diatrizoate/polysaccharide density gradient (Lymphoprep, Axis-Shield, Norway) at room temperature was aliquoted into a 50mL conical falcon tube. 25mls of peripheral blood was then layered slowly over the Lymphoprep using a pipette on the gravity-only setting, followed by centrifugation at 800g for 20 minutes with the centrifuge brake switched off. The interface layer, containing mononuclear cells, was then collected using a Pasteur pipette, placed in a clean 50mL Falcon tube and washed once in PBS. The mononuclear cells were resuspended in 1-3mls PBS and a cell count was performed using an automated counter (ABC Vet, ABX Montpellier, France). The cells were then used for purification of T-cells or CD34-positive cells, or for plating into semi-solid media.

The remaining plasma and Lymphoprep was removed down to the red cell/granulocyte layer. The red cell layer was resuspended in ice cold red cell lysis buffer (0.15M NH_4Cl, 10mM $NaHCO_3$ and 0.1mM EDTA in H_2O) and incubated on ice for 10-30 minutes. The cells were then centrifuged at 800g for 10 minutes and resuspended in 5mls PBS. In the event of incomplete red cell lysis, the above steps were repeated. A cell count was performed. In order to assess granulocyte purity, 10^5 cells in 100µL PBS were used to prepare a

cytospin slide. The cells were applied to a cytospin funnel mounted on a prelabelled glass slide and spun at 450rpm for 5 minutes on a cytocentrifuge (Cytospin 3, Shandon, UK). Following air-drying, the slides were fixed in methanol for 30sec and stained sequentially with haematoxylin (30sec) and methylene blue (30sec), and excess stain was rinsed off with tap water. Differential cell counts were performed by light microscopy in order to ensure that granulocyte purity was >95% before proceeding to cell lysis. Granulocytes were then pelleted by centrifugation and lysed in either Tri-reagent, DNA lysis buffer (containing 10mM EDTA, 50mM Tris-Cl and 0.5% sarkosyl) or RLT buffer (Qiagen, Hilden, Germany) and stored at -80^{o}C for RNA or -20^{o}C for DNA.

2.1.3 Purification of CD2-positive T-cells

Aliquots of 10^7 mononuclear cells in 1mL PBS were added to 25µL of prewashed anti-CD2 magnetic beads (Dynabeads, Dynal AS, Norway) in 2mL eppendorf tubes. Samples were incubated at 4^{o}C for 20 minutes with mixing. The tubes were applied to a magnet and inverted 10 times to capture the CD2-positive cells, allowing the PBS to be removed. Each sample was washed 5 times in PBS in this manner. A cell count was then performed using a Neubauer ruled haemocytometer, in order to enumerate total cells and the percentage bound to magnetic beads. If the bead-bound percentage was <95%, further washes were performed as above and the purity reassessed. Cells were then pelleted by centrifugation and lysed in either Tri-reagent, DNA lysis buffer or RLT buffer and stored at -80^{o}C for RNA or -20^{o}C for DNA.

2.1.4 Purification of CD34-positive progenitor cells

Aliquots of up to 10^8 mononuclear cells were resuspended in 300µL of buffer (containing 0.5% bovine serum albumin and 2mM EDTA in PBS), to which 100µL blocking reagent and 100µL CD34 magnetic beads were added (MACS system, Miltenyi, Germany). Samples were incubated on ice for 30 minutes, washed once in ice cold buffer and resuspended in 500µL ice cold buffer. A MS cell separation column (Miltenyi, Germany) was inserted into the magnet and rinsed with ice cold buffer. The sample was then applied to the column and

rinsed 3 times with 500µL aliquots of ice cold buffer. The column was removed from the magnet, 1mL buffer was added and the cells eluted off the column using the plunger. The eluted cells were washed once in Iscove's modified Dulbecco's medium (IMDM; Sigma, MO, USA) and a cell count was performed using a Neubauer ruled haemocytometer. Samples were then either lysed in RLT buffer or plated in semi-solid media.

2.2 Culture of human blood cells

2.2.1 Culture of erythroid and granulocyte-macrophage progenitors in semi-solid medium

The culture medium used was either Methocult GF H4531 (StemCell Technologies, Vancouver, Canada) containing 1% methylcellulose, 30% foetal bovine serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine and 10% agar leukocyte conditioned media (Agar-LCM) in Iscove's MDM; or Methocult GF H4534 (StemCell Technologies, Vancouver, Canada) containing 1% methylcellulose, 30% foetal bovine serum, 1% bovine serum albumin, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 50ng/mL recombinant stem cell factor, 10 ng/mL recombinant GM-CSF and 10ng/mL recombinant IL-3 in Iscove's MDM. 4mL aliquots of Methocult medium were prepared in 15mL tubes. Mononuclear cells in PBS obtained as per section 2.1.2 were adjusted to 6 x10^6/mL and 66-200µL cell suspension was added to each 4mL aliquot of medium (final concentration 1-3 x10^5/mL). Alternatively, CD34-positive cells were added to give a final concentration of 500-2,000/mL. Other cytokines and reagents were added as indicated. For blood samples sent from other institutions, penicillin and streptomycin were added to the culture medium (final concentration 1%). In no case was more than 10% volume added to the Methocult medium, in order to maintain optimal viscosity. Samples were vortexed for 30 seconds and allowed to stand for 5min to allow air bubbles to escape. Samples were then aliquoted into 6-well plates at 1.1mL per well, using a 5mL syringe and an 18g needle. Sterile water was dispensed between the

wells, and the plate was incubated at 37^{o}C with 5% CO_2 and high humidity for 14 days.

Erythroid and granulocyte colonies were identified by morphological characteristics and the red colour of the former. Individual colonies were harvested by visualising the plate under a dissecting microscope, and carefully aspirating individual colonies using a P200 pipette. For DNA extraction, individual colonies were lysed in 50µL of water, heated to 95^{o}C for 8min and stored at 4^{o}C. For RNA extraction, individual colonies were lysed in RLT buffer and stored at -80^{oC}.

2.2.2 Culture of megakaryocyte progenitors in semi-solid medium

The culture medium used was Megacult-C (StemCell Technologies, Vancouver, Canada). Mononuclear cells in PBS obtained as per section 2.1.2 were adjusted to 6 x10^{6}/mL and 50-200µL cell suspension was added to 1.7mL Megacult base medium. Cytokines were added as indicated at the following final concentrations: thrombopoietin 50ng/mL, IL-11 50ng/mL and IL-3 10ng/mL; the volume was then adjusted to 2.1mL with Iscove's MDM. 1.2mL of collagen solution was added to each sample to give a final concentration of 1.1 mg/mL bovine collagen, 1% bovine serum albumin, 10 mg/mL recombinant insulin, 200 mg/mL human transferrin (iron saturated), 10^{-4} M 2-Mercaptoethanol, 2 mM L-glutamine and 3-12 x10^{5}/mL mononuclear cells in Iscove's MDM. Samples were vortexed, aliquoted into slide chambers (StemCell Technologies, Vancouver, Canada) and incubated at 37^{o}C with 5% CO_2 and high humidity for 14 days.

After 14 days, the sides of the chambers were removed, and the gel matrix was dehydrated by layering a spacer and piece of pre-cut blotting paper over the slide. Slides were then fixed in 1:3 methanol:acetone solution for 20 minutes on ice. The spacers and blotting paper were carefully removed, and the fixed slides stored at -20^{o}C until staining. Before staining, slides were brought to room temperature and rehydrated with rinse buffer (containing 0.05M Tris and 0.135M NaCl at pH 7.6) for 20 minutes. Care was taken to ensure that the slides

remained covered with the relevant solution at all stages of the staining protocol. After each stage of the staining protocol, the applied solution was tipped off the slide, and the new solution was overlaid. Blocking buffer (containing 5% human serum in rinse buffer) was applied to the slides for 20 minutes in order to inhibit non-specific binding of the primary antibody. Mouse anti-human GPIIb/IIIa antibody (IgG2a) at 10 µg/mL (in 5% human serum, 0.05M Tris and 0.135M NaCl) was then applied to the slides for 30 minutes, following which the slides were rinsed three times with rinse buffer (3min per application). Biotin conjugated goat anti-mouse IgG at 10 µg/mL (in 1% bovine serum albumin, 0.05M Tris and 0.135M NaCl) was then applied to the slides for 30 minutes, following which the slides were rinsed three times with rinse buffer (3min per application). Avidin-Alkaline Phosphatase conjugate at 18 µg/mL (in 1% bovine serum albumin, 0.05M Tris and 0.135M NaCl) was then applied to the slides for 30 minutes, followed by three washes with rinse buffer (3min per application). Alkaline phosphatase substrate was prepared fresh and applied to the slides for 15 minutes, followed by three washes with rinse buffer (3min per application). Slides were counterstained for 10 minutes with 1% Evans blue in methanol and rinsed with distilled water until the water ran clear. Slides were then air-dried and kept at 4^{o}C until analysis.

Megakaryocytes colonies were identified by pale pink staining of their cytoplasm. They were differentiated from platelets which stain darker pink and appear as small anuclear fragments.

2.2.3 Culture and replating of acute myeloid leukaemia cells in semi-solid medium

Mononuclear cells from patients with AML (obtained from protocol 2.1.2) were plated in Methocult GF H4534 at a concentration of 10^3 to 10^5 cells/mL as outlined in section 2.2.1. Plates were examined for the presence of colonies after 7-14 days. Cytospins were prepared by dispersing single colonies in 100µL PBS as stained and outlined in section 2.1.2.

For replating experiments, each well was washed out twice with 3mL IMDM and transferred to a 15mL tube. The tube was made up to 14mL with IMDM and the cells pelleted by centrifugation at 800g for 8 minutes. The supernatant was removed down to 1ml above the cell pellet using a Pasteur pipette, and discarded. Following the addition of 12mL IMDM, the cell suspension was vortexed for 30sec and repelleted by centrifugation at 800g for 8 minutes. The supernatant was completely removed, the cells were resuspended in 500µL IMDM and a cell count was performed using an automated blood counter. Cells were then plated in fresh semi-solid media at a density dependent on the results of the initial plating experiment. Replating was repeated up to a further three times, and cytospin slides of individual colonies were prepared.

2.2.4 Propagation of acute myeloid leukaemia cells in liquid culture

Mononuclear cells from patients with AML (obtained from protocol 2.1.2) were cultured in IMDM containing 20% foetal calf serum, 100U/mL penicillin, 100mg/mL streptomycin and 10ng/mL IL-3 at 37^{o}C with 5% CO_2 and high humidity. Every 2-3 days the cells were transferred into a 15mL tube, pelleted by centrifugation at 800g for 8 minutes and resuspended in fresh culture medium. Cell counts were performed using a haemocytometer, and cell viability checked by trypan blue exclusion. Cytospins were prepared and stained as outlined in section 2.1.2.

2.2.5 Storage of viable human cells

For freezing of viable human cells, mononuclear cells at 1-5 x10^6 cells/mL in IMDM were chilled on ice. Cells were mixed in a one to one ratio with ice cold 20% dimethyl sulfoxide (DMSO) in 5% human albumin solution, and frozen slowly by insulating in a polystyrene casing at -80^{o}C. Cells were transferred to liquid nitrogen after around 3 days. To thaw viable cells, 1mL aliquots were rapidly thawed in a water bath at 37^{o}C, and diluted immediately on thawing into 14mL IMDM, followed by pelleting by centrifugation at 800g for 8min. Cells were then resuspended in IMDM and viability checked by trypan blue exclusion.

2.2.6 Obtaining images of progenitor colonies and cytospins

Images of erythroid and blast colonies were acquired from whole culture plates using a SMZ800 microscope (Nikon, Japan) with a Plan x1-x6.3 objective lens (Nikon, Japan) and Pro150ES camera (Pixera, California). Images of cytospins and stained megakaryocyte colonies were acquired using a U-TVO.5XC-2 microscope (Olympus, Japan) with a UplanApo 40x/0,85 objective lens (Olympus, Japan) and Penguin 600CL camera (Pixera, California). All images were captured using Viewfinder and Studio Software (Pixera, California).

2.3 Nucleic acid extraction from human blood cells

2.3.1 DNA extraction from DNA lysis buffer

Cell samples for DNA extraction were lysed in DNA lysis buffer (containing 10mM EDTA, 50mM Tris-Cl and 0.5% sarkosyl), incubated overnight at 55^{o}C and stored at -20^{o}C. Samples were allowed to come to room temperature before DNA extraction. 500µL of cell lysate was mixed with an equal volume of phenol-chloroform in a 2mL eppendorf tube and vortexed gently until the solution became cloudy. The sample was then centrifuged at 12,000g for 5min at room temperature. The supernatant (approximately 500µL) was transferred to a clean tube to which 1mL 100% ethanol and 150µL 3M sodium acetate was added. The sample was mixed by inversion. In most cases (with ≥10^6 cells) the DNA

could then be visualised as a stringy white precipitate, which was removed using a bacteria spreader and washed twice in 70% ethanol. The DNA pellet was then dried on the end of the bacteria spreader until just translucent and dissolved in 200µL of water. When the DNA could not be visualised, the sample was place at -80^{o}C for 30min and the DNA precipitated by centrifugation at 12,000g for 15min. The DNA pellet was then washed twice with 70% ethanol, air dried until just translucent and dissolved in 100µL water. DNA quantity and purity was assessed using a NanoDrop spectrophotometer (LabTech, UK). Purity was assessed by the $\lambda_{260}/\lambda_{280}$ absorbance ratio, with optimal values being in the range 1.7-2.0. DNA samples were stored at 4^{o}C.

2.3.2 DNA and RNA extraction from Tri-reagent

Samples in Tri-reagent were thawed at room temperate. 200µL chloroform was added to 1mL of sample in Tri-reagent followed by vigorous shaking for 15sec. The sample was allowed to stand for 10min at room temperature, followed by centrifugation at 12,000g at 4^{o}C for 20min. The upper phase was transferred to a clean tube, to which 500µL isopropanol and 1µL glycogen (Ambion, TX, USA) was added and mixed by inversion. After standing for the 10min at room temperature, the RNA was precipitated by centrifugation at 12,000g for 20min. The supernatant was discarded and the RNA pellet washed once with 70% ethanol in RNAse free water, followed by centrifugation at 12,000g for 10min. The RNA pellet was then air dried until just translucent and dissolved in 10-30µL RNAse free water. Samples that were not reverse-transcribed immediately were stored at -80^{o}C.

For DNA extraction, all traces of the upper phase were carefully removed. The lower phase was then mixed with 150µL 100% ethanol and 1µL glycogen by inversion and allowed to stand for 5min at room temperature. DNA was precipitated by centrifugation at 12,000g for 15min at 4^{o}C. The supernatant was discarded, 1mL 70% ethanol was added and the pellet washed by flicking the tube. The sample was allowed to stand for 15min at room temperature and repelleted by centrifugation at 12,000g for 10min. The wash step was repeated, following which all traces of ethanol were removed by pipetting and the DNA

pellet air dried until just translucent. 20-50µL water was added and the sample incubated at 55^{o}C until the DNA had fully dissolved. DNA quantity and purity was assessed as outlined in section 2.3.1. DNA samples were stored at 4^{o}C.

2.3.3 DNA and RNA extraction using spin columns

Qiagen RNeasy spin columns were used to isolate RNA from samples in RLT buffer. Samples were allowed to come to room temperature and mixed with an equal volume of 70% ethanol in RNAse free water. 700µL of sample was then applied to the spin column and centrifuged at 12,000g for 15sec. The flow-through was discarded and the process repeated as necessary until the whole sample had been applied to the column. The column was rinsed with 700µL RW1 buffer and two applications of 500µL RPE buffer, with centrifugation at 12,000g for 15sec and discarding of the follow-through after each application. Following the rinses, the column was placed in a clean collection tube and centrifuged for a further 1min to remove all traces of wash buffer. RNA was then eluted off the column into a clean tube in 30µL RNAse free water by centrifugation at 12,000g for 1min. Samples that were not reverse transcribed immediately were stored at -80^{o}C.

Qiagen AllPrep spin columns were used to isolate DNA and RNA in RLT or RLT plus buffer from small amounts of starting material, for example single erythroid colonies. Samples were thawed to room temperature and applied to the AllPrep column by centrifugation at 12,000g for 30sec. The AllPrep column, containing the DNA fraction, was set aside. The flow-through, containing the RNA fraction, was mixed with an equal volume of 70% ethanol in RNAse free water and applied to a RNeasy MiniElute column by centrifugation at 12,000g for 15sec. The column was rinsed sequentially with 700µL RW1 buffer, 500µL RPE buffer and 500µL 80% ethanol in RNAse free water by centrifugation at 12,000g for 15sec. The column was then transferred into a clean collection tube and centrifuged at 12,000g for 5min to removed residual traces of ethanol. RNA was eluted off the column into a clean tube in 12µL RNAse free water by centrifugation at 12,000g for 1min. Samples that were not reverse transcribed immediately were stored at -80^{o}C. The DNA bound to the AllPrep column was

rinsed sequentially with 500µL AW1 buffer and 500µL AW2 buffer by centrifugation at 12,000g for 15sec. The DNA was then eluted off the column into a clean tube in 50µL elution buffer at 70^{o}C by centrifugation at 12,000g for 1min. DNA samples were stored at 4^{o}C.

2.3.4 Preparation of complementary DNA (cDNA)

Frozen RNA samples were thawed at room temperature. 9.7µL of RNA solution was mixed with 2µL of 100µM random hexamers (Invitrogen, Paisley, UK). Following incubation for 5min at 70^{o}C, samples were placed immediately on ice for 2min. To each sample was added 4µL 5x reaction buffer, 2µL 100mM dithiothreitol, 0.8µL 25mM dNTP, 0.5µL 40U/L RNAse inhibitor (Rnasin, Promega, Southampton, UK) and 1µL reverse transcriptase (from murine Moloney leukaemia virus, Invitrogen, Paisley, UK). A control sample containing water instead of RNA template was also prepared in the same way to control for contamination of the reverse transcription reagents. Samples were incubated at 37^{o}C for 90min followed by an enzyme inactivation step of 70^{o}C for 10min. cDNA samples were stored at -20^{o}C.

2.4 Polymerase chain reaction (PCR) methods

2.4.1 Primers for direct sequencing: design, optimisation and sequencing protocol

Primers were designed using the Whitehead Institute on-line 'Primer 3' software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) on default settings with a product size of 150-500 bases. All DNA sequences were screened for regions of interspersed repeats of low complexity using an on-line repeat masker (http://www.repeatmasker.org/). In addition, primers were checked using an on-line in-silico PCR programme (http://genome.ucsc.edu/) to check that a single amplicon would be obtained. In assays designed to amplify intronic regions containing single nucleotide polymorphisms (SNPs), the predicted product was also checked for homology using an on-line bioinformatics site (http://genome.ucsc.edu/). In assays designed to amplify a cDNA product, the

primers were placed in different exons separated by an intron of at least 500 bases.

The basic PCR reaction consisted of 50µL volume containing 10-50ng template DNA or cDNA, 0.5U AmpliTaq Gold hot start taq polymerase (Applied Biosystems, CA, USA), 1µM dNTPs, 10mM Tris-Cl (pH 8.3), 50nM KCl, 1.5 mM $MgCl_2$ and 0.2 µM forward and reverse primers. Standard PCR conditions were 94^{o}C for 10min followed by 35 cycles of denaturing at 94^{o}C for 30sec, annealing at 55-65^{o}C for 30sec and extension at 72^{o}C for 30sec, followed by a final extension step of 72^{o}C for 10min. The optimum annealing temperature for each pair of primers was assessed by means of a 55-65^{o}C temperature gradient. Further optimisation was performed in the event of poor amplification, such as increasing the $MgCl_2$ concentration to 2-2.5mM, including 10% DMSO to the reaction or increasing the primer concentration. All PCR reactions included a positive control (a DNA or cDNA sample known to amplify well), a no-template control and in the case of cDNA amplification, a no-template control sample from the original reverse transcription reaction (as outlined in section 2.3.5). PCR products were visualised under ultraviolet light after running 10µL of the reaction on a 1-2% agarose electrophoresis gel stained with ethidium bromide.

For direct sequencing of PCR amplified DNA, the PCR product was first treated with ExoSAP-IT (USB, OH, USA), a mixture of Exonuclease 1 and Shrimp Alkaline Phosphatase to remove unincorporated primers and dNTPs, by incubating 5µL of PCR product with 2µL of ExoSAP-IT at 37^{o}C for 15min, followed by 80^{o}C for 15min to inactivate the enzyme. 2µL of treated PCR product was then added to the sequencing reaction, comprising 3µL reaction buffer (80mM Tris pH 9.0, 10mM $MgCl_2$), 1µL BigDye reaction mix (Applied Biosystems, CA, USA), 2.5µL of sequencing primer at 10µM concentration and 1.5µL H_2O. Standard PCR conditions for the sequencing reaction were 25 cycles of denaturing at 96^{o}C for 10sec, annealing at 50^{o}C for 5sec and extension at 60^{o}C for 4min. Products were then precipitated with 1µL 3M sodium acetate and 25µL 95% ethanol for 15min at room temperature, followed by centrifugation at 2,250g for 40min. Products were washed once with 70% ethanol, followed by centrifugation at 2,250g for 10min, and resuspended in

10μL formamide. Sequencing was performed on a 3730xL analyser (Applied Biosystems, CA, USA). Sequence traces were analysed manually using Chromas Lite software (version 2.0, Technelysium Pty Ltd, MN, USA).

2.4.2 Primers for pyrosequencing: design and optimisation

Pyrosequencing was used when accurate quantitation of alternative alleles was required, for example in the measurement of mutant allele burden or the genotyping of individual progenitor colonies for a specific mutation or SNP. Primers were designed using PSQ assay design software version 1.0.6 (Biotage, Uppsala, Sweden). Annealing temperature and primer conditions were optimised and PCR products examined after gel electrophoresis as outlined in section 2.4.1. For the pyrosequencing reaction, 3μL Streptavidin Sepharose HP beads (Amersham Biosciences, Chalfont St Giles, United Kingdom) were incubated with 37μL Binding buffer (10mM Tris-Cl pH 7.6, 2 M NaCl, 1mM EDTA and 0.1% Tween 20), 20μL PCR product and 20μL water for 10 minutes at room temperature with agitation. Biotinylated PCR products were then captured onto the filter probes of the Pyrosequencing Vacuum Prep Tool (Biotage, Uppsala, Sweden), washed with 70% ethanol for 5sec, denatured with 0.2M NaOH for 5sec and washed with10mM Tris-acetate pH 7.6 for 5sec. The vacuum was then released and the beads and bound single-stranded DNA template was dropped into a PSQ 96 Plate Low (Biotage) containing 45μL annealing buffer (20mM Tris-acetate, 2mM magnesium acetate, pH 7.6) with 0.3μM sequencing primer. The samples were heated to 80°C for 2 minutes and then allowed to cool to room temperature. Pyrosequencing reactions were performed using the PSQ 96 single nucleotide polymorphism reagent kit (Biotage), which contained the enzyme, substrate mixture and nucleotides.

Individual pyrosequencing assays were optimised by studying the following controls: biotinylated DNA template only to look for template looping and self priming, sequencing primer only and biotinylated primer only to look for primer dimerisation and extension, biotinylated primer and sequencing primer to look for aberrant extension from a primer-only template, and no template control from the original PCR reaction to look for interference from primer dimers. The

performance of each individual assay was assessed using PCR products cloned into the pGem-Teasy vector (see section 2.4.5) to generate a standard curve. For assays designed to quantitate mutant alleles where two or three adjacent bases were changed, a standard curve was generated using one of the following formulae:

two adjacent bases changed:

$$\frac{\frac{A}{2}}{\frac{A}{2}+T}$$

three adjacent bases changed:

$$\frac{\frac{A}{3}}{\frac{A}{3}+T}$$

where A and T are the pyrosequencing peak heights for the mutant and wild-type alleles respectively. The relative quantitation of the allele was then read from the graph.

2.4.3 Allele-specific polymerase chain reaction: design and optimisation

Allele-specific PCR assays were used as a sensitive method of detecting mutations in DNA and cDNA samples. Each assay consisted of a pair of control primers designed to produce an amplicon of around 400bp with both wild-type and mutant material, and an internal mutation specific primer designed to produce an amplicon of around 200bp with mutant samples. To ensure the specificity of the mutation specific primer, the 3' end was placed over the mutated base. In some instances, mismatches were introduced close to the 3' end of the primer to destabilise annealing and improve specificity for the mutant allele. It was generally necessary to screen many different mutation specific primers using different annealing temperatures to ensure both the sensitivity and specificity of the assay.

2.4.4 Real-time polymerase chain reaction (QPCR): design and optimisation

Real-time PCR (QPCR) was performed on a Stratagene Mx3000P machine and analysed using MxPro version 3.20 (Stratagene, CA, USA). Primers were designed using the Whitehead Institute on-line 'Primer 3' software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) on default settings with a product size of 80-110 bases for cDNA analysis, and a product size of around 50 bases for DNA copy number analysis. In assays designed to amplify a cDNA product, the primers were placed in different exons separated by an intron of at least 500 bases in order to avoid amplification of contaminating DNA. All reactions were run using Brilliant SYBR green 2x master mix (Stratagene, CA, USA), 30nM ROX reference dye, 100-300nM primers and 10-50ng template. No template controls were run for every primer pair to check for contamination and primer dimer formation. Cycling conditions used were 95^{o}C for 10min followed by 40 cycles of 95^{o}C for 15sec and 60^{0}C for 60sec followed by measurement of the dissociation curve with collection of fluorescence data for every 1^{o}C rise in temperature from 55^{o}C to 95^{o}C. Primers were optimised by preparing five serial 1 in 5 dilutions of a suitable template which was run in duplicate for each primer pair. This data was then used to generate a standard curve using the machine software. Such experiments were considered acceptable only where there was high precision between replicates (Rsq ≥0.985). Primers were considered acceptable where the slope of the curve gave a primer efficiency of 90-110% (Y value between -3.1 and -3.6 with optimum -3.3). The dissociation curve was examined for the presence of a single sharp peak (indicating the presence of a single amplified product). Primers producing more than one peak or a single poorly defined peak were redesigned. No template controls were studied for primer dimers, as suggested by the presence of poorly defined peaks at lower temperatures in the dissociation curve analysis. In such cases, optimisation was repeated with lower primer concentration or the primers were redesigned.

ABL and *GUSB* were used as housekeeping genes as studies have demonstrated little variability in their expression across different haematopoietic

cell types, both in normal and leukaemic samples[192]. One or more housekeeping genes were assessed in every experiment to control for the amount of input material, along with one or more genes of interest. All samples were run in duplicate, and an average was taken for the threshold value (Ct value).

In experiments looking for trends in gene expression relative to a specific sample (normaliser or calibrator sample), the expression of the gene of interest relative to the housekeeping gene was calculated using the ΔΔCt formula as below:

$$\Delta\Delta Ct = 2^{-(Ct\ gene\ of\ interest\ -\ Ct\ housekeeping\ gene)}$$

2.4.5 Cloning of polymerase chain reaction amplified products

DNA was amplified in a standard PCR reaction using a taq polymerase that leaves an A-overhang, such as AmpliTaq Gold. The PCR product was purified using a QiaQuick spin column (Qiagen, Germany) and visualised on an ethidium bromide stained agarose gel. A ligation reaction was then set up containing 2-4μL PCR product, 5μL 2x reaction buffer, 1μL T4 ligase, 1μL pGem-T Easy vector (Promega, WI, USA), and water to bring the reaction to 10μL. The reaction was left at room temperature for 1-4 hours. JM109 highly competent E-coli bacteria (Stratagene, CA, USA) were thawed on ice. 50μL of cells was mixed with 5μL of ligase reaction and incubated on ice for 30min. The sample was then heat shocked in a water bath at 42^{o}C without agitation for exactly 45sec, then transferred directly onto ice for 2min. 600μL of SOCS medium was added to each sample, which was then incubated at 37^{o}C for 45min. 100μL of 10mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 100μL of 2% 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-gal) was added to each sample, mixed by inversion and tipped onto individual LB agar plates containing ampicillin. The sample was spread evenly over the surface of the plate using a sterile bacterial spreader and left to dry completely at room temperature. The agar plates were then inverted and incubated overnight at 37^{o}C.

The pGem-T Easy vector contains an ampicillin resistance cassette such that only successfully transformed bacteria will produce colonies on an ampicillin impregnated plate. Successful ligase of DNA into the vector disrupts a β-galactosidase expression cassette, such that bacterial colonies that have taken up an empty vector will appear blue in the presence of X-gal and IPTG, whereas bacterial colonies containing the vector plus an insert will appear white. Individual white bacterial colonies were transferred into 50μL of SOCS medium using a pipette tip. To sequence the vector insert, a standard PCR reaction was set up using M13 primers (which anneal to the vector and allow amplification across the insert) and 2μL of bacterial colony in SOCS reagent. The PCR product was then prepared for direct sequencing.

When a bacterial colony containing the correct insert was identified, 10μL of the bacterial colony in SOCS medium was added to 3mL LB medium and incubated overnight at 37^{o}C with agitation at ~250rpm. Bacteria were pelleted from 2mL of culture by centrifugation at 6,000g for 5min and resuspended in 200μL ice cold resuspension buffer containing 50mM Tris-Cl pH 8.0, 10mM EDTA and 100μg/mL RNAse A. This was mixed by inversion with 200μL lysis buffer at room temperature, containing 200mM NaOH and 1% SDS, and left to stand at room temperature for 5min. 300μL ice cold neutralization buffer was then added, containing 3M potassium acetate pH 5.5, mixed by inversion and incubated on ice for 5min. Cellular debris was removed by centrifugation at 12,000g for 10min at 4^{o}C, and the supernatant transferred to a clean tube. DNA was precipitated by the addition of 900μL isopropanol, followed by incubation at room temperature for 20min and centrifugation at 12,000g for 20min at room temperature. The DNA pellet was washed once with 70% ethanol, repelleted by centrifugation at 12,000g for 5min and air dried until just translucent. The DNA was then dissolved in water, and quantity and purity of DNA were assessed as outlined in section 2.3.1. The sequence of the plasmid insertion was checked by direct sequencing using the M13 primer site within the vector. 500μL of bacterial culture were mixed with 500μL glycerol and stored at -20^{o}C for future use.

2.5 Summary of reagents used

Procedure	Reagent	Supplier
Density separation	Lymphoprep	Axis-Shield, Norway
Leucocyte depletion	CD45 magnetic beads	Dynal AS, Norway
HSC/AML enrichment	CD34 magnetic beads	MACS, Miltenyi, Germany
T-cell selection	CD2 magnetic beads	Dynabeads, Dynal AS, Norway
DNA & RNA isolation	TRI-reagent	Sigma, MO, USA
RNA isolation	RLT buffer & spin columns	Qiagen, Hilden, Germany
cDNA synthesis	Reverse transcriptase	Invitrogen, Paisley, UK
cDNA synthesis	RNAse inhibitor	Promega, Southampton, UK
Liquid cell culture	IMDM	Sigma, MO, USA
Semi-solid colony assays	Methocult	StemCell, Vancouver, Canada
Megakaryocyte colony assay	Megacult	StemCell, Vancouver, Canada
PCR primers	Oligonucleotides	Sigma-Aldrich, Poole, UK
PCR reaction	AmpliTaq gold	Applied Biosystems, CA, USA
Sequencing clean-up	ExoSAP-IT	USB, OH, USA
Sequencing reaction	Big Dye reagents	Applied Biosystems, CA, USA
Pyrosequencing	Pyro reagents	Biotage, Uppsala, Sweden
Real-time PCR	SYBR green 2x master mix	Stratagene, CA, USA
Cloning of PCR products	pGem-Teasy vector	Promega, WI, USA

2.6 Statistical analysis

Unpaired two-tailed *t* tests were used to compare the mean value of continuous variables between two groups. Fisher's exact test was used to compare categorical data in a 2x2 contingency table. Both tests were performed using Microsoft Excel 2002 and/or GraphPad Prism (http://www.graphpad.com/quickcalcs/).

Analysis of clinical and laboratory variables in the PT-1 cohort (Chapter 3, Tables 3.3, 3.4 and 3.5) was performed by Dr Peter Campbell. Pairwise univariate analyses comparing diagnostic variables between the *MPL*-mutant and either *JAK2 V617F*-positive or *JAK2 V617F*-negative groups were performed using the *t* test for continuous variables, Fisher's exact test for 2×2 tables, and Cochran-Armitage test for trend with exact p values for ordinal variables. Multivariate analyses for the associations of ordinal variables with mutation status, accounting for the effects of age, were performed using proportional odds logistic regression. Complication rates during follow-up were assessed using Kaplan-Meier life tables and log-rank analyses. Confidence intervals for odds ratios of complications in the year prior to diagnosis were calculated using the asymptotic formula 1/a + 1/b + 1/c + 1/d to estimate the variance of the log (OR). Multivariate survival analyses were performed using Cox proportional hazards models. S-plus v7.0 (Insightful Corp, Seattle, WA) was used for all statistical analyses, apart from the exact methods, for which SAS v9.0 (SAS, NC, USA) was used.

Chapter 3

Characterisation of mutations in *MPL*, the thrombopoietin receptor, in the human myeloproliferative disorders

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3.1 Introduction

Gain of function mutations in *MPL*, encoding the thrombopoietin receptor, have been reported in both inherited and acquired myeloid disorders, affecting the transmembrane and juxtamembrane regions of the protein (Figure 3.1A). An inherited mutation in the transmembrane domain (*MPL S505N*) has been reported in Japanese and Italian kindreds with familial thrombocytosis, but was not observed in cases of sporadic ET[172,193]. Although the precise molecular consequences of this alteration are unclear, expression of the *MPL S505N* allele conferred cytokine independent growth to the cytokine dependent BaF3 cell line[172]. Acquired mutations in the juxtamembrane region (*MPL W515L/K*) have been reported in 5-8% of patient with IMF[174,175]. This region, which is absent from the erythropoietin receptor (Figure 3.1A), is thought to play a role in negative regulation of MPL signalling[173]. Retrovirally-induced expression of the *MPL W515L* allele in murine bone marrow resulted in an MPD phenotype *in vivo* characterised by splenomegaly and marked thrombocytosis[174]. In IMF patients, *MPL* mutations are associated with lower haemoglobin levels and an increased risk of dependence on red cell transfusion when compared to both *JAK2 V617F*-positive and mutation negative patients[194]. The *MPL W515L* mutation was subsequently reported in around 1% of ET patients, although the small number of patients (n=4) precluded further phenotypic analysis[175].

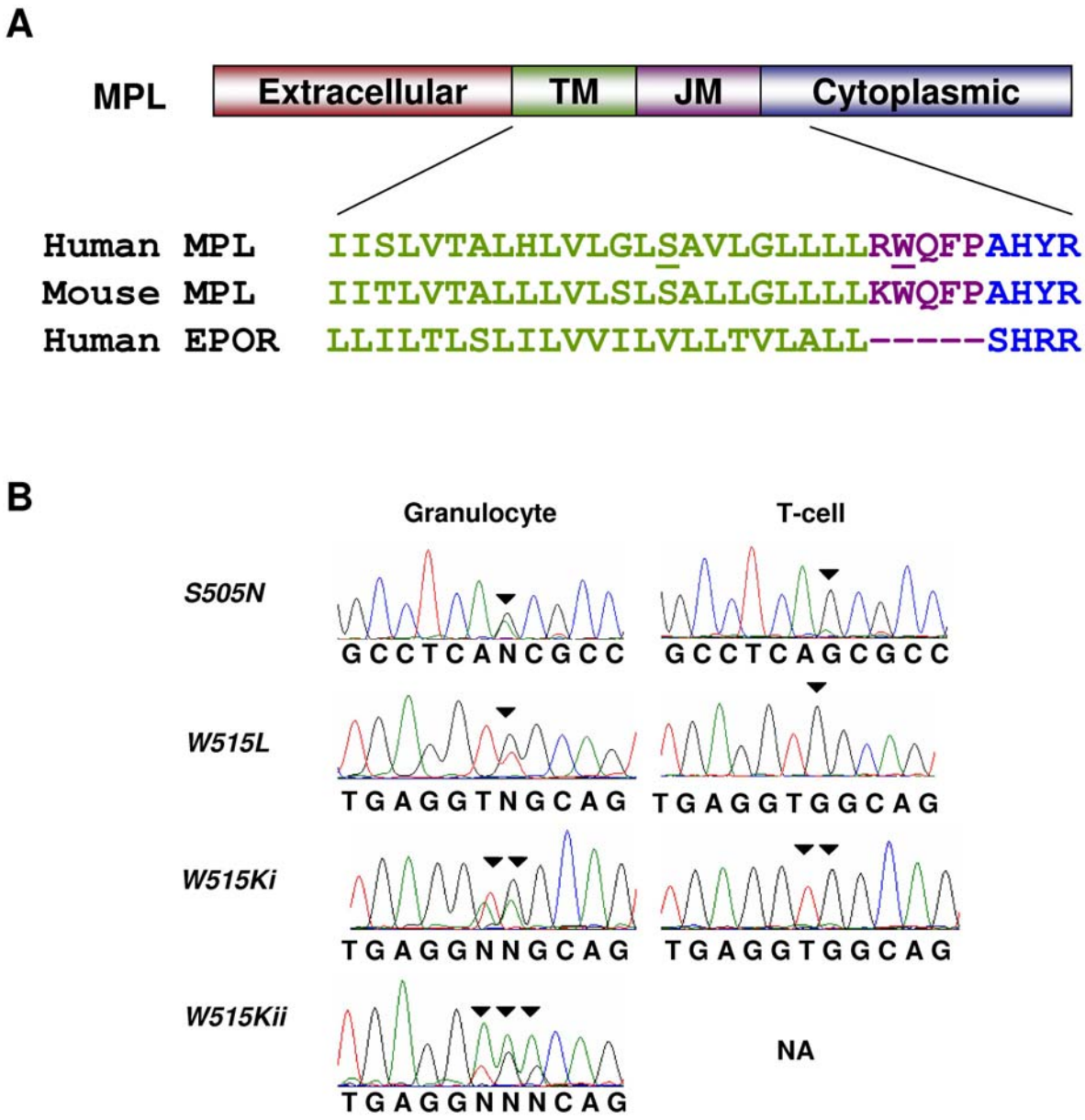

Figure 3.1. Mutations in *MPL* exon 10 in patients with a myeloproliferative disorder. (A) MPL structural domains and partial sequence alignment with murine MPL and the human erythropoietin receptor (EPOR). Residues mutated in patients with a myeloproliferative disorder are underlined. (B) Sequence traces showing the *S505N* allele as an acquired mutation, the previously reported *W515L* allele, and two different alleles resulting in a *W515K* substitution. TM: transmembrane; JM: juxtamembrane; NA: no sample available.

3.2 Aims of this chapter

This chapter addresses the following questions relating to *MPL* mutations in patients with an MPD:

1) Are mutations in *MPL* other than those previously described associated with the development of an MPD?
2) Are *MPL* mutations limited to the megakaryocyte lineage in a proportion of patients?
3) Are *MPL* mutations found in patients with polycythaemia vera?
4) What is the prevalence of *MPL* mutations in ET?
5) Do *MPL* mutations in ET define a distinct subset of patients with regard to laboratory, histological or clinical features?
6) Are *MPL* mutations associated with genuine ET or do *MPL* mutant ET patients represent an early stage of IMF?

3.3 Characterisation of mutations in *MPL*

To address the possibility that mutations in *MPL* may be restricted to the megakaryocyte lineage, or may occur elsewhere in the gene, platelet cDNA was prepared from 20 patients with a *JAK2 V617F*-negative MPD (18 ET and 2 IMF). The entire *MPL* coding region, spanning approximately 2Kb, was amplified by PCR in four overlapping fragments and assessed for mutations by bi-directional sequencing. Within this cohort, a single patient with an acquired *MPL W515L* mutation was identified, indicating that mutations outside exon 10 or mutations restricted to the megakaryocyte lineage are not a common cause of *JAK2 V617F*-negative MPD.

4 of 20 patients harboured a non-synonymous *MPL V114M* SNP. This SNP has been previously reported (rs12731981), but at a lower rate than observed in this cohort (heterozygous proportion 0.052, HapMap European cohort, n=116). As a non-synonymous SNP has previously been associated with higher platelet counts in a black-American cohort[195], further information was sought from the Bloodomics consortium. In their cohort of normal blood donors, the

heterozygosity rate for the rs12731981 SNP was 0.048 (Nick Watkins personal communication, n=291), in keeping with published data and significantly lower than observed in this MPD cohort (p=0.02, Fisher's exact test). In the Bloodomics cohort, however, the presence of the SNP was not associated with any difference in platelet count. In order to accurately characterise the frequency of this SNP in MPD patients, 381 ET patients from the PT-1 cohort were genotyped for the SNP using a pyrosequencing assay (Table 3.1). In the PT-1 cohort, the heterozygous proportion was no different from normal controls in Bloodomics cohort, suggesting that the over-representation of the SNP in the original cohort of 20 patients was a chance occurrence.

Table 3.1. Genotyping for the *MPL V114M* SNP in ET patients from the PT-1 cohort

	G/G (V/V)	**G/A (V/M)**	**Heterozygous proportion**
V617F-positive	198	10	0.048
V617F-negative	163	10	0.058
All patients	361	20	0.052

MPL exon 10 was further assessed in a retrospective cohort of 200 unselected patients with ET or IMF by direct sequencing of granulocyte-derived DNA. Exon 10 encodes both the transmembrane and juxtamembrane regions of the protein, and includes the *S505* and *W515* residues previously reported as sites of human mutation. This cohort was also genotyped for the *JAK2 V617F* mutation by allele-specific PCR[29]. *MPL* mutations were found in 8 IMF patients (7.1%) and 3 ET patients (3.4%), all of whom were negative for the *JAK2 V617F* mutation (Table 3.2).

Table 3.2. *JAK2 V617F* and *MPL* exon 10 mutations in a retrospective cohort of 200 MPD patients.

	JAK2 V617F-positive	*MPL* exon 10			Mutation negative
		S505N	*W515L*	*W515K*	
IMF - no.	57	1	5	2	47
ET - no.	41	1	2	0	44
Total	**98**	**2**	**7**	**2**	**91**

Three different alleles resulting in two different amino acid changes at the W515 residue were seen (Figure 3.1B), comprising the previously reported *MPL W515L* and *W515K* alleles. Two patients carried an *MPL S505N* mutation, previously reported as an inherited allele[172,193]. Constitutional material was available from one ET patient, and showed absence of the mutation in T-cell and buccal derived DNA, demonstrating that the mutation was acquired in this patient (Figure 3.1B). Moreover, the patient had had a number of normal blood counts in the years prior to diagnosis with ET, consistent with the presence of an acquired MPD.

