RESEARCH ARTICLE

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Circulating CD133+/–CD34– Have Increased c-*MYC* Expression in Myeloproliferative Neoplasms

Myeloproliferatif Neoplazilerde Dolaşımdaki CD133+/- CD34- Progenitörler Artmış *c-MYC* İfadelerine Sahiptir

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Abstract

Objective: Myeloproliferative neoplasms (MPNs) are hematopoietic stem cell (HSC)-originated diseases with clonal myeloproliferation. The constitutive activation of the JAK/STAT pathway is frequently detected in patients with Philadelphia chromosome-negative (Ph^-) MPNs with an acquired *JAK2*V617F mutation. The c-*MYC* proto-oncogene is associated with malignant growth and cellular transformation, and *JAK2*V617F was previously shown to induce constitutive expression of c-*MYC*. This study examines the expressional profile of c-*MYC* in Ph^- MPNs with *JAK2*V617F and highlights its hierarchical level of activation in circulating hematopoietic stem/progenitor cell (HSPC) subgroups.

Materials and Methods: Mononuclear cells (MNCs) of Ph^- MPNs were fluorochrome-labeled in situ with wild-type (wt) *JAK2* or *JAK2*V617F mRNA gold nanoparticle technology and sorted simultaneously. Isolated populations of *JAK2*wt or *JAK2*V617F were evaluated for their c-*MYC* expressions. The MNCs of 14 Ph^- MPNs were further isolated for the study of HSPC subgroups regarding their CD34 and CD133 expressions, evaluated for the presence of *JAK2*V617F, and compared to cord blood (CB) counterparts for the expression of c-*MYC*.

Results: The mRNA-labeled gold nanoparticle-treated MNCs were determined to have the highest ratio of c-*MYC* relative fold-change expression in the biallelic *JAK2*V617F compartment compared to *JAK2*wt. The relative c-*MYC* expression in MNCs of MPNs was significantly increased compared to CB (p=0.01). The circulating HSPCs of $CD133^{+/-}CD34^-$ MPNs had statistically significantly elevated c-*MYC* expression compared to CB.

Conclusion: This is the first study of circulating $CD133^{+/-}CD34^-$ cells in Ph^- MPNs and it has revealed elevated c-*MYC* expression levels in HSCs/endothelial progenitor cells (HSCs/EPCs) and EPCs. Furthermore, the steady increase in the expression of c-*MYC* within MNCs carrying no mutations and monoallelic or biallelic *JAK2*V617F transcripts was notable. The presence of *JAK2*V617F with respect to c-*MYC* expression in the circulating HSCs/EPCs and EPCs of MPNs might provide some

Öz

Amaç: Myeloproliferatif neoplazmalar (MPN), klonal myeloproliferasyon gösteren hematopoietik kök hücre (HKH) kaynaklı hastalıklardır. Philadelphia kromozom negatif (Ph^-) MPN hastalarında kazanılmış *JAK2*V617F mutasyonuna bağlı daimi JAK/STAT yolağı aktivasyonu sıklıkla saptanır. Proto-onkogen, c-*MYC*, malign büyüme ve hücresel transformasyon ile ilişkilidir ve daha önce *JAK2*V617F'nin, c-*MYC*'nin daimi anlatımını indüklediği gösterilmiştir. Bu çalışmada, *JAK2*V617F taşıyan Ph- MPN'lerde c-*MYC* anlatım profili incelenmiş ve dolaşımdaki hematopoietik kök/progenitör hücre (HKPH) alt gruplarında aktivasyonun hiyerarşik seviyesi araştırılmıştır.

Gereç ve Yöntemler: Mononükleer hücreler (MNH) mRNA-altın nanoparçacık teknolojisi ile in-situ yabanil-tip (yt) *JAK2* veya *JAK2*V617F florokromu ile etiketlendi ve izole edildi. *JAK2*yt veya *JAK2*V617F transkriptlerinin izole edilmiş popülasyonları, ilgili c-*MYC* ifadeleri açısından değerlendirildi. On dört MPN örneğinin MNH'leri, CD34 ve CD133 anlatımları kullanılarak dolaşımdaki herbir HKPH alt grubu için izole edildi, *JAK2*V617F durumu değerlendirildi ve karşılık gelen kordon kanı (KK) altgrubu ile c-*MYC* anlatımı karşılaştırıldı.

Bulgular: mRNA etiketli altın nanopartikül ile işaretlenmiş MNH'ler, *JAK2*yt'ye kıyasla bialelik-*JAK2*V617F bölmesinde en yüksek c-*MYC* nispi kat değişim ifadesi oranına sahip olduğu belirlendi. MPN MNH'lerindeki göreceli c-*MYC* anlatımı, KK'ye kıyasla anlamlı bir artış gösterdi (p=0,01). Dolaşan HKPH, $CD133^{+/-}CD34^-$, MPN'de KK'ye kıyasla istatistiksel anlamlı göreceli artmış c-*MYC* anlatımı gösterdi.

