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Targeting glutamine metabolism in myeloproliferative neoplasms

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Abstract

JAK2^{V617F} mutation can be detected in the majority of myeloproliferative neoplasm (MPN) patients. The JAK2 inhibitor Ruxolitinib is the first FDA-approved treatment for MPNs. However, its use is limited by various dose related toxicities. Here, we studied the metabolic state and glutamine metabolism of BaF3-hEPOR-JAK2V617F and BaF3-hEPOR-JAK2WT cells. We found that the *JAK2*^{V617F}-mutant cells were associated with increased oxygen consumption rate and extracellular acidification rate than the *JAK2*^{WT} cells and there was an increased glutamine metabolism in *JAK2*^{V617F}-mutant cells compared to wild-type cells. Glutaminase (GLS), the key enzyme in gluta-mine metabolism, was upregulated in the *JAK2*^{V617F}-mutant BaF3 cells compared to the JAK2^{WT} BaF3 cells. In MPN patient peripheral blood CD34+ cells, GLS expression was increased in *JAK2*^{V617F}-mutant progenitor cells compared to *JAK2* wild-type progenitor cells from the same patients and GLS levels were increased at the time of disease progression compared to at earlier time points. Moreover, GLS inhibitor increased the growth inhibitory effect of Ruxolitinib in both *JAK2*^{V617F}-mutant cell lines and peripheral blood CD34+ cells from MPN patients. Therefore, GLS inhibitor should be further explored to enhance the therapeutic effectiveness of JAK2 inhibitor and allow the administration of lower doses of the drug to avoid its toxicity.

Keywords

Glutamine; Cancer metabolism; Myeloproliferative neoplasm; Glutaminase inhibitor; JAK2 inhibitor

1. Introduction

The chronic Philadelphia chromosome negative myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), are clonal stem cell disorders characterized by dysregulated

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Author contribution

HZ designed the study, performed the research, and wrote the manuscript. KC and KD performed the research. SZ designed the experiment and edited the manuscript. All authors approved the submitted version of the manuscript.

hematopoiesis resulting in excessive production of red cells, white cells and/or platelets, a tendency to develop extramedullary sites of hematopoiesis, and possible evolution to acute leukemia or myelofibrosis. *JAK2*^{V617F} is an acquired activating mutation which can be detected in >95% PV patients and in 50–60% of ET and PMF patients [1–6]. Activated JAK2 causes dysregulated signaling through hematopoietic cytokine receptors and increased proliferation of hematopoietic cells and is associated with many of the phenotypic abnormalities of the MPNs. Ruxolitinib was the first FDA-approved JAK2 inhibitor for the treatment of MPNs; however, its use is limited by various toxicities due to its suppression of normal marrow function. Because its toxicity is related to dosage, combinations of drugs may enhance the therapeutic effectiveness of Ruxolitinib and permit the administration of lower doses of Ruxolitinib.

Over the past two decades, it has become appreciated that neoplastic diseases involve not only deregulated control of cell proliferation and survival but also metabolic transformations in order to fuel cancer cell growth and division. One hallmark of cancer is Otto Warburg's observation almost a century ago that cancers tend to use glucose at a high rate and convert it primarily to lactate (i.e. aerobic glycolysis or Warburg effect) rather than oxidizing it completely [7]. As a result of the Warburg effect, fewer glucose-derived metabolites feed into the tricarboxylic acid (TCA) cycle and cancer cells typically have an increased reliance on alternative nutrients to replenish the TCA cycle intermediates. One such nutrient is the amino acid glutamine which is the most abundant free amino acid in human blood [8] and has been known for more than half century as an essential ingredient for cultured cancer cells to proliferate [9–11]. Recent studies have suggested that cancer cells depend on a continued supply of glutamine to generate energy, amino acids, nucleotides, and glutathione for cell survival and proliferation [12–15]. The difference between cancer cells and normal cells in cell metabolism provides a promising cancer therapy target as pivotal metabolic enzymes are therapeutically accessible by small drug-like inhibitors [16]. However, glutamine metabolism is poorly understood in hematologic neoplasms.

After several decades of advances in our understanding of the genetics and molecular biology of cancer, it has become evident that targeting individual oncogene or tumor suppressor gene is not sufficient to eliminate cancer that has accumulated a host of mutations during its development and has the ability to shift and switch its vulnerability to oncogene-targeted therapy [17]. On the other hand, the constitutive active growth of cancer cells and their addiction to certain fuel sources makes them vulnerable to cell-killing agents that target cancer metabolism and bioenergetics. Recently, it was shown that the *JAK2*^{V617F}-mutant cells have an increased glucose uptake and glycolysis that can be partly inhibited by the JAK inhibitor [18]. Here, we investigated how the *JAK2*^{V617F} mutation affects glutamine metabolism and whether this could provide a therapeutic target to augment the effect of Ruxolitinib. We show that *JAK2*^{V617F} mutation is associated with an increased metabolic activity and an increased expression of the key enzyme in glutamine metabolism, glutaminase (GLS). Furthermore, GLS inhibitor increased the growth inhibitory effect of Ruxolitinib in both the *JAK2*^{V617F}-mutant cell lines and the peripheral blood CD34+ cells from MPN patients.