To investigate the possibility that mutations in *MPL* are also present in patients with PV, 120 patients were screened for mutations at *MPL W515* using a pyrosequencing assay with a sensitivity 10%. None of the PV patients, all of whom were positive for the *JAK2 V617F* mutation, harboured mutations in *MPL*, indicating that such mutations are limited to MPD patients with ET or IMF.

3.4 Development of sensitive assays for the detection and quantitation of *MPL* mutations

At the time of this study, nothing was known about the precise prevalence or clinical significance of *MPL* mutations in patients with ET. Mutations in *MPL* were therefore assessed in samples taken from patients entered into the PT-1 studies, for whom comprehensive diagnostic and prospectively acquired follow-up data was available. Although patients enrolled into the PT-1 trials had DNA

banked at trial entry, the samples were from unfractionated whole blood. Previous studies have indicated that in patients with a *JAK2 V617F* associated MPD, the clonal burden may be low, particularly in ET[29]. Moreover, *JAK2* and *MPL* mutations are absent from the majority of lymphocytes[58,196]. It was important, therefore, to develop sensitive assays for each mutant *MPL* allele. For genotyping of the PT-1 samples, allele-specific PCR assays were developed for each of the three *MPL* mutations, with the *MPL W515K* assay being able to detect both alleles found in the retrospective cohort.

In order to assess the sensitivity of each allele-specific PCR assay, pyrosequencing assays were developed to accurately quantitate the mutant allele burden in patient samples (Figure 3.2A). Each pyrosequencing assay was validated using a dilution series made by mixing cloned wild-type and mutant PCR products. Results from this analysis were then used to plot a standard curve, from which the allele burden was read (Figure 3.2B).

The pyrosequencing assays were used to assess the mutant allele burden in patient samples, and allele-specific PCR was then performed on dilutions corresponding to 1-9% mutant allele burden. The assays for *MPL S505N* and *MPL W515L* could detect a mutant allele burden of ~1%, whereas the assay for the *MPL W515Ki* and *MPL W515Kii* allele could detect a mutant allele burden of ~3-5% (Figure 3.3).

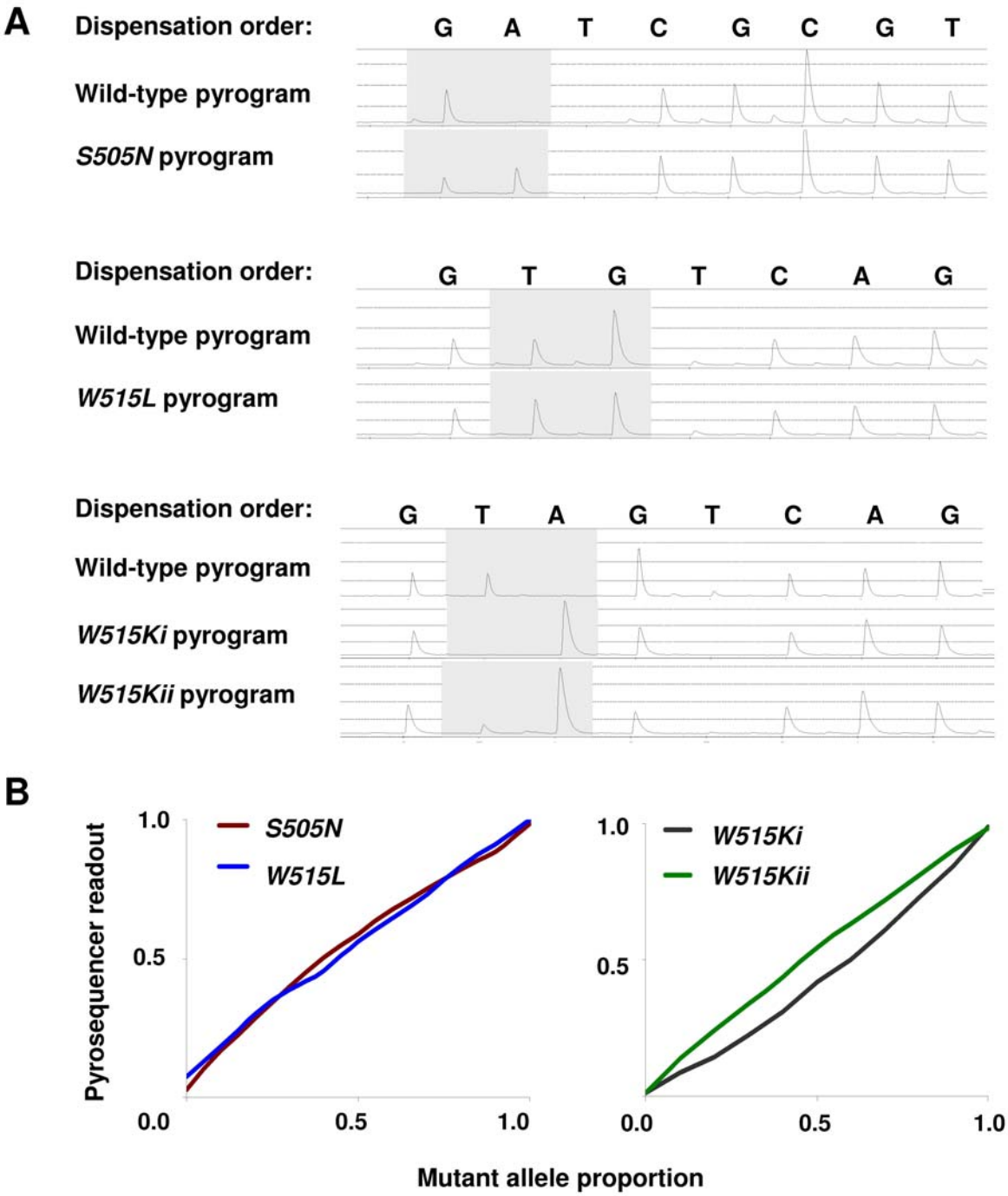

Figure 3.2. Pyrosequencing assays used for the quantitation of *MPL* exon 10 mutations. (A) Representative pyrograms for *MPL* exon 10 mutations showing the nucleotide dispensation order for the individual assays. Mutation sites are highlighted in grey. (B) Standard curves for the quantitative *MPL* exon 10 pyrosequencing assays generated by mixing cloned *MPL* wild-type and mutant PCR products.

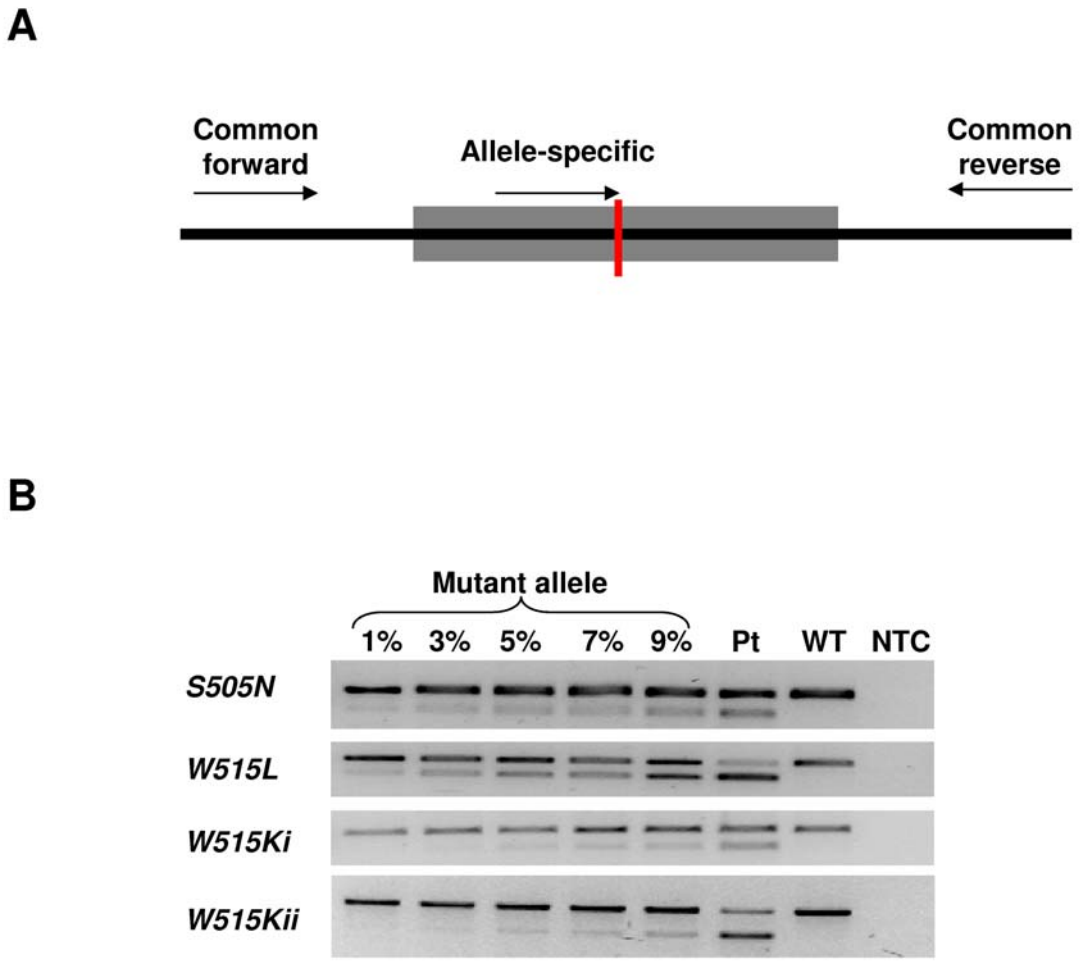

Figure 3.3. Allele-specific PCR assays used for the detection of *MPL* exon 10 mutations. (A) Primer design strategy for allele-specific PCR showing the common forward and reverse intronic primers and the allele-specific primer lying within *MPL* exon 10. (B) Mixing experiments performed with normal and mutant DNA demonstrating the sensitivity of each individual allele-specific PCR assay. PCR: polymerase chain reaction; Pt: patient sample; WT: wild-type control; NTC: no template control.

3.5 Clinical and laboratory features of *MPL* mutations in patients with essential thrombocythaemia

DNA samples were available from 776 patients enrolled into the PT-1 trials, comprising a randomised trial of hydroxycarbamide versus anagrelide in high risk ET, a randomised trial of hydroxycarbamide versus no cytoreductive therapy in intermediate risk ET and an observation trial of low risk ET (Figure 3.4A). Samples were genotyped for the *MPL S505N, MPL W515L* and *MPL W515K* mutations using allele-specific PCR. *MPL* mutations were detected in 32 patients, accounting for 4.1% of all ET patients (95% CI 2.9%-5.8%, Table 3.5) and 8.5% of *JAK2 V617F*-negative ET patients (Figure 3.4B). *MPL W515L* was the most common mutation, seen in 24 patients; 5 patients had *MPL W515K* (all *MPL W515Ki* allele) and 3 patients had *MPL S505N* mutations (Figure 3.4C).

In all patients with *MPL W515K* or *MPL S505N* alleles, the presence of the mutation was confirmed by both direct sequencing and pyrosequencing. Of the 24 *MPL W515L* mutations, 19 were detected by both direct sequencing and pyrosequencing, 3 were detected by pyrosequencing but not direct sequencing, and 2 were detected by allele-specific PCR alone. In these 2 patients, the presence of the mutation was confirmed by cloning an *MPL* exon 10 PCR amplicon, which was used for transformation of competent bacteria. Individual bacterial colonies were then picked and genotyped by direct sequencing. In both patients the mutant allele was detected in <1% of bacterial colonies screened, consistent with a low mutant allele burden. The allele-specific PCR result was also confirmed in both of these patients using an independent DNA sample. Of the 32 *MPL*-mutant patients, one also carried the *JAK2 V617F* mutation, and is included in the *MPL*-mutant group in the statistical analysis. No patient was positive for more than one *MPL*-mutant allele.

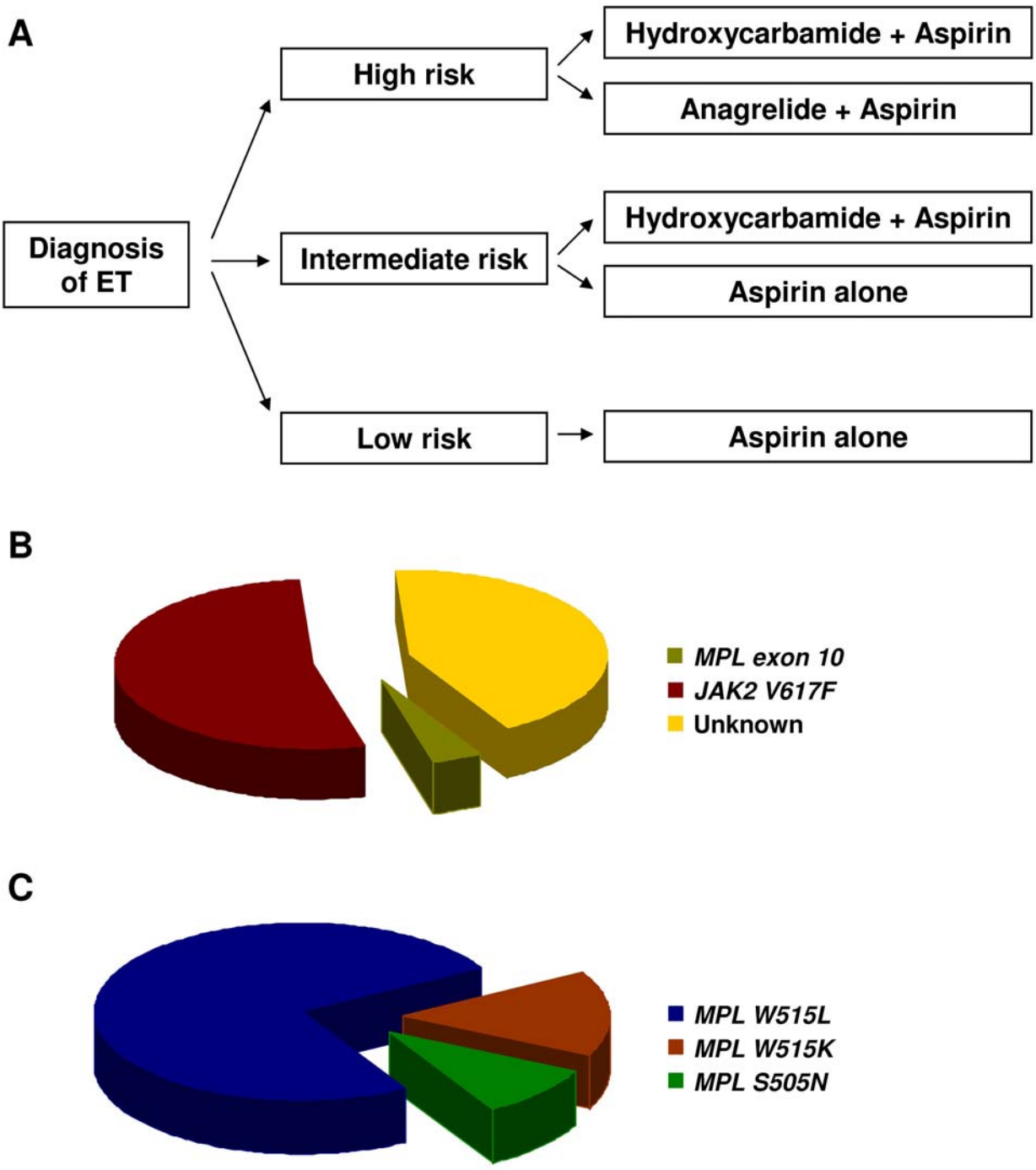

Figure 3.4. The PT-1 cohort: study design and mutation prevalence. (A) Study design of the three PT-1 trials. (B) Prevalence of *MPL* exon 10 and *JAK2 V617F* mutations within the PT-1 cohort. (C) Relative distribution of individual *MPL* exon 10 mutations within the PT-1 cohort. ET: essential thrombocythaemia.

Laboratory and clinical features of the *MPL*-mutant group were compared to both the *JAK2 V617F*-positive and *JAK2 V617F*-negative groups, both of which lack *MPL* mutations. The statistical analyses presented in Tables 3.3, 3.4 and 3.5 were performed by Dr Peter Campbell. *MPL*-mutant patients were significantly older at diagnosis than *JAK2 V617F*-negative patients. Compared to *JAK2 V617F*-positive patients, *MPL*-mutant patients had lower haemoglobin and higher platelet levels at diagnosis. There were no differences, however, in diagnostic blood counts between the *MPL*-mutant and *JAK2 V617F*-negative groups. There were also no significant differences in the presence of splenomegaly or bone marrow cytogenetic abnormalities between the groups (Table 3.3).

Two patients with *MPL* mutations harboured bone marrow cytogenetic abnormalities. A patient with an *MPL S505N* mutation harboured monosomy 7 and additional material on chromosome 6, and a patient with *MPL W515L* harboured trisomy 14. In both cases the abnormalities were detected at diagnosis prior to cytoreductive therapy, and neither case showed evidence of progression to myelofibrosis or acute leukaemia during trial follow-up. Trisomy 14 is a recognised recurrent abnormality in myeloid malignancy, although the majority of cases comprise either acute myeloid leukaemia or myelodysplasia[197]. Monosomy 7 is a frequent abnormality in myeloid malignancy, but again is generally associated with acute myeloid leukaemia or myelodysplasia.

Table 3.3. Laboratory and clinical features at diagnosis of 776 ET patients enrolled in the PT-1 trials.

	MPL mutant	*MPL* negative		p value (vs *V617F* pos)	p value (vs *V617F* neg)
		V617F positive	*V617F* negative		
Number	32 [1]	411	333		
Percentage	4.1%	53.0%	42.9%		
– (95% CI)	(2.9-5.8)	(49.4-56.5)	(39.4-46.5)		
Risk category					
Low risk[2]	0	26	31	0.2	0.09
Intermediate risk[3]	4	68	46	0.8	1.0
High risk[4]	28	339	273	0.6	0.6
Hydroxyurea + Aspirin	13	168	139	0.9	0.8
Anagrelide + Aspirin	15	171	134		
Demographics					
Female – no. (%)	17 (53%)	255 (62%)	192 (58%)	0.3	0.7
Male – no. (%)	15 (47%)	156 (38%)	141 (42%)		
Age (yr.)					
– median (10^{th}-90^{th} centile)	67 (48-77)	60 (39-77)	52 (32-74)	0.09	<0.0001
Disease duration[5] (mths.)					
– median (10^{th}-90^{th} centile)	18 (0-956)	38 (0-1106)	57 (0-2699)	0.2	0.7
Laboratory and clinical features at diagnosis					
Haemoglobin (g/L)					
– mean ± SD	133 ± 12	145 ± 14	135 ± 14	<0.0001	0.3
– median	136	145	136		
– (10^{th}-90^{th} centile)	(119 - 147)	(128 - 163)	(117 - 153)		
White cells (x10^9/L)					
– mean ± SD	9.9 ± 2.4	10.6 ± 3.4	9.3 ± 2.7	0.2	0.2
– median	9.7	10.0	8.8		
– (10^{th}-90^{th} centile)	(7.0 - 13.3)	(7.0 - 14.6)	(6.2 - 12.8)		
Neutrophils (x10^9/L)					
– mean ± SD	6.7 ± 2.1	7.4 ± 3.0	6.2 ± 2.2	0.3	0.2
– median	6.9	6.8	5.8		
– (10^{th}-90^{th} centile)	(4.4 - 9.6)	(4.2 - 11.0)	(3.8 - 9.1)		
Platelet count (x10^9/L)					
– mean ± SD	1040 ± 272	900 ± 274	1032 ± 350	0.006	0.9
– median	962	840	963		
– (10^{th}-90^{th} centile)	(752 - 1505)	(632 - 1221)	(666 - 1550)		
Splenomegaly – no. (%)	0/27 (0%)	11/326 (3%)	11/262 (4%)	0.7	0.6
Abnormal cytogenetics – no. (%)	2/24 (8%)	14/312 (4%)	10/262 (4%)	0.3	0.2

[1] One patient had both *JAK2 V617F* and *MPL W515L* mutations, and is included in the *MPL*-mutant group

[2] Includes 4 patients who subsequently enrolled in the intermediate risk arm, and 10 in the high risk arm

[3] Includes 4 patients who were previously enrolled in the low risk arm and 25 who subsequently enrolled in the high risk arm

[4] Includes 10 and 25 who were previously enrolled in the low and intermediate risk arms, respectively

[5] Refers to time elapsed between diagnosis and trial entry

Bone-marrow trephine biopsies at diagnosis were available from 311 patients, including 13 patients with *MPL* mutations, comprising 2 *S505N*, 2 *W515K* and 9 *W515L* patients. These were assessed independently by three haematopathologists (Dr Wendy Erber, Dr David Bareford and Dr Bridgit Wilkins) who were aware of the patient's age and sex but unaware of *JAK2* or *MPL* mutation status (Table 3.4). Given the known association between age and bone marrow cellularity, patient age was included as a variable in the statistical analyses for cellularity. There were no differences in the mean reticulin grade, megakaryocyte cellularity or the presence of megakaryocyte clusters and atypia between the *MPL*-mutant, *JAK2 V617F*-positive and *JAK2 V617F*-negative groups. However, trephine biopsies from the *MPL*-mutant group were less cellular than both the *JAK2 V617F*-positive and *JAK2 V617F*-negative groups (p=0.0001 (0.0003 with age) and p=0.005 (0.003 with age) respectively). Compared to the *JAK2 V617F*-positive group, both erythroid and granulocytic cellularity were reduced in the *MPL*-mutant group (p=0.0008 (0.0007 with age) and p=0.009 (0.02 with age) respectively). Compared to the *JAK2 V617F*-negative group, the *MPL* mutant group showed reduced erythroid cellularity (p=0.005 (0.004 with age)). Thus *MPL*-mutant patients exhibited a more isolated megakaryocytic proliferation at diagnosis, with a reduction in overall cellularity compared to both the *JAK2 V617F*-positive and *JAK2 V617F*-negative groups. There was, however, considerable overlap between the histological appearances observed in the three groups of patients, with *MPL*-mutant patients showing many features typical of ET, including increased megakaryocyte number with clustering and nuclear hyperlobation (Figure 3.5). As such, these results indicate that *MPL* mutations as a whole do not define a distinct histological subtype of ET. It remains formally possible that specific *MPL* mutations are associated with particular histological features, but the number of patients with each individual *MPL* mutation was too small to address this issue. Two *MPL* mutant patients developed IMF during trial follow-up (one *MPL S505N* and one *MPL W515K*). Of note, all *MPL*-mutant ET patients were classified as 'true-ET' at diagnosis, according to WHO criteria[74], with no cases showing features of pre-fibrotic or cellular phase myelofibrosis.

Table 3.4. Bone marrow trephine histology, erythropoietin levels and iron stores in patients enrolled in the PT-1 trials.

	MPL mutant	*MPL* negative		p value (vs *V617F* pos)	p value (vs *V617F* neg)
		V617F positive	*V617F* negative		
Bone marrow trephine histology[1]	n=13	n=168	n=130		
Reticulin grade – mean ± SD	1.8 ± 1.0	1.8 ± 0.8	1.9 ± 0.9	0.9	0.6
Megakaryocyte clusters and nuclear morphology					
Clusters (absent/loose/tight)	1 / 6 / 6	22 / 99 / 47	12 / 67 / 51	0.3	0.8
Pyknotic (absent/pres/predom)	6 / 6 / 1	55 / 112 / 1	48 / 79 / 3	0.8	0.9
Staghorn (absent/pres/predom)	2 / 10 / 1	31 / 132 / 5	23 / 102 / 5	0.7	0.7
Cloud-like (absent/pres/predom)	5 / 8 / 0	46 / 118 / 4	44 / 85 / 1	0.4	0.8
Dysplastic (absent/pres/predom)	4 / 8 / 1	57 / 110 / 1	48 / 80 / 2	0.6	0.6
Cellularity[2]					
Overall (dec/normal/inc)	3 / 5 / 5	1 / 36 / 131	2 / 43 / 85	0.0003	0.003
Erythroid (dec/ normal/inc)	3 / 9 / 1	5 / 90 / 73	6 / 77 / 47	0.0007	0.004
Granulocytic (dec/normal/inc)	3 / 6 / 4	3 / 74 / 91	2 / 76 / 52	0.02	0.07
Megakaryocytic (+/++/+++)	3 / 9 / 1	32 / 88 / 47	25 / 58 / 47	0.3	0.2
Erythropoietin levels and iron stores					
Erythropoietin[3] (U/L)					
– mean ± SD	20.7 ± 16.2	9.8 ± 10.8	23.8 ± 28.9	<0.0001	0.6
– median (10^{th}-90^{th} centile)	17.4 (6.3–33.8)	6.9 (3.2–17.3)	14.9 (7.9–42.7)		
Mean cell volume[4] (fL)					
– mean ± SD	89.6 ± 5.5	87.6 ± 6.4	89.4 ± 6.1	0.08	0.8
– median (10^{th}-90^{th} centile)	89.5 (83.1–96.9)	88.3 (79.8–94.2)	89.4 (83.5–95.2)		
Mean cell volume < 80fL[3] – no. (%)	2/32 (6.3%)	41/391 (10.5%)	4/312 (1.3%)	0.4	0.2
Ferritin[5] (µg/L) – mean ± SD	94 ± 79	90 ± 91	90.7 ± 90	0.8	0.9
– median	54	58	91		
– (10^{th}-90^{th} centile)	(19 – 213)	(23 – 192)	(29 – 201)		

[1] All bone marrow trephine biopsies were obtained at diagnosis
[2] Dec, decreased; inc, increased; +, ++, +++, increasing cellularity
[3] Based on 707 serum samples from trial entry. Normal range for serum erythropoietin, 5-25U/L
[4] Normal range for mean cell volume, 80-100fL
[5] Based on serum samples taken within 3 months of diagnosis (n=182). Normal range for ferritin, 20-300µg/L

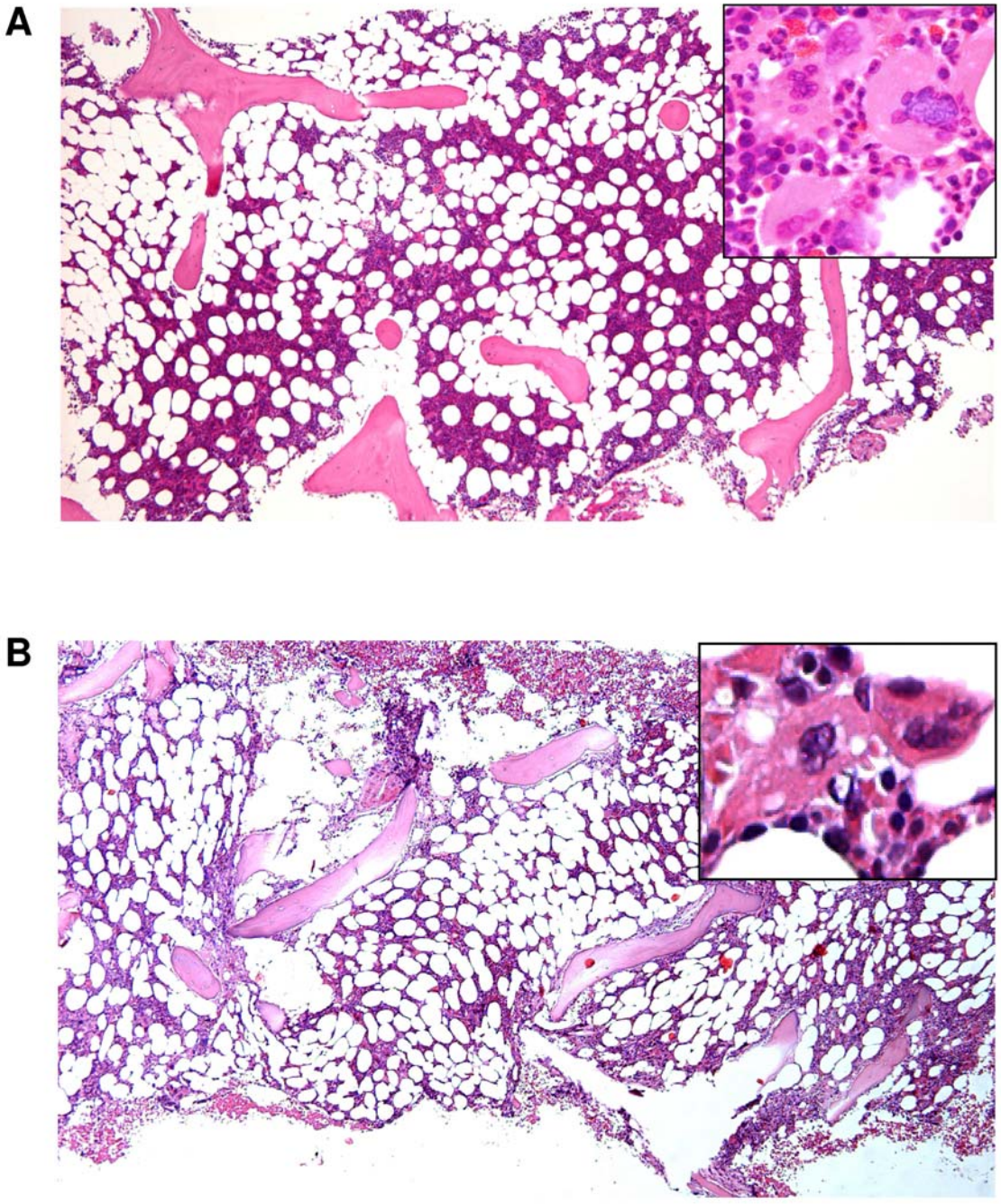

Figure 3.5. Bone marrow histology of *MPL* exon 10 mutations in essential thrombocythaemia . Haematoxylin and eosin stained bone marrow trephine biopsies from (A) an untreated 60 year old male with an *MPL W515L* mutation, showing increased megakaryocyte frequency with clustering (inset), and (B) an untreated 77 year old male with an *MPL S505N* mutation, showing reduced cellularity and occasional small megakaryocyte clusters (inset).

Serum erythropoietin levels at trial entry in *MPL*-mutant patients were significantly higher than *JAK2 V617F*-positive but not *JAK2 V617F*-negative patients. There were no significant differences in iron status between *MPL*-mutant patients and both comparator groups, as assessed by serum ferritin and erythrocyte mean cell volume (Table 3.4).

As part of the PT-1 studies, comprehensive clinical and outcome data were collected prospectively. Clinical events were independently adjudicated according to pre-defined criteria by a panel of experts blinded to treatment allocation[4]. The median follow-up was 36.5 months, although the relatively small numbers of patients in the *MPL*-mutant cohort meant that only a few end-point events were recorded in this group. There were no significant differences between the three comparator groups in rates of arterial thrombosis either before or after trial entry (Table 3.4). When compared to *JAK2 V617F*-negative patients, *MPL*-mutant patients had a higher rate of venous thrombosis after trial entry (odds ratio 34.3, 95% CI: 1.6-725, p=0.02). However, this association appeared weaker in multivariate analysis including patient age, sex and past history of venous thrombosis (Hazard ratio (HR), 4.7; 95% CI, 0.7-29.5; p=0.09), and there was no increased rate of venous thrombosis in the year before diagnosis. *MPL*-mutant patients had an increased rate of death compared to the *JAK2 V617F*-negative group on univariate analysis, but this was due to the significantly older age of the *MPL*-mutant patients (HR, 1.2; 95% CI, 0.5-3.5 after correction for patient age and history of arterial thrombosis; p=0.7).

Table 3.5. Thrombotic, haemorrhagic and transformation events after trial entry and in the year before diagnosis.

	MPL mutant n=32	*MPL* negative *V617F* pos n=411	*MPL* negative *V617F* neg n=333	OR vs *V617F* pos (95% CI)	OR vs *V617F* neg (95% CI)	p value[1] (vs *V617F* pos)	p value[1] (vs *V617F* neg)
Arterial thrombosis							
In year before diagnosis[2]	**4**	**38**	**20**	**1.4**	**2.2**	**0.5**	**0.2**
Myocardial infarction	0	6	5	**(0.5-4.2)**	**(0.7-7.0)**		
Stroke	1	10	4				
Transient ischaemic attack	3	23	13				
After trial entry[2]	**2**	**25**	**19**	**1.0**	**1.1**	**0.9**	**0.9**
Myocardial infarction	1	7	6	**(0.2-4.3)**	**(0.2-5.2)**		
Unstable angina	0	5	1				
Stroke	1	7	7				
Transient ischaemic attack	1	5	6				
Other[3]	0	2	1				
Venous thromboembolism							
In year before diagnosis[2]	**1**	**10**	**2**	**1.3**	**5.3**	**0.6**	**0.2**
Deep vein thrombosis	0	3	1	**(0.2-10.4)**	**(0.5-60.6)**		
Pulmonary embolism	0	3	1				
Splanchnic vein thrombosis	0	2	0				
Retinal vein thrombosis	1	2	1				
Cerebral sinus thrombosis	0	1	0				
After trial entry[2]	**2**	**11**	**3**	**2.6**	**34.3**	**0.3**	**0.02**
Deep vein thrombosis	2	5	3	**(0.4-19.3)**	**(1.6-725)**		**(0.09)[4]**
Pulmonary embolism	0	5	0				
Splanchnic vein thrombosis	0	2	0				
Major haemorrhage							
After trial entry[2]	**2**	**18**	**12**	**1.3**	**1.9**	**0.7**	**0.5**
Gastrointestinal	2	6	6	**(0.3-6.8)**	**(0.3-12.1)**		
Intracranial	0	3	4				
Epistaxis	0	5	0				
Other[5]	0	4	2				
Death	**5**	**34**	**18**	**1.8**	**4.5**	**0.3**	**0.04**
				(0.6-5.5)	**(1.1-18.4)**		**(0.7)[6]**
Myelofibrosis	**2**	**7**	**11**	**6.2**	**2.5**	**0.1**	**0.4**
				(0.6-66.6)	**(0.3-17.7)**		
Acute leukaemia/ MDS	**0**	**5**	**2**	**0.3**	**0.3**	**0.5**	**0.7**
				(0.02-7.2)	**(0.01-47.0)**		
Polycythaemia vera	**0**	**6**	**0**	**0.3**	**NA**	**0.5**	**NA**
				(0.02-6.5)			

[1] Assessed by Pearson's chi-squared test with Yates' continuity correction for events preceding diagnosis and log-rank test for events after trial entry
[2] Indicates total patients rather than total events; some patients had more than one event
[3] Lower limb arterial embolus (2); upper limb arterial thrombosis
[4] Multivariate analysis including patient age, sex and previous history of venous thrombosis
[5] Pericardial (2), urinary (2), post-operative and obstetric
[6] Multivariate analysis including patient age and previous history of arterial thrombosis

3.6 Biological aspects of *MPL* mutations

Cytokine-independent colony formation is one of the hallmark features of the MPD, with the presence of both endogenous megakaryocyte and erythroid colonies reported in patients carrying the *JAK2 V617F* mutation[29,198]. To investigate the association of *MPL* mutations and cytokine-independent colony formation, colony assays were performed using peripheral blood from two hydroxycarbamide-treated ET patients, and an IMF patient receiving transfusion support only, all of whom carried the *MPL W515L* mutation. These investigations demonstrated the presence of thrombopoietin-independent megakaryocyte colony growth in all three patients studied. In contrast to the *JAK2 V617F* mutation, however, endogenous erythroid colonies were not observed in 4 ET patients or 1 IMF patient with the *MPL W515L* mutation (Figure 3.6A).

To investigate possible differences between the *MPL W515L* and *MPL W515K* alleles, mutant allele burden was quantitated by pyrosequencing using whole blood DNA obtained at PT-1 trial entry. ET patients with *MPL W515K* had a significantly higher mutant allele proportion than those with *MPL W515L* (mean mutant allele burden 0.66 ± 0.26 versus 0.17 ± 0.11 respectively, p=<0.0001, Figure 3.6B). There were no significant differences in age at diagnosis (p=0.6), disease duration (p=0.5), use of cytoreductive therapy (p=0.2) or proportion of peripheral blood neutrophils at time of sampling (*W515L,* 0.67 ± 0.11; *W515K,* 0.75 ± 0.05; p=0.2) to account for this difference.

In order to examine the phenotypic correlates of the higher *MPL W515K* mutant allele burden, blood counts at diagnosis were compared between *MPL W515L* and *MPL W515K* ET patients in the PT-1 cohort. Despite the higher mutant allele burden in the *MPL W515K* group, there were no significant differences in diagnostic haemoglobin level, neutrophil count or platelet count (Table 3.6).

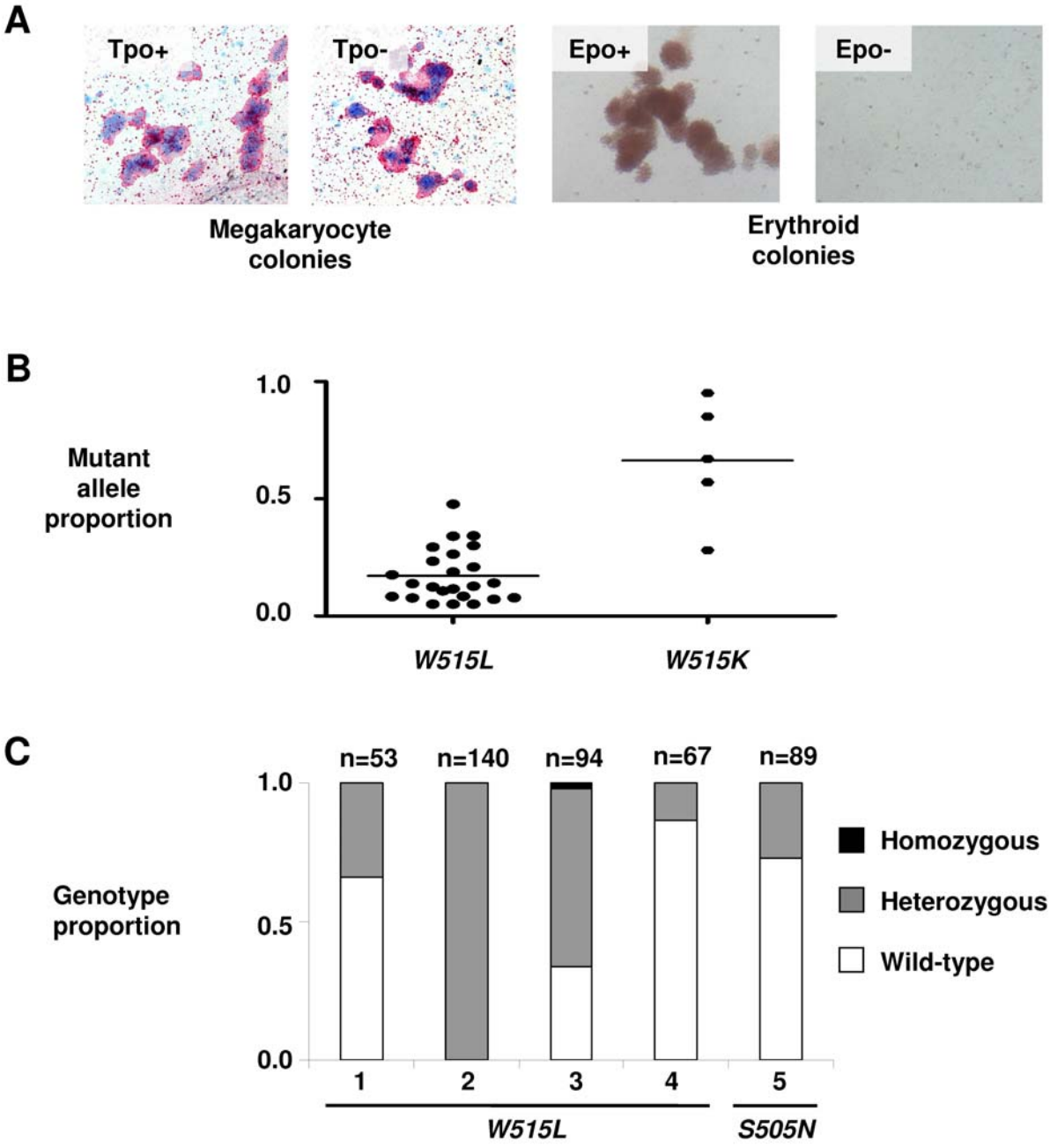

Figure 3.6. Progenitor assays, mutant allele burden and genotyping of progenitor colonies from essential thrombocythaemia patients with *MPL* exon 10 mutations. (A) Representative progenitor assays from an *MPL W515L* mutant ET patient showing cytokine independent megakaryocyte but not erythroid colony growth. (B) Assessment of peripheral blood mutant allele burden showing a higher mean mutant allele proportion in ET patients with the *MPL W515K* mutation. (C) Genotyping of individual erythroid colonies from 5 ET patients, 4 with *MPL W515L* and 1 with *MPL S505N* mutations; a single homozygous colony was identified in patient 3. Tpo: thrombopoietin; Epo: erythropoietin.

Table 3.6. Comparison of blood counts at diagnosis from ET patients carrying *MPL W515L* and *W515K* mutations.

	MPL W515L n=24	***MPL W515K*** n=5	**p-value**
Haemoglobin (g/L) - mean ± SD	13.2 ± 1.1	13.5 ± 1.6	0.7
White cell count (x10^9/L) - mean ± SD	9.7 ± 2.5	11.3 ± 2.8	0.2
Neutrophil count (x10^9/L) - mean ± SD	6.5 ± 2.0	8.7 ± 2.7	0.07
Platelet count (x10^9/L) - mean ± SD	1010 ± 242	1124 ± 397	0.4

3 of 5 ET patients with *MPL W515K* mutations had a mutant allele burden of greater than 50%, indicating the presence of a subclone in which either the wild-type allele had been deleted, or the mutant allele amplified, for example by mitotic recombination. Although none of 24 patients in the PT-1 cohort carrying the *MPL W515L* mutation had a mutant allele burden of greater than 50%, this does not exclude the presence of a subclone homozygous for the mutation[70]. Genotyping of single erythroid colonies was therefore used to look for the presence of such a subclone in 4 ET patients with *MPL W515L* mutations (Figure 3.6C). In one of the four patients, a single homozygous colony was found out of 94 colonies analysed; the other three patients had only heterozygous colonies, with or without wild-type colonies. Taken together with the mutant allele quantitation, these data suggest that ET patients carrying the *MPL W515K* allele commonly harbour clones in which the ratio of wild-type to mutant allele is reduced, but that such clones are less common in ET patients with the *MPL W515L* allele.

