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STUDY ON THE REGULATORY ROLES OF DEUBIQUITINASE GENES AND SOME SIGNALLING MOLECULES IN PATIENTS WITH POLYCYTHEMIA VERA

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INTRODUCTION

1. The necessity of research

Polycythemia vera is one of the malignant myeloproliferative neoplasms (MPNs) without the occurrence of Philadelphia chromosome segment, characterized by abnormal changes of hematopoietic bone marrow stem cells, causing an abnormal proliferation of blood cells such as white blood cells, red blood cells, and platelets, especially an increase in the number of red blood cells. Abnormally high levels of red blood cells lead to a decrease in the rate of circulation in the blood vessels, increasing the risk of blood clots. Many studies have shown that the V617F mutation of the *JAK2* gene (rs77375493) is responsible for the majority of cases of the disease and causes increased phosphorylation of *JAK2*.

Peripheral blood mononuclear cells (PBMCs) include many different types of immune cells such as lymphocytes, NK cells, dendritic cells, and monocytes. PBMC cells have a highly specialized function, playing a vital role in the immune system to help the body fight against foreign agents and keep the body healthy. Scientists have now studied and used dendritic cell immunotherapy (DCs) for the treatment of a number of cancers such as bile duct cancer, pancreatic cancer, ovarian cancer and lung cancer as certain success has been achieved.

The deubiquitinase (DUB) family of genes including the genes *A20*, *Otubain-1*, *CYLD*, and *Cezanne* plays an important role in deproteinization by cleaving their polyubiquitin chains. The role of the DUB gene group is diverse, in addition to its reduction function, it also participates in the regulation of tumor growth and plays an significant role in hematopoiesis, erythropoiesis and related abnormalities. Mice inactivating the *A20* and *CYLD* genes increase the expression of molecular signals such as NF-κB and STAT. Immunological studies have shown that the *A20* and *CYLD* genes are involved in blocking the inflammatory response and infiltration of leukocytes to the site of inflammation, thus they are implicated in the development of cancers. The study used PBMC peripheral blood mononuclear cells from healthy blood donors and patients with polycythemia vera to determine expression/polymorphism of DUB gene group, immune phenotype and some related molecular signals along with determining the function of some genes such as *Otubain-1* gene and *A20* gene involved in regulating PBMC cell activity, thereby contributing to a better understanding of the influence of the DUB gene group are metioned above that applied in combination of diagnosis and treatment of PV and some other blood malignancies.

In this study, we showed that PV patients had inactivated DUB genes including *A20*, *CYLD* and *Cezeanne* genes and elevated levels of cytokines IL6, TNF-α as well as increased number of $CD25^{+}$ CD4 T, Th1 and regulatory T cells in circulating blood. Genetic analysis of the *CYLD* gene identified 11 SNPs, including the previously unknown SNP W736G, which belongs to exon 15 and is highly pathogenic. The results of this study contribute significantly to the understanding of altered immune cell composition in the circulating blood of patients with PV, especially that elevated regulatory T cell counts are detrimental to the response immunity of patient. Changes in the composition of immune cell types, may result from the influence of functional genes that regulate immune cell activity such as *A20*, *CYLD* and *Cezanne* genes, when their expression is lower than normal. These genes are involved in suppressing inflammatory responses and suppressing immune cell overactivity. Thus, the expression of these genes are inactivated may be one of the reasons for the increased number of CD4 T, Th1 and T regulatory cells in the circulating blood of patients. Based on the results of this study, we have a scientific basis for further studies on the regulation mechanism of DUB genes, especially the *CYLD* gene, to the immune system activity of PV patients, from that could lead to applied research in the treatment of PV patients in combination with targeted gene therapy.

2. Research objective

1. Determining the role of *A20* gene and *Otubain-1* gene in regulating PBMC biological functions including cellular maturation, differentiation and apoptosis.
2. Determining expression levels/polymorphisms of some deubiquitinase and related signaling genes and immunophenotypes in PV patients and healthy people.

3. Research content

Study subjects were 77 patients with PV and 55 healthy individuals, using real-time PCR to determine gene expression levels of DUB group including (*A20, Otubain-1, Otubain-2, CYLD, Cezanne*), immune control genes (*Klotho, LAG3, CTLA4* and *PD1*) and related molecular signaling genes *SHP-1, SHP-2, IkB-α, STAT-1, STAT-3, STAT-5, STAT- 6*. Some DUB genes are identified with abnormal expression were re-examined by Sanger sequencing to identify gene polymorphisms/variants in PV patients. Immunophenotypic analysis of PV patients using flow cytometry and ELISA.

PBMC cells were isolated by Ficoll density centrifugation from 150ml blood samples of donors. Treating PBMC cells with siRNA-*A20* and siRNA-*Otubain-1* to inactivate two genes *A20* and *Otubain-1*, thereby determining the role of these two genes in regulating biological functions of PBMC cells including maturation, differentiation, cytokine secretion, migration and apoptosis and related molecular signals. Cells after treatment were extracted the RNA and transfered to cDNA, and then examined the expression level of related molecular signaling genes including (*SHP-1*, *SHP-2*, *IkB-α*, *STAT-1*, *STAT-3*, *STAT-5*, *STAT-6*). The suspension obtained was used to check the levels of cytokines by ELISA method. Physiological activity of PBMC cells changed under the influence of *A20* and *Otubain-1* gene inactivation was analyzed using flow cytometry.

CHAPTER 1: LITERATURE OVERVIEW

1.1 Introduction of polycytemia vera

Polycythaemia vera- PV is a blood cancer of the Myeloproliferative neoplasms (MPNs) group, characterized by the overgrowth of mature and immature myeloid cells. The excessive proliferation of myeloid cells leads to an excessive proliferation of blood cells including: red blood cells, white blood cells, and platelets. According to the World Health Organization (WHO), myeloproliferative diseases are classified into 6 diseases. PV is the most common myeloproliferative disease characterized by an abnormal increase in the number of red blood cells.

1.2 Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs are a diverse mixture of immune cells with highly specialized functions. PBMC plays a major role in the body's immune system or is an indispensable component in the body's defenses. In another way, PBMCs are round nucleated blood cells that are a heterogeneous population of lymphocytes (T cells, B cells and NK cells) with different numbers, dendritic cells (TBTs) and mononuclear cells depending on the different individual.

1.3. Several deubiquitinase proteins and the relationship with molecular signaling

The A20 protein or TNFAIP3 is a protein encoded by the *A20* gene and acts as a downregulator of the NF-κB molecule. This protein is encoded by the *A20* gene, and it is also a gene with a high rate of inactivation in B-cell lymphomas. Inactivation or deletion of the *A20* gene has been implicated in several malignancies, leading to cell proliferation in chronic T-lymphoid leukemia, primary mediastinal B-cell lymphoma, and classical Hodgkin lymphoma.

Otubain-1 protein is an ovarian tumor protein recently identified as a deubiquitating enzyme (DUBs) by cleaving the ubiquitin chain from TRAFs. Otubain-1 plays a vital role in cancer’s initiation and development. A growing body of literature demonstrates that Otubain-1 upregulates multiple cancer-related

signaling pathways including MAPK, ERa, EMT, RHOa, mTORC1, FOXM1 and P53, thereby promoting survival, proliferation, invasion and treatment resistance of cancer cells.

The CYLD protein encoded by the *CYLD* gene is located at position 16q12-13 on chromosome 16. CYLD has been identified as the main cause of cystic fibrosis. In addition, it is also associated with common benign tumors in the neck or many other locations. CYLD protein is a DUB of the USP family of proteins with tumor suppressor function in colon cancer, lung cancer, malignant tumor, and multiple myeloma. CYLD has been studied in acute T-lymphoid leukemia.

The Cezeanne protein is a DUB with a homologous sequence to A20, which has a role in upregulating the NF-κB signaling pathway. Activation of transcription factor NF-κB by cytokines such as TNF-α relies on ubiquitous alterations of receptor-interacting proteins including RIP1, leading to activation of downstream kinases. Overexpression of *Cezanne* results in a reduction of RIP secretion, however, this inhibitory effect of *Cezanne* is attributed to its nonspecific activity against Lys48 and Lys63 bindings.

CHAPTER 2: MATERIAL AND METHODS

2.1 Study subjects

The study subjects were 77 patients with confirmed diagnosis of PV and 55 clinically and subclinical healthy people at the National Institute of Hematology and Blood Transfusion and Military Hospital 103, Hanoi with average age is 58.7. PBMCs were isolated from the peripheral blood of volunteers in a sample volume of 150 ml.

2.2 Experimental diagram

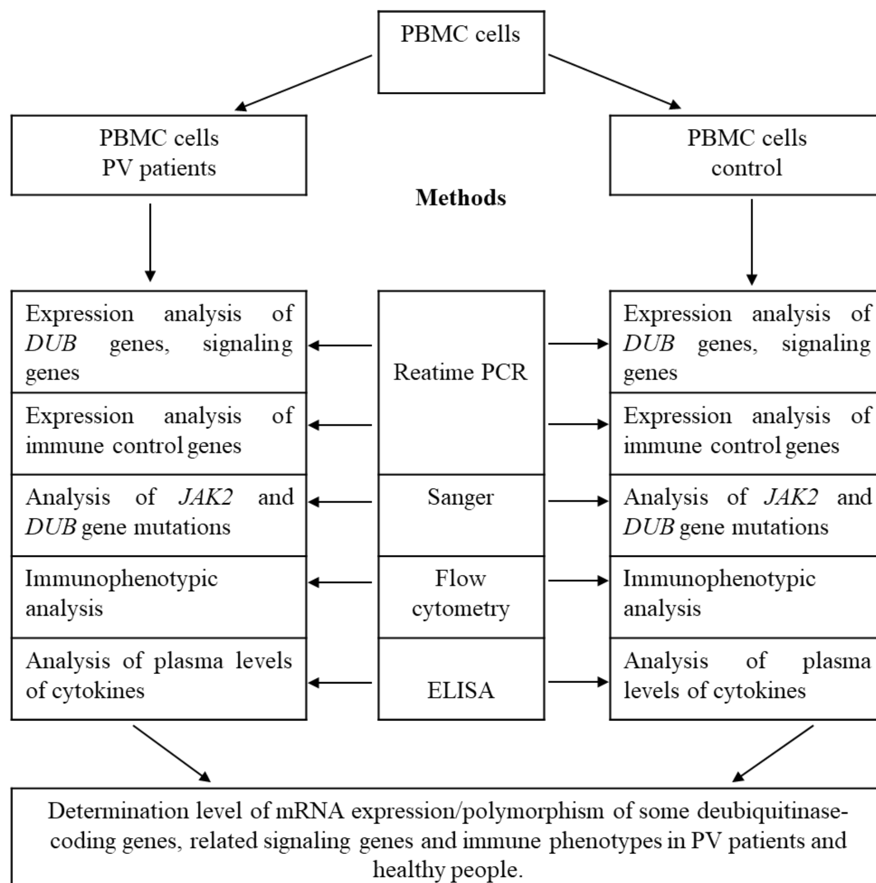


Figure 2.1. Diagram of the first experiment - PBMC cells were used to determine the role of several genes

