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# Apparent '*JAK2*-negative' polycythaemia vera due to compound mutations in exon 14

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A somatic activating mutation in exon 14 of *JAK2* involving the pseudokinase domain (c.1849G>T; p.V617F), is found in ~95% of patients with polycythaemia vera (PV), and is a major diagnostic criterion in the 2008 World Health Organization (WHO) classification (Swerdlow*, et al* 2008) and its proposed forthcoming revision (Barbui*, et al* 2015). A further 2-3% of PV patients have mutations in exon 12 of *JAK2*, so that very few PV patients are '*JAK2*-negative', lacking a somatic mutation in that gene. As there are many alternative causes of absolute or relative erythrocytosis the diagnosis sometimes remains in doubt when no *JAK2* mutation is identified.

Here we report a case of WHO-defined PV with no *JAK2* mutation detected on routine testing using a single nucleotide primer extension assay for *JAK2* V617F and direct sequencing of exon 12. Targeted next generation sequencing (NGS) of the entire coding region of *JAK2* revealed a novel mutation in exon 14 (c.1849_1853GTCTG>TTTCT; p.V617F/C618L). This mutation resulted in failure of the *JAK2* V617F mutation-specific primer to bind to the target, causing a false negative result with amplification only of the wild type allele. Direct sequencing of exon 14 in 7 additional cases of '*JAK2*-negative' polycythaemia without a conclusive diagnosis, identified one other compound mutation, demonstrating the diagnostic utility of broader sequencing of *JAK2* in such cases.

The index case (Patient 1) was a 58-year-old male who presented with itching for 2 years and recent onset night sweats. Red cell indices are shown in Table I. The bone marrow (BM) biopsy showed panmyelosis (Fig 1A) with grade 3/4 reticulin fibrosis and iron deficiency. The karyotype was normal, but single nucleotide polymorphism (SNP)-array analysis demonstrated a 23.6 MB block of uniparental disomy (UPD) at 9p24.3-p21.3, involving the *JAK2* gene. Targeted NGS identified the *JAK2* V617F mutation in 40% of 4362 reads, always with C618L *in cis* (data not shown), and confirmed by direct sequencing (Fig 1A).

Subsequently we tested 7 additional patients (summarized in Table I) who were referred by their treating clinicians with an elevated haemoglobin concentration, subnormal or low-normal serum erythropoietin (EPO) levels (<2x lower limit of normal) and no *JAK2* mutation in routine diagnostic testing (V617F and exon 12). Direct sequencing of *JAK2* exon 14 in genomic DNA extracted from peripheral blood leucocytes or BM aspirates revealed a compound mutation (c.1849G>T;1852T>C; p.V617F/C618R) in one additional patient (Patient 2). The other six patients had normal results. Patient 2 was a 76-year-old male with asymptomatic polycythaemia. The BM showed panmyelosis (Table I, Fig 1B) with iron deficiency and grade 2/4 reticulin fibrosis. The karyotype and SNP-array analysis were normal.

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Erythroid burst-forming units from Patients 1 and 2 were grown in the absence of EPO and the individual colonies sequenced. In Patient 1, the majority of colonies were homozygous for the compound mutation, suggesting that UPD was a secondary event, as is usual in classical PV (Wang*, et al* 2014). No V617F/C618R homozygous colonies were identified in Patient 2, consistent with the absence of UPD by SNP-array. Only ~20% of PV patients lack a substantial UPD clone (Godfrey*, et al* 2012). *JAK2* C618R has been reported as a sole mutation in a suspected myeloproliferative neoplasm (Ma*, et al* 2009), which could be consistent with the mutation either conferring a clonal advantage or co-existing with an unknown driver mutation. Altered JAK-STAT signalling has been reported for another compound mutation in PV, *JAK2* L611V/V617F (Cleyrat*, et al* 2010).

Multiple compound mutations in exon 14 of *JAK2* have been reported (Fig 1C): either point mutations *in cis* with V617F or small in-frame indels resulting in V617F with adjacent sequence variants. These variants are rare in cohorts of PV; yet the prevalence was 25% in our small series of highly selected patients with undiagnosed polycythaemia. The observation that V617F is common to all of the compound mutations highlights the biological importance of this specific mutation.

The WHO criteria enable a diagnosis of *JAK2*-negative PV in a patient with polycythaemia if two out of three minor criteria are met: subnormal serum EPO levels, endogenous erythroid colony (EEC) formation and BM hypercellularity with panmyelosis (Swerdlow*, et al* 2008). Alternative criteria of the British Committee for Standards in Haematology regard subnormal EPO and EEC as equivalent, and use other myeloproliferative features (splenomegaly, neutrophil leucocytosis and thrombocytosis as the minor criteria, rather than BM panmyelosis) (McMullin*, et al* 2005). Both of the patients that we identified with compound mutations in *JAK2* exon 14 had typical features of WHO-defined PV, highlighting the strong relationship between *JAK2* V617F and the associated clinical and morphological phenotype. Nevertheless, reliance on the minor criteria required for a diagnosis of *JAK2*-negative PV has its own limitations: the prevalence of a subnormal EPO level is method-dependent, reported in 30-80% of PV patients (Ancochea*, et al* 2014), EEC assays are not widely available, and the bone marrow biopsy is not without risk.

