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STUDY ON THE REGULATORY ROLE OF *A20* AND *CYLD* GENES ON CELL FUNCTION IN MYELOID LEUKEMIA PATIENTS

SUMMARY OF DISSERTATION IN BIOTECHNOLOGY

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INTRODUCTION

1. The necessity of research

Myeloid leukemia is one of the most common types of leukemia and occurs at any age but is most common in adults. There are two types of myeloid leukemia: acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). More than half of myeloid leukemia patients are found to be over 60 years of age, with a mean age of 64 patients. Currently, the treatment of myeloid leukemia patients is usually done with chemotherapy and allogeneic cell transplantation. However, the rate of remission after chemotherapy as well as the time of disease stabilization decreased, and the side effects of this treatment increased significantly in older patients. Therefore, at present, many domestic and foreign scientists are interested in studying the causes of myeloid leukemia.

The *A20, CYLD* genes encode proteins of the DUB (Deubiquitinase) group that play an important role in the regulation of proliferation and apoptosis. These DUB proteins cleave peptide or isopeptide bonds between the target protein and ubiquitin to reverse biological processes within the cell. The A20, CYLD proteins are involved in the negative regulation of the immune response and the release of inflammatory cytokines via the STAT signaling pathway. When *A20, CYLD* is abnormally expressed which may be involved in the formation and development of leukemia and lymphoma.

Recently, the proportion of people with myeloid leukemia has been increasing, while the treatment of this disease is still difficult, and A20, CYLD have been identified to be associated with the risk of CLL. and ALL. This is the reason that many scientists around the world and Vietnam are focusing on studying polymorphisms/mutations and gene expression A20, CYLD, inflammatory expression and related signaling genes in myeloid leukemia.

Vincristine is an alkaloid antineoplastic drug, extracted from the periwinkle plant (*Catharantus roseus*). Vincristine promotes the apoptosis of some cancer cells. Therefore, together with the identification of the *CYLD, A20* gene polymorphisms in myeloid leukemia, we continue to evaluate the role of these genes in the proliferation and apoptosis of blood cancer cells - K562 when treated with vincristine could represent a new step in finding a way to inhibit the growth of myeloid leukemia cell lines.

Besides, macrophages also have an important role in anti-inflammatory responses, tissue repair, homeostasis, and especially have the ability to inhibit tumor growth and apoptosis of cancer cells. letters. Therefore, finding out the role of the *CYLD* or *A20* gene in the function of macrophages is also an important task towards building effective cancer treatment support measures. From the above reasons, the thesis "Study on the regulatory role of *A20* and *CYLD* genes on cell function in myeloid leukemia patients" was carried out.

2. Research objective

- Determining polymorphisms, expression of *A20, CYLD* genes, inflammatory expression and some related signaling genes in myeloid leukemia patients.

- Determining the role of *A20* and *CYLD* gene in regulating the function of blood cancer cells and macrophages in myeloid leukemia patientss

3. Research content

- Determining some polymorphisms/mutations, expression of *A20, CYLD* genes; expression of signaling gene *STAT3* and levels of IL-6, TNF-α in myeloid leukemia patients

- Determining the role of *A20/CYLD* genes in proliferation and apoptosis of K562 cels and function of macrophages differentiated from PBMCs (Peripheral blood mononuclear cell) of AML patients when treated with fludarabine.

CHAPTER 1. LITERATURE OVERVIEW

1.1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a common leukemia in adults. AML is characterized by proliferation of blast cells, which mainly exist in the bone marrow, resulting in inhibition of normal hematopoiesis and bone marrow failure. Recently, the treatment of acute myeloid leukemia in young people has made significant progress, but the treatment of elderly patients still faces many obstacles. Although there have been improvements in the treatment of the disease, the prognosis of the disease is still very difficult.