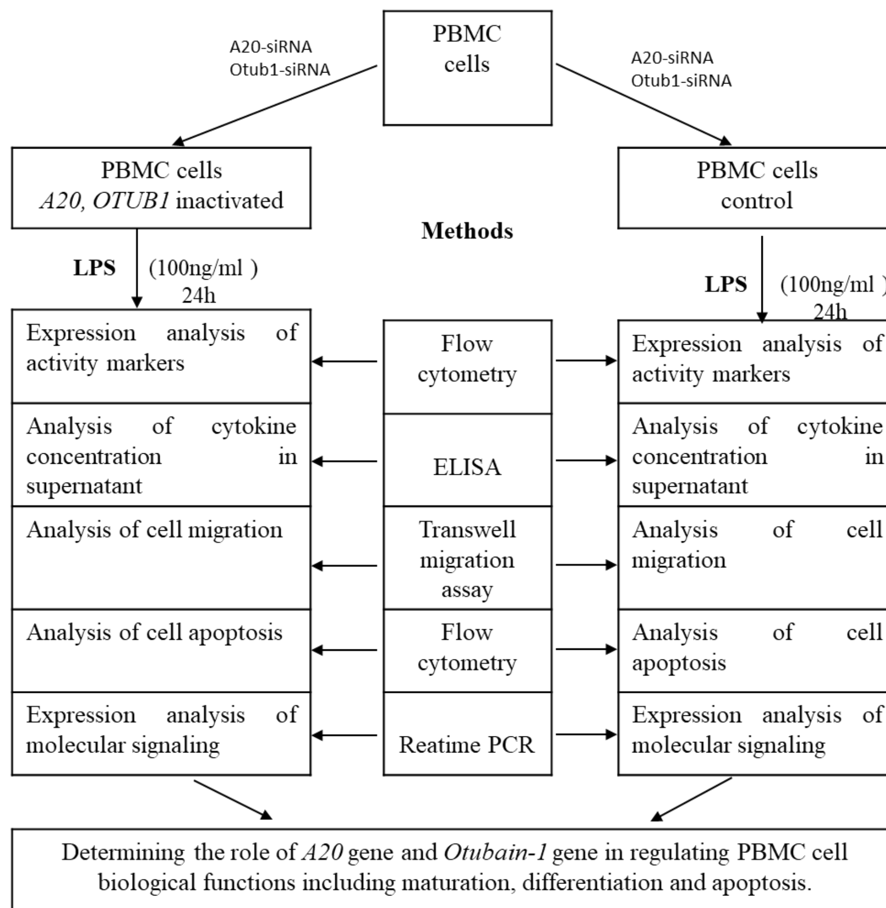


Figure 2.2. Diagram of the second experiment - PBMC cells cultured and exposed to LPS

2.3 Methodologies

2.3.1 Blood biochemistry test

2.3.1. Plasma separation

2.2.2 Total DNA and RNA extraction

2.2.3 PCR

2.2.4 Sanger sequencing and Data analysis

2.3.6. cDNA synthesis

2.3.7. Real-Time PCR

2.3.8 Bioinformatics methods used in the thesis

2.3.9. Insertion of siRNA into peripheral blood mononuclear cells

2.3.10. Isolation and culture of peripheral blood mononuclear cells

2.3.11 Flow cytometry

2.3.13 ELISA

2.3.14 Ethics Committee in Biomedical Research

CHAPTER 3. RESULTS

3.1. Research results on patients with polycythaemia vera

3.1.1 Blood biochemical index of patients with polycythaemia vera

The results of blood biochemical tests showed that the proportions of PV patients with uric acid index, indirect bilirubin, total protein, globulin and LDH were higher than healthy people (Table 3.1). High levels of these biological indicators indicate a high degree of influence of the disease on the damage of kidney, liver and some other organs. Elevated levels of LDH lead to increased blood lactate levels and tissue

damage. In addition, other biochemical indexes such as urea, glucose, ferritin, AST and ALT also have some patients with these indicators higher than normal.

Table 3.1: Percentage of PV patients with higher than normal blood biochemical test index

No.	Disease index	Patient (n)/Total Patient (77)	% Patient	Average value	Normal value
1	Ure (mmol/l)	12/77	**15,58**	7,5 ± 1,67	3,3-6,6
2	Glucose (mmol/l)	8/77	**10,38**	5,36 ± 0,7	3,9-5,6
3	Creatinin (µmol/l)	0/77	0	79,8 ± 20,5	50-110
4	Axid uric (µmol/l)	23/77	**29,87**	336,8 ± 112,2	< 420
5	Bilirubin Total (µmol/l)	10/77	**12,99**	14,15 ± 4,8	0-21
6	Bilirunbin Direct (µmol/l)	0/77	0	2,4 ± 1,2	0-5
7	Bilirubin Indirect (µmol/l)	19/77	**24,68**	11,75 ± 4,3	0-17
8	Protein Total (g/l)	35/77	**45,45**	80,8 ± 3,9	60-80
9	Albumin (g/l)	0/77	0	43,8 ± 2,48	35-50
10	Globulin (g/l)	19/77	**24,68**	37 ± 4	20-35
11	A/G Index	0/77	0	1,87 ± 2,7	>1
12	Ferritin	8/77	**10,38**	255 ± 230,69	10-300
13	Calci ion (mmol/l)	0/77	0	1,19 ± 0,07	1,7-2,5
14	AST (GOT) U/l-37°C	8/77	**10,38**	27,65 ± 6,8	0-40
15	ALT (GPT) U/l-37°C	16/77	**20,78**	29,3 ± 23,2	0-40
16	LDH U/l-37°C	46/77	**59,74**	600,4 ± 238,1	0-240
17	Canxi TP (mmol/l)	0/77	0	2,35 ± 0,12	1,7-2,5
18	Quantification of serum iron (µmol/l)	8/77	**10,38**	15,47 ± 6,7	5,5-25
19	Na+ (mmol/l)	0/77	0	140,11 ± 1,57	135-145
20	K+(mmol/l)	0/77	0	3,91 ± 0,34	3,5-5,0
21	Cl- (mmol/l)	0/77	0	104,08 ± 2,41	95-110

3.1.2 Analysis of gene expression and immunophenotypic profiles in PV patients

For expression of the DUB genes, we observed that mRNA levels of *A20*, *Cezanne* and *CYLD* were significantly downregulated in PV cells compared to the control groups; however, no difference in transcript expression of other DUB genes including *OTUB-1* and *OTUB-2,* between patient and control groups was detected (Figure 3.1B). The inactivated expression of *A20, CYLD* and *Cezanne* is predicted to be caused by their genetic alterations in PV.

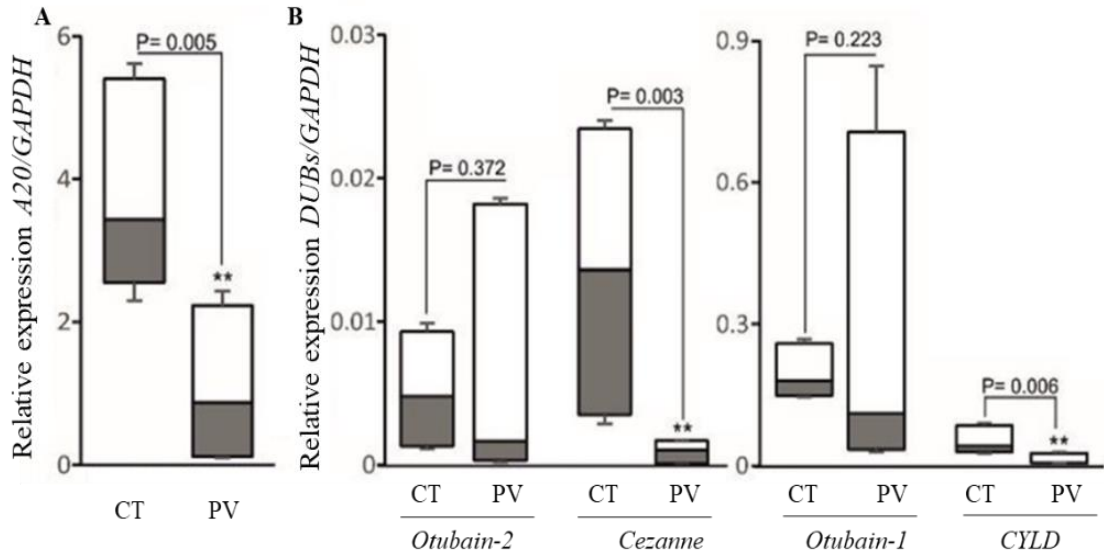


Figure 3.1. Gene expression in polycythaemia vera (PV) patients and controls.

A. Box plot graphs of transcript levels of *A20*,

B. Box plot graphs of transcript levels of *CYLD, OTUB1, OTUB2*, and *Cezanne* are shown for control and PV cells; ** ($p < 0.01$) indicates significant difference from healthy individuals (Mann–Whitney U test, $n = 15$). The box plots denote the median, interquartile range (IQR) and minimum and maximum values;

3.1.3 Expression levels of some signaling genes in patients with polycythaemia vera

Expression levels of genes involved in STAT molecular signaling including *STAT1, STAT3, STAT5* genes were all highly expressed in PV patients compared with control samples, reflecting that the *STAT* gene is especially important role in regulating the pathogenesis of PV. This is the first study to show increased expression of 3 genes *STAT1, STAT3, STAT5* in PV patients.

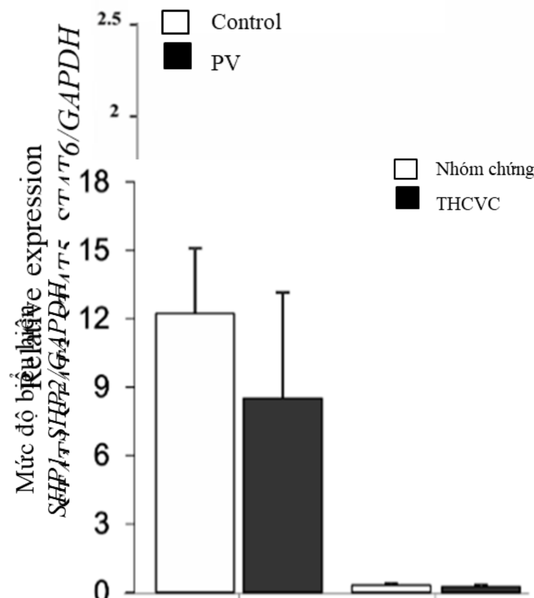


Figure 3.2. Expression levels of the molecular signaling genes STAT

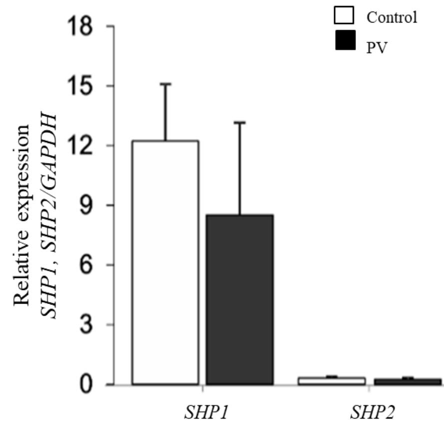


Figure 3.3. Expression of SHP1 and SHP2 genes in PV patients

Besides immunoregulatory genes, the *SH2* domain containing protein tyrosine phosphatase (*SHP*) gene family also plays an important role in the direct regulation of cellular physiological processes. Analysis of *SHP-1* and *SHP-2* gene expression associated with tyrosine phosphatase signaling activity showed that expression of the *SHP-2* gene was unchanged compared with healthy individuals, on the other hand, *SHP1* had a reduced expression compared with healthy subjects control sample. This shows that the expression of PV disease compared with leukemia is almost different in terms of mutations in some functional genes and the expression of some genes related to immune cell activity.

3.1.4 Expression of some immune control genes in patients with polycythaemia vera

The results of testing the expression levels of some genes involved in the regulation of T-cell activity including *LAG3, CTLA-4* and *PD-1* genes showed that these genes all had lower expression levels. The expression of *LAG3* was significantly lower than the control sample (Figure 3.4), indicating that the affected immune system of PV patients are less related to the immunoregulatory activity of the 3 genes *LAG3, CTLA-4* and *PD-1*.