Sonuç: Bu çalışma, Ph^- MPN'lerde, dolaşan $CD133^{+/-}CD34^-$ ile ilgili ilk çalışmadır. HKH/endotel progenitör hücreler (HKH/EPH) ve EPH'de yüksek c-*MYC* anlatım seviyesini ortaya koymaktadır. Ayrıca, mutasyon taşımayan, monoalelik veya bialelik-*JAK2*V617F transkriptleri olan MNH'lerde c-*MYC* ifadesinde görülen artış önemlidir. MPN'de dolaşımdaki HKH/EPH ve EPH'de c-*MYC* ifadesine göre *JAK2*V617F varlığı, *JAK2*V617F'nin oluşması ve hastalığın yayılması için bazı bilgiler sağlayabilir. Bu tür popülasyonlarda artan c-*MYC* ifadesinin anlaşılabilmesi için ileri çalışmalar gereklidir.

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Abstract

evidence for the initiation of *JAK2*V617F and propagation of disease. Further studies are needed to clarify the implications of increased c-*MYC* expression in such populations.

Keywords: Myeloproliferative neoplasms, *JAK2*V617F, Hematopoietic stem cells, c-*MYC*, CD133, CD34, Gold-nanoflare, Endothelial progenitor cell

Öz

Anahtar Sözcükler: Myeloproliferatif neoplazmalar, JAK2V617F, Hematopoietik kök hücreler, c-*MYC*, CD133, CD34, Altın nanoflare, Endotel progenitör hücre

Introduction

Philadelphia chromosome-negative (Ph^-) myeloproliferative neoplasms (MPNs) are clonal disorders originating from hematopoietic stem cells (HSCs) [1,2,3]. Patients with MPNs are characterized by increases in the myeloid, erythroid, and megakaryoid cells in peripheral blood, leading to varying phenotypic diseases, including polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) [4,5]. A cytosolic tyrosine kinase, Janus kinase 2 (*JAK2*), plays a significant role in hematopoiesis and immune responses in cytokine-dependent cancers [6]. An acquired mutation (V617F) of *JAK2* is frequently detected in patients with MPNs, causing constitutive activation of the intracellular JAK/STAT pathway and leading to uncontrolled myeloproliferation and cytokine-independent cell survival [7,8].

MYC is a family of basic helix-loop-helix leucine zipper transcription factors, including c-*MYC*, N-*MYC*, and L-*MYC*, that regulate 11%-15% of the human genome and play diverse roles in cell cycle progression, apoptosis, and cellular transformation [9,10,11]. *MYC* controls HSCs by fine-tuning the balance between self-renewal, differentiation, proliferation, survival, and hematopoiesis [12,13,14,15,16]. In nearly half of all human tumors, overexpression of *MYC* is associated with malignant growth [17], with which enhanced protein synthesis, energy metabolism, and genomic instability have been associated [9,18,19]. This most common oncogene is also frequently associated with hematopoietic tumors, such as lymphoma and leukemia [14,20,21], and its driving role in myeloid malignancies is increasingly being indicated [22,23,24]. Recent studies have demonstrated *JAK2*V617F-induced constitutive expression of c-*MYC*, which is mediated by STAT5 activation [25], and this activation is FERM domain-dependent [26]. Upregulated expression of c-*MYC* mRNA is detected in bone marrow cells in ET but not in PV [27]. In addition, knockdown of c-*MYC* significantly inhibits the proliferation of *JAK2*V617F-positive cells [25]. Since myeloproliferation is remarkable in MPNs, it might be interesting to estimate the hierarchical level of c-*MYC* activation in *JAK2*V617F-positive MPNs.

CD133 is a commonly expressed antigen at the cell surface of several somatic stem cells, particularly human hematopoietic stem/progenitor cells (HSPCs) [28]. Studies have shown that CD133 might further characterize HSPCs [29,30,31], with $CD133^+CD34^+CD45RA^-$ cell fractions being enriched for HSCs and multipotent progenitor cells (MPPs), giving rise to $CD133^{low}CD34^+CD45RA^-$ erythro-myeloid restricted progenitors (EMPs) [32]. This suggests that adult/postnatal multipotent HSCs/MPPs may be easily identified as $CD133^+CD34^+$ cells with erythroid potential [33,34]. Surprisingly, some other studies have revealed that $CD133^-CD34^-CD45RA^-$ cells show a stem cell ability making them compatible with both hematopoietic and endothelial potential by reconstituting hematopoietic tissue and generating functional HSCs and endothelial progenitor cells (EPCs) in vivo [35], and that $CD34^-CD133^+$ progenitors may further differentiate into EPCs [36].

In the present study, we establish various approaches for investigating the c-*MYC* expression of MPNs with *JAK2*V617F and its hierarchical activation level. Circulating $CD133^{+/-}CD34^-$ progenitors in MPNs have elevated c-*MYC* expression levels in such compartments. Additionally, genes known to be involved in the regulatory mechanisms of MPN pathogenesis related to c-*MYC*, including *STAT5A* and *STAT5B*, are investigated in this study at the mRNA level.

The results of this study may explain the development of clonal myelopoiesis, reveal the high frequency of EPCs in MPNs, and further illuminate the phenotypic characteristics of MPNs with *JAK2*V617F, which remain elusive.

Materials and Methods

Patients and Samples

Fourteen patients who met the revised 2008 World Health Organization diagnostic criteria for MPN [37] and five cord blood (CB) samples used as controls were included in this study. All patients were diagnosed in the Hematology Clinic of the İstanbul Medical Faculty, İstanbul University. Informed consent was obtained according to the guidelines outlined by the Ethical Review Board of the İstanbul Medical Faculty of İstanbul University.

