Arab American University

Faculty of Graduate Studies

WWOX Mutations and Expression Pattern in Myeloproliferative Neoplasms

By

Adleen Nazim Hussein Saffia

Supervisor

Dr. Zaidoun Mahmoud Hasan Salah

This thesis was submitted in partial fulfillment of the requirements for the Master`s degree in Molecular Genetics and Genetics Toxicology

July/ 2023

WWOX Mutations and Expression Pattern in Myeloproliferative Neoplasms

By

Adleen Nazim Hussein Saffia

This thesis was defended successfully on 22.07.2023 and approved by:

Committee members	**Signature**
1. Dr. Zaidoun Mahmoud Salah (Supervisor)	.....
2. Dr. Fekri Helmi Samarah (Internal Examiner)	.....
3. Dr. Mahmoud Abdel-Rahman Srour (External Examiner)	.....

Declaration

Here I declare that my MSc thesis entitled **"WWOX Mutations and Expression Pattern in Myeloproliferative Neoplasms"** is the result of my own research and was written independently with no other sources than quoted.

Adleen Nazim Hussein Saffia

Student's ID: 201812871

Signature: Adleen Saffia

Date: 05.10.2023

Dedication

To No One.

Acknowledgment

First of all, my sincere thanks and appreciation to Allah for aiding me in completing the work of this thesis.

Then, I would like to thank my supervisor, Dr. Zaidoun Salah, the best supervisor any student could hope for. He was very patient, helpful, and supportive in every step of this research. My experience of working with him was very useful. I learned so much from his huge experience, which helped me in improving my knowledge and skills in lab work specifically and research in general. I will always be thankful to him for tolerating my thoughts and being patient and always available to support and guide me.

I would also like to thank Mr. Husam Sallam and Ms. Rua' Thawabteh, the research assistants in the molecular genetics laboratory. Their aid and efforts are much appreciated.

Finally, I would like to thank all the patients and individuals, who agreed to volunteer to be a part of this research.

Abstract

Myeloproliferative neoplasms (MPNs) are disorders associated with the uncontrolled proliferation of at least one of the hematopoietic cell lineages. MPNs are classified into Philadelphia chromosome-positive (Ph+) and Philadelphia chromosome-negative (Ph-), where the latter lacks defining genetic alterations. Mutations in JAK2, MPL, and CALR are associated with Ph-ve MPNs. However, all these mutations are involved in the diagnosis of a wide array of Philadelphia chromosome-negative MPNs, regardless of whether their symptomatic manifestations are quite different. WWOX is a known regulator of apoptosis, tumor suppression, and transcription factors. Alterations in WWOX expression were reported in polycythemia vera, primary myelofibrosis, and many types of cancers. This project used; PCR to investigate the deletions in *WWOX* exons, sanger sequencing to identify single nucleotide polymorphisms, and real-time PCR and western blot to study the alterations in mRNA and protein levels in either cases carrying ph-ve MPNs and/or blood-derived cell lines, respectively. No macro deletions in the sequence of *WWOX* exons were observed. However, five variants; rs11545029, rs7201683, rs3764340, rs73572838, and rs76204496, were detected. WWOX mRNA levels of patient samples and the cell lines; NB4, HL60, HEL1, SET2, and KG1, were significantly reduced. This was also observed at the protein levels in all the hematopoietic cell lines. This marks the first study on WWOX expression in patients with Philadelphia chromosome-negative MPNs in the region. The reduction in WWOX expression might be a potential lead to a new marker in the diagnosis and pathogenesis of Philadelphia chromosome-negative MPNs. The mRNA levels in the cell lines; NB4, HL60,

HEL1, SET2, and KG1, were significantly reduced, which was also observed at the protein levels in all the hematopoietic cell lines. This marks the first study on WWOX expression in patients with Philadelphia chromosome-negative MPNs in our region. The reduction in WWOX expression might be a potential lead to a new marker in the diagnosis and pathogenesis of Philadelphia chromosome-negative MPNs.

Table of content

List of Tables

List of Figures

List of Abbreviation

Term	Abbreviation
Abelson proto-oncogene	ABL
Activating protein 2-gamma	AP2γ
Acute myeloid leukemia	AML
Additional sex combs like Transcriptional regulator 1	ASXL1
B-cell lymphoma 2	Bcl-2
B-cell lymphoma-extra large	Bcl-xL
Bone marrow	
Breakpoint cluster region	BCR
c-Jun N-terminal kinase1	JNK1
Calreticulin	CALR
Casitas B-lineage lymphoma	
DNA methyltransferase 3 alpha	DNMT3A
Endoplasmic reticulum	
Enhancer of zeste homolog 2	EZH2
Erb-B2 Receptor Tyrosine Kinase 4	ErbB4
Erythropoietin	
Essential thrombocytosis	
Fetal calf serum	
Fibroblast growth factor receptor 1	FGFR1
Granulocyte macrophage-colony stimulating factor	GM-CSF
Granulocyte-colony stimulating factor	G-CSF
Growth hormone	
Hematopoietic stem cell	
Human leukocyte antigen	HLA
Hydroxyurea	
IKAROS family zinc finger 1	IKZF1
Interferon-gamma	IFN-γ
Interleukin 3	
Isocitrate dehydrogenase ½	IDH1/2
kinase domain	
pseudokinase domain	
Janus kinase 2	JAK2
Lymphocyte-specific adaptor protein	LNK
Mitogen-activated protein kinase	MAPK
Myeloproliferative leukemia protein	MPL
Myeloproliferative neoplasm	MPN
Philadelphia chromosome-negative	
Philadelphia chromosome-positive	
Phosphatidylinositol 3-kinase	PI3K
Platelet-derived growth factor receptor alpha	PDGFRA

Platelet-derived growth factor receptor beta	PDGFRB
Polycythemia vera	PV
Primary myelofibrosis	PMF
Protein kinase B	AKT
Rat sarcoma virus	RAS
Runt-related transcription factor 2	RUNX2
Serine/arginine-rich splicing factor 2	SRSF2
Short-chain dehydrogenase/reductase	SDR
Signal transducers and activators of transcription	STAT
Ten-eleven translocation-2	TET2
Thrombopoietin	TPO
TNF receptor-associated death domain protein	TRADD
Tumor protein p53	p53
U2 small nuclear RNA auxiliary factor 1	U2AF1
World health organization	WHO
WW domain-containing oxidoreductase	WWOX
Zinc finger-like protein	Zfra

CHAPTER 1: INTRODUCTION

1.1 Philadelphia Chromosome Negative-Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) are a group of disorders known for the uncontrolled proliferation of at least one of the hematopoietic cell lineages. They are classified into Philadelphia chromosome-positive (Ph+) and Philadelphia chromosome-negative depending on the presence or absence of Philadelphia chromosome (Boddu et al., 2018).

Philadelphia chromosome is the result of the balanced translocation between the Breakpoint Cluster Region (*BCR*) gene on chromosome 22 and the Abelson proto-oncogene (*ABL*) on chromosome 9. The product of this fusion is a constitutively active tyrosine kinase that aids in the development of various types of cancer. This genetic marker aided in the successful development of drugs for the treatment of Ph+ myeloproliferative neoplasms. Unfortunately, similar progress was not as easily achieved in discovering potential therapies for the treatment of Ph- disorders. Mainly due to the lack of a definitive marker protein for each Ph-ve MPN. Recently, two activating mutations have been identified in the signaling pathways of some types of Ph-ve MPNs, including Janus kinase 2 (*JAK2*), and the thrombopoietin receptor, known as Myeloproliferative leukemia protein (*MPL*). This led to an increase in the efforts of finding inhibitors that target these two markers (reviewed by Campregher et al., 2012).

Most of the mutations that aid in the diagnosis and treatment of Ph-ve MPNs

subtypes are categorized into three different groups; mutations related to the activation of signal transducer and activator of transcription 3 (STAT3) and STAT5, mutations related to transcriptional regulation, and mutations linked to the progression to acute myeloid leukemia (AML) (Campregher et al., 2012).

The worldwide incidence of Ph-ve MPNs ranges between 0.44 – 5.8 in one hundred thousand cases (Moulard et al., 2014). Thiele has summarized the World Health Organization (WHO) classification of Ph-ve MPNs into 8 groups (Thiele, 2009): Chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia (not otherwise specified), mastocytosis, unclassifiable myeloproliferative neoplasms, myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of platelet-derived growth factor receptor alpha (PDGFRA), platelet-derived growth factor receptor beta (PDGFRB), or fibroblast growth factor receptor 1 (FGFR1).

1.1.1 Polycythemia Vera

Polycythemia vera is the most common type of MPNs. It is a chronic myeloproliferative neoplasm that has the same hematopoietic stem cell (HSC) as other subtypes of MPNs, such as ET, and PMF. These neoplasms also share the ability to overproduce blood cells due to the constitutive hematopoiesis process that does not cause any morphological changes. They also tend to undergo extramedullary hematopoiesis and transform into bone marrow (BM) failure (Iurlo et al., 2020).

Symptoms of PV include; dizziness, erythromelalgia, headache, paresthesia, ischemic attack, visual disturbances, and arterial and venous thrombosis (Raedler, 2014). A study of 1,638 patients with PV has shown that fatal and non-fatal thrombotic events account for 5.5% of cases annually, and the annual mortality of PV patients is around 3.7%, which might be a result of transformation or cardiovascular events like thrombosis (Marchioli et al., 2005).

The diagnosis of PV requires the verification of three major criteria or two major criteria with a minor criterion as reviewed by Arber et al., 2016. The first major criterion is an increase in red blood cell mass or elevated hemoglobin levels higher than 16.5 g/dL in men (hematocrit > 49%) or 16 g/dL in women (hematocrit > 48). The second major criterion is characterized by the trilineage proliferation of BM cells with the presence of pleomorphic mature megakaryocytes. The third major criterion is the detection of *JAK2* mutation. Meanwhile, the only minor criterion is lower levels of serum erythropoietin.

The first mutation to be identified and correlated with PV is the JAK2V617F in exon 14 (Levine et al., 2005). Later on, other mutations in the *JAK2* gene, specifically in exon 12, have been identified in patients with PV (Scott et al., 2007). Mutations in these exons were found in 96% and 2-3% of the PV cases, respectively (Stein et al., 2015). Also, both nonsense and missense mutations in Lymphocyte-specific adaptor (*LNK*) were found to associate with the disease (Lasho et al., 2010). This gene expresses a negative regulator of JAK signaling pathway. All of these mutations point to the leading role of JAK2 in the pathogenesis of PV, while it also raises questions about the diverse clinical

impact of JAK2V617F as it appears in patients diagnosed with ET and PMF as well (Stein et al., 2015). PV patients with low thrombosis risk are subjected to phlebotomy with antiplatelet therapy, while high-risk patients are treated with aspirin and cytoreductive agents (Barbui et al., 2018). Hydroxyurea (HU) is the first choice for cytoreduction in PV patients. It works as an oral inhibitor of ribonucleoside reductase which functions normally in DNA synthesis. However, it was reported that 13% and 11% of patients have not benefited from the use of HU due to intolerance and resistance, respectively (Alvarez-Larrán et al., 2012).

Therefore, alternative treatment options are under study to alleviate the clinical complications of PV, especially those associated with thrombosis. Recombinant interferon-alpha and the JAK1/2 inhibitor ruxolitinib are used for treating patients who suffer from HU intolerance or resistance (Barbui et al., 2018).

1.1.2 Essential Thrombocythemia

Essential thrombocythemia is a myeloproliferative disorder marked by a persistent increase in platelet count. This disorder is thought to be of a multipotent stem cell origin. Thrombocytosis is a consequence of the increased proliferation of megakaryocytes (Brière, 2007).

Thrombosis is a frequent occurrence in ET patients and it is the main adverse outcome of this disease (Fenaux et al., 1990). It can lead to venous and arterial thrombus as well as strokes and ischemic attacks (Chuzi & Stein, 2017). The risk factors of thrombosis can be classified into host factors and ET-specific factors.

The former includes thrombophilia, advanced age, previous history of thrombosis, and cardiovascular problems, whereas the latter includes high platelets and leukocyte count, platelets abnormalities, JAK2V617F mutation, coagulation, and leukocytes-platelet interactions (Cervantes, 2011). Neurological symptoms are also recurrent in ET patients and might include headache, dizziness, disturbance in vision, consciousness loss, and neurologic thrombotic symptoms (Billot et al., 2011). The transformation of ET to a leukemic or myelofibrotic state is rare and occurs as a late complication of the disease. A study on 605 patients reported that 2.3% of the cases developed leukemia after a median time of 11 years, while 2.8% of them developed myelofibrosis after a median time of 9.1 years (Passamonti et al., 2008). Still, the average life expectancy for patients of ET is not significantly lower than their counterparts in the general population (Passamonti et al., 2008).

Major criteria for the diagnosis of ET include; a platelet count $\geq 450 \times 10^9$/L, the proliferation of megakaryocytes in BM that appears in loose clusters, not meeting the diagnostic criteria for other similar myeloproliferative neoplasms, and the detection of mutation in JAK2, MPL, or calreticulin (CALR) (Tefferi & Barbui, 2019). The minor criterion for the diagnosis of ET requires the presence of other clonal markers or the absence of reactive thrombocytosis. The certified diagnosis of ET requires the fulfillment of all the previously mentioned major criteria or the first three major ones with the minor criterion (Tefferi & Barbui, 2019).

JAK2V617F is one of the genetic markers that can be found in ET patients. Variations in the occurrence percentage of ET patients carrying JAK2V617F

were reported. One study has reported the presence of this mutation in 39% out of 115 ET cases (Levine et al., 2005). While another study in the same year reported a percentage of 57% out of 130 cases (Antonioli et al., 2005).

The second common genetic marker is the MPLW515L/K mutation which results in the conversion of tryptophan to either leucine or lysine. The rate of this mutation in the ET patient is somewhat low. In 994 ET patients, this mutation was identified in only 3%, where a quarter of them co-existed with the JAK2V617F mutation (Vannucchi et al., 2008). Mutations in exon 9 of *CALR* represent the third genetic anomaly of ET patients. Such mutations were reported in 67% out of 289 ET cases that lack mutations in both *JAK2* and *MPL* (Klampfl et al., 2013).

The treatment strategy of ET is highly dependent on the risk level of each patient. Low-risk patients require aspirin prescription or observation-only, intermediate-risk patients require the administration of aspirin and HU, while high-risk patients require aspirin, HU, and/or anticoagulants (Tefferi et al., 2018). Other treatment options arise in response to the failure of first-line therapeutic agents, such as; anagrelide (a selective agent to decrease platelet count), and imetelstat (a telomerase inhibitor) (Gisslinger et al., 2013; Elisabeth Oppliger Leibundgut et al., 2020).2013; Elisabeth Oppliger Leibundgut et al., 2020).

1.1.3 Primary Myelofibrosis

Primary myelofibrosis is a chronic myeloproliferative disorder characterized by

fibrosis in BM and abnormal hyperplasia of megakaryocytes. This leads to an increase in reticulin fibrosis, which subsequently progresses to collagen fibrosis and osteosclerosis (Gangat & Tefferi, 2020).

The clinical complications of this neoplasm appear as fatigue (85%), symptomatic splenomegaly (76%), night sweats (55%), bone pain (51%), itching (39%), severe weight loss (30%), and fever (19%) (Mesa et al., 2009). Anemia, leukoerythroblastosis, and musculoskeletal symptoms are also common in PMF cases (O. I. Abdel-Wahab & Levine, 2009).

The diagnosis of *JAK2*-positive PMF requires two essential factors; a mutation in *JAK2* and grade 3 or more of reticulin, in addition to two of the following minor factors; splenomegaly, leukoerythroblastosis, extramedullary hematopoiesis, unclear anemia source, teardrop-shaped red cells, and systemic symptoms (Campbell et al., 2006).

A study of 617 patients with PMF has reported 64.7% of JAK2V617F, 22.7% of *CALR* exon 9 indel, 4% of MPLW515 mutation, and 8.6% with none of these mutations (Rumi et al., 2014). Mutations in other genes were also correlated with PMF, such as; Additional Sex Combs Like Transcriptional Regulator 1 (*ASXL1*), Serine/arginine-rich splicing factor 2 (*SRSF2*), Enhancer of zeste homolog 2 (*EZH2*), Ten-Eleven Translocation-2 (*TET2*), DNA Methyltransferase 3 Alpha (*DNMT3A*), Casitas B-lineage Lymphoma (*CBL*), and isocitrate dehydrogenase 1/2 (*IDH1/2*) (Vannucchi et al., 2013).

Treatment of PMF aims mainly to alleviate symptomatic complications. Thrombosis can be treated with aspirin, anemia can be treated with corticosteroids, androgens, or danazol, whereas splenomegaly is treated initially with HU, cladibrine, and lastly with a surgical splenectomy (Vannucchi, 2011). Ruxolitinib was associated with improved PMF-related symptoms in comparison with the conventional treatment (Harrison et al., 2013).

Allogeneic hematopoietic cell transplantation is the only available intervention that can cure PMF. A percentage of 37% survival after 5 years of follow-up was reported after transplantation from siblings with matched human leukocyte antigen (HLA) (Ballen et al., 2010). The overall survival of allogeneic transplantation is negatively affected by the presence of mutations in *DNMT3A* and U2 small nuclear RNA auxiliary factor 1 (*U2AF1*) (Ballen et al., 2010).

1.2 Genomic Alteration in Philadelphia Chromosome Negative Myeloproliferative Neoplasms

Genetic markers can be used in the diagnosis and prognosis of different diseases. *JAK2*, *MPL*, and *CALR* are the most studied genetic markers, which are used in the diagnosis of PV, ET, and PMF. Other mutations are always considered to cover incomplete information about causative mutations in these disorders.

1.2.1 JAK2

JAK2 gene maps to the short arm of chromosome 9, specifically on 9p24. It encodes for JAK2 protein, which has a JAK kinase domain (JH1) and a JAK pseudokinase domain JH2. Unlike JH1 which has catalytic activity, JH2 is

responsible for the negative regulation of JAK2 activity (Saharinen et al., 2000). The JAK2 enzyme is a cytoplasmic tyrosine kinase that has a function in the cytokine signaling pathways in the hematopoietic processes. JAK2 and its family of proteins are also involved in cellular proliferation, differentiation, survival, and migration (Gäbler et al., 2013).

The signaling pathway of JAK2 was reviewed by Sandberg (Sandberg et al., 2004). It starts with the binding of a cytokine to its receptor on the outer membrane of the cell. This leads to dimerization of the receptors, which activates the non-covalently bound JAK2. Subsequently, activated JAK2 molecules phosphorylate the extended tails of the receptors inside the cytoplasm on fixed tyrosine residues. The tail conformation changes, then, to accommodate proteins like signal transducers and activators of transcription (STAT), which are also phosphorylated by JAK2 on tyrosine residues. The phosphorylation allows STATs to de-attach themselves and bind to form dimers, which will translocate to the nucleus. Inside the nucleus, the dimers bind to specific elements to control the transcription of specific genes. Figure 1 demonstrates the mechanism of action of JAK/ STAT pathway from ligand binding to transcription (Dodington et al., 2018).