1.2. Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a type of cancer that originates in certain blood-forming cells found in the bone marrow. CML is

characterized by proliferation of differentiated granulocytes. Initially, granulocytes are elevated in the peripheral blood, but they function relatively normally. However, as the disease progresses, immature white blood cells (myeloblasts) begin to accumulate in the blood and bone marrow. The overgrowth of myeloblasts reduces the growth of other blood cells, resulting in a deficiency of red blood cells and platelets. This change induces *BCR-ABL* gene fusion, which in turn overproduces tyrosine kinase that causes CML. White blood cells develop in the bone marrow, then enter the bloodstream and travel to other parts of the body. CML develops quite slowly and can turn into acute leukemia very quickly and is difficult to treat.

1.3. General introduction of deubiquitinase A20 and CYLD

1.3.1. A20 Protein

A20 is a tumor necrosis factor-stimulating factor 3 protein that is encoded by the TNFAIP3 gene. *A20* is located on chromosome 6q23.3, cDNA sequence is 4,440 bp long with open reading frame of 2,370 Nu encoding for protein containing 790 amino acids. A20 acts as a negative regulator of NF-κB in response to multiple stimuli and is considered a tumor suppressor. A20 dysfunction may be associated with lymphoproliferative malignancy. Furthermore, A20 and A20 binding proteins can be used as biomarkers and as novel therapeutic targets in lymphocyte malignancies.

1.3.2. CYLD Protein

CYLD is a deubiquitinase enzyme encoded by the cylindromatosis (*CYLD*) gene. *CYLD* is located on human chromosome 16q12.1, is 60 kb in size, and encodes for the enzyme thioesterase containing 956 amino acids with three conserved protein-glycine (Cap-Gly) regions to interact with target proteins in humans. NF-κB signaling pathway. CYLD protein acts as a tumor suppressor, so when *CYLD* is mutated or reduced in expression, it promotes the growth of various types of tumors. CYLD has an inhibitory role on tumorigenesis through the regulation of apoptosis, and cell necrosis. These findings suggest that CYLD can be considered a biomarker, as well as a factor to promote more effective leukemia treatment.

1.4. STAT1 and STAT3 signals

STAT1 participates in signaling by both type I and II IFNs in inflammatory responses after viral infection and protects the host from bacterial and parasitic attack; Activated STAT3 promotes the formation of head and neck cancers, multiple myeloma cells, some solid tumours, leukemias, and lymphomas.

1.5. Vincristine's role

Vincristine is a substance extracted from the periwinkle plant (Cantharanthus roseus) that has an effective inhibitory effect on some cancers. Vincristine's mechanism of action was determined to be similar to that of specific cell cycle-based antineoplastic agents. During treatment, vincristine is often toxic to the nervous system, which then releases proinflammatory mediators such as IL-6 and TNF-α. By activating the NF-κB/STAT signaling pathway, vincristine is a partial regulator of antitumor activity.

1.6. Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs are the main cell type in the human immune system. PBMCs are used for research in infectious diseases, vaccine development, immunology, hematologic malignancies, and transplant therapy. Essentially, PBMC studies under in vitro conditions provide information regarding cellular function, biomarker recognition, and disease patterns. The use of human PBMCs to promote the recovery of immunocompromised mice is considered a basis for studying the human immune system and the response of these cells to pathogens, toxins, and pathogens. or cancer in an in vivo model.

CHAPTER 2. MATERIAL AND METHODS

2.1. Study subjects

The study subjects were 92 patients diagnosed with AML and 50 patients diagnosed with CML (but untreated). The control group was 80 healthy volunteers.

K562 chronic myeloid leukemia cell was purchased from an ATCC (American type culture collection) organization.

Macrophages were differentiated from PBMCs of healthy and patients with AML.