Mononuclear Cell Isolation and FACS Sorting of the Cells

Mononuclear cells (MNCs) were isolated using the Ficoll gradient centrifugation technique (Ficoll-Paque Premium, GE Healthcare,

Uppsala, Sweden) from the peripheral blood of patients with MPNs after phlebotomy and from CB samples in blood bags containing 10% sodium citrate.

Following centrifugation at 400x g and 20 °C for 30 min, the collected MNCs were counted and resuspended in phosphate-buffered saline (PBS)-based buffer containing 500 mL PBS with 7.5% bovine serum albumin +0.5 mM EDTA, referred to hereafter as "buffer."

The MNCs were labeled with CD45-FITC (BD Pharmingen, San Diego, CA, USA), CD34-PE (BD Pharmingen), and CD133-APC (BD Pharmingen). Each antibody was added to 1 µL ($1x10^6$) of cells. After 30 min of incubation with the antibodies on ice, the MNCs were washed twice with buffer and resuspended in 500 µL of buffer containing 0.1% propidium iodide (PI) (Sigma Aldrich, St. Louis, MO, USA). Each sample was acquired on a FACSAria II instrument (BD Bioscience, Franklin Lakes, NJ, USA). The acquisition was performed with a CD45-negative gate using the following steps: 1) dead cells were excluded using PI; 2) doublets were excluded by gating outliers on SSC-A vs. SSC-H and FSC-A vs. FSC-H plots; 3) negative CD45 populations were segregated into four different quadrants ($CD133^-CD34^+$, $CD133^+CD34^+$, $CD133^-CD34^-$, and $CD133^+CD34^-$) for subpopulation analysis. Each gate was then sorted with ~98% purity.

JAK2 Wild-Type and *JAK2V617F* mRNA-Labeled Gold Nanoflare Infection of MPN Cells and FACS Sorting

Seven different probes were applied for the detection of *JAK2* wild-type (wt) and *JAK2*V617F transcripts in live cells as follows: two target-specific probes (*JAK2*wt-Cy5 and *JAK2*V617F-Cy3) with a housekeeping control; beta-actin human-Cy3 and beta-actin human-Cy5; and three control probes (JAK2 Scramble-Cy3, Uptake-Cy3, and Scramble-Cy3+Uptake-Cy5) (EMD Millipore Company, Merck KGaA, Darmstadt, Germany), as explained elsewhere [38]. In brief, the MNCs were incubated with either the specific or control probes using 30000 cells at 200 µL/well in 96-well plates. After 16 h of incubation at 37 °C in 5% CO_2 with ≥95% humidity, the cells were collected and resuspended in 500 µL of buffer using a FACSAria II instrument (BD Bioscience) with ~90% purity.

Allele-Specific Nested PCR

Genomic DNA was extracted using the Quick gDNA Micro Prep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The *JAK2*V617F mutation was detected by nested allele-specific PCR for the 14 MPN patients and the sorted compartments of 12 MPN patients as described previously [39]. Agarose gel electrophoresis revealed a band for a mutant allele of *JAK2*V617F with 279 bp and a wt allele of *JAK2* with 229 bp with a 50-bp ladder (Bio-Rad, Hercules, CA, USA).

RNA Isolation and Quantitative Real-Time Reverse Transcription PCR

Total RNA was extracted using a Pico Pure RNA Extraction Kit (Applied Biosystems, Bedford, MA, USA). According to the manufacturer's instructions, first-strand complementary DNA (cDNA) was synthesized from 60 ng of total RNA using a SCRIPT cDNA Synthesis Kit (Jena Bioscience, Jena, Germany). Differences in each gene expression were detected by applying Universal Probe Library (UPL) probes (Roche, Basel, Switzerland). Expression analysis was performed using a primer designed for the c-*MYC* gene of the NM_002467.4 transcript variant with forward primer 5'-GCT GCT TAG ACG CTG GAT TT-3' and reverse primer 5'-TAA CGT TGA GGG GCA TCG-3' combined with UPL probe #66 (Roche). The beta-actin gene (*ACTB*) was applied as a reference housekeeping gene for quantitative real-time PCR with the NM_001101.3 transcript variant using forward primer 5'-CCA ACC GCG AGA AGA TGA-3' and reverse primer 5'-CCA GAG GCG TAC AGG GAT AG-3' with UPL probe #64 (Roche). A total volume of 20 µL, composed of 1X ORA qPCR Probe Mix (HighQu, Kraichtal, Germany), forward primer (200 nM), reverse primer (200 nM), UPL probe (0.04 U), and dH_2O, was prepared and run with an initial denaturation of 2 min at 95 °C, then 40 cycles of 5 s at 95 °C and 40 cycles of 20 s at 60 °C, ending with 30 s at 40 °C using a real-time quantitative RT-PCR instrument (LightCycler II 480, Roche).

In order to detect expressional gene changes for *JAK2*, *STAT5A*, and *STAT5B*, the commercially available PCR Array for the Human JAK/STAT Signaling Pathway (QIAGEN, Dusseldorf, Germany) was used. Comparative measurements with real-time RT-PCR were performed using SYBR Green (RT^2 SYBR Green qPCR Mastermix, QIAGEN). Five different housekeeping genes were applied as the reference genes included in the array and run on the instrument.