The ligands that can activate the JAK2 pathway include many cytokines and growth factors, such as; interleukin 3 (IL-3), IL-5, IL-6, IL-12, interferon-gamma (IFN-γ), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), erythropoietin (EPO), growth

hormone (GH), and thrombopoietin (TPO) (Kisseleva et al., 2002)

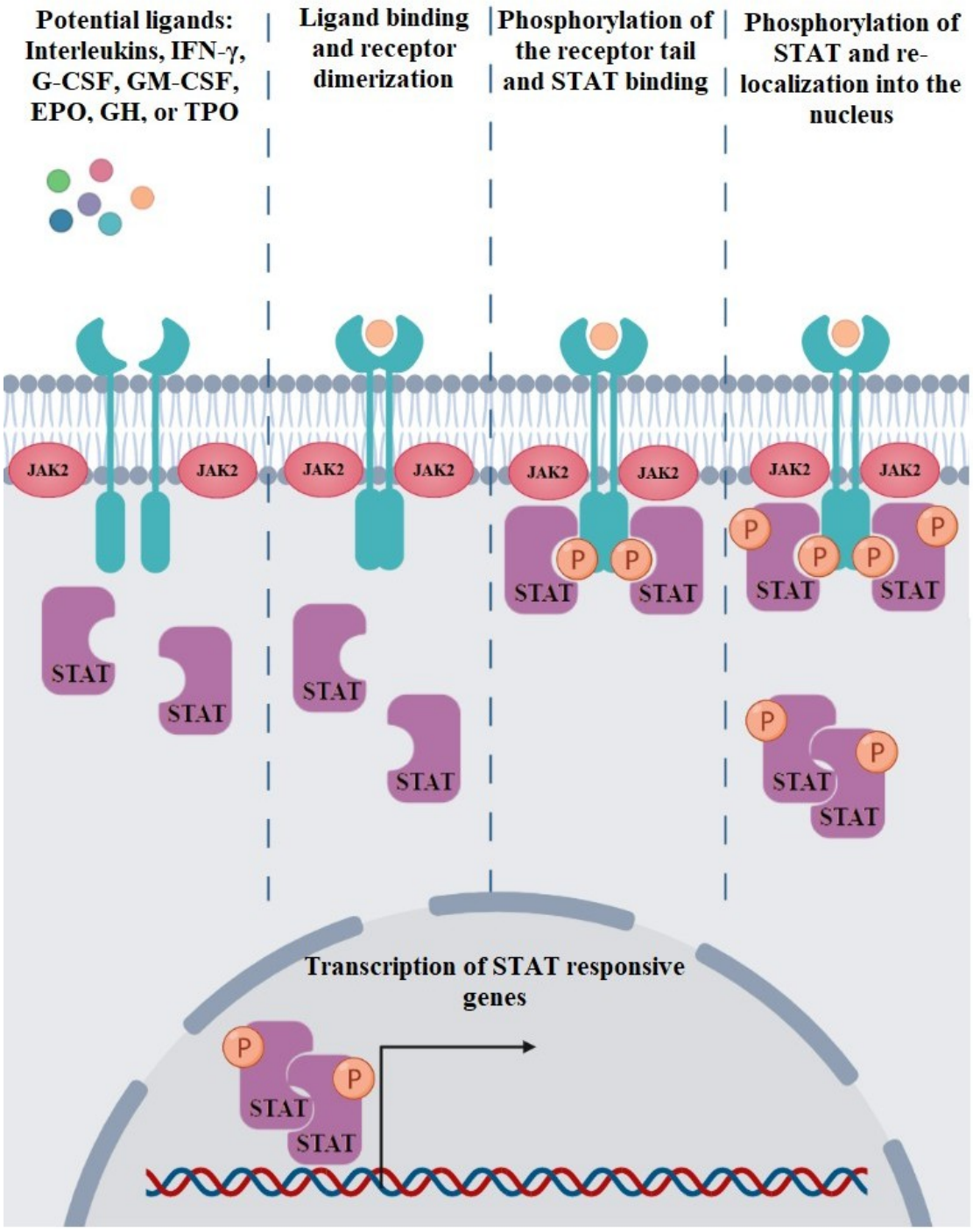


Figure 1: The activation of JAK2/STAT pathway upon exposure to certain cytokines. The ligand binding drives the dimerization of the receptor and the phosphorylation of the two attached JAK proteins. The activated JAK proteins phosphorylate the tails of the receptor and then the STAT proteins. The phosphorylated STAT proteins dimerize and translocate to the nucleus to modulate the transcription of downstream target genes.

JAK2V617F is an exon 14, point mutation recognized in PV, ET, and PMF (Kralovics et al., 2005). This mutation was detected in the hematopoietic progenitor cells, which define their ability in producing mutated erythroid and myeloid lineages (Baxter et al., 2005). It is located in the JH2 domain, which leads to constitutive activation of the JAK2 protein as a result of the substitution of the valine amino acid at location 617 to phenylalanine (Baxter et al., 2005). JAK2V617F is responsible for the activated STAT, mitogen-activated protein kinase (MAPK), and Protein kinase B (AKT) pathways, which promote the transcription and translation of antiapoptotic and cell regulatory proteins (Gäbler et al., 2013).

Many other *JAK2* mutations have been discovered so far, specifically in exon 12 near the beginning of the JH2 domain. These mutations vary between substitution (11 mutations), deletion (20 mutations), and duplication (6 mutations), where the most widely detected mutations are the two *JAK2* deletion mutations; N542-E543del, and E543-D544del, the two deletion-insertion mutations; F537-K539delinsL, and R541- E543delinsK, and the substitution mutation JAK2K539L (Scott, 2011).

JAK2 can be targeted to treat Ph-ve MPNs due to the severity of its persistent activity on the normal process that happens inside the cells. Promising results have been reported for the effect of several selective JAK2 inhibitors in-vitro and in-vivo regarding the symptoms of Ph-ve MPNs, including; Lestaurtinib, TG101348, and INCB01842. (Hexner et al., 2008; Wernig et al., 2008; Verstovsek et al., 2010; A. Pardanani et al., 2011).

1.2.2 MPL

MPL protein is a hematopoietin receptor expressed from 12 exons from the genomic sequence; 1p34 (Coniat et al., 1989). The activation of this receptor is involved in the proliferation of differentiated blood cells including megakaryocytes (Tefferi, 2008).

Three of the identified mutations in *MPL* alter codon 515 from translating into tryptophan to either leucine (MPLW515L), lysine (MPLW515K), or serine (MPLW515S) (A. D. Pardanani et al., 2006; Pikman et al., 2006; Schnittger et al., 2007). The mutation MPLW515L was associated with high-risk leukemic transformation, with the loss of functional MPL due to mitotic recombination (Beer et al., 2010).

MPLS505N is another mutation that was reported in patients diagnosed with ET (Beer et al., 2008). This mutation has a deleterious impact on ET patients, as it increases the serious symptoms, such as; splenomegaly, thrombotic events, and BM fibrosis (Teofili et al., 2010). These aforementioned mutations occur in exon 10 of the MPL gene (Beer et al., 2008). The substitution mutation of codon 515 was reported to be obtained early by the hematopoietic progenitors (A. Pardanani et al., 2007). The proposed mechanism of action of MPL mutation includes constitutive signal transduction by the JAK/STAT, rat sarcoma virus (RAS)/MAPK, and, phosphatidylinositol 3-kinase (PI3K)/AKT axis (Pikman et al., 2006). Two other mutations, MPLS204P and MPLY591N, revealed a weakly constitutive activity of MPL due to independence or hypersensitivity to thrombopoietin (Cabagnols et al., 2016).

1.2.3 CALR

The CALR gene spans 17 kb on the genomic position 19p13.2, and its transcript consists of 9 exons (Persson et al., 2002). It encodes for a Calcium-binding protein, that weighs 46 kDa. This protein consists of a globular N-domain, proline-rich P-domain, C- domain, as well as a 4 amino acid domain in the C-terminus that is involved in endoplasmic reticulum (ER) translocation. The former two domains are essential for the chaperoning of the protein, whereas, the C-domain consists of negatively charged amino acids that act as a calcium store (Michalak et al., 2009).

CALR proteins are located in the ER and aid in chaperoning the newly formed polypeptides through the ER (Michalak et al., 2009). CALR is important for the correct folding of proteins and glycoproteins, as it prevents aggregate formation (Michalak et al., 2009). In addition, CALR was reported to be linked with calcium metabolism, cell adhesion, immunity and phagocytosis, and the quality regulation of proteins (Michalak et al., 2009).

One study has identified 148 mutations in exon 9 of CALR, which result in one-base frameshift that could alter the C-terminus of the protein (Nangalia et al., 2013). Moreover, it was reported in the same study that these mutations were presented in the hematopoietic progenitors and stem cells (Nangalia et al., 2013). A different study has reported two mutations responsible for 80% of the mutated CALR sequences; a deletion mutation of 52bp from nucleotide 1092 to 1143, and an insertion mutation of 5bp between nucleotide numbers 1154 and 1155 (Klampfl et al., 2013). The 52bp deletion (type 1 mutation) substitutes most of

the negatively charged residues of the CALR protein, while the 5bp insertion (type 2 mutation) substitutes half of the negatively charged amino acids (Klampfl et al., 2013). In both cases, the ER retention signal, which consists of lysine, aspartic acid, glutamic acid, and leucine (KDEL), is removed (Klampfl et al., 2013).

In contrast to wild-type CALR, mutated CALR is capable of interacting with MPL that is already bound to the JAK2, via the activation of JAK/STAT pathway (Araki et al., 2016). CALR mutation alone could confer an MPN-like state in mice, which might be blocked by the administration of JAK2 inhibitors (Elf et al., 2016)

1.2.4 Sub-Clonal Mutations

Various other mutations have been discovered that might affect the diagnosis, prognosis, progression, and treatment plan of PV, ET, and PMF. These mutations might co-occur with each other, or with JAK2, MPL, and CALR mutations (Accurso et al., 2020). In this section, some of the most widely studied mutations are addressed.

TET2 is involved in the conversion of 5-methylated cytosine to 5-hydroxymethylated cytosine, which might aid in the demethylation of the genome (O. Abdel-Wahab et al., 2012). ASXL1 function in mammalian blood cells is not well understood, but mutations in this gene have been detected in PMF, ET, and PV (O. Abdel-Wahab et al., 2012).

CBL mutations were also identified in patients with MPNs, as this protein normally regulates the E3 ubiquitin ligase signaling pathway (Tefferi, 2010), which is involved in metabolism, DNA repair, and programmed cell death (Humphreys et al., 2021).

IKAROS family zinc finger 1 (IKZF1) regulates the differentiation process of lymphoid tissue, in which mutations were associated with MPNs (Tefferi, 2010). Other mutations that affect MPNs occur in *IDH*, *SRSF2*, *EZH2*, and *DNMT3A*, which affect TET2 function, RNA splicing, histone methylation, and DNA methylation, respectively (Tefferi, 2016).

1.3 WW Domain-Containing Oxidoreductase (WWOX)

The genomic sequence, as well as the protein structure of the *WWOX* gene, is highly conserved among different species. The fragile site FRA16D that contains the *WWOX* gene has big similarities with a mouse ortholog called Fra8E1. The expressed proteins from these two regions share similar domains as well (Krummel et al., 2002). These genes show similarities in the expressed products that rise to almost 96% (Aldaz et al., 2014). Even, the intronic regions in the *WWOX* sequence are consensus with their coordinate match in other species, which proposes the high importance of conserving this gene throughout the genomic evolution (Aldaz et al., 2014).

1.3.1 The Human *WWOX* Gene

The human *WWOX* gene spans a huge sequence, of more than 1.1 million bases

despite transcribing only nine small exons. It is located on chromosome 16q23, which crosses a common fragile site known as FRA16D. This fragile site is highly susceptible to genetic mutations and breaks, as this instability highlights its role in cancer development (Ried, 2000).

The complete *WWOX* transcript can be alternatively spliced into seven isoforms. The dominant mRNA form consists of 1245 base pairs and encodes for the full-length protein (Bednarek et al., 2000). Since the gene is transcribed into a huge pre-mRNA, one or few complete transcripts might be generated in the time of completing a full cell cycle. The short versions of this transcript might compete with it and negatively regulate its function (Richards et al., 2015). It was suggested that the expression of truncated WWOX protein that lacks functional segments might be inhibiting the tumor suppressor activity of WWOX leading to the development of different types of cancer. (Paige et al., 2001).

1.3.2 WWOX Protein Expression and Localization

WWOX sequence consists of three domains; WW1, WW2, and a short-chain dehydrogenase/reductase (SDR) domain. The SDR domain of the WWOX protein has a substrate-binding region in addition to its catalytic site, but the specific ligands that can bind to this domain in WWOX are yet to be determined (Richards et al., 2015).

The two WW domains consist of almost 40 amino acids, which are characterized by two tryptophan residues set 20-22 amino acids apart (Sudol et al., 2005). The abbreviation WW came from the symbols of these two tryptophan residues. Upon

binding to a ligand, the 3D conformation of WW domains changes, where WW2 only acts as a chaperone to stabilize the ligand interaction with WW1 (McDonald et al., 2012). The ligands that bind to the WW1 domain are categorized into four groups depending on the amino acid residues that directly interact with the WW1 domain. The motifs usually possess proline residues, and they include; sequence of PPxY, sequence of PPLP, poly-P surrounded with arginine or lysine, and short sequences of phosphoserine or phosphothreonine (Salah et al., 2010).

Exposure to stressful stimuli was found to phosphorylate Tyr33 of WWOX (Chang et al., 2003). This resulted in the formation of a complex between WWOX and tumor protein Tp53 (p53) and its subsequent translocation to the mitochondria (Chang et al., 2003). A sequence between the two WW domains is responsible for the nuclear localization of the WWOX protein in mice, which is highly similar in structure to its human counterpart (Chang et al., 2001). Another study has demonstrated that WWOX is also present in the Golgi apparatus, as it translocates to it via the SDR sequence (Mahmud et al., 2021). The aberrant proteins which are translated from mRNA variants that lack exons 5-8 or 6-8 translocate to the nucleus rather than their usual location in Golgi, as they lack the necessary exons for functional SDR (Bednarek et al., 2001; Ludes-Meyers et al., 2003). This protein can also be found in the cytoplasm of normal as well as cancerous cells (Aqeilan & Croce, 2007).

1.3.3 Functions of WWOX Protein

WWOX has a wide array of indirect functions because it can bind to a range of transcription factors to regulate their activity and translocation to the nucleus

(Aqeilan & Croce, 2007). This protein is linked to steroidogenesis, as *WWOX* knockout mice failed to express essential enzymes involved in steroid production (Aqeilan et al., 2009). Moreover, this protein is associated with proper lipid metabolism, as it affects high-density lipoprotein levels (Iatan et al., 2014). Some of the major functions of the WWOX protein involve; tumor suppression activity, control of apoptosis, and regulation of proteins and transcription factors.

1.3.3.1 Tumor Suppressor Functions of WWOX

Overexpression and knockout experiments have proven the ability of WWOX to function as a tumor suppressor. Exogenous overexpression of WWOX in breast cancer cell lines has inhibited their growth and tumorigenicity (Bednarek et al., 2001). Variants of WWOX proteins that appear in cancerous tissue cause an abnormal localization of WWOX into the nucleus (Bednarek et al., 2001). In pancreatic cancer cell lines, ectopic expression of the WWOX protein inhibited the formation of colonies (Kuroki et al., 2004).

WWOX knockout mice are reported to be more susceptible to developing lymphoma, lung, and bone tumors naturally or with the aid of inducers compared with littermate controls (Aqeilan et al., 2007). The WWOX protein regulates the function of Runt-related transcription factor 2 (RUNX2), as overexpression of this gene coincides with the loss of WWOX (Kurek et al., 2010). RUNX2 is a transcription factor that functions normally in bone formation and becomes upregulated in osteosarcoma (Kurek et al., 2010). This might be a mechanism by which the WWOX protein exerts its tumor suppressor activity.

1.3.3.2 WWOX Regulates Cell Apoptosis

WWOX can regulate apoptosis of the cell due to its ability to upregulate the pro-apoptotic protein, p53, and downregulate the anti-apoptotic proteins, B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-xL) (Chang et al., 2001). The interaction between p53 and WWOX is related to the proline-rich area of p53 that can be recognized by the WW domain (Chang et al., 2001). Blocking the translation of murine WWOX mRNAs was sufficient to abolish the p53-dependant apoptosis (Chang et al., 2001). In response to stressful stimuli, both WWOX and p53 are phosphorylated, respectively, on Tyr33 and Ser46, which were found to form a complex and translocate to the nucleolus to drive the apoptosis of cancerous cells (Chang et al., 2005). The nuclear localization of WWOX can also be achieved by its polyubiquitination on lys63 by E3 ubiquitin ligase (Abu-Odeh et al., 2014). This signal was also associated with an increase in cellular death (Abu-Odeh et al., 2014).

The role of WWOX in apoptosis is also supported by the activation of both caspase-3 and caspase-9 after WWOX overexpression in cancerous cells, as they are involved in the apoptosis driven by p53 (Hu, et al, 2012). WWOX overexpression was also able to delay the transition of cells from the G1 to the S phase, which indicates inhibition of cellular proliferation (Hu *et al.*, 2012).

1.3.3.3 WWOX Controls the Functional Activity of different Proteins and Transcription Factors

WWOX regulates the activity of a wide range of proteins. It interacts physically with c- Jun N-terminal kinase1 (JNK1), which inhibits the antiproliferative

property of WWOX (Chang, Doherty, and Ensign, 2003). c-Jun is a transcription factor that regulates major cellular processes, such as; proliferation, differentiation, and apoptosis. This protein was found to interact with WWOX upon phosphorylation, which results in preventing WWOX from translocating from the cytoplasm (Gaudio *et al.*, 2006). Similar behavior was observed with the interaction between zinc finger-like protein (Zfra) and WWOX. Zfra is another protein that regulates cellular apoptosis, but in response to stress, it can prevent nuclear translocation of WWOX, which itself can control the apoptosis process (Hong *et al.*, 2007), thus, it suppresses the apoptotic function of WWOX (Hong *et al.*, 2007). WWOX interactions with TNF receptor-associated death domain protein (TRADD) could enhance the cell death rate as well (Chang *et al.*, 2001).

WWOX can regulate the function of the proto-oncogene, Erb-B2 Receptor Tyrosine Kinase 4 (ErbB4), which is sequestered in the cytoplasm and blocked from interacting with its target gene upon interaction with WWOX (Aqeilan and Croce, 2007). WWOX suppresses the transactivation activity of RUNX2 as well, which is involved in the proper differentiation of bone tissues (Aqeilan *et al.*, 2008). Activating protein 2- gamma (AP2γ) is another transcription factor, whose function is suppressed by the WWOX protein (Aqeilan *et al.*, 2004).

1.4 Contribution of *WWOX* to Cancer Progression

As discussed above, WWOX regulates a wide array of proteins and transcription factors related to major cellular pathways and processes, including cellular proliferation and apoptosis. Due to its various functions, loss of *WWOX* expression was reported in many types of cancers.

1.4.1 Breast Cancer

WWOX mRNAs and protein levels were reported to be reduced in breast cancer tissues (Nunez et al., 2005; Aqeilan, et al., 2007; Wang et al., 2009). This decreased expression was associated with methylation of CpG islands of the gene (Wang *et al.*, 2009). Low WWOX expression is correlated with active STAT3, as ectopic overexpression of WWOX can suppress this activity (Chang *et al.*, 2018). In addition, WWOX coexpression with the ErbB4 receptor in breast cancer patients had more favorable outcomes compared to patients who had lost WWOX expression (Aqeilan, et al., 2007). Furthermore, the reduced WWOX expression is highly linked to better disease-free survival in women with breast cancer (Płuciennik *et al.,* 2006).

