Screening of *JAK2* V617F and *MPL* W515 K/L negative essential thrombocythaemia patients for mutations in *SESN2, DNAJC17, ST13, TOP1MT,* and *NTRK1*

Essential thrombocythaemia (ET) is a myeloproliferative neoplasm characterized by a sustained elevation of the platelet count and a tendency for thrombosis and haemorrhage. Cytogenetic abnormalities are rare in ET, accounting for <5% of cases. Molecular abnormalities include *JAK2* V617F [Mendelian Inheritance in Man (MIM) reference 614521], a mutation found in 50% of ET patients (Kralovics *et al*, 2005). In addition, a small fraction of *JAK2* V617F-negative ET patients (about 10%) have activating point mutations in the thrombopoietin receptor gene (*MPL*, MIM 159530) (Kilpivaara & Levine, 2008). Other genes were also found to be mutated in ET, such as *TET2, LNK* and *ASXL1,* but at a low frequency. As such, the underlying molecular cause remains to be discovered in a substantial fraction of ET cases.

Recently, Hou *et al* (2012) developed a high-throughput single-cell sequencing method for sequencing the cancer genome with high accuracy at the nucleotide level to facilitate the analysis of tumour evolution in cancers. They applied their method on single cells derived from an ET *JAK2* V617F-negative patient and identified several mutated genes. Based on the type, frequency, *in silico* analysis and score of the detected mutations, eight genes were identified as potential candidate drivers: *SESN2*, *DNAJC17*, *ST13*, *TOP1MT*, *NTRK1*, *ABCB5*, *FRG1*, and *ASNS*. However, their recurrence rate in ET was not determined. As none of these genes have been linked to ET before, our aim was to screen a large cohort of ET patients to verify the recurrence rate of these mutations.

Peripheral blood (n = 31) or bone marrow (n = 33) was collected from a cohort of 64 patients diagnosed with ET according to the World Health Organization criteria (Vardiman *et al*, 2009) and verified to be *JAK2* V617F-negative and *MPL* wild-type by allele-specific polymerase chain reaction (PCR) and Sanger sequencing, respectively. We checked the presence and recurrence of the mutations in *SESN2*, *DNAJC17*, *ST13* and *TOP1MT*, which exhibited the highest scores in the study reported by Hou *et al* (2012), and *NTRK1*, which encodes a tyrosine kinase, in a large cohort of 64 *JAK2* V617F- and *MPL* W515L/K-negative ET patients. PCR primers were designed to amplify the full exonic region encompassing the reported mutations: SESN2 p.P87S, TOP1MT p.S479L, ST13 p.Q349*, NTRK1 p.N323S, DNAJC17 p.A292P (Table I). The generated amplicons were analysed by Sanger sequence analysis.

Our sequencing experiments did not reveal any of the mutations reported by Hou *et al* (2012). However, our sequencing covered the exon involved in each gene, detecting two other novel heterozygous mutations. The first mutation was in exon 11 of *DNAJC17* (c.877C>T, p.R293W), and was predicted to be benign by polyphen 2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei *et al*, 2010). This mutation was also detected in the buccal swab of the patient indicating that it is

Table I. Overview of the investigated genes, exons, and mutations.

Gene	Exon	Nucleotide change	AA change	Amplicon size	Primers
SESN2	3	c.259C>T	P87S	311	TTTGATGAGTGGGGCAGGA
					CAAAGGACAGAACCCACCA
TOP1MT	11	c.1436T>C	S479L	416	GTGCCTGTGTGAGTGGAAG
					CAGGAAAAGGGTCCAGACAG
ST13	12	c.1045C>T	Q349*	306	CACTCATCTAAACCCCATT
					TCTAGGTTGCTTTTCCTTC
NTRK1	8	c.878A>G	N323S	667	CCTGTGGGGCTGTGACTTAT
					GCAGGAAGTTGGCTGTAACC
DNAJC17	11	c.874G>C	A292P	333	GAGACTCTTAGAACGCAGCA
					CCCAGCACCTTATACCTAT

cDNA sequence of *SESN2*, *TOP1MT*, *ST13*, *NTRK1*, and *DNAJC17* are available at Refseq nucleic NM_031459.4, NM_052963.2, NM_003932.4, NM_001012331.1, NM_018163.2, respectively, in which nucleotide 1 is the A of the ATG-translation initiation codon.
*Indicates a stop codon.