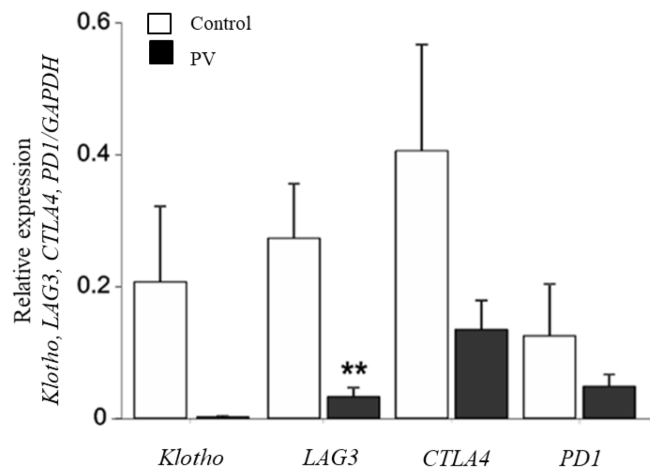


Figure 3.4: Expression of Klotho, LAG3, CTLA4, PD1 genes in PV patients

3.1.5 Analysis of JAK2 mutation in patients with polycythaemia vera

3.1.5.1 ***Mutational analysis of JAK2 gene in PV patients***

In this study, we investigated genotype frequency of this mutation to determine the correlation among mutations in the *JAK2*. We observed that 51/77 (66.23%) untreated PV cases were positive with $JAK2^{V617F}$

(Figure 3.5). The JAK2^{V617F} mutation occurs due to a missense mutation at nucleotide position 1849 on the cDNA sequence that converts Guanine to Thymine. Sanger sequencing results from purified PCR products containing mutations showed clear, sharp sequencing signals, no noise or foreign signals. The results can be used to easily distinguish homozygous GG, heterozygous GT or homozygous TT genotypes without causing confusion (Table 3.2; 3.3).

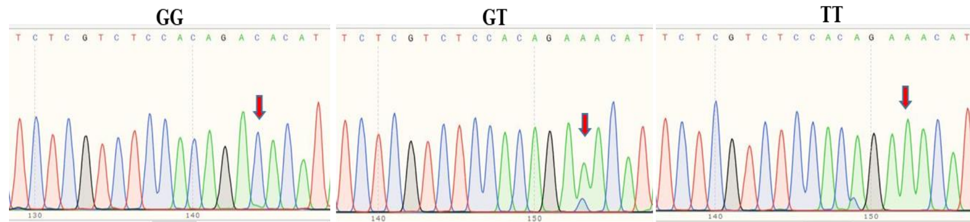


Figure 3.5. Partial sequence chromatograms of JAK2 gene from healthy individuals

Table 3.2. General information on single nucleotide polymorphisms (SNPs) of JAK2 gene in polycythaemia vera (PV) patients and controls

Gen/SNP	SNP ID GenBank	Type of variant	Allele	MAF in PV group	HWE in PV group (*p*)	MAF In control group	HWE In control group (*p*)	HWE In all population(*p*)
JAK2/rs77375493	9:5073770	Missense	G>A	0.442	0.656	0	NaN	0.051

Table 3.3. Comparison of genotype frequencies of JAK2 gene between polycythaemia vera (PV) patients and controls SNP Gene Test model Controls (n = 55) PV patients (n = 77) p-value

SNP	Gene	Test model	Controls (n=55)	PV patients (n=77)	*P- value*
rs77375493	*JAK2*	GG	55 (100%)	26 (33.76%)	**<0.001(2)**
		GT/TT	0 (0%)	51 (66.24%)	

3.1.5.2 In-silico prediction results of JAK2 gene

Analysis results using software such as PredictSNP, PhD-SNP, Polyphen-2, SIFT, SNAP all predict that JAK2^{V617F} mutation has a high ability to change the 3D structure of the protein, thereby reducing the activity of the corresponding enzyme (deleterious). In addition, the amino acid position 617 (Valine) in the *JAK2* gene also shows great conservatism between different animals, suggesting that this amino acid sequence region plays an important role in structure and function of Janus kinase 2 (Figure 3.6.).

Species							JAK2 V617		
Homo sapiens (Human)	V	L	N	Y	G	V	C	**V**	C	D
Monodelphis domestica (Gray short-tailed opossum)	V	L	N	Y	G	V	C	**V**	C	E
Rattus norvegicus (Rat)	V	L	N	Y	G	V	C	**V**	C	E
Myotis lucifugus (Little brown bat)	V	L	N	Y	G	V	C	**V**	C	E
Meleagris gallopavo (Wild turkey)	V	L	N	Y	G	V	C	**V**	C	E
Loxodonata Africana (African elephant)	V	L	N	Y	G	V	C	**V**	C	E
Gallus gallus (Chicken)	V	L	N	Y	G	V	C	**V**	C	E
Xenopus tropicalis (Western clawed)	I	L	N	Y	G	V	C	**V**	C	E
Tetraodon nigroviridis (Spotted green pufferfish)	V	L	T	Y	G	I	C	**V**	C	D

Figure 3.6. Comparison of amino acid sequences at the JAK2^{V617F} position between different species

3.1.6 Analysis of CYLD gene mutations in patients with polycythaemia vera

3.1.6.1 CYLD gene mutations in patients with polycythaemia vera

Sequencing of the *CYLD* gene identified 6 nucleotide changes in exon 15 (Figure 3.7 A), in which 5 out of 6 SNPs including p.A705P, including p.A705P (c.2355 G > C), p.Q731H (c.2435 G) > C), p.E735K (c.2445 G > A), p.W736G (c.2448 T > G) and p.E747K (c.2481 G > A) were missense, resulting in amino acid substitutions and a remaining SNP p.E723E (c.2411G> A) was silent. Next, 5 intronic nucleotide changes including 2 SNPs c.2351-118delA and c.2351-31 T>G in intron 14 and 3 SNPs c.2483+6 T>G, c. 2483+39 T>G and c. 2483+53 G>A in intron 15 were found (Fig. 3.7 B).

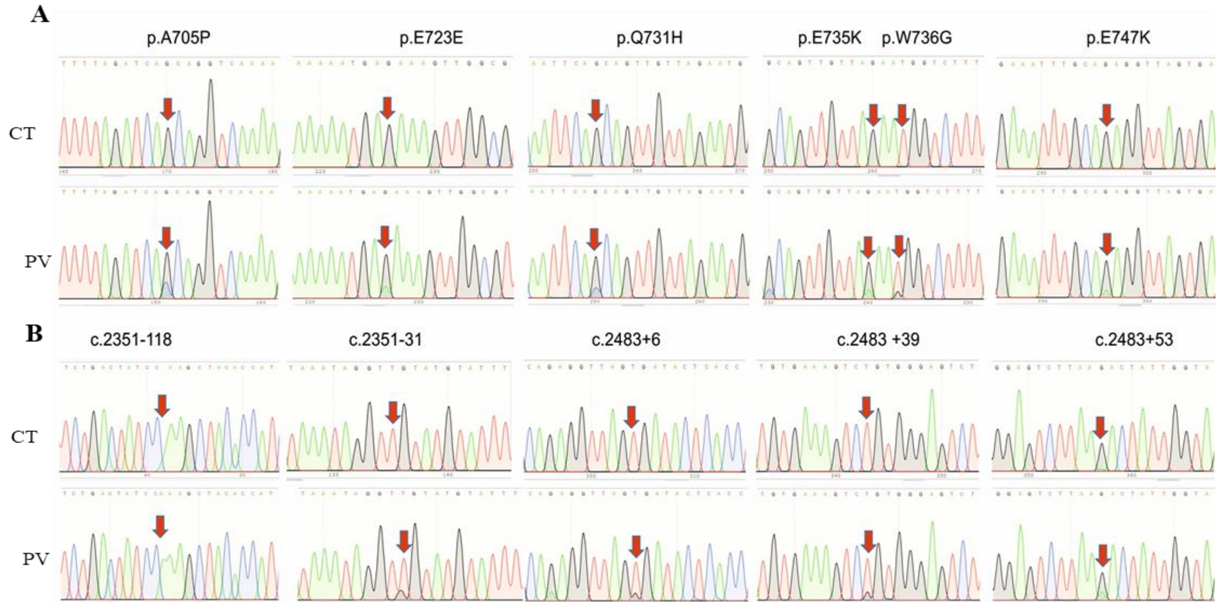


Figure 3.7. Polymorphism scores of the CYLD gene in PV patients and controls.

Among the 11 SNPs, the genotype distributions of all, except for the 2 p.E747K and c.2483+53 G>A SNPs were in agreement with Hardy-Weinberg equilibrium (HWE) ($p > 0.05$). The minor allele frequency (MAF) of the p.W736G variant was significantly higher in the PV group as compared to control group ($p = 0.022$) and the difference in the MAFs for the 10 remaining SNPs between two groups was not found (Table 3.4).

Table 3.4. General information on single nucleotide polymorphisms (SNPs) of CYLD gene in polycythaemia vera (PV) patients and controls

Gen/SNP	SNP ID GenBank	Type of variant	Allele	MAF in PV group	HWE in PV group (*p*)	MAF in control group	HWE in control group (*p*)	HWE in all population (*p*)
CYLD/ c.2351-118	16:50791440	Intron	Del A	0	0.993	**0**	0.913	0.938
CYLD/c.2351-31	16:50791527	Intron	T>G	0.006	0.998	0	NaN	0.999
CYLD/c.2355 p.A705P	16:50791562	Exon	G>C	0.02	0.985	0	NaN	0.991
CYLD/c.2411 p.E723E	16:50791618	Exon	G>A	0.033	0.958	0.055	0.913	0.883
CYLD/c.2435 p.Q731H	16:50791642	Exon	G>C	0.111	0.553	0.137	0.504	0.285
CYLD/ c.2445 p.E735K	16:50791652	Exon	G>A	0.13	0.424	0.173	0.302	0.138
CYLD/c.2448 p.W736G	16:50791655	Exon	T>G	0.091	0.681	0.01	0.998	0.787
CYLD/c.2481 p.E747K	16:50791688	Exon	G>A	0.169	0.204	0.219	0.118	0.027
CYLD/ c.2483+6	16:50791696	Intron	T>G	0.026	0.973	0	NaN	0.985
CYLD/ c.2483 +39	16:50791729	Intron	T>G	0.039	0.939	0.01	0.998	0.952
CYLD/c.2483+53	16:50791743	Intron	G>A	0.182	0.149	0.219	0.118	0.019

For determination of susceptibility to PV by evaluating the deleterious effect of the nsSNPs in CYLD gene, the results indicated that among the 5 nsSNPs, only the W736G nsSNP was predicted to be probably damaging by PolyPhen-2 [230] with score of 0.9456 (score range: 0–1; sensitivity: 0.8; specificity: 0.95) (Fig. 2D) (Figure 3.8). Therefore, variant p.W736G may be one of the non-synonymous SNPs present in the *CYLD* gene.

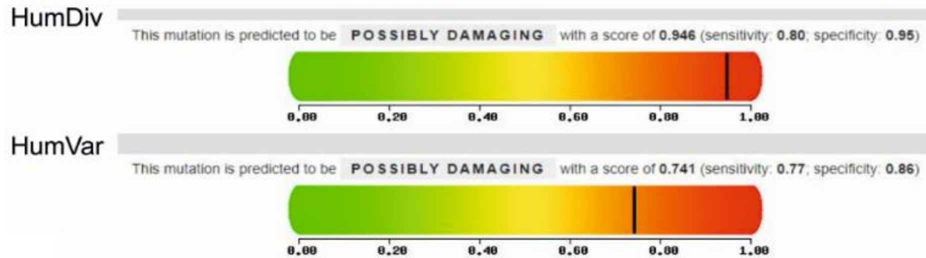


Figure 3.8. Prediction of the p.W736G variant function using the program Polyphen-2

For the p.W736G mutation, the TG genotype was detected in 14 patients and 01 healthy individual (18.2%) than in healthy subjects (1.81%; p = 0.018, Table 3.5), whereas, 10 other SNPs in the *CYLD* gene were not significantly associated with the PV phenotype. Evidence suggests that the p.W736G mutation in the *CYLD* gene may be the mutation caused PV.