2. Materials and methods

2.1. Patient sample and cell preparation

8 MPN patients (4 PV, 2 ET and 2 PMF) were included in the study. All patients met the World Health Organization diagnosis criteria for PV, ET, and PMF. All patients gave written consent for venipuncture and the study protocol was approved by the Institutional Review Board of Northport VA Medical Center. Peripheral blood CD34+ cells from MPN patients were isolated from the Ficoll buffy coats using immunomagnetic beads (Miltenyi®) as previously described [19]. The purity of the CD34+ cell population was analyzed using a FACSCalibur flow cytometer (BD Biosciences) and it was >90% positive for CD34+. Peripheral blood CD34+ cells from healthy controls were purchased from AllCells™. Peripheral blood CD34+ cells from healthy controls were purchased from AllCells™.

2.2. Cell lines

The *JAK2*^{V617F}-mutant human erythroleukemia (HEL) cell line was provided by Dr. Ronald Hoffman (Mount Sinai School of Medicine, NY) and was maintained in RPMI 1640 medium (Cellgro®) supplemented with 10% FBS and 1% penicillin–streptomycin. The interleukin-3 (IL-3)-dependent murine prolymphoid cell line BaF3 expressing human EPOR and wild-type or mutant *JAK2* (BaF3-hEPOR-JAK2^{WT} and BaF3-hEPOR-JAK2^{V617F}) was generated by Dr. Ian Hitchcock (Stony Brook University, NY) [20]. All BaF3 cells (BaF3 parental, BaF3-hEPOR-JAK2^{WT}, and BaF3-hEPOR-JAK2^{V617F}) were maintained in RPMI1640 medium supplemented with 10% FBS, 1% penicillin–streptomycin, and IL-3 (2 μl/mL of conditioned medium from IL-3-producing baby hamster kidney cells) during all the experiments [20,21].

2.3. Seahorse XF^{e}96 extracellular flux analysis

Mitochondrial respiration (indicated by oxygen consumption rate, OCR) and glycolysis (indicated by extracellular acidification rate, ECAR) of the BaF3-hEPOR-JAK2^{WT} and BaF3-hEPOR-JAK2^{V617F} cells were measured using the XF^{e}96 Cell Bioanalyzer (Seahorse Biosciences®). Briefly, 40,000 cells per well were seeded in non-buffered assay medium on Cell-Tak (Corning®) coated XF^{e} 96 Cell Culture Microplate. Cells were then incubated in a 37°C non-CO2 incubator for 30 min. Basal OCR and ECAR were measured within 1 h.

2.4. Gas chromotography–mass spectrometry analysis

5×10^5 BaF3 cells expressing the wild type or mutant *JAK2* (BaF3-hEPOR-JAK2^{WT} and BaF3-hEPOR-JAK2^{V617F}) were grown in $^{13}C_5$-glutamine (4 mM) medium (Sigma®) for 24 h. At the end of the culture period, cells were collected and metabolites were extracted together with Na-2-oxobutyrate (Sigma®) (100 nmol per sample) and derivatized with Tri-Sil HTP Reagent (Thermo Scientific®) (100 μl per sample) before GC–MS analysis. Data were analyzed using the MassHunter software (Agilent Technologies®).

2.5. Cell proliferation assay

For cell lines, HEL and BaF3 cells were treated with DMSO, glutaminase inhibitor BPTES (Sigma®), and JAK2 inhibitor Ruxolitinib (Selleckchem®) for 72–96 h and cell

proliferation was assessed by counting bright live cells in a hemocytometer using trypan blue dye to exclude the dead cells. Each experiment was performed in triplicate samples and was repeated at least twice.

For CD34+ cell in vitro proliferation, 1×10^5 peripheral blood CD34+ cells from MPN patient were cultured in serum-free medium with cytokine stem cell factor (100 ng/mL), Flt3-ligand (100 ng/mL), and thrombopoietin (20 ng/mL) in the presence of DMSO, BPTES (2 μM) and/or Ruxolitinib (250 nM). Cells were harvested on day 8, and the corresponding cell numbers were counted using trypan blue dye exclusion. Each experiment was performed in triplicate.

2.6. Hematopoietic progenitor cell assay

Peripheral blood CD34+ cells from MPN patients were cultured using Methocult H4230 semisolid media (StemCell Technologies®) with growth factors as described previously [19]. Briefly, 1000 CD34+ cells were plated per dish in duplicate cultures to which SCF (50 ng/mL), IL-3 (50 ng/mL), GM-SCF (50 ng/mL), and erythropoietin (2 U/mL) were added. After 14 days of incubation, individual colonies (both burst forming unit-erythroid (BFUe) and colony forming unit-granulocyte macrophage (CFU-GM) derived colonies) were plucked and genotyped for *JAK2*^{V617F} status.

For drug treatment experiments, cells were cultured using Methocult H4230 in the presence of DMSO, BPTES (2 μM), and/or Ruxolitinib (250 nM). Each experiment was performed in duplicate. After 14 days of culture, the morphology and number of colonies was determined using an inverted light microscope.