2.2. Experimental diagram

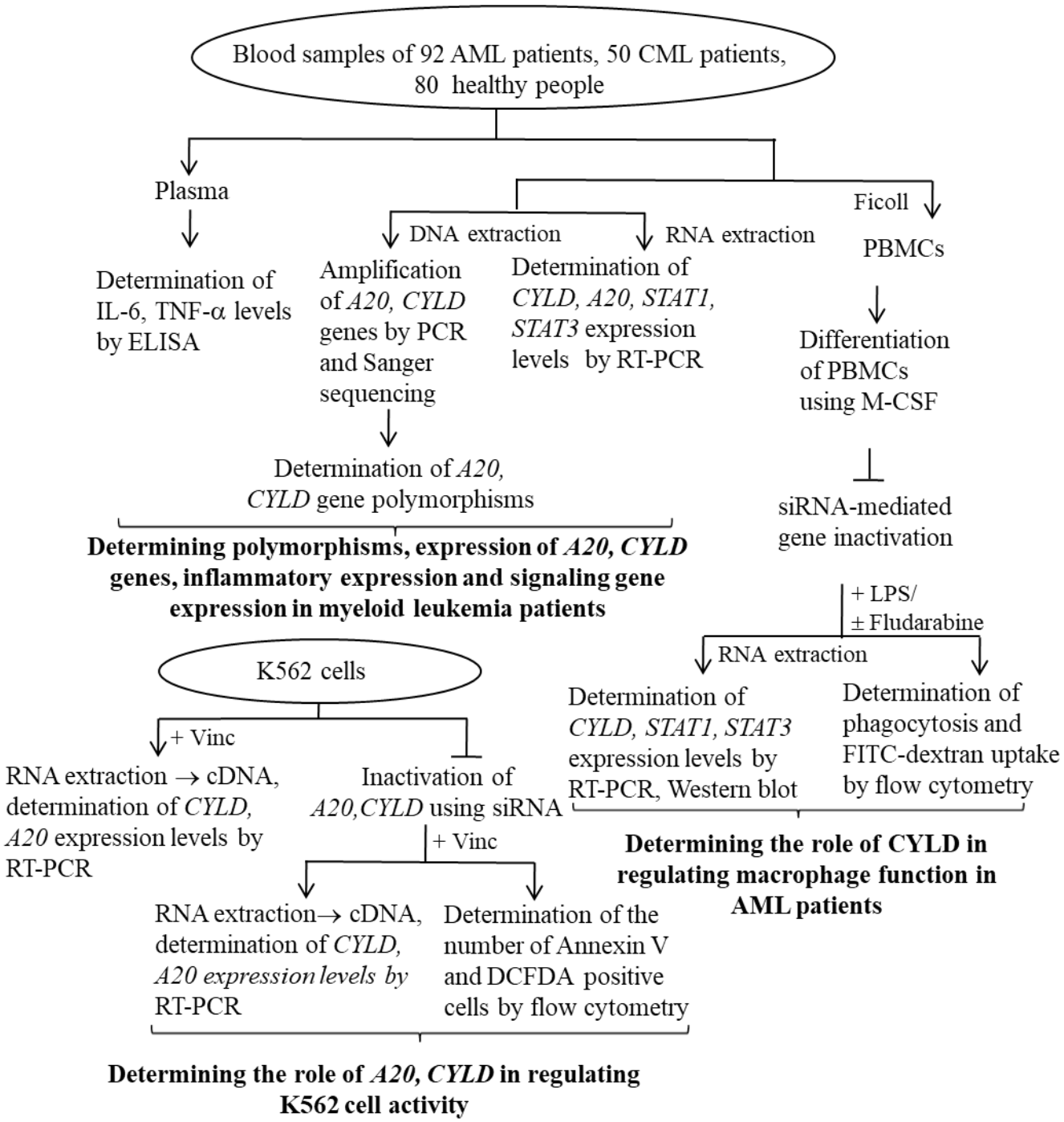


Figure 2.1. Research diagram

2.3 Methodologies

- ✓ Sample collection
- ✓ DNA extraction and *A20, CYLD* gene amplification (PCR)
- ✓ Sanger sequencing and polymorphism determination
- ✓ RNA extraction, cDNA synthesis and Realtime-PCR

- ✓ Culture and treatment of K562 cells by vincristine
- ✓ Isolation and differentiation of PBMCs using M-CSF
- ✓ siRNA-mediated gene inactivation
- ✓ ELISA
- ✓ Western blot
- ✓ Flow cytometry
- ✓ Data analysis