In contrast to patients with ET, 4 of 6 IMF patients with an *MPL W515L* mutation had a mutant allele burden of greater than 50%, indicating that subclones with reduced or lost wild-type allele can occur with this mutation, and may be associated with progression to myelofibrosis. There are four mechanisms by which such subclones may arise: (a) mitotic recombination, (b) loss of the wild-type allele, (c) duplication of the chromosome bearing the mutant allele, or (d) second mutation (Figure 3.7A). These mechanisms were investigated in a *W515L*-positive IMF patient with a mutant allele burden of 90%. Purified constitutional material was not available from this patient, and although peripheral blood mononuclear cells contained a significant proportion of *MPL*-mutant DNA, comparison with granulocyte DNA revealed loss of heterozygosity for four informative SNPs close to the *MPL* gene, thus excluding models (c) and (d) (Figure 3.7B). In order to distinguish between models (a) and (b), *MPL* copy number was assessed by a real-time PCR assay using patient granulocyte DNA. Two independent samples from this patient showed no change in *MPL* copy number compared to normal controls (Figure 3.7C). Taken together, these data indicate that in this patient mitotic recombination had led to duplication of the mutant allele (model (a), Figure 3.7A).

Three ET patients in the PT-1 cohort harboured an *MPL S505N* mutation, with mutant allele burdens of 62%, 47% and 17%. Further samples were available from the latter patient which demonstrated the absence of the mutation in buccal cells, and the presence of both wild-type and heterozygous erythroid colonies (Figure 3.6C), indicative of an acquired mutation.

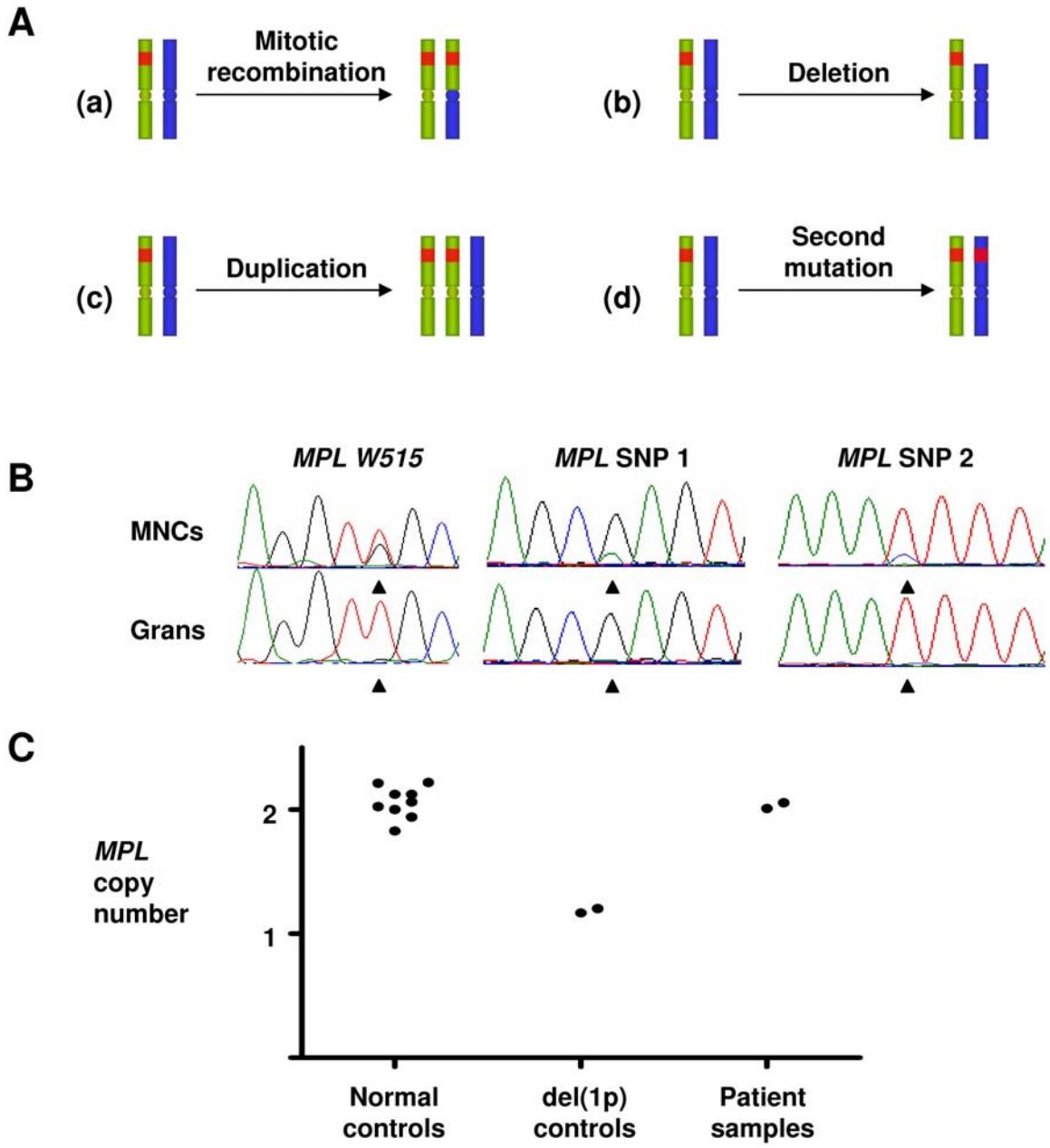

Figure 3.7. Duplication of the *MPL W515L* allele by mitotic recombination. (A) Models to explain the development of a clone with a mutant allele proportion of greater than 0.5. (B) Direct sequencing of granulocyte and mononuclear cell (MNC) derived DNA from a patient with an *MPL W515L* mutation showing loss of heterozygosity for two single nucleotide polymorphisms (SNPs) close to the *MPL* gene. (C) Real-time PCR analysis demonstrating no alteration in *MPL* copy number, confirming mitotic recombination (model a) as the mechanism for *MPL W515L* homozygosity in this patient.

3.7 Progression to acute myeloid leukaemia in an *MPL W515L*-positive ET patient

A 64 year old female patient with *MPL W515L*-positive ET developed rapidly progressive pancytopaenia without clinical splenomegaly. She had been diagnosed with ET 5 years previously, and was treated with hydroxycarbamide. Bone marrow cytogenetic analysis was normal at diagnosis, and there was no history of thromboembolic or haemorrhagic complications. At the time of disease progression, analysis of peripheral blood showed haemoglobin 8.0 g/dl, white cell count 32 $x10^9$/l, blast cell count 8.64 $x10^9$/L and platelet count 15 $x10^9$/L. Bone marrow could not be aspirated. Cytogenetic analysis performed on peripheral blood showed complex karyotypic abnormalities[†]. Bone marrow trephine biopsy showed markedly increased cellularity with abundant dysplastic and immature megakaryocytes, grade III reticulin fibrosis and clusters of $CD34^+$ progenitor cells (Figure 3.8A), in keeping with a diagnosis of acute panmyelosis with myelofibrosis, a subtype of acute myeloid leukaemia[74].

The patient had been found to harbour an *MPL W515L* mutation in a sample taken 1 year prior to transformation. Erythroid colony analysis performed 1 year prior to transformation had shown wild-type and heterozygous colonies only (Patient 1, Figure 3.6C). At the time of transformation to acute leukaemia, blast colonies were cultured from peripheral blood mononuclear cells. Cytospin preparations from individual blast colonies confirmed the absence of differentiation after 14 days in culture. Moreover, blast colonies cultured over 4 rounds of serial replating retained an undifferentiated appearance (Figure 3.8B&C), confirming transformation to an acute leukaemia. Sequencing of DNA and cDNA derived from single blast colonies indicated the leukaemic transformation was heterozygous for the *MPL W515L* mutation (Figure 3.8C).

[†] 44-49,XX,-5,+6,del(6)(q?21q?23),+8,t(11;21)(q13;q22),+der(11)t(11;21),del(13)(q?14q?22), +del(13)(q?14q?22),der(15;17)t(15;17)(q?15;p?12),-17,+1-4mar[cp11]/46,XX[4]

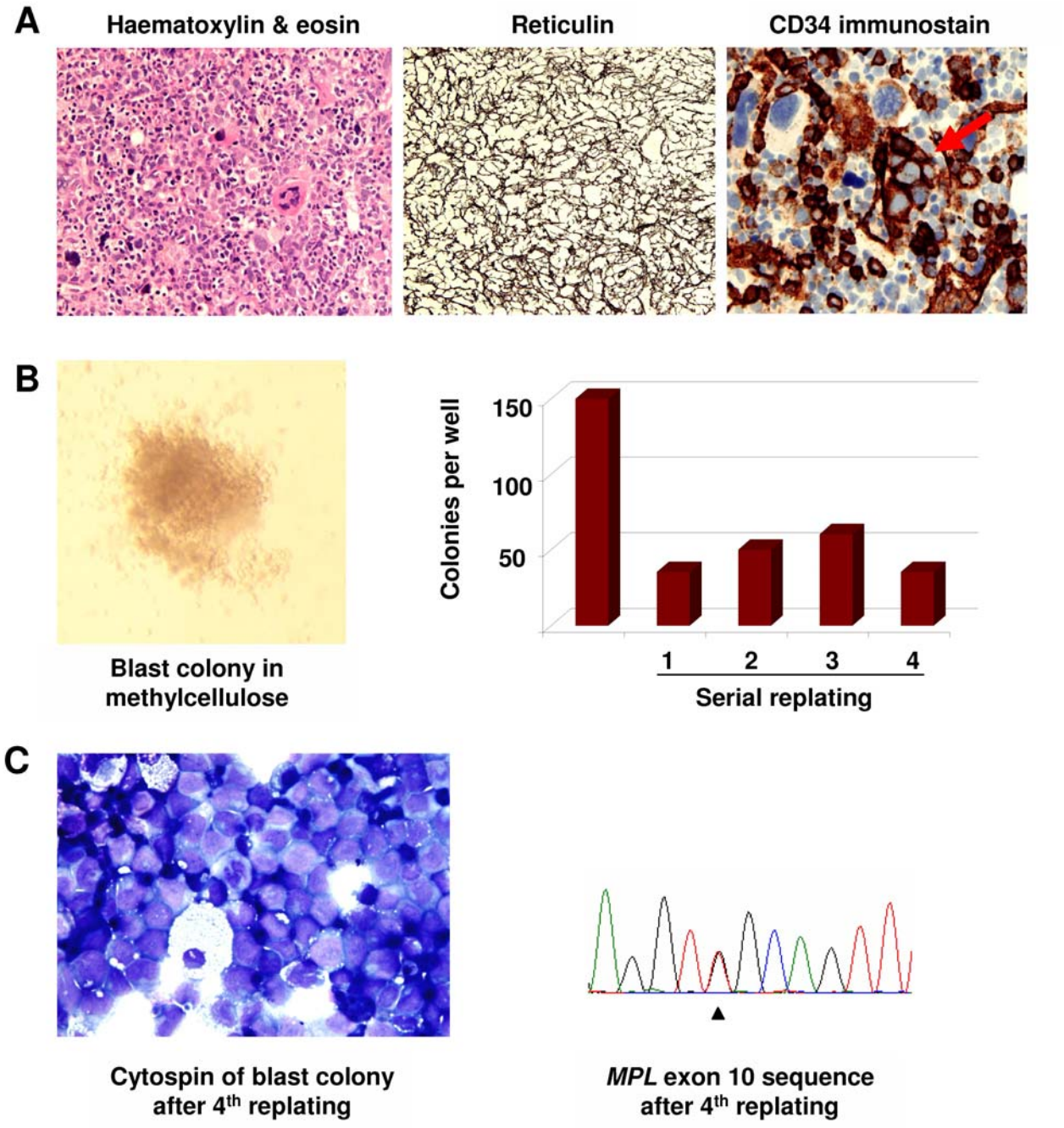

Figure 3.8. Transformation to acute myeloid leukaemia following *MPL W515L* positive essential thrombocythaemia. (A) Bone marrow trephine biopsy at the time of transformation showing panmyeloisis, reticulin fibrosis and clusters of CD34+ cells by immunostaining (arrow). (B) Representative blast colony grown in methylcellulose; serial replating studies demonstrating colony growth to the fourth plate. (C) Cytospin preparation of blast colony following 4 rounds of replating showing lack of cellular differentiation; sequencing of *MPL* exon 10 from blast colony derived cDNA showing heterozygosity for the *MPL W515L* mutation.

Analysis of blast colony DNA for mutations known to be associated with acute myeloid leukaemia (Table 5.4) revealed a homozygous mutation within the p53 DNA binding domain (*TP53 R248Q*). These data demonstrate that an *MPL W515L*-positive MPD may progress to acute leukaemia which harbours the *MPL W515L* mutation.

3.8 Discussion

These studies identified four mutant *MPL* alleles in patients with an MPD. No mutations were detected outside exon 10 despite analysis of platelet cDNA, which excludes the possibility that such mutations might be restricted to the megakaryocyte lineage. These results are consistent with and extend two previous studies using genomic DNA from 14 adults with an MPD[199] and 9 children with ET[200], both of which failed to identify mutations in *MPL*, and demonstrate that mutations outside *MPL* exon 10 are not a common cause of *JAK2 V617F*-negative ET. Changes elsewhere in *MPL* have recently been reported, although it is not yet clear if they are acquired mutations or inherited polymorphisms[201]. Previous studies have reported the *W515L* and *W515K* alleles in IMF[175,194] but only the *W515L* allele in ET[175]. This work demonstrates the presence of the *W515K* allele in patients with ET and also two different mutations capable of generating a *W515K* substitution.

In two patients with ET, an *MPL S505N* allele, previously reported as an inherited mutation[172,193], was detected in granulocytes but was absent from buccal cells. Moreover in one of the patients the mutation was only present in a minority of erythroid colonies, and the other patient had a normal platelet count over several years prior to presentation with ET, making germline mosaicism unlikely. Taken together, these findings strongly suggest that the *MPL S505N* allele can occur as both an inherited and acquired mutation. Mutations in the *KIT* gene have been reported in patients with both sporadic and familial mast cell proliferations. However the alleles involved are different, with inherited mutations not seen in acquired disease and *vice versa*[202-211]. Other inherited alleles associated with familial MPD, including mutations in the thrombopoietin and erythropoietin receptor genes, have not been reported in patients with an

acquired MPD[212,213]. To my knowledge, this is the first example of a single allele being associated with both acquired and inherited forms of an MPD.

MPL mutations were detected in 7% of IMF patients, comprising 15% of *JAK2 V617F*-negative patients. These data are similar to previous reports describing a prevalence of 5.5% or 8.5% in all patients[175,194] and 9% in *JAK2 V617F*-negative patients[174]. In ET the prevalence of *MPL* mutations was 3.4% in the retrospective cohort and 4.1% in the prospective PT-1 cohort, accounting for 8.5% of *JAK2 V617F*-negative patients. These results are somewhat higher than the 1% overall prevalence previously reported in ET[175], and likely reflect the use of more sensitive assays for mutation detection in this study.

ET patients with the *W515K* allele had significantly higher mutant allele burdens than those with the *W515L* allele. This result is reminiscent of the observation that most PV patients with a *JAK2 V617F* mutation harbour a homozygous clone[70], whereas such clones are rarer in PV patients with a *JAK2* exon 12 mutation[105]. *JAK2* exon 12 mutations appear to signal more strongly than *JAK2 V617F* mutations[105] but it is not clear whether the difference between the *W515L* and *W515K* mutations reflects quantitative or qualitative alterations in signalling. Interestingly a mutant allele burden of >50% was found in 0/24 ET patients with the *W515L* allele, but in 4/6 IMF patients. An allele burden of >50% indicates the existence of one or more additional events giving rise to homozygous or hemizygous mutant clones. In one patient where suitable material was available, duplication of the *MPL W515L* allele had arisen by mitotic recombination, giving rise to a *W515L*-homozygous clone. These results suggest that duplication of the *MPL W515L* allele may be associated with progression to IMF. A patient with *MPL W515L*-positive ET developed acute myeloid leukaemia which also harboured the *MPL* mutation. Thus the *MPL W515L* allele may be associated with ET, IMF and progression to acute leukaemia.

The development of sensitive assays for each *MPL* mutation allowed analysis of samples from the PT1 cohort together with an assessment of the clinical and laboratory features. This large cohort includes ET patients in all risk categories, with centralized review of endpoints, comprehensive follow-up and the

participation of a large number of secondary and tertiary centres. As such, these results are likely to be of general relevance to ET patients with *MPL* mutations. Compared to *JAK2 V167F*-positive patients, those carrying an *MPL* mutation exhibited lower haemoglobin levels and higher platelet counts at diagnosis, higher serum erythropoietin levels and reduced bone marrow erythroid and overall cellularity. Moreover it was possible to grow thrombopoietin-independent megakaryocyte colonies but not erythropoietin-independent erythroid colonies from ET patients with an *MPL* mutation, an observation consistent with previous data from patients with IMF[196,214]. Taken together, these features suggest that patients with *MPL* mutations have a lower drive towards erythroid differentiation, accompanied by a more isolated megakaryocyte proliferation. There were no consistent differences in other histological features including megakaryocyte morphology and reticulin grade. Compared to *JAK2 V617F*-negative patients, those carrying an *MPL* mutation were older at diagnosis with reduced bone marrow cellularity that remained significant when patient age was taken into account. However, it was not possible to identify other clinical, histological or additional laboratory features which allowed the *MPL*-mutant subgroup to be distinguished from the remaining *JAK2 V617F*-negative patients. Furthermore, *MPL*-mutant patients did not exhibit altered rates of thrombosis, major haemorrhage, transformation or death when compared to *V617F*-positive or *V617F*-negative patients.

Known mutations in *MPL* and *JAK2* account for 57% of the PT-1 patients presented here. Comparisons between this group and the remaining 43% who lack an identified mutation did not reveal any significant differences in the prevalence of splenomegaly, abnormal cytogenetics, myelofibrotic transformation or acute myeloid leukaemia. There were also no differences between the mutation-negative and mutation-positive groups in histological features other than cellularity. Taken together these findings suggest that mutation-negative patients do have a genuine myeloproliferative disorder, the molecular basis for which remains obscure.

Chapter 4

Clonal diversity in the myeloproliferative disorders

4.1 Introduction

Neoplasia is thought to be initiated in a single cell, with step-wise accumulation of genetic lesions leading to a malignant phenotype[215]. However, divergent clones bearing dissimilar patterns of somatic mutation have been observed in epithelial malignancies including multifocal bladder tumours and Barrett's disease, a premalignant tumour of the lower oesophagus[216-220]. In some cases, an overlap in somatic mutation pattern was observed, indicating that divergent clones were the phylogenetically related progeny of a shared founder clone[216,217]. In other cases, no such overlap could be identified, raising the possibility that some tumours are comprised of more than one genetically unrelated clone[220].

In the human MPD, recurrent genetic alterations, other than those activating a tyrosine kinase, are uncommon in early disease, but genetic events accumulate with progression through an accelerated phase of disease to acute leukaemia[69,221-226]. As such, these disorders serve as an experimentally tractable paradigm for the study of early stage tumourigenesis. Circumstantial evidence suggests that some MPD patients harbour genetically dissimilar clones: cytogenetically abnormal but *BCR-ABL1* negative clones have been reported in patients with CML following imatinib therapy[190]; patients with *JAK2*-mutant disease can progress to a *JAK2* wild-type acute leukaemia[102,103]; and two different tyrosine kinase mutations may be found in the same patient[60,175,185,227]. With respect to the latter observation, analysis of individual progenitor colonies has been reported in only two cases, and although one case suggested the presence of biclonal disease, evolution from a shared founder clone could not be excluded[185,227].

4.2 Aims of this chapter

This chapter addresses the following questions relating to the presence of clonal diversity in the MPD:

1) In patients with two or more acquired genetic events, what is the clonal relationship of the lesions?
2) When two lesions are acquired sequentially within the same clone, is there a predictable order of mutation acquisition?
3) When a second clonal expansion is present, what is the phylogenetic relationship of the two clones?

4.3 Identification and analysis of patients with two or more acquired genetic lesions

Patients were sought with either a tyrosine kinase pathway mutation and a cytogenetic abnormality or two tyrosine kinase pathway mutations. Cytogenetic data were screened from patients attending clinics in Cambridge, London, Birmingham and Belfast and from those enrolled in the PT-1 studies. This information was then compared to *JAK2* and *MPL* mutation status. Following the identification of an ET patient harbouring mutations in both *JAK2* and *MPL*, *JAK2 V617F*-positive ET and IMF patients in the Cambridge cohort were screened for mutations in *MPL* exon 10 by allele specific PCR as described in Chapter 3 (part of this screening was performed by Dr Elaine Boyd). Patients harbouring mutations in *MPL* or *JAK2* exon 12 were also screened for the *JAK2 V617F* mutation by Dr Anthony Bench and Miss Andrea Goday-Fernandez using a highly sensitive real-time PCR assay[178], an approach that identified 3 patients with two tyrosine kinase pathways mutations.

Table 4.1. Clinical features of patients with two or more acquired genetic lesions.

Pt	Diagnosis	Sex	At diagnosis				Prior Therapy	Disease duration	Lesion 1	Lesion 2
			Age yrs	Hb g/L	WCC $x10^9$/L	Plts $x10^9$/L				
1	PV	F	61	158†	20.2	671	HC	3 yrs	*JAK2 V617F*	Trisomy 9
2	PV	F	65	198†	7.2	542	HC	8 yrs	*JAK2 V617F*	Trisomy 9 Trisomy 8
3	PV	M	71	182	10.4	977	HC	1 yr	*JAK2 V617F*	Trisomy 9
4	PV	M	34	182	64.3	200	HC	12 yrs	*JAK2 V617F*	Trisomy 9
5	PV	M	66	177†	10.6	553	HC	4 yrs	*JAK2 V617F*	Trisomy 8
6	CML	M	52	134	26.8	167	IM	9 yrs	*BCR-ABL1*	del(20q)
7	IMF	M	59	94	3.3	68	None	9 yrs	*JAK2 V617F*	del(20q)
8	ET	M	69	135	7.9	1016	HC	12 yrs	*JAK2 V617F*	del(20q)
9	ET	F	92	134	10.8	1240	HC	2 yrs	*JAK2 V617F*	*MPL W515L*
10	ET	F	28	140	12.8	1221	HC, AN	20 yrs	*JAK2 V617F*	*MPL W515L*
11	ET	M	72	128	6.7	1258	HC	1.5 yrs	*JAK2 V617F*	*MPL W515L*
12	ET	M	74	140	6.6	979	None	0 yrs	*JAK2 V617F*	*MPL W515L*
13	SM	M	43	147	10.1	306	NL, PK, HC	3.5 yrs	*JAK2 V617F*	*KIT D816V*
14	PV	F	73	172†	5.3	314	Ven	8 yrs	*JAK2 V617F*	*JAK2 E543-D544del*

Pt: patient number; Hb: haemoglobin; WCC: white cell count; Plts: platelet count; PV: polycythaemia vera; CML: chronic myeloid leukaemia; ET: essential thrombocythaemia; SM: systemic mastocytosis; HC: hydroxycarbamide; IM: imatinib; AN: anagrelide; NL: nilotinib; PK: PKC412; Ven: venesection alone; † increased red cell mass confirmed by radio-isotope studies

Progenitor colonies were cultured from peripheral blood mononuclear cells obtained from 14 patients harbouring two or more acquired genetic lesions (Table 4.1). In patients with trisomy 8 or trisomy 9, colonies were cultured from $CD34^+$ progenitor cells isolated from the peripheral blood mononuclear fraction in order to reduce the number of contaminating cells present in each individual colony and thereby improve the accuracy of colony genotyping. Patient 12 was enrolled in the PT-1 study, and colonies were cultured from a sample of cryopreserved bone marrow obtained 10 years previously, at the time of diagnosis with ET.

4.4 Analysis of patients with a tyrosine kinase mutation and a cytogenetic abnormality

A previously reported pyrosequencing assay[64] was validated for the quantitation of the *JAK2 V617F* allele by mixing experiments using cloned mutant and wild-type PCR products (Figure 4.1A). The assay was then used to measure the ratio of wild-type to mutant *JAK2* in individual erythroid colonies from 4 *JAK2 V617F*-positive PV patients with trisomy 9 (Figure 4.1B). All four patients harboured colonies with a 1:2 ratio of wild-type to mutant allele, indicating (i) the *JAK2* mutation and trisomy 9 were in the same clone, and (ii) trisomy 9 reflected duplication of the chromosome carrying the mutant *JAK2* allele (Figure 4.1C).

In patients 1-3, *V617F*-homozygous colonies were not identified. This is contrast to PV patients without trisomy 9, the majority of whom harbour a *V617F*-homozygous clone[70]. In patient 4, however, colony genotyping identified an additional clone with loss of wild-type *JAK2*. There are at least three different models to explain this occurrence (Figure 4.2A). In model (i) mitotic recombination within a trisomy 9 cell leads to duplication of the mutant allele resulting three copies of mutant *JAK2.* In model (ii) mitotic recombination within a *V617F*-heterozygous cell leads to duplication of the mutant allele resulting in two copies of mutant *JAK2.* In model (iii) the chromosome 9 bearing the wild-type *JAK2* allele is lost. In order to distinguish these possibilities, a pyrosequencing assay was developed to assess the allelic ratio of an informative SNP on chromosome 9q. As expected, colonies with a 1:2 wild-type

to mutant *JAK2* ratio also had a 1:2 ratio of the 9q SNP, consistent with the presence of three copies of chromosome 9. A 1:2 9q SNP ratio was also observed in all *V617F*-homozygous colonies, indicating these colonies also had three copies of chromosome 9. These findings indicate that in patient 4, non-dysjunction for chromosome 9 was followed by mitotic recombination in a trisomy 9 cell, giving rise to a clone in which cells harboured three copies of the mutant *JAK2* allele (model (i), Figure 4.2A).

Quantitative pyrosequencing also allowed discrimination of colonies disomic or trisomic for chromosome 8 in two *JAK2 V617F*-positive patients. Two pyrosequencing assays were developed for SNPs on chromosome 8, and validated by mixing experiments using whole blood DNA from individuals known to be homozygous for alternative alleles of each SNP (Figure 4.3A). The two assays were used to quantitate the mean allelic ratio of each chromosome 8 SNP in individual erythroid colonies. Colonies with a mean allele proportion of 0.45 - 0.55 were classed as disomic, and those with a mean allele proportion of 0.61 - 0.71 were classed as trisomic (Figure 4.3B). In both patients the two molecular lesions were present within the same clone, and in both patients all *V617F*-negative colonies were chromosome 8 disomic. In patient 2, who harboured a *JAK2* mutation with trisomy 8 and trisomy 9, a proportion of trisomy 8 colonies were disomic for chromosome 9 and a proportion were trisomic for chromosome 9 (Table 4.2). Thus acquisition of trisomy 8 had preceded the acquisition of trisomy 9 (Figure 4.3C).

Table 4.2. Genotyping of erythroid colonies from a patient 2.

	JAK2 V617F		
	Wild-type	Heterozygous	Trisomy 9
Disomy 8	38	0	0
Trisomy 8	0	8	85

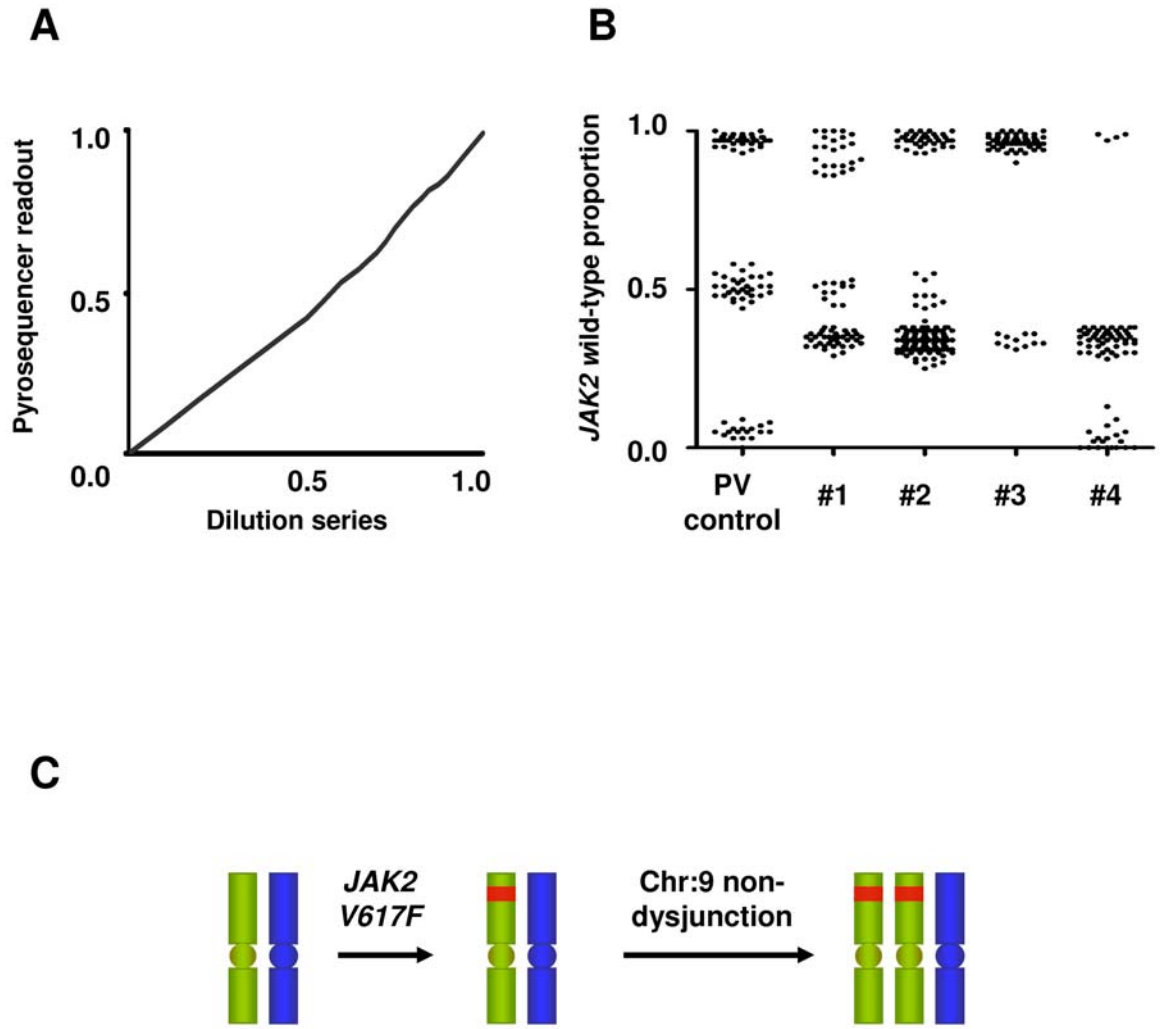

Figure 4.1. Duplication of the *JAK2 V617F* allele in patients with trisomy 9. (A) Standard curve for the quantitative *JAK2 V617F* pyrosequencing assay generated by mixing cloned *JAK2* wild-type and mutant PCR products. (B) Quantitation of wild-type to mutant *JAK2* ratio in individual erythroid colonies from 4 patients with trisomy 9; analysis of erythroid colonies from a PV patient without trisomy 9 is shown as a control. (C) Mechanism of clonal evolution in trisomy 9 patients, with chromosome 9 non-dysjunction leading to duplication of the mutant *JAK2* allele.

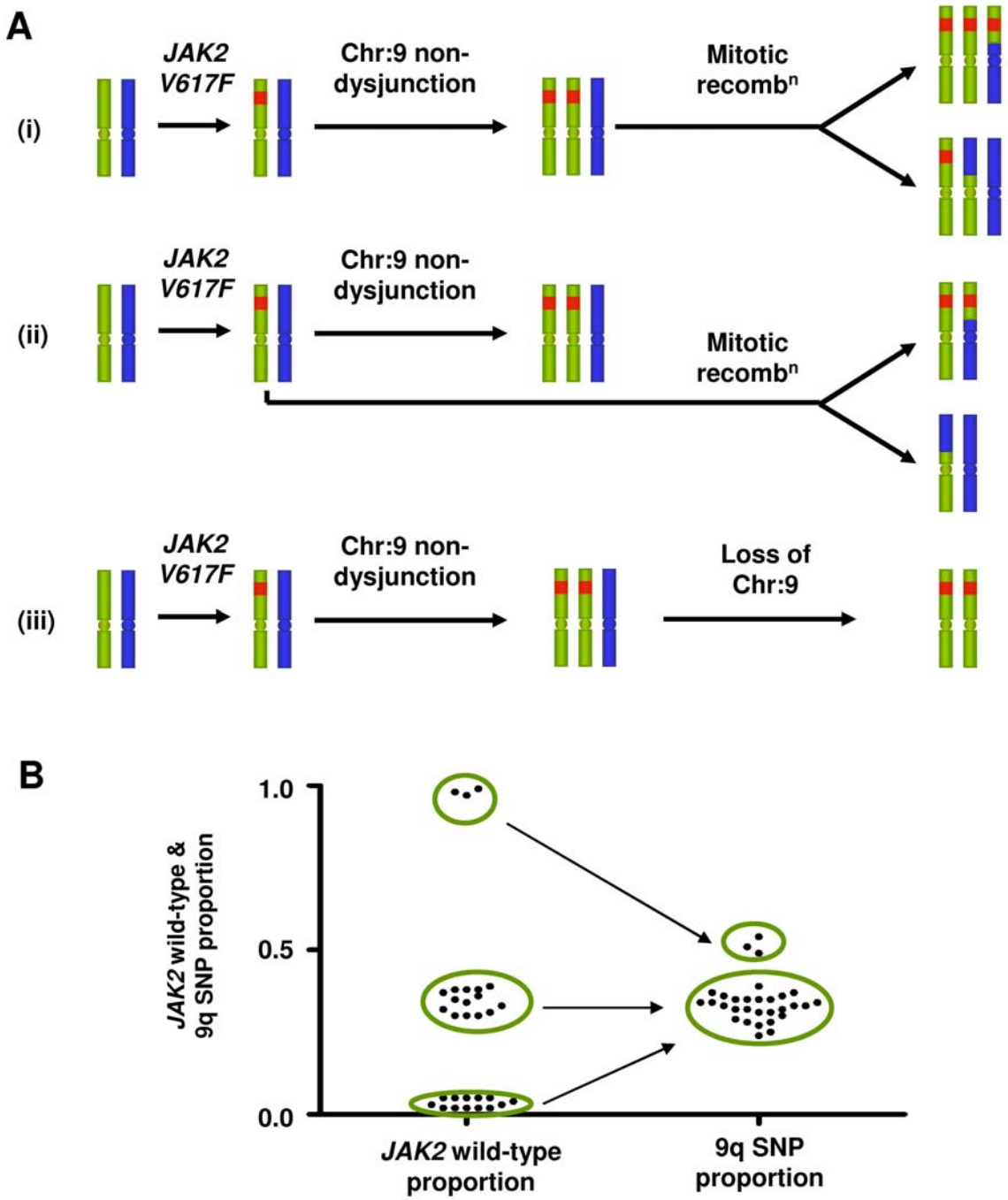

Figure 4.2. Mechanism of clonal evolution in a patient with trisomy 9 and a *JAK2 V617F* homozygous clone. (A) Models to explain the coexistence of trisomy 9 and a *JAK2 V617F* homozygous clone. (B) Quantitation of both *JAK2 V617F* and a chromosome 9q single nucleotide polymorphism (SNP) in individual erythroid colonies, demonstrating a 2:1 9q SNP ratio in all *JAK2 V617F* homozygous colonies, consistent with model (i).

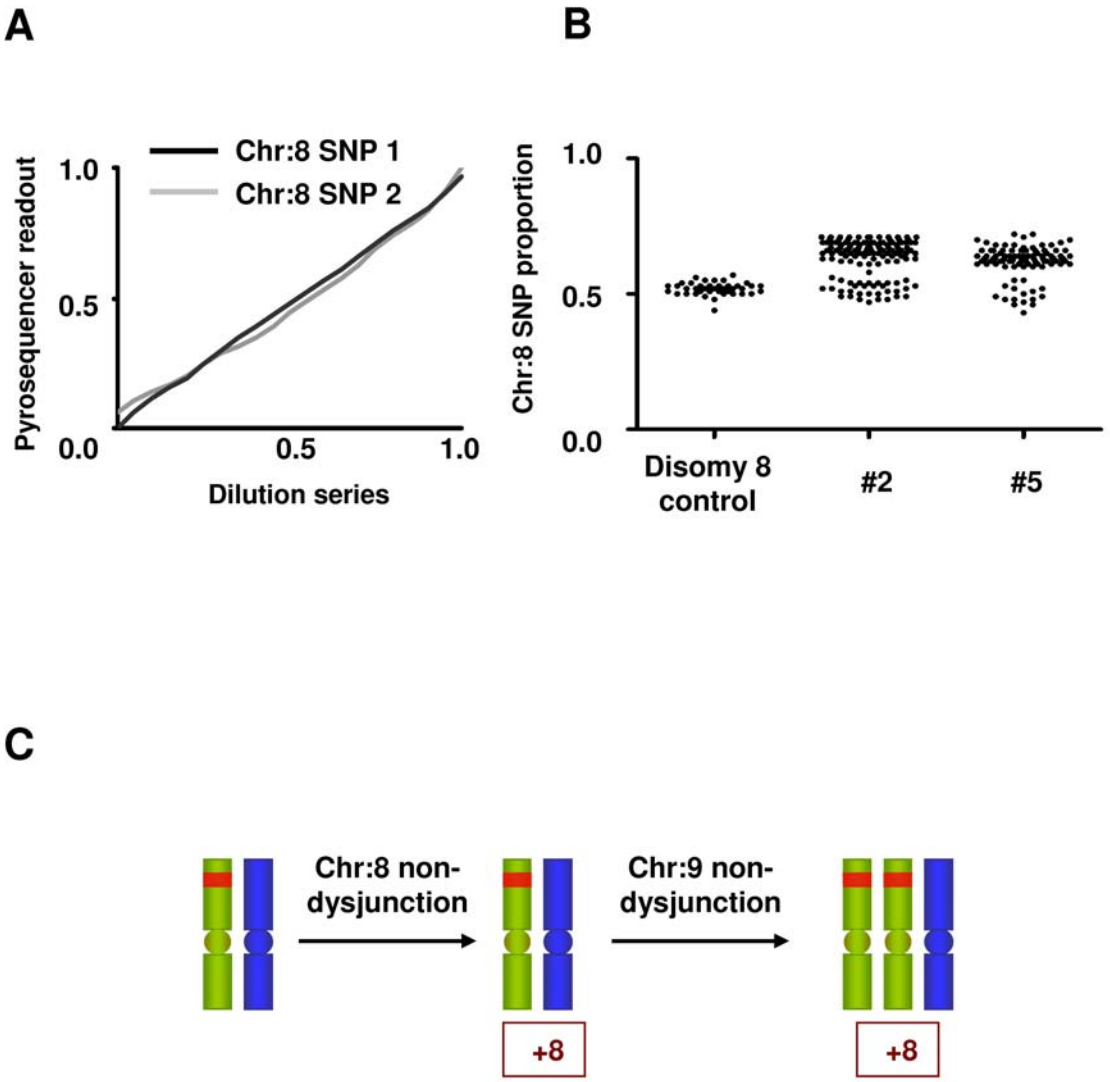

Figure 4.3. Coexistence of trisomy 8 and the *JAK2 V617F* mutation in the same clone. (A) Standard curve for the quantitative chromosome 8 single nucleotide polymorphism (SNP) pyrosequencing assays generated by mixing DNA from normal individuals homozygous for the alternative alleles. (B) Mean chromosome 8 SNP proportion in individual erythroid colonies from 2 patients with trisomy 8; analysis of erythroid colonies from a patient without trisomy 8 is shown as a control. (C) Mechanism of clonal evolution in patient 2, with sequential duplication of chromosome 8 and chromosome 9.

Three patients were identified with a deletion of chromosome 20q in association with a tyrosine kinase alteration. Granulocyte-macrophage colonies from a patient with CML (Patient 6, Table 4.1) were genotyped for the deletion of 20q by loss of heterozygosity (LOH) studies (Figure 4.4A) and for *BCR-ABL1* using an RT-PCR assay (Figure 4.4B). Although colonies were identified harbouring both the 20q deletion and *BCR-ABL1*, the 20q deletion was also present on its own in a proportion of colonies (Table 4.3).

Table 4.3. Genotyping of granulocyte-macrophage colonies from patient 6.

	BCR-ABL1	
	Negative	Positive
20q wild-type	25	0
del(20q)	7	3

There are two models to explain this result (Figure 4.4C). In model (i), the 20q deletion preceded the acquisition of *BCR-ABL1*. In model (ii), *BCR-ABL1* was the first event, followed by the 20q deletion, with subsequent mitotic recombination involving chromosome 22q giving rise to a del(20q) *BCR-ABL1* negative daughter clone. As such a clone would also harbour loss of heterozygosity for chromosome 9q, individual colonies were genotyped for an informative 9q SNP distal to the *ABL1* locus. All del(20q) *BCR-ABL1* negative colonies were heterozygous for the 9q SNP, indicating that the 20q deletion had arisen before *BCR-ABL1* (model (i) Figure 4.4C). Although deletions of 20q are occasionally seen in CML patients, often with progression to accelerated phase disease[228], to my knowledge a 20q deletion preceding acquisition of *BCR-ABL1* has not previously been reported. Subsequent to these studies the patient received therapy with the tyrosine kinase inhibitor nilotinib. After 12 months of treatment, the *BCR-ABL1* clone was no longer detectable by fluorescent *in-situ* hybridisation analysis, whereas the del(20q) clone persisted (Table 4.4), consistent with the del(20q) occurring prior to *BCR-ABL1* (analysis performed by Bridget Manasse, Department of Haematology, Addenbrooke's Hospital).

