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ORIGINAL ARTICLE

Next-generation sequencing for JAK2 mutation testing: advantages and pitfalls

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Abstract

The JAK2^{V617F} mutation is part of the major criteria for diagnosis of myeloproliferative neoplasms (MPN). Allele-specific quantitative PCR (qPCR) is the most prevalent method used in laboratories but with the advent of next-generation sequencing (NGS) techniques, we felt necessary to evaluate this approach for *JAK2* mutations testing. Among DNA samples from 427 patients analyzed by qPCR and NGS, we found an excellent concordance between both methods when allelic burden was superior to 2% (the detection limit of our NGS assay). Only one sample among 298 was found negative by NGS while allelic burden by qPCR was 3%. Because NGS detection limit is higher, sensitivity was lower as exemplified by 21 samples found negative whereas qPCR measured allelic burdens between 0.1 and 1%. Importantly, quantitative data of samples found positive by both techniques were highly correlated ($R^2 = 0.9477$). We also evaluated 40 samples tested for *JAK2* exon 12 mutations by HRM. The concordance with NGS was of 100%. Using NGS, the full coding region of *JAK2* was analyzed leading to identification of several variants outside of exon 12 and 14 which were previously described or not. Interestingly, we found one somatic mutation (c.1034A>T p.H345L) which induced constitutive activation of the JAK/STAT pathway leading to an increased proliferation of BaF/3 cells with low-dose EPO. This study showed that NGS is a robust method highly correlated to qPCR, although less sensitive, but providing the opportunity to identify other JAK2 variants with potential impact on disease initiation or evolution.

Keywords JAK2 · Next-generation sequencing · Mutation · Diagnosis · Myeloproliferative neoplasm

Introduction

The classical myeloproliferative neoplasms (MPNs), also called BCR-ABL negative MPNs, are characterized by excessive production of terminally differentiated and functional blood cells. MPNs have been subdivided into three entities: polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The diagnosis of MPNs has changed dramatically with the discovery of a somatic point mutation in the *JAK2* gene (c.1849G>T or JAK2^{V617F}) [1]. One major criterion for the diagnosis of MPNs is the detection of this mutation [2] which can be found at the heterozygous state or present on both *JAK2* alleles due to mitotic recombination [3, 4]. Variation of mutant allele burden thus reflects both the size of the myeloproliferative clone and the homozygous or heterozygous status of clonal cells. It has been shown that JAK2^{V617F} allele burdens (%JAK2VF) are higher in PV than in ET [5, 6]. In addition, a high %JAK2VF has been associated with increased risk of thrombotic events in ET, increased risk of cardiovascular events, larger spleen and greater treatment requirement in PV, higher leukocytosis, and risk of acute transformation in PMF [7–9]. Although these findings have not been confirmed in every study [10] and no requirements are indicated in the WHO recommendations, such association between %JAK2VF and disease evolution argues for the need to precisely assess the %JAK2VF. Furthermore, quantitative follow-up of JAK2^{V617F} allelic burden is informative for minimal residual disease evaluation after

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allogeneic bone marrow transplantation [11] but also in patients undergoing interferon alpha [12–14] or JAK2 inhibitor therapies [15]. Several methods have been developed throughout time to quantify the %JAK2VF in peripheral blood leukocytes, most of them based on real-time allele-specific PCR have been compared in a joint European collaborative study [16].

Several other mutations in the *JAK2* gene have been described throughout time. First, somatic activating mutations in the exon 12 of the *JAK2* gene were recurrently described in $JAK2^{V617F}$-negative PV patients [17, 18]. Then, several germline or somatic *JAK2* variants were described and some of them shown to confer cytokine independency to hematopoietic cells [19–24]. It is thus considered that such variants could at least participate to leukemogenesis in MPN patients, although animal models are lacking in most cases. In the recent studies, these *JAK2* variants were identified using next-generation sequencing (NGS) techniques which allow an easy sequencing of the whole coding regions of this gene. These techniques are more and more accessible to many laboratories and are seen as potentially replacing the real-time PCR methods.

In this context, we aimed at comparing the allele-specific $JAK2^{V617F}$ quantitative PCR technique routinely used in our laboratory to the NGS method we recently introduced for the molecular screening of MPNs patients. We further scrutinized the non-exon 12 or 14 *JAK2* variants identified using NGS method and modeled one non-described somatic variant.