A strong correlation between loss of WWOX and the expression of estrogen receptor was also reported, which highlights the role of WWOX in steroidogenesis (Nunez *et al.*, 2005). Moreover, in WWOX mammary gland conditional knockout mice, the loss of WWOX leads to the formation of basal-like breast cancer by altering the p53 activity (Abdeen and Aqeilan, 2019).

1.4.2 Ovarian Cancer

The *WWOX* gene is downregulated in women with ovarian cancers as well as ovarian cancer cell lines (Gourley *et al.*, 2005; Paige *et al.*, 2010; Yu *et al.*, 2019). In this case, the loss of WWOX expression is related to the hypermethylation of the promoter region (Yan and Sun, 2013). In ovarian cancer, low WWOX expression correlated with lymph node metastases, advanced stage, and poor survival (Paige *et al.*, 2010; Lan *et al.*, 2012). On the other hand,

overexpression of WWOX could inhibit tumorigenicity and induce apoptosis of ovarian cancers (Gourley *et a l* . , 2009; Xiong, Hu, and Wang, 2010).

This WWOX-induced apoptosis was found to be easily achieved in detached ovarian cancer cells when the cell interactions with the extracellular matrix are reduced (Gourley *et al.*, 2009). Another mechanism by which WWOX might induce cellular apoptosis in ovarian cancer cells, is by upregulating the levels of JNK, and caspase-3, as all of them are involved in the apoptosis (Yan *et al.*, 2015).

1.4.3 Prostate Cancer

Reduction of WWOX expression was reported in prostate cancer as well (Qin *et al.*, 2006; Lin *et al.*, 2015). This downregulation was attributed to epigenetic methylation of the regulatory regions of the gene (Qin *et al.*, 2006). WWOX is able to downregulate cyclin D1 in animal and cell models, suggesting the arrest of prostate cancer cells in G1 phase (Lin *et al.*, 2015). Moreover, ectopic expression of WWOX was associated with an increase in apoptosis and a decrease in cellular growth (Qin *et al.*, 2006). This overexpression was also associated with the suppression of ErbB2 expression, a known activator of the androgen receptor pathway that is involved in the pathogenesis of prostate cancer (Qin *et al.*, 2007).

1.4.4 Liver Cancer

The levels of mRNA transcripts and proteins of *WWOX* were reported to be lost in hepatocellular carcinoma cells (Park *et al.*, 2004). The loss of expression was

investigated in a mouse model, as knockout mice showed an increase in the proliferation and regeneration of liver cells (Abu-Remaileh *et al.*, 2018). Whereas, overexpression of WWOX was able to decrease the proliferation and induce apoptosis in hepatic carcinoma cells (Hu *et al.*, 2012). A new potential therapeutic approach to treat hepatocellular carcinoma was developed to introduce long non-coding RNA, known as TSLD8, in cancerous cells, where they stabilize the WWOX protein (Hu *et al.*, 2012). Using evodiamine, a potential anti-cancerous agent, in vivo and in vitro experiments have shown an increase in *WWOX* expression, which was associated with a decrease in the growth ability of liver cancerous cells (Hu *et al.*, 2017).

1.4.5 Osteosarcoma

Deletion in the *WWOX* gene sequence and protein expression was observed in osteosarcoma samples (Kurek *et al.*, 2010; Yang *et al.*, 2010, 2013), but the loss of expression does not affect the overall survival of osteosarcoma patients (Yang *et al.*, 2010). In addition to *WWOX* deletion, *WWOX* reduced expression is also related to an increase in methylation of *WWOX* (Liu *et al.*, 2020). The overexpression of WWOX inhibited cellular proliferation and decreased cell migration and invasion (Kurek *et al.*, 2010; Del Mare and Aqeilan, 2015; Liu *et al.*, 2017). On the mechanistic level, the overexpression of WWOX inhibited cell adhesion and metastasis by decreasing the activity of the transcription factor; RUNX2, which is associated with osteosarcoma metastasis (Kurek *et al.*, 2010; Del Mare and Aqeilan, 2015).

1.4.6 Hematopoietic Malignancies

Reduction in the expression of *WWOX* was associated with both acute myeloid and lymphoblastic leukemias (Chen *et al.*, 2013; Elbossaty, Malak, and Elghanam, 2019). An increase in the methylation levels of *WWOX* in acute lymphoblastic leukemia was detected and associated with a reduction in the activity of the pro-apoptotic protein (Chen *et al.*, 2013). *WWOX* gene rearrangements were also observed in polycythemia vera (Luís Vieira *et al.*, 2011). In addition, genetic alterations in the region that have the *WWOX* gene were detected in PMF patients leading to the authors' suggestion of its potential role in PMF pathogenesis. (Visani *et al.*, 2009). Moreover, a mutation in *WWOX* involving the variant; WWOXR188H, was reported in 47.37% of PMF in comparison with 10.4% in the control group (Klampfl *et al.*, 2013).

Therefore, for all mentioned before, *WWOX* is a worthy candidate to study in the progression of MPNs, firstly; due to the mutations and gene rearrangements that were detected in patients with MPNs. (Luís Vieira *et al.*, 2011; Klampfl *et al.,* 2011; Klampfl *et al.*, 2013), and secondly; due to the involvement of WWOX in cancer metastasis by utilizing the JAK2 pathway. (Chang et al., 2018).

1.5 Objective of the study

The contribution of *WWOX* to many solid cancers was thoroughly tackled by researchers, while there is a clear lack of studies on hematopoietic neoplasms and disorders. Therefore, the main objective of this research project was conducted to further enhance the overall knowledge of the role of the *WWOX* gene in MPNs, such as PV, ET, and PMF.

The genetic diagnosis of the Ph-ve MPNs, such as; Polycythemia vera, essential thrombocythemia, and primary myelofibrosis, is complicated. They share common genetic markers (JAK2, MPL, CALR) with an overlap in the diagnosis process, which cannot explain why the same mutations result in symptomatically different conditions. Thus, studying the role of other genetic markers might be essential in differentiating between these disorders. So far, many sub-clonal mutations have been identified, but none was common among the majority of patients diagnosed with either PV, ET, or PMF.

This project aims to investigate the potential role of WWOX in MPNs by studying:

- Potential macro deletions in *WWOX* exons of MPNs.
- Potential exonic variants in *WWOX* gene.
- Upregulation or downregulation of *WWOX* transcription in patients and hematopoietic cell lines by real-time PCR.
- Increased or decreased WWOX protein levels in MPN cell lines.

In this study, deletions in the sequence of WWOX exons, the presence of harmful variants, and alterations in either the levels of the mRNA and/or the protein were expected in accordance with the literature.

Chapter 2: Materials and Methods

2.1 Study Design

This case-control study was planned to investigate deletions in the *WWOX* gene exons and single nucleotide polymorphisms, as well as study its mRNA and protein levels in the myeloproliferative hematopoietic cell lines and Ph-ve MPNs patient samples (Figure 2). Therefore, EDTA blood samples were collected to extract the DNA in order to test for any deletions in *WWOX* exons by conventional PCR. Sanger sequencing was also used to determine the occurrence of genetic variants in these samples.

Moreover, RNA was extracted from both cell lines and blood samples in order to study WWOX expression patterns by using real-time PCR. Furthermore, the myeloproliferative cell lines, HEL1, HL60, KG1, NB4, SET2, and UKE1, were used to detect the levels of WWOX protein by western blot analysis.

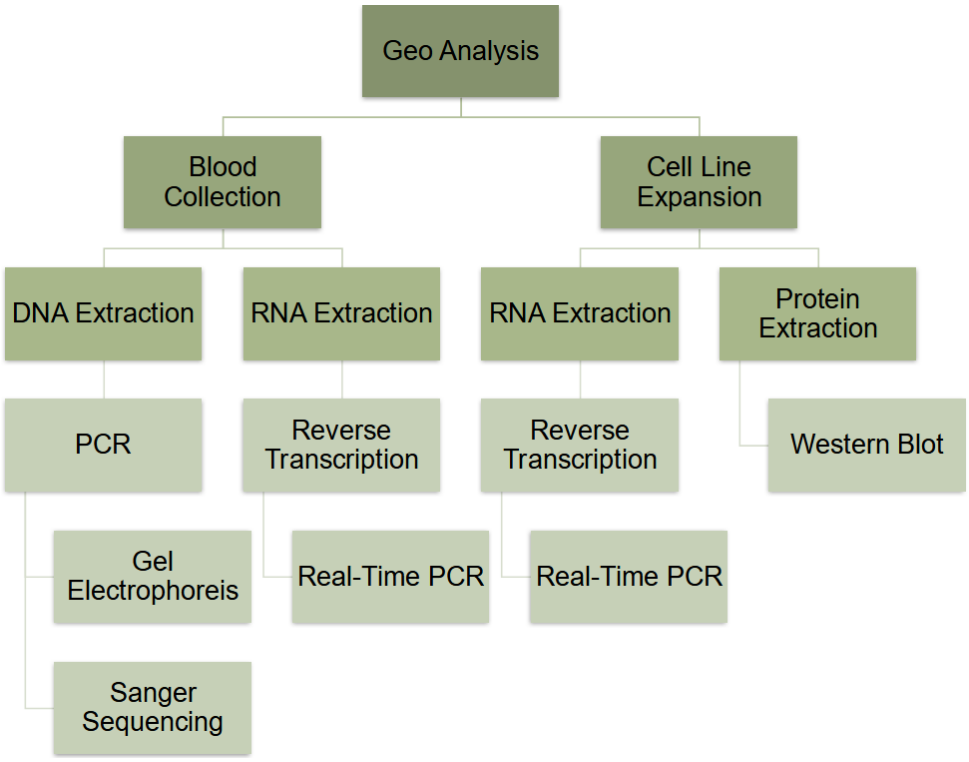


Figure 2: The workflow of investigating exonic deletions of *WWOX*, potential variants, and alterations in mRNA and protein levels.

2.2 Sample Collection and Ethical Consideration

Blood samples were collected in (Ethylenediaminetetraacetic acid) EDTA tubes from control subjects who do not have a history of MPN, and patients with JAK2V617F. The MPN patients and control subjects were sex and age-matched. About 28 MPN patient samples were used for DNA extraction. Another 15 control blood samples and 20 samples of patients carrying Ph-ve MPNs were collected for RNA extraction. Access to the complete data of the patients was not possible, except for the fact that they were Philadelphia chromosome negative and JAK2V617F positive, which were recruited from patients` files.

These samples were gathered in collaboration with Medicare Labs, Ramallah. Written consent forms from each volunteer were required before blood withdrawal. All the volunteers' data were secured and processed with high confidentiality.

2.3 Cell Lines Growth Conditions

Six hematopoietic cancerous cell lines were used including HEL1, HL60, KG1, NB4, SET2, and UKE1. The cell lines were donated by Sheba Medical Center, Tel Hashomer Hospital. Table 1 represents the mutation state of the used cell lines.

HEL, NB4, KG1, and HL60 were grown in (Roswell Park Memorial Institute) RPMI media supplemented with 1% L-glutamine, 1% penicillin-streptomycin, and 10% fetal calf serum (FCS). UKE1 cells were grown in RPMI media with 1% glutamine, 1% penicillin-streptomycin, and 10% fetal calf serum (FCS) in addition to 1 μM hydrocortisone. SET2 cells were grown in RPMI media containing 1% glutamine, 1% penicillin-streptomycin, and 20% FCS. All cell lines were grown at 37°C, and 5% CO2. All the mentioned materials in this paragraph were provided by Biological Industries, Israel.

Table 1: The status of *JAK2* mutations in the used hematopoietic cell lines.

Cell Line	**Mutation of *JAK2***
HEL1	V617F
HL60	wild-type
KG1	wild-type
NB4	wild-type
SET2	V617F
UKE1	V617F

2.4 Passaging, Freezing, and Thawing of cell lines

Cells were passaged when cell clumps appeared under the microscope or when the media turned yellowish. Between 1 to 2 mL of the crowded cells were transferred to a new flask containing fresh media. Cells were split twice a week per need.

For cell preservation, healthy dividing cells were frozen in freezing medium containing 10% (Dimethyl sulfoxide) DMSO, 20% FCS, and 70% growth media of that specific cell line. The mixture was added to cryovials and the temperature was decreased gradually in a freezing container, then they were stored in the vapor phase of a liquid nitrogen tank. When new cells were needed, the cryovial was placed in a 37°C water bath until a small ice crystal remained. Then, the content of the cryovial was added to 5ml of fresh media in a conical tube in order to get rid of DMSO, the cells were centrifuged for 5 minutes at 1500 RPM. Finally, cell pellet was resuspended in fresh medium and transferred to a new flask.

2.5 DNA Extraction

The DNA of the collected blood samples was extracted using Promega Wizard® Genomic DNA Purification Kit (Promega, USA). From each sample, 300 µL of blood was mixed with 900 µL of Cell Lysis Solution. After incubating the tubes for 10 min at room temperature, they were centrifuged at 14,000 rpm for 30 sec. The supernatant was discarded, and the pellet was resuspended in 300µl Nuclei Lysis Solution. The samples were then incubated at 37°C for approximately 10 min until all the clumps were dissolved. Following the addition of 100µL of Protein Precipitation Solution, the samples were centrifuged at 14,000 rpm for 3 min. The supernatant of this centrifugation step was transferred and mixed with 300µl of isopropanol (BioLab, Israel). Threads of the DNA strands became visible by gentle inversion of the tubes. These strands were precipitated by centrifugation at 14,000 rpm for 3 min. The supernatant was then replaced by 300µL of 70% ethanol (BioLab, Israel) and samples were centrifuged at 14,000 rpm for 3 min. The ethanol was discarded and tubes were inverted on an absorbent paper until the pellets were completely dry. The pellets were resuspended with 50µL of DNA Rehydration Solution and incubated at 65°C for 1hr with continuous shaking. Finally, DNA concentration and purity were determined by a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000c, Thermo Fisher Scientific, USA).

2.6 Primer Design

Ten sets of primers were designed to amplify the nine *WWOX* gene exons (Accession number: NC_000016). The sequence of each exon was obtained from

the NCBI (https://www.ncbi.nlm.nih.gov/gene/51741). Two sets of primers were designed for exons nine because of its larger size. Primer design was done using the online primer design software, Primer 3 (https://primer3.ut.ee/). The best-fitting sets of primers were determined depending on melting temperature (Tm), GC content %, product size, and spanning the whole exon in addition to a small sequence from the intron flanking region. The specificity of each set of primers was further confirmed by UCSC in-silico PCR tool (https://genome.ucsc.edu/cgi-bin/hgPcr). Table 2 shows the sequences of all the primers used in this research.

2.7 Polymerase Chain Reaction

Deletions in any of the nine *WWOX* exons in the 28 samples were determined by PCR. The reactions were performed, using GoTaq® Green Master Mix (Promega, USA), according to the manufacturer's provided recommendations. The PCR reactions were prepared by adding 12.5µL of 2X master mix, 1µL (10µM) forward primer, 1 µL (10µM) reverse primer (specific to each exon), 100ng DNA, and ultrapure water (Biological Industries, Israel) in a total reaction volume of 25ul. All PCR reactions were run with negative control, where the DNA sample was replaced with water. PCR amplification process was achieved by the thermocycler machine (FlexCycler2 PCR Thermal Cycler, Analytik Jena, Germany). The PCR program was as follows; initial denaturation step: 95℃ for 2 min, 36 cycles of the amplification step: 95℃ for 30 sec, 58℃ for 30 sec, 72℃ for 45 sec, and a final extension step: 72℃ for 5 mi

Table 2: The sequence of forward and reverse primers used to amplify WWOX exons.

Exon Number	Product size		Primer Sequence (5'→ 3')	Length	Tm	GC%	Self complementary
1	698	F	GTC ATA TCC AAT TGC CGG GCT C	22	61.71	54.55	6.00
		R	GCC CTT TTC CCT GAA GCT CCT	21	62.36	57.14	4.00
2	294	F	GTT GGG GTC ACA GTC CTC TTT C	22	60.81	54.55	3.00
		R	GTA ACC TGT CAC CTC TCT GCC	21	60.07	57.14	4.00
3	274	F	GTT TAC TTC TCC CTG GCA CCT G	22	60.88	54.55	3.00
		R	GGC GAT GTT AAA AGT TCC AGC G	22	60.73	50.00	4.00
4	407	F	GTC TGT GTT CAT TGC TGT GGG T	22	61.33	50.00	3.00
		R	GCA CAG GCT TCC ATG ACA ACA	21	61.42	52.38	4.00
5	240	F	GTC CGG TAA AGG CCA TTC AAC A	22	61.14	50.00	6.00
		R	GTA GCC CGG CAT GTG TAT TTG	21	59.67	52.38	5.00
6	353	F	GTC TGG GCG TCT TAT ATT AAA CAG G	25	59.53	44.00	6.00
		R	GAA CAG GGC CAA TAT AAA TGA GCC	24	60.02	45.83	6.00
7	362	F	GTC CTT GGT TGT AGT GTT TAT GTC C	25	59.82	44.00	2.00
		R	GCT GAG TCC ACC CAC ATG TCT	21	62.06	57.14	6.00
8	462	F	GTC CAC TCG TCT AAG ACT CCC A	22	60.88	54.55	7.00
		R	GGC AGG TTT CTC TAG GAC CCA	21	61.18	57.14	4.00
9-a	614	F	GTT GCT ATG CCA AGA TCC AGC T	22	61.01	50.00	4.00
		R	GTC CAG GGA GAT ACG GAA CCT	21	60.69	57.14	4.00
9-b	780	F	GTG TGG TGG CCT GTT TGA AAG T	22	61.79	50.00	5.00
		R	GAG AAT CGT GTT TTC CTG GCA	21	58.85	47.62	3.00

2.8 Gel Electrophoresis

The samples were run on 1% (weight/volume) agarose gel (SigmaAldrich, St.Louis, USA) prepared in 1X TAE buffer (Thermo Scientific™, Lithuania). Ethidium bromide (HyLabs, Israel) was added to each gel at a concentration of 0.01% from 10mg/ml stock. The prepared gels were loaded with 5 µL of each PCR product alongside 10 µL of 100 base pair DNA ladder (HyLabs, Israel). The gels were set to run for 45 min at 100 volts. The acquired bands were visualized using UV light with the ChemiDoc imaging system (ChemiDoc™, BioRad, USA).

2.9 Sanger Sequencing Techniques

2.9.1 Clean-up and Amplification

PCR products were cleaned for sequencing by mixing 1 µL of EPPiC fast mixture (A&A Biotechnology, Poland) with 5 µL of the PCR product. EPPiC fast mixture is composed of exonuclease I enzyme and alkaline phosphatase enzyme; the former breaks down the residual primers in the mix, whereas the latter dephosphorylates the dNTPs. The mixture was then incubated for 15 minutes in the thermal cycler (Biometra TONE thermal cycler, Analytik Jena, Germany); the first 10 min at 37.0 ℃ to activate the enzymes, followed by 1 minute at 80.0℃ to deactivate the enzymes.