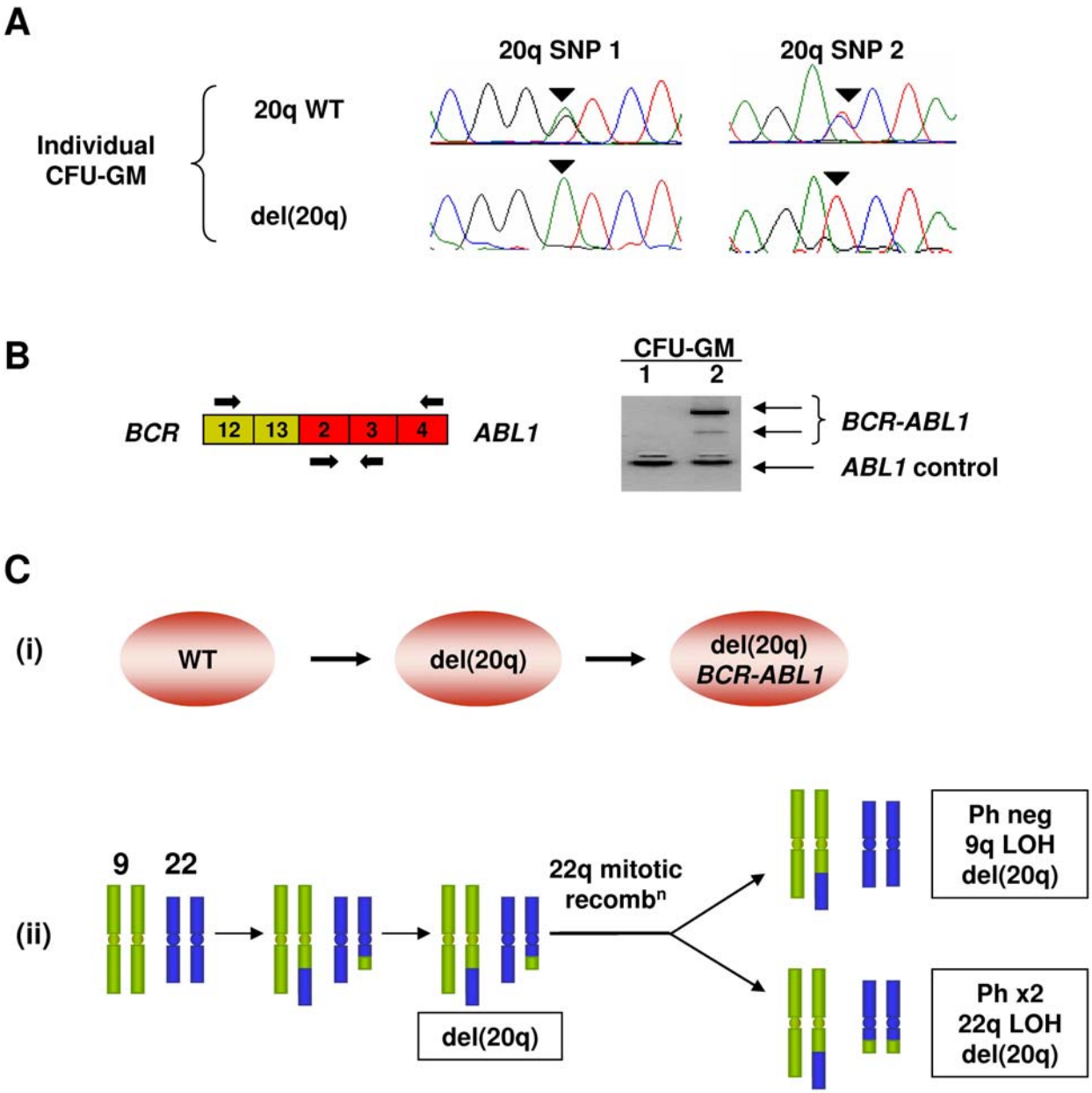

Figure 4.4. Deletion of chromosome 20q may precede acquisition of a *BCR-ABL1* fusion in chronic myeloid leukaemia. (A) Examples of individual granulocyte-macrophage colonies (CFU-GM) with and without a deletion of chromosome 20q, as assessed by loss of heterozygosity (LOH) for two single nucleotide polymorphisms (SNP) within the 20q common deleted region. (B) Primer strategy for detection of *BCR-ABL1* by reverse-transcriptase PCR; examples of individual colonies demonstrating a single *ABL1* control band in a *BCR-ABL1* negative colony (CFU-GM 1) and two additional *BCR-ABL1*-specific bands in a *BCR-ABL1* positive colony (CFU-GM 2). (C) Models to explain the coexistence of *BCR-ABL1* and a deletion of chromosome 20q; 9q LOH was not seen in any *BCR-ABL1* negative colony, consistent with the 20q deletion preceding the acquisition of *BCR-ABL1* (model (i)). WT: wild-type; Ph: Philadelphia chromosome; neg: negative.

Table 4.4. Bone marrow fluorescent *in-situ* hybridisation studies from patient 6.

	Pre-treatment	**12 months nilotinib**
BCR-ABL1	67%	0%
del(20q)	77%	20%

In an IMF patient with *JAK2 V617F* and a 20q deletion (Patient 7, Table 4.1), the two lesions were found within the same clone. A single *V617F*-positive 20q wild-type colony was identified, indicating the *JAK2* mutation preceded acquisition of the 20q deletion (Table 4.5).

Table 4.5. Genotyping of individual erythroid colonies from patient 7.

	JAK2 V617F	
	Negative	Heterozygous
20q wild-type	434	1
del(20q)	0	34

By contrast to the previous seven cases, *JAK2 V617F* and a 20q deletion were present in separate clones in a patient with ET (Table 4.6, Figure 4.5A). There are two possible explanations for these findings (Figure 4.5B). In model (i) the two lesions arose in separate clones. In model (ii) the two lesions were initially in the same clone, with mitotic recombination affecting chromosome 9p giving rising to *V617F*-homozygous and *V617F*-negative daughter clones, both of which would carry the 20q deletion and loss of heterozygosity for 9p. These mechanisms were further investigated by genotyping del(20q) colonies for 9p LOH using an informative SNP close to the *JAK2* locus. No del(20q) colonies harboured 9p LOH, thus proving that the two lesions were present in separate clones, consistent with model (i) in Figure 4.5B.

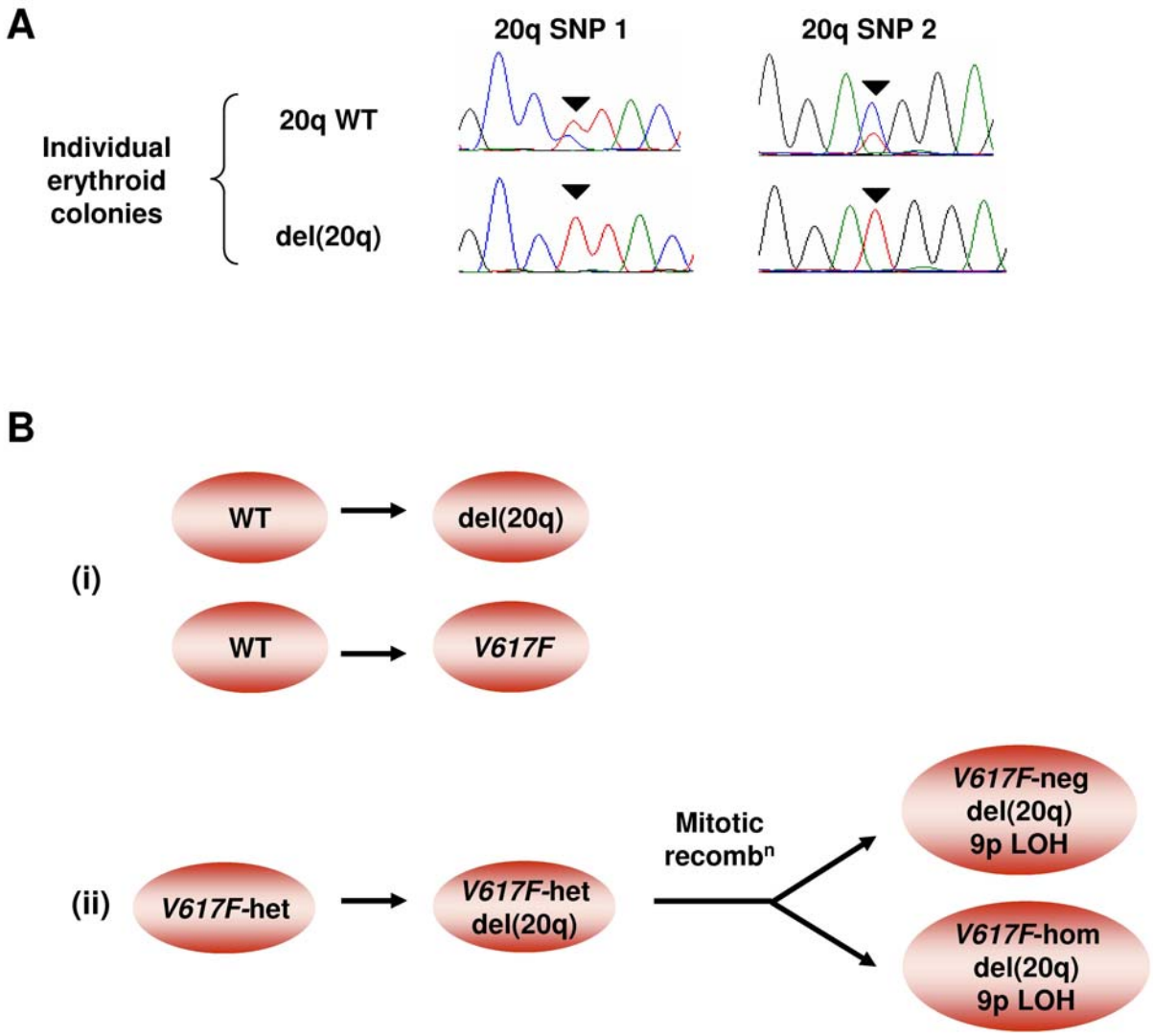

Figure 4.5. Deletion of chromosome 20q and a *JAK2 V617F* mutation in separate clones. (A) Examples of individual erythroid colonies with and without a deletion of chromosome 20q, as assessed by loss of heterozygosity for two single nucleotide polymorphisms (SNP) within the 20q common deleted region. (B) Models to explain the coexistence of deletion of chromosome 20q and a *JAK2 V617F* mutation; 9p LOH was not seen in any del(20q) colonies, consistent with the 20q deletion and *JAK2* mutation arising in separate clones (model (i)). WT: wild-type; het: heterozygous; hom: homozygous; neg: negative.

Table 4.6. Genotyping of individual erythroid colonies from patient 8.

	JAK2 V617F	
	Negative	Heterozygous
20q wild-type	2	12
del(20q)	59	0

Taken as a whole, this analysis of patients with a tyrosine kinase mutation and a cytogenetic aberration demonstrates that cytogenetic lesions generally occur within the clone carrying an oncogenic kinase mutation. However in one of eight cases the cytogenetic abnormality reflected the presence of a separate clonal expansion.

4.5 Analysis of patients with two tyrosine kinase pathway mutations

Studies were performed on a further six patients who harboured *JAK2 V617F* together with a second tyrosine kinase pathway mutation (4 *MPL W515L*, 1 *KIT D816V* and 1 *JAK2 E543-D544del;* patients 9-14, Table 4.1). Analysis of colonies from patient 13 was performed by Dr Amy Jones in the laboratory of Professor Nick Cross in Salisbury, UK. Individual progenitor colonies were genotyped for the relevant mutations by either direct sequencing or pyrosequencing. In all six cases, analysis of individual colonies demonstrated that *JAK2 V617F* and the second mutation were mutually exclusive (Figure 4.6A). Thus in all six patients, the two lesions were present in separate clones.

Differences were apparent in the proportion of colonies harbouring each mutation. The *JAK2 V617F*-positive clone was smaller than the second mutation bearing clone in all of the six cases, and the mean proportion of *JAK2 V617F* mutant colonies was significantly smaller overall (proportion of mutant colonies: *JAK2 V617F* 0.11 ± 0.08, second mutation 0.37 ± 0.23; p=0.03) (Figure 4.6B).

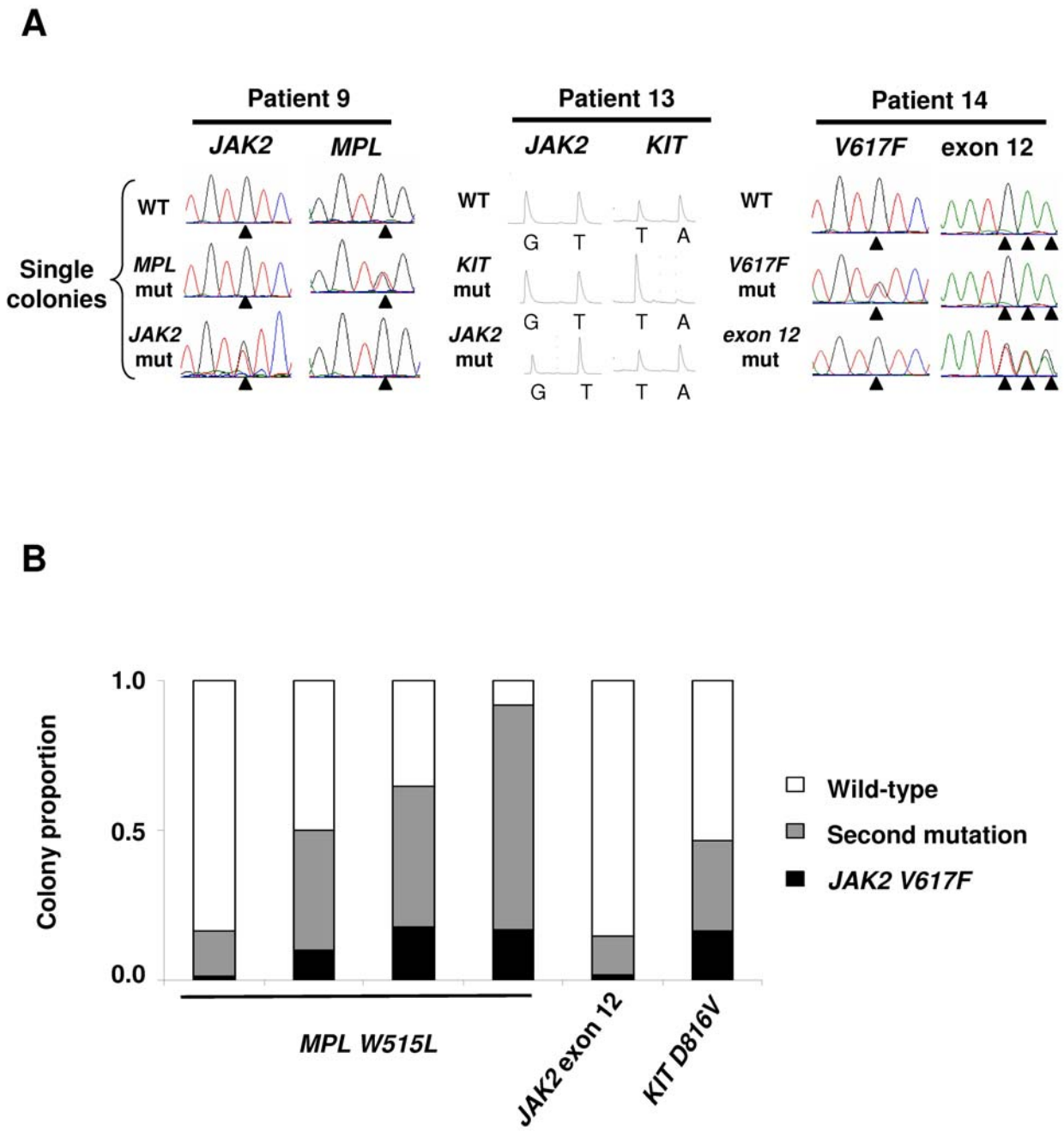

Figure 4.6. Genotyping of progenitor colonies from patients with two tyrosine kinase pathway mutations. (A) Examples of single colonies from patient 9 showing genotyping for *JAK2 V617F* and *MPL W515L* by direct sequencing, from patient 13 showing genotyping for *JAK2 V617F* and *KIT D816V* by pyrosequencing and from patient 14 showing genotyping for *JAK2 V617F* and *JAK2 E543-D544del* (exon 12) by direct sequencing. (B) Proportion of wild-type and mutant colonies in patients with two different tyrosine kinase pathway mutations.

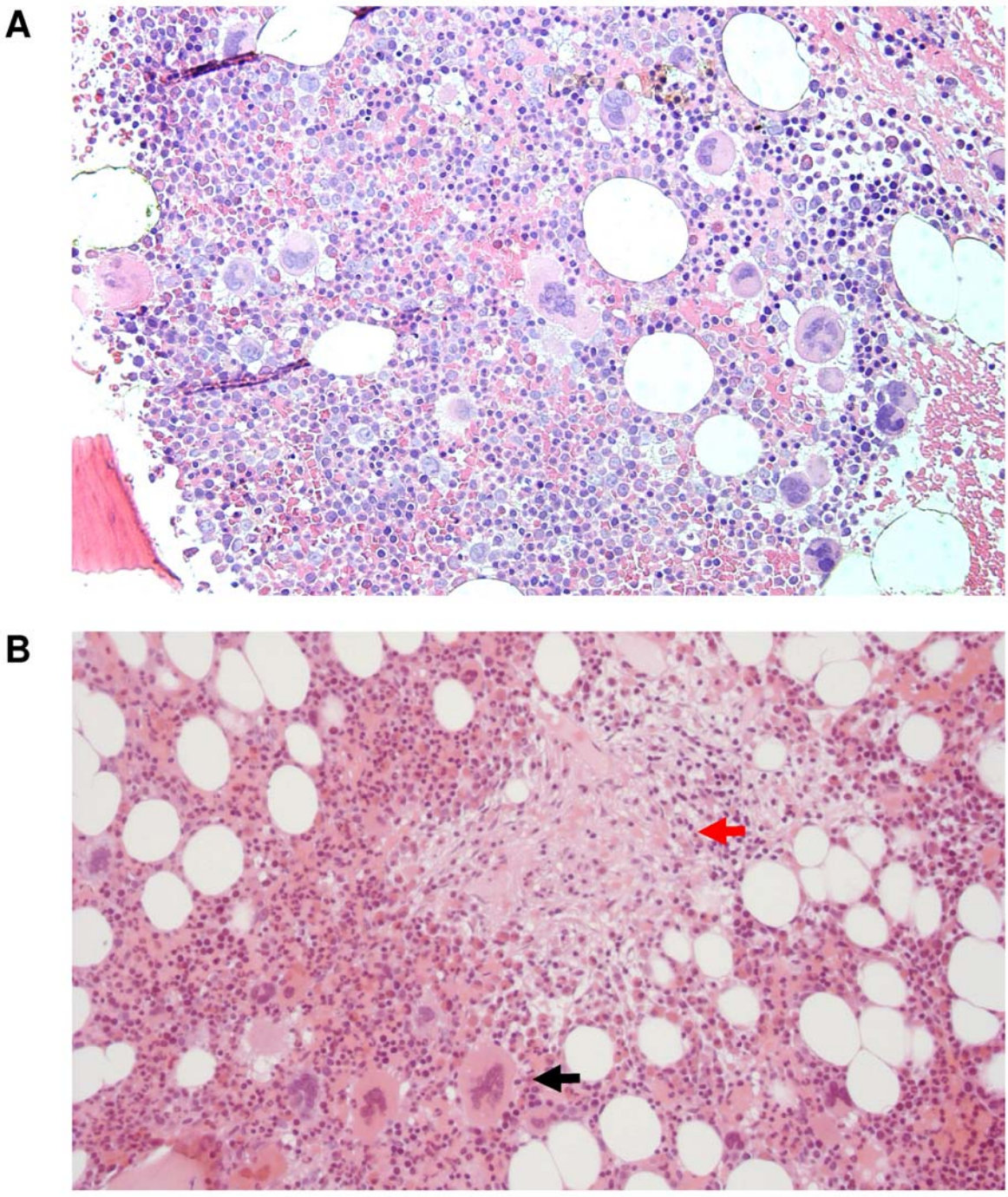

Figure 4.7. Bone marrow histology of two patients with a biclonal myeloproliferative disorder. Haematoxylin and eosin stained bone marrow trephine biopsies from (A) a hydroxycarbamide treated 42 year old female with *MPL W515L* and *JAK2 V617F* mutations showing increased cellularity and large megakaryocytes, and (B) an untreated 42 year old male with *JAK2 V617F* and *KIT D816V* mutations showing hyperlobated megakaryocytes typical of essential thrombocythaemia (black arrow) and mast cell aggregates typical of systemic mastocytosis (red arrow).

Bone marrow trephine sections were examined to assess the morphological features of patients with a biclonal MPD. In patients harbouring *JAK2 V617F* and *MPL W515L* or a *JAK2* exon 12 mutation, no unusual histological features were identified (Figure 4.7A). This is not surprising given the considerable overlap in bone marrow appearances in ET patients with *JAK2 V617F* or *MPL W515L* (Chapter 3) and the lack of specific morphological features associated with *JAK2* exon 12 mutations[105]. By contrast coexisting *JAK2*-mutant and *KIT*-mutant clones were associated with distinctive histological features, showing typical features of both ET (large, hyperlobated megakaryocytes) and systemic mastocytosis (discreet infiltrates of mast cells embedded in fibrotic tissue) (Figure 4.7B). In such cases, therefore, the presence of biclonal disease may be suspected from the unusual bone marrow morphology.

There are two possible explanations to explain the existence of biclonal disease in these patients: either the two clones are the related progeny of a shared founder clone, or the two genetics lesions arose independently in unrelated HSCs (Figure 4.8A). To distinguish between these possibilities, X-chromosome inactivation patterns were studied in the three female patients with biclonal disease (Figure 4.8B). In patient 9 both *MPL*-mutant and *JAK2*-mutant erythroid colonies expressed the same allele of the X-linked *LANCL3* gene, a result consistent with either model in Figure 4.8A. However in patient 10, the *MPL*-mutant and *JAK2*-mutant colonies expressed different alleles of the X-linked *IDS* gene. Furthermore, in patient 14 the *JAK2 V617F*-positive and exon 12-positive colonies expressed different alleles of the *MAGED2* gene. These data provide formal proof that patients 10 and 14 both harbour two clones that arose independently and not from a shared founder clone.

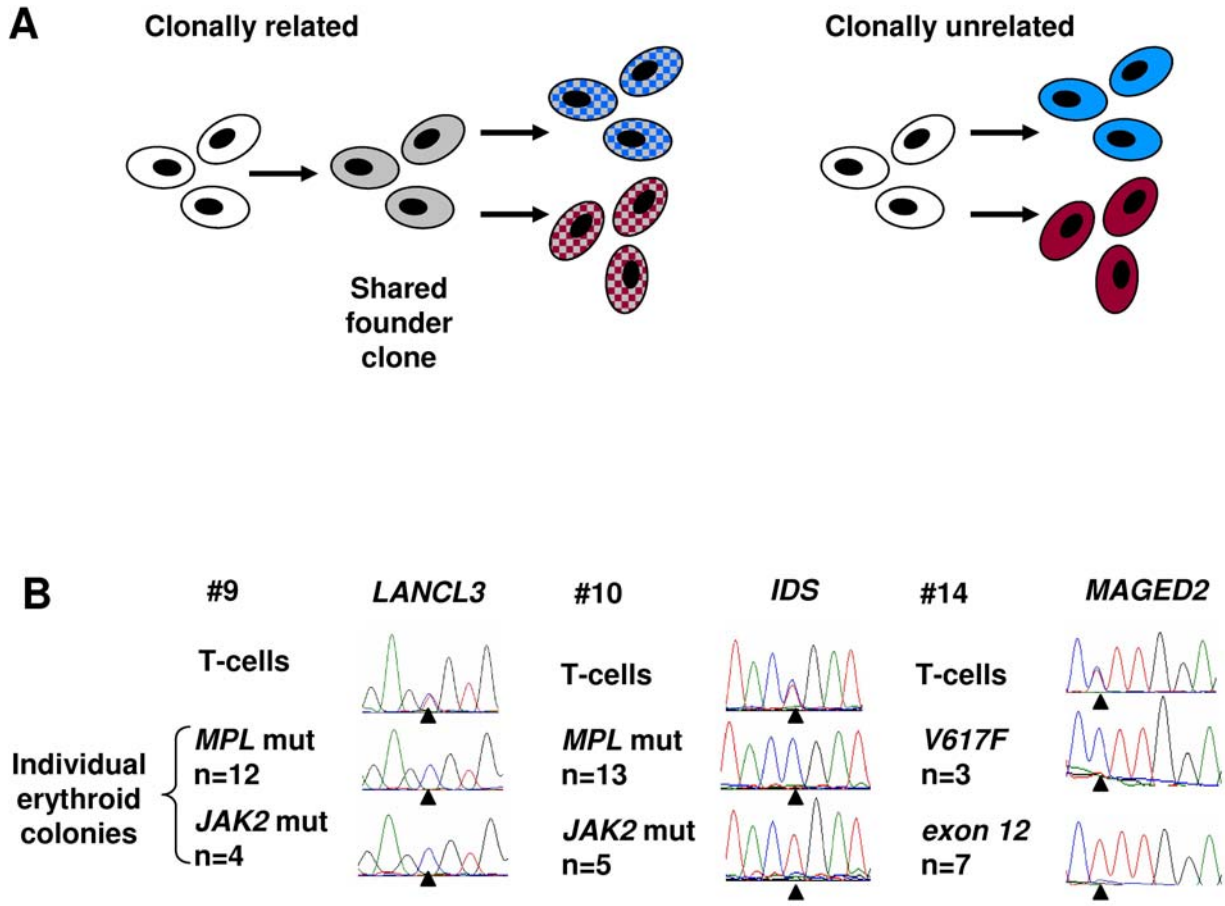

Figure 4.8. Independent origins of genetically distinct clones in patients with a myeloproliferative disorder. (A) Models to explain the coexistence of two mutation-bearing clones, showing clonally related proliferations derived from a shared founder clone and clonally unrelated proliferations derived from independent haematopoietic stem cells. (B) X-chromosome inactivation patterns in the mutation-bearing clones from patients 9, 10 and 14. In patients 10 and 14 the mutation-bearing clones express different alleles of an X-linked single nucleotide polymorphism, indicating that the mutations had arisen in unrelated haematopoietic stem cells. mut: mutant.

4.6 Discussion

In these studies, 14 patients with two or more acquired genetic lesions were identified, and the clonal relationship of the two lesions was investigated. An additional cytogenetic abnormality was present in the same clone as a tyrosine kinase mutation in 7 of 8 patients (Figure 4.9). In three patients with a deletion of chromosome 20q, no consistent pattern was observed in the timing of the cytogenetic abnormality, with the 20q deletion acquired before or after the tyrosine kinase mutation, or present in a separate clone (patients 6, 7 and 8 respectively). Although 20q deletions are amongst the most common recurrent genetic aberrations in myeloid malignancy[229], their pathological consequences are currently unknown. Moreover differences in disease phenotype or clinical outcome between patients with and without a 20q deletion have not been identified[78-80]. The acquisition of a 20q deletion prior to *BCR-ABL1* (patient 6) or in a separate clone to *JAK2 V617F* (patient 8) raises the possibility that deletions of 20q may act as clone-initiating events, and that the onset of clonal haematopoiesis may precede the acquisition of an oncogenic tyrosine kinase in some patients. The occurrence of a 20q deletion after the acquisition of a *JAK2 V617F* mutation, however, is consistent with a role for del(20q) in modulation of oncogenic tyrosine kinase activity. Alternatively deletions of 20q may serve as markers of a genetically unstable clone, with little or no direct effects on disease biology. Consistent with the heterogeneity observed in this study, a recent report identified 20q deletions occurring before or after acquisition of a *JAK2 V617F* mutation[230].

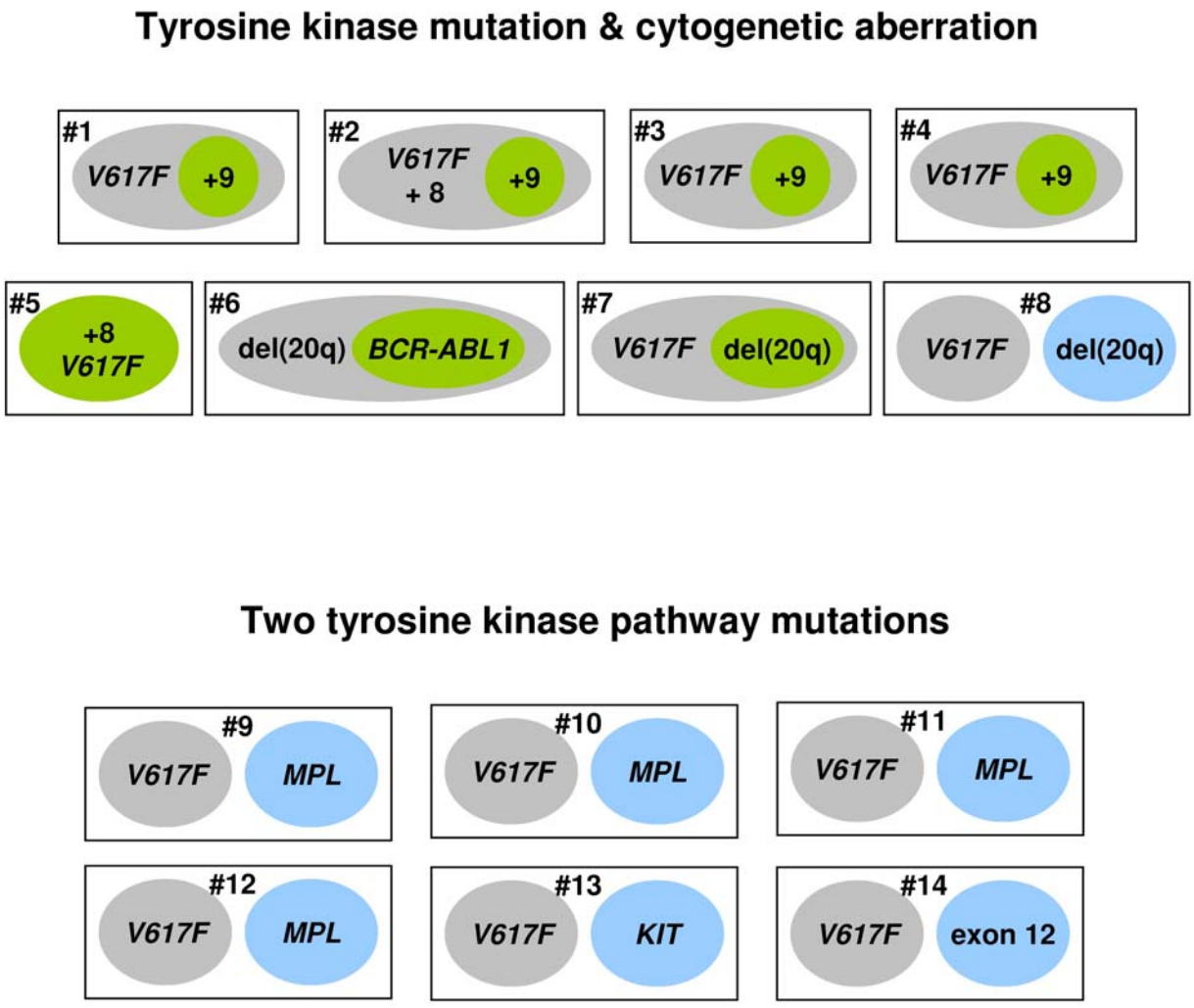

Figure 4.9. Clonal relationship of genetic lesions in patients with more than one acquired genetic event. Summary of individual colony analysis demonstrating that a cytogenetic aberration usually occurred within the same clone as a tyrosine kinase mutation, whereas the presence of two tyrosine kinase pathway mutations indicated the presence of biclonal disease.

In contrast to patients with an additional cytogenetic aberration, detection of a second tyrosine kinase pathway mutation reflected the presence of a separate clonal expansion in all six cases studied, with the two mutations being mutually exclusive in single haematopoietic colonies (Figure 4.9). This is consistent with a previous single case report, where different *JAK2* mutations were present in separate clones[185]. It is possible that additional signalling pathway mutations may not confer any further clonal advantage, as is the case in acute myeloid leukaemia[231] and juvenile myelomonocytic leukaemia[232] where different tyrosine kinase pathway mutations are generally mutually exclusive[232]. However this may be counter-intuitive given that homozygosity for the *JAK2 V617F* mutation may confer such a selective advantage[70,233], and in a single case report, *JAK2 V617F* and *BCR-ABL1* were found within the same clone[227].

The number of patients in this study harbouring a second clonal expansion appears surprisingly high. Seven such cases were identified from around 1,000 patients screened. Although the precise denominator is unclear, this seems much greater than expected, either from the prevalence of each individual MPD[8] or considering the probability of an individual acquiring two independent mutations by chance alone[234]. Given that the molecular cause for around half of patients with ET or IMF is currently unknown, the true prevalence of multiple clones may be even higher.

The presence of diverse clones could account for several observations which are currently unexplained, including (1) patients in whom the proportion of *V617F*-positive granulocytes is less than the proportion that appear clonally-derived by X-chromosome inactivation patterns or a cytogenetic marker[177,178]; (2) patients who develop a *V617F*-negative acute leukaemia following a *V617F*-positive MPD[102,103] and (3) the culture of *V617F*-negative erythropoietin-independent erythroid colonies from *V617F*-positive patients[184,185]. It is possible that such cases represent biclonal disease in which the second clonal expansion is negative for the *JAK2 V617F* mutation.

Most importantly, analysis of X-chromosome inactivation patterns were used to demonstrate that patients may harbour diverse clones that have arisen independently and not from a shared founder clone. This phenomenon has been observed in multifocal thyroid carcinoma[235], but to my knowledge this is the first demonstration of coexisting, genetically unrelated clones in haematological malignancy. These data suggest a role for either an inherited allele or an environmental mutagen in promoting the acquisition or survival of such clones. A familial association with MPD has been reported[236] and often shows incomplete penetrance[15,16], and a recent report has implicated environmental exposure in MPD pathogenesis[12].

Chapter 5

Progression to acute myeloid leukaemia following a *JAK2*-mutant myeloproliferative disorder

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5.1 Introduction

Progression to AML occurs in 2-5% of patients with ET or PV[76,77,92] and 15-30% of patients with IMF[75,83]. Risk of AML in ET and PV increases with patient age[92] and prior use of genotoxic agents[92-94] particularly when used sequentially with hydroxycarbamide[92,95,96]. The leukaemogenic potential of hydroxycarbamide when used as single agent therapy remains controversial[97]. In IMF, risk factors for transformation include karyotypic abnormalities (other than del(20q) or del(13q))[83,101], higher peripheral blood blast count[84] and prior splectomy[84]. Acute leukaemia has been reported in several MPD patients who have never received cytotoxic therapy[92,98-100], suggesting that progression to leukaemia is part of the natural history of these disorders.

In contrast to CML, where the blastic phase of disease almost invariably harbours the *BCR-ABL1* fusion[223], a significant proportion of leukaemic transformations occurring in patients with a *JAK2*-mutant MPD are negative for the *JAK2* mutation[102,103]. At present, however, the mechanisms leading to the development of *JAK2* wild-type leukaemia are not known. Data on the genetic events associated with evolution to post-MPD leukaemia are limited, with an *AML1-ETO* fusion reported in a *JAK2*-mutant transformation[237] and mutations in *NPM* or *FLT3* reported in single cases of *JAK2* wild-type transformation[103,104].

5.2 Aims of this chapter

This chapter addresses the following questions relating to AML following a *JAK2*-mutant MPD:

1) What proportion of AML transformations are negative for the *JAK2* mutation?

2) What are the mechanisms leading to development of a *JAK2* wild-type leukaemia following a *JAK2*-mutant MPD?

3) What are the genetic events involved in the progression from MPD to AML?

4) Are there any clinical or biological differences between patients who progress to *JAK2* wild-type versus *JAK2*-mutant leukaemia?

5.3 Sample preparation and *JAK2* genotyping

Samples were obtained from 16 patients with AML and evidence of a preceding *JAK2*-mutant MPD (15 patients with *JAK2 V617F* and 1 patient with a *JAK2* exon 12 mutation: *N542-E543del*; Table 5.1). In order to ascertain the *JAK2* status of each AML, it was important to obtain a sample of leukaemic blasts free from contamination by the preceding MPD. Where possible, peripheral blood samples were collected prospectively and leukaemia cells were purified by magnetic selection of $CD34^+$ cells from the mononuclear fraction. Mononuclear cells were also plated in semi-solid medium in order to obtain individual blast colonies; in some cases erythroid progenitor colonies were also obtained (Figure 5.1A). In all cases, leukaemia samples consisted of at least 90% blast cells by morphological criteria (Figure 5.1B). In the majority of cases, material representative of the preceding MPD was also available (Table 5.1).

Each sample was genotyped for the relevant *JAK2* mutation by direct sequencing of blast cell DNA. Of the 16 AML transformations, 1 was *JAK2 V617F*-heterozygous, 6 were *JAK2 V617F*-homozygous and 9 were *JAK2* wild-type, including the patient with a preceding *JAK2* exon 12 mutant MPD (Figure 5.3). Therefore in over half the cases studied the leukaemic transformation was negative for the *JAK2* mutation.

Table 5.1. Clinical details and sample availability of 16 patients progressing to acute leukaemia following a *JAK2*-mutant myeloproliferative disorder.

Pt	Diagnosis	Sex	*JAK2* mutation	Age at diagnosis (years)	Therapy	Time to AML (years)	MPD sample	AML sample
1	IMF	M	V617F	65	HC, AN	2	Diagnostic BM	PB $CD34^+$ cells
2	ET->IMF	M	V617F	63	HC	16	Grans 1 yr before AML	PB $CD34^+$ cells
3	PV->IMF	F	V617F	57	HC, IFN	10	Grans 1 yr before AML	PB $CD34^+$ cells
4	IMF	M	V617F	59	none	1	Not available	PB $CD34^+$ cells
5	Probable IMF	M	V617F	70	none	-	Erythroid colonies	PB $CD34^+$ cells
6	IMF	M	V617F	80	HC	8	Grans 1 yr before AML	PB $CD34^+$ cells
7	ET->IMF	M	V617F	73	HC	6	Grans 1 yr before AML	PB $CD34^+$ cells
8	PV	M	exon 12	69	P32, BU	26	Not available	PB $CD34^+$ cells
9	IMF	M	V617F	82	HC	3	Grans 1 yr before AML	PB $CD34^+$ cells
10	PV	F	V617F	87	HC	12	Not available	PB MNC
11	PV	F	V617F	78	HC	14	PB 1 yr before AML	BM
12	RARS-T	M	V617F	84	HC	8	Grans 1 yr before AML	PB $CD34^+$ cells
13	PV	M	V617F	93	HC, BU, P32	26	Grans 3 yr before AML	PB $CD34^+$ cells
14	PV	M	V617F	75	HC	10	PB 1 yr before AML	PB $CD34^+$ cells
15	PV	F	V617F	61	HC	3	Grans 1 yr before AML	PB $CD34^+$ cells
16	ET	F	V617F	86	HC, BU	10	PB 1 yr before AML	BM

Pt: patient; IMF: idiopathic myelofibrosis; ET: essential thrombocythaemia; PV: polycythaemia vera; RARS-T: refractory anaemia with ringed sideroblasts and thrombocytosis; M: male; F: female; HC: hydroxycarbamide; IFN: interferon-alpha; AN: anagrelide; P32: radioactive phosphorous; BU: busulphan; BM: bone marrow; Grans: purified granulocytes; PB: peripheral blood; MNC: mononuclear cells.

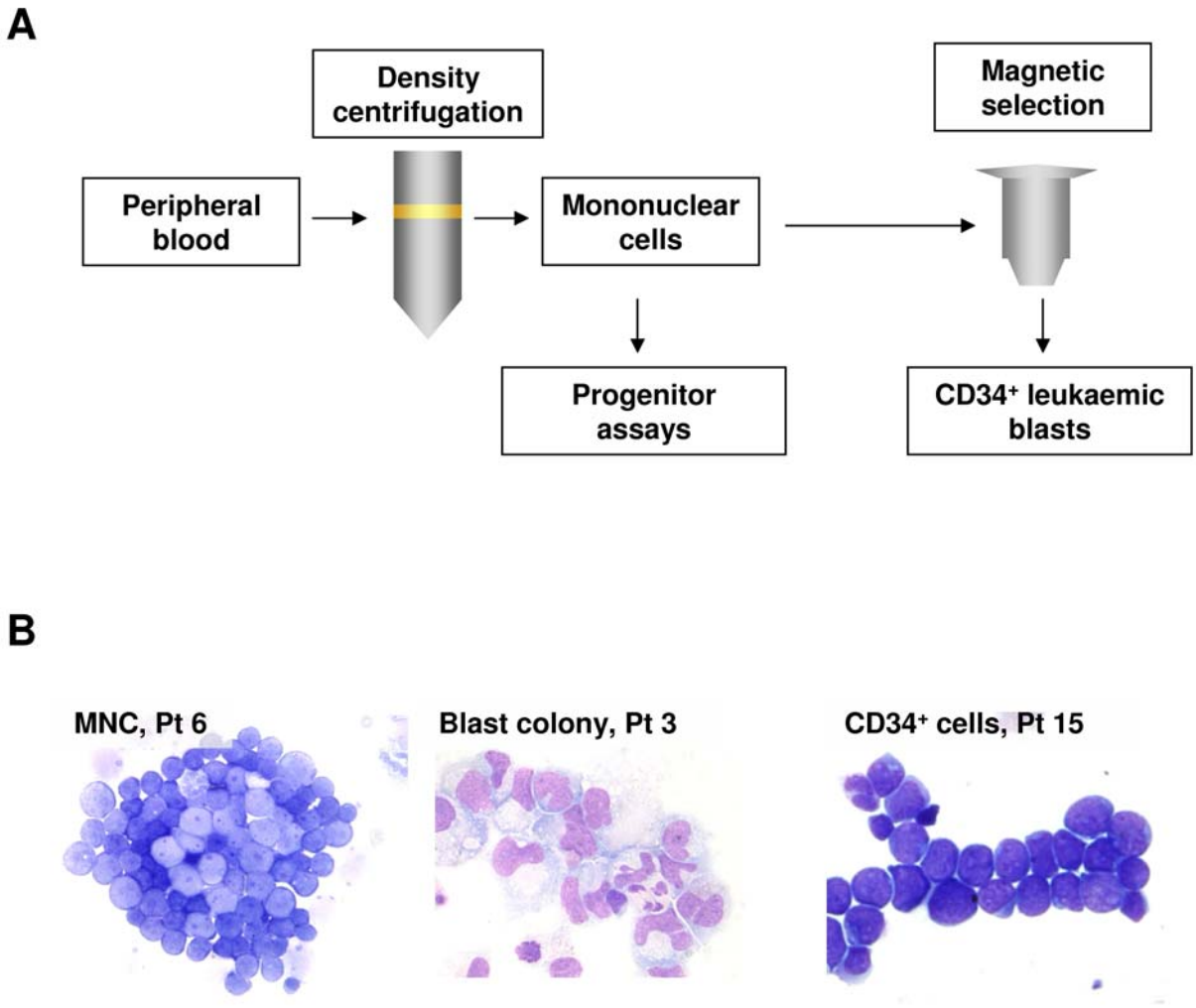

Figure 5.1. Purification of leukaemic blasts from patients developing acute myeloid leukaemia following a *JAK2*-mutant myeloproliferative disorder. (A) Strategy for purification of leukaemia cells. (B) Cytospin preparations of leukaemia cells obtained by different purification methods. MNC: mononuclear cells.