Materials and methods

Samples

From January 2013 to September 2017, NGS was performed in our laboratory at Saint-Louis Hospital on 473 consecutive DNA samples extracted from peripheral blood of patients known or suspected to develop MPN. The same DNA samples were also tested in our laboratory for the quantification of the $JAK2^{V617F}$ mutation.

$JAK2^{V617F}$ and other *JAK2* variants

The $JAK2^{V617F}$ mutation was quantified using a quantitative real-time allele-specific PCR (qPCR) method (Ipsogen JAK2 MutaQuant kit, Qiagen, Germany) according to the manufacturer's recommendations. Detection limit of this assay is set at 0.1%. An NGS panel was developed in our laboratory using the TruSeq Custom Amplicon method (TSCA; Illumina, San Diego, CA, USA). Specific oligo probes were designed for 27 genes among which the full coding regions of the *JAK2* gene were comprised. Total blood DNA was quantified using a Qubit instrument and 250 ng were used as input for amplicon-based library preparation according to manufacturer's instructions. Libraries were loaded on a MiSeq instrument (Illumina) at 10pM and run using a MiSeq Reagent kit v2 (500 cycles) (Illumina). Obtained sequences were aligned to the reference genome (GRCh37/hg19) and variants were called and annotated using a pipeline developed by Sophia Genetics (Sophia Genetics, Switzerland). Variants were retained according to the following criteria: exonic non-synonymous variants; minimum coverage of 500×; minimum variant allele frequency (VAF) of 2%; removal of known polymorphisms (SNPs) presenting a frequency > 0.01% (1000 genome, ExAC).

Site-directed mutagenesis

Both p.H345L and p.V617F JAK2 mutations were individually introduced into the TMJ-JAK2-GFP WT (kindly provided by Chloé James) plasmid using QuikChange® II site-directed mutagenesis kit (Agilent Technologies Inc., Santa Clara, CA). The following oligonucleotides serving as templates for the synthesis of the mutant plasmids were synthesized by Eurogentec. The reaction sample was prepared by mixing 5 μL of 10× reaction buffer, 20 ng of plasmid, 125 ng of each oligonucleotide (c.1034C>T 8F: GCCGAGTTGTAACTATCCTTAAGCAA GATGGTAAAAATC, 8R: GATTTTTACCATCTTGCTTA AGGATAGTTACAACTCGG) and (c.1849G>T 14F: AGCATTTGGTTTTAAATTATGGAGTATGTTTCTGTGGA GACGAGA; 14R: TCTCGTCTCCACAGAAACATACTCCA TAATTTAAAACCAAATGCT), 1.5 μL of Quicksolution reagent, 1 mL of QuickChange Lightning Enzyme and H2O for a reaction volume of 50 μL.

Strand synthesis was carried out according to a protocol of amplification recommended by the manufacturer, with a final extension of the strand of 7 min. Enzymatic digestion of the non-mutated strand was performed by adding 2 μL of Dpn-I enzyme (Agilent) to the reaction medium and incubating for 5 min at 37 °C. Plasmids were transformed into XL-10 Gold Ultracompetent (Agilent) cells.

In each case, the presence of the mutation was verified by Sanger sequencing using the following primers. For the c.1034C>T p.H345L mutant: forward primer, 5′-GGAA ACTCTGCAGTCTGCCT-3′, and reverse primer: 5′-GGGC CATGACAGTTGCTTTG-3′. For the c.1849G>T p.V617F mutant, forward primer, 5′-TCTTTCTTTGAAGCAGCAAG TATG-3′ and reverse primer: 5′-TGGCCCATGCCAAC TGTTTA-3′.

Cell lines and culture

The BaF/3 cell line overexpressing the human erythropoietin receptor (BaF/3 R-EPO) was grown at 37 °C in RPMI (Gibco), 10% FBS, 1% Penicillin-Streptomycin, 1% Glutamax, and EPO (1 IU/mL) under 5% CO_2. Cells were transfected using the Amaxa instrument (Lonza). Briefly, 2×10^6 cells were placed in the presence of a mixture consisting of 2 μg of TMJ-JAK2-GFP of each type described

above and Amaxa Cell Line Nucleofactor Kit V solution (Lonza). After the electroporation was performed, cells were cultured without antibiotic for 24 h and the transfection rate was assessed at 24 h by measuring GFP-positive cells by FACS analysis (FACS Canto II, Becton Dickinson).