The sequencing reaction was prepared in a 96-well PCR plate by combining 2 µL of the cleaned-up PCR products, 3.5µL of sequencing buffer, 2µL (5 pM stock) of either the forward or reverse primer, 1µL of BigDye™ terminator (Thermo

Fisher Scientific, USA), and 11.5µL of DEPC-treated water. After spinning the plate down, it was incubated in the thermal cycler for 25 repetitive cycles of; 70 sec at 96.0 ℃, 5 sec at 50.0℃, and 180 sec at 60.0℃.

2.9.2 DNA Precipitation and Sanger Sequencing

The resulting DNA product was precipitated by adding 5 µL of 0.5 M EDTA solution and 60 µL of absolute Ethanol to each well. The plate was then vortexed, incubated at 4℃ for 12 min, and centrifuged at 2200 x g for 12 min. After that, another 80µL of absolute ethanol was added and centrifuged for 15 min at 1600 x g. After inverting the plate to get rid of ethanol and let air-dry for 15 min, 10µL of formamide (Thermo Fisher Scientific, USA) was added to the wells. The plate was then incubated in a dry heat bath at 95°C for 3 min, then on ice for 3 min. Finally, the Sanger sequencing machine (Hitachi3500 Genetic Analyzer, Thermo Fisher Scientific, USA) was used to read the plate.

The sequence results were analyzed against their counterpart reference sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) by using the Sequence Scanner™ Software V 2.0.

2.10 RNA Extraction

Concerning the cell lines, the suspended cells were centrifuged and the pellet was resuspended with 1ml of TRIzol (Thermo Fisher Scientific, USA). For patient samples, blood samples were centrifuged at 1800 rpm for 10 min. After that, the buffy coat was collected in 15 ml falcon tubes and mixed with 13 ml RBC Lysis

Buffer (prepared by dissolving 8.26 g of ammonium chloride, 1 g of potassium bicarbonate, 37 mg of EDTA in 100 ml of water). The tubes were incubated in ice for 15-30 min and were centrifuged another time using the same parameters. A second RBCs lysis step was performed if RBCs were still seen in the formed pellet. The pellet was washed with 5 ml of phosphate buffer saline (PBS), and centrifuged at 1800 rpm for 10 min. The pellet was then mixed with 1 ml of TRIzol Reagent. Afterward, cellular and blood samples were pipetted up and down gently and mixed with 0.2ml of chloroform (Frutarom, Israel). The samples were vortexed, incubated for 10 min in ice, and centrifuged for 15 min at 12,000 x g at 4 °C. The mixture was separated into 3 layers. The upper layer was collected in a newly labeled tube and mixed with 0.5 ml of isopropanol. Then the tubes were vortexed and incubated at 4°C for 10 min, and centrifuged for another 10 min at 12,000 x g at 4 °C. The supernatant was aspirated and the pellet containing RNA was resuspended in 1 ml of 70% ethanol. Afterward, the samples were vortexed briefly and centrifuged for 5 min at 7,500 x g at 4 °C. Then the supernatant was discarded and the pellet was air-dried for 10 min. Finally, the pellet was resuspended in 50 µL of DEPC-treated water, and the concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000c, Thermo Fisher Scientific, USA).

2.11 Reverse Transcription

The reverse transcription of the mRNA samples was carried out by mixing 4µL of 5X Maxima H Minus cDNA Synthesis Master Mix (Thermo Fisher Scientific, USA) with 1 µg RNA, and the volume was completed up to 20µL with nuclease-

free water. The reactions were carried out on the following program: 25°C for 10 minutes, 50°C for 15 minutes, and 85°C for 5 minutes.

2.12 Real-Time PCR

Mixtures of 3µL of 1:10 diluted cDNA, 1 µL of the forward primer (10µM), 1 µL of the reverse primer (10µM), 12.5 2x SYPR Green Master Mix (Applied Biosystems, Lithuania), and 2.5µL DEPC-treated water were prepared. WWOX forward primer: TGA AGC AGT GTC ACG CAT TT, WWOX reverse primer: TGG TGA GAC TCC AGG GTA GA. HUBC forward primer: GTC GCA GTT CTT GTT TGT GG. HUBC reverse primer: GAT GGT GTC ACT GGG CTC AA, were used. The PCR program was performed in 7500 Real-Time PCR System (Applied Biosystems, Singapore) as follows; holding stage: 50°C for 2 min, 95°C for 10 min, 40 cycles of: 95°C for 1 sec, 60°C for 1 min, melt curve stage: 95°C for 15 sec, 60°C for 1 min, 95°C for 30sec and 60°C for 15 sec.

2.13 Western Blot analysis

2.13.1 Protein Extraction

Lysis of the cells was achieved by using RIPA lysis buffer prepared with a final concentration of 150 mM NaCl, 50 mM Tris (pH=8), 5 mM EDTA (pH=8), 1% NP-40 (IGEPAL CA-630), 0.5% sodium deoxycholate, 0.1% SDS, and 1X Halt Protease Phosphatase Inhibitor Cocktail to prevent protein degradation. The mixture was always fresh and ice-cooled before adding to the cells. Suspended cells were cultured in T25 flasks until they became crowded. After centrifugation, the cell pellet was resuspended with 0.5 ml of the cold RIPA

buffer. The acquired mixture was collected in an Eppendorf tube, centrifuged for 5 min at 13,000 x g, then stored at -80°C until they were used.

The protein concentration of each sample was measured by Bradford assay (BioRad, USA). Equal quantities of the protein from each sample were combined with 4X sample buffer. This buffer was prepared by mixing 20µL DTT, 100µL Bromophenol Blue, 50µL β- mercaptoethanol (5%), 460 mg SDS, 1.26 ml of 1 M Tris/HCl (pH=6.8), 5.4 ml of 75% Glycerol and 2.8mL H2O. Finally, the proteins were denatured by incubation at 95°C for 10 min.

2.13.2 Western Blotting

The gel casting apparatus was assembled and pre-cast SDS-polyacrylamide gel was mounted inside. The chamber was filled with 1X running buffer (3 g Tris Base, 14.4 g Glycine, 1 g SDS for 1 L of ddH2O). A protein marker was loaded alongside the boiled samples on the gel. The gel was set to run at 80 volts for 30 min then at 120 volts for 1.5-2 hrs. After that, the gel was blotted to a nitrocellulose membrane for 7 min using transblot turbo (BioRad, USA). Membranes were blocked with 5% Skim milk (Sigma Aldrich), and placed on a rocking platform for 30 min. After incubating the membrane overnight at 4°C with the primary antibody (anti-WWOX, Cell signaling), it was washed three times with Tween-Tris Buffered Saline (TTBS). The TTBS was left to soak the membrane for 10 min each time. The membrane was then incubated with a secondary anti-rabbit horse raddish peroxidase-conjugated antibody (Bethyl, A120-101P) according to the manufacturer's instructions. The signal was

developed by the membrane incubation in Clarity Western ECL (enhanced luminol-based chemiluminescent) Substrate (BioRad), and the protein presence was detected by a gel documentation system (ChemiDoc™, BioRad, USA).

2.14 In-silico analysis

At the beginning of the study, GEO analysis (https://www.ncbi.nlm.nih.gov/geo/) was used to study the expression pattern of the WWOX gene in studies conducted on MPN patients. Following Sanger sequencing, the Ensemble platform was used to determine the allele frequency of each variant in the population. The probable effect of each variant was also predicted using a number of in-silico tools, including;

- ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).
- PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/).
- MutationTaster (https://www.mutationtaster.org/).
- PROVEAN (http://provean.jcvi.org/index.php).
- SIFT (https://sift.bii.a-star.edu.sg/).
- GVGD (http://agvgd.hci.utah.edu/).

The protein-protein interactions were investigated using NDEx Integrated Query website (https://www.ndexbio.org/iquery/).

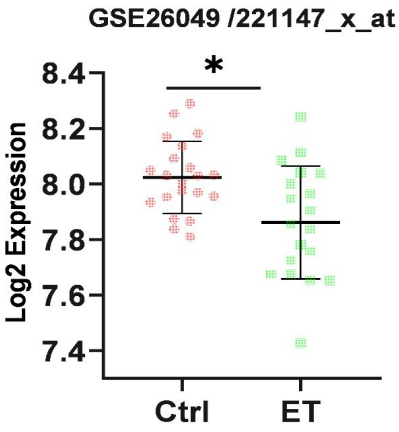
Chapter Three: Results

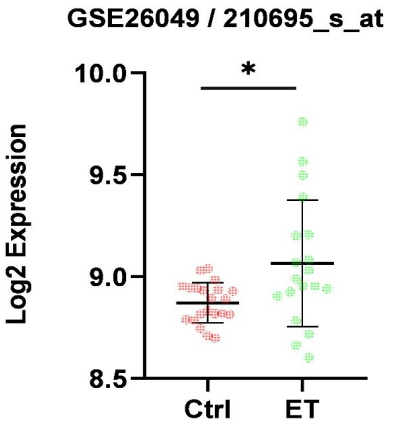
3. 1 GEO analysis of previous studies on MPNs.

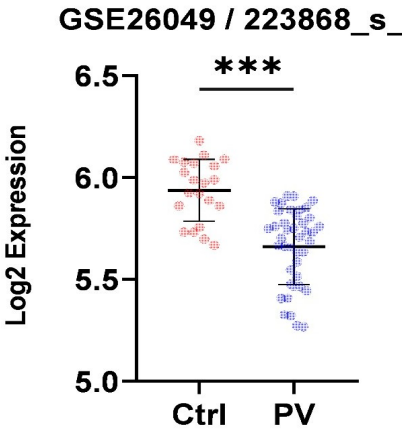
In order to know if there was a consistent pattern in *WWOX* expression in MPNs, GEO analysis was performed on previous microarray studies conducted on patient samples with PV, ET, or PMF. Variations in upregulation and downregulation were observed in these studies (Table 3, Figure 3, and Figure 4). Different probes were used for *WWOX* depending on the used microarray. These probes, sometimes, have shown opposite results. While one probe detected a rise in the expression another detected a decrease in the expression. This can be noticed in the study GSE26049 when comparing control with either PMF, ET, or all the MPN conditions (Figure 3). However, the other three studies revealed a decrease in the expression of WWOX in PV and PMF (Figure 4). As these studies didn't show a consistent pattern of *WWOX* expression, thus, studying the expression of *WWOX* in patients with MPNs and hematological cell lines might be valuable in determining the real expression pattern of WWOX in PV, ET, and PMF.

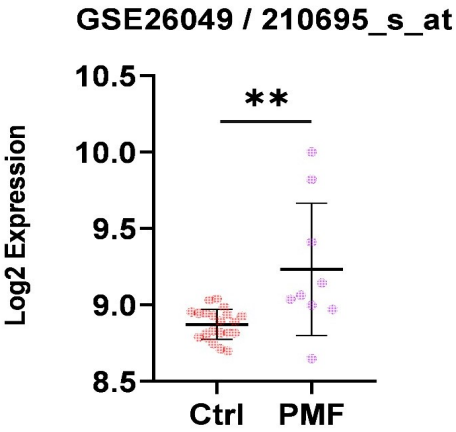
Table 3: Studies with a significant difference in WWOX expression between control and MPN conditions by GEO analysis.

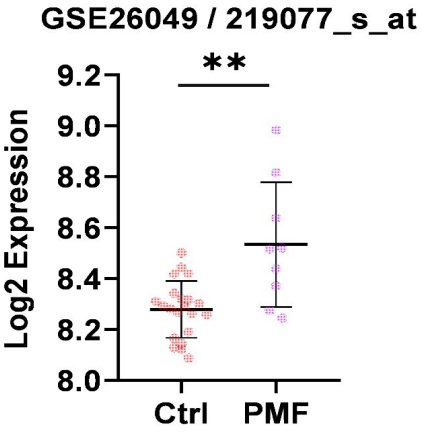
GSE	Group 1	Group 2	WWOX probe ID/ transcript	Adjusted p-value	GEO Title	Fig.
26049	21 Control	9 PMF	219077_s_at	0.00341	Expression data from patients with Essential Thrombocythemia (ET), Polycythemia Vera (PV), Primary Myelofibrosis (PMF), and control subjects	3
			210695_s_at	0.00366		
			223747_x_at	0.0211		
		41 PV	223868_s_at	0.00000249		
		19 ET	221147_x_at	0.0152		
			210695_s_at	0.0238		
		69 MPN	223868_s_at	0.000811		
			210695_s_at	0.00752		
			223747_x_at	0.0114		
			219077_s_at	0.0464		
103176	15 Control	26 PV	11749046_a_at	0.0369	Gene and miRNA expression profiles in Polycythemia Vera and Essential Thrombocythemia according to CALR and JAK2 mutations	4
61629	15 Control	21 PV	210695_s_at	0.0457	Expression data from patients with Essential Thrombocythemia (ET), Polycythemia Vera (PV), Primary Myelofibrosis (PMF) (untreated)	
53482	16 Control	42 PMF	11730156_a_at	0.00352	Integrative Analysis of Gene and miRNA expression profiles in Primary Myelofibrosis CD34+ Cells	
			11726214_x_at	0.0253		

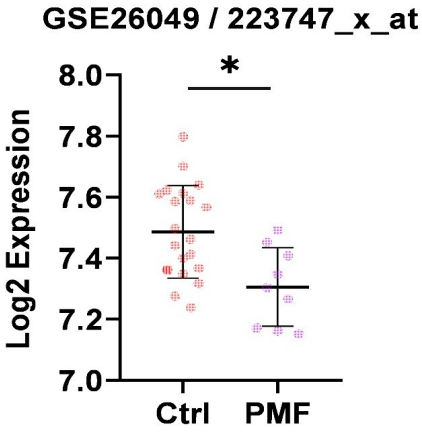

GSE26049 /221147_x_at
Log2 Expression
8.4
8.2
8.0
7.8
7.6
7.4
*
Ctrl
ET


GSE26049 / 210695_s_at
Log2 Expression
10.0
9.5
9.0
8.5
*
Ctrl
ET


GSE26049 / 223868_s_at
Log2 Expression
6.5
6.0
5.5
5.0
***
Ctrl
PV


GSE26049 / 210695_s_at
Log2 Expression
10.5
10.0
9.5
9.0
8.5
**
Ctrl
PMF


GSE26049 / 219077_s_at
Log2 Expression
9.2
9.0
8.8
8.6
8.4
8.2
8.0
**
Ctrl
PMF


GSE26049 / 223747_x_at
Log2 Expression
8.0
7.8
7.6
7.4
7.2
7.0
*
Ctrl
PMF

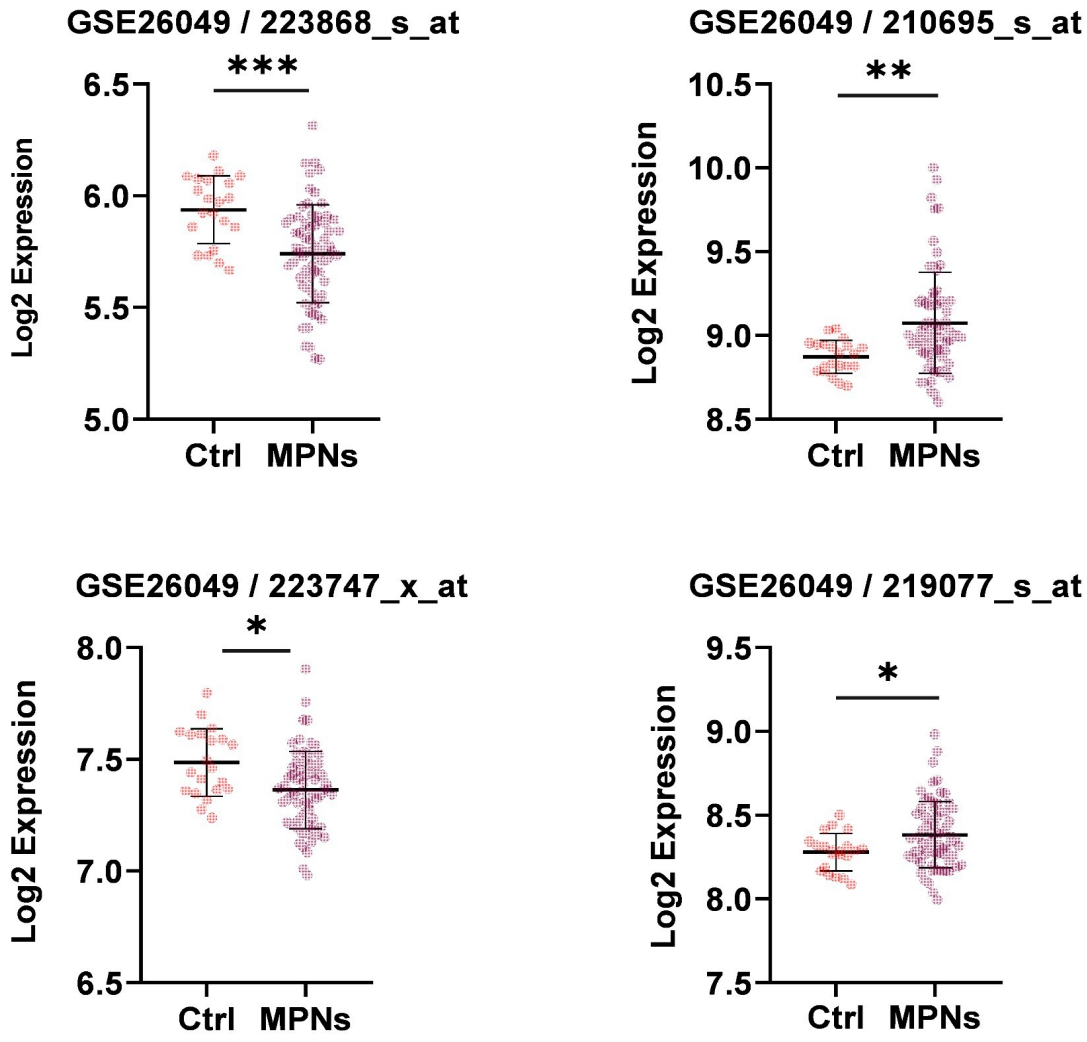


Figure 3: GEO analysis of GSE26049 study. Significant upregulation or downregulation in *WWOX* expression could be observed.

* p-value < 0.5. ** p-value < 0.1. *** p-value < 0.01.