2.7. Nested allele-specific PCR for JAK2^{V617F}

Genomic DNA was isolated from plucked colonies using the Extract-N-Amp Blood PCR Kits (Sigma-Aldrich®). *JAK2*^{V617F} was detected using a nested allele-specific PCR as described previously [22,23]. The final PCR products were analyzed on 2.0% agarose gels. The nested PCR product had a size of 453 bp. A 279-bp product indicated allele-specific *JAK2*^{V617F}-positive, whereas a 229-bp product denoted allele-specific wild-type product.

2.8. Quantitative real-time polymerase chain reaction

The TaqMan® Gene Expression Assay (Applied Biosystems) was used for quantitative real-time polymerase chain reaction (qRT-PCR) to verify differential GLS expression (Hs00248163_m1 for human GLS and Mm01257297_m1 for mouse GLS), glutamate dehydrogenase (Mm00492353_m1), Glutamic pyruvate transaminase (alanine aminotransferase) 2 (Mm00558028_m1), on an ABI ViiA™ 7 Real-Time PCR machine (Applied Biosystems). The GLS expression levels were normalized to 18S (human) or Transferrin receptor (mouse) expression (Applied Biosystems) and relative fold changes was calculated by the $2^{\Delta\Delta CT}$ method. All assays were performed in triplicate.

2.9. Statistical analysis

Statistical analyses were performed using Student's unpaired, 2-tailed t tests using Excel software (Microsoft). A *P* value of less than 0.05 was considered significant. For all bar graphs, data are represented as mean ± standard error of the mean (SEM).

3. Results

3.1. JAK2^{V617F} mutation is associated with increased metabolic activity in BaF3 cells

To assess how *JAK2*^{V617F} mutation affects cellular metabolism, we studied an isogenic pair of *JAK2*^{V617F} mutant and *JAK2* wild-type BaF3 cell lines (BaF3-hEPOR-JAK2^{V617F} and BaF3-hEPOR-JAK2^{WT}) using the Seahorse XF^{e}96 Cell Bioanalyzer (Seahorse Biosciences®), which measures oxygen consumption rate (OCR) as an indicator of oxidative phosphorylation and extracellular acidification rate (ECAR) as an indicator of glycolytic conversion of glucose to lactate. Compared to *JAK2*^{WT} cells, *JAK2*^{V617F} cells had increased cell growth (2.5-fold, p = 0.013), cellular OCR (1.5-fold, p = 0.0007), and cellular ECAR (2-fold, p = 0.0002). This indicated that the *JAK2*^{V617F}-mutant cells had higher mitochondrial respiration and glycolytic activity than the *JAK2*^{WT} cells (Fig. 1A). The mutant cells also had a lower OCR to ECAR ratio than the wild-type cells (4.04 v.s. 5.05), which is consistent with an increased glycolysis associated with the *JAK2*^{V617F} mutation (Fig. 1B). We did not notice any difference in the metabolic activity between the BaF3-hEPOR-JAK2^{WT} cells and the parental BaF3 cells.

3.2. JAK2^{V617F} mutation is associated with increased glutamine metabolism

Previously, it was shown that the *JAK2*^{V617F}-mutant cells have increased glucose uptake and glycolytic lactate production associated with the induction of key glycolytic enzymes [18]. It has been reported that the glycolytic cancer cells increase their consumption of glutamine to feed the TCA cycle and produce the bioenergetic and biosynthetic substrates [15,24]. We used ^{13}C stable isotope analysis to determine the differences in glutamine utilization between *JAK2*^{V617F}-mutant and *JAK2* wild-type cells. Briefly, equal numbers of BaF3-hEPOR-JAK2^{WT} cell and BaF3-hEPOR-JAK2^{V617F} cells were grown in [U5]-^{13}C glutamine for 24 h and metabolites were extracted. We found that there were increased glutamate (1.9-fold), citrate (4.2-fold), and lactate (4.4-fold) abundance in *JAK2*^{V617F}-mutant cells compared to *JAK2*^{WT} cells, suggesting that the *JAK2*^{V617F}-mutation was associated with increased glutamine use and flux through the TCA cycle (Fig. 1C).

3.3. JAK2^{V617F} mutation is associated with increased glutaminase expression

The central role of glutamine metabolism in cancer cell growth makes glutaminolytic enzymes attractive targets for cancer therapy. Glutamine can be converted to glutamate by the enzyme glutaminase (GLS). Glutamate can then be converted to α-ketoglutarate by the enzyme glutamate dehydrogenase or transaminases [25]. Using quantitative real-time PCR, we found that GLS was upregulated (2-fold) and glutamate dehydrogenase was down regulated (3.3-fold) in the *JAK2*^{V617F}-mutant BaF3 cells compared to the *JAK2*^{WT} BaF3 cells. There was no difference in glutamic pyruvate transaminase between the *JAK2*^{V617F}-mutant and the *JAK2*^{WT} cells. (Fig. 2A)