Table 3.5 Comparison of genotype frequencies of CYLD gene between polycythaemia vera (PV) patients and controls

SNP	Gene	Test model	Controls (n=55)	PV patients (n=77)	*P- value*
c.2351-118	*CYLD*	AA	49 (89.09%)	75(97.4%)	
		DelA	6 (10.91 %)	2 (2.6%)	0.052(1)
c.2351-31	*CYLD*	TT	55 (100%)	76 (98.7%)	
		TG	0	1 (1.3%)	1(1)
c.2355 p.A705P	*CYLD*	GG	55 (100%)	74 (96.1%)	
		GC	0 (0%)	3 (3.9%)	0.121(1)
c.2411 p.E723E	*CYLD*	GG	49 (89.09%)	72 (93.5%)	
		GA	6 (10.91 %)	5 (6.5%)	0.335(1)
c.2435 p.Q731H	*CYLD*	GG	40 (72.73%)	60 (77.9%)	
		GC	15 (27.27%)	17 (22.1%)	0.511(1)
c.2445 p.E735K	*CYLD*	GG	36 (65.46%)	57 (44%)	
		GA	19 (34.54%)	20 (56%)	0.055(1)
c.2448 p.W736G	*CYLD*	TT	54 (98.19%)	63 (81.8%)	
		TG	1 (1.81%)	14 (18.2%)	**<0.001(1)**
c.2481 p.E747K	*CYLD*	GG	31 (56.36%)	51 (66.2%)	
		GA	24 (43.64%)	26 (33.8%)	0.147(2)
c.2483+6	*CYLD*	TT	55 (100%)	73 (94.8%)	
		TG	0 (0%)	4 (5.2%)	0.059(1)
c.2483 +39	*CYLD*	TT	54 (98.19%)	71(92.2%)	
		TG	1 (1.81%)	6 (7.8%)	0.101(1)
c.2483+53	*CYLD*	GG	31 (56.37%)	49 (63.6%)	
		GA	24 (43.63%)	28 (36.4%)	0.248(2)

3.1.6.2 In-silico prediction results of CYLD gene

For the analysis of the possible impact of the 5 intronic SNPs in CYLD gene on splicing, only the SNP c. 2483+6 T>G was predicted as an aberrant splicing according to the SD-Score prediction program (Table 3.6 A). Besides, the MaxEntScan32 splicing prediction through the score analysis of MaxENT (Maximum Entropy Model), MDD (Maximum Dependence Decomposition Model), MM (First-order Markov Model),

and WMM (Weight Matrix Model) indicated that the mutant scores of the SNP c.2483+6 T>G were lower than the wild-type scores (Table 3.6 B) suggesting that the SNP c.2483+6 T>G may be a splice-donor-site mutation.

Table 3.6. The PV pathogenic effect of SNP c.2483 + 6 T > G. Predicted by the software program SD-Score (A) or MaxEntScan (B).

A

Wt.Seq					Mt.Seq				Difference			
pos	Wt.Seq (9bases)	SD-Score	Ri	CV	Mt.Seq (9bases)	SD-Score	Ri	CV	ΔSD-Score	ΔRi	ΔCV	Prediction
4	GAGGTTAGT	-3.08	7.568	0.87	GAGGTTAGG	-4.163	6.274	0.823	-1.087	-1.294	-0.049	Aberrant

B

Genotype	Sequence	MaxENT	MDD	MM	WMM
Wild type	gagGTTAGT	7.15	12.78	6.8	6.92
Mutant	gagGTTAGG	4.36	8.98	5.11	5.99

Further analysis of an alignment of CYLD protein using the PolyPhen-2 software showed that the p.W736 residue is a highly conserved site among different species, including humans *(Homo sapiens),* rat (Rattus norvegicus), gray shorttailed opossum *(Monodelphis domestica)*, Tasmanian devil (Sarcophilus harrisii), duckbill platypus *(Ornithorhynchus anatinus)*, little brown bat *(Myotis lucifugus),* chicken *(Gallus gallus)*, wild turkey *(Meleagris gallopavo)*, western clawed frog *(Xenopus tropicalis)*, nile tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), three-spined stickleback (*Gasterosteus aculeatus*), african elephant (*Loxodonta africana*), and spotted green pufferfish (*Tetraodon nigroviridis*) (Figure 3.9).

Species					p.W 736						
Homo sapiens (Human)	Q	L	L	E	**W**	S	F	I	N	S	N
Rattus norvegicus (Rat)	Q	L	L	E	**W**	S	F	I	N	S	N
Monodelphis domestica (Gray short-tailed opossum)	Q	L	L	E	**W**	S	F	I	N	S	N
Sarcophilus harrisii (Tasmanian devil)	Q	L	L	E	**W**	S	F	I	N	S	N
rnithorhynchus anatinus (Duckbill platypus)	Q	L	L	E	**W**	S	F	I	N	S	S
Myotis lucifugus (Little brown bat)	Q	L	L	E	**W**	S	F	I	N	S	N
Gallus gallus (Chicken)	Q	L	L	E	**W**	S	F	I	N	S	N
Meleagris gallopavo (Wild turkey)	Q	L	L	E	**W**	S	F	I	N	S	N
Xenopus tropicalis (Western clawed frog)	Q	L	L	E	**W**	S	F	I	N	S	S
Oreochromis niloticus (Nile tilapia)	Q	L	L	E	**W**	S	F	I	N	S	D
Danio rerio (Zebrafish)	Q	L	L	E	**W**	S	F	I	N	S	D
Gasterosteus aculeatus (Three-spined stickleback)	Q	L	L	E	**W**	S	F	I	N	S	D
Loxodonta africana (African elephant)	Q	L	L	E	**W**	S	F	I	N	S	N
Tetraodon nigroviridis (Spotted green pufferfish)	Q	L	L	E	**W**	S	F	I	N	S	D

Figure 3.9. Comparison of amino acid sequences at position p.W736 in CYLD protein

3.1.7 Analysis of A20 gene mutation in patients with polycythaemia vera and healthy people

Next, the sequencing of *A20* gene identified 3 nucleotide changes, including rs776591390 G>T, rs141376366 G>A and rs745670694 G>A in exon 7 (Figure 3.10). The genotype distributions of the 3 SNPs in this gene were in accordance with HWE ($p > 0.05$) (Table 3.8). The MAF for the SNP rs776591390 was slightly higher, whereas the MAFs for the 2 remaining SNPs were lower in PV patients than in the control group. Among the 3 SNPs, the missense SNP rs776591390 of A20 gene was identified in 1 out of 77 PV

patients (1.81%) and the 2 remaining SNPs rs141376366 and rs745670694 were found only in control individuals with the carrier frequencies of 1.81% and 3.63%, respectively (Table 3.7).

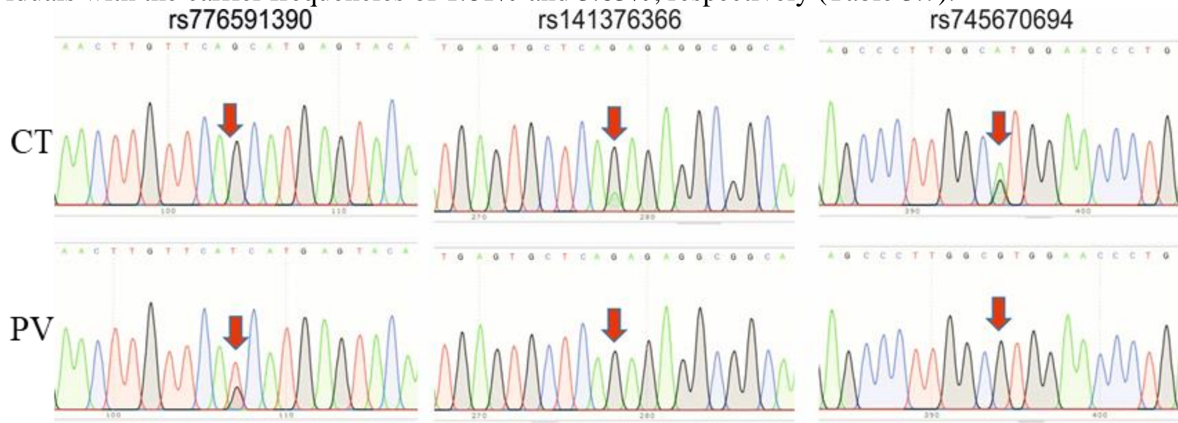


Figure 3.10. Polymorphism of A20 in patients with PV and control group

Table 3.7. General information on single nucleotide polymorphisms (SNPs) of A20 gene in polycythaemia vera (PV) patients and controls

SNP	Gene	Test model	Controls (n=55)	PV patients (n=77)	*P- value*
rs776591390	*A20*	GG	55 (100%)	76 (98.71%)	
		TG	0	1 (1.29%)	1(1)
rs141376366	*A20*	GG	54 (98.19%)	77 (100%)	
		GA	1 (1.81%)	0	0.497(1)
rs745670694	*A20*	GG	53 (96.37%)	77 (100%)	
		AG	2 (3.63%)	0 (0%)	0.121(1)

Table 3.8. Comparison of genotype frequencies of A20 gene between polycythaemia vera (PV) patients and controls SNP Gene Test model Controls (n = 55) PV patients (n = 77) p-value

Gen/SNP	SNP ID GenBank	Type of variant	Allele	MAF in PV group	HWE in PV group (*p*)	MAF in control group	HWE in control group (*p*)	HWE In all population (*p*)
A20/ rs776591390	6:137878495	Missense	G>T	0.006	0.998	0	NaN	0.999
A20/ rs141376366	6:137878670	Exon	G>A	0	NaN	0.01	0.998	0.999
A20/ rs745670694	6:137878786	Synonymous	G>A	0	NaN	0.108	0.991	0.996

The results showed that there was no association between the above variants with PV disease (p> 0.05).

3.1.8 Analysis of Cezanne gene mutations in patients with polycythaemia vera and healthy people

Finally, the sequencing of *Cezanne* gene identified 7 nucleotide changes in intron 10, 3 out of the 7 intronic SNPs (c.1584-287 C>G, c.1584-167 G>T and c.1584122 G>T) were unidentified SNPs and the 4 remaining intronic SNPs (rs1168285629 A>G, rs1394369937 A>G, rs1158787149 C>G, and rs1553772411 C>G) are reported in NCBI SNP database (Figure 3.11).

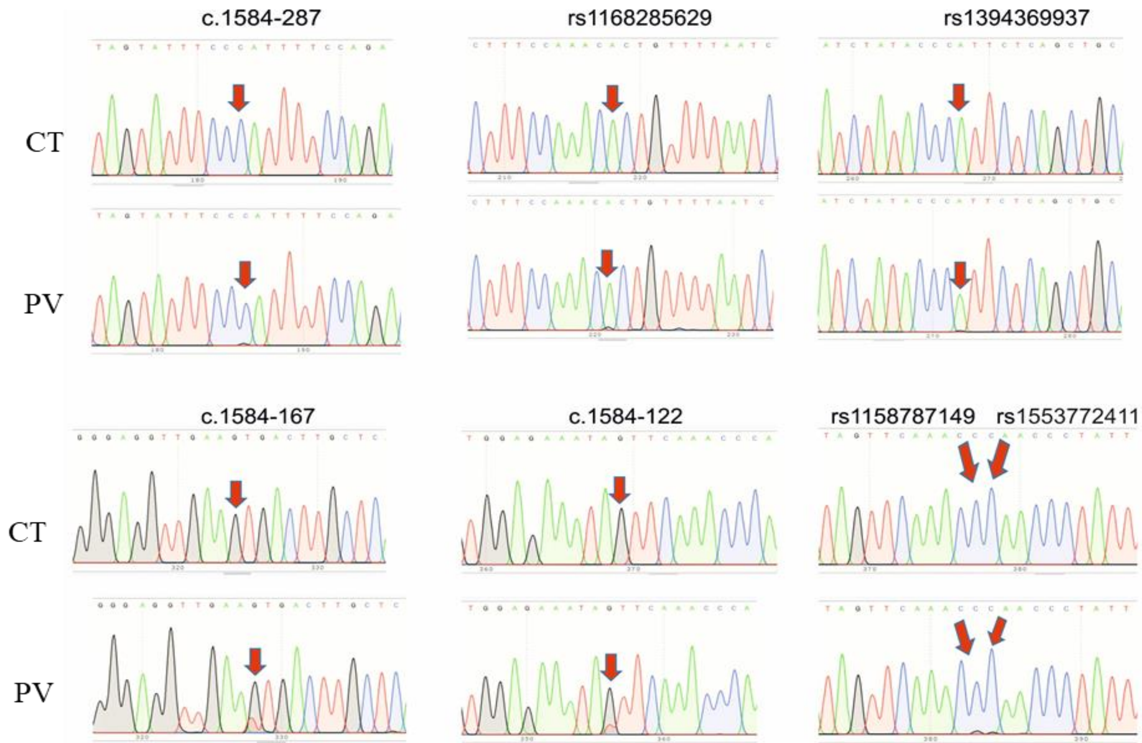


Figure 3.11. Cezanne polymorphism in PV patients and controls

The genotype distributions of the 7 SNPs in *Cezanne* gene were in accordance with HWE ($p > 0.05$) (Table 3.9). The MAFs of the 7 intronic SNPs were slightly higher in the PV group than in healthy individuals. Among these SNPs, 5 out the 7 intronic SNPs (c.1584-287 C>G, rs1394369937 A>G, c.1584-167 G>T, rs1158787149 C>G and rs1553772411 C>G) appeared in PV patients, but not in the control group, with the carrier frequencies of 7.79%, 5.19%, 2.6%, 5.19%, and 3.89%, respectively (Table 16). Importantly, 7 out of 77 (9.09%) PV patients carried at least 2 SNPs in *Cezanne* gene.