Statistical Analysis

Gene expression fold changes were examined for MNCs and sorted stem cell compartments of patients and CB. The specified primers were applied for amplification. The fluorescence emitted by dye above the baseline signal was detected using the software in real time, recorded, and presented as the cycle threshold (C_T). The arithmetic mean values of C_Ts, obtained twice, were calculated. All samples were studied in duplicate. The relative c-*MYC* gene expressions were calculated using the formula of $2^{-\Delta Ct} = 2^{-(CTExample - CTReferance)}$ and applying the *ACTB* reference gene for samples [40]. GraphPad Prism 8.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis of gene expression data.

Significant differences in HSPC subgroups were determined using the Mann-Whitney U test as a nonparametric statistical test to compare the relative mRNA levels of patients and CB

samples. The variables used included mean values, standard deviation of the mean (SD), 95% confidence intervals, and percentage and frequency values. Statistical significance was set at $p<0.05$ (**) and $p<0.01$ (***).

Results

The clinical characteristics of the patients with Ph⁻ MPNs enrolled in this study are summarized in Table 1.

JAK2V617F-Positive Cells Have High Relative c-MYC Gene Expression

In order to ascertain whether c-*MYC* expression has an association with *JAK2*V617F mutation, a previously established method for live cell analysis of *JAK2*V617F mRNA and *JAK2*wt transcripts with fluorochrome-conjugated gold nanoparticles [38,41] was applied to the MNCs of two Ph⁻ MPN samples that had monoallelic *JAK2*V617F. The *JAK2*V617F mRNA- and JAK2wt mRNA-labeled gold nanoparticle-treated MNCs of MPN patients were sorted with a fluorescence-activated cell sorter for the *JAK2*V617F and *JAK2*wt transcripts, followed by analysis for relative c-*MYC* gene expression. Representative images of the flow cytometry analysis of distinct populations of *JAK2*wt⁺ and *JAK2*V617F⁺ were obtained (Figure 1). The MNCs of MPN patients were initially determined as monoallelic *JAK2*V617F by nested PCR analysis (Table 1).

Surprisingly, there were heterogeneous subgroups of transcripts within the MNCs, including solely monoallelic and biallelic

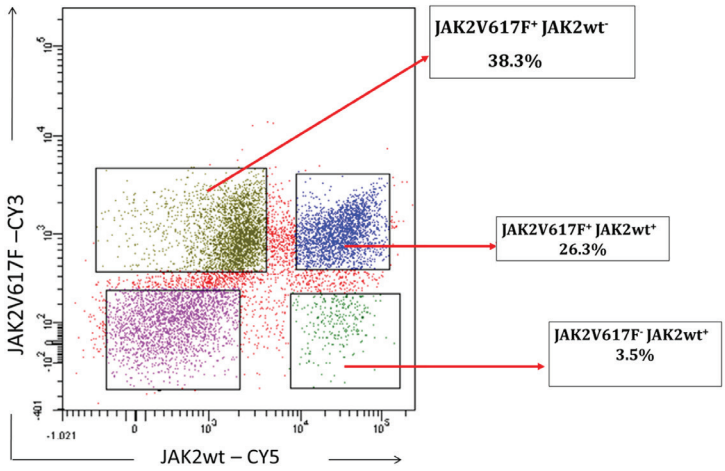

Figure 1. Fluorescence-activated cell sorting (FACS) plot of peripheral blood mononuclear cells of a patient (P29) with Philadelphia chromosome-negative Ph⁻ myeloproliferative neoplasm with polycythemia vera using gold nanoparticle probes. For all analyses, the main population of cells was gated excluding dead and fractured cells. After settings were completed for the background and control probes, including uptake fluorescence, analysis and sorting of the gold nanoparticle-treated cells were performed according to their signals in channels of FL-2 for *JAK2*V617F-CY3 and FL-4 for *JAK2* wild-type (wt)-CY5 transcripts. The FACS of the gold nanoparticle-treated cells was performed in four quadrants: *JAK2*V617F⁻*JAK2*wt- for the negative control, *JAK2*V617F⁺*JAK2*wt⁻ for *JAK2*V617F mutant mRNA, *JAK2*V617F⁺*JAK2*wt⁺ for both JAK2wt and JAK2V617F mRNA-positive cells, and *JAK2*V617F⁻*JAK2*wt⁺ for *JAK2*wt mRNA.

Table 1. Clinical characteristics of the patients with Philadelphia chromosome-negative myeloproliferative neoplasms included in this study.