Glutaminase (GLS) is the key enzyme in glutamine metabolism and is frequently upregulated in various human cancers [16,26–28]. GLS suppression reduces cell proliferation and blocks oncogenic transformation [15,25,29,30]. We investigated whether GLS expression was altered in MPN patients. In peripheral blood CD34+ cells, we did not detect any significant difference in GLS mRNA levels between healthy donors (n = 2) and MPN patients (n = 6) (Fig. 2B). As we know, the stem cell compartment in MPN is heterogeneous with the presence of both *JAK2* wild-type clones and $JAK2^{V617F}$-mutant clones co-existing in most MPN patients and there are widely varying proportions of wild-type and mutant clones present in samples obtained from different MPN patients. The $JAK2^{V617F}$ clone has the capacity to expand over time and become dominant over the normal clones [31–34]. Since cancer cell proliferation depends on a continued supply of glutamine [12–14], we hypothesized that MPN clonal expansion and disease progression would be associated with a concomitant induction of GLS levels to support the metabolic transformation needed. To test this hypothesis, we first studied GLS expression in clonally-derived erythroid progenitor cells (Burst Forming Unit-Erythoid, BFUe) from five $JAK2^{V617F}$-positive MPN patients (3 PV, 1 ET, and 1 PMF). We found that GLS expression was increased by an average of 36% (range 9%–83%, p = 0.02) in the $JAK2^{V617F}$-mutant BFUe colony cells compared to the *JAK2* wild-type BFUe colony cells from the same patients (Fig. 2C). Second, in serial peripheral blood CD34+ cell samples from two PMF patients (one $JAK2^{V617F}$-mutant and one $JAK2^{WT}$) whose disease progressed during follow up (i.e. worsening constitutional symptoms and/or increasing peripheral blood blast counts), GLS mRNA levels were increased by a mean of 1.7-fold at the time of disease progression compared to earlier time points (Fig. 2D). Although our study could not provide the definitive answer regarding the role of glutaminase in MPN clonal expansion and disease progression due to the small sample size, these findings prompted us to further explore the potential of GLS inhibitor in MPN therapy.

3.4. Glutaminase inhibitors increased the growth inhibitory effect of JAK2 inhibitors in $JAK2^{V617F}$-mutant cell lines

Previously it was shown that the $JAK2^{V617F}$-mutant cells have an increased glucose uptake and glycolysis that can be partially inhibited by a JAK inhibitor [18]. Our current study has shown that the $JAK2^{V617F}$ mutation is associated with an increased glutamine use and the key glutamine metabolism enzyme glutaminase is increased in $JAK2^{V617F}$-mutant cells. Since both glucose and glutamine are essential nutrients for cancer cell growth and proliferation, we hypothesized that combined JAK inhibitor and GLS inhibitor treatment will have more suppression effect on MPN cell growth by inhibiting both the glucose and glutamine metabolism than JAK inhibitor alone. We tested Ruxolutinib (Selleckchem®), the first FDA-approved JAK2 inhibitor for the treatment of MPNs [35,36], and a small molecule GLS inhibitor, BPTES (bis-2-[5-phenylacetamido-1,3,4-thiadiazol-2-yl]ethyl sulfide) (Sigma®), which is a specific GLS inhibitor with minimal off-target effects due to its unique chemical structure [25,29,37,38]. We observed a dose-dependent effect of BPTES on inhibiting the HEL cell growth (Fig. 3A). While low-dose BPTES (5 μM) had little effect on HEL cell growth, the combination of BPTES (5 μM) and Ruxolitinib (1.5 μM) significantly inhibited HEL cell growth by 66% while Ruxolitinib alone inhibited cell growth by only

35% (p = 0.04) (Fig. 3B). Similar results were obtained with the BaF3-hEPOR-JAK2^{V617F} cells (data not shown).

3.5. Glutaminase inhibitor increased the effect of Ruxolitinib in suppressing the MPN patient CD34+ cell growth

Finally, we tested the combination therapy of BPTES and Ruxolitinib in peripheral blood CD34+ cells from healthy donor and MPN patients in both the serum-free liquid culture medium (to assess the effect on CD34+ cell in vitro proliferation) and the methylcellulose semi-solid medium (to assess the effect on CD34+ cell clonogenic growth). Since MPN patients without the *JAK2*^{V617F} mutation are also characterized by activated JAK2 signaling [39], we hypothesized that the combination therapy of BPTES and Ruxolitinib would be effective in both *JAK2*^{V617F}*-mutant* and *JAK2 wild-type* MPN patients. In serum-free liquid culture medium of two MPN patient peripheral blood CD34+ cells, BPTES and Ruxolitinib suppressed cell proliferation more significantly than Ruxolitinib alone did in both the *JAK2*^{V617F}-mutant (patient #1) and the *JAK2 wild-type* (patient #2) MPN patients, while there was no significant difference between Ruxolitinib alone and Ruxolitinib + BPTES in healthy donor CD34+ cells. (Fig. 4A) We then tested the combination therapy on peripheral blood CD34+ cell clonogenic growth in the methylcellulose colony formation assay. The results from the same healthy donor, *JAK2*^{V617F}-mutant patient (patient #1) and *JAK2* wild-type patient (patient #2) as above were shown in Fig. 4B. Overall in four MPN patients (one *JAK2*^{V617F}-mutant and three *JAK2* wild-type) tested (Fig. 4C), BPTES (2 μM) and Ruxolitinib (250 nM) suppressed total hematopoietic colony growth of MPN CD34+ cells by 91% in comparison to 68% inhibition by Ruxolitinib alone (p = 0.007). Both BFUe and CFU-GM colonies were suppressed by the combination therapy more than by Ruxolitinib alone although it was only significant for the CFU-GM colony formation (p = 0.025). In addition, BPTES co-treatment resulted in smaller colony sizes than Ruxolitinib alone did (data not shown).