CHAPTER 3. RESULTS

3.2. Evaluation of polymorphisms on *CYLD, A20* genes

3.2.1. CYLD gene polymorphism in myeloid leukemia

3.2.1.1. CYLD gene polymorphism in acute myeloid leukemia

4 nucleotides on exon 16 were identified, including 3 SNPs (p.Q723H/c.2435 G>C; p.E735K/c.2445 G>A; p.E747K/c.2481 G>A)) is a non-synonymous SNP that causes the old amino acid to be replaced by a new amino acid (Figure 3.1) and SNP (p.E723E/c.2411 G>A) is a synonymous SNP. Among these polymorphisms, the genotypic distribution at p.E723E, p.Q731H and p.E735K positions obeyed the HWE-equilibrium in both the disease group, the control group and the whole study population ($p > 0.05$).

For the polymorphism at p.Q731H position, a significant difference in GC genotype between AML patients and healthy controls was detected when compared with GG genotype ($p = 0.0024$). In addition, the frequency of C allele was also shown to be associated with a high risk of AML ($p = 0.0032$).

To determine whether the impact of non-synonymous SNPs on *CYLD* gene alters amino acids to protein structure and function, this study used the tool Polyphen-2. Based on the predictable results, SNP p.Q731H position on *CYLD* gene is predicted to be the causative agent of the disease (Figure 3.2).

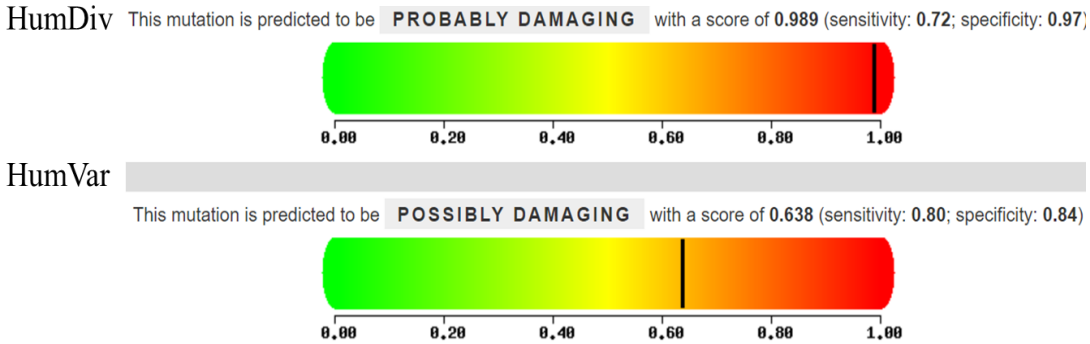


Figure 3.2. Prediction of pathogenicity of non-synonymous SNP p.Q731H on *CYLD* gene by Polyphen-2

3.2.1.2. *CYLD* gene polymorphism in CML

Identified 2 nucleotides on exon 16 changed, in which SNP p.Q731H (c.2435 G>C) is a non-synonymous SNP that causes the old amino acid to be replaced by a new amino acid and SNP p.V725V (c.2417 T>G) is a synonymous SNP (Figure 3.3). Among these polymorphisms, the genotypic distribution at p.V725V and p.Q731H positions follows the HWE-equilibrium over the entire study population ($p > 0.05$) (Table 3.3).

For the polymorphism at p.V725V position, TG genotype between CML patients and healthy controls was found to have no significant difference when compared with TT genotype ($p > 0.05$) and allele frequency G was not associated with a reduced risk of CML. Meanwhile, at p.Q731H position, GC genotype between CML patients and healthy controls was found to be significantly different when compared with GG genotype ($p = 0.0275$). In addition, the frequency of C allele was also shown to be associated with an increased risk of high CML ($p = 0.0336$). Based on the predictable results, SNP p.Q731H on *CYLD* gene is also predicted to be pathogenic.