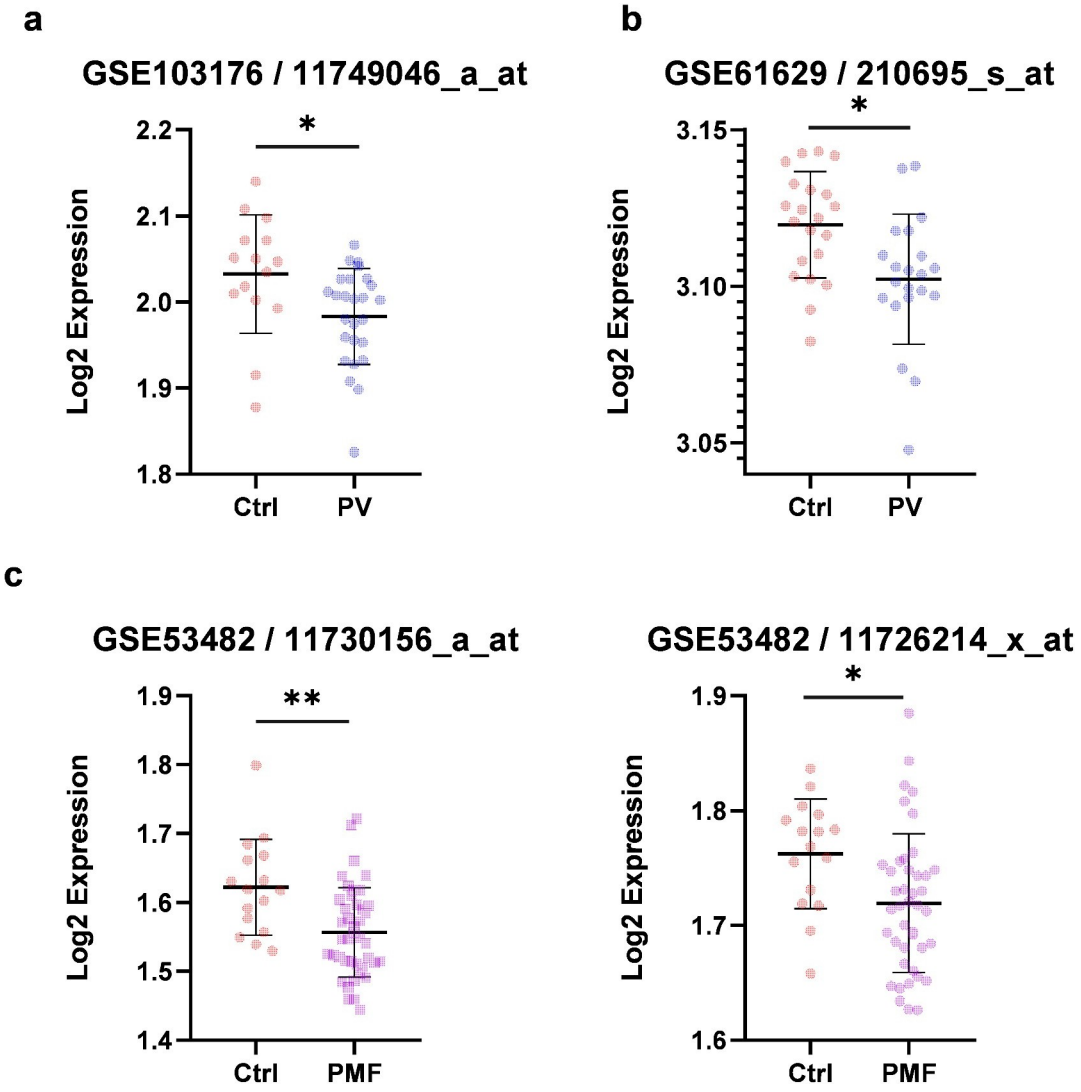


Figure 4: GEO analysis of three different microarray studies. Significant upregulation or downregulation of *WWOX* expression by GEO analysis in 3 studies. **a**: GSE103176 study. **b**: GSE61629 study. **c**: GSE53482 study. * p-value < 0.5. ** p- value < 0.1.

3. 2 *WWOX* gene exon deletion analysis in MPN patients

As mentioned earlier, *WWOX* falls in a fragile genomic locus and could be thus subject to deletions, we tested for the presence of large *WWOX* gene exonic deletions by amplifying each exon and intronic flanking regions. Our results show that there was no WWOX exon deletion in any of our studied samples (Fig.

5).

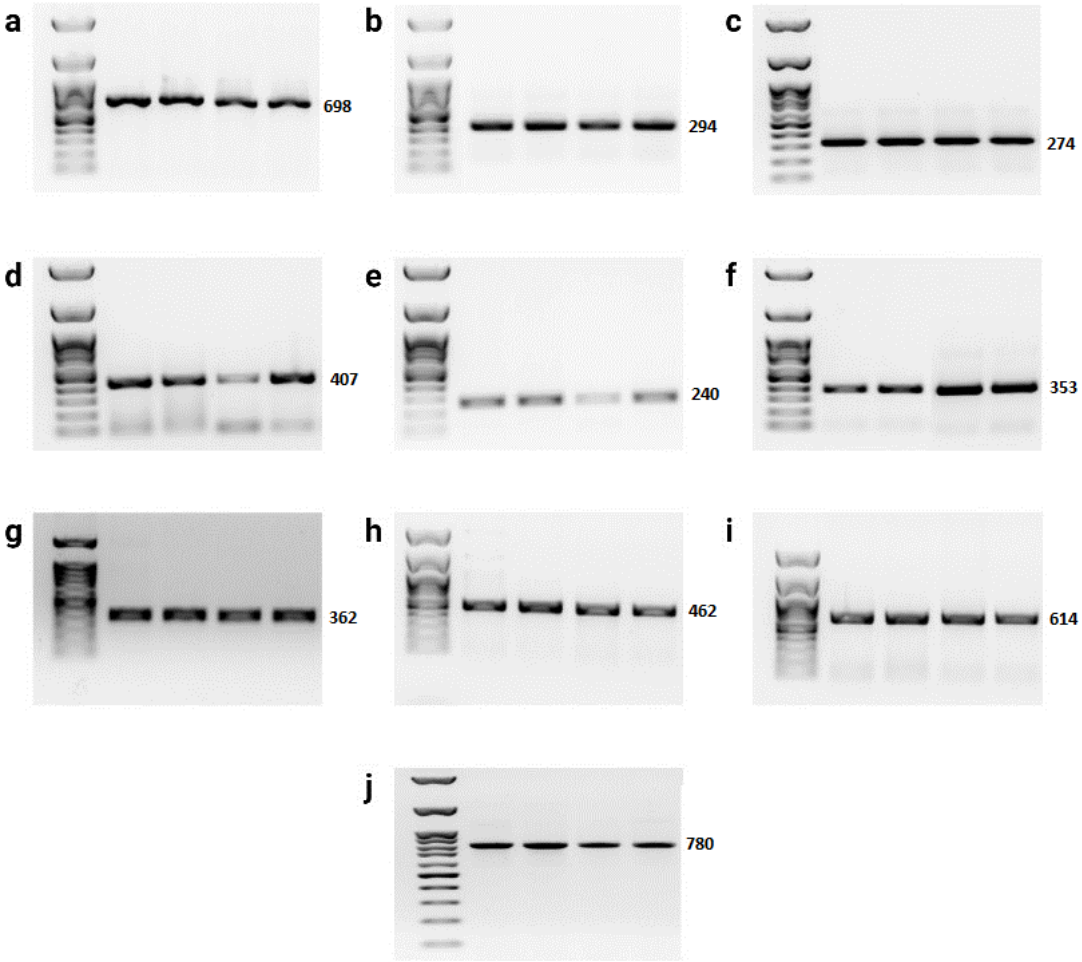


Figure 5: WWOX exon deletions analysis. Representative PCR products for all *WWOX* exons. **a:** PCR product of exon 1 that spans 698 nucleotides. **b:** PCR product of exon 2 that spans 294 nucleotides. **c:** PCR product of exon 3 that spans 274 nucleotides. **d:** PCR product of exon 4 that spans 407 nucleotides. **e:** PCR product of exon 5 that spans 240 nucleotides. **f:** PCR product of exon 6 that spans 353 nucleotides. **g:** PCR product of exon 7 that spans 362 nucleotides. **h:** PCR product of exon 8 that spans 462 nucleotides. **i:** PCR product of part 1 of exon 9 that spans 614 nucleotides. **j:** PCR product of part 2 of exon 9 that spans 780 nucleotides.

3.3 Genetic variants in the *WWOX* gene in MPN patients

To determine if there are single nucleotide polymorphisms (SNPs) or other small insertion-deletion variations in *WWOX* gene exons, all *WWOX* gene exons were

sequenced using Sanger sequencing. Twenty-six *(*26) samples of Ph-ve MPNs were analyzed by sequencing and five different variants were identified as follows (Figure 6); rs11545029 (p.Ala179Thr), rs7201683 (p.Leu216Val), rs3764340 (p.Pro282Ala), rs73572838 (p.Arg314His), and rs76204496 (p.Gly410Cys), in exons 6, 7, 8, 8, and 9 respectively (Table 4). Of the previously mentioned variants, four are located in the ADH/SDR domain, while the last one is located at the end of the amino acid sequence of the WWOX protein (Figure 7).

The p.Ala179Thr variant has appeared in 18 samples, representing 69.23% of the 26 samples. Around 55.55% of the samples carrying this variant were homozygous for the polymorphism, while 44.44% were heterozygous for the polymorphism. The two variants in exon 8 were reported in only 1 sample each. The variant of exon 7 and the variant of exon 9 each have existed in 2 patients each, accounting for 7.69% of the samples. The allele frequency of the identified variants was retrieved from the Ensemble platform (Table 4). The variants; p.Ala179Thr, p.Leu216Val, p.Pro282Ala, and p.Arg314His, appear in the normal population, but with a lower frequency than that of the standard allele. Whereas, p.Gly410Cys does not appear in the normal population, therefore, it might have a deleterious impact effect.

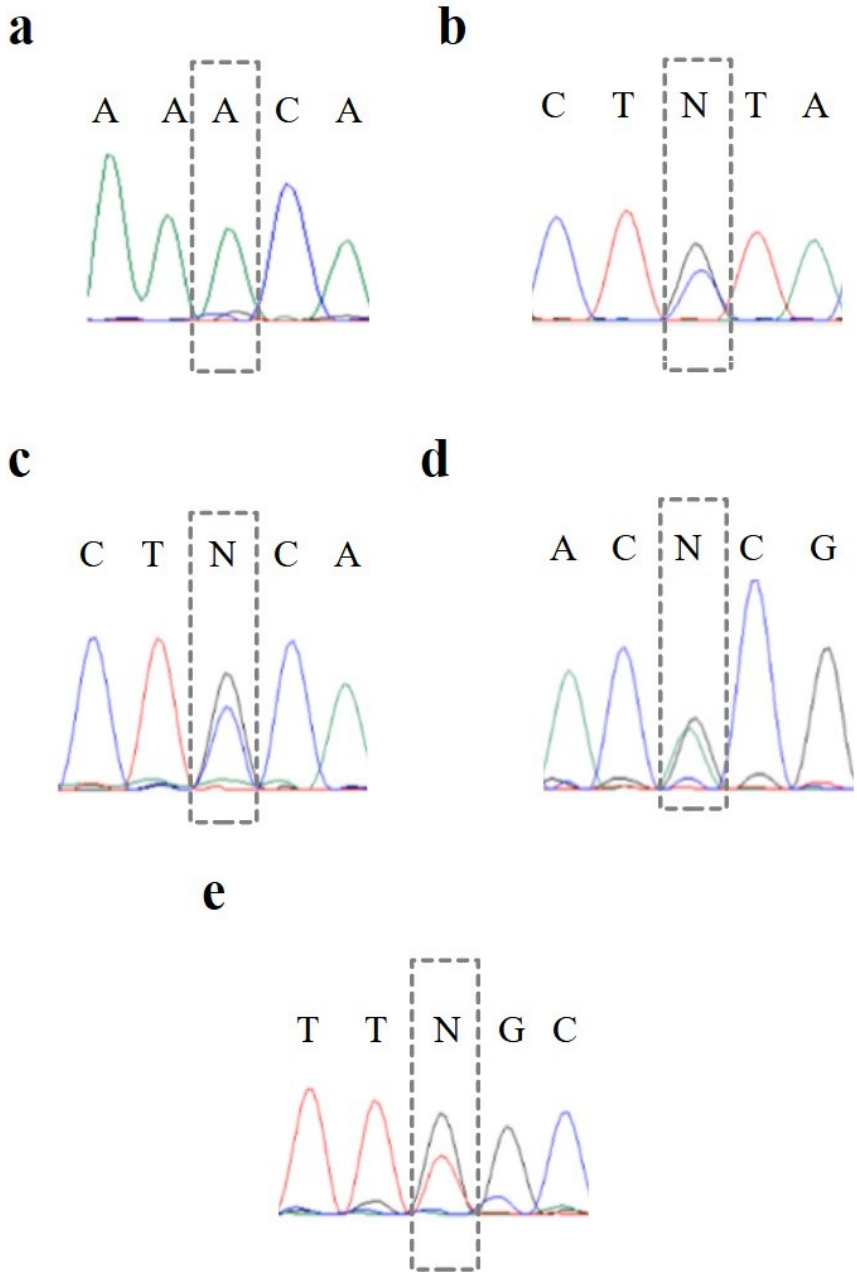


Figure 6: *WWOX* genotyping by Sanger sequencing. **a**: homozygous rs11545029 (c.535G>A). **b**: heterozygous rs7201683 (c.646C>G). **c**: heterozygous rs3764340 (c.844C>G). **d**: heterozygous rs73572838 (c.941G>A). **e**: heterozygous rs76204496 (c.1228G>T).**e**: heterozygous rs76204496 (c.1228G>T)

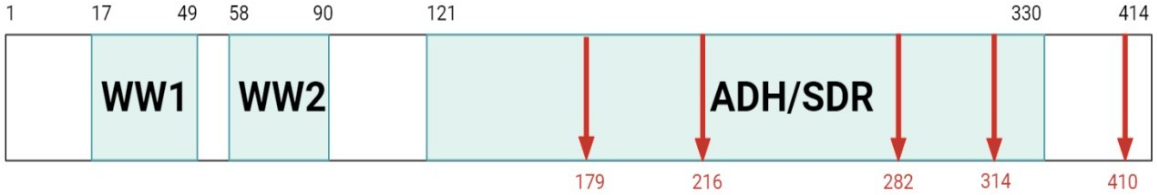


Figure 7: Mapping of the five detected variants to their position in the WWOX protein. The red color represents the variants from left to right; rs11545029, rs7201683, rs3764340, rs73572838, and rs76204496. Numbers in red color represent the number of the changed amino acid.

Table 4: The variants identified, their position, and frequency in patients diagnosed with MPNs.

Reference ID	**Exon**	**Amino Acid Change**	**CDS Position**	**Genotype**	**Number of samples carrying SNP**	**Allele frequency (Ensemble)**
rs11545029	6	p.Ala179Thr	c.535G>A	AA (55.56%) AG (44.44%)	18	G: 74% A: 26%
rs7201683	7	p.Leu216Val	c.646C>G	CG (100%)	2	C:96% G:4%
rs3764340	8	p.Pro282Ala	c.844C>G	CG (100%)	1	C: 91% G: 9%
rs73572838	8	p.Arg314His	c.941G>A	AG (100%)	1	G: 97% A: 3%
rs76204496	9	p.Gly410Cys	c.1228G>T	GT (100%)	2	G:100%
The accession number of the used sequence is NC_000016.						

To determine if these variants have any damaging impact on the protein, online in-silico tools were used. Table 5 summarizes the predicted effects of the five variants using ClinVar, PolyPhen-2, MutationTaster, PROVEAN, SIFT, and GVGD. The variant rs11545029 was predicted to interfere with the WWOX

protein function by GVGD. The polymorphism rs3764340 was predicted to be damaging by both PolyPhen-2 and PROVEAN, while rs76204496 was predicted to negatively affect the protein function by PolyPhen-2, Mutation Taster, SIFT, and GVGD. The other two variants were not likely to cause any deleterious impact by any of the used tools. Further investigations on larger sample populations are needed to determine if any of these mutations have a significant impact on the pathogenesis of Ph-ve MPNs.

3.4 Levels of *WWOX* transcripts in samples from Ph -ve MPN patients

To determine any variations in *WWOX* expression at the mRNA level, real-time PCR was performed using *WWOX* specific primers. In this experiment, 14 control samples and 19 patient samples were analyzed. The fold change of the mRNA transcripts in the cases group was 0.4328±0.4309 compared to 1.341±1.021 WWOX mRNA level expression in normal cases (Figure 8). This suggests an average of about 3 folds reduction in *WWOX* mRNA in the blood cells of MPN patients. The two groups of data passed the D'Agostino & Pearson test normality test as well as the Kolmogorov-Smirnov normality test. The reduction in the transcription in the cases is considered significant as the p-value is 0.0065 according to the unpaired t-test with Welche's correction. These results indicate that WWOX expression is downregulated in MPN cases.

Table 5: The probable effect of the identified variants on WWOX protein using in-silico tools

Reference ID	**Clin Var**	**Poly Phen-2**	**Mutation Taster**	**PROVEAN**	**SIFT**	**GVGD**
rs11545029	Benign	BENIGN (0.098)	polymorphism	Neutral	Tolerated	Class C55(most likely to interfere with protein function)
rs7201683	Benign	BENIGN (0.011)	polymorphism	Neutral	Tolerated	Class C25(less likely to interfere with protein function)
rs3764340	Benign	PROBABLY DAMAGING (0.994)	polymorphism	Deleterio-us	Tolerated	Class C25(less likely to interfere with protein function)
rs73572838	Benign	BENIGN (0.005)	polymorphism	Neutral	Tolerated	Class C25(less likely to interfere with protein function)
rs76204496	Likely benign	PROBABLY DAMAGING (0.961)	Disease-causing	Neutral	Damaging	Class C65(most likely to interfere with protein function)

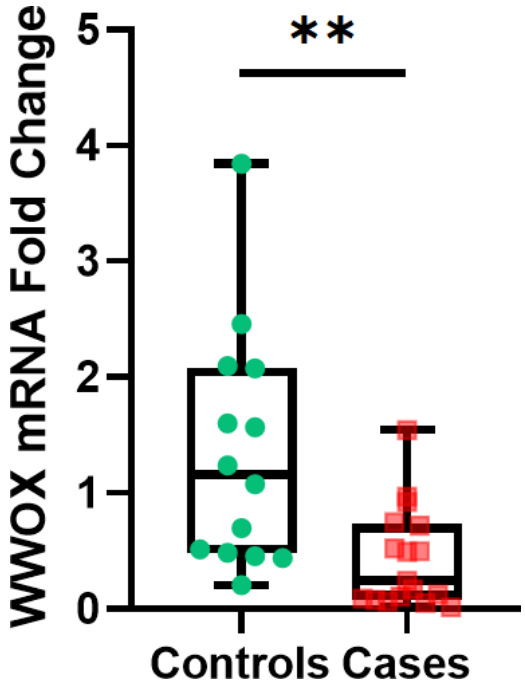


Figure 8: Comparison of WWOX expression between MPN cases and normal samples. Demonstration of WWOX mRNA fold change between a controls group (n=14) and cases group (n=17). The mean of the controls equals 1.341±1.021, while in the cases is 0.4328±0.4309. ** p-value < 0.01.

3.5 *WWOX* mRNA expression levels in MPN cell lines

To evaluate the level of WWOX mRNA expression in different hematopoietic cell lines, real-time PCR was performed on RNA extracted from HEL1, HL60, KG1, NB4, SET2, and UKE1 cell lines. Three independent experiments were performed for each cell line. The essential thrombocythemia cell line, UKE1, was used as a reference point to compare the expression of WWOX in other cell lines, as it has the highest level of transcripts among the tested cell lines. A significant reduction in the mRNA transcript of WWOX was observed in some cell lines according to the unpaired t-test with Welche's correction (Figure 9). The Megakaryoblastic cell line, SET2, showed the lowest expression with a p-value of 0.003. The transcripts level of WWOX in this cell line is very low in

comparison with UKE1 cells. The acute promyelocytic leukemia cell line, NB4, and the acute myeloid leukemia cell line, HL60, respectively showed 78% and 73% lower levels compared to UKE1. HEL1 WWOX level is almost one-sixth of the WWOX transcripts in UKE1. The macrophage-derived cell lines, KG1, showed a moderate reduction (about 50%) in its *WWOX* level compared to UKE1 cells. Altogether, these data suggest that *WWOX* expression is reduced in some MPN cell lines.