Cell sorting

At 24-h post-transfection GFP-positive cells (MFI > 10^4) were sorted (FACSAria II, Becton Dickinson) into wells containing RPMI (Gibco), 10% FBS, 1% Penicillin-Streptomycin (Gibco), 1% Glutamax (Gibco), and EPO (1 IU/mL). One week after sorting, the percentage of GFP-positive cells was verified to be > 99% by FACS analysis (FACSCanto II, Becton Dickinson).

Proliferation assay

Each of the BaF/3 clones overexpressing one of the JAK2 variants (WT, p.V617F, and p.H345L) was cultured at 2 × 10^5/mL in triplicate. Cell proliferation was assessed by daily counting from day 1 to day 4 using trypan blue staining in a TC20 automatic counter (Biorad).

Results

Detection and quantification of the JAK2^{V617F} mutation in MPNs samples using qPCR or NGS

We analyzed the data obtained from 473 samples evaluated by NGS and qPCR, corresponding to 427 patients suspected to develop MPN. The cohort consisted of 215 men and 212 women (sex ratio = 1.01), mean age was 51 years (ranging from 15 to 89 years). The majority of samples (N = 185, 44%) were from primary or secondary myelofibrosis patients while PV and ET patients accounted for 26% (N = 112) and 25% (N = 108) respectively. MPN/MDS represented 4.5% of the cohort, and 2 patients tested for recurrent thrombosis were found JAK2^{V617F} positive at low level (VAF: 0.5%). In 46 patients, 2 different samples were evaluated to follow disease evolution.

We compared the accuracy of both methods in terms of JAK2^{V617F} detection and quantification. A positive result was found by the two techniques in 276 samples (Table 1), in which the comparison of quantitative results showed a positive correlation (R^2 = 0.9477, slope = 0.9911, Fig. 1), with a Lin concordance coefficient estimated at 0.975 which confirms an excellent concordance between the two techniques [25].

Discordant results were found in 25 samples: 22 of them were found negative by NGS whereas the mutation was detected at low level by qPCR. Indeed, 21 samples were found below the %JAK2VF threshold of 2% that we established for NGS detection. Only 1 sample was really discordant among the 22, as it was assigned with a 3% allele burden by qPCR. We did not identify any explanation for this discrepancy and concluded to false negativity with NGS in this sample. On the contrary, 3 samples were found positive by NGS but negative by qPCR (Table 1). In one of these discrepant cases, the JAK2^{V617F} mutation resulted from a genetic alteration different from the usual one. Instead of the classical c.1849G>T substitution, NGS analysis identified two substitutions targeting nucleotides 1848 and 1849 (c.1848_1849delTGinsCT). Although the resulting amino acid change was identical (p.V617F), the DNA sequence was different. The second discrepant case harbored both the classical c.1849G>T (p.V617F) mutation together with a substitution located 11 nucleotides away (c.1860C>A) targeting codon 620 (p.D620E). Such mutant has already been described [26]. Finally, we also identified by NGS a double mutant in the third sample. In addition to the classical JAK2^{V617F} mutation, the