Patient no.	First-line treatment	Current				Thrombosis history	Sex	Age	Age at diagnosis	Diagnosis	JAK2V617F allele status
		Hb (g/dL)	HCT (%)	WBC (10^3/mm^3)	PLT (10^3/mm^3)						
P7	P-H-A	14.9	46.3	7.1	369.0	N	M	59	46	PV	Monoallelic
P8	P-H-A	14	41,6	8.2	83.0	N	F	65	57	PV	Monoallelic
P10	P-A	16.5	48.3	9.8	241.0	N	M	32	21	PV	Wild-type
P11	P-H-A	12.70	38	39	162.0	NA	M	64	59	PMF	Monoallelic
P15	P-H-A	13.4	38	8.8	405.0	N	F	72	63	PV	Monoallelic
P23	P-H-W	14.9	48	11.6	229.0	Y	F	63	35	PV	Monoallelic
P24	P-A	15.8	47	5.7	277.0	N	M	67	54	PV	Wild-type
P26	P-H-T-U-A	14.4	44.6	6.6	646.0	Y	F	75	55	ET	Monoallelic
P27	P-H-A-f	12.8	38.4	4.8	506.0	N	F	55	40	ET	Monoallelic
P29	P-H-A-U	15.3	48.8	9.1	206.0	N	M	89	67	PV	Monoallelic
P34	P- H- A	13.2	42.5	9.1	241.0	Y	M	62	56	PV	Wild-type
P35	P-H-A-U	14.8	44	6.09	200.0	N	M	75	68	PV	Wild-type
P66	P-H	18.5	53.4	10.6	156.0	N	M	45	37	PV, PMF	Biallelic
P68	P-H-U	14.9	48	5.3	238.0	N	F	46	36	PV, PMF	Biallelic

Hb: Hemoglobin, HCT: hematocrit, WBC: white blood cells; PLT: platelets; P: phlebotomy, H: hydroxyurea, T: thromboreductin, U: uricolysis, A: acetylsalicylic acid, W: warfarin, f: Folbiol, N: no; Y: yes; NA: not available; F: female, M: male; PV: polycythemia vera; PMF: primary myelofibrosis; ET: essential thrombocythemia.

*JAK2*V617F and *JAK2*wt transcripts. In addition, populations of *JAK2*wt$^+$*JAK2*V617F$^+$, *JAK2*wt$^+$*JAK2*V617F$^-$, and *JAK2*wt$^-$ *JAK2*V617F$^+$ were detected at rates of 26.3±4.1%, 3.5±2.3%, and 38.5±5.7%, respectively. Each of the sorted cell populations was analyzed for relative changes in expressions of c-*MYC*, *JAK2*, *STAT5A*, and *STAT5B*. The ratio of the relative fold change of c-*MYC* was found to be highest (5.9-fold) in the *JAK2*V617F$^+$ compartment compared to the *JAK2*wt$^+$ compartment (biallelic/wt). The relative fold change of *JAK2* expression in the same compartment was determined to be 3.85-fold, while the *STAT5A* and *STAT5B* expressions were 2.78-fold and 4.87-fold, respectively (Table 2). Surprisingly, the ratio of gene expression fold changes in the compartments of biallelic/monoallelic *JAK2*V617F was below 2-fold. However, the relative gene expression fold changes for *c-MYC, JAK2*, and *STAT5B* in *JAK2*V617F monoallelic/wt compartments were 3.03-, 2.53-, and 3.89-fold, respectively.

JAK2V617F Is Detected in MPP, EMP, HSC/EPC, and EPC Populations

To evaluate the circulating HSPCs (MPP, EMP, HSC/EPC, and EPC populations) of Ph$^-$ MPN samples and the status of *JAK2*V617F in each population, MNCs labeled with CD45, CD34, and CD133 antibodies were sorted accordingly and further analyzed for the presence of *JAK2*V617F.

Table 2. Relative gene expression fold changes of JAK2 wild-type and JAK2V617F mRNA sorted Philadelphia chromosome-negative myeloproliferative neoplasms.

Gene	Relative gene expression fold change		
	Biallelic/wt	Biallelic/monoallelic	Monoallelic/wt
c-MYC	5.9	1.9	3.03
JAK2	3.85	1.52	2.53
STAT 5A	2.78	1.40	1.99
STAT 5B	4.87	1.25	3.89

wt: Wild-type.

Table 3. Percentage of sorted cell compartments of CD133 and CD34 cells of cord blood and mononuclear cells of Philadelphia chromosome-negative myeloproliferative. neoplasms.

Sorted cell compartments	Cord blood, % (mean ± SD; n=5)	MPN, % (mean ± SD; n=14)
CD133$^+$CD34$^+$ (MPPs)	0.16±0.09	0.22±0.18
CD133$^-$CD34$^+$ (EMPs)	1.42±0.8	4.58±3.61
CD133$^-$CD34$^-$ (HSCs/EPCs)	47.16±20.55	29.54±16.43
CD133$^+$CD34$^-$ (EPCs)	0.93±0.41	0.79±0.42

MPN: Myeloproliferative neoplasm; MPPs: multipotent progenitor cells; EMPs: erythro-myeloid restricted progenitors; HSCs/EPCs: hematopoietic stem cells and endothelial progenitor cells; EPCs: endothelial progenitor cells; SD: standard deviation.

In the cohort of 14 Ph$^-$ MPN samples, MPPs (CD133$^+$CD34$^+$) accounted for 0.22±0.18% of the total cells, EMPs (CD133$^-$ CD34$^+$) accounted for 4.58±3.61%, and EPCs (CD133$^+$CD34$^-$) accounted for 0.79±0.42% (Table 3). HSCs/EPCs (CD133$^-$ CD34$^-$) demonstrated the highest percentage in this compartmentalization.

Results from allele-specific nested PCR analysis are summarized in Tables 2 and 4. The MNCs of 8 of the 14 patients had monoallelic *JAK2*V617F, 2 patients had biallelic *JAK2*V617F, and 4 patients had the *JAK2*wt allele.