4. Discussion

MPN are hematopoietic disorders characterized by dysregulated clonal hematopoiesis resulting in excessive production of red cells, white cells and/or platelets, and possible evolution to acute leukemia or myelofibrosis. Activated JAK2 signaling is seen in all MPN patients. Ruxolitinib was the first FDA-approved JAK2 inhibitor for the treatment of MPNs; however, its use is limited by various toxicities including anemia and thrombocytopenia due to its suppression of normal bone marrow function. Combinations of drugs to enhance the therapeutic effectiveness of Ruxolitinib will permit the use of lower doses of the drug to avoid the toxicities. We showed that there was an increased glutamine metabolism in *JAK2*^{V617F}-mutant cells compared to wild-type cells and the key glutamine metabolism enzyme, glutaminase, was upregulated in *JAK2*^{V617F}-mutant cells compared to *JAK2* wild-type cells. We demonstrated that the GLS inhibitor BPTES significantly increased the growth inhibition of the JAK2 inhibitor Ruxolitinib in both the *JAK2*^{V617F}-mutant cell lines and the peripheral blood CD34+ cells from MPN patients, while BPTES did not enhance the Ruxolitinib effect in healthy donor CD34+ cells. Therefore, the combination therapy of GLS inhibitor and JAK2 inhibitor should be further explored to not only enhance the therapeutic

effectiveness of JAK2 inhibitor but also allow the administration of lower doses of JAK2 inhibitor to avoid its toxicity. In our study, the combination therapy BPTES and Ruxolitinib was effective in both *JAK2*^{V617F}-mutant and *JAK2* wild-type MPN patients. This is not surprising as activated JAK2 signaling is seen in all MPN patients regardless of clinical phenotype or mutational status [39–41]. Recently it has been reported that somatic calreticulin (CALR) mutations occur in the vast majority of patients with nonmutated *JAK2* and is associated with an activated JAK2 transcriptional signature [39,42,43]. It is very likely that some of our *JAK2* wild-type MPN patients harbor the *CALR* mutation although this was not tested at the time of sample collection.

The stem cell compartment in MPN is heterogeneous with both *JAK2* wild-type clones and *JAK2*^{V617F}-mutant clones co-existing in most MPN patients. The *JAK2*^{V617F} clone has the capacity to expand over time and become dominant over the normal clones [31–34]. The mechanism(s) that contribute to the *JAK2*^{V617F}-positive hematopoietic stem/progenitor cell (HSPC) proliferation and expansion in MPN is poorly understood. In our study, we found that GLS expression was increased by an average of 36% in the *JAK2*^{V617F}-mutant erythroid progenitor cells compared to the *JAK2* wild-type erythroid progenitor cells in five MPN patients and there was an up-regulation of GLS levels (mean ~1.7-fold) at the time of disease progression compared to earlier follow up samples in two PMF patients (Fig. 2), suggesting that increased GLS levels may be associated with MPN clonal expansion and disease progression to support the metabolic transformation needed in MPN pathogenesis. Although our study was limited by the small patient sample size and therefore could not provide the definitive answer to the roles of glutaminase in MPN metabolic transformation, malignant clone expansion and disease progression, it showed that the constitutive growth of MPN cancer cells and their addiction to glucose and glutamine make them vulnerable to agents (e.g. glutaminase inhibitor) that target its unique cancer metabolism. Indeed, GLS has proved to be a critical enzyme in a number of cancer types [15,30,37,44] and pharmacological inhibition of GLS slowed the proliferation in several cancer cell types in vitro [37,45,46] and diminished tumorigenesis in several animal models [15,25,44]. Our work in JAK2^{V617F}-mutant cell lines and primary MPN patient samples suggest that MPN would respond to glutaminase inhibition and GLS inhibitor could be used to improve the therapeutic efficacy of Ruxolitinib the only FDA approved therapy in MPNs. Further testing will be required to explore whether GLS inhibitor would impact the JAK-STAT signaling and if the GLS inhibitor would allow dose reduction of JAK inhibitors in vivo.