Table 3.9. General information on single nucleotide polymorphisms (SNPs) of Cezeanne gene in polycythaemia vera (PV) patients and controls

Gen/SNP	SNP ID GenBank	Type of variant	Allele	MAF in PV group	HWE in PV group (*p*)	MAF in control group	HWE in control group (*p*)	HWE In all population (*p*)
Cezanne/c.1584-287	1:149947622	Intron	C>G	0.039	0.998	0	NaN	0.965
Cezanne/ rs1168285629	1:149947587	Intron	A>G	0.046	0.916	0.019	0.991	0.921
Cezanne/rs1394369937	1:149947537	Intron	A>G	0.026	0.973	0	NaN	0.985
Cezanne/c.1584-167	1:149947502	Intron	G>T	0.013	0.993	0	NaN	0.996
Cezanne/c.1584-122	1:149947457	Intron	G>T	0.026	0.973	0.010	0.998	0.976
Cezanne/rs1158787149	1:149947449	Intron	C>G	0.026	0.987	0	NaN	0.985
Cezanne/rs1553772411	1:149947450	Intron	C>G	0.019	0.993	0	NaN	0.991

In addition, to determine the association between mutations in the *CYLD* and *Cezanne* genes, we also observed that PV samples carrying the p.W736G and/or c.2483+6 variant in the *CYLD* gene had no variants in the *Cezanne* gene.

Table 3.10. Comparison of genotype frequencies of Cezanne gene between polycythaemia vera (PV) patients and controls SNP Gene Test model Controls (n = 55) PV patients (n = 77) p-value

SNP	Gene	Test model	Controls (n=55)	PV patients (n=77)	*P- value*
c.1584-287	*Cezanne*	CC	55 (100%)	71 (92.21%)	
		CG	0 (0%)	6 (7.79%)	**0.007(1)**
rs1168285629	*Cezanne*	AA	53 (96.36%)	70 (90.91%)	
		AG	2 (3.64%)	7 (9.09%)	0.251(1)
rs1394369937	*Cezanne*	AA	55 (100%)	73 (94.81%)	
		AG	0 (0%)	4 (5.19%)	0.059(1)
c.1584-167	*Cezanne*	GG	55 (100%)	75 (97.4%)	
		GT	0 (0%)	2 (2.60%)	0.246(1)
c.1584-122	*Cezanne*	GG	54 (98.18%)	73 (94.81%)	
		GT	1 (1.82%)	4 (5.19%)	0.445(1)
rs1158787149	*Cezanne*	CC	55 (100%)	73 (94.81%)	
		CG	0 (0%)	4 (5.19%)	0.059(1)
rs1553772411	*Cezanne*	CC	55 (100%)	74 (96.11%)	
		CG	0 (0%)	3 (3.89%)	0.121(1)

For the 7 variants tested in the table above, based on the odds ratio and confidence interval, the p-value showed no association between the above variants with PV disease ($p > 0.05$) (Table 3.10).

For determination of the correlation among the SNPs in *JAK2* and the DUB genes, we observed that 10 out of 14 (71.4%) PV cases carrying the W736G nsSNP and 3 out of 3 (100%) carriers of the intronic SNP c. 2483+6 T>G in *CYLD* gene were positive for the JAK2^{V617F} mutation (data not shown), whereas, the JAK2^{V617F}-positive rate of PV carriers of the other SNPs in the DUB genes were similar to healthy individuals (data not shown). The evidence indicated that there was a significant positive correlation between PV carriers of the W736G nsSNP or/and the intronic SNP c.2483+6 T>G and PV patients with JAK2^{V617F} mutation. Interestingly, out of the 14 patients carrying the W736G nsSNP in *CYLD* gene, 13 (92.8%) cases were found to carry the SNP p.E747K; 11 (78.6%) cases had both the SNP p.Q731H G>C and the SNP c.2483+53 G>A; 9 (64.3%) cases were found to carry the SNP p.E735K; 3 (21.4%) carriers of the SNP c.2483+6 T>G and 5 (35.7%) cases infected with the SNP c.2483+39 T>G were observed (Figure 3.12). According to the Kruskal–Wallis test results, significant relevance was observed among the SNPs in *CYLD* gene ($\chi 2=7.364$, $p = 0.007$), suggesting that PV carriers of the W736G nsSNP had multiple SNPs in *CYLD* gene. Additionally, we observed that PV carriers of the W736G nsSNP and the intronic SNP c.2483+6 T>G in *CYLD* gene did not have SNP in *A20* or *Cezanne* gene (Figure 3.12).

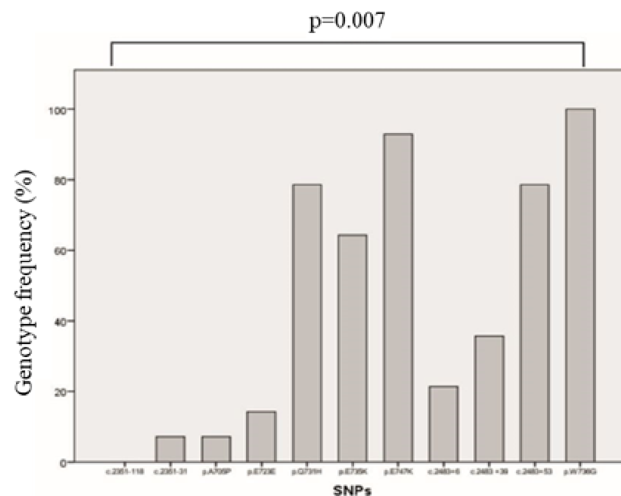


Figure 3.12. The relevance of polycythaemia vera (PV) carriers of the W736G nsSNP and the other 9 SNPs in CYLD gene.

3.1.9 Immune phenotype in patients with polycythaemia vera

In our results, $CD45^+$ cells considered as leukocytes were gated in all experiments. Flow cytometry analysis revealed an increased percentage of $CD11b^+$ cells in the circulating blood of PV patients (Figure 3.13A, 3.14A). Activation of $CD3^+CD4^+$ T cells was enhanced in PV patients (Figure 3.13A). The number of $CD3^+CD4^+CD44^+$ cells did not appear to be increased in PV patients and controls (Figure 3.13 B, 3.14 B).

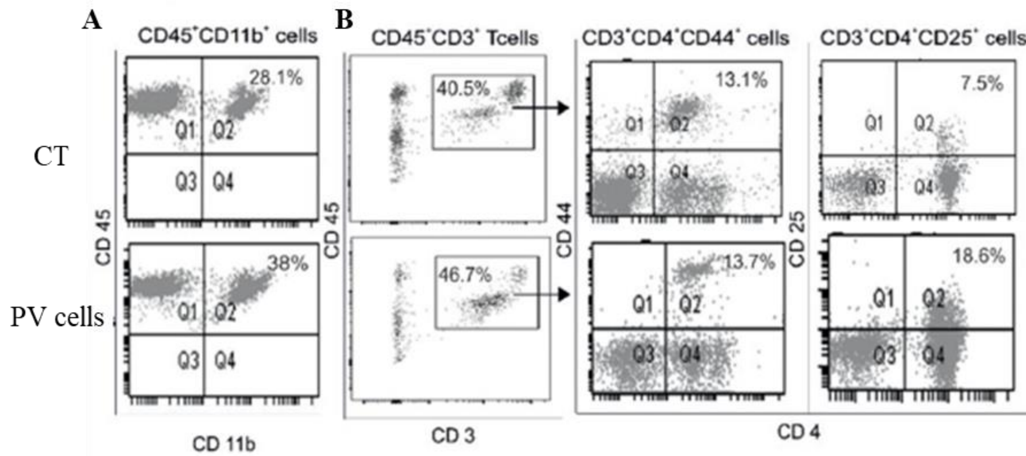


Figure 3.13. Original dot plots of $CD45^+CD11b^+$ (A), $CD45^+CD3^+$, $CD3^+CD4^+CD44^+$ and $CD3^+CD4^+CD25^+$ (B) expressing cells are shown for control (upper panels) and PV cells (lower panels). All samples were gated with $CD45^+$ live cells;

However, activation of $CD11b^+$ myeloid cells in PV cases was found to be similar to that in healthy subjects, as the number of cells expressing $CD11b^+CD86^+$ and $CD11b^+CD40^+$ remained unchanged in these patients (Figure 3.14A).

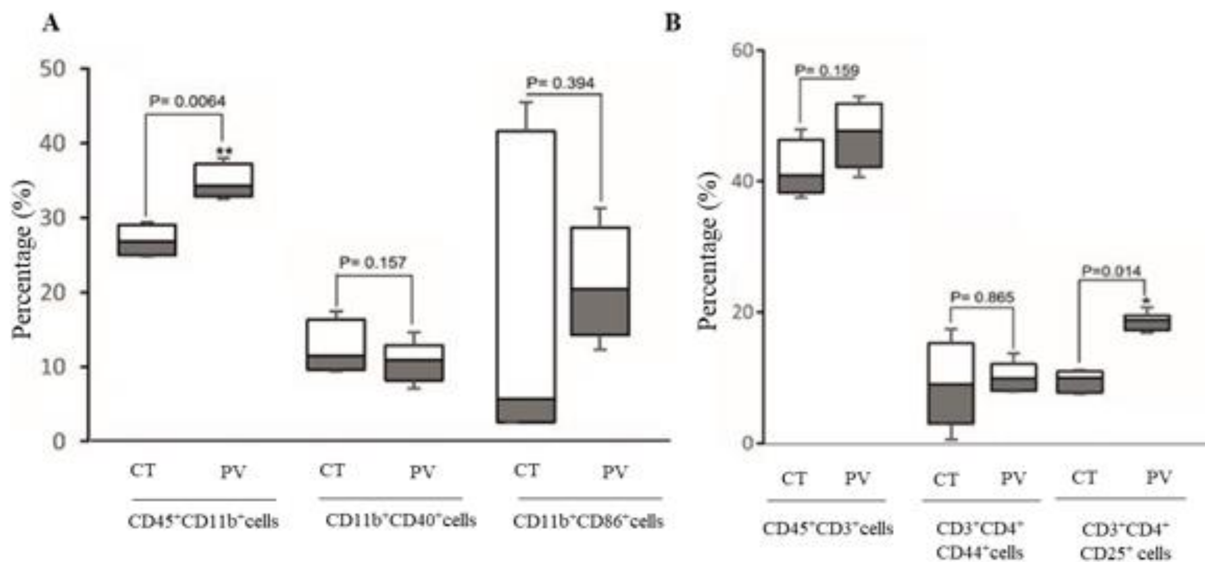


Figure 3.14. Expression plot of the percentage of cells in PV patients compared with control samples

Next, analysis of CD4 T cells subsets showed that the $CD3^+CD4^+CD25^+FoxP3^+$ (Treg) and $CD3^+CD4^+IFN\text{-}\gamma^+$ (Th1) expressing cells were recruited into the circulation, whereas the number of $CD3^+CD4^+IL\text{-}4^+$ (Th2) and $CD3^+CD4^+IL\text{-}17^+$ (Th17) expressing cells was unaltered in PV cases (Figure 3.15). In agreement with a previous study, Treg cells are recruited to circulatory system to facilitate the development of PV,26 suggesting that the inactivated expression of *A20, CYLD* and *Cezanne* genes in PV patients could be involved in the recruitment of $CD25^+$ CD4 T, T regulatory and Th1 cells into blood.