3.2.2. A20 gene polymorphism in myeloid leukemia

3.2.2.1. Polymorphisms in the A20 gene in AML

4 nucleotides were identified in exon 7 (p.L335S/c.1303 T>C; p.K337Q/c.1308 A>C; p.K354N/c.1361 G>T; p.S376T/c.1425 T>A) are changed and are all non-synonymous SNPs, causing the old amino acid to be replaced by a new amino acid (Figure 3.4). Genotypic distribution at p.L335S, p.K337Q, p.K354N and p.S376T positions all obeyed HWE-equilibrium on the disease group and on the whole study population ($p > 0.05$) (Table 3.5). Besides, polymorphism at p.L335S, p.K337Q, p.K354N

and p.S376T positions, TC, AC, GT and TA genotypes did not have significant difference when compared with TT, AA, GG and TT genotypes respectively ($p < 0.05$), and the allele frequencies C (p.L335S), C (p.K337Q), T (p.K354N) and A (p.S376T) are also not associated with the likelihood of AML.

3.2.2.2. Polymorphisms on *A20* gene in CML

It was identified that 2 nucleotides on exon 7 (rs374721883/p.G456V; rs200878487/p.S466G) were changed and both are non-synonymous SNPs, causing the old amino acid to be replaced by a new amino acid (Figure 3.5). Besides, the genotypic distribution at positions rs374721883 and rs200878487 both obeyed HWE-equilibrium on the disease group and the whole study population ($p > 0.05$) (Table 3.7). For the polymorphism score at rs374721883 and rs200878487, the assessment of genotype-CML association was only tested in the dominant phenotype because TT and GG genotypes were not present in the study population, respectively. GT (rs374721883), CG (rs200878487) genotypes between CML patients and healthy controls were found to have no significant difference when compared with the GG (rs374721883), CC (rs200878487) genotypes ($p > 0,05$) and allele frequency was also not related to the likelihood of CML (Table 3.9).