We report a novel somatic mutation in *JAK2* exon 14, and show that sequencing of exon 14 can identify a small proportion of PV-associated mutations that would be missed by most diagnostic laboratories. Failure to diagnose PV may lead to under-treatment of false-negative cases, and may also lead to unnecessary investigations to rule out secondary

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causes of erythrocytosis. Our experience highlights the potential utility of NGS in a routine diagnostic service. Many diagnostic laboratories, like our own, use a two-step approach for the detection of *JAK2* mutations, using allele-specific polymerase chain reaction for V617F and an alternative assay, such as direct sequencing or high-resolution melting curve analysis, for the diverse mutations in exon 12. Direct sequencing lacks sensitivity and will occasionally fail to detect mutations with a low allelic burden, while melting curve analysis is more complex to interpret and cannot reliably identify the specific variant present (Rapado*, et al* 2009). Sequencing of all *JAK2* exons by NGS is highly sensitive and overcomes these limitations; it can also identify rare mutations, such as those that we describe in exon 14. These advantages were highlighted in a recent review of molecular diagnostic tests for myeloproliferative neoplasms (Langabeer*, et al* 2015). As targeted sequencing panels based on NGS become cheaper and more widely available, it is likely that NGS will become the preferred secondary assay for the detection of *JAK2* mutations in polycythaemia after a specific test for V617F is negative.

**Author contributions**

IST and DMR designed the study and wrote the manuscript. IST, DAC, SM, TN, MVV, HSS, CNH and RJD performed the experiments and analysed data; IST, DZ, and DMR contributed essential clinical data. All authors critically reviewed the paper.

**Competing interests**

The authors declare no conflict of interest.

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**Table I. Key clinical features of the 8 patients with '*JAK2*-negative' polycythaemia.**

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| Patient | Age (years) /sex | Haemoglobin (g/l) (Male: 135-175) (Female: 115-155) | Haematocrit (Male: 0.40-0.50) (Female: 0.35-0.45) | Erythropoietin (mu/l) (4-29) (2.5-18.5)* | Bone marrow findings |
|---|---|---|---|---|---|
| **1** | 58/Male | 196 | 0.59 | 1 | Panmyelosis |
| **2** | 76/ Male | 215 | 0.66 | 1 | Panmyelosis |
| **3** | 38/ Male | 200 | 0.57 | 2 | Mildly hypercellular |
| **4** | 58/ Male | 211 | 0.58 | 1 | Mildly hypercellular |
| **5** | 50/Female | 187 | 0.54 | 1 | Not done |
| **6** | 91/ Male | 196 | 0.63 | 1 | Not done |
| **7** | 41/ Male | 203 | 0.57 | 4.4* | Mildly hypercellular |
| **8** | 67/ Male | 188 | 0.52 | 3.1* | Mildly hypercellular |

*Different reference range from Monash Health Pathology, Clayton, Victoria, Australia.

**Figure 1. Bone marrow histology and sequencing results of two patients with compound mutations in *JAK2* exon 14.**

**(A)** Patient 1: Bone marrow (BM) histology showed panmyelosis with pronounced megakaryocyte clustering and fibrosis. Direct sequencing of *JAK2* exon 14 showed a homozygous mutation (c.1849_1853GTCTG>TTTCT; p.V617F/C618L) in the majority of erythroid colonies (heterozygous chromatogram shown). **(B)** Patient 2: BM histology showed panmyelosis. Sequencing showed either heterozygous mutation (c.1849T>C;1852T>C; p.V617F/C618R) or wild type; no homozygous colonies were detected. **(C)** Summary of

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these and other *JAK2* exon 14 mutations reported in myeloproliferative neoplasms in the COSMIC database (<http://cancer.sanger.ac.uk/cosmic>, accessed 21 January 2015).