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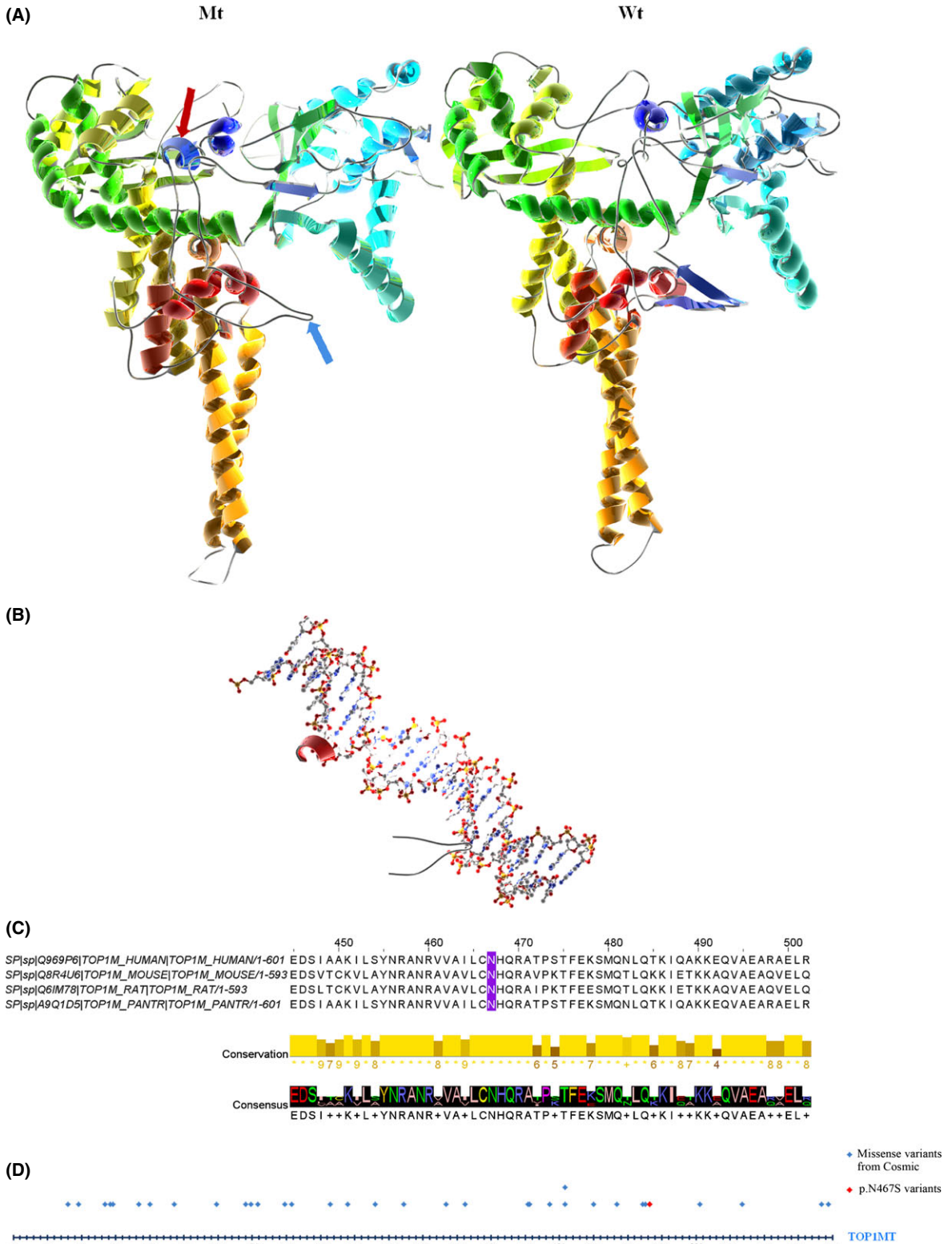

Fig 1. TOP1MT p.N467S leads to a structural change that might affect the interaction of TOP1MT with DNA. In comparison to wild-type (Wt) TOP1MT, the mutated (Mt) TOP1MT gained an α helix (red arrows) and lost a β strand (blue arrows) in addition to other conformational changes (A). The sites of structural change are in close proximity to the DNA molecule when bound to TOP1MT (B). Alignment of TOP1MT (amino acid 445 to 502) from UNIPROT on Jalview for human, mouse, rat, and chimpanzee shows that asparagine (N, in purple) at position 467 is conserved between these organisms (C). Representation of amino acid changes in TOP1MT as a result of missense mutations, collected from the COSMIC database, indicates that p.N467S is a new variant located in close proximity to other reported mutations from other cancers (D).

germline. The second mutation was in exon 11 of *TOP1MT* (c.1400A>G, p.N467S). It was predicted to be damaging by polyphen 2 and SIFT (http://sift.bii.a-star.edu.sg/) and was not present in the buccal swab corroborating it as an acquired mutation. *TOP1MT* is localized on human chromosome 8q24.3. It is highly homologous to the nuclear *TOP1* gene and consists of 14 exons. TOP1MT is a mitochondrial topoisomerase encoded by the genomic DNA. It is a type IB enzyme, which sustains the befitting conformation of DNA during replication, transcription, recombination and repair. In addition to the main form of *TOP1MT* mRNA, two alternatively spliced forms were found, which appeared more frequent in renal cell carcinoma compared to normal adjacent tissue (Zhang *et al*, 2007). TOP1MT was found to be overexpressed in haematological cancer cell lines in parallel with MYC overexpression leading to the assumption that MYC expression induces TOP1MT expression (Zoppoli *et al*, 2011).