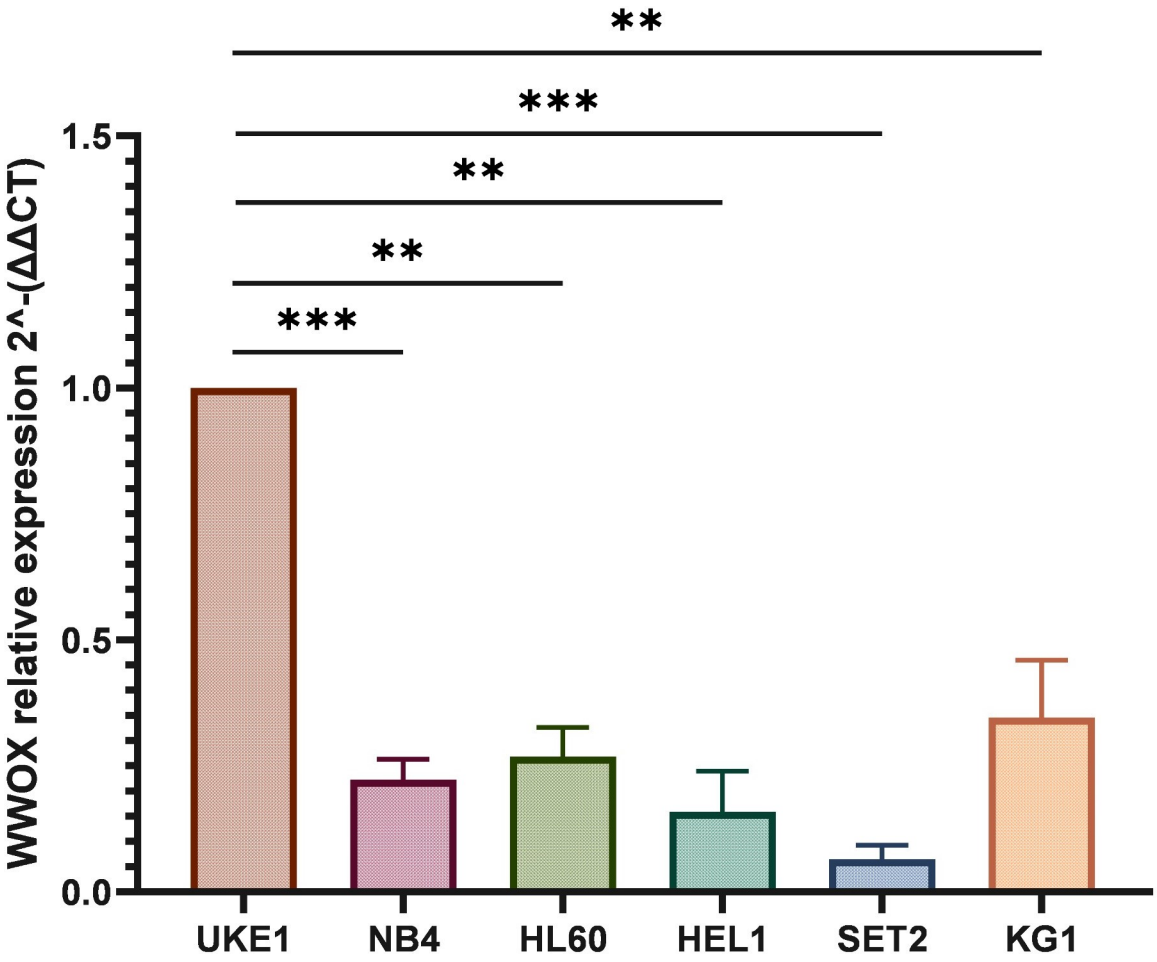


Figure 9: WWOX expression level in MPN cell lines. qPCR results showing relative WWOX mRNA expression level. WWOX expression in UKE1 cells was used as a reference point. Unpaired t-test with Welche's correction was used to determine the level of significance between UKE1 and each cell line. ** p-value < 0.01. *** p-value < 0.001. Bars represent standard error of the mean.

3.6 WWOX protein expression in MPN cell lines.

The protein is the functional molecule in the cell, thus it is important to determine the protein expression level of genes. To determine WWOX protein levels in MPN cell lines, we used Western blot analysis. The breast cancer cell line, MCF7, was used as a positive control due to its known high WWOX expression. All the hematopoietic cell lines showed relatively lower WWOX levels compared to MCF7 cells (Figure 10). The results demonstrate that SET2 cells have the lowest expression level, where the expression of WWOX was almost completely lost. HL60 also had a very low expression of WWOX in comparison with MCF7. KG1 and HEL1 showed similar low expression, whereas UKE1 displayed a slightly higher expression level. NB4 showed the highest expression level but this may be because the actin level is also higher than in other cells. Therefore, our results showed that the protein expression of the *WWOX* gene is reduced in hematopoietic-derived cell lines.

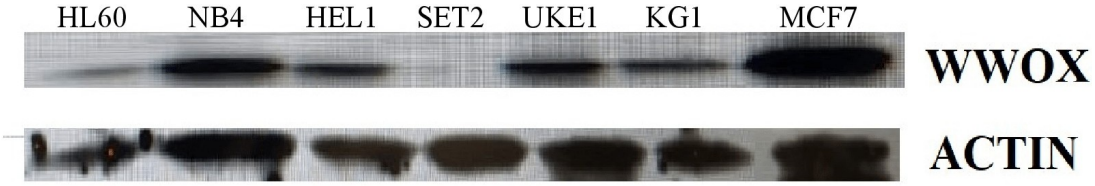


Figure 10: WWOX protein expression level. Western blot analysis showing WWOX protein level in the indicated MPN cell lines. MCF7 was used as a positive control. Actin was used as protein loading control.

3.7 WWOX interactions using in-silico tools.

WWOX was shown previously to affect other proteins through protein-protein interactions, so we used NDEx Integrated Query website to study WWOX protein-protein interactions. Two interaction networks involving WWOX were found (Figure. 11). The left side represents an ErbB4 signaling pathway events and the right side represents a p73 transcription factor network. The yellow-marked proteins are the ones that are expected to interact with WWOX.

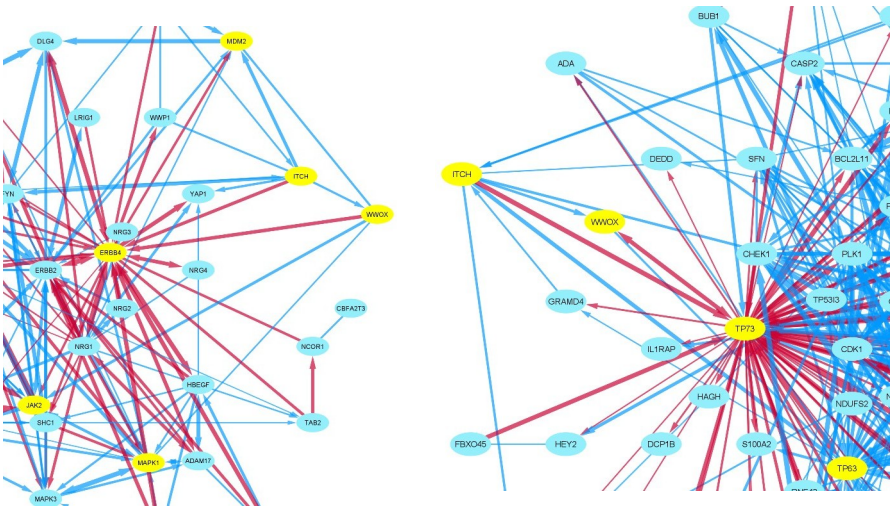


Figure 11: The expected interactions of various proteins with WWOX by NDEx Integrated Query website. Left side: ErbB4 signaling events (v2.0). Right side: p73 transcription factor network (v2.0).

In the first network, WWOX was found to interact with JAK2, MAPK1, MDM2, ITCH, and ERBB4. While on the second one, it was found to interact with ITCH, TP73, and TP63. JAK2 has an already established role in the pathogenesis of Ph-ve MPNs. Constitutive activation of MAP kinase was also reported in the progression of MPNs (Chung, Hsu, and Kondo, 2011). The mutation, JAK2V617F, was also associated with an increase in MDM2 accumulation and the subsequent degradation

of the apoptotic protein p53 in MPNs. (Nakatake *et al.,* 2012) Also, mice with ITCH deficiency developed a phenotype similar to MPNs (Jutzi *et al.*, 2014). Meanwhile, no link was found in the literature between MPNs with TP73 and TP63. Therefore, more investigations on the link between these proteins and MPNs might expand the knowledge of the involved markers in the development of such disorders. Furthermore, studies on the connection between all these proteins and WWOX in Ph-ve MPNs might be of great use in understanding the WWOX involvement in MPNs more clearly.

Chapter Four: Discussion

The genetic diagnosis criteria of different Ph-ve MPNs disorders are somehow similar and overlapping, as these disorders share the three main genetic markers (JAK2, MPL, CALR). However, this fails to clarify the different symptomatic manifestations. Many subclonal mutations have been identified, but none of them were common among the majority of patients diagnosed with either PV, ET, or PMF. Aberrations in the *WWOX* gene were detected in PV and PMF (Vieira et al., 2012; Klampfl et al., 2013). Therefore, this study aimed to find alterations in *WWOX* expression that might be of significance in understanding the pathogenesis of MPNs, specifically, Ph-ve MPNs.

Several studies have shown downregulation of *WWOX* expression in multiple types of solid cancers (Park et al., 2004; Pospiech et al., 2018; Wen et al., 2017). Alteration in the expression was also noted in hematopoietic malignancies; such as acute myeloid leukemia and acute lymphoblastic leukemia (Chen et al., 2013; Elbossaty et al., 2019). The preliminary data we obtained about expression depending on GEO analysis failed to show any consistent trend toward reduction or elevation in *WWOX* expression in any of the tested conditions in the study with the biggest number of samples (GSE26049). These discrepancies might be caused by the detection of alternatively spliced isoforms of *WWOX* by the different probes used in the study. This was obvious because WWOX showed different expression levels when using different probes. The higher WWOX expression in some cases might be of no biological significance, as some WWOX isoforms are not expressed or not functional, which might lead

to the expression of truncated proteins. Although in the GEO study GSE26049 inconsistent patterns of upregulation or downregulation were observed with the different probes, significantly lower levels of WWOX were observed in the patient groups of the other three studies; GSE10317, GSE61629, and GSE53482, which might point to the fact that WWOX expression tends to be lower in MPN cases compared to normal subjects.

Deletions in *WWOX* sequence were reported to affect the tumor suppressor function of the protein (Abdeen et al., 2018; McBride et al., 2019). Our results demonstrated no large deletions in the exons of the two alleles of *WWOX* in the majority of cells extracted from each sample. This means that, deletions in WWOX exons are not the reason for lower WWOX expression observed at the mRNA and protein levels. Moreover, the original sequence is huge, consisting of more than 1.1 million bases, while the mRNA form is composed of only 1245 bases. Therefore, the introns and regulatory sequences of the *WWOX* are quite large, where numerous and large deletions can occur anywhere, without being detected by exon-focused PCR. These hypothetical deletions might affect the expression rate of transcription, as they might upregulate or downregulate the transcription process, and they might be able to abolish the expression overall.

In fact, other factors might affect the expression of this gene rather than sequence deletions. These factors include epigenetic regulation or alteration in the amino acids of the protein that might change the tertiary conformation and subsequently the functionality of the protein. The epigenetic chemical modifications might occur on the regulatory regions of the gene or they might be presented on

the DNA-associated histones. This will either drive the transcription machinery away from the promoter of the gene or toward it.

Moreover, variants can easily avoid detection by PCR alone. These variants might be deleterious, as they might affect the translation process or the proper folding of the protein. Screening of 26 patients led to the detection of five different variants; rs11545029 (p.Ala179Thr), rs7201683 (p.Leu216Val), rs3764340 (p.Pro282Ala), rs73572838 (p.Arg314His), and rs76204496 (p.Gly410Cys). Four of these variants are positioned in the ADH/SDR domain of WWOX, whereas the fifth detected variant; rs76204496, is located toward the end of the amino acid sequence. The allele T of this same variant was not found in the ensemble platform, as the G allele frequency is 100%. This might be the cause of its identification as probably damaging by four in-silico tools. This variant was also reported in consanguineous Arab families with intellectual disability (Alkhateeb et al., 2016).

The variant; rs11545029, was detected as most likely to interfere with protein function in only GVGD. Whereas, rs3764340 was predicted to be damaging by PolyPhen-2 and PROVEAN. This variant was also detected in various cancer types; lung (Huang et al., 2013; Wang et al., 2016), osteosarcoma (Zhu et al., 2016), esophagus (Guo, Wang, et al., 2013), thyroid (Cancemi et al., 2011), and liver (Lee et al., 2017). It was also detected in patients with oral cancer (Cheng et al., 2016), urothelial cell carcinoma (Hung et al., 2020), gastric cardia adenocarcinoma (Guo, Dong, et al., 2013), and uterine cervical cancer (Lin et al., 2018). These correlations with this variant highlight its role as possibly

deleterious.

The variants; rs7201683 and rs73572838 were not shown as possibly damaging in any of the in-silico tools. Also, the allele frequencies of both variants were reported in the normal population. However, rs7201683 was reported in Multiple Sclerosis and Mycophenolate Related Leukopenia (Jacobson et al., 2011; Ziliotto et al., 2019).

The alteration of the identified variants might not be of a significant effect because the exchange in rs7201683, rs3764340, rs73572838, and rs76204496 variants happens in amino acids with similar properties. For example; the alteration of the variant p.Leu216Val exchanges leucine with valine where both have hydrophobic side chains. The same is applied to p.Pro282Ala and p.Gly410Cys, as the amino acids involved in these two variants are in the non-polar group. p.Arg314His is another case of alteration between similar amino acids, as both arginine and histidine have positive charges in their side chain. their side chain.

The transcript levels of *WWOX* in patients with MPNs were significantly reduced in comparison with the control group, with around 3-fold change and a p-value of 0.0065. The decrease in the mRNA might affect the function of WWOX and lead to the development of various types of MPNs, especially, since its known that WWOX possesses a tumor suppression activity (Aqeilan et al., 2007; Kuroki et al., 2004). Also, a decrease in the mRNA levels of WWOX might drive harmful effects and lead to the transformation of Ph-ve MPNs into leukemia. Furthermore, WWOX loss was reported to aid the metastasis of breast cancer and

hepatocellular carcinoma via JAK2 pathway; the known driver in Ph-ve MPNs (Chang et al., 2018). This might be due to epigenetic regulation of the WWOX on the mRNA levels in MPNs, as an increase in the methylation of WWOX was reported in acute lymphoblastic leukemia. (Chen *et al.*, 2013). *et al.*, 2013).

The transcription levels were tested in various hematopoietic cell lines as well, where KG1, NB4, HL60, HEL1, and SET2 showed a significant reduction in *WWOX* mRNA in comparison to UKE1. The cell line; SET2, a representative of ET, showed the most reduction in the *WWOX* expression, suggesting a significant role of WWOX in the pathogenesis of ET. Both leukemic cell lines (NB4, and HL60) also presented similar results, highlighting a potential role of *WWOX* in leukemic transformation. In two independent previous studies, real-time PCR was performed and showed a reduction in *WWOX* levels in acute myeloid leukemia and acute lymphoblastic leukemia (Chen et al., 2013; Elbossaty et al., 2019).

Western blot results supported the real-time PCR data to some extent, as the protein levels of WWOX were relatively reduced when compared with MCF7. The cell line, SET2, once again recorded the least amount of expression. HL60 and KG1 revealed more decrease in the protein levels than the mRNA levels when compared to the other cell lines. This might be justified by post-transcription regulatory processes, that might have affected the final amount of expressed protein. Surprisingly, NB4 expressed the highest amount of protein among the 6 hematopoietic cell lines, when it had been the third with the least amount of mRNA. In fact, this is not the first type of tumor where WWOX is

downregulated. Moreover, the downregulation of WWOX in MPNs might enhance the established JAK2 function which leads to more prominent tumor progression, as crosstalk between WWOX and JAK2 pathway was reported before (Chang et al., 2018).

Further investigations that might highlight the role of WWOX in Ph-ve MPNs could lead to the development of a new therapeutic approach to relieve the patients from the symptoms and enhance the quality of their lives, as the restoration of the normal levels of WWOX protein might be promising. A recent study focusing on the ectopic expression of WWOX in an animal model of WOREE disease; a fatal neuronal disease characterized by loss of WWOX, alleviated the severity of the symptoms. (Repudi *et al.*, 2021)

Overall, our results support the main claim of this study, as the expression of WWOX is reduced on both the mRNA and the protein levels. Also, to our current knowledge, this marks the first research to study the alteration in WWOX expression in patients with Ph-ve MPN and hematopoietic cell lines. The causes behind these alterations could be further investigated in the future, prioritizing post-transcription regulation and crosstalks between WWOX and other known markers of Ph-ve MPNs like JAK2, CALR, and MPL.

References

Abdeen, S. K. and Aqeilan, R. I. (2019) 'Decoding the link between WWOX and p53 in aggressive breast cancer', *Cell Cycle*, 18(11), pp. 1177–1186. doi: 10.1080/15384101.2019.1616998.

Abdeen, S.K. *et al.* (2018) 'Somatic loss of WWOX is associated with TP53 perturbation in basal-like breast cancer', *Cell Death & Disease*, 9(8), p. 832. Available at: https://doi.org/10.1038/s41419-018-0896-z.

Abdel-Wahab, O. I. and Levine, R. L. (2009) 'Primary Myelofibrosis: Update on definition, pathogenesis, and treatment', *Annual Review of Medicine*, 60, pp. 233–245. doi: 10.1146/annurev.med.60.041707.160528.

Abdel-Wahab, O., Tefferi, A. and Levine, R. L. (2012) 'Role of TET2 and ASXL1 Mutations in the Pathogenesis of Myeloproliferative Neoplasms', *Hematology/Oncology Clinics of North America*, 26(5), pp. 1053–1064. doi: 10.1016/j.hoc.2012.07.006.

Abu-Odeh, M. *et al.* (2014) 'Characterizing WW domain interactions of tumor suppressor WWOX reveals its association with multiprotein networks', *Journal of Biological Chemistry*, 289(13), pp. 8865–8880. doi: 10.1074/jbc.M113.506790.

Abu-Remaileh, M. *et al.* (2018) 'WWOX controls hepatic HIF1α to suppress hepatocyte proliferation and neoplasia', *Cell Death and Disease*, 9(5), p. 511. doi: 10.1038/s41419-018-0510-4.

Accurso, V. *et al.* (2020) 'The Essential Thrombocythemia in 2020: What We Know and Where We Still Have to Dig Deep', *Clinical Medicine Insights: Blood Disorders*, 13, p. 2634853520978210. doi: 10.1177/2634853520978210.

Aldaz, C. M., Ferguson, B. W. and Abba, M. C. (2014) 'WWOX at the crossroads of cancer, metabolic syndrome related traits and CNS pathologies', *Biochimica et Biophysica Acta - Reviews on Cancer*, 1846(1), pp. 188–200. doi: 10.1016/j.bbcan.2014.06.001.