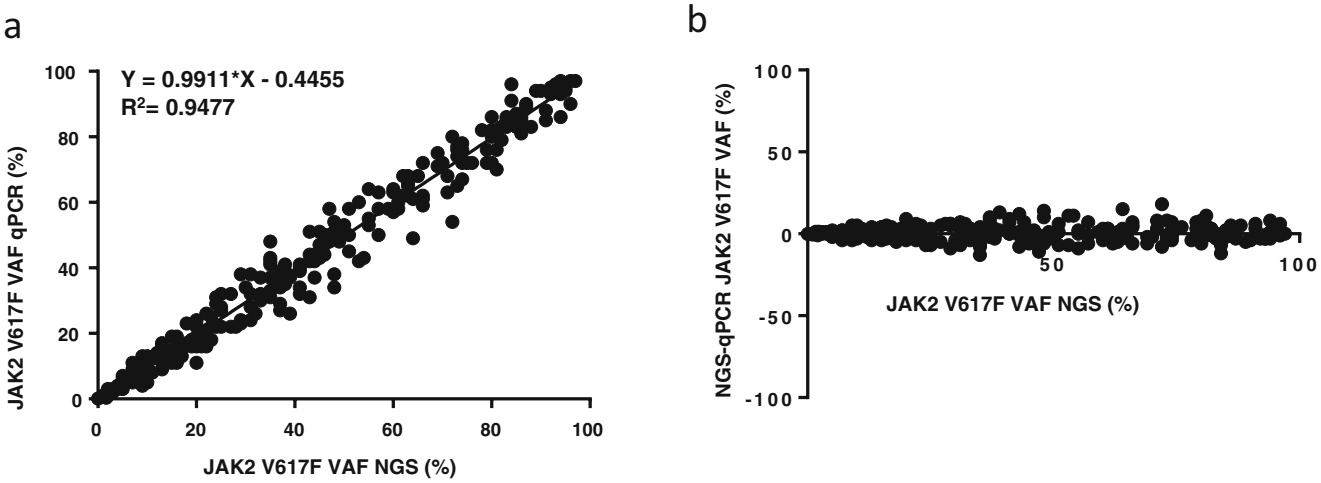

Fig. 1 Comparison of qPCR and NGS techniques for the detection and quantification of the JAK2^{V617F} mutation. **a** Correlation curve showing variant allelic fraction (VAF) found in qPCR (Mutaquant, Ipsogen), and in NGS (TSCA, Illumina) in 276 MPN samples found positive for JAK2^{V617F} mutation in both techniques. **b** Difference of VAFs between NGS and qPCR for the 276 samples

Table 1 Results of $JAK2^{V617F}$ mutation detection in 473 samples using qPCR and NGS

	Negative NGS	Positive NGS	Total
Positive qPCR	22	276	298
Negative qPCR	172	3	175
Total	194	279	473

c.1843G>T (p.V615L) was present. This association of p.V615L with p.V617F has already been reported [27]. All these mutations were confirmed by Sanger sequencing. Although the $JAK2^{V617F}$ mutation was present in these three samples, the presence of additional nucleotide changes probably interfered with the mutation-specific primer annealing, explaining false negativity by qPCR [26, 28].

Interestingly, we also had the opportunity to compare the results for mutations located in the exon 12 of the *JAK2* gene. Among 40 samples analyzed with HRM (high-resolution melting curve analysis) and NGS techniques, the correlation was excellent since the 4 HRM-positive samples were correctly detected using NGS technique, without false-positive result (Table 2).

NGS allows detection of other rare JAK2 variants in MPNs

Compared to allele-specific PCR or HRM, the NGS approach allows covering much larger genomic regions, making easy to sequence the full coding regions of the *JAK2* gene. The analysis of the 473 samples identified both already known and previously undescribed variants (Table 3). In total, 28 different variants were present in 52 distinct patients among the cohort of 427 patients (12%). These variants were concomitantly present with the $JAK2^{V617F}$ mutation in 24 patients (45%) or with CALR mutants in 8 patients (16%) but never with an MPL mutant. Several variants were previously described as germline mutations (Table 3). Among these, c.2538G>C (p.E846D) and c.3323A>G (p.N1108S) were identified in two and four patients respectively while the c.3188G>A (p.R1063H) was found with a high frequency in our cohort (18 patients, 4%), significantly higher than the minor allelic fraction described in dbSNP (0.4%). Three variants identified in few patients in this study are referenced as rare single nucleotide polymorphisms (SNPs) in genomic databases such as dbSNP (c.98A>C, p.K33T; c.380G>A, p.G127D or c.731A>G, p.K244R). For two variants of unknown significance (c.415G>A, p.G139S or c.448A>C, p.M150L), the VAFs were close to 50% and thus compatible with SNPs. However, we identified four variants present with VAFs below 30%, thus suggesting a somatic acquisition (c.2251G>C, p.D751H VAF 9%; c.841G>A, p.G281S VAF 21%; c.1034A>T p.H345L VAF 29%; c.2449_2451delACA, p.T817del VAF 5%).