We assessed the effect of the mutation p.N467S on the structure of the protein by implementing *in silico* analysis. Amino acid sequences of wild-type and mutated TOP1MT sequences (Refseq: NP_443195; Uniprot: Q969P6) were supplied to the automated I-TASSER server (http://zhanglab.ccmb.med.umich.edu/) (Roy *et al*, 2010). The obtained 3D structures were viewed on DeepView–Swiss-PdbViewer (http://www.expasy.org/spdbv/) (Guex & Peitsch, 1997). Models were energy minimized by performing two cycles of steepest descent with 50 steps each and one cycle of conjugate gradient of 200 steps with a minimum ΔE of 0·01 kJ/mol together with a harmonic constraint of 100 kJ/mol. The resulting models were further refined by ModRefiner (http://zhanglab.ccmb.med.umich.edu/ModRefiner) in order to obtain the best possible conformation of TOP1MT (Xu & Zhang, 2011). The final images were rendered on POV-Ray v3.6 (http://www.povray.org). By comparison, the major changes caused by p.N467S mutation were the gain of an α helix and the loss of a β strand (Fig 1A) which are in close proximity to the bound DNA molecule. This suggests that this mutation might affect the interaction of TOP1MT with the DNA molecule (Fig 1B).

Moreover, TOP1MT is a highly conserved protein; by aligning TOP1MT from different species on Jalview we could confirm that Asparagine at position 467 is conserved (Fig 1C). Moreover, although this mutation has not been reported before in the catalogue of somatic mutations in cancer (COSMIC), two other mutations were reported in close proximity, c.1384G>A p.V462M and c.1390A>G p.I464V in adenocarcinoma and endometrioid carcinoma, respectively (Fig 1D). To check the recurrence of the p.N467S in ET, we screened exon 11 of *TOP1MT* gene in 38 additional *JAK2* V617F-negative *MPL* wild-type ET cases, but did not detect any additional mutant cases.

In summary, in this series of *JAK2* V617F and *MPL* W515K/L negative ET cases, we did not detect any of the mutations that were previously proposed as potential candidate drivers (Hou *et al*, 2012). However, a novel mutation in exon 11 of *TOP1MT* was found. This establishes this gene as recurrently mutated in *JAK2* V617F and *MPL* W515K/L negative ET, and suggests that *TOP1MT* mutations are involved, at low frequency, in the pathogenesis of ET. However, its functional consequences remain to be investigated. Given that the candidate mutations, as proposed by Hou *et al* (2012) were predicted to be protein damaging, the possibility remains that other exons of these genes might harbour loss of function mutations. The quest for the full complement of driver mutations in ET therefore remains open.

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Author contributions

Carla AL Assaf performed the experiments and wrote the manuscript. Timothy Devos, Johan Billiet, and Carlos Graux provided the ET samples. Petros Papadopoulos and Els Lierman reviewed the manuscript. Peter Vandenberghe provided patient samples, designed the experiments and wrote the manuscript.

Note added in proof

After submission of this paper, two independent groups published that *CALR* mutations are recurrent in *JAK2* V617F and *MPL* W515 K/L negative ET (Klampfl *et al*, 2013; Nangalia *et al*, 2013). Our case with the *TOP1MT* mutation discussed here, was indeed found to have a *CALR* exon 9 insertion p.K385fs*47. The interaction between these mutations remains to be investigated.

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An old and simple solution for a new problem - more on clinical staging and evaluation of response in B-cell chronic lymphocytic leukaemia in the era of new therapies

The exciting progress in the therapy of B-cell chronic lymphocytic leukaemia (B-CLL) with inhibitors of B-cell receptor (BCR) signalling has posed a new problem in response classification. B-cell receptor signalling inhibitors (ibrutinib, idelalisib and others) cause pronounced redistribution of B-CLL lymphocytes that doesn't fit into the current International Workshop on CLL (iwCLL) response criteria guidelines (Hallek *et al*, 2008) addressing clinical research and practice in the era of chemo-immunotherapy (Oscier *et al*, 2012). With start of BCR signalling inhibitor therapy there is regularly a significant increase in peripheral blood lymphocytosis with simultaneous marked shrinkage of lymph nodes and spleen (Byrd *et al*, 2013). The failure of the current response criteria to adequately evaluate response to this new class of drugs led to proposals for new categories of response, such as partial response with lymphocytosis, nodal response or objective response, and calls for revision of the guidelines (Cheson *et al*, 2012). Although current guidelines are quantitative and precise in evaluating tumour burden separately in different compartments, they fail to measure integral tumour mass, by disregarding the fact that the tumour as a whole may not differ in size but may differ only in distribution pattern. It is therefore reasonable to conclude that a parameter that combines all particular measures in a single one may overcome the problem. In addition, quantification of lymphocyte redistribution may supplement the definition of overall response.

One old and simple solution in line with this concept is the Total Tumour Mass score (TTM) (Jaksic & Vitale, 1981). The TTM scoring system uses exactly the same basic parameters as the current iwCLL guidelines, but combines them differently in a single, comprehensive parameter. TTM is a simple clinical parameter that takes into account tumour burden in major tumour cell compartments (Fig 1A). It has been validated in prognostic studies and in several multicentre international clinical trials both for disease progression rate and responsiveness to therapy, and proved to be useful because of its continuous quantitative character based on tumour parameters and not on bone marrow failure parameters