5.4 Clonal origins of *JAK2* wild-type AML

The clonal origin of *JAK2* wild-type AML following a *JAK2*-mutant MPD is currently unknown. A proportion of patients with a *JAK2*-mutant MPD harbour clones that are homozygous for the *JAK2 V617F* mutation[70], with such clones generated by a process of mitotic recombination in a *V617F*-heterozygous cell[28-31] which gives rise to both *V617F*-homozygous and *JAK2* wild-type daughter clones (Figure 5.2A). It is possible, therefore, that a mitotically recombined *JAK2* wild-type clone could represent the cell of origin for a subsequent *JAK2* wild-type leukaemia. As both the *V617F*-homozygous and *JAK2* wild-type mitotically recombined clones will harbour loss of heterozygosity (LOH) around the *JAK2* locus, LOH studies were used to investigate whether such clones persist *in vivo*. Individual erythroid progenitors from a separate cohort of PV patients known to harbour a *V617F*-homozygous clone were genotyped for the *JAK2 V617F* mutation and an informative SNP within or close to the *JAK2* locus. LOH was not identified in any of 1,195 *JAK2* wild-type erythroid colonies from 10 PV patients (Table 5.2). These data suggest that following mitotic recombination, the *JAK2* wild-type daughter clone does not commonly expand to detectable levels.

Table 5.2. Genotyping of individual erythroid colonies from patients with polycythaemia vera for 9p loss of heterozygosity.

		Individual erythroid colonies					
		JAK2 wild-type		*V617F*-het		*V617F*-hom	
Patient	9p SNP	9p het	9p LOH	9p het	9p LOH	9p het	9p LOH
a	rs465514	160	0	0	0	0	5
b	rs428111	154	0	19	0	0	5
c	rs428111	56	0	2	0	0	2
d	rs428111	78	0	9	0	0	6
e	rs2230724	64	0	3	0	0	5
f	rs428111	58	0	3	0	0	1
g	rs428111	64	0	5	0	0	1
h	rs7847141	119	0	15	0	0	1
i	rs428111	334	0	2	0	0	49
j	rs428111	108	0	9	0	0	1
Total		**1195**	**0**	**67**	**0**	**0**	**76**

LOH studies were also performed on leukaemic blasts using multiple informative SNPs within or close to the *JAK2* locus (Figure 5.2B). As expected all informative *V617F*-homozygous leukaemias showed 9p LOH, consistent with prior mitotic recombination. No evidence for LOH, however, was found in any of 9 *JAK2* wild-type leukaemias (Figure 5.2C and Figure 5.3). These data exclude reversion to wild-type either by mitotic recombination, consistent with 3 cases previously reported[102,103], or by a more localised gene conversion or gene deletion event.

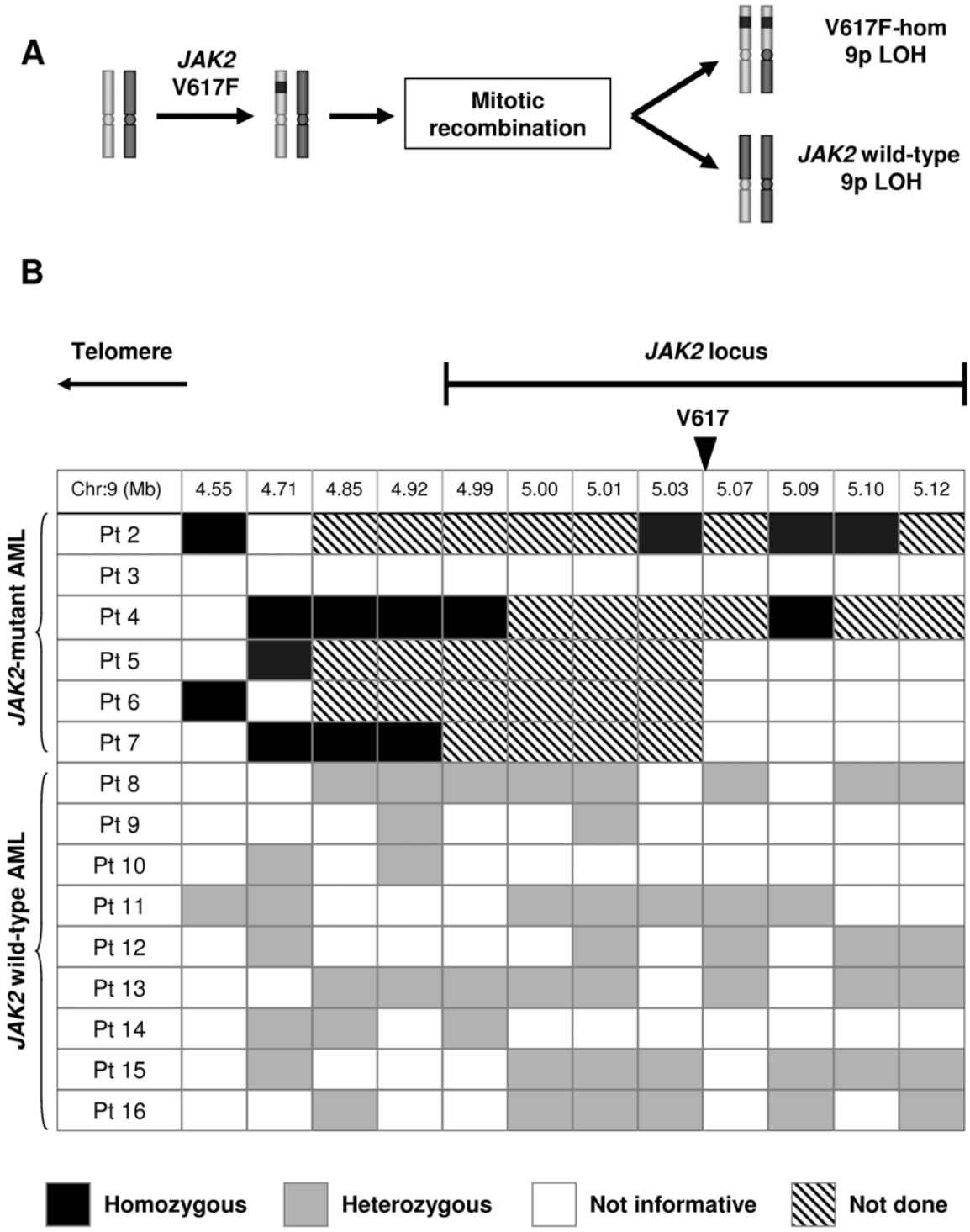

Figure 5.2. Reversion to wild-type is not a common mechanism of progression from a *JAK2*-mutant myeloproliferative neoplasm to a *JAK2* wild-type leukemia. (A) Mitotic recombination in a *JAK2* V617F-heterozygous cell gives rise to both V617F-homozygous and *JAK2* wild-type daughter cells, both of which harbor LOH for chromosome 9p. (B) Summary of SNP analysis, showing SNP location relative to the *JAK2* locus and genotyping results in post-MPN leukemias; whereas all informative *JAK2* V617F-homozygous leukemias showed LOH close to *JAK2*, this was not observed in any of 9 *JAK2* wild-type leukemias. Patient 1 is not included in this analysis as leukemic blasts were heterozygous for the *JAK2* V617F mutation. SNP: single nucleotide polymorphism; LOH: loss of heterozygosity.

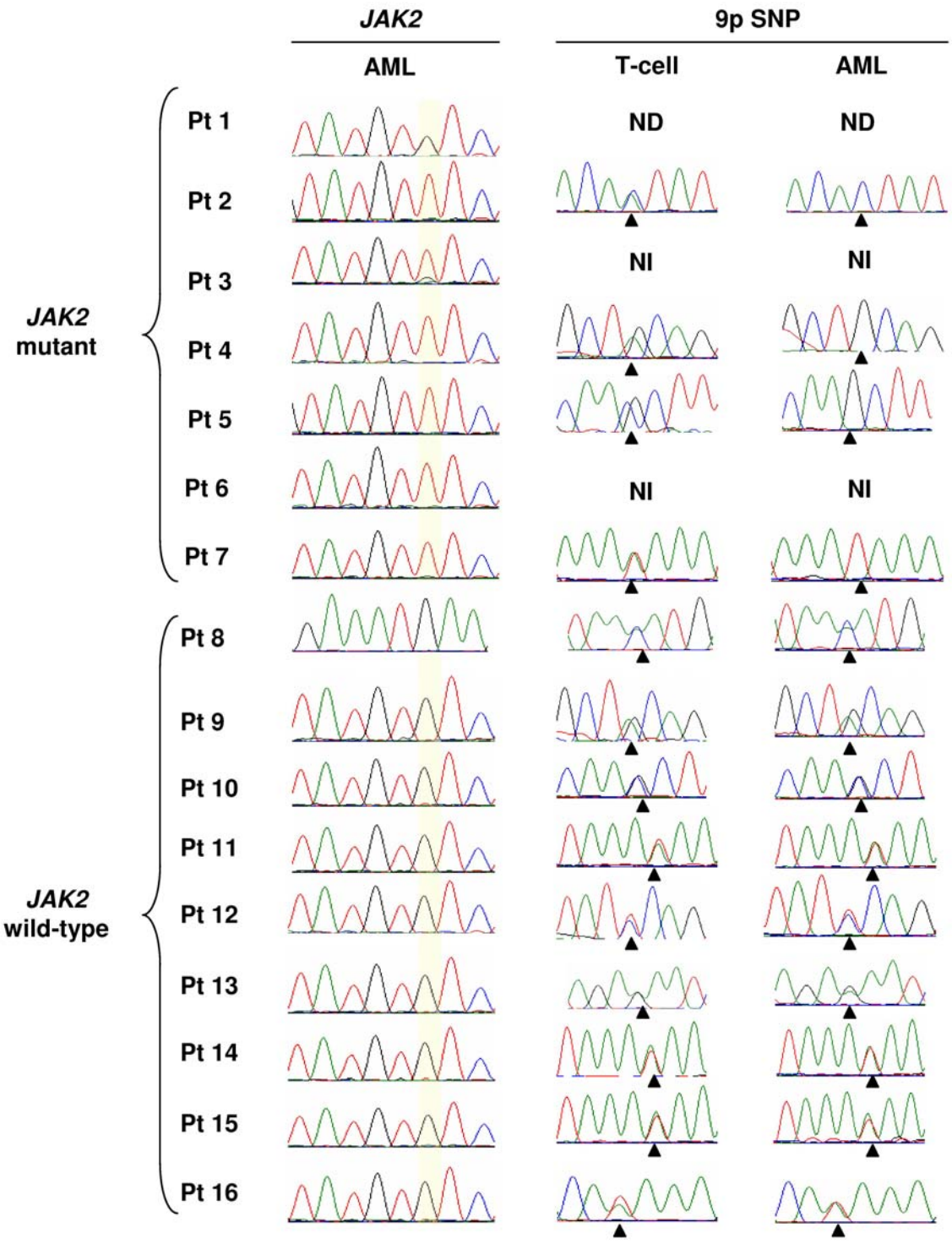

Figure 5.3. *JAK2* mutation status and 9p loss of heterozygosity in leukaemic blasts. Genotyping for the *JAK2 V617F* (highlighted in yellow) or *JAK2* exon 12 mutation (patient 8) and examples of single nucleotide polymorphisms used in 9p loss of heterozygosity studies. NI: not informative; ND: not done.

In order to assess the clonal relationship of *JAK2*-mutant leukaemia and *JAK2* wild-type MPD, X-inactivation patterns (XCIP) were studied in a female patient from whom the necessary material was available (patient 15, Table 5.1). In this patient, the same XCIP was observed in *JAK2*-mutant erythroid colonies, peripheral blood granulocytes obtained prior to transformation and *JAK2* wild-type leukaemic blasts (Figure 5.4A), a result consistent with the MPD and AML arising either from a shared founder clone or from independent stem cells.

5.5 Additional genetic events involved in progression to AML

5.5.1 Amplification of JAK2

In CML, progression to accelerated and blastic phase disease has been associated with an increase in both *BCR-ABL1* copy number and expression[238]. *JAK2* copy number was therefore assessed in *JAK2*-mutant leukaemic blasts by comparing amplification of the *JAK2* locus by real-time PCR with amplification of control regions on chromosomes 13q and 1p (Figure 5.4B). No increase in *JAK2* copy number was seen in any of 7 *JAK2*-mutant leukaemias compared to normal controls. Analysis of two cells lines known to harbour a chromosome 9p deletion (MDA-MB-361 & NB16) and a cell line known to harbour multiple copies of *JAK2* (HEL) are shown as controls.

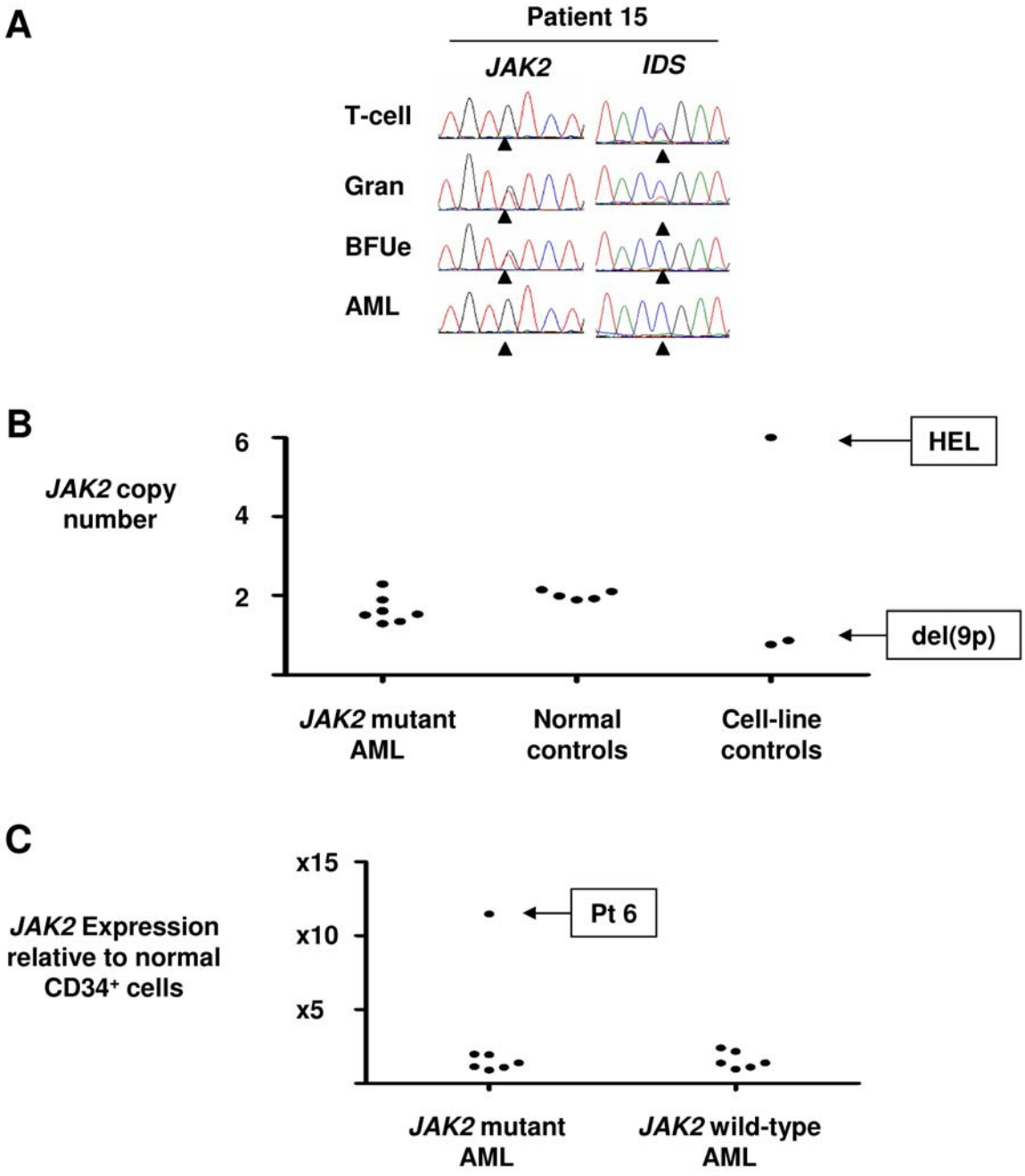

Figure 5.4. Clonality studies, *JAK2* copy number and *JAK2* expression in leukaemic blasts. (A) X-chromosome inactivation patterns in a patient progressing to *JAK2* wild-type leukaemia following a *JAK2 V617F*-positive MPD. (B) Real-time PCR showing no alterations in *JAK2* copy number in *JAK2* mutant leukaemic blasts. (C) Analysis of *JAK2* expression in leukaemic blasts by real-time PCR showing a 12 fold increase in a patient with *JAK2 V617F*-homozygous leukaemia. Gran: granulocytes; BFUe: burst forming unit erythroid; AML: acute myeloid leukaemia.

Real-time PCR was also used to quantitate expression of total *JAK2* in *JAK2*-mutant and *JAK2* wild-type leukaemias. Expression of *ABL1* was used as a housekeeping gene, and *JAK2* expression was normalised to two independent samples of buffy coat derived $CD34^+$ cells. No significant alterations in *JAK2* expression were seen in any *JAK2* wild-type leukaemia. A single *V617F*-homozygous leukaemia, however, showed a 12-fold increase in *JAK2* expression compared to normal $CD34^+$ cells (Figure 5.4C). There are several possible explanations for the over-expression of *JAK2* observed in this patient, including (a) leukaemic differentiation towards a high *JAK2* expressing lineage such as erythroid (although this was not apparent morphologically; see Figure 5.1B), (b) genetic rearrangement placing *JAK2* under the control of a stronger promoter or enhancer, or (c) epigenetic alteration of the *JAK2* promoter/enhancer region. It is therefore possible that amplification of *JAK2* expression plays a role in disease progression in a proportion of patients.

5.5.2 Karyotypic abnormalities

Cytogenetic analysis at the time of transformation to AML was available for 8 of the 16 patients (Table 5.3). Of these patients, 7 harboured unbalanced karyotypic abnormalities, including two patients with complex chromosomal rearrangements. Patient 7 harboured an unbalanced translocation resulting a loss of material from chromosome 17p including the *TP53* locus, an abnormality often associated with mutation of the remaining *TP53* allele. Although patient 8 harboured a deletion of chromosome 20q at the time of transformation, the abnormality was absent from the *JAK2* wild-type leukaemia as assessed by LOH studies (Figure 5.5A), suggesting the presence of del(20q) in the preceding *JAK2*-mutant MPD. Similarly in patient 15, detection of trisomy 9 at transformation represented the preceding *JAK2*-mutant MPD (Chapter 4, patient 1).

A single patient harboured a balanced inversion of chromosome 3 (patient 2, Table 5.3). This rearrangement is associated with increased expression of *EVI1* due to translocation of the entire reading frame of *EVI1* in proximity to the enhancer of *RPN1*, a constitutively expressed housekeeping gene[239] (Figure 5.5B). Analysis of leukaemic blasts from patient 2 confirmed a 17-fold increase in *EVI1* expression compared to normal $CD34^+$ cells, with the remaining AML samples showing either no expression of *EVI1*, or expression comparable to normal $CD34^+$ cells (Figure 5.5C).

Table 5.3. *JAK2* mutation status and cytogenetic analysis of leukaemic blasts from 16 patients with a preceding *JAK2*-mutant myeloproliferative disorder.

Pt	Prior MPD	Prior therapy	Sex	At transformation to AML	
				***JAK2* mutation**	**Cytogenetics**
1	IMF	HC, AN	M	heterozygous	add(21)
2	ET->IMF	HC	M	homozygous	inv(3), del(7)
3	PV->IMF	HC, IFN	F	homozygous	ND
4	IMF	none	M	homozygous	ND
5	Probable IMF	none	M	homozygous	add(8), del(7q)
6	IMF	HC	M	homozygous	ND
7	ET->IMF	HC	M	homozygous	der(5)t(5;17)
8	PV	P32, BU	M	wild-type	del(20q)
9	IMF	HC	M	wild-type	ND
10	PV	HC	F	wild-type	ND
11	PV	HC	F	wild-type	ND
12	RARS-T	HC	M	wild-type	complex[¶]
13	PV	HC, BU, P32	M	wild-type	ND
14	PV	HC	M	wild-type	complex[Δ]
15	PV	HC	F	wild-type	add(9)
16	ET	HC, BU	F	wild-type	ND

Pt: patient; IMF: idiopathic myelofibrosis; ET: essential thrombocythaemia; PV: polycythaemia vera; RARS-T: refractory anaemia with ringed sideroblasts and thrombocytosis; HC: hydroxycarbamide; IFN: interferon-alpha; AN: anagrelide; P32: radioactive phosphorous; BU: busulphan; M: male; F: female; ND: not done

[¶] 43,XY,del(5)(q1q3),-7,-12,-16 [5], 43,XY,idem,del(6)(q1q2) [3], 46,XY [2]

[Δ] 45-46,XY,del(5)(q1?),add(16)(q2?), -17, -17, -22, +2~3 mar [10]

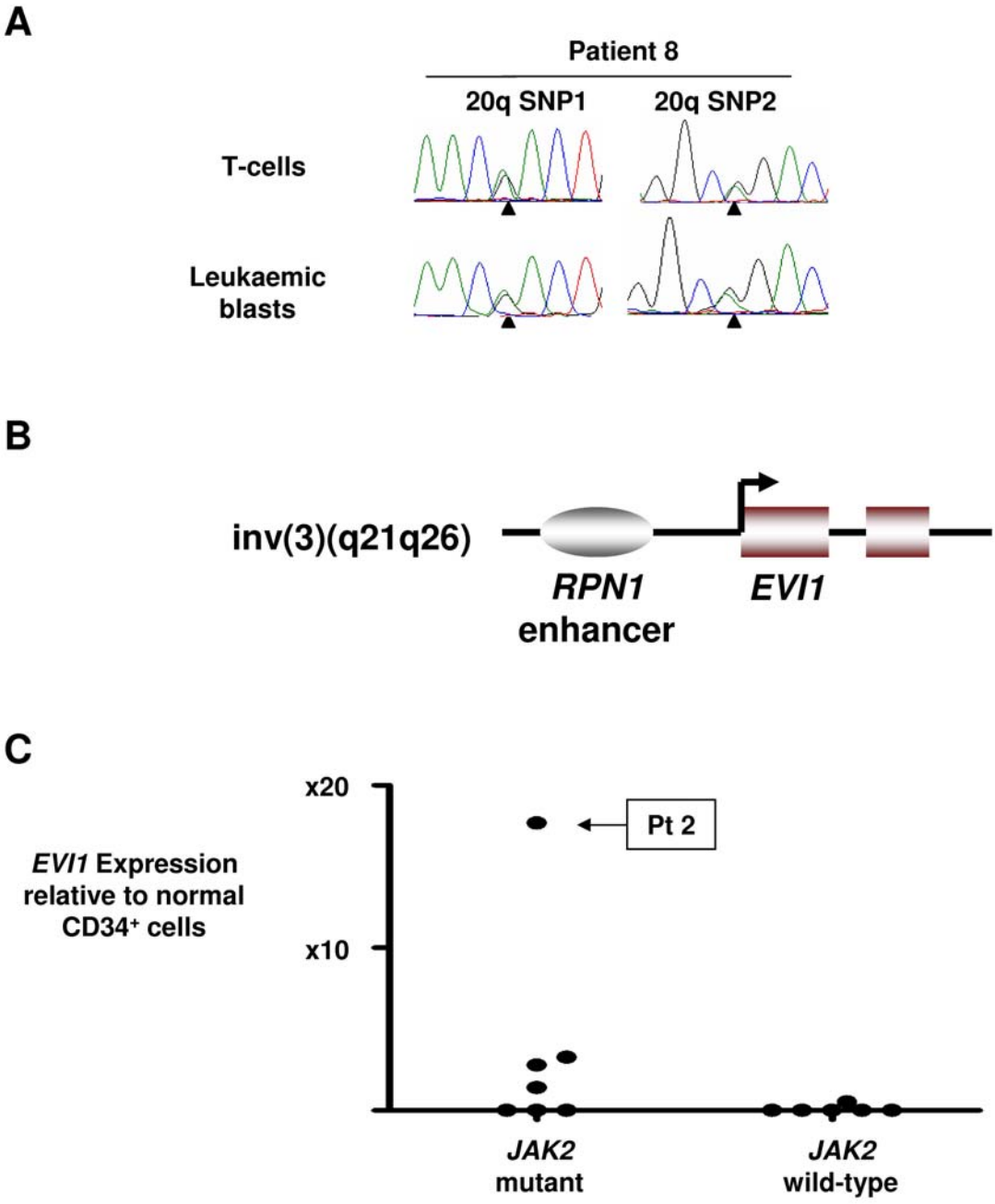

Figure 5.5. Deletion of chromocomse 20q and rearrangement of *EVI1* in two patients with acute leukaemia. (A) Single nucleotide polymorphism studies in a patient with a 20q deletion and *JAK2* wild-type leukaemia showing no loss of heterozygosity for 20q in leukaemic blasts. (B) Schematic representation of inv(3) whereby expression of *EVI1* comes under the control of the *RPN1* enhancer. (C) Expression of *EVI1* in leukaemic blasts relative to normal CD34+ cells showing a 17 fold increase in a patient harbouring inv(3).

5.5.3 Mutations in leukaemia associated genes

To further investigate the genetic events involved in progression to acute leukaemia, DNA from leukaemic blasts was assessed for known leukaemia associated mutations involved in increased proliferation (*KRAS*, *NRAS* and *FLT3*), block in differentiation (*CEBPA*, *RUNX1*, *GATA2*, *NPM* and *WT1*) or genomic instability (*TP53*) (Table 5.4). 2 of 7 *JAK2*-mutant leukaemias harboured mutations in *NRAS*, (*NRAS G12S* and *NRAS G12D*, patients 1 & 2 respectively). Both mutations were heterozygous and affected a conserved residue within the GTP-binding domain of the protein (Figure 5.6A & B). A single *JAK2* wild-type leukaemia harboured a *FLT3-ITD* (Figure 5.6C).

Point mutations in *RUNX1* were identified in 1 of 9 *JAK2* wild-type and 4 of 7 *JAK2* mutant leukaemias. In all cases, *RUNX1* mutations were acquired and affected the DNA binding domain of the protein, with retention of the wild-type allele (Figure 5.7A & B).

Mutations in *TP53* were detected in 1 of 7 *JAK2* mutant and 3 of 9 *JAK2* wild-type leukaemias. In all cases the mutations were acquired and affected the p53 DNA binding domain, with loss of the wild-type allele in the leukaemia samples (Figure 5.7C & D).

In summary, in *JAK2*-mutant leukaemias genetic alterations associated with a block in cellular differentiation were detected in 5 of 7 cases, comprising 4 patients with *RUNX1* point mutations and 1 patient with over-expression of *EVI1*. Two patients with *JAK2*-mutant leukaemia also harboured activating mutations in *NRAS*. Mutations of *TP53* with loss of the wild-type allele were detected in 25% of this cohort.

Table 5.4. Screening for leukaemia associated mutations in acute leukaemia following a myeloproliferative disorder

Gene	Mutation frequency in *de novo* AML	Structural domain altered	Region screened
Proliferation			
KRAS	5% AML[240]	GTP binding domains	Codons 12, 13 & 61
NRAS	12% AML[240,241]	GTP binding domains	Codons 12, 13 & 61
FLT3	30% NC-AML[241]	Juxtamembrane domain	Exon 11
Differentiation			
CEBPA	15% NC-AML[242]	DNA binding domain or Loss of full-length protein	Exon 1
RUNX1	33% CML-BC with +21[243] 1-5% AML[244,245]	DNA binding domain	Exons 3 - 5
GATA2	10% CML-BC[246]	DNA binding domain	Exon 4
NPM	50% NC-AML[247]	C-terminus	Exon 12
WT1	10% NC-AML [248,249]	DNA binding domain	Exons 7 & 9
Genomic stability			
TP53	50-70% AML with complex karyotype[250,251]	DNA binding domain	Exons 4-8

AML: acute myeloid leukaemia; NC-AML: acute myeloid leukaemia with normal cytogenetics; CML-BC: chronic myeloid leukaemia in blast crisis

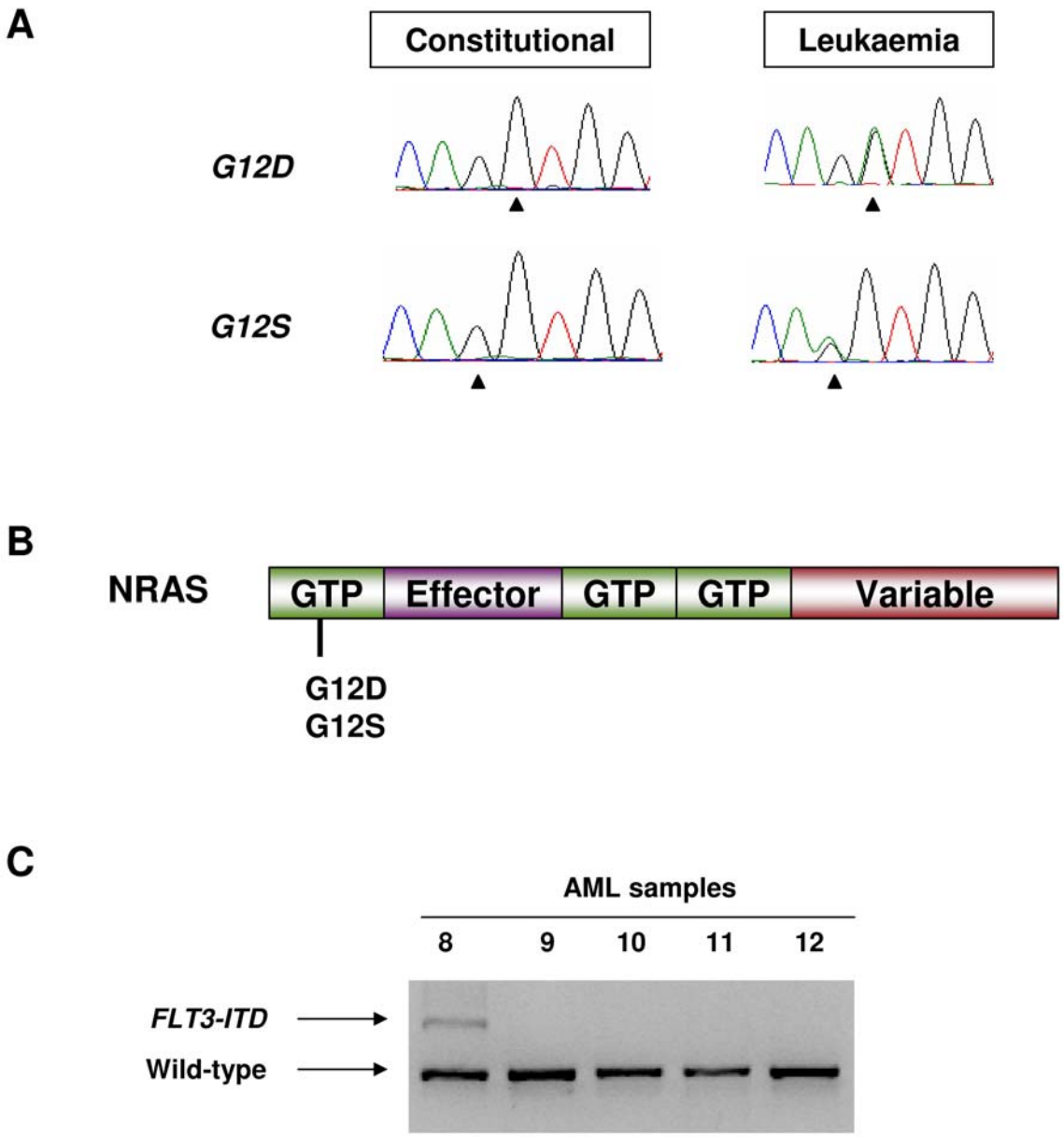

Figure 5.6. Alterations of *NRAS* and *FLT3* in patients progressing to acute myeloid leukaemia. (A) Heterozygous mutations in *NRAS* in two patients with a *JAK2*-mutant leukaemia. (B) Location of *NRAS* mutations within the GTP binding domain of the protein. (C) PCR analysis showing an internal tandem duplication (ITD) of *FLT3* in a patient with a *JAK2* wild-type leukaemia.

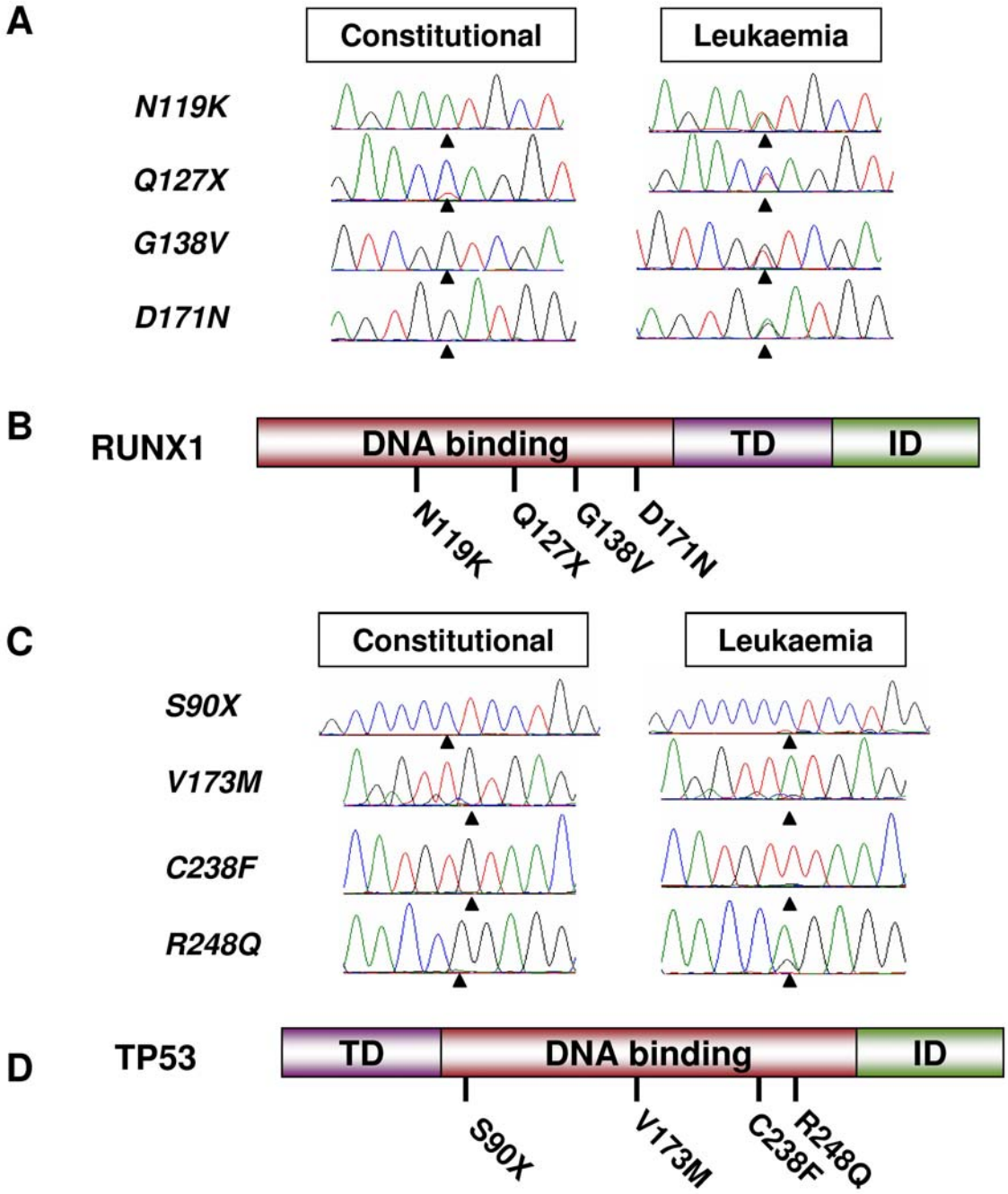

Figure 5.7. Mutations in *RUNX1* and *TP53* in patients progressing to acute myeloid leukaemia. (A) Acquired mutations in *RUNX1* in patients progressing to acute leukaemia. (B) Position of *RUNX1* mutations within the DNA binding domain of the protein. (C) Acquired mutations of *TP53* in patients progressing to acute leukaemia. (D) Position of *TP53* mutations within the DNA binding domain of the protein. TD: transactivation domain; ID: inhibitory domain.

5.6 Acquisition timing of additional genetic events

In patient 1, an *NRAS* mutation was present in both leukaemic blasts and in bone marrow DNA obtained 2 years prior to the development of AML (Figure 5.8A). In patient 2 an *NRAS* mutation was present in both leukaemic blasts and erythroid colonies at the time of transformation, but absent from granulocyte DNA obtained 1 year prior to AML as assessed by both direct sequencing and allele-specific PCR (Figure 5.8B & C). The presence of an *NRAS* mutation prior to the development of AML or in erythroid colonies suggests that oncogenic RAS may be involved in the pathogenesis of the preceding MPD rather than progression to AML.

In patients 1, 4 and 5, *RUNX1* mutations were present in leukaemic blasts only and not in samples representative of the preceding MPD (Figures 5.8A & 5.9A), indicating that such mutations are associated with evolution to acute leukaemia. In patient 1, in whom cytogenetic analysis had indicated duplication of chromosome 21, direct sequencing of *RUNX1* in leukaemic blasts showed a predominant mutant peak, consistent with duplication of the mutant *RUNX1* allele (Figure 5.8A). In patients 7 and 13, *TP53* mutations were similarly present in leukaemic blasts and absent from the preceding MPD. By contrast in patient 10 a *TP53* mutation could be detected in peripheral blood obtained 1 year prior to transformation to AML, at which time the patient was well and the full blood count was normal (Figure 5.9B).

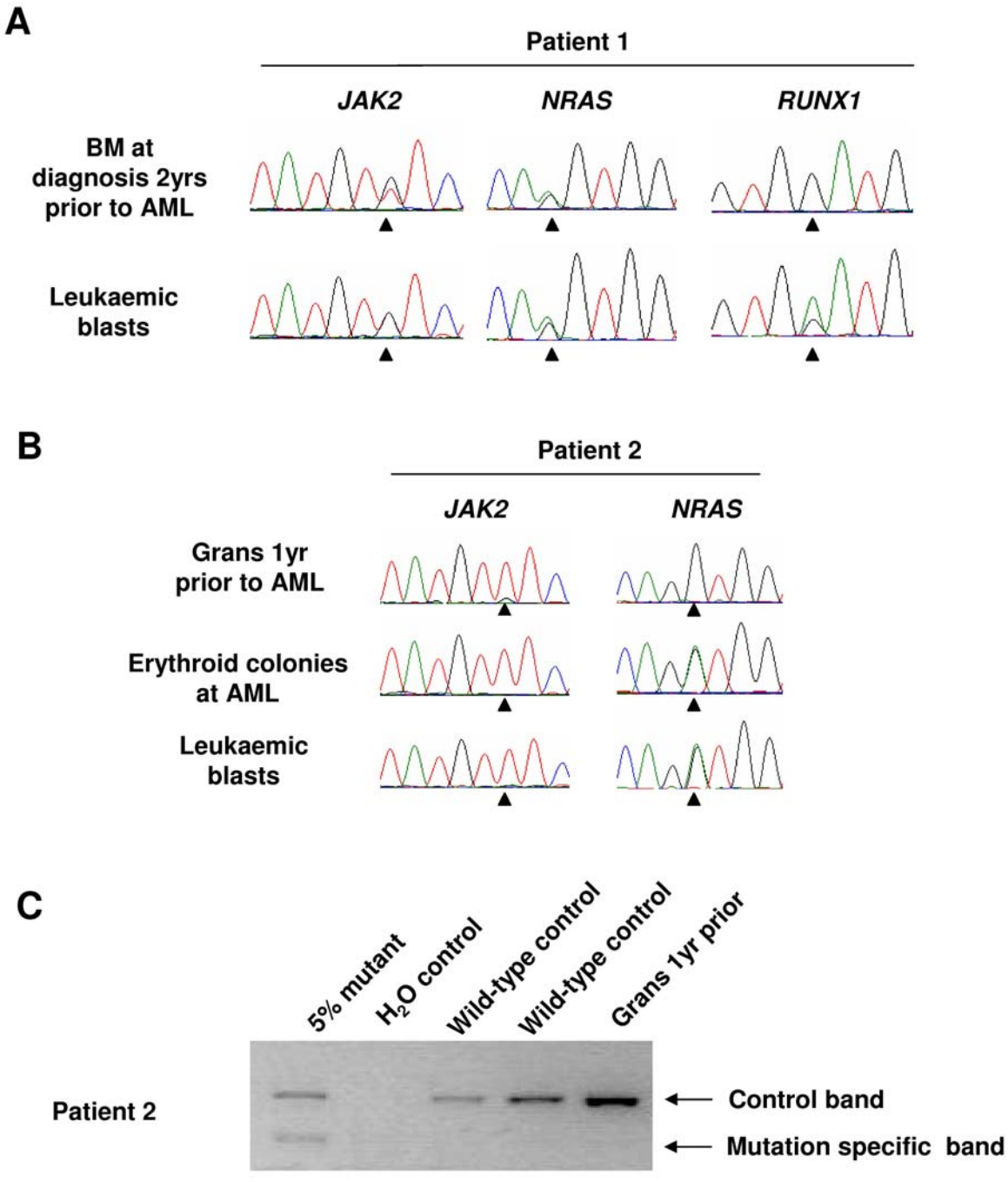

Figure 5.8. Sequential acquisition of mutations in *NRAS* and *RUNX1* in patients progressing to acute leukaemia. (A) Sequence of acquisition of mutations in *NRAS* and *RUNX1* in a patient with *JAK2 V617F*-heterozygous leukaemia. (B) Sequence of acquisition of an *NRAS* mutation in a patient with *JAK2 V617F*-homozygous leukaemia. (C) Allele-specific PCR analysis showing absence of *NRAS* mutation in granulocytes obtained 1 year prior to transformation in patient 2.