In addition, the number of cells expressing $CD3^+CD4^+IL\text{-}4^+$ (Th2) in PV patients was 0.9% compared with 0.6% in the control sample. The amount of $CD3^+CD4^+IL\text{-}17^+$ (Th17) cells in the circulatory system of

PV patients was 1.2% while that in the controls was 0.8% (Figure 3.15, 3.16). The results indicated that there was no major change of these two cell types in PV patients.

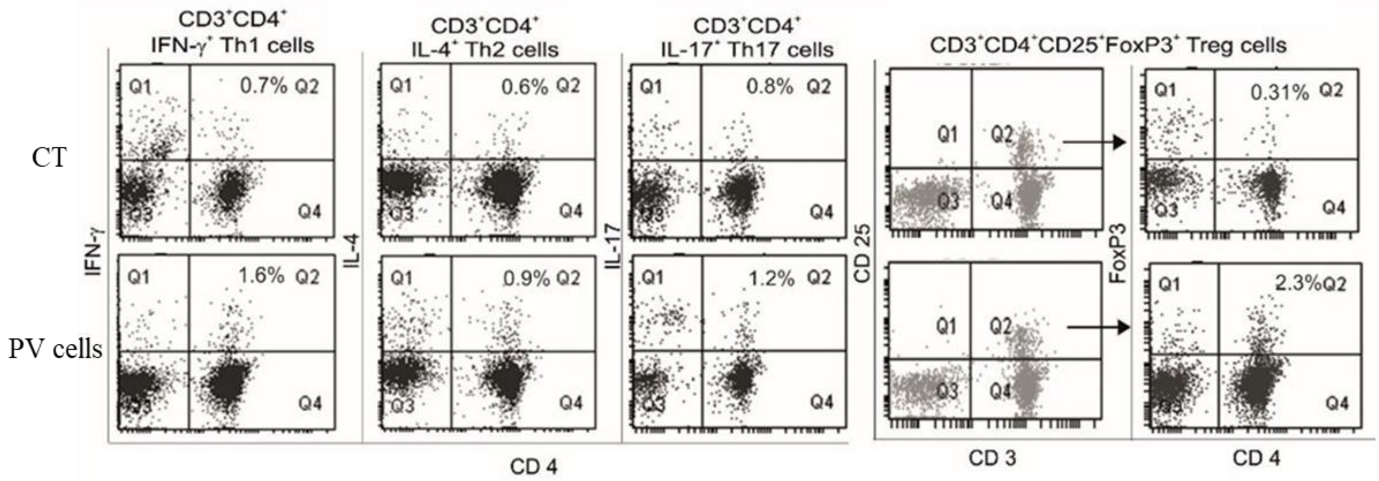


Figure 3.15 Dot blot shows the secretory ability of T cell types in PV patients.

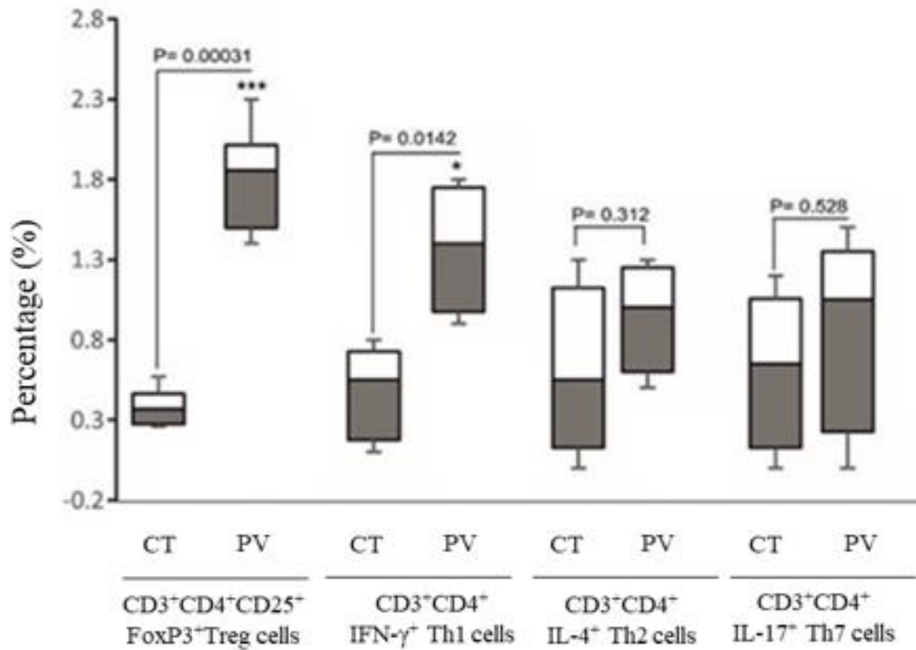


Figure 3.16. Expression of some immune cells in PV patients compared with control samples

3.1.10 Cytokine secretion and cancer antigens in patients with polycythaemia vera

The A20 and CYLD are also known as inhibitors of inflammatory reaction; thus, cytokine production in sera of PV patients was examined. Similar to a recent study, we also observed that levels of IL-6 and TNF-α in PV patients were found higher than in control individuals; however, these patients showed no change in the serum level of IL-1β (Figure 3.17).

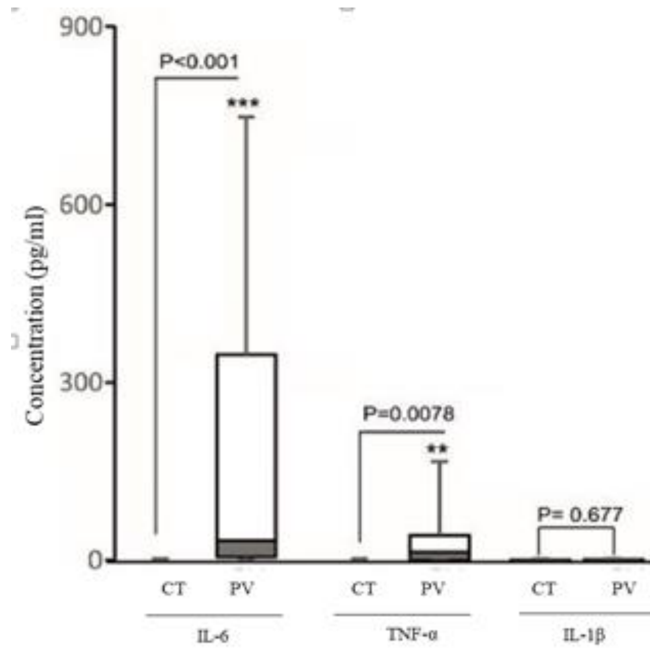
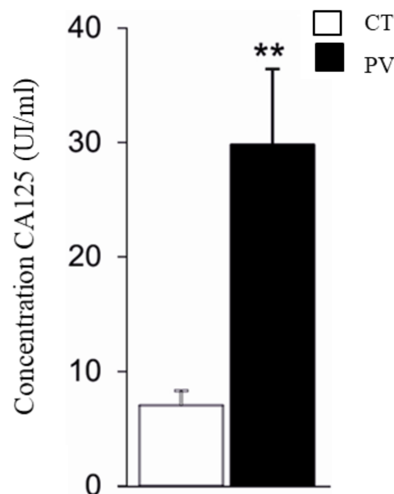


Figure 3.17. Results of measuring IL-6, TNF-α, IL-1β levels by ELISA method.

Finally, level CA-125 was examined by ELISA since content of this cancer antigen was revealed to be higher in elderly female patients of ALL at the age of 50-75 in our recent study. This marker is considered as a marker involved in the development and pathogenesis of leukemia. As expected, CA-125 level was significantly higher in PV patients than healthy controls (Figure 3.18) and 18 of 77 (23.4%) PV patients had CA125 level higher than the clinical cutoff, 35UI/mL.



Hình 3.18. Kết quả đo nồng độ CA125 bằng phương pháp ELISA.

3.2 Research results on peripheral blood mononuclear cells

3.2.1 Results of isolation and culture of peripheral blood mononuclear cells

Based on the density of Ficoll 1.077 g/mL and the density of blood cells is higher than that of lymphocytes but lower than that of red blood cells and granulocytes. When centrifuged, red blood cells and granulocytes settle to the bottom of the centrifuge tube, while lymphocytes and other monocytes are on top of the Ficoll layer. Collect the cell layer above the Ficoll layer which is rich in hematopoietic stem cells. (Figure 3.19)

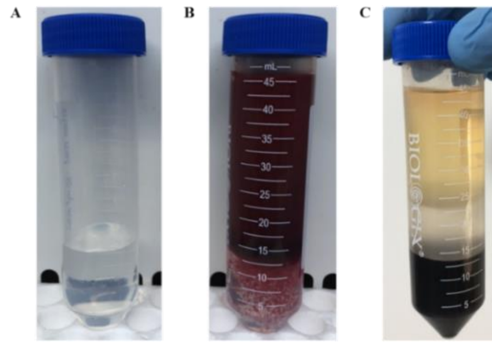


Figure 3.19. Isolation of PBMCs by density layered centrifugation.

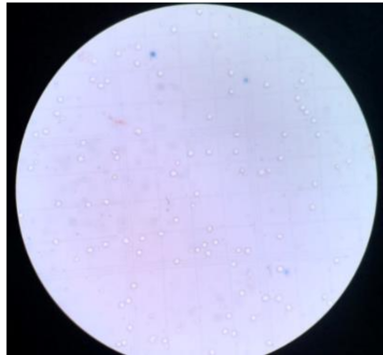


Figure 3.20. PBMC cells observed under the microscope (40x)

3.2.2 A20 regulates the immune phenotype of peripheral blood mononuclear cells

Analysis of cell surface marker expression was conducted to evaluate the effect of *A20* on specific immune cell populations. The cells were transfected with control or *A20* siRNA for 48h and followed by treatment of the cells without or with LPS for another 24h. The protein level of *A20* gene expression level was observed to be downregulated in *A20*-silenced PBMCs (Figure 3.21A).

For the determination of immunophenotypes, the $CD45^+$ gated population was analyzed for percentages and activation of PBMCs. Results revealed that LPS treatment results in activation of myeloid, T and B cells (Figure 3.21B-C) and downregulation of *A20* by using *A20* siRNA further enhanced the proportion of $CD11b^+$ $CD40^+$ (activated myeloid cells), $CD19^+$ $CD25^+$ (activated B cells) and $CD3^+$ $CD25^+$ (activated T cells) expressing cells, however, the numbers of $CD11b^+$ $CD86^+$, $CD19^+$ $CD44^+$ and $CD3^+$ $CD44^+$ cells were similar to control group (Figure 3.21B-C). The evidence suggested that *A20* partially inhibited activation of myeloid, T and B cell populations.