Table 2 Results of JAK2 exon 12 mutation detection in 40 samples using HRM and NGS

	Negative NGS	Positive NGS	Total
Positive HRM	0	4	4
Negative HRM	41	0	41
Total	41	4	45

The JAK2 p.H345L variant detected in NGS is associated with cytokine hypersensitivity

Among the variants of unknown significance identified in this study, we were particularly interested in the p.H345L mutant because several mutants have been described in close vicinity at codons 317 (p.Y317H) and 340 (p.R340Q) [24]. The mutation was identified in a 65-year-old man diagnosed with PMF. This patient also presented with a type 1 *CALR* mutation (VAF 41%) and an *IDH1* mutation (c.395G>A, p.R132H VAF 30%). We first excluded a potential germline origin of the mutation by studying DNA extracted from patient's nails, in which only the wild-type sequence of *JAK2* was detectable. To evaluate the functional consequences of this mutant, BaF/3 cells expressing the EPO receptor were transfected with plasmids containing the *JAK2* wild-type ($JAK2^{WT}$), $JAK2^{V617F}$, or $JAK2^{H345L}$ genes. At low concentration of EPO, the cells expressing $JAK2^{H345L}$ grew significantly better compared to $JAK2^{WT}$ cells (Fig. 2a) demonstrating that the presence of the p.H345L mutation conferred cytokine hypersensitivity to the BaF/3 cells. The dose-response curve at increasing EPO concentrations showed a maximal proliferation rate achieved at doses 5 to 10 times lower in BaF/3 cells expressing $JAK2^{H345L}$ compared to $JAK2^{WT}$ cells, approaching results of $JAK2^{V617F}$ expressing cells (Fig. 2b). As a confirmation, we found that the JAK2/STAT5 pathway was over-activated by $JAK2^{H345L}$. Indeed, STAT5 was constitutively phosphorylated in $JAK2^{H345L}$ BaF/3 cells in the absence of EPO. Furthermore, significantly higher levels of phospho-STAT5 were observed after EPO stimulation (Fig. 2c, d). In conclusion, these elements strongly suggest that JAK2 p.H345L is a gain-of-function mutant which induces a stronger stimulation of the JAK2/STAT5 pathway resulting in a proliferation advantage compared to the JAK2 WT cells.

Discussion

Identification of the $JAK2^{V617F}$ mutation using allele-specific qPCR techniques has been a gold standard for the diagnosis and monitoring of patients for about 10 years because of its high sensitivity and its accuracy for quantifying allelic burden in patients [16]. Confirming previous studies [29, 30], we

Table 3 Other JAK2 variants found by NGS in a cohort of 427 MPN patients

JAK2 variant	Protein	Germline/ somatic	MAF	Reference	Number of patients
c.98A>C	p.K33T	NA	NA	rs538474116	1
c.380G>A	p.G127D	Germline	0.20%	rs56118985	1
c.415G>A	p.G139S	NA	NA	NA	1
c.448A>C	p.M150L	NA	NA	NA	1
c.731A>G	p.K244R	Germline	0.20%	rs62637625	2
c.841G>A	p.G281S	NA	NA	NA	1
c.901G>A	p.G301R	NA	NA	NA	1
c.1009A>C	p.N337D	NA	0.20%	rs149683525	1
c.1019G>A	p.R340Q	Somatic	0.003%	COSM88204	2
c.1034A>T	p.H345	NA	NA	NA	1
c.1075T>A	p.L359I	NA	NA	NA	1
c.1711G>A	p.G571S	Somatic	0.01%	COSM29107	2
c.1718T>C	p.L573P	NA	NA	NA	1
c.1805G>C	p.S602T	NA	NA	NA	1
c.1843G>T	p.V615L	NA	NA	Lippert 2014	1
c.1848_1849delTGinsCT	p.V617F	NA	NA	NA	1
c.1860C>A	p.D620E	NA	NA	Grünebach 2006	1
c.2008A>G	p.I670V	NA	0.003%	rs771779649	1
c.2251G>C	p.D751H	NA	NA	NA	1
c.2438A>G	p.Y813C	NA	NA	NA	2
c.2449_2451delACA	p.T817del	NA	NA	NA	1
c.2538G>C	p.E846D	Germline	0.05%	rs150221602	2
c.2696T>C	p.I899T	NA	0.01%	rs200282557	1
c.2767C>T	p.R923C	NA	0.003%	COSM4384408	1
c.3152T>C	p.I1051T	NA	0.01%	rs375671491	1
c.3163A>G	p.K1055E	NA	0.002%	rs774539976	1
c.3188G>A	p.R1063H	Germline	0.40%	rs41316003	18
c.3323A>G	p.N1108S	Germline	0.20%	COSM33708	4