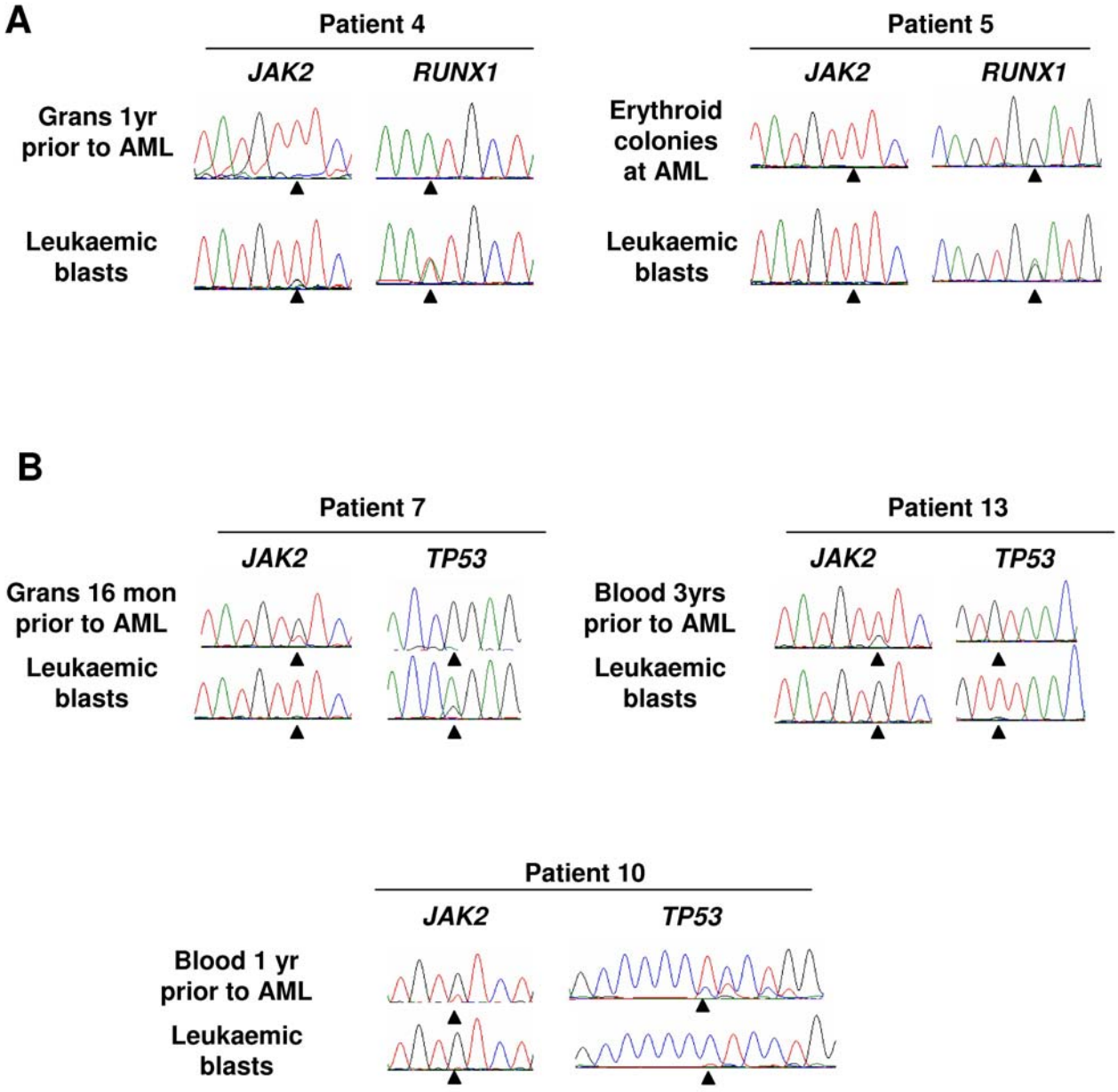

Figure 5.9. Sequential acquisition of mutations in *RUNX1* and *TP53* in patients progressing to acute leukaemia. (A) Sequence of acquisition of mutations in *RUNX1* in two patients with *JAK2 V617F*-homozygous leukaemia. (B) Sequence of acquisition of mutations in *TP53* in three patients progressing to acute leukaemia.

At the time of presentation with AML, patient 5 gave no history of a prior blood disorder. However, the presence of splenomegaly palpable to the umbilicus raised the possibility of an undiagnosed preceding MPD. Erythroid colonies were cultured from a peripheral blood sample obtained at transformation to AML. Whereas leukaemic blasts harboured mutations in both *JAK2* and *RUNX1*, erythroid colonies were either *JAK2 V617F*-homozygous (n=43) or *JAK2* wild-type (n=3), but none were positive for the *RUNX1* mutation (Figure 5.9A), demonstrating the presence of an established *JAK2*-mutant clone prior to the acquisition of the *RUNX1* mutation. Bone marrow trephine biopsy at time of AML showed clusters of hyperlobated megakaryocytes and dense reticulin fibrosis (Figure 5.10A & B). Taken together, these findings strongly suggest the presence of a *JAK2*-mutant MPD prior to the development of acute leukaemia.

5.7 Clinical characteristics of patients progressing to *JAK2*-mutant and *JAK2* wild-type AML.

There was no difference in age at diagnosis of initial MPD between the 16 patients who progressed to AML when compared to *JAK2*-mutant patients in the Cambridge MPD cohort (AML patients 65 ± 11 yrs, Cambridge cohort 60 ± 16 yrs; p=0.2). There were no differences between the *JAK2*-mutant and wild-type leukaemias with regard to gender (p=0.6), age at diagnosis (p=0.5) or prior use of cytoreductive therapy (p=0.2). Although not statistically significant, it should be noted that therapeutic use of known leukaemogenic agents (radioactive phosphorous or busulphan) was associated with progression to a *JAK2* wild-type leukaemia in all 3 cases. In contrast to a previous study[103], no significant differences were observed in the duration of the preceding MPD (*JAK2*-mutant AML 7 ± 6yrs, *JAK2* wild-type AML 12 ± 9yrs; p=0.2). Patients with a *JAK2* wild-type leukaemia, however, were significantly older at time of progression to AML (*JAK2*-mutant AML 67 ± 8yrs, *JAK2* wild-type AML 79 ± 10yrs; p=0.02).

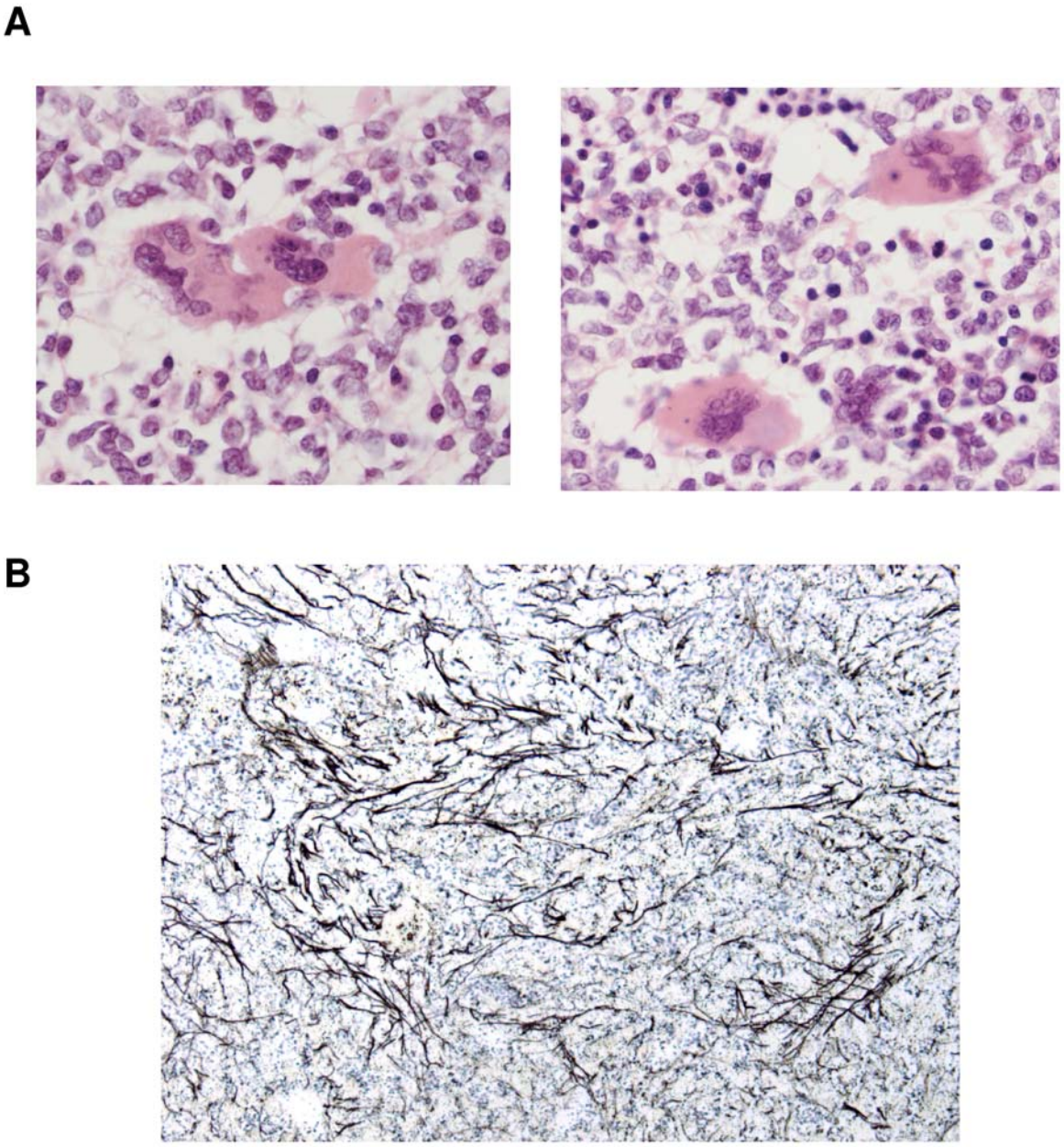

Figure 5.10. Bone marrow histological appearances of a patient with *JAK2 V617F*-homozygous *de novo* acute myeloid leukaemia. (A) Haematoxylin and eosin stained trephine section showing large hyperlobated megakaryocytes surrounded by leukaemic blasts. (B) Silver stained trephine section showing a dense network of coarse reticulin fibres.

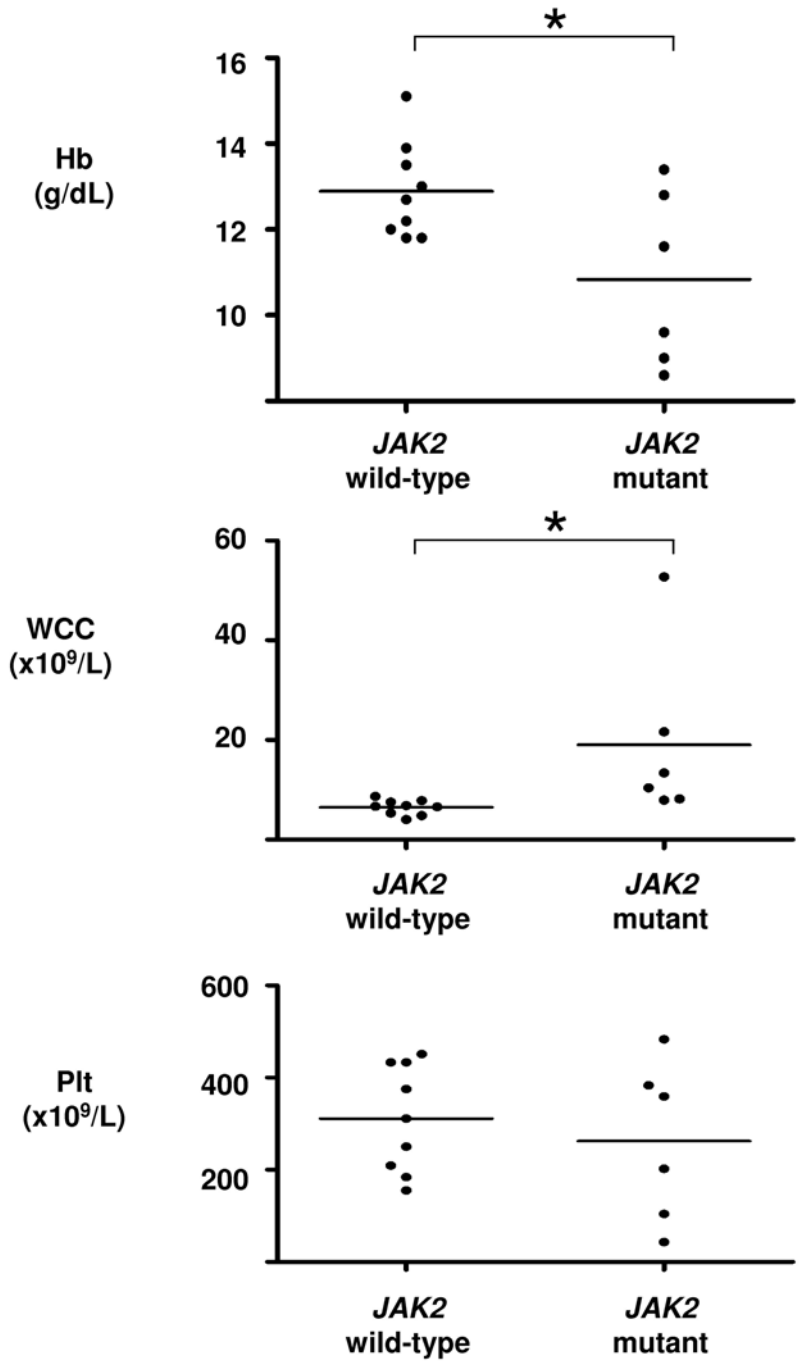

Figure 5.11. Blood counts obtained 1 year prior to transformation to acute leukaemia in patients with a preceding myeloproliferative disorder. Hb: haemoglobin; WCC: white cell count; Plt: platelet count; *: p=<0.05.

A striking contrast was observed between *JAK2*-mutant and *JAK2* wild-type leukaemias with respect to the phenotype of the preceding MPD. All 6 patients with *JAK2*-mutant AML and a characterised preceding MPD had prior evidence of either post ET/PV myelofibrosis or *de novo* IMF (Table 5.3), compared to only 1 of 9 patients with *JAK2* wild-type AML (p=0.001; Fisher's exact test). Consistent with this difference in prior disease phenotype, patients progressing to *JAK2*-mutant AML had significantly lower haemoglobin levels (a finding not explained by a difference in gender distribution) and higher white cell counts one year prior to transformation when compared to those who developed a *JAK2* wild-type leukaemia (*JAK2*-mutant AML: Hb 10.8 ± 2.0g/dl, *JAK2* wild-type AML: Hb 12.9 ± 1.0g/dl; p=0.03; *JAK2*-mutant AML: WCC 19.0 ± 17.0x10^9/L, *JAK2* wild-type AML: WCC 6.5 ± 1.5x10^9/L; p=0.045) (Figure 5.11). Therefore whereas patients with PV or ET generally transformed directly to a *JAK2* wild-type AML, *JAK2*-mutant leukaemia was usually preceded by evidence of disease evolution to myelofibrosis.

5.8 Discussion

This study examined 16 patients with AML and evidence of a preceding *JAK2*-mutant MPD. In over half of the cases, the leukaemia was negative for the *JAK2* mutation, a proportion consistent with two previous reports[102,103]. Differences were observed in the phenotype of the preceding MPD, with *JAK2*-mutant leukaemias evolving from either *de novo* or secondary myelofibrosis and *JAK2* wild-type leukaemias usually arising directly from ET or PV. This finding suggests that the mechanism of disease evolution is different in *JAK2*-mutant versus *JAK2* wild-type leukaemia.

In *JAK2*-mutant AML, the sequential acquisition of mutations suggests that accumulation of genetic events within the *JAK2*-mutant clone is associated with progression from ET/PV to myelofibrosis and eventually acute leukaemia. 6 of 7 *JAK2*-mutant leukaemias were homozygous for the *JAK2* mutation (Table 5.5), suggesting that either loss of wild-type or gain of mutant *JAK2* is important in disease evolution. This finding is consistent with two previous series describing 6 patients with a *JAK2*-mutant AML, all of whom had a mutant allele burden of

greater than 50%[102,103]. 2 of 7 *JAK2*-mutant leukaemias also harboured activating mutations in *NRAS*, which is perhaps surprising given that genetic alterations with similar biological consequences are generally mutually exclusive in *de novo* AML[231]. The detection of mutant *RAS* prior to transformation in patient 1 and in erythroid colonies at the time of transformation in patient 2, however, suggests these mutations may be involved in the pathogenesis of the preceding MPD rather than progression to AML.

Potential cooperating genetic lesions were identified in 5 of 7 *JAK2*-mutant leukaemias, comprising 1 patient with rearrangement of *EVI1* and 4 patients with point mutations in *RUNX1* (Table 5.5). EVI1 is an important haematopoietic transcription factor[252], with over-expression resulting in differentiation block and accumulation of immature cells[253,254]. Increased expression of *EVI1* in seen in *de novo* AML[255], CML blast crisis[256] and juvenile myelomonocytic leukaemia (JMML) with progressive disease[257]. Of note, JMML is commonly associated with gain-of-function mutations in the RAS signalling pathway[232], suggesting possible cooperation between mutant RAS and *EVI1* over-expression in the development of acute leukaemia.

Table 5.5. Summary of genetic lesions in patients progressing to acute leukaemia following a *JAK2*-mutant myeloproliferative disorder

Pt	Type 1 lesion (Cellular proliferation)	Type 2 lesion (Differentiation block)	*TP53* mutation (genomic instability)
1	*JAK2 V617F* (het) *NRAS G12S*	*RUNX1 D171N*	
2	*JAK2 V617F* (hom) *NRAS G12D*	↑*EVI1* expression	
3	*JAK2 V617F* (hom)	*RUNX1 N119K*	
4	*JAK2 V617F* (hom)	*RUNX1 G138V*	
5	*JAK2 V617F* (hom)	*RUNX1 D171N*	
6	*JAK2 V617F* (hom) ↑*JAK2* expression		
7	*JAK2 V617F* (hom)		*R248Q*
8			
9			
10	*FLT3 ITD*	*RUNX1 Q127X*	
11			*S90X*
12			*V173M*
13			*C238F*
14			
15			
16			

Pt: patient; hom: homozygous; het: heterozygous; ↑: increased

The RUNX1 transcription factor complex is essential for the initiation of definitive haematopoiesis in the embryo[258]. Loss of RUNX1 expression in adult bone marrow results in impaired differentiation and accumulation of immature cells[259]. Alteration of RUNX1 activity is common in *de novo* AML, due to chromosomal translocations in 15-25% of cases (*AML1-ETO* or *CBFB-MYH11*) or point mutations in 1-5% of cases[244,245,260]. The functional consequences of these alterations appear similar, resulting in defective DNA binding and dominant negative effects over the wild-type RUNX1 protein complex[260-262], although additional genetic events appear necessary for the development of a fully malignant phenotype[263]. The association of *JAK2 V617F* with both *RUNX1* rearrangements in *de novo* AML[61,65,66] and *RUNX1* point mutations in post-MPD AML (present study) suggests that alterations of the RUNX1 complex cooperate with mutant *JAK2* in the development of acute leukaemia.

The sequential acquisition of genetic events associated with progression from ET or PV to IMF and subsequently acute leukaemia is reminiscent of the disease pattern observed in CML, where patients progress from chronic phase via an accelerated phase to blastic phase disease. Of note, patients with CML occasionally have features of accelerated or blastic phase disease at initial presentation. Thus patients with IMF may be considered as presenting with accelerated phase disease, and patient 5 in this study as presenting in blast crisis.

The mechanisms involved in progression from a *JAK2*-mutant MPD to a *JAK2* wild-type AML are currently unknown. To investigate the possibility that the two phases of disease are clonally related by reversion to wild-type of a heterozygous *JAK2*-mutant clone, LOH studies were performed on both erythroid colonies from PV patients and *JAK2* wild-type leukaemia samples. These data indicate that (1) mitotically recombined, *JAK2* wild-type daughter clones do not commonly persist *in vivo*, and (2) reversion to wild-type is not a common mechanism for the development of *JAK2* wild-type leukaemia. These data extend the findings of 3 reported cases where reversion to wild-type by mitotic recombination was excluded[102,103]. Moreover, analysis of SNPs close to the *JAK2* locus also excludes a more localised gene conversion or gene deletion event in the 9 cases presented herein.

As such, there are two models to explain the development of *JAK2* wild-type leukaemia (Figure 5.12). In model (i), the *JAK2*-mutant MPD and *JAK2* wild-type AML arise in independent stem cells, as a result of either a shared environmental insult, an inherited predisposition to develop myeloid malignancies or secondary to the clastogenic effects of drugs used to manage the MPD phase of disease. Environmental exposures such as radiation and proximity to toxic waste dumps have been implicated in the development of an MPD[9,11,12], and exposure to radiation and benzene have been implicated in the pathogenesis of AML[264]. Studies of both registry data and affected kindreds have indicated a familial tendency to develop an MPD, although the inherited alleles have yet to be identified[15-19]. A familial tendency to AML is also recognised, and includes kindreds with inherited mutations in *CEBPA* or *RUNX1*, and kindreds in whom an inherited allele has not been identified[265]. The use of alkylating and radiomimetic agents in MPD therapy increases the risk of progression to AML[92-94]. In this study, 3 of 9 patients with *JAK2* wild-type AML had received such agents (busulphan or radioactive phosphorous). The remaining 6 cases, however, had received only hydroxycarbamide therapy. The association of hydroxycarbamide and acute leukaemia remains controversial[97] and definitive prospective data are lacking. Hydroxycarbamide does not appear leukaemogenic when used to treat sickle cell disease, an inherited non-clonal disorder of erythroid cells[130], although the younger age and shorter treatment duration of these patients may mask a true leukaemogenic effect. Hydroxycarbamide use has been linked to mutations in *TP53*[132,266], which were present in 1 of 7 *JAK2*-mutant and 3 of 9 *JAK2* wild-type leukaemias in this study (Table 5.5). Although all 4 *TP53*-mutant patients in this series had received therapy with hydroxycarbamide, the common usage of this drug in both the present cohort (13 of 16 patients) and in MPD patients in general means that a direct link between hydroxycarbamide use and *TP53* mutations, or indeed progression to acute leukaemia, will be difficult to establish.

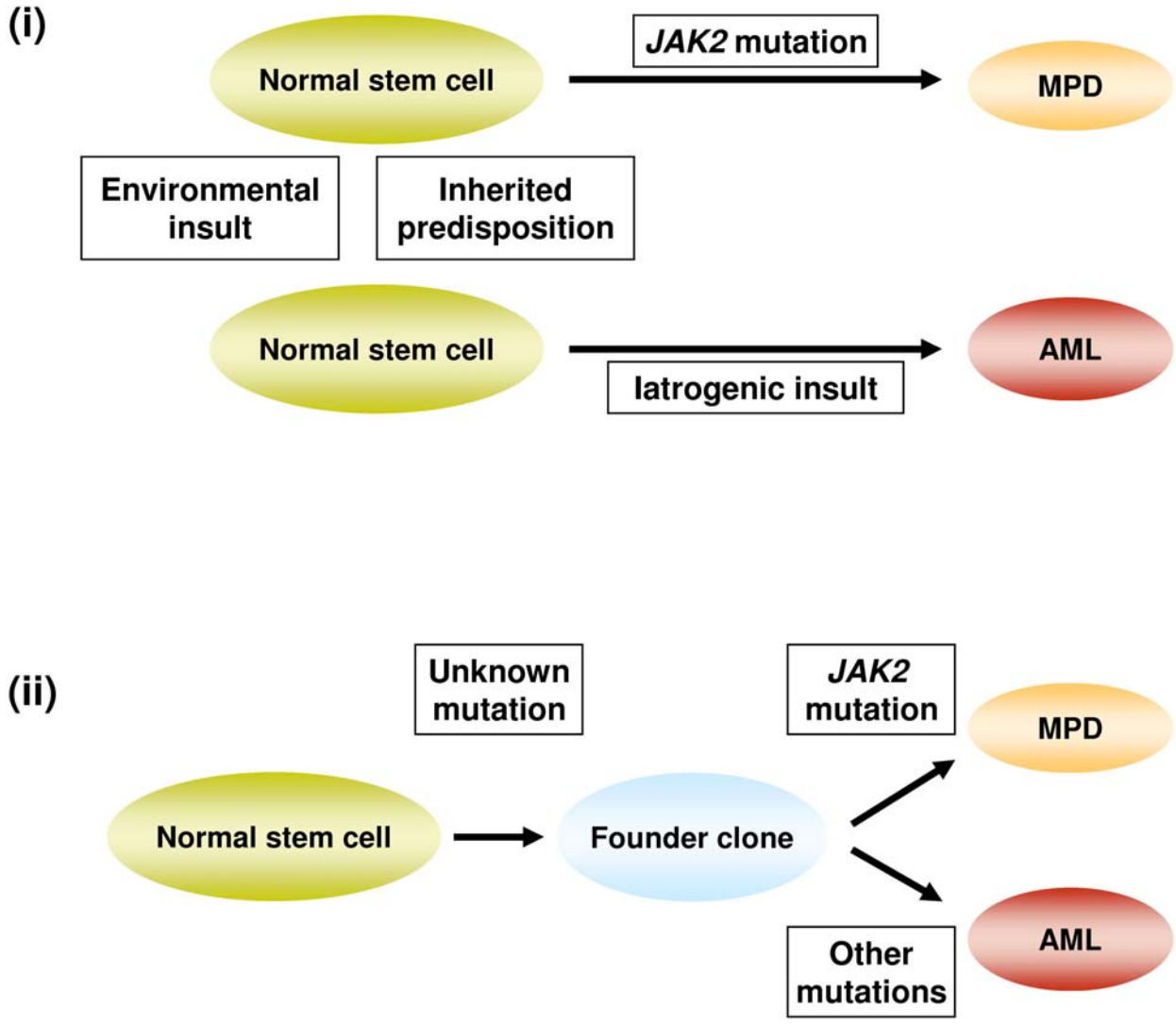

Figure 5.12. Models to explain the development of a *JAK2* wild-type leukaemia following a *JAK2*-mutant myeloproliferative disorder. In model (i), the *JAK2*-mutant MPD and *JAK2* wild-type AML arise in independent stem cells, due to an inherited predisposition, an environmental insult or an iatrogenic insult by an agent used to treat the MPD. In model (ii), the *JAK2*-mutant MPD and *JAK2* wild-type AML arise from a shared founder clone, initiated by an unknown mutation.

In model (ii), the *JAK2*-mutant MPD and *JAK2* wild-type AML are clonally related, having both arisen from a shared founder clone initiated by an unknown genetic event. In a female patient in this study, patterns of X-chromosome inactivation in the *JAK2*-mutant MPD and *JAK2* wild-type AML were consistent with either model (i) or (ii). At the present time, direct evidence supporting either of these two models is lacking.

Chapter 6

Molecular events in the myeloproliferative disorders: relationship of mutation genotype to disease phenotype

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6.1 Introduction

The *JAK2 V617F* mutation is found in the majority of patients with PV and around half of those with ET, posing the question: how is the same mutation associated with two apparently distinct phenotypes? Studies of progenitor colonies from MPD patients identified *JAK2 V617F*-homozygous clones in the majority of patients with PV, but not in patients with ET[70]. This difference suggests that duplication of the mutant *JAK2* allele and/or loss of wild-type *JAK2* may play a role in the determination of disease phenotype. In keeping with this observation, studies of transgenic mice have suggested that *JAK2 V617F* expression level modulates the disease phenotype, with a high mutant to wild-type ratio associated with erythrocytosis and low mutant to wild-type ratio associated with thrombocytosis[53,54]. *In vitro* studies of normal human progenitors have identified STAT5, an important downstream target of both wild-type and mutant JAK2, as a dynamic modulator of lineage determination, with higher levels of activated STAT5 favouring erythroid differentiation and lower levels favouring megakaryocyte differentiation[71]. Taken together, these studies imply a role for mutant *JAK2* gene dosage in determining whether an individual patient manifests an ET or PV phenotype. However a proportion of ET patients have a mutant allele burden of greater than 50%[123,267], and homozygous colonies were not identified in some patients with PV[49,70], suggesting that determination of disease phenotype may be more complex than the presence or absence of a homozygous clone.

Although activating mutations in *JAK2* or *MPL* are found in the majority of patients with an MPD, the molecular cause of around a third of ET and IMF is unknown. Recently, mutations elsewhere in *JAK2* and *MPL* have been reported in patients with acute lymphoblastic leukaemia, acute megakaryocytic leukaemia and in an

acute megakaryocytic leukaemia cell line. Of note, these mutations gave rise to an MPD phenotype when expressed in a murine bone marrow transplant system, raising the possibility that such mutations may be associated with human MPD[106-108,268]. Previous studies have also identified oncogenic *RAS* mutations in a proportion of MPD patients, although patient numbers were small and the relationship of mutant *RAS* to mutations in *JAK2* or *MPL* is not known[269,270].

6.2 Aims of this chapter

This chapter addresses the following questions concerning the association between genetic mutation and disease phenotype in the MPD:

1) What is the relationship of *JAK2 V617F*-positive ET to *JAK2 V617F*-positive PV with respect to haematological parameters and the presence of a homozygous clone?
2) Does the association of mutations elsewhere in *JAK2* or *MPL* with an MPD phenotype in mouse models predict their association with a human MPD?
3) What is the role of oncogenic *RAS* in the phenotype of the human MPD?

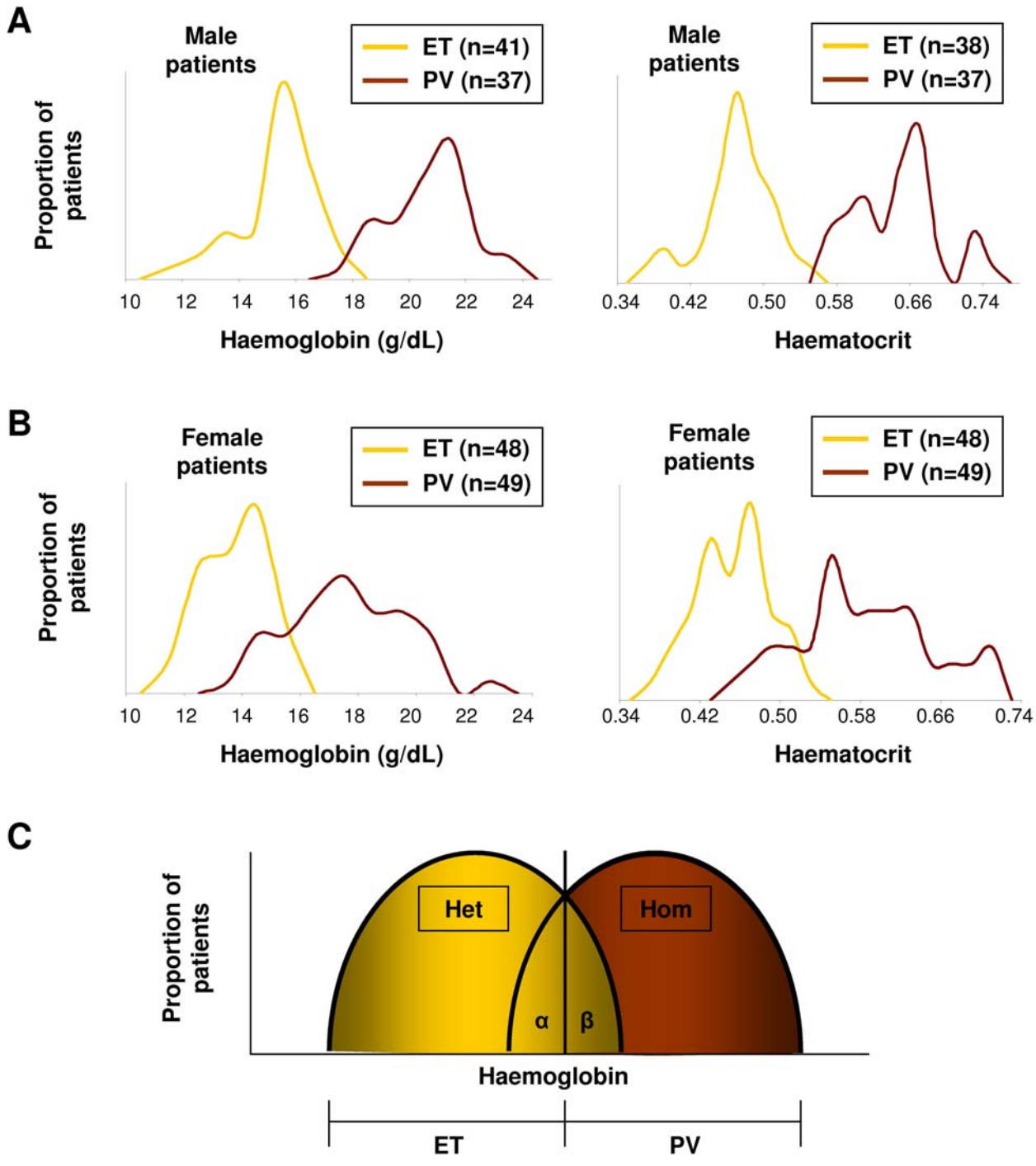

Figure 6.1. Haemoglobin levels in patients with ET or PV show overlap in female but not male patients. Haemoglobin and haematocrit levels for (A) male and (B) female patients with ET or PV diagnosed according to PVSG criteria. (C) Model to explain the overlap in haemoglobin levels between female ET and PV patients. Het: heterozygous *JAK2 V617F* clone; Hom: homozygous *JAK2 V617F* clone; α: ET patients with a *JAK2 V617F* homozygous clone; β: PV patients without a *JAK2 V617F* heterozygous clone.

6.3 Homozygosity for the *JAK2 V617F* mutation in polycythaemia vera and essential thrombocythaemia: relationship of genotype to phenotype

To investigate the phenotypic relationship of ET to PV, blood parameters were analysed from *JAK2 V617F*-positive patients in the Cambridge MPD cohort, all of whom met the PVSG diagnostic criteria for ET or PV[271,272]. Graphical plots of haemoglobin and haematocrit levels for male patients indicated good discrimination of ET from PV (Figure 6.1A). In females, however, there was an overlap in haemoglobin and haematocrit levels between patients with ET and PV (Figure 6.1B). These data would be consistent with a model whereby disease phenotype is related to the presence or absence of a homozygous clone (Figure 6.1C). Moreover the overlap in haemoglobin levels seen in female patients would predict that ET patients falling in the α region in Figure 6.1C may harbour a homozygous clone, and that such patients will have higher haemoglobin levels than ET patients without a homozygous clone. Conversely patients within the β region in Figure 6.1C may manifest a PV phenotype in the absence of a homozygous clone.

Studies were undertaken to investigate the existence of α region patients (ET patients with a homozygous clone). At the time of these studies, the effect of cytoreductive therapy such as hydroxycarbamide on mutant allele burden was unknown. Analysis of ET patients enrolled in the PT-1 study had indicated that *JAK2 V617F*-positive ET patients are more sensitive to the effects of hydroxycarbamide when compared to *JAK2 V617F*-negative patients[68], raising the possibility that this agent has a differential effect on the *V617F*-positive clone. Therefore only patients who had never received cytoreductive therapy were included in the present study. Peripheral blood samples were obtained from 20 *JAK2 V617F*-positive patients meeting the PVSG diagnostic criteria for ET. Erythroid colonies were cultured in semi-solid medium with saturating erythropoietin (1U/mL). Individual colonies were then genotyped for the *JAK2 V617F* mutation using a quantitative pyrosequencing assay; colonies with a wild-type proportion of ≥0.8 were classed as wild-type, 0.4-0.6 as heterozygous and ≤0.2 as homozygous. The genotype of homozygous colonies was confirmed in all

cases using a different assay: by direct sequencing of PCR products generated by a different primer pair.

A mean of 88 individual colonies were genotyped for each of 20 treatment naïve ET patients (Table 6.1). 5 of 20 patients (25%) harboured at least one colony that was homozygous for the *JAK2 V617F* mutation by both pyrosequencing and direct sequencing (Figure 6.2A). Of note, whereas 5 of 10 female patients harboured a homozygous clone, such clones were not seen in any of 10 male patients (p=0.03, Fisher's exact test; Table 6.1). Clinical and laboratory features were compared for patients with and without a homozygous clone. Given the known sex differences in haemoglobin and haematocrit levels, female patients were also analysed separately (Table 6.2). When compared to female patients without a homozygous clone, females with a homozygous clone had significantly higher haemoglobin and haematocrit levels (Table 6.2, Figure 6.2B). No differences in other clinical or laboratory features were identified (Table 6.2, Figure 6.2C).

In summary, these data indicate that *JAK2 V617F*-homozygous clones are present in a significant proportion of females with ET, but appear rare in male ET patients. Moreover, females with a homozygous clone have a higher mean haematocrit level compared to those without. The proportion of female patients harbouring a homozygous clone appears higher than would be predicted from the overlap in haematocrit levels observed between ET and PV patients. It is likely, therefore, that other factors are at play to restrain erythropoiesis in female ET patients bearing a *JAK2 V617F*-homozygous clone.

Table 6.1. Comparison of untreated *JAK2* V617F-positive ET patients with and without a homozygous clone.

		Colonies genotyped	Female	Age at diagnosis (years)	Thrombotic event	At time of study							At diagnosis	
						Disease duration (months)	Hb (g/dL)	Hct	MCV	WCC (x10^9/L)	Neut (x10^9/L)	Plts (x10^9/L)	Ferritin (µg/L)	Retic
het only	Mean	89	33%	52	7%	24	14.6	0.43	86	7.8	5.4	844	76	1.1
n=15	±SD	±5		±15		±40	±1.4	±0.04	±4	±1.4	±1.2	±177	±62	±0.9
het & hom	Mean	85	100%	65	40%	1	14.2	0.43	87	9.2	6.4	768	95	1.9
n=5	±SD	±7		±11		±0	±0.7	±0.02	±3	±2.7	±2.2	±209	±67	±0.5
	p-value	0.2	0.03	0.1	0.2	0.2	0.6	0.8	0.5	0.2	0.2	0.4	0.6	

Table 6.2. Comparison of untreated female *JAK2* V617F-positive ET patients with and without a homozygous clone

		Colonies genotyped	Age at diagnosis (years)	Thrombotic event	At time of study							At diagnosis	
					Disease duration (months)	Hb (g/dL)	Hct	MCV	WCC (x10^9/L)	Neut (x10^9/L)	Plts (x10^9/L)	Ferritin (µg/L)	Retic
het only	Mean	89	47	0%	18	13.2	0.39	88	7.3	5.1	840	48	1.0
n=5	±SD	±5	±22		±26	±0.7	±0.03	±4	±1.6	±1.3	±182	±39	±0.8
het & hom	Mean	85	65	40%	1	14.2	0.43	87	9.2	6.4	768	70	1.9
n=5	±SD	±7	±11		±0	±0.7	±0.02	±3	±2.7	±2.2	±209	±39	±0.5
	p-value	0.2	0.1	0.4	0.2	0.04	0.03	0.7	0.2	0.3	0.6	0.4	

Hb: haemoglobin; Hct: haematocrit; MCV: mean cellular volume; WCC: white cell count; Neut: neutrophil count; Plts: platelet count; Retic: bone marrow reticulin (graded 0 to 4).

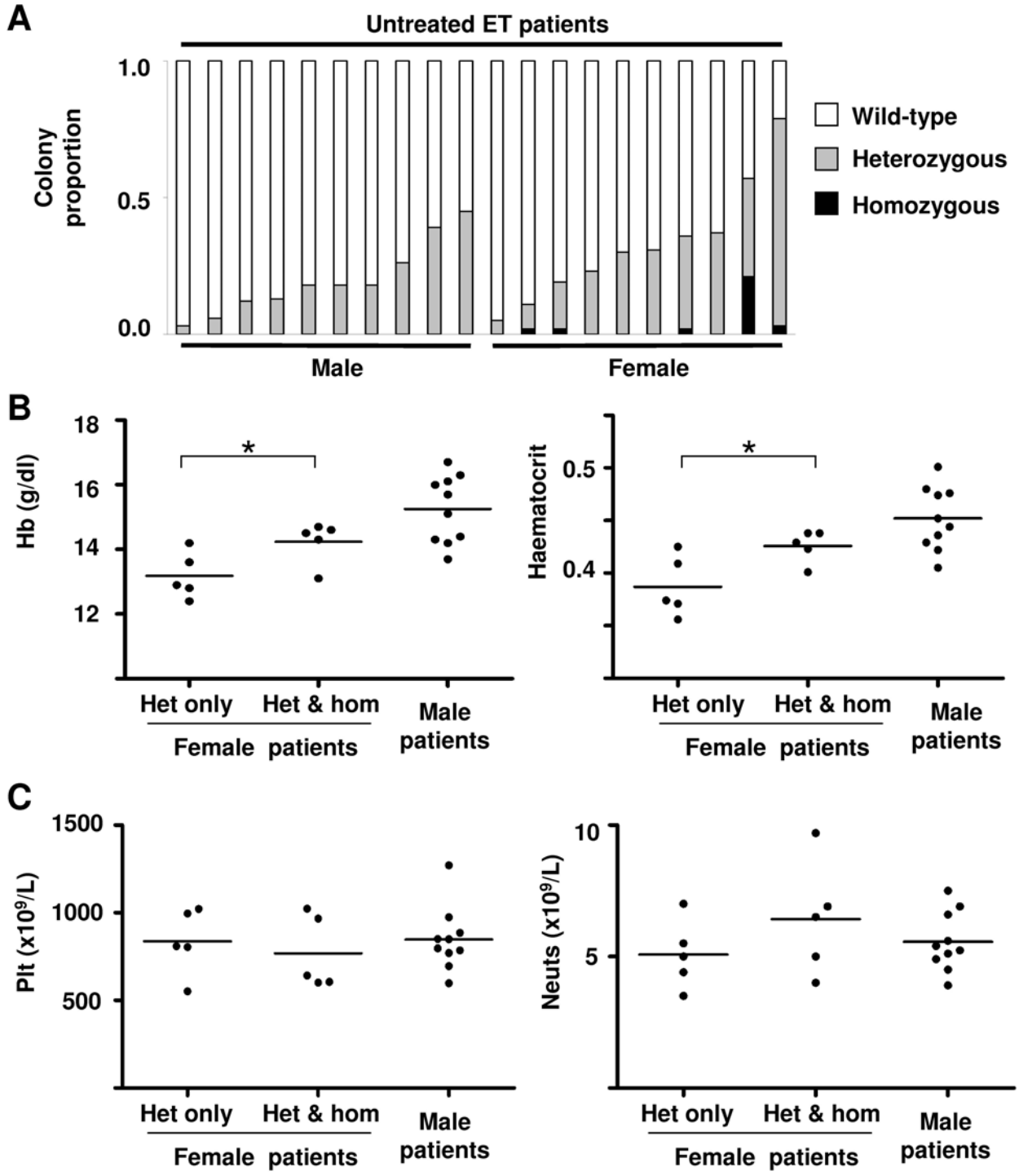

Figure 6.2. Progenitor colony genotyping and blood parameters from 20 untreated patients with essential thrombocythaemia. (A) Genotyping of erythroid colonies for the *JAK2 V617F* mutation. (B) Haemoglobin (Hb) and haematocrit levels at time of colony analysis. (C) Platelet (Plt) and neutrophil count (Neut) at time of colony analysis. *: p=<0.05

6.4 Genetic lesions in the myeloproliferative disorders: other mutations in *JAK2* and *MPL*

Acquired gain-of-function mutations in *MPL* or *JAK2* have been associated with the development of ET, PV or IMF. The *MPL W515L*, *JAK2 V617F* and *JAK2* exon 12 mutations produce an MPD-like phenotype *in vivo* when expressed in murine bone marrow, with expression of mutant JAK2 leading to erythrocytosis, expression of mutant *MPL* associated with thrombocytosis and expression of either allele associated with the development of bone marrow fibrosis[28,105,174]. As such, expression of MPD associated alleles in the mouse can recapitulate features of the counterpart human disease. More recently, further mutations in *JAK2* and *MPL* have been reported in haematological malignancies (Figure 6.4A & B). An acquired mutation in the MPL extracellular domain (*MPL T487A*) was reported in a single case of non-Down's syndrome childhood acute megakaryoblastic leukaemia[268]. A mutation in the JAK2 kinase domain (*JAK2 T875N*) was observed in an acute megakaryocytic leukaemia cell line[106]. Finally, a cluster of mutations centred around *JAK2 R683* have been reported in ALL in patients with Down's syndrome or in association with an additional copy of chromosome 21q[107,108]. All three mutations conferred cytokine independence to cytokine dependent cell lines, although the *JAK2* mutations were only transforming in the presence of a type 1 homodimeric receptor (as is the case for both the *JAK2 V617F* and *JAK2* exon 12 mutations[48,105]). Of particular note, all three alleles produced an MPD-like phenotype in retrovirally-induced murine bone marrow transplant models[106,108,268]. Screening for *JAK2 R683* mutations has previously been reported in a cohort of ET patients[107], although these studies were performed using DNA from unfractionated blood which may be diluted by the presence of non-clonal lymphoid cells. At the time of writing, there were no reports of the other two mutations in human MPDs.