3.3. Gene expression levels in myeloid leukemia patients

3.3.1. Expression of CYLD, A20 genes in myeloid leukemia patients

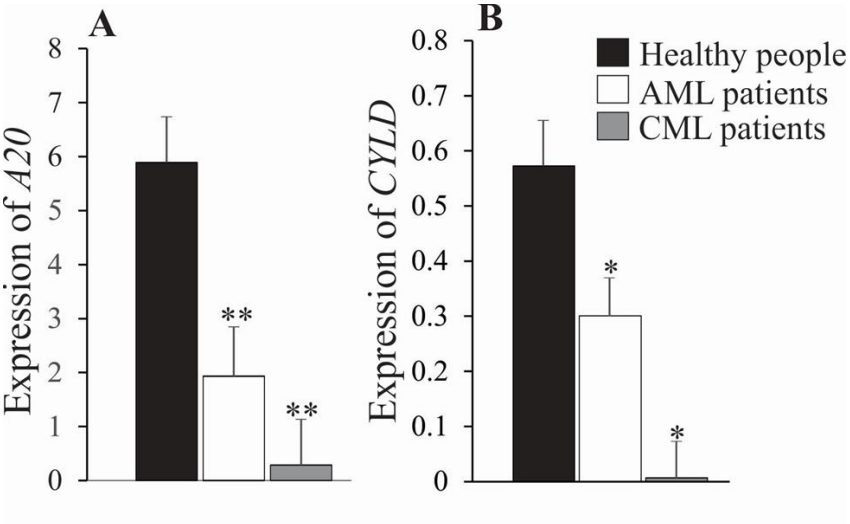


Figure 3.6. Expression of *A20* and *CYLD* genes in myeloid leukemia

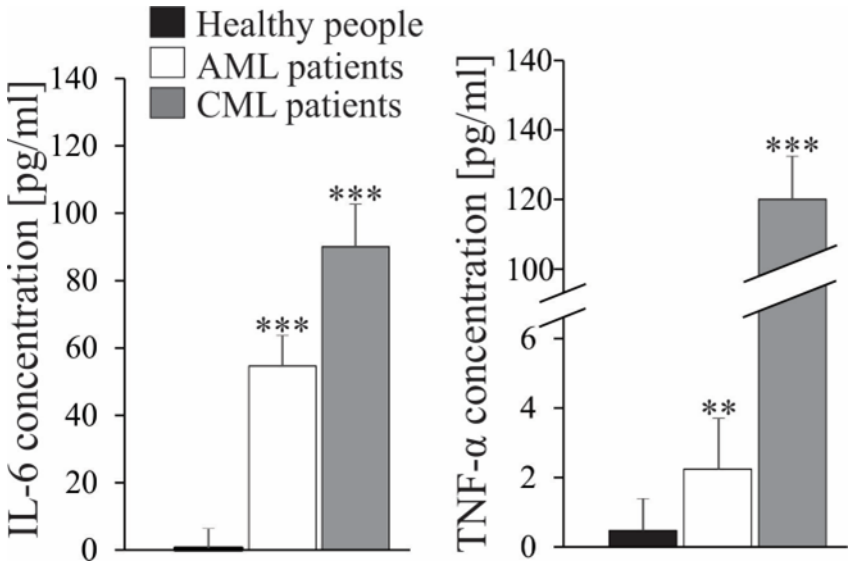
** ($p < 0.05$), ** ($p < 0.01$) indicates a statistically significant difference between the healthy group and the patient group*

mRNA expression levels of *A20* gene were decreased by about 3-fold in acute myeloid leukemia patients and 39-fold in chronic myeloid leukemia patients compared with healthy subjects (Figure 3.6A). Similarly, mRNA expression of *CYLD* gene in healthy subjects was approximately 2-fold and 89-fold higher in acute myeloid leukemia and chronic myeloid leukemia patients, respectively (Figure 3.6B). Thus, in myeloid leukemia patients, the expression of *A20* and *CYLD* genes was lower than in healthy subjects and this difference was statistically significant ($p < 0.01$ and $p < 0,05$)

3.3.2. Expression of STAT1, STAT3 genes in myeloid leukemia patients

Expression levels of *STAT1, STAT3* genes were increased in AML patients compared with healthy controls. However, this change was not statistically significant ($p > 0.05$). Similarly, although the data showed that *STAT1* expression was increased and *STAT3* expression decreased in CML patients compared with healthy subjects, this difference was not statistically significant ($p > 0.05$).

3.3.3. IL-6, TNF-α concentration in myeloid leukemia patients



Hinh 3.8. Levels of IL-6, TNF-α expression in myeloid leukemia patients

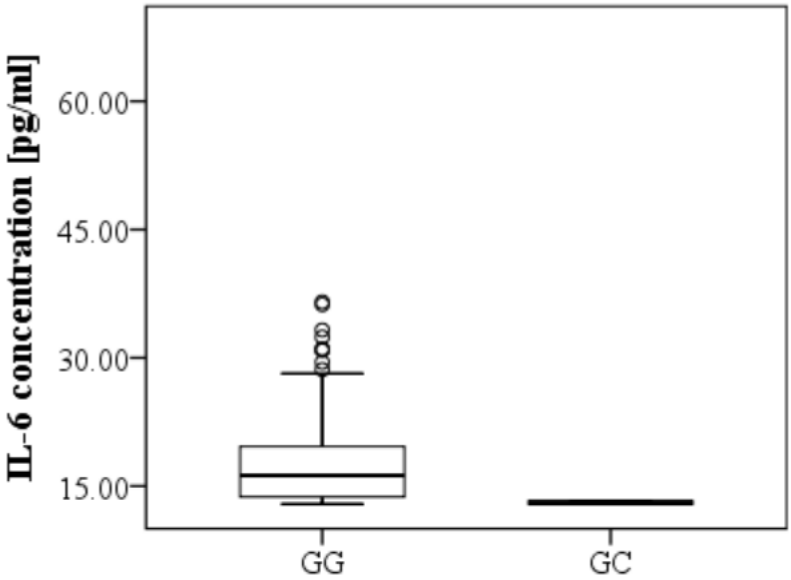
*** (p < 0.01), *** (p < 0.001) indicates a statistically significant difference between the healthy group and the patient group*

IL-6 expression levels increased in both AML and CML patients about 84- and 138-fold, respectively, compared with healthy subjects. This change was found to be statistically significant ($p<0.05$). Similarly, TNF-α concentration secreted in myeloid leukemia (AML and CML) patients were

approximately 5 and 269 times higher, respectively, than in healthy subjects. This change was also statistically significant ($p < 0.05$).