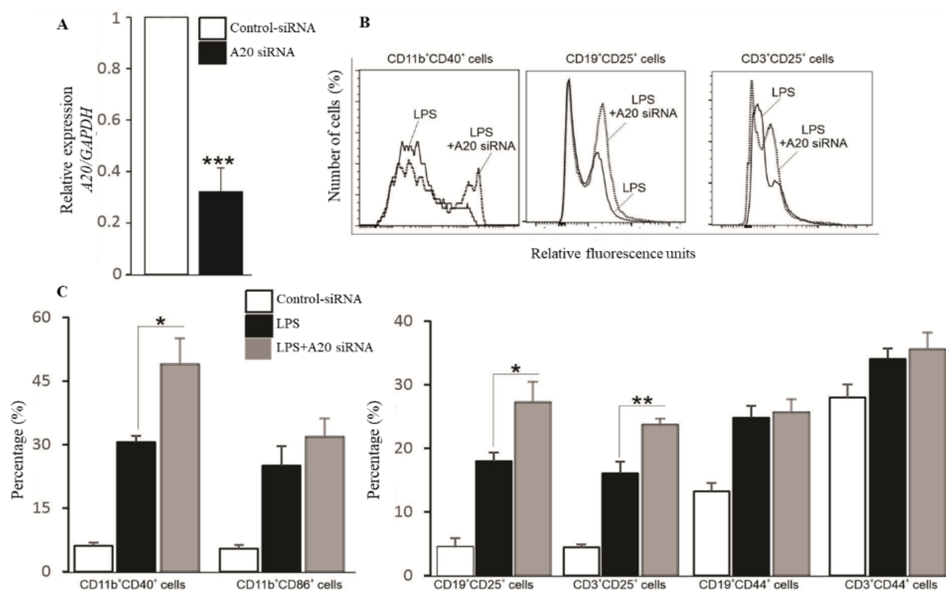


Figure 3.21. Immunophenotypic profile of A20-silenced PBMCs

3.2.3 A20 regulates cytokine secretion

We next examined cytokine productions secreted by LPS-induced PBMCs. LPS treatment leads to increased release of inflammatory cytokines by PBMCs, however, *A20*-silenced mature PBMCs produced higher TNF-α and IL-1β cytokines as compared to control siRNA-treated PBMCs (Figure 3.22), pointing out that the release of TNF-α and IL-1β was sensitive to the presence of *A20* in PBMCs.

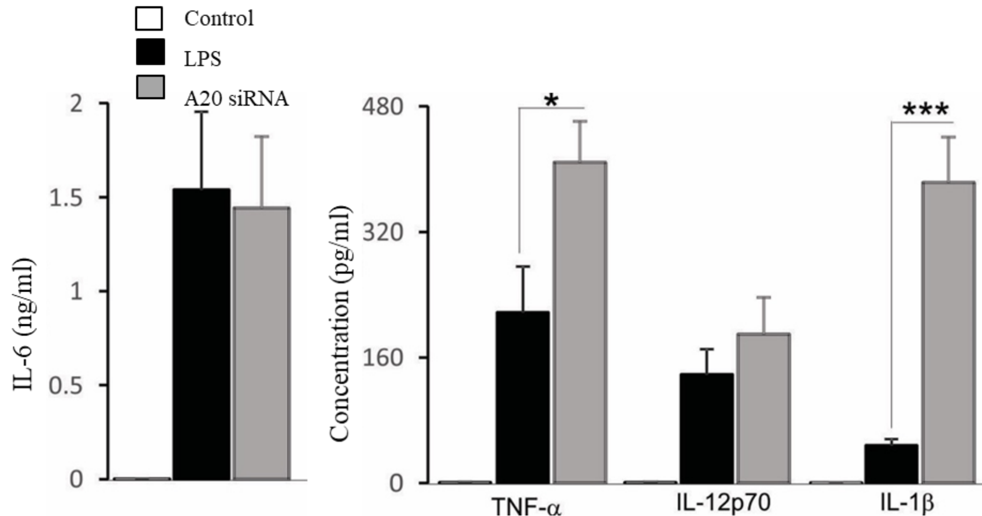


Figure 3.22. Results of cytokine release from PBMCs inactivating the A20 gene.

3.2.4 A20 regulates peripheral blood mononuclear cell migration

In addition of regulating immune responses by secretion of various cytokines, cell migration is also a functional hallmark of activated PBMCs. With regard to the inhibitory effect of *A20* on functional maturation of PBMCs, the migration of *A20*-silenced PBMCs was increased compared to control siRNA treated PBMCs (Figure 3.23).

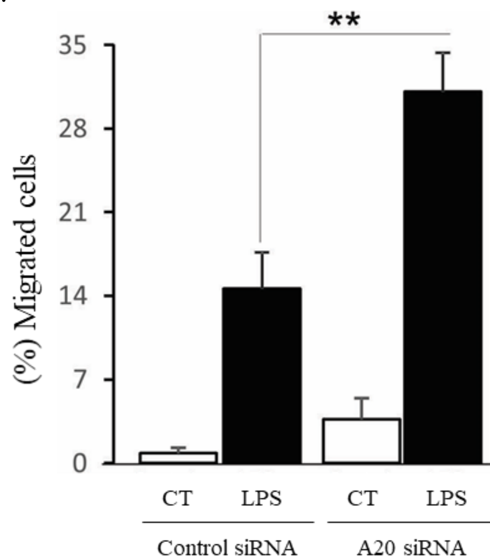


Figure 3.23. Effect of A20 on the migration of PBMCs

3.2.5 A20 regulates peripheral blood mononuclear cell apoptosis

Finally, cell viability was checked by measuring annexin V binding to the cell membrane and caspase 3 activity. As shown in Figure 3.24 that LPS protects PBMCs against suicidal cell death, as percentages of annexin V^{+} and caspase 3^{+} PBMCs were reduced when treated with LPS. The effects were remained unaltered in the presence of *A20* siRNA, pointing out that the presence of *A20* was not sensitive to apoptotic signaling in PBMCs.

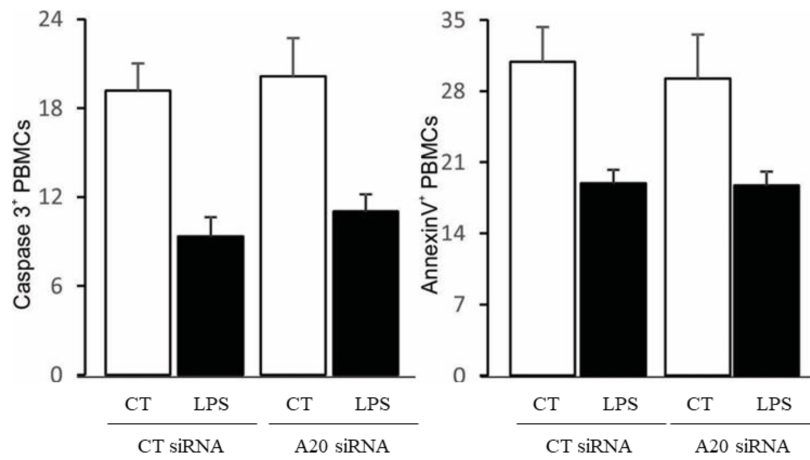


Figure 3.24. Effect of A20 on apoptosis of PBMCs.

3.2.6 Otubain-1 in regulating peripheral blood mononuclear cell function

To understand how distinct immune cells are affected by *OTUB1*, cells were transfected with control or *OTUB1* siRNAs and followed by treatment with LPS. The efficacy of transcriptional silencing was tested by real-time PCR, and the results showed that *OTUB1* siRNA effectively blocked expression of *OTUB1* in PBMCs (Figure 3.25A). For determination of cellular immunophenotype, $CD45^+$ gated cell population was analysed for activation of the immune cells. Flow cytometry analysis showed that down-regulation of *OTUB1* by using *OTUB1* siRNA significantly enhanced number of $CD56^+CD44^+$ (activated NK cells) expressing cells in PBMCs (Figure 3.25 C-D) and did not affect proportion of all $CD11b^+CD40^+$, $CD11b^+CD86^+$ (activated myeloid cells), $CD11c^+CD40^+$, $CD11c^+CD86^+$ (activated dendritic cells), $CD19^+CD25^+$, $CD19^+CD44^+$ (activated B cells), $CD3^+CD4^+CD25^+$, $CD3^+CD4^+CD44^+$ (activated CD4 T cells), $CD3^+CD8^+CD25^+$, $CD3^+CD8^+CD44^+$ (activated CD8 T cells), and $CD56^+CD25^+$ (activated NK cells) expressing cells as compared to those in control PBMCs (data not shown). The evidence indicated that *OTUB1* supressed recruitment of $CD56^+CD44^+$ expressing cells into circulatory system in the exposure to LPS.

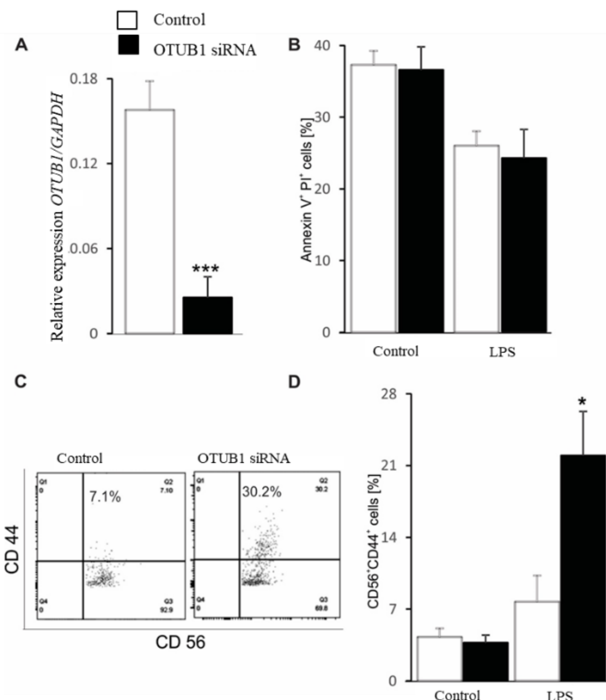


Figure 3.25. Effect of Otubain-1 on the activation and apoptosis of PBMCs

With respect to NK cell activity, we next examined cytokine productions in the response with LPS. The results indicated that release of inflammatory cytokines TNF-α, IL-6 and IL-1β were enhanced by LPS-stimulated PBMCs and however, concentration of these cytokines was unchanged when the cells were exposed to *OTUB1*-siRNA (data not shown). Finally, to ask whether apoptosis of PBMCs is modulated by the presence of *OTUB1*, percentage of annexinV+PI- cells was determined by staining PBMCs with APC annexinV antibody and PI to detect phosphatidylserine (PS) exposure to the extracellular environment. As shown in Figure 3.25 B, number of annexinV+PI- cells remained unaltered in *OTUB1*-silenced PBMCs, pointing out that apoptosis of PBMCs was not sensitive to *OTUB1* upon LPS challenge.

3.2.7 Otubain-1 in the regulation of cytokine products

We continue to examine cytokine products in response to LPS to check the cytokine-secreting activity of PBMC cells.

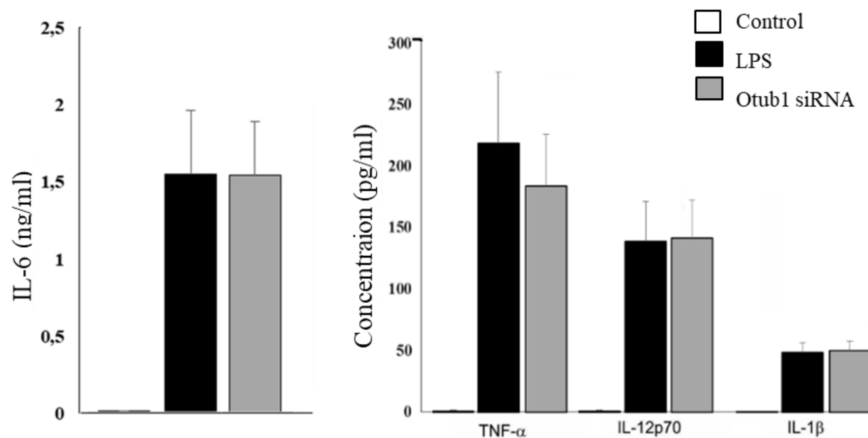


Figure 3.26. Results of cytokine release from Otubain-1 gene inactivated PBMCs

The results illustrated in Figure 3.26 indicate that, after treating PBMC cells with LPS, the ability to produce the pro-inflammatory cytokines IL6, TNF-α, IL-12p70 and IL-1β increases. However, the release of these cytokines did not change compared with the control sample in the absence of the *Otubain-1* gene. The results indicated that the presence of *Otubain-1* was insensitive to the secretion of IL6, TNF-α, IL-12p70 and IL-1β by PBMC cells.

3.2.8 Otubain-1 to signaling in peripheral blood mononuclear cells

To test signalling molecules involved in regulating activation of NK cells by *OTUB1*, mRNA level of IκB-α, SHPs (SHP1 and SHP2) and STATs (STAT1, STAT3, STAT5 and STAT6) signals was examined by real time PCR. The results indicated that among the genes examined, mRNA levels of SHP1 and STAT6 in *OTUB1*-silenced PBMCs were significantly overexpressed as compared to control cells. In addition, expression of IκB-α, SHP2, STAT1, STAT3 and STAT5 genes were unchanged in the absence of OTUB1 gene (Figure 3.27).