*JAK2*V617F characterization was performed for the sorted cell compartments of 12 Ph$^-$ MPN samples. *JAK2*V617F was not detected in the sorted cell compartments of 4 patients who did not carry the mutation in their MNCs. These 4 patients also did not carry the *JAK2* exon 12 mutation. The Ph$^-$ MPN patients who carried monoallelic *JAK2*V617F in their MNCs had varying presentations of mutation, including no mutation (wt) or monoallelic *JAK2*V617F, in each sorted cell compartment. All patients with a mutation in the MNC samples carried the monoallelic *JAK2*V617F mutation in the EMP (CD133$^-$CD34$^+$) cell population. Three of 8 MPN samples had no mutation in the MPP (CD133$^+$CD34$^+$) population, 2 of 8 monoallelic *JAK2*V617F Ph$^-$ MPN samples had no mutation in the EPC (CD133$^+$CD34$^-$) population, and 1 of 8 Ph$^-$ MPN patients had no mutation in the HSC/EPC (CD133$^+$CD34$^-$) population (Table 4).

Relative c-MYC Expression Increased in Progenitor Cell Populations and MNCs

To identify the activation level of c-*MYC* expressions in the HSPC subgroups, 14 patients with Ph$^-$ MPNs and 5 CB samples were studied. The overall analysis of the relative c-*MYC* expression in the MNCs of the MPN samples and CB showed increases of 0.026±0.015 and 0.009±0.004, respectively (p=0.01) (Figure 2A).

Relative gene expression analysis was performed for Ph$^-$ MPNs and CB in the MPP (CD133$^+$CD34$^+$), EMP (CD133$^-$CD34$^+$), HSC/EPC (CD133$^-$CD34$^-$), and EPC (CD133$^+$CD34$^-$) populations. The relative c-*MYC* expression levels detected in the MPPs of the MPN and CB samples were 0.083±0.060 and 0.057±0.017, respectively (p>0.05) (Figure 2B). In the HSCs/EPCs, relative c-*MYC* expression was determined as being significantly higher in MPNs compared to CB, at 0.026±0.021 and 0.003±0.004, respectively (p=0.01) (Figure 2D). In the EPCs of MPNs, relative c-*MYC* expression was statistically significant compared to the CB samples, at 0.019±0.013 and 0.004±0.004, respectively (p=0.03) (Figure 2E). Contrarily, EMPs had higher relative c-*MYC* expression in the CB than the MPN samples, at 0.037±0.015 and 0.018±0.013, respectively (p=0.02) (Figure 2C).

Discussion

The increased oncogenic activity of c-*MYC* has been previously associated with *JAK2*V617F but the exact level of upregulation

Table 4. *JAK2*V617F allele status of mononuclear cells and sorted populations of Philadelphia chromosome-negative myeloproliferative neoplasm samples.

Patient no.	MNCs	MPPs ($CD133^+CD34^+$)	EMPs ($CD133^-CD34^+$)	HSCs/EPCs ($CD133^-CD34^-$)	EPCs ($CD133^+CD34^-$)
P7	Monoallelic	Wild-type	Monoallelic	Wild-type	Wild-type
P8	Monoallelic	Monoallelic	Monoallelic	Monoallelic	Monoallelic
P11	Monoallelic	Monoallelic	Monoallelic	Monoallelic	Monoallelic
P15	Monoallelic	Wild-type	Monoallelic	Monoallelic	Monoallelic
P23	Monoallelic	Wild-type	Monoallelic	Monoallelic	Monoallelic
P26	Monoallelic	Monoallelic	Monoallelic	Monoallelic	Wild-type
P27	Monoallelic	Monoallelic	Monoallelic	Monoallelic	Monoallelic
P29	Monoallelic	Monoallelic	Monoallelic	Monoallelic	Monoallelic
P24	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type
P10	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type
P34	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type
P35	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type

MNC: Mononuclear cells, MPPs: multipotent progenitor cells; EMPs: erythro-myeloid restricted progenitors; HSCs/EPCs: hematopoietic stem cells and endothelial progenitor cells; EPCs: endothelial progenitor cells.