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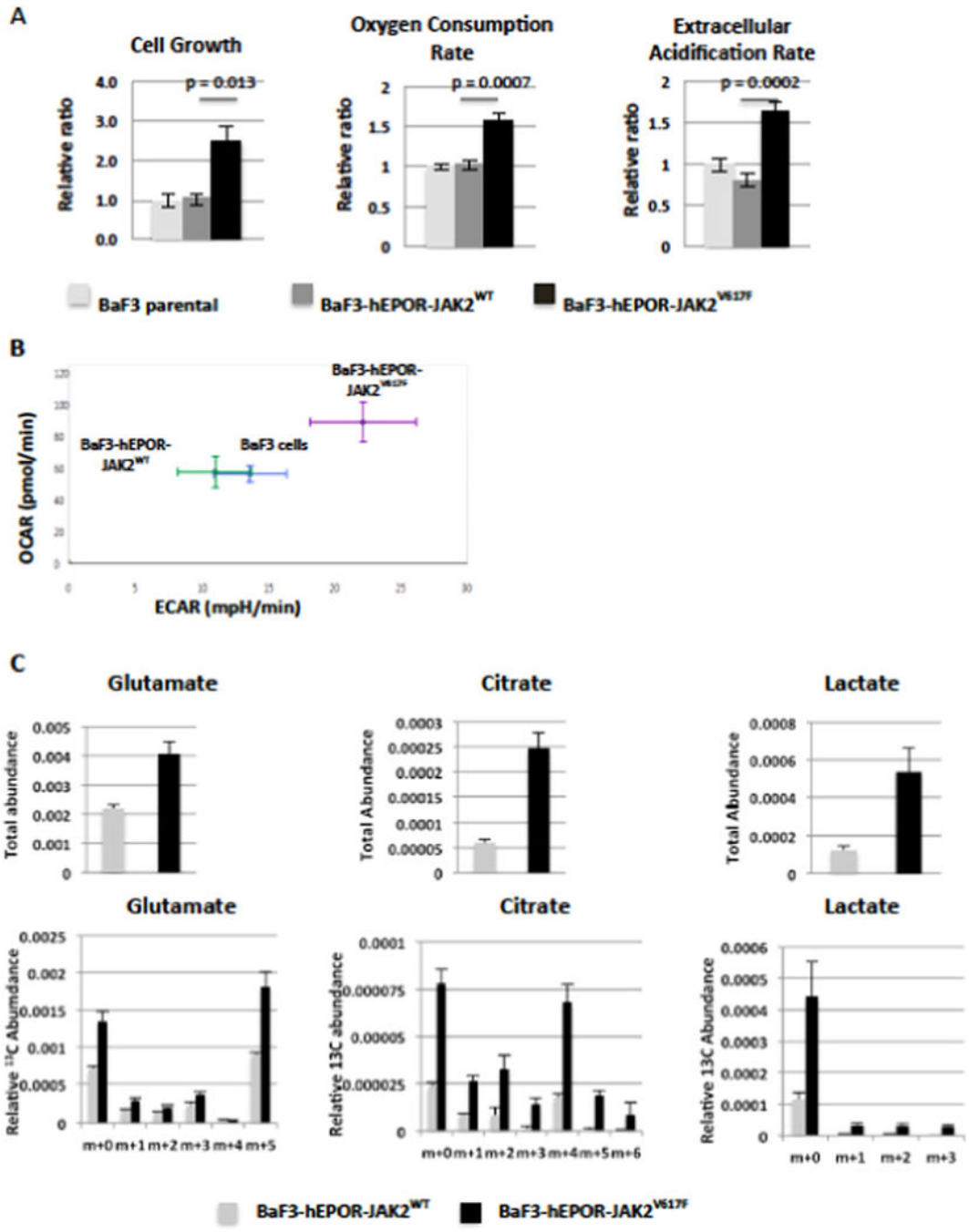

Fig. 1.

JAK2^{V617F} mutation is associated with increased metabolic activity in BaF3 cells. (A–B) JAK2^{V617F}-mutant cells had increased cell growth, oxygen consumption rate, and extracellular acidification rate compared to JAK2^{WT} cells (top), suggesting that the JAK2^{V617F} mutation is associated with increased mitochondrial respiration and glycolytic activity (bottom). (C) GC–MS analysis of glutamine metabolism in BaF3-hEPOR-JAK2^{wt} cells and BaF3-hEPOR-JAK2^{V617F} cells using stable ^{13}C isotope analysis. Top panel: increased total abundance of glutamate (1.9-fold), citrate (4.2-fold), and lactate (4.4-fold) in

JAK2^{V617F}-mutant cells compared to JAK2^{WT} cells. Bottom panel: relative ^{13}C isotopologue abundance of glutamate, citrate, and lactate in JAK2^{WT} and JAK2^{V617F}-mutant cells.