NA not available

show here that this mutation can be accurately detected by a NGS approach. By comparing results obtained using NGS and qPCR on a large cohort of 427 patients, we found an excellent correlation of quantitative data. The main limitation of NGS in our hands was sensitivity. Indeed, with a higher threshold of detection compared to qPCR, several samples with low allelic burdens were found negative. It appeared that qPCR is better suited to the evaluation of minimal residual disease under treatment or after allogeneic bone marrow transplantation [11]. However, using highly sensitive approaches, NGS proved to be able to detect $JAK2^{V617F}$ at low levels [29].

Since the discovery of the $JAK2^{V617F}$ mutation in 2005 [1] and the JAK2 exon 12 mutations in 2007 [17], several other mutants have been reported in MPNs. These mutations can affect the FERM domain or the kinase domain, can be found isolated or associated with driver mutations in *JAK2* or *CALR*, and may be germline or somatically acquired [19, 20, 22, 26, 31–33]. Mutants altering the kinase domain are usually associated with thrombocytosis or polycythemia [21, 24]. Some of the alternative mutations were functionally characterized showing in some cases a moderate to high gain of function as determined by the ability of cell lines to grow in the presence of low EPO concentrations and by activation of the JAK/STAT pathway. Among the mutants, we have identified in our cohort, the p.R1063H and the p.E846D germline mutants have been shown to weakly induce JAK/STAT signaling when tested separately [23]. However, when both mutants were co-expressed in the same cells, they cooperated to induce a stronger activation [23]. Although animal models are lacking, these results suggest that these germline mutants may cooperate together and probably with $JAK2^{V617F}$ as well, and may participate to the disease initiation or evolution. Interestingly, in our cohort, the p.R1063H was present in 4% of patients (in 39% of cases associated with $JAK2^{V617F}$ and in 17% with a *CALR* mutant). It would thus be interesting to evaluate