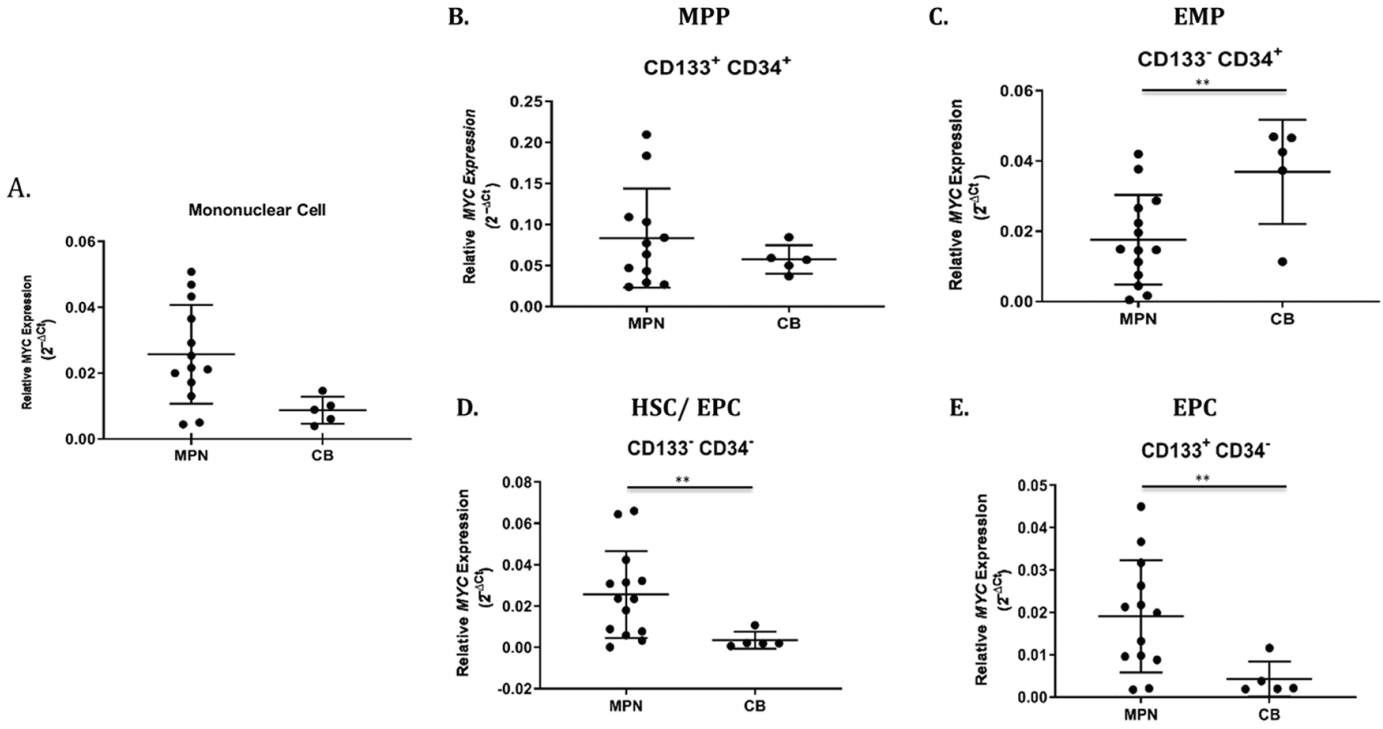

Figure 2. Relative c-*MYC* gene expression analysis of (**A**) mononuclear cells (p=0.01, Mann-Whitney U), (**B**) multipotent progenitor cells (MPPs, $CD133^+CD34^+$) (p>0.05, Mann-Whitney U), (**C**) erythro-myeloid restricted progenitors (EMPs, $CD133^-CD34^+$) (p=0.02 Mann-Whitney U), (**D**) hematopoietic and endothelial progenitor cells (HSCs/EPCs, $CD133^-CD34^-$) (p=0.01, Mann-Whitney U), and (**E**) endothelial progenitor cells (EPCs, $CD133^+CD34^-$) (p=0.03, Mann-Whitney U) in samples from 14 patients with myeloproliferative neoplasms and 5 control samples of cord blood. Relative c-*MYC* gene expressions were calculated with the formula $2^{-\Delta Ct} = 2^{-(CTExample - CTReferance)}$ applying *ACTB* as a reference gene. GraphPad Prism 8.0 software was used for the statistical analysis of gene expression data; in each figure, the standard deviation of the mean (SD) is shown with a bar; **p< 0.05.

of HSCs is lacking [42]. The determination of such a process might link the initiation and progression of Ph^- MPNs with *JAK2*V617F.

The transcript-labeled gold nanoparticle technology method was applied in this study for the mutant and wild-type allele-specific isolation of cells and distinct Ph^- MPN MNC populations of *JAK2*wt^+ and *JAK2*$V617F^+$ were created [38]. This showed that

the c-*MYC*, *JAK2*, *STAT5A*, and *STAT5B* genes were overexpressed in *JAK2*V617F$^+$ compared to *JAK2*wt$^+$.

It is well known that *JAK2*V617F induces aberrant activation of a transcription factor, *STAT5*, which plays a role in the JAK/STAT pathway, is critical for antiapoptotic and oncogenic activities [25], and directly regulates the synthesis of proteins in growth and survival [20]. Activation of the JAK/STAT pathway is required to induce the MYC protein level whereby MYC protein synthesis is found to be due to JAK2 kinase activation [26]. Therefore, the increase in c-*MYC*, *JAK2*, and *STAT5B* expressions in the presence of *JAK2*V617F in MNCs shown here is in concordance with previous findings. Furthermore, the steady increase in the expression of these genes within MNCs carrying no mutation (*JAK2*wt) and monoallelic or biallelic *JAK2*V617F transcripts was notable.

The *JAK2*V617F allele varies in the HSC compartments of MPNs, and the role of c-*MYC* expression in such environments is not clear. Therefore, we further evaluated the c-*MYC* expression in circulating HSPCs and their subgroups. CD34$^+$ cells are commonly detected in peripheral blood circulation [33] and are found to be increased among inpatients with MPNs [43]. CD133 expression among CD34$^+$ cells has been reported to enrich the stem cell fraction of circulating cells [31,44] and could also be applied as a marker of neoplastic stem cell activity in the peripheral blood circulation of patients with PMF [45]. Thus, the presence of *JAK2*V617F with respect to c-*MYC* expression in the circulating HSC subpopulation including the MPPs, EMPs, HSCs/EPCs, and EPCs of MPNs might provide evidence for the initiation of *JAK2*V617F and the propagation of disease.