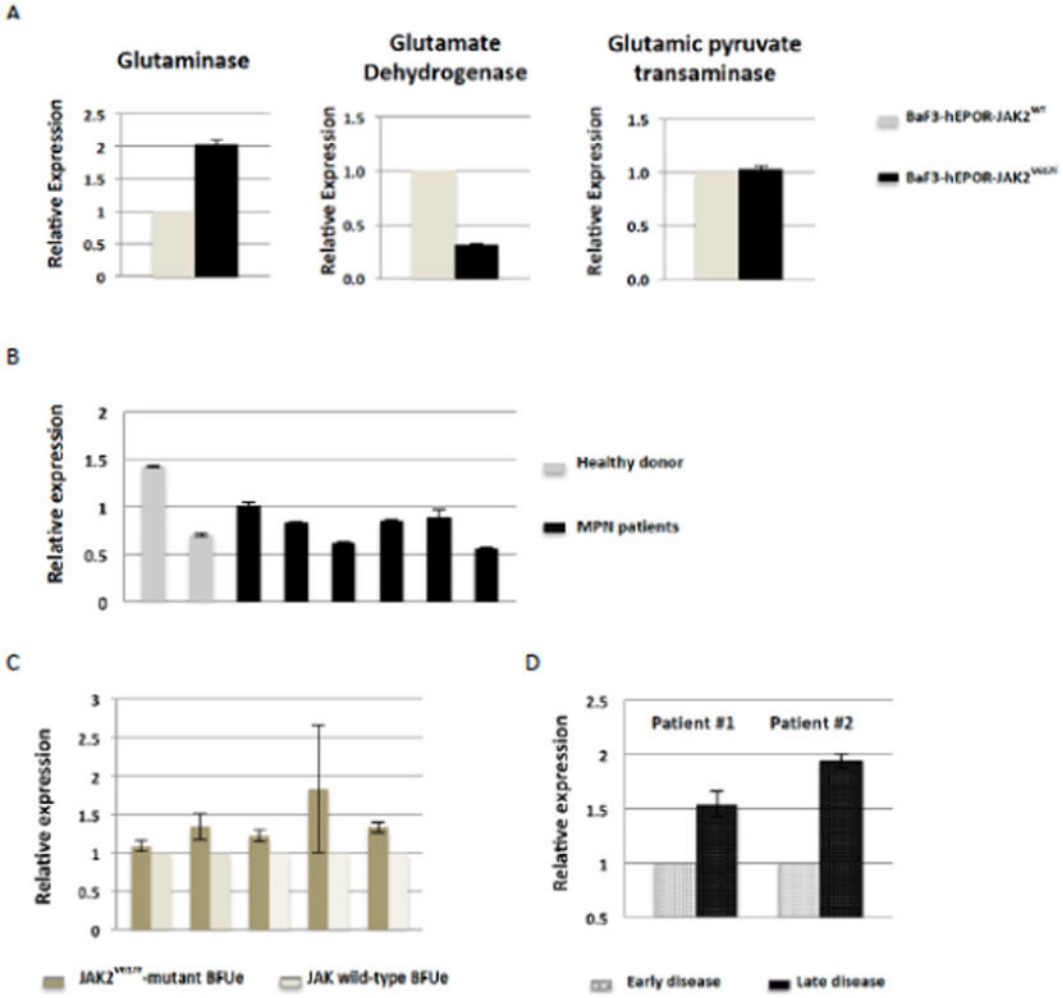

Fig. 2.

Glutaminase expression is upregulated in the *JAK2*^{V617F}-mutant cells compared to the *JAK2*^{WT} cells. (A) Glutaminase (GLS), glutamate dehydrogenase, and glutamic pyruvate transaminase mRNA expression in BaF3-hEPOR-JAK2^{WT} and BaF3-hEPOR-JAK2^{V617F} cells. Their expressions in the JAK2^{V617F}-mutant cells were shown as the fold-change compared to the JAK2 wild-type cells which was set as “1”. (B) GLS expression in peripheral blood CD34+ cells from healthy donors (n = 2) and MPN patients (n = 6). The individual sample miRNA expression was shown as the fold-change compared to the average healthy control expression which was set as “1”. (C) GLS expression in the Burst Forming Unit-Erythoid (BFUe) colonies derived from peripheral blood CD34+ cells of five JAK2^{V617F}-positive MPN patients. GLS expressions in JAK2^{V617F}-mutant BFUe colonies were shown as the fold-change compared to the JAK2 wild-type BFUe colonies from the same patient which was set as “1”. (D) GLS expression in serial peripheral blood CD34+ cell samples from two primary myelofibrosis patients during early follow up (“early disease”) and at the time of disease progression (“late disease”). GLS expressions in late disease samples were shown as the fold-change compared to the “early disease” samples from the same patient which was set as “1”.

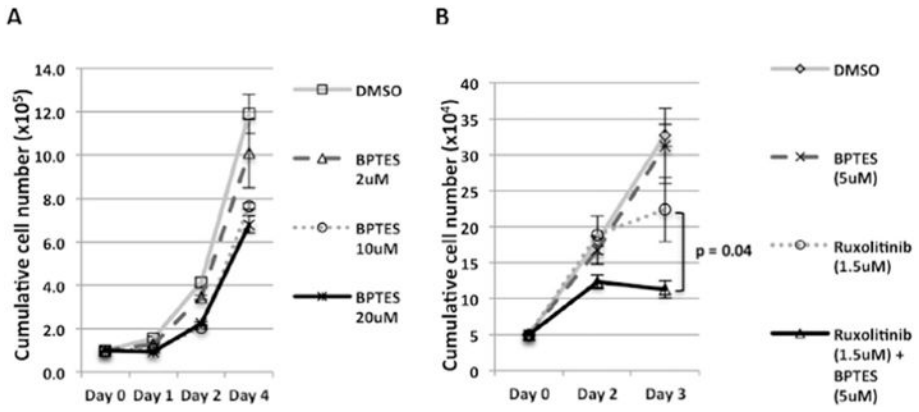

Fig. 3.

Glutaminase (GLS) inhibitor BPTES increased the growth inhibition of Ruxolitinib in $JAK2^{V617F}$-mutant cell lines. (A) BPTES induced a dose-dependent growth inhibition in the *JAK2*^{V617F}-mutant human erythroleukemia (HEL) cell line. (B) BPTES enhanced the growth inhibition of Ruxolitinib in the HEL cells.