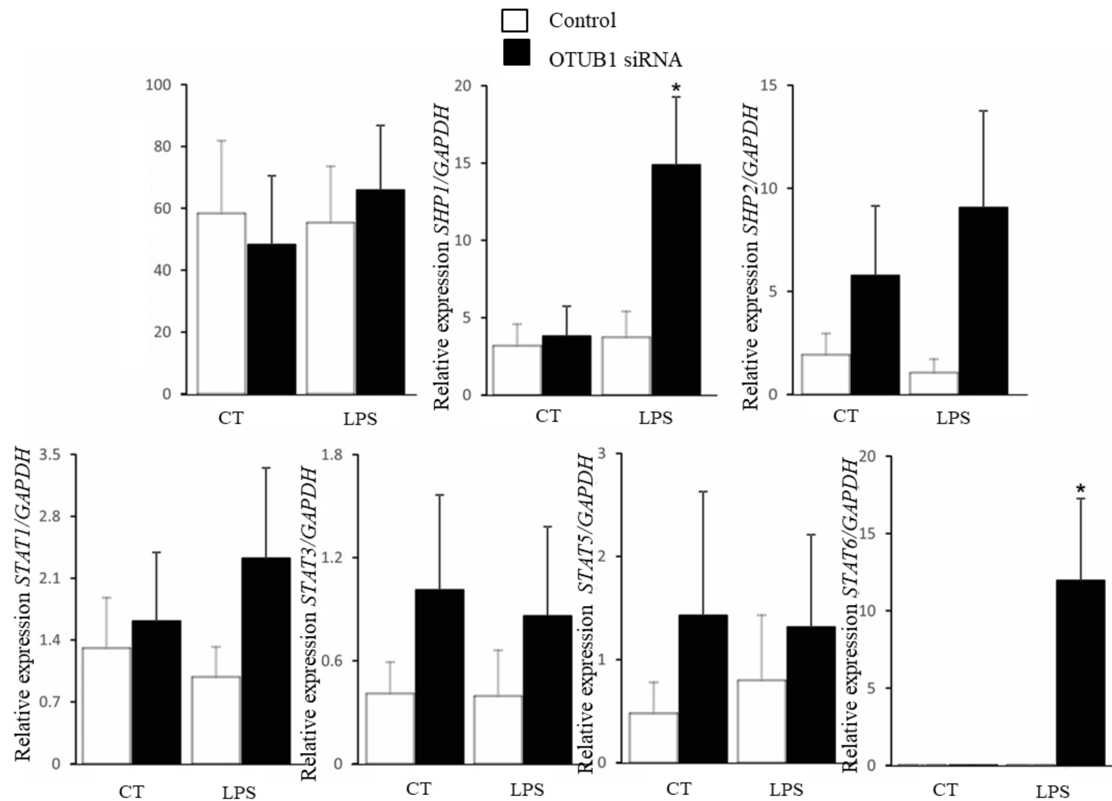


Figure 3.27. Effect of Otubain-1 on signaling pathways in PBMCs

CHAPTER 4: DISCUSION

4.1 Polymorphisms and expression of *deubiquitinase* genes, *JAK2* genes and immune phenotypes in polycythaemia vera

The presence of JAK2^{V617F} mutation in PV patients was conducted to determine the association of mutations in *JAK2* gene with *DUB* genes. The JAK2^{V617F} mutation occurred in 66.67% of PV patients diagnosed in this study, whereas another study indicated the presence of this mutation in ≥90% of PV cases. This study observed that 11 SNPs in the *CYLD* gene, of which p.W736G and c.2483+6 T>G were predicted to be novel mutations related to the pathogenesis of PV. Importantly, these 2 mutations were not found in the 90 samples of CML patients, 32 samples of AML patients, 16 samples of ALL patients, and 21 samples of CLL patients in this survey (unpublished data), showed that the two mutations p.W736G and c.2483+6 T>G may be associated with the risk of developing PV but may not some other leukemias. Other studies of genetic alterations in the *A20* gene indicate that mutations in the *A20* gene in exons 5, 6, and 7 have a high risk for autoimmune disease and lymphocytic leukemia. In this study, SNP *rs776591390* of the *A20* gene was detected in PV patients with a frequency of 1.29%, suggesting that the *A20* gene polymorphisms associated with the progression of leukemia are different. Unlike *A20* and *CYLD*, the regulatory effect of *Cezanne* on the activation of JAK/STAT signaling as well as the association of SNPs with possible risk of leukemia has not yet been elucidated, although expression Cezanne is linked to poor prognosis in HCC. This study revealed for the first time that changes of 7 nucleotides in intron 10 in the *Cezanne* gene were found in PV patients and that 7/77 (9.09%) PV patients carried at least 2 SNPs in this gene. Similar to the 2 abnormal mutations in the *CYLD* gene, the 7 SNPs in the *Cezanne* gene were not carried by AML and CML patients (unpublished data). In addition, the MAFs of the 7 SNPs had a slightly higher frequency in the PV groups compared with the controls, suggesting that carriers of SNPs in *Cezanne* gene tend to be at risk for the development of PV.

Increases in the number and activity of helper T cells ($CD3^+CD4^+CD25^+$ positive cells) and regulatory T cells (Treg, $CD3^+CD4^+CD25^+$ $FoxP3^+$ positive cells) enter the circulatory system in PV patients may be related to the regulatory activity of 2 *CYLD* gene mutations. Abnormal expression of CD25 on helper T cells is associated with poor prognosis in AML and CML patients. A previous study showed that PV disease is associated with thrombosis including: increased hematocrit, thrombocytosis, platelet activation and leukocyte activation, suggesting the effect of 2 mutations in *CYLD* gene on the development of thrombosis in PV patients.

4.2 Discuss the role of *A20* gene in regulating peripheral blood mononuclear cell function

Up to now, A20 protein has been studied to participate in inhibiting functional activation of some immune cells including T cells and B cells, TBT, but the role of A20 in PBMC cells is still unknown. has not been mentioned. In addition to the presence of TBT, T, and B cells, PBMCs also include other cell types such as myeloid progenitor cells, NK cells, and monocytes. Furthermore, the association between cell number and their activated expression needs to be further analyzed to determine the functional role of each cell type in the PBMC population. Therefore, we observed that decreased *A20* expression on PBMCs resulted in increased expression of CD25 on B and T cells and higher CD40 levels on myeloid progenitor cells as well as enhanced release of inflammatory cytokines of TNF-α and IL-1β.

In summary, the present study indicates that decreased *A20* leads to increased partial activation of myeloid progenitor cells, B and T cells, and release of TNF-α and IL-1β from PBMC cells. However, PBMC apoptosis was independent of the presence of *A20* in PBMCs. *A20* expression level may regulate immune response in autoimmune diseases and cancer.

4.3 Discuss the role of *Otubain-1* gene in regulating peripheral blood mononuclear cell function

The role of *Otubain-1* gene in regulating PBMC cells function has not been studied. *Otubain-1* participates in the activation of CD8 T cell and NK cell function in immune responses against infection and cancer. In this study, inactivation of the *Otubain-1* gene that activates NK cells presenting in PBMC cells ($CD56^+CD44^+$ cells) and does not alter myeloid, B-cell and T-cell activity and neither affects cytokine release and apoptosis of PBMC cells (Figure 3.25-3.26). NK cell activation through enhanced expression of CD44 has been shown to increase the cytotoxic activity of NK cells. Similarly, a recent study also showed that the *Otubain-1* gene is involved in the regulation of maturation and activation of NK cells.

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

• Polymorphisms, gene expression and immune phenotypes in PV patients

- Genetic analysis on a total of 77 PV patients of CYLD gene identified 11 SNPs, of which SNP p. W736G was identified in exon 15 as a novel pathogenic mutation and a c.2483 + 6 T > G SNP belonging to intron 15 was predicted to be a mutation in the splicing region with a frequency of 18.2% and 5.2% respectively. *Cezanne* gene sequencing identified 7 SNPs in the intron 10 region, and PV patients carried at least 2 SNPs in this gene. In particular, PV patients carrying the SNP p.W736G had many SNPs in the *CYLD* gene, but no SNP in the *A20* or *Cezanne* gene.

- It has been determined that the expression levels of some genes related to immune system activity including *Klotho* gene and *LAG3* gene have decreased in PV patients. Expression levels of STAT molecular signaling genes including *STAT1*, *STAT3* and *STAT5* genes were all highly elevated, while expression of *SHP1* and *SHP2* signaling genes were unaffected in PV patients.

- PV patients have inactivated *A20*, *CYLD* and *Cezanne* genes; elevated levels of IL-6 and TNF-α; the number of $CD25^+CD4$ activated T cells, Th1 cells and regulatory T cells increased significantly compared

with healthy subjects. In addition, levels of the cancer antigen CA125 were increased in the blood in PV patients.

• The role of the *A20* gene in regulating peripheral blood mononuclear cell function

- PBMC cells that were inactivated with the *A20* gene had an increased density and activation level of cells expressing $CD3^+CD25^+$, $CD19^+CD25^+$ and $CD11b^+CD40^+$; increased release of several cytokines TNF-α and IL-1β; and enhanced cell migration. However, PBMC cell apoptosis did not depend on the presence of the *A20* gene. Therefore, *A20* expression may be involved in immune responses in autoimmune disease and cancer.

• The role of Otubain-1 in regulating peripheral blood mononuclear cell function

- *Otubain-1* gene inactivated PBMC cells increased the expression level of *SHP1* and *STAT6* genes and did not change the expression of *IκB*-α, *SHP2*, *STAT1*, *STAT3* and *STAT5* signaling genes.

- Inactivation of *Otubain-1* gene activates NK cells ($CD56^+CD44^+$); and did not alter the myeloid, B-cell and T-cell activity present in PBMC cells nor affect the release of cytokines IL-6, IL-12p70, TNF-α and IL-1β and apoptosis of PBMC cells.

5.2 Recommendations

The CYLD gene mutation in PV patients needs to be functionally determined through gene transfer techniques to be able to determine the role of this mutation in the activity of cancer cells.

NEW FINDINGS OF THE THESIS

➢ PV patient has two SNPs p.W736G, c.2483 + 6 T>G belonging to the *CYLD* gene which are predicted to be potentially related to the disease. In particular, PV patients carrying the SNP p.W736G had many SNPs in *CYLD*, but no SNPs in the *A20* or *Cezanne* genes.

➢ Patients with PV have inactivated genes *A20, CYLD, Cezanne* and *Klotho*; elevated levels of IL-6 and TNF-α; The number of $CD25^+CD4$ activated T cells, Th1 cells and regulatory T cells increased significantly compared with healthy subjects.

➢ The *A20* gene inhibits T, B and myeloid cell activity ($CD11b^+CD40^+$, $CD3^+CD25^+$ and $CD19^+CD25^+$), TNF-α, IL-1β secretion and PBMC cell migration. The Otubain-1 gene inhibits NK cell activity ($CD56^+$ $CD44^+$) and expression of the *SHP1* and *STAT6* signaling genes.

LIST OF PUBLICATIONS

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➢ Do Thi Trang, Nguyen Thi Xuan. *Biểu hiện một số gen kiểm soát miễn dịch và nồng độ CA125 ở bệnh nhân tăng hồng cầu vô căn.* Tạp chí công nghệ sinh học, 2020, 42(1): 1.

➢ Nguyễn Thy Ngọc, Bùi Bích Hậu, Phạm Hoàng Nam, Trần Tuấn Anh, Đỗ Thị Trang, Nguyễn Thị Xuân. (2021) *Đánh giá một số phương pháp xác định đột biến $JAK2^{V617F}$ phục vụ việc dự đoán nguy cơ mắc bệnh đa hồng cầu và một số căn bệnh tăng sinh tủy ác tính khác*. Tạp chí Công nghệ Sinh học, 19(3):p433-440, ISSN: 1811-4989.