Alkhateeb, A.M. *et al.* (2016) 'Novel mutations in WWOX, RARS2, and C10orf2 genes in consanguineous Arab families with intellectual disability', *Metabolic Brain Disease*, 31(4), pp. 901–907. Available at: https://doi.org/10.1007/s11011-016- 9827-9.

Alvarez-Larrán, A. *et al.* (2012) 'Assessment and prognostic value of the European leukemiaNet criteria for clinicohematologic response, resistance, and intolerance to hydroxyurea in polycythemia vera', *Blood*, 119(6), pp. 1363–1369. doi: 10.1182/blood-2011-10-387787.

Antonioli, E. *et al.* (2005) 'Clinical implications of the JAK2 V617F mutation in essential thrombocythemia', *Leukemia*, 19(10), pp. 1847–1849. doi: 10.1038/sj.leu.2403902.

Aqeilan, R. I. *et al.* (2004) 'Physical and functional interactions between the Wwox tumor suppressor protein and the AP-2γ transcription factor', *Cancer Research*, 64(22), pp. 8256–8261. doi: 10.1158/0008-5472.CAN-04-2055.

Aqeilan, R. I., Donati, V., *et al.* (2007) 'Association of Wwox with ErbB4 in breast cancer', *Cancer Research*, 67(19), pp. 9330–9336. doi: 10.1158/0008- 5472.CAN-07-2147.

Aqeilan, R. I., Trapasso, F., *et al.* (2007) 'Targeted deletion of Wwox reveals a tumor suppressor function', *Proceedings of the National Academy of Sciences of the United States of America*, 104(10), pp. 3949–3954. doi: 10.1073/pnas.0609783104.

Aqeilan, R. I. *et al.* (2008) 'The WWOX tumor suppressor is essential for postnatal survival and normal bone metabolism', *Journal of Biological Chemistry*, 283(31), pp. 21629–21639. doi: 10.1074/jbc.M800855200.

Aqeilan, R. I. *et al.* (2009) 'Targeted ablation of the WW domain-containing oxidoreductase tumor suppressor leads to impaired steroidogenesis', *Endocrinology*, 150(3), pp. 1530–1535. doi: 10.1210/en.2008-1087.

Aqeilan, R. I. and Croce, C. M. (2007) 'WWOX in Biological Control and Tumorigenesis', *Journal Cellular Physiology*, 212(May), pp. 307–310. doi: 10.1002/JCP.

Araki, M. *et al.* (2016) 'Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms', *Blood*, 127(10), pp. 1307– 1316. doi: 10.1182/blood-2015-09-671172.

Arber, D. A. *et al.* (2016) 'The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia', *Blood*, 127(20), pp. 2391–2405. doi: 10.1182/blood-2016-03-643544.

Ballen, K. K. *et al.* (2010) 'Outcome of Transplantation for Myelofibrosis', *Biology of Blood and Marrow Transplantation*, 16(3), pp. 358–367. doi: 10.1016/j.bbmt.2009.10.025.

Barbui, T. *et al.* (2018) 'Philadelphia chromosome-negative classical myeloproliferative neoplasms: revised management recommendations from European LeukemiaNet', *Leukemia*, 32(5), pp. 1057–1069. doi: 10.1038/s41375-018- 0077-1.Philadelphia.

Baxter, E. J. *et al.* (2005) 'Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders', *The Lancet*, 365(9464), pp. 1054–1061. doi: 10.1016/s0140-6736(05)71142-9.

Bednarek, A. K. *et al.* (2000) 'WWOX, a novel WW domain-containing protein mapping to human chromosome 16q23.3-24.1, a region frequently affected in breast cancer', *Cancer Research*, 60(8), pp. 2140–2145.

Bednarek, A. K. *et al.* (2001) 'WWOX, the FRA16D gene, behaves as a suppressor of tumor growth', *Cancer Research*, 61(22), pp. 8068–8073.

Beer, P. A. *et al.* (2008) 'MPL mutations in myeloproliferative disorders: Analysis of the PT-1 cohort', *Blood*, 112(1), pp. 141–149. doi: 10.1182/blood-2008-01- 131664.

Beer, P. A. *et al.* (2010) 'Molecular mechanisms associated with leukemic transformation of MPL-mutant myeloproliferative neoplasms', *Haematologica*, 95(12), pp. 2153–2156. doi: 10.3324/haematol.2010.029306.

Billot, S. *et al.* (2011) 'Neurological disorders in essential thrombocythemia', *Haematologica*, 96(12), pp. 1866–1869. doi: 10.3324/haematol.2011.050005.

Boddu, P., Chihara, D., Masarova, L., Pemmaraju, N., Patel, K. P., & Verstovsek, S. (2018). The co-occurrence of driver mutations in chronic myeloproliferative neoplasms. Annals of hematology, 97(11), 2071–2080. https://doi.org/10.1007/s00277-018-3402-x

Brière J. B. (2007). Essential thrombocythemia. *Orphanet journal of rare diseases*, 2, 3. https://doi.org/10.1186/1750-1172-2-3

Cabagnols, X. *et al.* (2016) 'Presence of atypical thrombopoietin receptor (MPL) mutations in triple-negative essential thrombocythemia patients', *Blood*, 127(3), pp. 333–342. doi: 10.1182/blood-2015-07-661983.

Campbell, P. J. and Green, A. R. (2006) 'The myeloproliferative disorders', *The New Zealand medical journal*, 355(23), pp. 2452–2466. doi: 10.1177/003693306501000606.

Campregher, P. V. *et al.* (2012) 'Molecular biology of Philadelphia-negative myeloproliferative neoplasms', *Revista Brasileira de Hematologia e Hemoterapia*, 34(2), pp. 150–155. doi: 10.5581/1516-8484.20120035.

Cancemi, L. *et al.* (2011) 'Evidences that the polymorphism Pro-282-Ala within the tumor suppressor gene WWOX is a new risk factor for differentiated thyroid carcinoma', *International Journal of Cancer*, 129(12), pp. 2816–2824. Available at: https://doi.org/10.1002/ijc.25937.

Cervantes, F. (2011) 'Management of essential thrombocythemia', *Hematology Am Soc Hematol Educ Program*, 2011, pp. 215–221. doi: 10.3109/9781420061635-6.

Chang, N. S. *et al.* (2001) 'Hyaluronidase Induction of a WW Domain-containing Oxidoreductase that Enhances Tumor Necrosis Factor Cytotoxicity', *Journal of Biological Chemistry*, 276(5), pp. 3361–3370. doi: 10.1074/jbc.M007140200.

Chang, N. S. *et al.* (2003) 'Molecular mechanisms underlying WOX1 activation during apoptotic and stress responses', *Biochemical Pharmacology*, 66(8), pp. 1347– 1354. doi: 10.1016/S0006-2952(03)00484-2.

Chang, N. S. *et al.* (2005) 'WOX1 is essential for tumor necrosis factor-, UV light-, staurosporine-, and p53-mediated cell death, and its tyrosine 33-phosphorylated form binds and stabilizes serine 46-phosphorylated p53', *Journal of Biological Chemistry*, 280(52), pp. 43100–43108. doi: 10.1074/jbc.M505590200.

Chang, N. S., Doherty, J. and Ensign, A. (2003) 'JNK1 physically interacts with WW domain-containing oxidoreductase (WOX1) and inhibits WOX1-mediated apoptosis', *Journal of Biological Chemistry*, 278(11), pp. 9195–9202. doi: 10.1074/jbc.M208373200.

Chang, R. *et al.* (2018) 'Loss of Wwox drives metastasis in triple-negative breast cancer by JAK2/STAT3 axis', *Nature Communications*, 9(1), p. 3486. doi: 10.1038/s41467-018-05852-8.

Chen, X. *et al.* (2013) 'Gene expression of WWOX, FHIT and p73 in acute lymphoblastic leukemia', *Oncology Letters*, 6(4), pp. 963–969. doi: 10.3892/ol.2013.1514.

Cheng, H.-L. *et al.* (2016) 'Functional genetic variant in the Kozak sequence of WW domain-containing oxidoreductase (WWOX) gene is associated with oral cancer risk', *Oncotarget*, 7(43), pp. 69384–69396. Available at: https://doi.org/10.18632/oncotarget.12082.

Chung, E., Hsu, C.-L. and Kondo, M. (2011) 'Constitutive MAP Kinase Activation in Hematopoietic Stem Cells Induces a Myeloproliferative Disorder', *PLoS ONE*. Edited by D.T. Covas, 6(12), p. e28350. Available at: https://doi.org/10.1371/journal.pone.0028350.

Chuzi, S. and Stein, B. L. (2017) 'Essential thrombocythemia: a review of the clinical features, diagnostic challenges, and treatment modalities in the era of molecular discovery', *Leukemia and Lymphoma*, 58(12), pp. 2786–2798. doi: 10.1080/10428194.2017.1312371.

Coniat, M. Le *et al.* (1989) 'The human homolog of the myeloproliferative

virus maps to chromosome band 1p34', *Human Genetics*, 83(2), pp. 194–196. doi: 10.1007/BF00286717.

Dodington, D. W., Desai, H. R. and Woo, M. (2018) 'JAK/STAT – Emerging Players in Metabolism', *Trends in Endocrinology and Metabolism*, 29(1), pp. 55–65. doi: 10.1016/j.tem.2017.11.001.

Elbossaty, W. F., Malak, C. A. A. and Elghanam, D. (2019) 'High and low WWOX gene expression levels in acute myeloid leukemia - A real time RT-PCR study', *Clinical Laboratory*, 65(9), pp. 1607–1613. doi: 10.7754/Clin.Lab.2019.190119.

Elf, S. *et al.* (2016) 'Mutant calreticulin requires both its mutant C-terminus and the thrombopoietin receptor for oncogenic transformation', *Cancer Discov*, 6(4), pp. 368–381. doi: 10.1158/2159-8290.CD-15-1434.Mutant.

Fenaux, P. *et al.* (1990) 'Clinical course of essential thrombocythemia in 147 cases', *Cancer*, 66(3), pp. 549–556. doi: 10.1002/1097-0142(19900801)66:3<549::AID-CNCR2820660324>3.0.CO;2-6.

Gäbler, K., Behrmann, I. and Haan, C. (2013) 'JAK2 mutants (e.g., JAK2V617F) and their importance as drug targets in myeloproliferative neoplasms', *Jak-Stat*, 2(3), p. e25025. doi: 10.4161/jkst.25025.

Gangat, N. and Tefferi, A. (2020) 'Myelofibrosis biology and contemporary management', *British Journal of Haematology*, 191(2), pp. 152–170. doi: 10.1111/bjh.16576.

Gaudio, E. *et al.* (2006) 'Physical association with WWOX suppresses c-Jun transcriptional activity', *Cancer Research*, 66(24), pp. 11585–11589. doi: 10.1158/0008-5472.CAN-06-3376.

Gisslinger, H. *et al.* (2013) 'Anagrelide compared with hydroxyurea in WHO-classified essential thrombocythemia: The ANAHYDRET Study, a randomized controlled trial', *Blood*, 121(10), pp. 1720–1728. doi: 10.1182/blood-2012-07-443770.

Gourley, C. *et al.* (2005) 'WWOX mRNA expression profile in epithelial ovarian cancer supports the role of WWOX variant 1 as a tumour suppressor, although the role of variant 4 remains unclear.', *International journal of oncology*, 26(6), pp. 1681–1689. doi: 10.3892/ijo.26.6.1681.

Gourley, C. *et al.* (2009) 'WWOX gene expression abolishes ovarian cancer tumorigenicity in vivo and decreases attachment to fibronectin via integrin α3', *Cancer Research*, 69(11), pp. 4835–4842. doi: 10.1158/0008-5472.CAN-08- 2974.

Guo, W., Wang, G., *et al.* (2013) 'Decreased expression of WWOX in the development of esophageal squamous cell carcinoma: DEVELOPMENT OF ESCC', *Molecular Carcinogenesis*, 52(4), pp. 265–274. Available at: https://doi.org/10.1002/mc.21853.

Guo, W., Dong, Z., *et al.* (2013) 'Genetic and epigenetic alterations of *WWOX* in the development of gastric cardia adenocarcinoma', *Environmental and Molecular Mutagenesis*, 54(2), pp. 112–123. Available at: https://doi.org/10.1002/em.21748.

Harrison, C. N. *et al.* (2013) 'Health-related quality of life and symptoms in patients with myelofibrosis treated with ruxolitinib versus best available therapy', *British Journal of Haematology*, 162(2), pp. 229–239. doi: 10.1111/bjh.12375.

Hexner, E. O. *et al.* (2008) 'Lestaurtinib (CEP701) is a JAK2 inhibitor that suppresses JAK2/STAT5 signaling and the proliferation of primary erythroid cells from patients with myeloproliferative disorders', *Blood*, 111(12), pp. 5663–5671. doi: 10.1182/blood-2007-04-083402.

Hong, Q. *et al.* (2007) 'Zfra affects TNF-mediated cell death by interacting with death domain protein TRADD and negatively regulates the activation of NF-κB, JNK1, p53 and WOX1 during stress response', *BMC Molecular Biology*, 8, p. 50. doi: 10.1186/1471-2199-8-50.

Hu, B. S. *et al.* (2012) 'WWOX induces apoptosis and inhibits proliferation of human hepatoma cell line SMMC-7721', *World Journal of Gastroenterology*, 18(23), pp. 3020–3026. doi: 10.3748/wjg.v18.i23.3020.

Hu, C. Y. *et al.* (2017) 'Evodiamine Exerts an Anti-Hepatocellular Carcinoma Activity through a WWOX-Dependent Pathway', *Molecules (Basel, Switzerland)*, 22(7), p. 1175. doi: 10.3390/molecules22071175.

Huang, D. *et al.* (2013) 'The polymorphisms and haplotypes of WWOX gene are associated with the risk of lung cancer in southern and eastern chinese populations: *WWOX* GENETIC VARIANTS AND LUNG CANCER RISK', *Molecular Carcinogenesis*, 52(S1), pp. 19–27. Available at: https://doi.org/10.1002/mc.21934.

Hung, S.-C. *et al.* (2020) 'Functional genetic variant of WW domain containing oxidoreductase gene associated with urothelial cell carcinoma clinicopathologic characteristics and long-term survival', *Urologic Oncology: Seminars and Original Investigations*, 38(2), p. 41.e1-41.e9. Available at: https://doi.org/10.1016/j.urolonc.2019.07.016.

Iatan, I. *et al.* (2014) 'The WWOX gene modulates high-density lipoprotein and lipid metabolism', *Circulation: Cardiovascular Genetics*, 7(4), pp. 491–504. doi: 10.1161/CIRCGENETICS.113.000248.

Iurlo, A. *et al.* (2020) 'New perspectives on polycythemia vera: From diagnosis to therapy', *International Journal of Molecular Sciences*. MDPI AG, p. 5805. doi: 10.3390/ijms21165805.

Jacobson, P.A. *et al.* (2011) 'Genetic Determinants of Mycophenolate-Related Anemia and Leukopenia After Transplantation', *Transplantation*, 91(3), pp. 309–316. Available at: https://doi.org/10.1097/TP.0b013e318200e971.

Kisseleva, T. *et al.* (2002) 'Signaling through the JAK/STAT pathway, recent advances and future challenges', *Gene*, 285(1–2), pp. 1–24. doi: 10.1016/S0378- 1119(02)00398-0.

Klampfl, T. *et al.* (2013) 'Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms', *New England Journal of Medicine*, 369(25), pp. 2379–2390. doi: 10.1056/nejmoa1311347.

Kralovics, R. *et al.* (2005) 'A Gain-of-Function Mutation of JAK2 in Myeloproliferative Disorders', *N Engl J Med*, 352(17), pp. 1779–1790.

Krummel, K. A. *et al.* (2002) 'The common fragile site FRA16D and its associated gene WWOX are highly conserved in the mouse at Fra8E1', *Genes Chromosomes and Cancer*, 34(2), pp. 154–167. doi: 10.1002/gcc.10047.

Kurek, K. C. *et al.* (2010) 'Frequent attenuation of the WWOX tumor suppressor in osteosarcoma is associated with increased tumorigenicity and aberrant RUNX2 expression', *Cancer Research*, 70(13), pp. 5577–5586. doi: 10.1158/0008- 5472.CAN-09-4602.

Kuroki, T. *et al.* (2004) 'The Tumor Suppressor Gene WWOX at FRA16D Is Involved in Pancreatic Carcinogenesis', *Clinical Cancer Research*, 10(7), pp. 2459–2465. doi: 10.1158/1078-0432.CCR-03-0096.

Lan, C. *et al.* (2012) 'Aberrant expression of WWOX protein in epithelial ovarian cancer: A clinicopathologic and immunohistochemical study', *International Journal of Gynecological Pathology*, 31(2), pp. 125–132. doi: 10.1097/PGP.0b013e3182297fd2.

Lasho, T. L., Pardanani, A. and Tefferi, A. (2010) 'LNK Mutations in JAK2 Mutation– Negative Erythrocytosis ', *New England Journal of Medicine*, 363(12), pp. 1189–1190. doi: 10.1056/nejmc1006966.

Lee, H.-L. *et al.* (2017) 'Functional genetic variant of WW domain-containing

oxidoreductase (WWOX) gene is associated with hepatocellular carcinoma risk', *PLOS ONE*. Edited by S.-N. Lu, 12(4), p. e0176141. Available at: https://doi.org/10.1371/journal.pone.0176141.

Leibundgut, E. O. *et al.* (2021) 'Dynamics of mutations in patients with essential thrombocythemia treated with imetelstat', *Haematologica*, 106(9), pp. 2397– 2404. doi: 10.3324/haematol.2020.252817.

Levine, R. L. *et al.* (2005) 'Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis', *Cancer Cell*, 7(4), pp. 387–397. doi: 10.1016/j.ccr.2005.03.023.

Lin, J. T. *et al.* (2015) 'WWOX suppresses prostate cancer cell progression through cyclin D1-mediated cell cycle arrest in the G1 phase', *Cell Cycle*, 14(3), pp. 408–416. doi: 10.4161/15384101.2014.977103.

Lin, Y.-H. *et al.* (2018) 'Relationship of genetic variant distributions of WW domain- containing oxidoreductase gene with uterine cervical cancer', *International Journal of Medical Sciences*, 15(10), pp. 1005–1013. Available at: https://doi.org/10.7150/ijms.25553.

Liu, P. *et al.* (2017) 'Correlation between osteosarcoma and the expression of WWOX and p53', *Oncology Letters*, 14(4), pp. 4779–4783. doi: 10.3892/ol.2017.6747.

Liu, Y. *et al.* (2020) 'Methylation of WWOX gene promotes proliferation of osteosarcoma cells', *Journal of B.U.ON.*, 25(6), pp. 2708–2713.

Ludes-Meyers, J. H. *et al.* (2003) 'WWOX, the common chromosomal fragile site, FRA16D, cancer gene', *Cytogenetic and Genome Research*, 100(0), pp. 101– 110. doi: 10.1159/000072844.

Luís Vieira *et al.* (2011) 'Three-way translocation (X;20;16)(p11;q13;q23) in essential thrombocythemia implicates NFATC2 in dysregulation of CSF2 expression and megakaryocyte proliferation', *Cancer*, 396(January), pp. 389–396. doi: 10.1002/gcc.

Mahmud, M. A. Al *et al.* (2021) 'Cellular Expression and Subcellular Localization of Wwox Protein During Testicular Development and Spermatogenesis in Rats', *Journal of Histochemistry and Cytochemistry*, 69(4), pp. 257–270. doi: 10.1369/0022155421991629.