Our study revealed that a minor proportion of circulating HSCs are co-expressed with CD133. However, HSCs appear to be roughly split between circulating CD133$^+$ and CD133$^-$ cells. The majority of the cells closely related to HSCs, MPPs, and EMPs are also divided between CD133$^+$ and CD133$^-$ cells and predominantly enhanced in the CD133$^-$ population. Although each population needs further characterization with in vivo xenograft experiments, the indication of circulating CD133$^+$ cells being more enriched for a more primitive state of HSPCs might be partially correct [44]. Furthermore, the determination of the least common population being the CD133$^+$CD34$^+$ phenotype [31,46] is consistent with a previous study in which patients with PMF had more circulating CD133$^+$CD34$^+$ cells than healthy donors [45].

The *JAK2*V617F allele statuses of the MNCs and the circulating MPP, EMP, HSC/EPC, and EPC populations of the MPN cohort, including 9 PV, 2 ET, and 3 PMF cases, were compared in the present study. The allele-specific mutational analysis of the subpopulations of HSCs of 12 MPN samples that did not have mutations in the MNCs suggests that mutations other than *JAK2*V617F also affect disease development [47].

Regarding circulating EMPs with the CD133$^-$CD34$^+$ immunophenotype, all 8 cases with monoallelic *JAK2*V617F in MNCs revealed the monoallelic *JAK2*V617F genotype. Considering malignant clones with myeloid proliferation and progression, this was anticipated. For the more primitive population of circulating MPPs with the CD133$^+$CD34$^+$ phenotype, the mutant monoallelic genotype was detected in 5 of 8 MPN samples.

Surprisingly, the circulating CD34$^-$ cells with the CD133$^{+/-}$ phenotype with the capacity to differentiate into endothelial cell (ECs) revealed *JAK2*V617F-positive cases. Since the discovery of *JAK2*V617F-positive ECs [48], the presence of *JAK2*V617F in ECs has led to many theories about the origin of malignant clones based on experiments with CD34$^+$ MPN cells [39,49,50]. Although circulating HSCs/EPCs and EPCs require further conformational studies, this preliminary finding is exciting because high levels of EPCs in MPNs [51] might originate from circulating CD34$^-$ cells.

An abundance of c-MYC protein increases as HSCs differentiate into myelo-erythroid and myeloid progenitor lineages [16] and cause cells to leave the niche [10]. An in vivo study performed by Franke et al. [52] showed that the constitutive overexpression of c-*MYC* in the HSPC compartment resulted in a myeloproliferative disorder. The present study showed increased c-*MYC* expression in MNCs and circulating MPPs, HSCs/EPCs, and EPCs of Ph$^-$ MPNs compared to CB. Remarkably, the relative c-*MYC* expression in the circulating EMPs of Ph$^-$ MPN cases was lower compared to the EMPs of CB. Previous studies have revealed that CB cells enriched with CD34 have increased c-*MYC* expression compared to the adult bone marrow cells of healthy donors [53]. Possible limitations of the present study might include the comparison of c-*MYC* expression with CB, which could be deceptive in some cases. Although there are examples of studies in the literature utilizing CB cells as controls [53], better optimization with more appropriate controls or more Ph$^-$ MPN cases with no mutations and detailed analysis for other driver mutations might be necessary.

Nevertheless, the elevated circulating HSC/EPC and EPC populations in MPNs might still be driven by c-*MYC*. One study in which c-*MYC* mRNA was determined in formalin-fixed and paraffin-embedded bone marrow samples from patients with ET revealed c-*MYC* overexpression. However, a lack of significant expression in PV might also be related to the phenotype of the bone marrow cells analyzed, as bone marrow cells in cases of PV might be enriched with CD133$^-$CD34$^+$ [27]. Furthermore, the regulation of gene expression profiles and methylation patterns vary significantly between bone marrow cells and circulating HSPCs, such that the comparative effect of *JAK2*V617F with c-*MYC* expression in varying cell types might be diverse. Since more than 95% of PV cases carry *JAK2*V617F, and *JAK2*V617F is known to induce c-*MYC* expression in cell lines [14,27], the

present study further confirms the increased expression of the *JAK2*V617F population.

Conclusion

The data obtained in this work support the increased c-*MYC* expression in Ph^- MPNs with *JAK2*V617F and further reveal the overexpression of c-*MYC* in HSCs/EPCs and EPCs of MPNs. The effects of c-*MYC* on the molecular mechanisms of these compartments and its functional relevance remain elusive and require further study.

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Ethics

Ethics Committee Approval: All patients were diagnosed in the Hematology Clinic of the İstanbul Medical Faculty, İstanbul University.

Informed Consent: Informed consent was obtained according to the guidelines outlined by the Ethical Review Board of the İstanbul Medical Faculty of İstanbul University.

Authorship Contributions

Surgical and Medical Practices: A.S.Y.; Concept: İ.U.B., S.S.T.; Design: S.S.T.; Data Collection or Processing: İ.U.B., B.T.; Analysis or Interpretation: İ.U.B., B.T., A.S.Y., S.S.T.; Literature Search: İ.U.B., S.S.T.; Writing: İ.U.B., A.S.Y., S.S.T.

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