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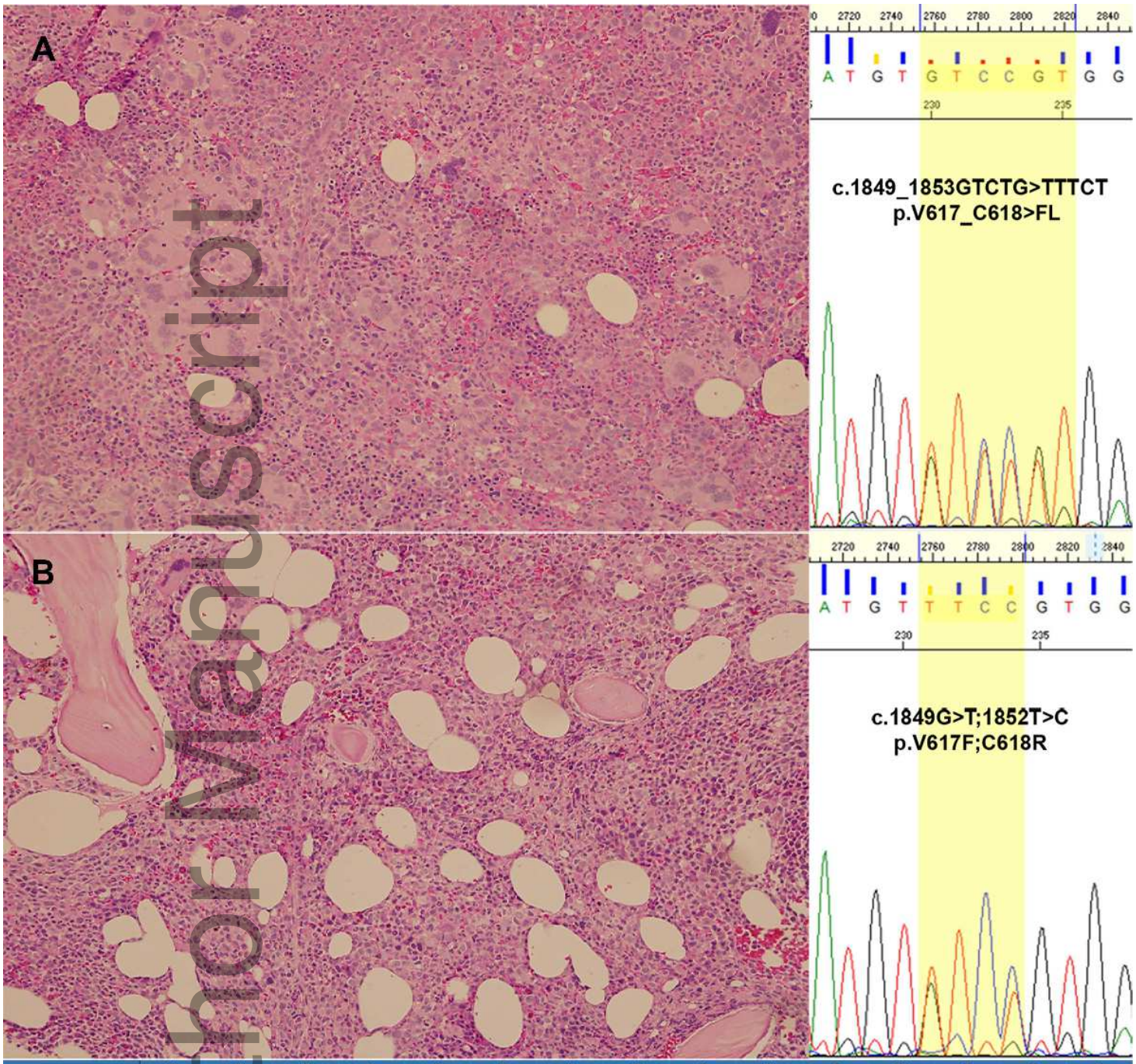


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| | H | K | H | L | V | L | N | Y | G | V | C | V | C | G | D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CAC | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | GTC | TGT | GGA | GAC |
| H606Q | CA**A** | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | GTC | TGT | GGA | GAC |
| H608Y | CAC | AAG | **T**AT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | GTC | TGT | GGA | GAC |
| L611S | CAA | AAG | CAT | TTG | GTT | T**C**A | AAT | TAT | GGA | GTA | TGT | GTC | TGT | GGA | GAC |
| V615L | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | **T**TA | TGT | GTC | TGT | GGA | GAC |
| C616Y | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | ??? | TGT | GTC | TGT | GGA | GAC |
| V617I | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | **A**TC | TGT | GGA | GAC |
| C618R | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | GTC | **C**GT | GGA | GAC |
| D620E | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | GTC | TGT | GGA | GA**A** |
| L611V/V617F | CAA | AAG | CAT | TTG | GTT | **G**TA | AAT | TAT | GGA | GTA | TGT | **T**TC | TGT | GGA | GAC |
| L611S/V617F | CAA | AAG | CAT | TTG | GTT | T**C**A | AAT | TAT | GGA | GTA | TGT | **T**TC | TGT | GGA | GAC |
| V615L/V617F | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | ??? | TGT | ??? | TGT | GGA | GAC |
| V617F/C618R (Patient 2) | | | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | **T**TC | **C**GT | GGA | GAC |
| V617F/C618F | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | **T**TC | T**T**T | GGA | GAC |
| V617F/D620E | CAA | AAG | CAT | TTG | GTT | TTA | AAT | TAT | GGA | GTA | TGT | **T**TC | TGT | GGA | GA**A** |

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