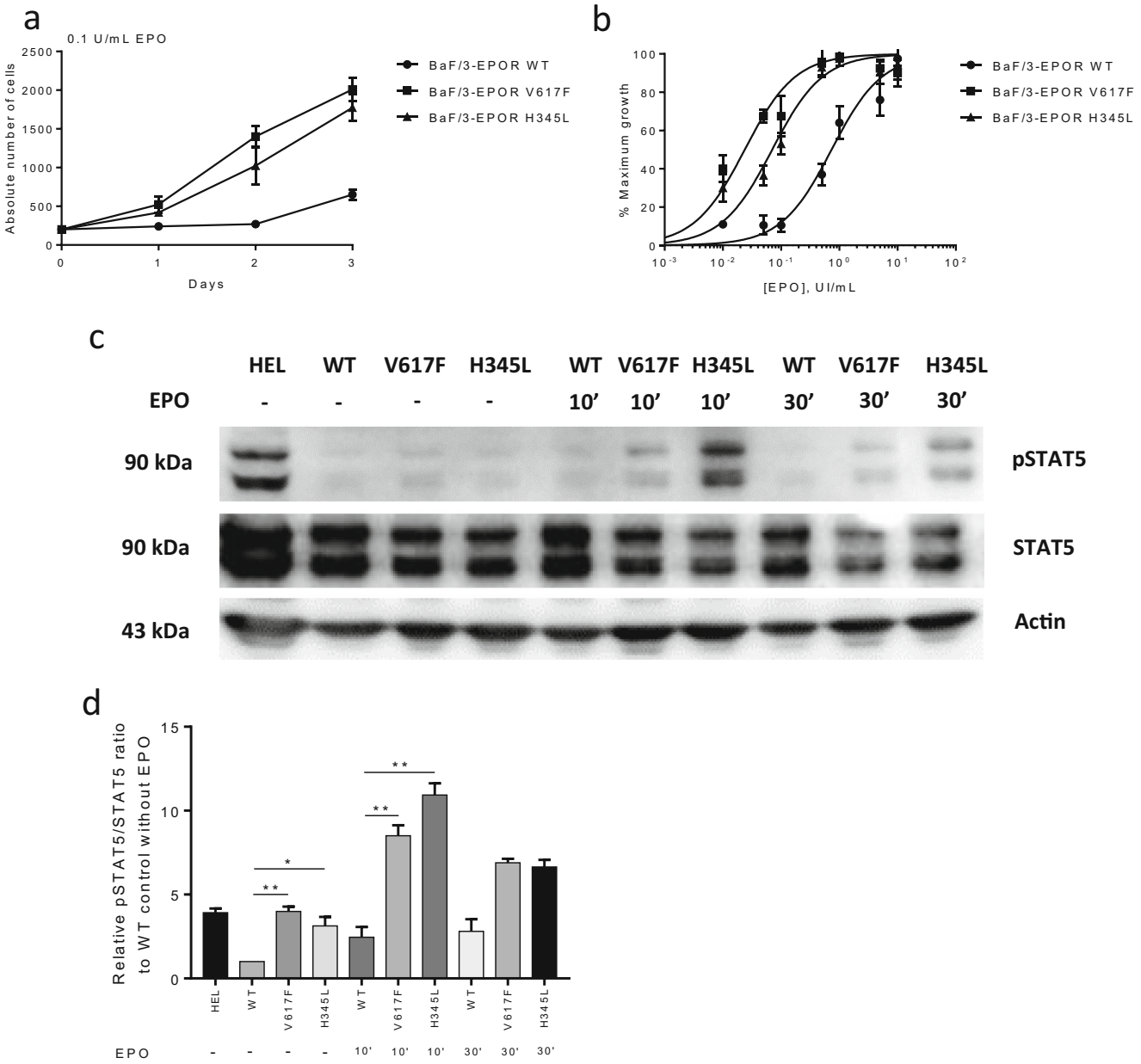

Fig. 2 The JAK2^{H345L} variant is associated with cytokine hypersensitivity. **a** Absolute numbers of cells at low dose of EPO (0.1 IU/mL) in BaF/3 cells stably expressing JAK2 wild-type (WT), JAK2^{V617F}, or JAK2^{H345L}. **b** Dose response to increasing doses of EPO curve BaF/3 cells stably expressing the different forms of JAK2. **c**, **d** Western blot of phopho-STAT5, total STAT5, and actin expression before and after treatment with EPO of BaF/3 cells stably expressing the different forms of JAK2. The JAK2^{V617F}-positive HEL cell line is used as control

whether such patients evolve differently than patients with JAK2^{V617F} alone.

We identified one novel somatic variant exhibiting an obvious gain of function. This p.H345L mutant affects the FERM domain of the JAK2 protein, responsible for the interaction with cytokine receptors. This could explain its impact on kinase activity and cell growth. Several mutants have been reported in this region, in positions 293, 317, 337, and 340 respectively and we identified in the present study another mutant in position 301 (p.G301R, VAF 8%). Although p.R340Q did not appear to modify cell proliferation or JAK/STAT signaling, p.Y317H was shown to be a gain-of-function mutant giving a proliferative advantage to mutated cells [24]. Together with our results on the p.H345L, this suggests that acquired mutations in the FERM domain of JAK2 could be a novel region of interest for MPN pathogenesis. The clinical outcome of patients with such mutations should be scrutinized in future studies.

The p.H345L mutant we identified was present in a patient with a *CALR* mutation. According to the VAFs of each

mutation (37% for CALR and 29% for $JAK2^{H345L}$), it is highly likely that both mutations were present in the same clone. Although the coexistence of both $JAK2^{V617F}$ and *CALR* mutations in the same patients have already been described [34], the functional impact due to the potential cooperation between these mutants has not been tested yet. These results together with our observation deserve further studies to characterize the consequences of the co-occurrence of these mutations in the same cells, which could lead to the activation of the MPL-dependent signaling by two different routes.

In conclusion, we show that NGS allows the detection of the $JAK2^{V617F}$ mutation with comparable performances, but weaker sensitivity, to qPCR as well as the detection of new potentially pathogenic *JAK2* variants in MPN patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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