To investigate the possibility that the *MPL T487A* mutation is associated with an MPD phenotype in humans, *MPL* exon 9 was assessed by direct sequencing using granulocyte DNA from 172 unselected patients with IMF or ET (Table 6.3). No mutations in exon 9 were detected in this cohort.

Table 6.3. Cohort of patients with a myeloproliferative disorder screened for the *MPL T487A* mutation

	***JAK2 V617F*-positive**	***MPL* mutation positive**	**Mutation negative**	**Total**
IMF	43	8	45	96
ET	19	1	56	76
Total	62	9	101	**172**

Direct sequencing of granulocyte DNA was also used to screen for the *JAK2 T875N* and *R683* mutations in a cohort of 110 unselected IMF patients. (Table 6.4). No mutations were detected in this cohort.

Table 6.4. Cohort of patients with a myeloproliferative disorder screened for the *JAK2 T875N* and *R683* mutations.

	***JAK2 V617F*-positive**	***MPL* mutation positive**	**Mutation negative**	**Total**
IMF	50	7	53	**110**

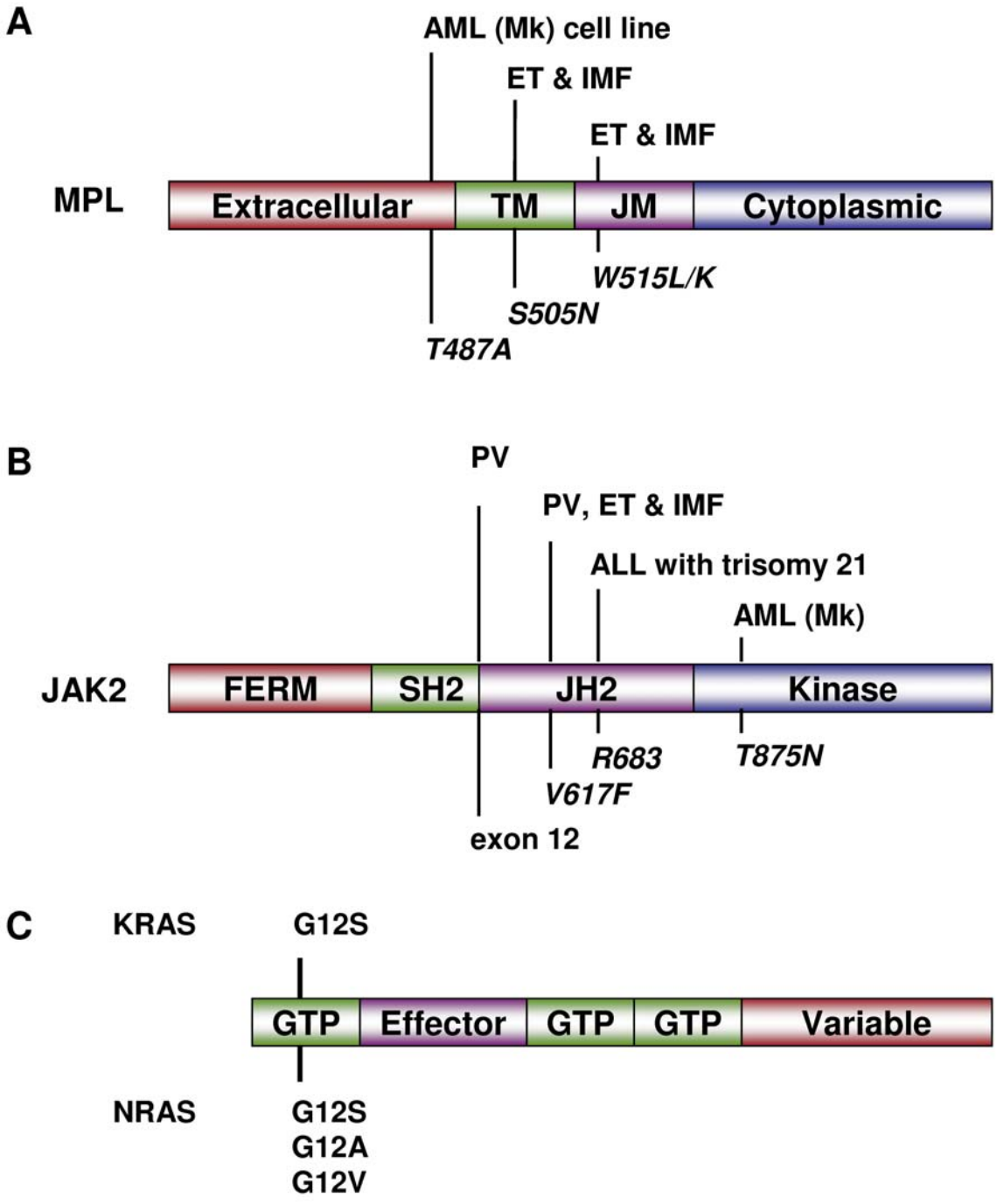

Figure 6.3. *MPL*, *JAK2* and *RAS* mutations in haematopoietic malignancy. (A) *MPL* mutations and their associated haematopoietic malignancies. (B) *JAK2* mutations and their associated haematopoietic malignancies. (C) Mutations in *KRAS* and *NRAS* in patients with myelofibrosis. AML: acute myeloid leukaemia; Mk: megakaryocytic; ET: essential thrombocythaemia; IMF: idiopathic myelofibrosis; PV: polycythaemia vera; ALL: acute lymphoblastic leukaemia; TM: transmembrane; JM: juxtamembrane; GTP: GTPase binding domain.

In summary, *MPL T487A, JAK2 T875N* or *JAK2 R683* mutations were not identified in granulocyte DNA from MPD patients. The use of direct sequencing, a relatively insensitive method of mutation detection, means that patients with a low mutant allele burden may have been missed. For the *JAK2 V617F* and *MPL W515L* mutations, however, the mutant allele burden in IMF patients is high enough to be detectable by direct sequencing in the majority of patients (Chapter 3 and [29]). It seems unlikely, therefore, that the *MPL T487A, JAK2 T875N* or *JAK2 R683* alleles are a common cause of human MPD.

6.5 Genetic lesions associated with accelerated phase disease: a potential role for oncogenic RAS mutations

Various lines of evidence suggest that IMF is associated with the accumulation of further genetic damage, including an increased prevalence of cytogenetic abnormalities, increasing perturbation of haematopoiesis, increased rates of progression to AML and significantly worse survival compared to both ET and PV[75-77,81,83,84,224,273]. At present, however, the specific genetic events associated with disease evolution are unknown. Studies of MPD patients who had transformed to acute leukaemia identified two patients who harboured both *JAK2 V617F* and *RAS* mutations within the leukaemic clone. In one of these patients the *RAS* mutation was present 2 years prior to the development of AML and in the second patient the *RAS* mutation was present in erythroid colonies (Chapter 5), suggesting that mutant RAS may be involved in the MPD phase of disease. Of note, both these patients had IMF prior to the development of AML.

To investigate the possibility that mutations in *RAS* are associated with disease evolution in the MPD, codons 12, 13 and 61 of *NRAS* and *KRAS* were assessed by direct sequencing using granulocyte DNA from a cohort of unselected patients with ET or IMF (Table 6.5).

Table 6.5. *RAS* mutations in a cohort of patients with ET or IMF

		JAK2 V617F-positive	*MPL* mutation positive	Mutation negative	Total
IMF	***RAS* mutant**	2	1	1	4
	***RAS* wild-type**	49	4	51	104
ET	***RAS* mutant**	0	0	0	0
	***RAS* wild-type**	58	5	60	123

Mutations in *RAS* were found in 4 of 104 IMF patients (including patient 1, Chapter 5), comprising 3 mutations at *NRAS* codon 12 and 1 mutation at *KRAS* codon 12 (Figure 6.4C). By contrast, *RAS* mutations were not identified in any of 123 patients with ET (p=0.04; Fisher's exact test). These data suggest that mutations in *RAS* may be preferentially associated with manifestation of a myelofibrosis phenotype.

6.6 Discussion

Analysis of individual progenitor colonies from 20 untreated ET patients identified a homozygous clone in 5 of 10 females but in none of 10 males. Of note, females with a homozygous clone had significantly higher haemoglobin and haematocrit levels compared to those without. Two factors may explain the discrepancy between these results and a previous report in which homozygous clones were not identified in ET patients[70]. Firstly, the mean number of colonies genotyped per patient was higher in this study (88 versus 40), suggesting that small homozygous clones may have been missed in the previous study. Secondly, the majority of patients in the previous study were receiving hydroxycarbamide. A recent report has shown a fall in peripheral blood *JAK2 V617F* mutant allele burden during hydroxycarbamide therapy[274], raising the possibility that this agent may reduce the proportion of homozygous and/or heterozygous colonies obtained in progenitor assays.

The observation that 50% of females with *JAK2 V617F*-positive ET harbour a *V617F*-homozygous clone, together with previous studies indicating that a proportion of PV patients do not have a homozygous clone[49,70], suggests the molecular relationship of PV to ET is more complex than the presence or absence of a *V617F*-homozygous

clone. Although biochemical and mouse studies have suggested a role for wild-type to mutant *JAK2* ratio in determining disease phenotype[53,54,71], it is likely that additional factors interplay to determine whether MPD patients manifest ET or PV. Inter-individual differences in erythropoietin and iron homeostasis may restrict erythropoiesis in some individuals, resulting in an ET phenotype despite the presence of a homozygous clone. Low iron stores are found in up to 40% of pre-menopausal and around 10% of post-menopausal females, but are rare in adult males[275,276], offering a potential explanation for the gender difference observed in this study. Genetic background may also play a role in phenotypic modulation, as specific single nucleotide polymorphisms in *JAK2* or the erythropoietin receptor have been associated with preferential development of ET or PV[73], and mouse models of *JAK2 V617F* associated disease show strain specific differences in phenotype[51,277]. It is also possible that a proportion of patients diagnosed with ET actually have PV, with a concomitant increase in plasma volume masking a raised red cell mass. A raised plasma volume is present in up to a third of patients with a raised red cell mass, particularly in the presence of splenomegaly, and may result in normal peripheral blood haemoglobin and haematocrit levels due to haemodilution[278-280]. Finally, although expression of *JAK2 V617F* is sufficient to produce an ET or PV phenotype in mouse models[53,54], it remains formally possible that additional acquired genetic lesions are present in human disease, and that such lesions play a role in altering the disease phenotype.

Despite their association with an MPD-like phenotype in murine models, *MPL T487A, JAK2 T875N* and *JAK2 R683* mutations were not identified in a large cohort of MPD patients. Mouse models differ from human disease in several important respects. In most mouse models, the oncogene is activated in a large number of cells, whereas human disease is thought to be initiated in a single cell with subsequent clonal expansion leading to manifestation of disease. Moreover in retroviral and transgenic models, both the timing and level of oncogene expression may be altered compared to expression from the endogenous locus.

Expression of the AML associated *FLT3-ITD* mutation in different mouse models is associated with subtle variations in disease phenotype (Table 6.6). This variability is likely to reflect differences in both the timing and magnitude of *FLT3* expression as well as variability in mouse genetic background. Of note, *FLT3-ITD* mutations have not been observed in human MPD, despite producing an ET-like phenotype in a transgenic model[281-283], but do occur as rare events in chronic myelomonocytic leukaemia, an MPD-MDS overlap syndrome with a similar phenotype to the knock-in mouse[284].

Table 6.6. Mouse models of FLT3-ITD associated disease

Method	Mouse strain	Phenotype
Retroviral transduction[285]	Balb/c	Neutrophilia, bone marrow fibrosis, splenomegaly
Transgenic: *vav* promoter[286]	FVB	Thrombocytosis, splenomegaly
Endogenous locus[284,287]	C57BL/6 or Balb/c	Neutrophilia, monocytosis, splenomegaly

There are several possible explanations for the absence of *MPL T487A, JAK2 R683* and *JAK2 T875N* mutations in human MPD. The mutations may be unable to initiate a clonal expansion when present in a single cell, or may occur only as secondary events within a malignant clone. Alternatively, such mutations may give rise to a different phenotype in humans to that observed in murine systems. Taken together, these findings suggest that mouse models may be limited in their ability to predict the precise phenotype of human disease.

RAS proteins are ubiquitously expressed components of intracellular signalling pathways. Oncogenic *RAS* mutations are amongst the most common alterations in human malignancy and result in constitutive activation of signalling cascades with consequent dysregulation of cellular processes such as proliferation and differentiation[288]. A vital role for RAS proteins in the haematopoietic system is suggested by murine knock-out models, with $KRAS^{-/-}$ and $KRAS^{+/-}NRAS^{-/-}$ mice dying during early embryogenesis due to severe anaemia[289], although both $NRAS^{-/-}$ and $HRAS^{-/-}$ mice develop without apparent abnormalities in the blood system[290,291]. RAS signalling downstream of the erythropoietin receptor appears to have a negative regulatory effect on erythroid differentiation, with chemical inhibition accelerating

erythroid differentiation[26] and expression of oncogenic RAS (*NRAS G12D*[292], *KRAS G12D*[293,294] or *HRAS G12*[295]) inhibiting erythroid differentiation with a block at the late erythroblast stage. By contrast, RAS signalling downstream of the MPL receptor appears to enhance megakaryocyte differentiation[296,297], with chemical inhibition leading to alterations in megakaryocyte ploidy[298,299] and expression of oncogenic RAS (*HRAS G12V*) promoting megakaryocyte differentiation and reducing nuclear ploidy[296]. Thus expression of oncogenic *RAS* in the haematopoietic system mimics several phenotypic features of human IMF, including suppression of erythropoiesis and enhanced megakaryopoiesis with reduced nuclear ploidy.

RAS mutations have previously been reported in a small proportion of patients with IMF, but not in association with ET or PV[269,270]. In this study, *RAS* mutations were detected in IMF patients with *JAK2* or *MPL* mutations, but not in patients with ET. Taken together with the effects of mutant *RAS* on haematopoiesis outlined above, these findings suggest that increased signalling via the RAS pathway may cooperate with mutations such as *JAK2 V617F* in the development of an IMF phenotype.

Chapter 7

Discussion: Models of disease progression and clonal diversity in the myeloproliferative disorders

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7.1 Introduction

The MPD are a group of stem cell neoplasms with similarities at the phenotypic and molecular level (Table 7.1). Phenotypically, these disorders are characterised by over-production of one or more mature myeloid elements with a variable tendency to develop acute leukaemia. At the molecular level, they are characterised by genetic alterations in signalling pathway molecules due to localised mutations or chromosomal alterations. Localised mutations result in either increased activity of the tyrosine kinase domain (*KIT D816V*)[300] or decreased activity of autoinhibitory regions (mutations in *JAK2* or *MPL*)[28,173]. Chromosomal alterations result in expression of a fusion protein which retains the signalling domain of the tyrosine kinase molecule, with the fusion partner usually providing a protein-protein interaction domain which mediates dimerisation (rearrangement of *ABL1, PDGFRB* or *FGFR1*)[223,301-307]. In some cases, however, the fusion partner contains no recognised dimerisation motif; rather the rearrangement results in loss of an autoinhibitory region from the tyrosine kinase partner (rearrangement of *PDGFRA*)[308]. The consequence of these genetic events is dimerisation of the signalling pathway molecule in the absence of cognate ligand binding, resulting in constitutive activation of downstream pathways and consequent dysregulation of vital cellular processes such as proliferation and differentiation.

Table 7.1. The chronic myeloproliferative disorders

Disease	Lineage involvement	Molecular lesions
Chronic myeloid leukaemia	Granulocyte +/- Platelet	*BCR-ABL1*
Chronic neutrophilic leukaemia	Neutrophil	*JAK2 V617F* (occasional cases)
Chronic eosinophilic leukaemia	Eosinophil +/- Mast cell	Rearrangement of: *PDGFRA* *PDGFRB* *FGFR1*
Polycythaemia vera	Erythrocyte +/- Platelet +/- Granulocyte	*JAK2 V617F* *JAK2* exon 12
Idiopathic myelofibrosis	Megakaryocyte +/- Granulocyte	*JAK2 V617F* *MPL* exon 10
Essential thrombocythaemia	Platelet +/- Granulocyte	*JAK2 V617F* *MPL* exon 10
Systemic mastocytosis	Mast cell +/- Eosinophil +/- Granulocyte	*KIT D816V*

The classical MPD, comprising ET, PV and IMF, share a tendency to undergo phenotypic shift, such that patients with ET may develop PV, and either ET or PV may progress to IMF. Thus ET, PV and IMF have traditionally been considered as three separate but related diseases, with a variable tendency to transform to AML (Figure 7.1). The identification of the same *JAK2 V617F* mutation in patients with ET, PV or IMF, however, has led to a rethinking of how these disorders are best classified. Specifically, are these three conditions separate diseases, or does the occurrence of the same genetic event in all three imply they are different phenotypic manifestations of the same disease process?

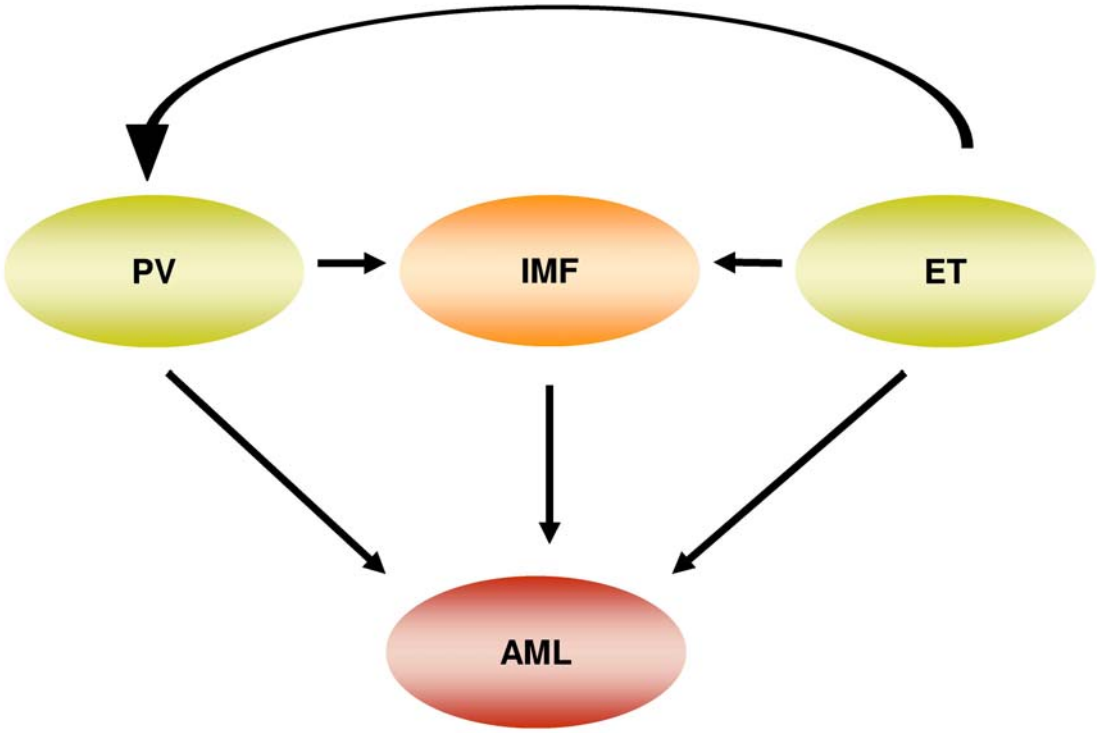

Figure 7.1. Traditional model to explain the relationship of the classical myeloproliferative disorders. PV: polycythaemia vera; IMF: idiopathic myelofibrosis; ET: essential thrombocythaemia; AML: acute myeloid leukaemia.

A precedent for a single genetic event in association with different phenotypic manifestations comes from CML (Table 7.2). CML is invariably associated with expression of a *BCR-ABL1* fusion gene, usually due to a balanced translocation between chromosomes 9 and 22. Prior to the advent of tyrosine kinase inhibitor therapy, the natural history of CML consisted of three phenotypically distinct phases. Most patients presented with chronic phase disease, characterised by over-production of mature myeloid elements with a good response to cytotoxic agents such as hydroxycarbamide. At a variable time from presentation, usually between 3 and 5 years, changes in the disease phenotype were observed, including an increase in immature cells or basophils, failure of agents such as hydroxycarbamide to control the myeloid proliferation or progressive bone marrow fibrosis. This accelerated phase of disease, lasting in the order of 3-6 months, was followed by the development of a fully malignant acute leukaemia (blastic phase disease). Of note, patients with CML may manifest chronic, accelerated or occasionally blastic phase disease at the time of presentation.

Table 7.2. Features of triphasic disease in chronic myeloid leukaemia

Chronic phase	Myeloid hyperplasia Good response to cytoreductive therapy
Accelerated phase	Increased proportion of immature cells Marked basophilia Refractory leucocytosis, thrombocytosis or splenomegaly Bone marrow fibrosis
Blastic phase	Acute leukaemia Extramedullary blast proliferation

Genetic lesions other than the *BCR-ABL1* fusion are uncommon in patients in chronic phase, with accumulation of karyotypic abnormalities associated with progression to accelerated and blastic phase disease[222,223]. Although the genetic events involved in disease progression are not fully understood[223], dysregulation of the WNT/β-catenin pathway may play an important role[309,310]. The gradual accumulation of genetic damage appears to be driven in part by the *BCR-ABL1* oncogene, expression of which leads to increased levels of intracellular reactive oxygen species with resultant damage to genomic DNA[311]. *BCR-ABL1* expression also modulates DNA repair pathways[312,313] and impairs the usual apoptotic response to genomic damage[314].

By analogy to CML, *JAK2 V617F*-positive ET, PV and IMF may also be considered as different phases of the same disease process (Figure 7.2). In this model, ET and PV represent phenotypic manifestations of chronic phase disease, characterised by the over-production of red cells and/or platelets with a good response to cytotoxic agents such as hydroxycarbamide (Table 7.3). Development of an IMF phenotype represents progression to accelerated phase disease, characterised by progressive bone marrow fibrosis and reduced therapeutic efficacy. Finally a proportion of patients will develop blastic phase disease, almost invariably AML. In common with CML, patients may manifest chronic or accelerated phase disease at initial presentation.

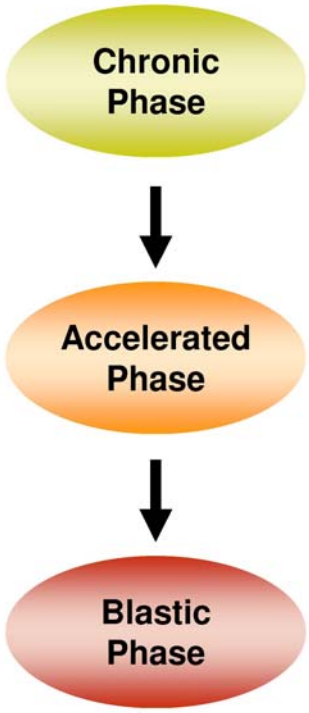

Figure 7.2. Triphasic model to explain the relationship of the classical myeloproliferative disorders.

Genetic lesions other than the *JAK2 V617F* mutation and associated chromosome 9p uniparental disomy are uncommon in chronic phase[69,221], with the prevalence of karyotypic aberrations increasing in patients with accelerated phase disease[224,273]. The precise events involved in progression to accelerated and blastic phase disease, however, are currently unknown. *JAK2 V617F* is associated with both increased DNA damage and modulation of DNA repair pathways, suggesting that expression of mutant JAK2 may lead to the acquisition of further genetic lesions[86]. It should be noted that whereas progression to blastic phase disease was seen in the vast majority of CML patients prior to the advent of tyrosine kinase inhibitor therapy, this is not the case in the MPD, where blastic phase disease complicates only 2-5% of ET or PV[76,77,92] and 15-30% of IMF[75,83]. It is probable, therefore, that *BCR-ABL1* and *JAK2 V167F* differ in their ability to drive the accumulation of further genetic damage.

Table 7.3. Features of triphasic disease in the classical myeloproliferative disorders

Chronic phase	Erythrocytosis Thrombocytosis
Accelerated phase	Bone marrow fibrosis Circulating progenitor cells Splenomegaly and extramedullary haematopoiesis Treatment refractory leucocytosis or splenomegaly Cytopenias unrelated to therapy
Blastic phase	Acute leukaemia Extramedullary blast proliferation

The first part of this section examines how data presented in this thesis contributes to a triphasic model of disease progression in the MPD, with reference to the association of *JAK2 V617F* with both ET and PV, the role of mutant *MPL* in MPD pathogenesis and the genetic lesions involved in progression to accelerated and blastic phase disease. The second part of this section discusses work presented in this thesis describing clonal diversity in the MPD, and how these findings can be integrated into current models of disease pathogenesis.

7.2 Towards a triphasic model of MPD pathogenesis

7.2.1 The JAK2 V617F mutation in chronic phase disease

The *JAK2 V617F* mutation is found in the majority of patients with PV and around half of those with ET. Biochemical studies[71] and mouse models[53,54] suggest the level of mutant *JAK2* expression may play a role in modulating disease phenotype, with lower levels favouring platelet over-production and higher levels favouring over-production of erythrocytes. Consistent with these observations, studies of progenitor colonies in human disease identified a *V617F*-homozygous clone in the majority of PV patients but not in those with ET[70].

Homozygosity for the *JAK2* mutation arises through a process of mitotic recombination between homologous chromosomes[28-31]. Background rates of mitotic

recombination in normal subjects differ over several orders of magnitude[72], suggesting that some individuals may be predisposed to undergo mitotic recombination and thus generate homozygous clones. Rates of mitotic recombination also differ between sexes, with higher rates reported in females[72]. Given the association of PV with the presence of a homozygous clone, however, this difference would predict an excess of PV in females; in reality PV appears more common in males[8]. Cell line studies have suggested that *JAK2 V617F* expression may increase the rate of mitotic recombination within the mutant clone[86]. If mitotic recombination was entirely driven by mutant JAK2, however, it would be expected that most if not all patients would harbour a homozygous clone. As such, it remains unclear why some patients generate *V617F*-homozygous clones and others do not.

The identification of *V617F*-homozygous progenitor colonies in 50% of female ET patients indicates that disease phenotype is more complex than the simple presence or absence of a homozygous clone (Chapter 6). ET patients with a homozygous clone had significantly higher haemoglobin levels than those without, consistent with an increased mutant to wild-type *JAK2* ratio resulting in a bias towards erythroid differentiation. The lack of a PV phenotype in these patients suggests that erythropoiesis may be restrained, for example by reduced iron stores. Sub-clinical depletion of storage iron is observed not infrequently in normal females, including post-menopausal subjects, but is a rare phenomenon in males[275,276]. Alternatively a raised red cell mass may be masked by an increase in plasma volume. Although assessment of cardiovascular parameters suggested that aging in normal females (but not males) may be associated with an increase in plasma volume[315], direct measurements were not made, and gender differences in the prevalence of masked PV have not been observed[278-280]. Other potential contributing factors include differences in genetic background[73] or the presence of additional phenotype modulating lesions, for example secondary to 9p loss of heterozygosity. Whereas LOH in *V617F*-homozygous clones invariably extends from the *JAK2* locus to the telomere, extension in the centromeric direction is highly variable, covering between 2 and 40 megabases[31,316]. Given that polymorphisms within this region will become homozygous and expression of imprinted genes will be altered, it is theoretically possible that 9p LOH generates additional genetic alterations that act in concert with mutant JAK2. As such, the extent of 9p LOH may play a role in modulating disease phenotype.

Within the Cambridge MPD cohort, trisomy 9 was present in 6% of *JAK2 V617F*-positive patients, usually associated with a PV phenotype (Table 7.4). Trisomy 9 results in duplication of the chromosome bearing the *JAK2 V617F* mutation (Chapter 4), and as such plays a similar role to mitotic recombination, giving rise to a subclone bearing two copies of the mutant *JAK2* allele. Although cell line studies have suggested expression of wild-type *JAK2* may inhibit the transforming effects of *JAK2 V617F*[28], PV patients with trisomy 9 retain the wild-type allele, and thus loss of wild-type *JAK2* is not essential for the development of PV. Of note, trisomy 9 was present in a female patient with ET, in whom diagnostic haemoglobin and haematocrit levels were within normal limits. Similar to patients with a *V617F*-homozygous clone, therefore, duplication of the mutant *JAK2* allele in patients with trisomy 9 is not invariably associated with a PV phenotype.

Table 7.4. Prevalence of trisomy 9 in Cambridge MPD patients with ET or PV

	JAK2 V617F	*JAK2*-exon 12	*JAK2*-wild-type
PV	6/68 (9%)	0/10 (0%)	-
ET	1/44 (2%)	-	0/48 (0%)
Total	7/112 (6%)	0/10 (0%)	0/48 (0%)

In summary, studies presented herein indicate that female ET patients may harbour a clone that is homozygous for the *JAK2 V617F* mutation. Similarly the presence of an additional copy of chromosome 9, leading to duplication of the *JAK2 V617F* allele, may be associated with either PV or ET. Although biochemical and murine studies have indicated a role for mutant allele gene dosage in the determination of disease phenotype, other factors are likely to be of importance, including erythropoietin and iron homeostasis, patient genetic background, changes in plasma volume and possibly the presence of additional acquired genetic lesions (Figure 7.3).

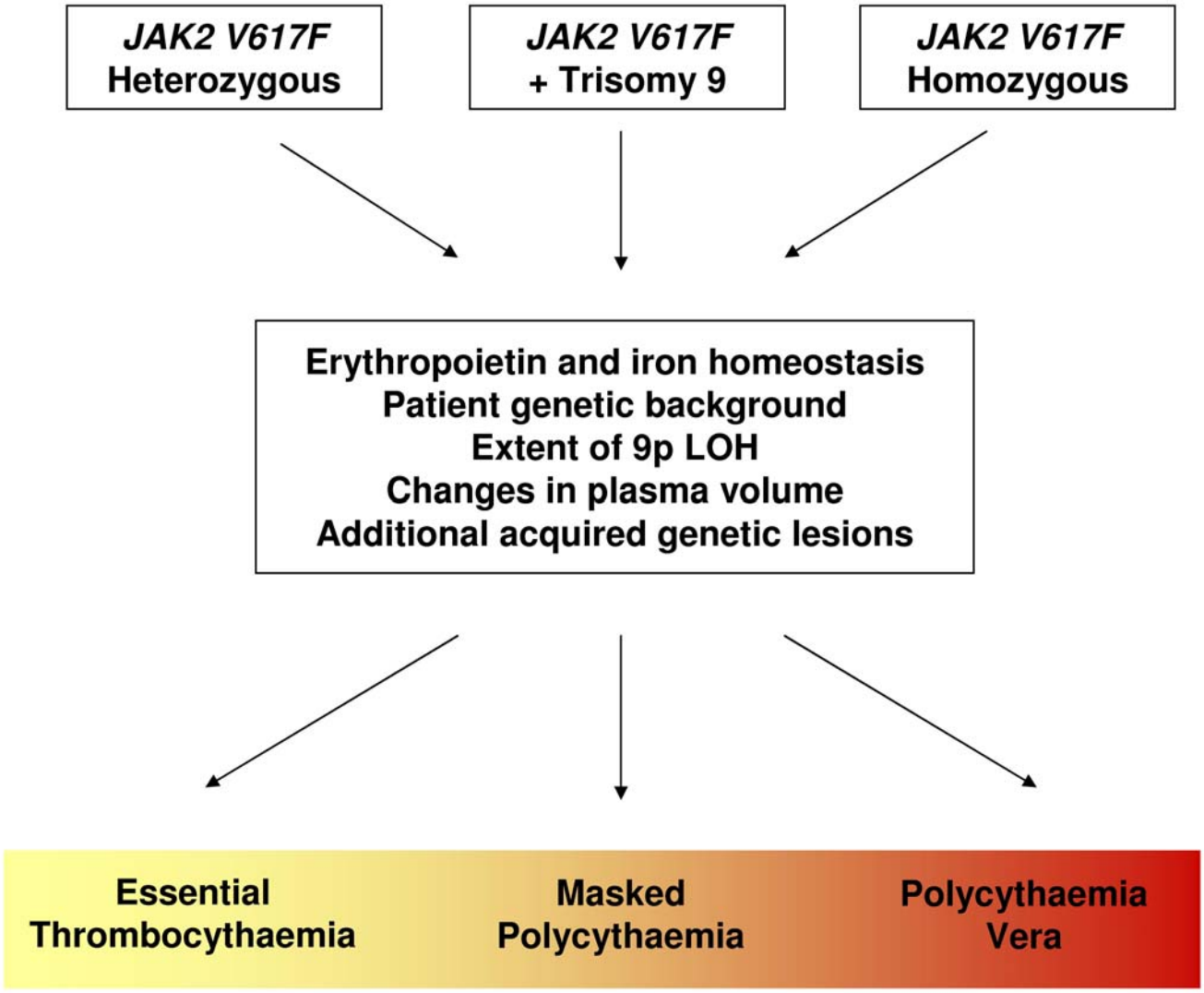

Figure 7.3. Interplay of genetic lesions and modulating factors in the determination of a polycythaemic versus thrombocythaemic phenotype. LOH: loss of heterozygosity.

7.2.2 MPL exon 10 mutations in essential thrombocythaemia

At the time of these studies, little was known about the prevalence and clinical significance of *MPL* mutations in patients with ET. Three different mutations in *MPL* exon 10 were seen in patients with ET or IMF (*S505N, W515L* and *W515K*) whereas mutations outside *MPL* exon 10, restricted to the megakaryocyte lineage or in patients with PV were not identified (Chapter 3). A previous study had found *MPL* mutations rarely in patients with ET, leading to the suggestion that these alleles may be specifically associated with IMF[175]. Using sensitive assays, however, *MPL* mutations were identified in 8.5% of *JAK2 V617F*-negative ET patients. Moreover *MPL* mutant patients showed typical laboratory and histological features of ET without an increased risk of progression to IMF, indicating that *MPL* mutations are associated with genuine ET. Although *MPL* mutations do not appear to define a specific clinical or histopathological sub-type of ET, their detection provides a useful new tool in the diagnostic armamentarium.

An *MPL S505N* mutation, previously reported as an inherited allele in familial thrombocytosis[172], was identified as an acquired mutation in MPD patients (Chapter 3). Mutations in *KIT* or *KRAS* are also associated with both inherited and acquired forms of myeloid disease, occurring in mast cell disease[202-211] and juvenile myelomonocytic leukaemia[317-319] respectively. In contrast to the *MPL S505N* allele, however, inherited and acquired mutations in *KIT* or *KRAS* are mutually exclusive, with inherited mutations not found in acquired disease and *vice versa* (Figure 7.4). Although inherited and acquired *KRAS* alleles are associated with similar biological sequelae such as increased cellular proliferation and cytokine hypersensitivity, inherited alleles are consistently weaker in their transforming effects[232,317]. It may be that more strongly activating acquired alleles would not be tolerated in the germline, or conversely that weaker inherited alleles may not be of sufficient strength to initiate malignancy if acquired in a single cell. As such there are at least two potential explanations for the occurrence of the *MPL S505N* mutation in both acquired and inherited forms of disease. Firstly, the *S505N* allele may be weak enough to be tolerated in the germline but require additional genetic events in order to drive an acquired clonal expansion. Secondly, the restriction of *MPL* expression to the haematopoietic compartment (in contrast to both *KIT* and *KRAS*) may allow a

mutation of sufficient strength to drive an acquired clonal expansion to be tolerated as a germline mutation.

7.2.3 MPD patients without an identified mutation

The *JAK2 V617F* mutation is found in the vast majority of patients with PV, and many of the remainder harbour mutations in *JAK2* exon 12[29,105]. Analysis of the Cambridge MPD cohort identified *JAK2* mutations in all 114 patients who met PVSG criteria for PV, with 97% harbouring a *JAK2 V617F* mutation and 3% a *JAK2* exon 12 mutation[176]. Therefore most, if not all, PV patients harbour mutations in *JAK2*.

The prevalence of both *JAK2 V617F* and *MPL* exon 10 mutations is similar in ET compared to IMF, with known mutations accounting for around 60% of such patients (Chapter 3). Despite their association with an MPD-phenotype in mouse models, *MPL T487A, JAK2 T875N* and *JAK2 R683* mutations were not identified in MPD patients (Chapter 6). Analysis of clinical and laboratory features of mutation negative ET patients in the PT-1 cohort revealed similar laboratory features to the *MPL*-mutant patients (Chapter 3). Specifically, mutation negative ET patients manifest a megakaryocyte restricted MPD, in contrast to the trilineage myeloid proliferation associated with the *JAK2 V617F* mutation[68]. Mutation negative ET may therefore be considered a phenocopy of *MPL*-mutant ET, with the initiating genetic events likely affecting components of the MPL signalling pathway.

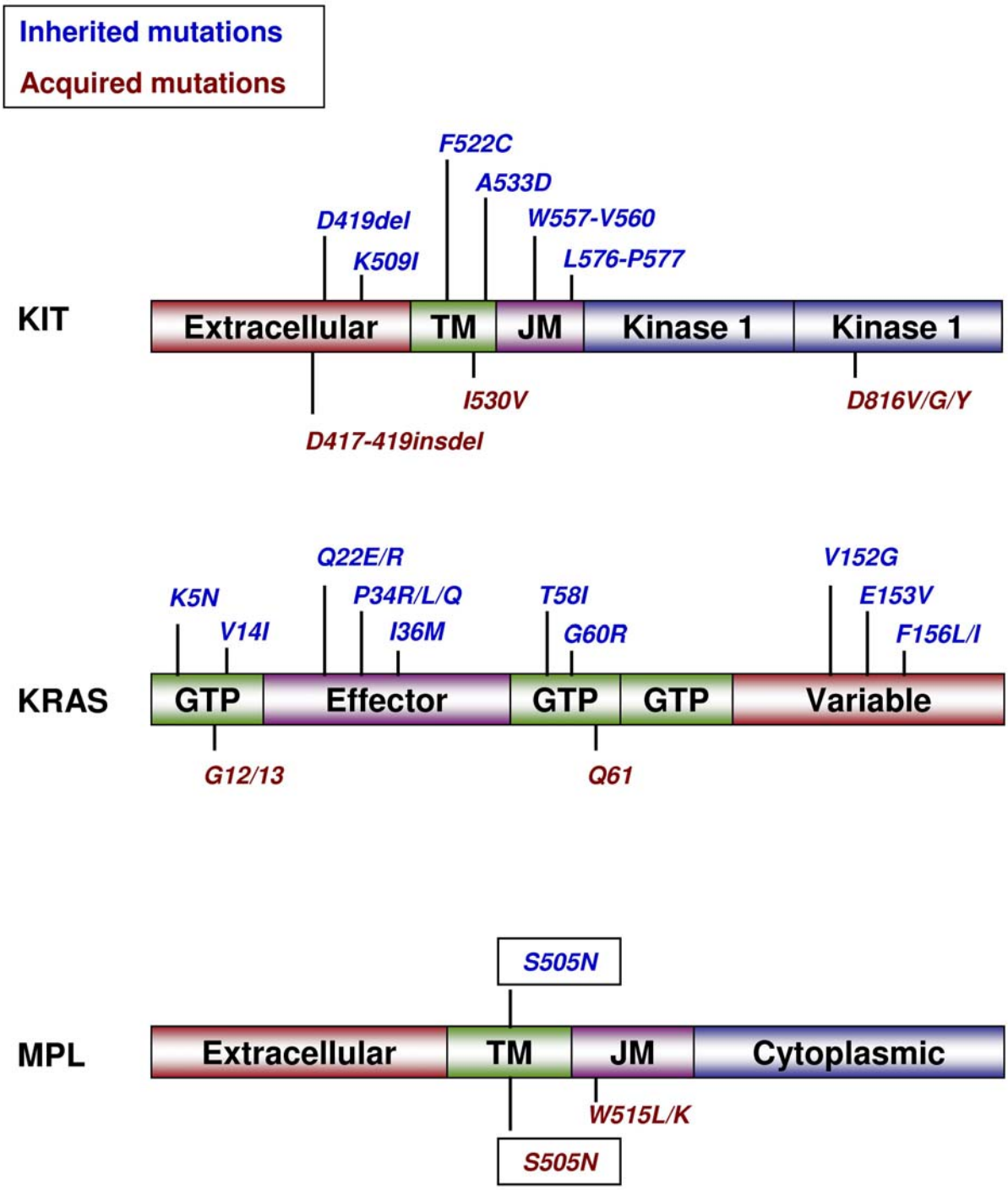

Figure 7.4. Acquired and inherited mutations in *KIT*, *KRAS* and *MPL*.

7.2.4 Molecular lesions associated with accelerated phase disease

Little is known about the precise genetic events associated with the development of accelerated and blastic phase disease. Expression of either *JAK2 V617F* or *MPL W515L* in mouse models leads to an IMF-like syndrome including bone marrow fibrosis, splenomegaly and falling haemoglobin levels[50,53,174]. The relatively short latency between disease initiation and development of bone marrow fibrosis in these models is consistent with a direct role for JAK2 signalling in disease progression, although it should be noted that retroviral models are generally associated with oncogene over-expression. A role for increased JAK2 activity in human MPD is suggested by studies demonstrating methylation of *SOCS* family gene promoters[320,321]. SOCS proteins (suppressors of cytokine signalling) function as negative regulators of JAK2, such that decreased SOCS expression may be expected to result in increased JAK2 activity[322,323]. Data are conflicting, however, as to whether SOCS proteins are able to suppress the activity of mutant JAK2[41,320]. Although it is possible that increased activity of mutant JAK2 is associated with progression to IMF, this is yet to be demonstrated in human disease.