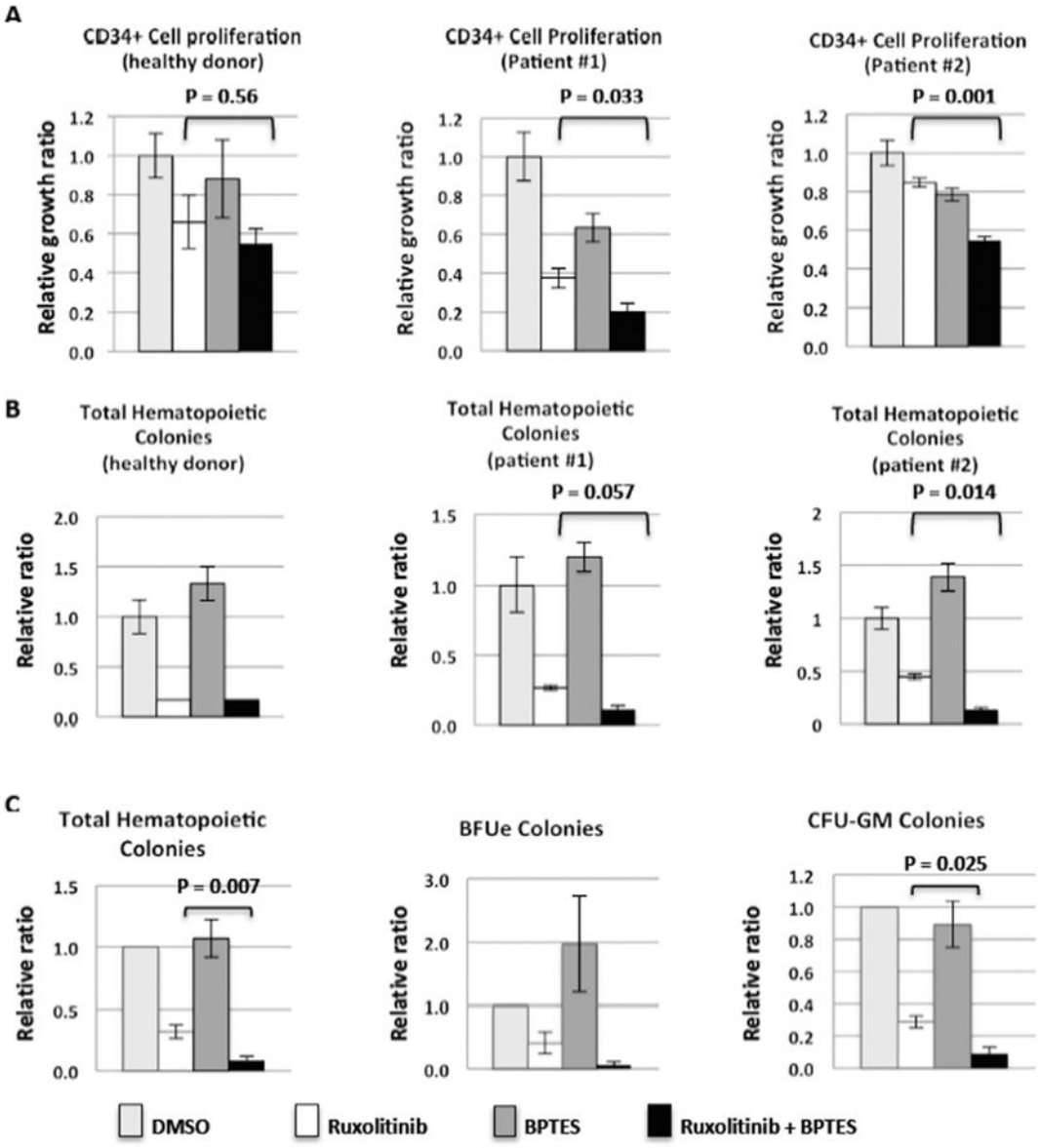

Fig. 4.

Glutaminase inhibitor BPTES enhanced Ruxolitinib-mediated inhibition of cell proliferation in MPN patients. (A) Peripheral blood CD34+ cells from a healthy donor, a $JAK2^{V617F}$-mutant (patient #1) and a JAK2 wild-type (patient #2) MPN patients were cultured in serum free medium with cytokines and treated with BPTES (2 μM) and/or Ruxolitinib (250 nM) and cumulative cell numbers were counted at the end of the 8-day culture. Cell proliferation was measured compared to cells treated by DMSO alone which was set as "1". Three independent experiments were performed. (B) Peripheral blood CD34+ cells from the same healthy donor, $JAK2^{V617F}$-mutant (patient #1) and JAK2 wild-type (patient #2) MPN patients as shown in (A) were cultured in methylcellulose semi-solid medium with growth factors in the presence of DMSO, Ruxolitinib (250 nM), BPTES (2 μM), and Ruxolitinib + BPTES. Total hematopoietic colonies were enumerated after 14 days of incubation and compared to the DMSO control which was set as "1". (C) Total hematopoietic colonies,

Burst Forming Unit-Erythroid (BFUe) colonies, and Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) colonies from four MPN patients (including the two patients shown in (A–B)). Peripheral blood CD34+ cells were cultured in methylcellulose semi-solid medium with growth factors in the presence of DMSO, Ruxolitinib (250 nM), BPTES (2 μM), and Ruxolitinib + BPTES. Each assay was performed in duplicate. The average colony growth was shown as the fold-change compared to the DMSO control which was set as “1”.