Marchioli, R. *et al.* (2005) 'Vascular and neoplastic risk in a large cohort of patients with polycythemia vera', *Journal of Clinical Oncology*, 23(10), pp. 2224–2232. doi: 10.1200/JCO.2005.07.062.

Del Mare, S. and Aqeilan, R. I. (2015) 'Tumor Suppressor WWOX inhibits osteosarcoma metastasis by modulating RUNX2 function', *Scientific Reports*, 5, p. 12959. doi: 10.1038/srep12959.

McBride, K.M. *et al.* (2019) 'Wwox Deletion in Mouse B Cells Leads to Genomic Instability, Neoplastic Transformation, and Monoclonal Gammopathies', *Frontiers in Oncology*, 9, p. 517. Available at: https://doi.org/10.3389/fonc.2019.00517.

McDonald, C. B. *et al.* (2012) 'Biophysical basis of the binding of WWOX tumor suppressor to WBP1 and WBP2 adaptors', *Journal of Molecular Biology*, 422(1), pp. 58–74. doi: 10.1016/j.jmb.2012.05.015.

Mesa, R. A. *et al.* (2009) 'The Myelofibrosis Symptom Assessment Form (MFSAF): An evidence-based brief inventory to measure quality of life and symptomatic response to treatment in myelofibrosis', *Leukemia Research*, 33(9), pp. 1199– 1203. doi: 10.1016/j.leukres.2009.01.035.

Michalak, M. *et al.* (2009) 'Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum', *Biochemical Journal*, 417(3), pp. 651–666. doi: 10.1042/BJ20081847.

Moulard, O. *et al.* (2014) 'Epidemiology of myelofibrosis, essential thrombocythemia, and polycythemia vera in the European Union', *European Journal of Haematology*, 92(4), pp. 289–297. doi: 10.1111/ejh.12256.

Nangalia, J. *et al.* (2013) 'Somatic CALR Mutations in Myeloproliferative Neoplasms with Nonmutated JAK2', *The New England journal of medicine*, 369(25), pp. 2391–2405. doi: 10.1056/NEJMoa1312542.Somatic.

Nunez, M. I. *et al.* (2005) 'Frequent loss of WWOX expression in breast cancer: Correlation with estrogen receptor status', *Breast Cancer Research and Treatment*, 89(2), pp. 99–105. doi: 10.1007/s10549-004-1474-x.

Paige, A. J. W. *et al.* (2001) 'WWOX: A candidate tumor suppressor gene involved in multiple tumor types', *Proceedings of the National Academy of Sciences of the United States of America*, 98(20), pp. 11417–11422. doi: 10.1073/pnas.191175898.

Paige, A. J. W. *et al.* (2010) 'WWOX tumour suppressor gene polymorphisms and ovarian cancer pathology and prognosis', *European Journal of Cancer*, 46(4), pp. 818–825. doi: 10.1016/j.ejca.2009.12.021.

Pardanani, A. *et al.* (2007) ' Extending Jak2 V617F and Mpl W515 Mutation Analysis to Single Hematopoietic Colonies and B and T Lymphocytes ',

Stem Cells, 25(9), pp. 2358–2362. doi: 10.1634/stemcells.2007-0175.

Pardanani, A. *et al.* (2011) 'Safety and efficacy of TG101348, a selective JAK2 inhibitor, in myelofibrosis', *Journal of Clinical Oncology*, 29(7), pp. 789–796. doi: 10.1200/JCO.2010.32.8021.

Pardanani, A. D. *et al.* (2006) 'MPL515 mutations in myeloproliferative and other myeloid disorders: A study of 1182 patients', *Blood*, 108(10), pp. 3472–3476. doi: 10.1182/blood-2006-04-018879.

Park, S. W. *et al.* (2004) 'Frequent downregulation and loss of WWOX gene expression in human hepatocellular carcinoma', *British Journal of Cancer*, 91(4), pp. 753– 759. doi: 10.1038/sj.bjc.6602023.

Passamonti, F. *et al.* (2008) 'Prognostic factors for thrombosis, myelofibrosis, and leukemia in essential thrombocythemia: A study of 605 patients', *Haematologica*, 93(11), pp. 1645–1651. doi: 10.3324/haematol.13346.

Persson, S., Rosenquist, M. and Sommarin, M. (2002) 'Identification of a novel calreticulin isoform (Crt2) in human and mouse', *Gene*, 297(1–2), pp. 151–158. doi: 10.1016/S0378-1119(02)00880-6.

Pikman, Y. *et al.* (2006) 'MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia', *PLoS Medicine*, 3(7), p. e270. doi: 10.1371/journal.pmed.0030270.

Płuciennik, E. *et al.* (2006) 'WWOX - The FRA16D cancer gene: Expression correlation with breast cancer progression and prognosis', *European Journal of Surgical Oncology*, 32(2), pp. 153–157. doi: 10.1016/j.ejso.2005.11.002.

Pospiech, K., Płuciennik, E. and Bednarek, A.K. (2018) 'WWOX Tumor Suppressor Gene in Breast Cancer, a Historical Perspective and Future Directions', *Frontiers in Oncology*, 8, p. 345. Available at: https://doi.org/10.3389/fonc.2018.00345.

Qin, H. R. *et al.* (2006) 'A role for the WWOX gene in prostate cancer', *Cancer Research*, 66(13), pp. 6477–6481. doi: 10.1158/0008-5472.CAN-06-0956.

Qin, H. R. *et al.* (2007) 'Wwox suppresses prostate cancer cell growth through modulation of ErbB2-mediated androgen receptor signaling', *Molecular Cancer Research*, 5(9), pp. 957–965. doi: 10.1158/1541-7786.MCR-07-0211.

Raedler, L. A. (2014) 'Diagnosis and Management of Polycythemia Vera', *Am Health Drug Benefits*, 7(7 suppl3), pp. S36–S47. doi: 10.1055/s-2006-939438.

Repudi, S. *et al.* (2021) 'Neonatal neuronal WWOX gene therapy rescues *Wwox* null phenotypes', *EMBO Molecular Medicine*, 13(12). Available at: https://doi.org/10.15252/emmm.202114599.

Richards, R. I. *et al.* (2015) 'WWOX, the chromosomal fragile site FRA16D spanning gene: Its role in metabolism and contribution to cancer', *Experimental Biology and Medicine*, 240(3), pp. 338–344. doi: 10.1177/1535370214565990.

Ried, K. *et al.* (2000) 'Common chromosomal fragile site FRA16D sequence: Identification of the FOR gene spanning FRA16D and homozygous deletions and translocation breakpoints in cancer cells', *Human Molecular Genetics*, 9(11), pp. 1651–1663. doi: 10.1093/hmg/9.11.1651.

Rumi, E. *et al.* (2014) 'Clinical effect of driver mutations of JAK2, CALR, or MPL in primary myelofibrosis', *Blood*, 124(7), pp. 1062–1069. doi: 10.1182/blood- 2014-05-578435.

Saharinen, P., Takaluoma, K. and Silvennoinen, O. (2000) 'Regulation of the Jak2 Tyrosine Kinase by Its Pseudokinase Domain', *Molecular and Cellular Biology*, 20(10), pp. 3387–3395. doi: 10.1128/mcb.20.10.3387-3395.2000.

Salah, Z., Aqeilan, R. and Huebner, K. (2010) 'WWOX gene and gene product: tumor suppression through specific protein interactions.', *Future oncology*, 6(2), pp. 249–259. doi: 10.2217/fon.09.152.

Sandberg, E. M. *et al.* (2004) 'Jak2 tyrosine kinase: a true jak of all trades?', *Cell Biochemistry and Biophysics*, 41(2), pp. 207–231.

Schnittger, S. *et al.* (2007) 'Detection of Three Different MPLW515 Mutations in',
Blood, 110(11), p. 2546. doi: 10.1182/blood.V110.11.2546.2546.

Scott, L. M. *et al.* (2007) ' JAK2 Exon 12 Mutations in Polycythemia Vera and Idiopathic Erythrocytosis ', *New England Journal of Medicine*, 356(5), pp. 459– 468. doi: 10.1056/nejmoa065202.

Scott, L. M. (2011) 'The JAK2 exon 12 mutations: A comprehensive review', *American Journal of Hematology*, 86(8), pp. 668–676. doi: 10.1002/ajh.22063.

Stein, B. L. *et al.* (2015) 'Polycythemia vera: An appraisal of the biology and management 10 years after the discovery of JAK2 V617F', *Journal of Clinical Oncology*, 33(33), pp. 3953–3960. doi: 10.1200/JCO.2015.61.6474.

Sudol, M. *et al.* (2005) 'WW or WoW: The WW domains in a union of bliss',

IUBMB Life, 57(12), pp. 773–778. doi: 10.1080/15216540500389039.

Tefferi, A. (2008) 'JAK and MPL mutations in myeloid malignancies', *Leukemia and Lymphoma*, 49(3), pp. 388–397. doi: 10.1080/10428190801895360.

Tefferi, A. (2010) 'Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1', *Leukemia*, 24(6), pp. 1128–1138. doi: 10.1038/leu.2010.69.

Tefferi, A. (2016) 'Primary myelofibrosis: 2017 update on diagnosis, risk-stratification, and management', *American Journal of Hematology*, 91(12), pp. 1262–1271. doi: 10.1002/ajh.24592.

Tefferi, A. and Barbui, T. (2019) 'Polycythemia vera and essential thrombocythemia: 2019 update on diagnosis, risk-stratification and management', *American Journal of Hematology*, 94(1), pp. 133–143. doi: 10.1002/ajh.25303.

Tefferi, A., Vannucchi, A. M. and Barbui, T. (2018) 'Essential thrombocythemia treatment algorithm 2018', *Blood Cancer Journal*, 8(1), p. 2. doi: 10.1038/s41408-017-0041-8.

Teofili, L. *et al.* (2010) 'Hereditary thrombocytosis caused by MPLSer505Asn is associated with a high thrombotic risk, splenomegaly and progression to bone marrow fibrosis', *Haematologica*, 95(1), pp. 65–70. doi: 10.3324/haematol.2009.007542.

Thiele, J. (2009) 'Philadelphia chromosome-negative chronic myeloproliferative disease', in *American Journal of Clinical Pathology*, pp. 261–280. doi: 10.1309/AJCPR8GINMBDG9YG.

Vannucchi, A. M. *et al.* (2008) 'Characteristics and clinical correlates of MPL 515W>L/K mutation in essential thrombocythemia', *Blood*, 112(3), pp. 844– 847. doi: 10.1182/blood-2008-01-135897.

Vannucchi, A. M. (2011) 'Management of myelofibrosis', *Hematology Am Soc Hematol Educ Program*, 2011, pp. 222–230. doi: 10.1159/000496889.

Vannucchi, A. M. *et al.* (2013) 'Mutations and prognosis in primary myelofibrosis',
Leukemia, 27(9), pp. 1861–1869. doi: 10.1038/leu.2013.119.

Verstovsek, S. *et al.* (2010) 'Safety and Efficacy of INCB018424, a JAK1 and JAK2 Inhibitor, in Myelofibrosis', *N Engl J Med*, 363(12), pp. 1117–1127. doi: 10.1038/leu.2013.71.

Vieira, L. *et al.* (2012) 'Three-way translocation (X;20;16)(p11;q13;q23) in essential thrombocythemia implicates *NFATC2* in dysregulation of *CSF2* expression and megakaryocyte proliferation', *Genes, Chromosomes and Cancer*, 51(12), pp. 1093–1108. Available at: https://doi.org/10.1002/gcc.21994.

Visani, G. *et al.* (2009) 'Identification of Novel Cryptic Chromosomal Abnormalities in Primary Myelofibrosis by Single-Nucleotide Polymorphism Oligonucleotide Microarray', *Blood*, 114(22), p. 1890.

Wang, X. *et al.* (2009) 'Association between CpG island methylation of the WWOX gene and its expression in breast cancers', *Tumor Biology*, 30(1), pp. 8–14. doi: 10.1159/000197911.

Wang, X. *et al.* (2016) 'Combining Telomerase Reverse Transcriptase Genetic Variant rs2736100 with Epidemiologic Factors in the Prediction of Lung Cancer Susceptibility', *Journal of Cancer*, 7(7), pp. 846–853. Available at: https://doi.org/10.7150/jca.13437.

Wen, J. *et al.* (2017) 'Decreased WWOX expression promotes angiogenesis in osteosarcoma', *Oncotarget*, 8(37), pp. 60917–60932. Available at: https://doi.org/10.18632/oncotarget.17126.

Wernig, G. *et al.* (2008) 'Efficacy of TG101348, a Selective JAK2 Inhibitor, in Treatment of a Murine Model of JAK2V617F-Induced Polycythemia Vera', *Cancer Cell*, 13(4), pp. 311–320. doi: 10.1016/j.ccr.2008.02.009.

Xiong, Z., Hu, S. and Wang, Z. (2010) 'Cloning of WWOX gene and its growth-inhibiting effects on ovarian cancer cells', *Journal of Huazhong University of Science and Technology - Medical Science*, 30(3), pp. 365–369. doi: 10.1007/s11596-010-0358-z.

Yan, H. *et al.* (2015) 'Effect of the WWOX gene on the regulation of the cell cycle and apoptosis in human ovarian cancer stem cells', *Molecular medicine reports*, 12(2), pp. 1783–1788. doi: 10.3892/mmr.2015.3640.

YAN, H. and SUN, J. (2013) 'Methylation status of WWOX gene promoter CpG islands in epithelial ovarian cancer and its clinical significance', *Biomedical Reports*, 1(3), pp. 375–378. doi: 10.3892/br.2013.86.

Yang, J. *et al.* (2010) 'Deletion of the WWOX gene and frequent loss of its protein expression in human osteosarcoma', *Cancer Letters*, 291(1), pp. 31–38. doi: 10.1016/j.canlet.2009.09.018.

Yang, J. *et al.* (2013) 'Correlation of WWOX, RUNX2 and VEGFA protein expression in human osteosarcoma', *BMC Medical Genomics*, 6, p. 56. doi: 10.1186/1755- 8794-6-56.

Yu, L. *et al.* (2019) 'The correlation of the expressions of WWOX , LGR5 and vasohibin-1 in epithelial ovarian cancer and their clinical significance', *Int J Clin Exp Pathol*, 12(1), pp. 327–336.

Zhu, Y. *et al.* (2016) 'Association of polymorphisms in WWOX gene with risk and outcome of osteosarcoma in a sample of the young Chinese population', *OncoTargets and Therapy*, p. 807. Available at: https://doi.org/10.2147/OTT.S99106.

Ziliotto, N. *et al.* (2019) 'C6orf10 Low-Frequency and Rare Variants in Italian Multiple Sclerosis Patients', *Frontiers in Genetics*, 10, p. 573. Available at: https://doi.org/10.3389/fgene.2019.00573.

APPENDIX: Consent Form

موافقة اشتراك في بحث علمي

فريق البحث. زيدون صلاح، ادلين صفية.
البحث: دراسة طفرات ونمط التعبير للجين ووكس في الأورام التكاثرية النخاعية.
مكان اجراء البحث: الجامعة العربية الامريكية.

المراجع الكريم:
انت مدعو للموافقة على اجراء فحوصات اضافية على عيناتك حيث تاتي هذه الفحوصات في اطار دراسة علمية يجريها فريقنا بهدف البحث العلمي، فنأمل من حضرتك الموافقة حيث نتعهد من طرفنا بضمان خصوصية المعلومات الطبية بشكل تام.

سنقوم نحن كفريق من الباحثين باخذ جزء من العينات المأخوذة منك لغرض التشخيص المطلوب من طبيبك واجراء فحوصات أخرى عليها بصورة موسعه على ان يتم ادراجها في الدراسة مع المحافظة التامة على الخصوصية وابقاء نتائج الفحوصات واسماء المشاركين طي الكتمان.

بامكانكم طلب اي ايضاحات او معلومات اضافية من الباحث الذي قد يتواصل مع طبيبكم لمعرفة السبب المباشر لطلب الفحوصات، تجدر الاشارة انه لا يترتب على مشاركتك في هذه الدراسة اي اثار جانبية او تكاليف للفحوصات التي سيتم اجراؤها.

ختاما، ان مشاركتك في البحث تسهم في فهم أفضل للعوامل الجينية التي تلعب دور في الأورام التكاثرية النخاعية والتي قد تفيد مستقبلا في اتخاذ الاجراء المناسب بخصوص كل حالة.

موافقة المشترك

لقد قرأت استمارة القبول هذه وفهمت مضمونها، وبعد الاجابة على كل الاستفسارات التي احتاج اليها. وعليه فإنني حرا مختارا اوافق على الاشتراك في البحث واستخدام العينات والنتائج لاغراض البحث العلمي

اسم المشترك : توقيع المشترك: التاريخ:

Abstract in Arabic

الملخص

تعرف أورام التكاثر النخاعي بأنها اضطرابات مرتبطة بانتشار غير منضبط لواحد من سلالات الخلايا المكونة للدم على الأقل. يتم تصنيفها إلى نوعين، هما؛ اضطرابات موجبة كروموسوم فيلادلفيا واضطرابات سلبية كروموسوم فيلادلفيا، حيث يفتقر الأخير إلى تغيرات وراثية واضحة ومحددة مرتبطة خصوصا به. مع ذلك، فلقد اكتشفت عدد من الطفرات التي ترتبط بنشأة هذا النوع من الاضطرابات، والتي تقع في الجينات؛ JAK2 و MPL و CALR. ولكن جميع هذه الطفرات تشارك في تشخيص مجموعة واسعة من اضطرابات أورام التكاثر النخاعي سلبية كروموسوم فيلادلفيا، بغض النظر عن اختلاف أعراضها كليا. يعرف البروتين WWOX بوظائفه في تنظيم موت الخلايا المبرمج، وقمع الأورام، وتنظيم عوامل النسخ. تم الإبلاغ سابقا عن تغيرات في التعبير الجيني لهذا البروتين في اضطراب كثرة الكريات الحمر الحقيقية واضطراب التليف النقي. بحثت هذه الدراسة في وجود عمليات حذف في إكسونات WWOX، اختلافات مفردة في سلسلة النيوكليوتيدات، وتغييرات في مستويات الحمض النووي الريبوزي المرسال والبروتين في كل من مرضى أورام التكاثر النخاعي سلبية كروموسوم فيلادلفي و/أو السلالات الخلوية المشتقة من الدم. لم تستطع الدراسة الكشف عن أي عمليات حذف كبيرة في الإكسونات التسعة لجين هذا البروتين. ولكن، تم إيجاد خمسة اختلافات مفردة في سلسلة النيوكليوتيدات. كما تمت ملاحظة نقصان واضح في مستويات الحمض النووي الريبوزي المرسال في كل من عينات المرضى والسلالات الخلوية المستخدمة. وقد لوحظ هذا أيضا في مستويات البروتين في السلالات الخلوية المشتقة من الدم. تمثل هذه الدراسة الأولى من نوعها حول التعبير الجيني

للـبروتين WWOX في المرضـى الـذين يعـانون من أورام التكـاثر النخـاعي سـلبية كروموسـوم فيلادلفيـا. وقـد يمثـل الانخفـاض الملاحـظ في التعبـير الجيـني لهـذا الـبروتين علامـة جديـدة في تشـخيص أورام التكـاثر النخـاعي سـلبية كروموسـوم فيلادلفيا.