Both RAS and STAT5 signalling pathways are activated by mutant JAK2, with studies suggesting a central role for STAT5 activation in the phenotype of chronic phase disease[42,71]. Oncogenic *RAS* mutations were identified in 4% of patients with IMF but not in those with ET (Chapter 6). In murine models, expression of oncogenic RAS results in suppression of erythroid differentiation, augmentation of granulocyte proliferation and increased megakaryocyte proliferation with abnormalities of nuclear ploidy[292-295], features also associated with human IMF. Taken together, these findings suggest a potential role for increased signalling via the RAS pathway in progression to accelerated phase disease (Figure 7.5).

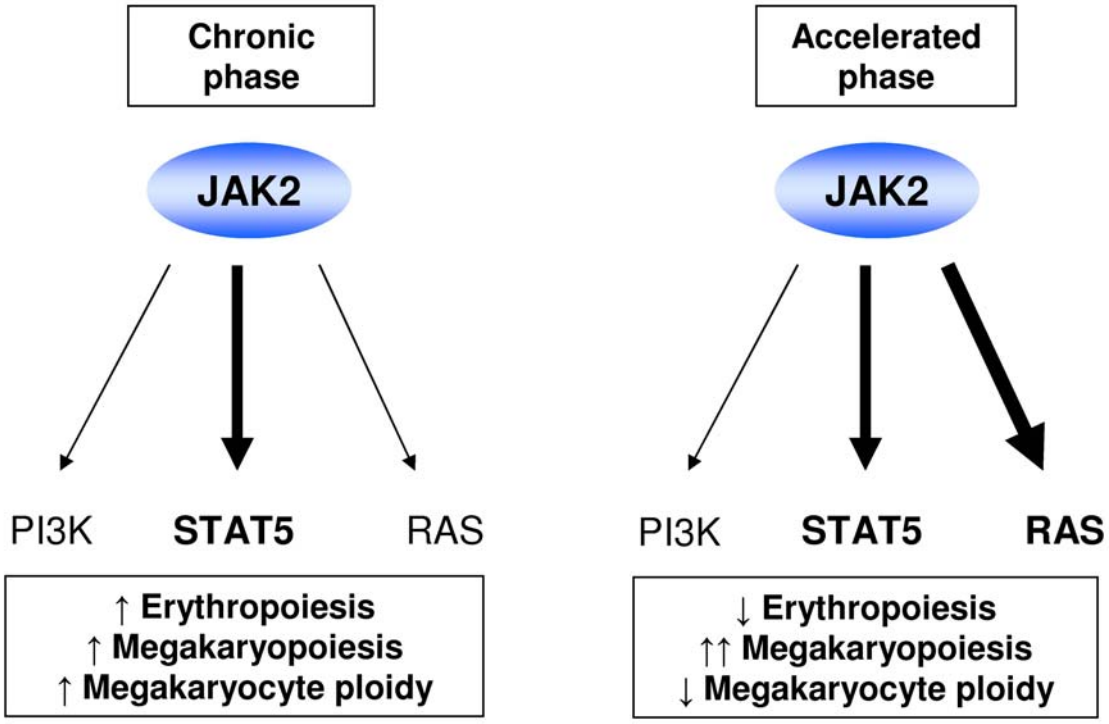

Figure 7.5. Increased signalling via the RAS pathway may play a role in progression to accelerated phase disease.

In *MPL W515L*-positive disease, a higher mutant allele burden was seen in IMF compared to ET patients (0.59 ± 0.27 versus 0.17 ± 0.11 respectively; p=<0.0001). Specifically, 4 of 6 IMF patients had a mutant allele burden of greater than 50%, indicating the presence of subclones with reduced or absent wild-type allele. Studies identified mitotic recombination leading to duplication of the mutant allele as the mechanism responsible in one of these patients. Homozygous clones, however, were rare in *MPL W515L*-positive ET patients (Chapter 3), suggesting that homozygosity for the *MPL W515L* mutation may be associated with the development of myelofibrosis.

7.2.5 Molecular lesions associated with blastic phase disease

Patients with *de novo* AML often harbour genetic lesions of two distinct types. Type 1 lesions, resulting in signalling pathway hyperactivity, lead to increased cellular proliferation. Type 2 lesions, often due to altered activity of DNA transcription factors, lead to a block in cellular differentiation[231]. In this model, *JAK2 V617F* could function as a type 1 lesion driving the proliferation of the leukaemic clone, and indeed a small proportion of *de novo* leukaemias are *JAK2 V617F*-positive[60,61,65,66]. Of 16 *JAK2 V617F*-positive patients who developed blastic phase disease, 7 (44%) were positive for the *JAK2 V617F* mutation (Chapter 5). In 6 of these 7, leukaemic blasts were homozygous for the *JAK2 V617F* mutation, suggesting that either loss of wild-type or increased mutant JAK2 may be important for the development of blastic phase disease. Type 2 lesions were identified in 5 of 7 patients with a *JAK2* mutant leukaemia, comprising 4 with *RUNX1* point mutations and 1 with over-expression of *EVI1*. Of note, genetic lesions leading to disruption of the RUNX1 DNA-binding complex have also been associated with *JAK2 V617F* in *de novo* AML[61,65,66], suggesting that mutant JAK2 and RUNX1 may cooperate in the development of blastic phase disease.

6 of 7 patients with *V617F*-positive AML had a phenotypically defined preceding MPD, with all cases showing evidence of accelerated phase disease. This finding lends support to a triphasic model of *JAK2 V617F* associated disease, where accumulation of genetic events is associated with phenotypic progression from chronic phase via an accelerated phase to blastic phase disease. In a patient with *de novo* AML, bone marrow histological appearances and established *JAK2 V617F*-positive erythropoiesis suggested the presence of a prior MPD. Thus patients with a *JAK2 V617F*-positive MPD may present with blastic phase disease, a situation also observed in patients with CML. 2 of 7 patients with *JAK2 V617F*-positive leukaemia had received no prior cytoreductive therapy, indicating that progression to blastic phase is part of the natural history of *JAK2 V617F*-positive disease, and not necessarily the consequence of previous therapy[97]. Mirroring the situation with *JAK2 V617F*, the *MPL W515L* mutation may also be present in chronic, accelerated and blastic phase disease (Chapter 3).

7.2.6 Towards a triphasic model of MPD pathogenesis: summary

In conventional models of MPD pathogenesis, ET, PV and IMF were considered as distinct but related disorders. In the revised, triphasic model of disease the three different phenotypes, along with progression to acute leukaemia, are considered as different manifestations of a single disease process. Consistent with this notion, both the *JAK2 V617F* and *MPL W515L* mutations may be present in chronic, accelerated and blastic phase disease (Figure 7.6). It is currently unclear how the *JAK2 V617F* mutation is associated with either an ET or PV phenotype, although the explanation appears more complex than the presence or absence of a homozygous clone. Potential events associated with the development of accelerated phase disease include increased dosage of the *MPL W515L* allele or increased signalling via the RAS pathway. Mutations in *RUNX1* occur as recurrent lesions in blastic phase disease, and may cooperate with mutant JAK2 in the development of acute leukaemia. *JAK2 V617F*-positive patients who develop blastic phase disease generally progress via a phenotypically apparent accelerated phase, consistent with a step-wise acquisition of genetic events driving disease progression.

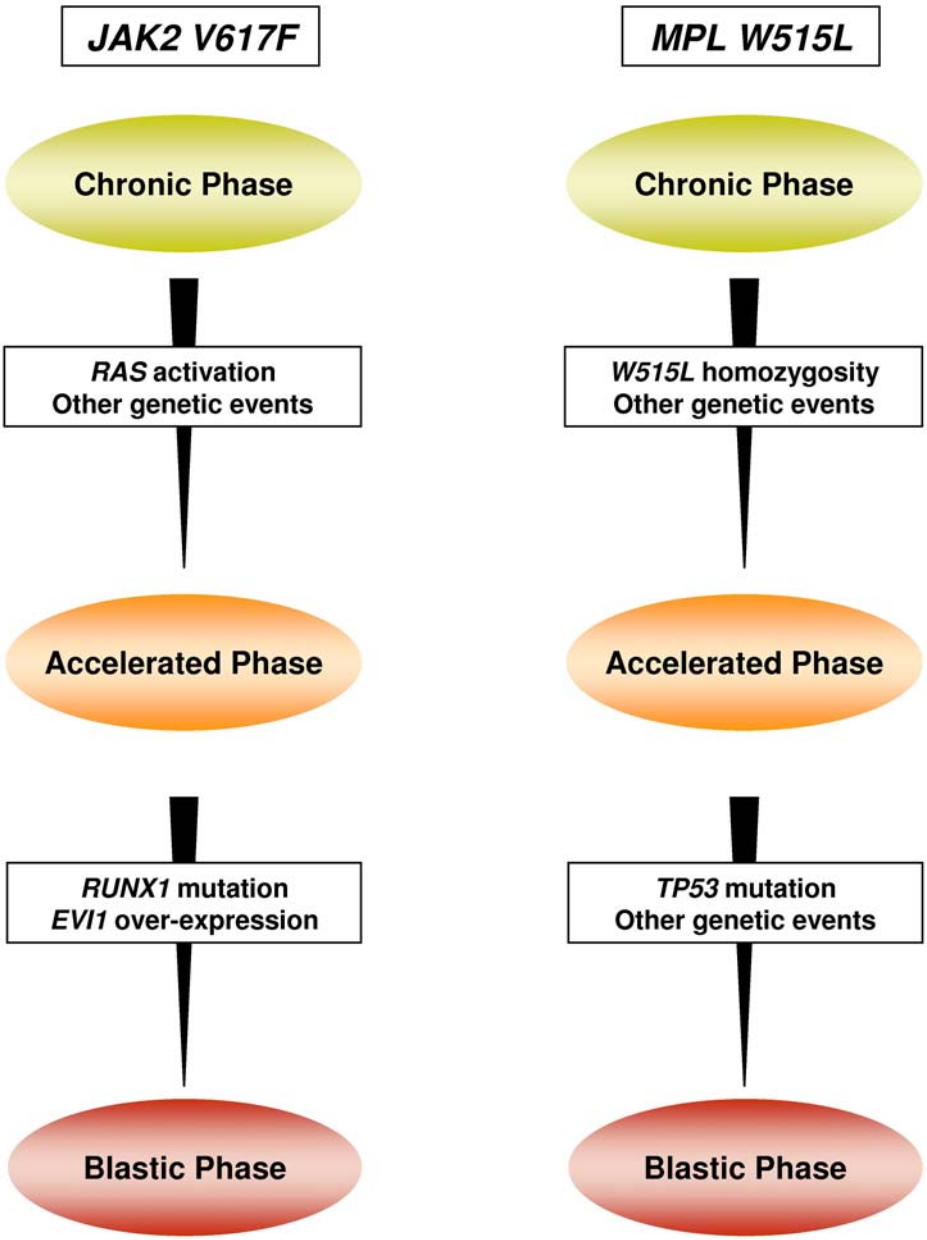

Figure 7.6. *JAK2 V617F* and *MPL W515L* mutations may be associated with chronic, accelerated or blastic phase disease, with the accumulation of further genetic events associated with disease progression.

7.3 Clonal diversity in the myeloproliferative disorders

Neoplastic proliferations are thought to be initiated in a single cell, with step-wise accumulation of additional genetic events leading to a fully malignant phenotype[215]. CML and the MPD are premalignant haematopoietic disorders often associated with constitutive activation of intracellular signalling pathways. In CML, the *BCR-ABL1* fusion results in activation of the ABL1 cytoplasmic tyrosine kinase[223], and the majority of MPD patients harbour activating mutations in *JAK2* or *MPL*[29,176] (Chapter 3). The clonal nature of both CML and the MPD has been established by studies of X-chromosome inactivation patterns in female patients[21,22,324], and expression of *BCR-ABL1*, mutant *JAK2* or mutant *MPL* in murine bone marrow recapitulates many features of the counterpart human disease[50,105,174,325]. Taken together, these studies imply a single cell of origin for CML and the MPD, and support a central role for alterations of *ABL1*, *JAK2* or *MPL* in disease pathogenesis.

Although a triphasic model of disease progression explains many of the biological and phenotypic features of CML and the MPD, studies have suggested that a higher degree of complexity exists in human disease. A familial tendency to develop an MPD has been identified by registry data and kindred studies, with affected family members showing clonal haematopoiesis and acquired mutations such as *JAK2 V617F*, indicative of a genuine MPD[15-19]. These findings have led to the suggestion that mutations in *JAK2* are insufficient alone to produce an MPD phenotype, with additional genetic alterations being inherited in familial cases and acquired in sporadic disease. In a proportion of patients with CML or an MPD, studies of X-chromosome inactivation pattern (XCIP) in immortalised B-cells (CML)[186,187] or granulocytes (MPD)[177,178] have suggested clonal haematopoiesis in excess of that accounted for by the mutation bearing clone (*BCR-ABL1* or *JAK2 V617F* respectively). CML patients treated with imatinib (a tyrosine kinase inhibitor) may develop *BCR-ABL1* negative clones which harbour acquired chromosomal abnormalities[188-190], and along with the XCIP studies may be interpreted as evidence for clonal haematopoiesis prior to the acquisition of *BCR-ABL1* or a *JAK2* mutation. In the MPD, growth of *JAK2* wild-type endogenous erythroid colonies (EEC) has been reported in patients with a *JAK2 V617F*-positive MPD[184,185]. As EEC growth is not a feature of normal haematopoiesis, such findings suggest the presence of additional genetic alterations in these patients. Similarly patients with a *JAK2 V617F*-

positive MPD may progress to acute leukaemia that is negative for the *JAK2* mutation[102,103]. Finally, a proportion of patients may harbour more than one disease associated genetic lesion, including *JAK2 V617F* in combination with *BCR-ABL1*, *MPL* or *JAK2* exon 12 mutations[60,175,185,227].

There are two main explanations for the observations outlined above: (1) MPD associated genetic lesions are not the disease initiating events, and/or (2) a proportion of patients harbour more than one clonal expansion. In order to examine the role of clonal heterogeneity in MPD pathogenesis, studies were undertaken in two groups of patients: those with more than one acquired genetic alteration (Chapter 4) and those with progression to acute leukaemia following a *JAK2 V617F*-positive MPD (Chapter 5).

In patients with more than one acquired genetic alteration, cytogenetic abnormalities were usually found in the same clone as a signalling pathway mutation (*BCR-ABL1* or mutations in *JAK2* or *MPL*). In one case, a deletion of chromosome 20q preceded the acquisition of *BCR-ABL1*. As such, *BCR-ABL1* was acquired as a secondary event in an abnormal haematopoietic clone. Deletions of 20q are the most common chromosomal alteration in the MPD[229], and have also been reported in CML[228]. The functional consequences of this abnormality, however, are currently unknown. Of note, in a second patient a *JAK2 V617F* mutation and a 20q deletion were present in separate clones. It is possible, therefore, that 20q deletions may serve as clone-initiating events, with subsequent mutations such as *BCR-ABL1* giving rise to the specific disease phenotype. However acquisition of a 20q deletion after a *JAK2* mutation was also observed in these studies. There are at least three potential explanations for this inconsistency: (i) both the 20q deletion and *JAK2* mutation are able to initiate clonal haematopoiesis, (ii) both lesions are acquired as secondary events within a pre-existing clonal expansion, or (iii) deletions of 20q are markers of a genetically unstable clone with little or no functional consequence.

In contrast to patients with cytogenetic aberrations, the presence of two signalling pathway mutations (mutations in *JAK2* or *MPL*) indicated the presence of a second clonal expansion in all cases studied. Moreover in 2 patients, studies of XCIP demonstrated that the two mutations had arisen in unrelated stem cells. In the

remaining 4 patients, it is not known whether the two clones arose independently or from a shared founder clone.

In studies of patients progressing to acute leukaemia following a *JAK2 V617F*-positive MPD, over half were negative for the *JAK2* mutation in leukaemic blasts (Chapter 5). Reversion to wild-type by mitotic recombination, gene conversion or gene deletion was excluded in all cases. Differences in the clinical features of *V617F*-negative versus *V617F*-positive transformations may provide clues to the biological differences between the groups. Firstly, patients progressing to *V617F*-negative AML were significantly older at the time of disease progression. Secondly, whereas patients with *V617F*-positive AML displayed features of accelerated phase disease prior to transformation, this was not necessarily the case with *V617F*-negative AML, with the majority of patients progressing directly from chronic to blastic phase disease. The lack of accelerated phase disease suggests that the MPD and AML clones developed as parallel and separate entities; whilst evolution within the AML clone leads to the development of a fully malignant leukaemia phenotype, the MPD clone may remain genetically stable without evidence of disease progression. As such, there are two possible explanations for the development of *V617F*-negative AML following a *V617F*-positive MPD: either (i) the two phases of disease are genetically related, representing the progeny of shared founder clone, or (ii) the diseases are unrelated, having arisen in independent stem cells.

The coexistence of separate MPD clones and progression to *JAK2 V617F*-negative AML may both be conceived as manifestations of a biclonal disease process (Figure 7.7). In this model the two clones, which may be phylogenetically related or unrelated, harbour phenotypic mutations resulting in either an MPD or AML phenotype. Genetically related clones represent the progeny of a shared founder clone. This founder clone harbours a ‘clone-initiating mutation’: a hypothetical genetic event leading to the onset of clonal haematopoiesis, but not necessarily associated with the phenotypic manifestations of the disease. Different phenotypic mutations are subsequently acquired in progeny of the founder clone, giving rise to genetically diverse but related daughter clones. The phenotypic mutations, whilst directly implicated in the biological and clinical features of the resulting disease, may not be directly involved in the proliferation of the abnormal stem cell clone. Indirect evidence cited in support of clone-initiating mutations includes discrepant XCIP patterns in

CML and MPD patients, cytogenetically abnormal *BCR-ABL1* negative clones in CML and the growth of *JAK2 V617F*-negative EEC from patients with a *JAK2 V617F*-positive MPD. Each of these observations, however, is equally well explained by the presence of a second clonal expansion which is not necessarily genetically related to the original clone. Recent work presented in abstract form identified mutations in the *TET2* gene in a proportion of patients with an MPD[326]. Of note, *TET2* mutations preceded the acquisition of a phenotypic mutation (in this case *JAK2 V617F*). Little is known about the TET2 protein, although the identification of nonsense and biallelic mutations suggests tumour suppressor activity. These preliminary data suggest that alterations in *TET2* may serve as clone-initiating mutations in a proportion of patients, and work is underway to identify *TET2* mutations in patients with biclonal disease and *JAK2 V617F*-negative leukaemia following a *JAK2 V617F*-positive MPD.

In the alternative model, phenotypic mutations are acquired in genetically unrelated stem cells (Figure 7.7). Given that the prevalence of biclonal disease (either two MPD clones or progression to *V617F*-negative AML) appears higher than expected by chance alone, this model would predict a predisposition to the acquisition or survival of such clones. It is possible that exposure to an environmental clastogen leads to the acquisition of both phenotypic mutations. Alternatively patients may harbour an inherited stem cell defect that leads to an increased rate of acquisition and/or survival of abnormal clones. Data presented in this thesis provides direct evidence for the presence of genetically unrelated clones in patients with two phenotypic mutations (Chapter 4).

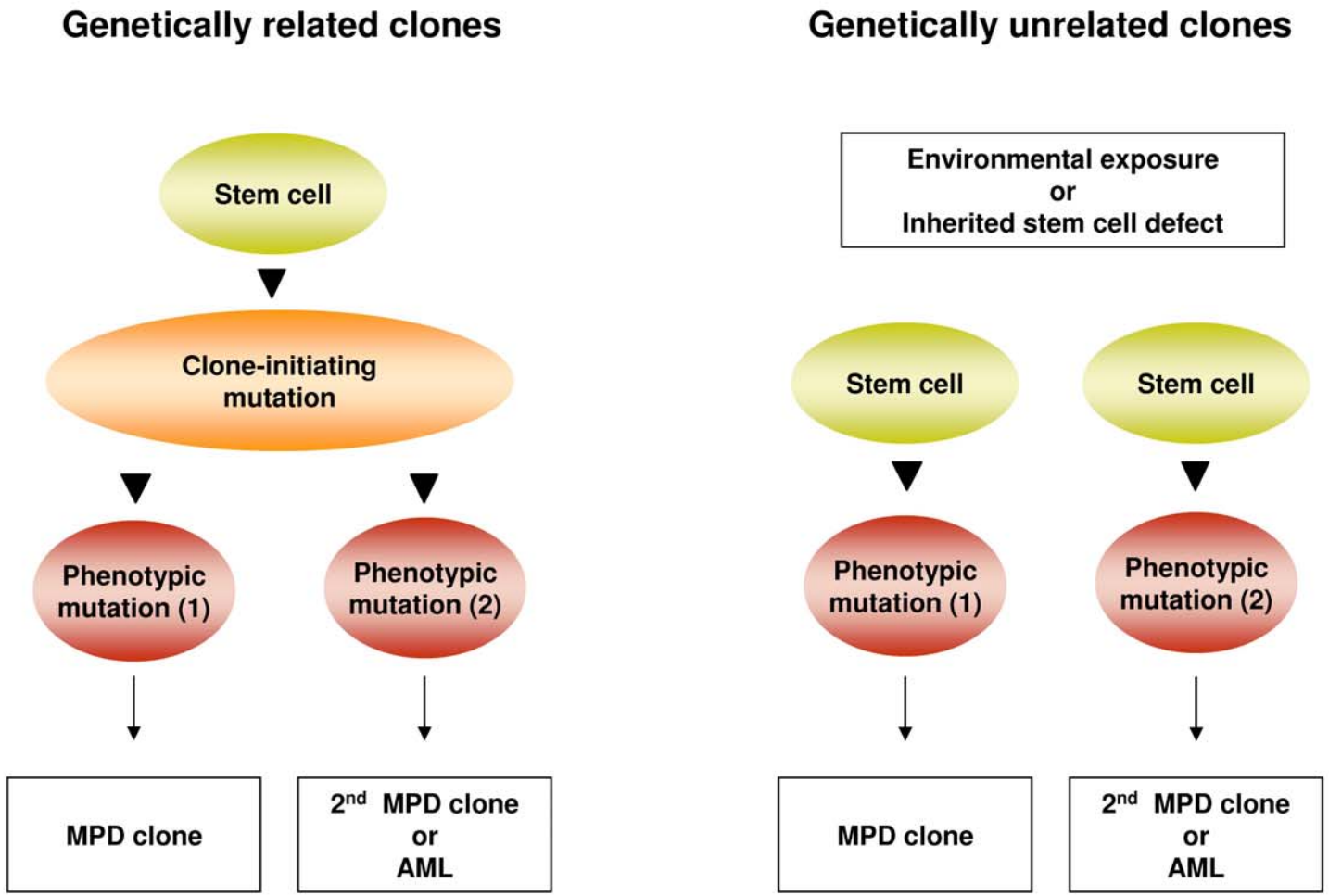

Figure 7.7. Origins of biclonal disease from a shared founder clone or genetically unrelated stem cells

7.4 Conclusions

Our understanding of MPD pathogenesis has advanced considerably since the identification of the *JAK2 V617F* mutation. As well as leading to improvements in patient diagnosis and raising the hope of targeted molecular therapy, the association of specific mutations such as *JAK2 V617F* with apparently distinct MPD phenotypes has stimulated discussion as to how these disorders are best classified. A triphasic model of disease progression explains many of the phenotypic features of the MPD, with *JAK2 V617F* and *MPL W515L* mutations present in chronic, accelerated and blastic phase disease. The mechanisms by which the *JAK2 V617F* mutation is associated with two distinct manifestations of chronic phase (ET or PV) remain to be fully elucidated, but appear more complex than the presence or absence of a homozygous clone. Progression to accelerated phase disease may be associated with increased signalling via the RAS pathway or an increase in mutant to wild-type ratio in patients with an *MPL W515L* mutation. In blastic phase disease, genetic lesions involving transcription factors such as RUNX1 or EVI1 likely cooperate with mutant JAK2 to produce a fully malignant acute leukaemia phenotype.

The presence of clonal diversity, however, suggests that a triphasic model represents an oversimplification of disease pathogenesis in a proportion of patients. In some cases, the presence of two genetically unrelated clones implicates either environmental exposure or an inherited stem cell defect in the genesis of disease. In other cases, the phenotypic mutation appears not to be the clone-initiating event. These observations are particularly prescient in the era of targeted molecular therapy, as the use of JAK2 inhibitors may permit continued proliferation of either a founder clone or a second clonal proliferation. The identification and study of potential clone-initiating mutations and inherited predisposition alleles will not only improve our understanding of disease biology, but also help to guide the development of effective molecular therapies.

Appendix 1

PCR primers

Chapter 3

Primer name	Primer sequence	Product size	Annealing temp	PCR conditions
Mutation detection (direct sequencing)				
Mpl_FL_cDNA_Fa	ACACAGTGGCGGAGAAGATG			
Mpl_FL_cDNA_Ra	AGACTGGTCCAGAGCAGAGG	644	62	Standard
Mpl_FL_cDNA_Fb	GAGCCAGCTCCAGAAATCAG			
Mpl_FL_cDNA_Rb	GAGCCCAGGTAGCTGTGAA	677	62	Standard
Mpl_FL_cDNA_Fc	TACCCCATCTGGGAGAACTG			
Mpl_FL_cDNA_Rc	GAAACTGCCACCTCAGCAG	571	62	Standard
Mpl_FL_cDNA_Fd	GCGATCTCGCTACCGTTTAC			
Mpl_FL_cDNA_Rd	AGGGAACTGGGAGTGAGGAG	577	62	Standard
MPL_e10_DNA_F	AGTAGGGGCTGGCTGGAT			
MPL_e10_DNA_R	GCGGTATAGTGGGCGTGTTA	364	58	Standard
MPL_e10_cDNA_F	GCCCCACCTACCAAGGTC			
MPL_e10_cDNA_R	GGCTGCAGTGTCCCTAAGGT	245	58	Standard
SNP Genotyping				
MPL_SNP_1_F	GCTGGCAAAAACGTCTCCT			
MPL_SNP_1_R	TCATTGATGGACATTTAGGGTTT	623	58	Standard
MPL_SNP_2_F	GACGTAGGCTCCTCAACACC			
MPL_SNP_2_R	TTAAGTCAAGGCGAGTGCAG	482	58	Standard
Allele-specific PCR				
MPL_e10_control_F	AGTAGGGGCTGGCTGGAT			
MPL_e10_control_R	GCGGTATAGTGGGCGTGTTA			
MPL_W515L	CCTGCTGCTGCTGAGGTT	364+234	58	Standard
MPL_W515K	CCTGCTGCTGCTGAGGAA	364+234	58	Standard
MPL_S505N	TCTAGTGCTGGGCATCAA	364+263	58	Standard
JAK2_e14_F	ATCTATAGTCATGCTGAAAGTAGGAGAAAG			
JAK2_e14_R	CTGAATAGTCCTACAGTGTTTTCAGTTTCA			
JAK2_e14_V617F	AGCATTTGGTTTTAAATTATGGAGTATATT	364+206	58	Standard
Pyrosequencing				
W515_PY_F1	TTGGTGACCGCTCTGCATCT			
W515_PY_R3	7GCGGTGGACGGAGATCTG	193	60	Standard
W515_PY_S	CCTGCTGCTGCTGAG			
S505N_PY_F	GTCTGACCCTTTTTGTCTCCTAGC			
S505N_PY_R	7GGGGCGGTACCTGTAGTGT	130	60	Standard
S505N_PY_S	TAGTGCTGGGCCTCA			
MPLV114M_PY_F	TGCACCTCTGGGTGAAGAA			
MPLV114M_PY_R	7TGACAGGAGGATGGCTCTTA	93	58	2mM Mg^{++}
MPLV114M_PY_S	CCTCTGGGTGAAGAAT			
Real-time PCR				
C13_copy_F	CCATGGACGACGGGTTTCT			
C13_copy_R	TGTACAGGACGTAGGAGGGTGA	51	60	100nM primer
MPL_copy_F1	CCAGCTCCAGCCCTACATAA			
MPL_copy_R1	TCTCACTGCCTGGGATAGGT	51	60	100nM primer
Cloning				
M13F	GTAAAACGACGGCCAGT			
M13R	CAGGAAACAGCTATGAC	variable	56	

Chapter 4

Primer name	Primer sequence	Product size	Annealing temp	PCR conditions
***BCR-ABL1* detection**				
BCR_b2_F	CAGATGCTGACCAACTCGTGT			
ABL_a3_R	TTCCCCATTGTGATTATAGCCTA			
ABL_cDNA_F	TGCGTGAGAGTGAGAGCAGT	119 +	65	2.5mM Mg^{++}
ABL_cDNA_R	GCTCTCGGAGGAGACGTAGA	200-400		1' ext/ann
Mutation detection (direct sequencing)				
JAK2_e14_F	ATCTATAGTCATGCTGAAAGTAGGAGAAAG			
JAK2_e14_R	CTGAATAGTCCTACAGTGTTTTCAGTTTCA	364	58	Standard
JAK2_e12_F	CTCCTCTTTGGAGCAATTCA			
JAK2_e12_R	GAGAACTTGGGAGTTGCGATA	496	58	Standard
MPL_e10_F	AGTAGGGGCTGGCTGGAT			
MPL_e10_R	GCGGTATAGTGGGCGTGTTA	364	58	Standard
Pyrosequencing				
JAK2_PRYO_F	7GAAGCAGCAAGTATGATGAGCA			
JAK2_PRYO_R	TGCTCTGAGAAAGGCATTAGAA	120	58	Standard
JAK2_PRYO_S	TCTCGTCTCCACAGA			
8p6_PY_F	7CTTTTCGGTTCGTGCATGT			
8p6_PY_R1	GGCAGAGTATTACGCTTTTGTTAA	174	59	Standard
8p6_PY_S	GATTTTCAAATTTTCTCCTA			
8p7_PY4_F1	TCATTCGGGCTGGAGCAG			
8p7_PY4_R1	7GTTCCCCACTGTGGCCTAGAC	108	58	Standard
8p7_PY4_S	GCCCGGCCCTCCGCT			
9q12.5_PY_F	7GTCTCTGCATCAACCAGTCATT			
9q12.5_PY_R	ATTCCTTCATTTGGCAAGAGTC	131	58	2mM Mg^{++}
9q12.5_PY_S	TTTGCCTAGCTGAAAGT			
20q4_PY_F	CAAATGCATGTAAGGGGCTTAG			
20q4_PY_R	7CAGCCTAGGAATCCCACAAG	163	60	Standard
20q4_PY_S1	CCCAGGGTCTGAACT			
SNP genotyping				
C20orf111_cDNA_F1	GAGCACCAGTCAGAACAGCA			
C20orf111_cDNA_R1	TGGCTTTTGTGCTTGAGTTG	214	60	Standard
20qF4Sx	CATGTAAGGGGCTTAGCACA			
20qR4Sx	ATGCATGTCCGAGGCTATTC	135	58	Standard
20qF14Sx	CCCCACTGTCGTGTTTCTTT			
20qR14Sx	TGCCCAGGGAAATACAACCT	112	58	Standard
NUP214_cDNA_F	GCTGCTTCATTCTCCTTTGG			
NUP214_cDNA_R	GCGCTCTGGGAGCTACTAAC	191	58	Standard
9pF2Sx	AAAAACAATTCTCTTTAACATTTCTCA			
9pR2Sx	TCAAACACATGCATTTCAGC	123	58	Standard
IDS_e4_F	TGGAAACTTCTCCACCATCC			
IDS_e4_R	AACATCCAGCACATCCACAG	235	58	Standard
LANCL3_F	GCTCACGGCTTGTCGTCTAT			
LANCL3_R	CAGTGCACCAGCTCATTCTC	176	58	Standard
MAGED2_e4_F	CCAAGGTCAAAGCCAAGAAA			
MAGED2_e4_R	AGCCAACCGAGTCCTTGAT	197	58	Standard

Chapter 5 (i)

Primer name	Primer sequence	Product size	Annealing temp	PCR conditions
Real-time PCR				
C13_copy_F	CCATGGACGACGGGTTTCT			
C13_copy_R	TGTACAGGACGTAGGAGGGTGA	51	60	100nM primer
MPL_copy_F1	CCAGCTCCAGCCCTACATAA			
MPL_copy_R1	TCTCACTGCCTGGGATAGGT	51	60	100nM primer
JAK2_copy_F	TCCTCAGAACGTTGATGGCA			
JAK2_copy_R	GTGCTTTGGTCCCTTTATATGGA	51	60	100nM primer
ABL_cDNA_F	TGCGTGAGAGTGAGAGCAGT			
ABL_cDNA_R	GCTCTCGGAGGAGACGTAGA	119	60	300nM primer
JAK2_cDNA_F1	GCAGGCAACAGGAACAAGAT			
JAK2_cDNA_R1	CCATTCCCATGCAGAGTCTT	109	60	300nM primer
EVI1_cDNA_RTF	TGGAAGCTGGCTCAAGTACA			
EVI1_cDNA_RTR	GCGCAATGTCTGCAACTACT	110	60	300nM primer
Mutation detection (direct sequencing)				
JAK2_e14_F	ATCTATAGTCATGCTGAAAGTAGGAGAAAG			
JAK2_e14_R	CTGAATAGTCCTACAGTGTTTTCAGTTTCA	364	58	Standard
NRAS1213_F	AGAACCAAATGGAAGGTCACA			
NRAS1213_R	TGCATAACTGAATGTATACCCAAAA	370	58	Standard
NRAS61_F	GGCAGAAATGGGCTTGAATA			
NRAS61_R	AAAGCTCTATCTTCCCTAGTGTGG	397	58	Standard
KRAS1213_F	CGTCTGCAGTCAACTGGAAT			
KRAS1213_R	CCCTGACATACTCCCAAGGA	492	58	Standard
KRAS61_F	CCAGACTGTGTTTCTCCCTTC			
KRAS61_R	CACTGCTCTAATCCCCCAAG	377	58	Standard
CFMS969_F	GTGTTAATGGCCCCTGGAC			
CFMS969_R	ATGACCGAAGGCAGAGTTTG	524	58	Standard
WT1_e7_F	GCCACCCCTTCTTTGGATA			
WT1_e7_R	CTGGAAATAACCTGGGTCCT	348	58	Standard
WT1_e9_F	AGGAAATGCTGGGCTCCT			
WT1_e9_R	GCCACGCACTATTCCTTCTC	333	64	Standard
CEBPA_DNA_F1	GCCGGGAGAACTCTAACTCC			
CEBPA_DNA_R1	AGCCTGCCGTCCAGGTAG	451	58	10% DMSO
CEBPA_DNA_F2	TGGACAAGAACAGCAACGAG			
CEBPA_DNA_R2	GTTGCCCATGGCCTTGAC	236	58	Standard
RUNX1_e3_F	GAGCTGCTTGCTGAAGATCC			
RUNX1_e3_R	CATCCCAAGCTAGGAAGACC	496	58	10% DMSO
RUNX1_e4_F	CATTGCTATTCCTCTGCAACC			
RUNX1_e4_R	GGGATTCCATCACAGAAATCA	387	58	Standard
RUNX1_e5_F	GAAGGGCTGGACAGCATAAA			
RUNX1_e5_R	TCTGAGACATGGTCCCTGAG	283	58	Standard
GATA2_e4_F	TTAGCCCTCCTTGACTGAGC			
GATA2_e4_R	AGCCAAGCTGGATATTGTGG	334	58	Standard
TP53_e4_F	CTGGTAAGGACAAGGGTTGG			
TP53_e4_R	GCCAAAGGGTGAAGAGGAAT	495	58	
TP53_e5-6_F	GTTTCTTTGCTGCCGTCTTC			Standard
TP53_e5-6_R	TTGCACATCTCATGGGGTTA	590	58	1' ext/ann
TP53_e7_F				
TP53_e7_R				
TP53_e8_F	TGTGGCTTCTCCTCCACCTA			
TP53_e8_R	AGGAAAGAGGCAAGGAAAGG	378	58	Standard
***FLT3-ITD* detection**				
FLT3_ITD_F	GCAAATTAGGTATGAAAGCCAGC			
FLT3_ITD_R	CTTTCAGCATTTTGACGGCAACC	329	56	1' ext/ann

Chapter 5 (ii)

Primer name	Primer sequence	Product size	Annealing temp	PCR conditions
Pyrosequencing				
9p1_pyro_F	ATCAGCGCAACTCCTAACACA			
9p1_pyro_R	7GAGCAGCAGGGGTTTGTTAC	82	58	Standard
9p1_pyro_S	ACACACTCCTTGGCA			
9p2_pyro_F	7CATGTTACCAAATATGAGGATAGG			
9p2_pyro_R	AACTGTGTAGGATCCCGGTCT	72	58	Standard
9p2_pyro_S	AAAGGCACCAGAAAAC			
9p3_pyro_F	7GGCAGCACTCAGAACTAAAAGAGG			
9p3_pyro_R	CTAGGGTCTTTCCACTCCAACAAA	129	58	Standard
9p3_pyro_S	TTTGTCTTATTTTCATGTCA			
9p5_pyro_F	7CTTCTCACTTCACTTGGCAGGTAT			
9p5_pyro_R	GAAAAGGGGTGTTTGATATTGGG	108	58	Standard
9p5_pyro_S	TATGGCTGGGGGCTT			
SNP Genotyping				
9pF1	TCCCCAGTCAGGTCATCAAT			
9pR1	GCCAGTGGCCGAGTCTATAA	668	63	Standard
9pF2	CTCCCAGCCAAAAAGACCT			
9pR2	CCTTGCCAAGTTGCTGTAGA	677	58	Standard
9pF4	GACAGCCCTTTCAAATCTGG			
9pR4	CAACTATCTGAAAAAGGAGAAAAGG	579	58	Standard
9pF5	CAGGATCCTTTGTGGGTCTT			
9pR5	TGATGTTACCACCACGCAGT	579	58	Standard
9pF12	GTTCCATCCATGTTGCTGTG			
9pR12	CGGCCCAAGCTAAAAATCT	667	58	Standard
9pF24	GCAATGCATGAAGGTTCTCA			
9pR24	GACGCAGCACTGCTCTTCTT	219	58	Stabdrad
J2_5_F	TCATCGTCCTCAATTAGGTTGT			
J2_5_R	GGCACCCTTCATACACAAAGA	178	58	Standard
J2_10_F	TCCCAAAGTGCTGGGATTAC			
J2_10_R	TTTGGGACCCATAATATTGACTTT	220	58	Standard
IDS_cDNA_e4_F	TGGAAACTTCTCCACCATCC			
IDS_cDNA_e4_R	AACATCCAGCACATCCACAG	235	58	Standard

Chapter 6

Primer name	Primer sequence	Product size	Annealing temp	PCR conditions
Pyrosequencing				
JAK2_PRYO_F	7GAAGCAGCAAGTATGATGAGCA			
JAK2_PRYO_R	TGCTCTGAGAAAGGCATTAGAA	120	58	Standard
JAK2_PRYO_S	TCTCGTCTCCACAGA			
Mutation detection (direct sequencing)				
JAK2_col_seq_F1	GTCTTTCTTTGAAGCAGCAAG			
JAK2_col_seq_R1	GGCATTAGAAAGCCTGTAGTTTT	118	57	Standard
JAK2_R683_F	CAATGCATGCCTCCAAATTA			
JAK2_R683_R	CAAATTAAATGGAATTCAAGGAAAA	369	56	2mM Mg^{++}
JAK2_T875_F	AATTTGCCTTGAAAACTTGGT			
JAK2_T875_R	CTCTGGGCATTGGCATAAGT	349	58	2mM Mg^{++}
MPL_e9_F2	CAAACAGACGCTGGGCTATC			
MPL_e9_R1	AGCCAGCCCCTACTCTGG	422	62	2.5% formamide
NRAS1213_F	AGAACCAAATGGAAGGTCACA			
NRAS1213_R	TGCATAACTGAATGTATACCCAAAA	370	58	Standard
NRAS61_F	GGCAGAAATGGGCTTGAATA			
NRAS61_R	AAAGCTCTATCTTCCCTAGTGTGG	397	58	Standard
KRAS1213_F	CGTCTGCAGTCAACTGGAAT			
KRAS1213_R	CCCTGACATACTCCCAAGGA	492	58	Standard
KRAS61_F	CCAGACTGTGTTTCTCCCTTC			
KRAS61_R	CACTGCTCTAATCCCCCAAG	377	58	Standard

Note: primer prefix 7 denoted biotinylated primer

Appendix 2

Single nucleotide polymorphisms

Patient ID	SNPs	PCR primers
Chapter 3		
PT-1 cohort	rs12731981	MPLV114M_PY
W515L-hom IMF	rs7537577 & rs1870509 rs10737413 & rs1762830	1p6 1p7
Chapter 4		
Patient 4	rs1329624	9q12.5_PY
Patients 2 & 5	rs1729178 rs867858	8p6_PY 8p7_PY
Patient 6	rs9346 rs103612	C20orf111 NUP214
Patient 7	rs6031491	20q_PY
Patient 8	rs6130271 rs7875908	20qF4Sx 9p2Sx
Patient 9	rs12009345	LANCL3
Patient 10	rs11549009	IDS_e4
Patient 14	rs2071932	MAGED2_e4
Chapter 5		
Pts 4, 5, 7, 10 - 12, 14 & 15	rs465514, rs456374, rs465793, rs17761666, rs16921966	9p1
Pts 8, 11, 12 & 13	rs7875908, rs7034539, rs2230724	9p2
Patient 2	rs9886808, rs10974623, rs9886720, rs9886723	9p4
Pts 2, 15 & 16	rs7847141, rs7847294, rs3780372	9p5
Patient 12	rs11794708, rs7043489, rs11794778	9p12
Pts 8, 9 & 13	rs7031315, rs7046183, rs7034721	9p24
Pts 9, 11, 15 & 16	rs7034753	J2_5
Pts 4, 8, 13 & 14	rs7030260	J2_10
Pt 8	rs6030071 & rs2299976	20q1 & 20q8

Appendix 3

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Appendix 4

Publications relating to the thesis

Beer PA, Jones AV, Bench AJ, Goday-Fernandez A, Boyd EM, Vaghela KJ, Erber WN, Odeh B, Wright C, McMullin MF, Cullis J, Huntly BJP, Harrison CN, Cross NCP and Green AR. Clonal diversity in the myeloproliferative neoplasms: independent origins of genetically distinct clones. Br J Haematol. 2009 Jan 16. [Epub ahead of print]

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Appendix 5

First author journal articles resulting from work presented in the thesis

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