3.4. Association between SNPs, gene expression and cytokine levels in myeloid leukemia patients

Based on Mann-Whitney U test, we determined the association between p.Q731H position on *CYLD* gene and IL-6 concentration in AML patients ($p < 0.05$) (Table 3.11 , Figure 3.9), but no relationship was found between the genotypic distribution of polymorphism at p.Q731H position on *CYLD* gene and *CYLD* gene expression (Table 3.9); *STAT1* signaling gene expression (Table 3.10) and TNF-α concentration in myeloid leukemia patients.



Figurre 3.9. Association between polymorphism at p.G731H position on *CYLD* gene and IL-6 concentration in AML

Besides, based on Spearman test, there was no association between expression of *A20, CYLD* genes and expression of *STAT1* signaling gene in myeloid leukemia (AML and CML) (Table 3.12).

3.5. Determining the role of *A20, CYLD* in proliferation and apoptosis of K562 cells

3.5.1. Determining the role of A20, CYLD genes in proliferation of K562 cells

In this study, when K562 cells were treated with vincristine at different concentrations (60-300 nM), mRNA expression levels of *A20* and *CYLD* genes were significantly higher than those of the control group, especially in the control group. when the cells were treated with vincristine

at a concentration of 300 nM (Figure 3.10). Based on CFSE fluorescence plot obtained from K562 cells, it can be seen that K562 proliferation is significantly reduced when treated with 300nM vincristine. However, in the case of *A20* and *CYLD* inactivated cells, the inhibitory effect of vincristine on cell proliferation was no longer (Figure 3.11). Thus, vincristine inhibits cell proliferation through expression of *A20, CYLD* genes.

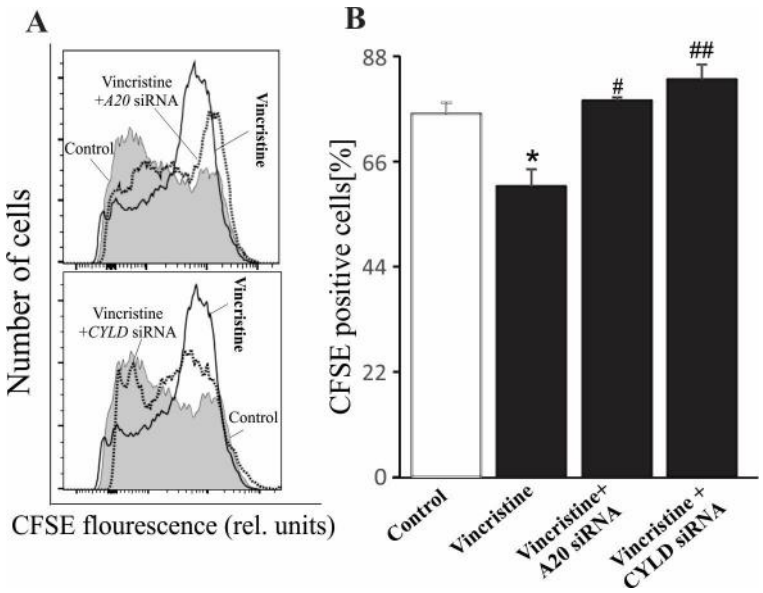


Figure 3.11. Effect of *A20, CYLD* in proliferation of K562 cells

** (p<0.05) indicates a statistically significant difference between vincristine treated and untreated K562 cells; # (p<0.05) and ## (p<0.01) indicate a statistically significant difference between K562 cells treated with vincristine in the case of inactivation and non-inactivation of A20, CYLD*

3.5.2. Determining the role of A20, CYLD in apoptosis of K562 cells

Based on the fluorescence plot, the treatment with vincristine (300 nM) significantly increased the number of Annexin V^+/PI^- positive K562 cells and the accumulation of ROS. However, when K562 cells were inactivated with *A20* gene, the number of cells positive for Annexin V^+/PI^- antibodies and ROS accumulation was significantly reduced compared with cells without gene inactivation and alteration. was found to be statistically significant ($p < 0.05$), and when *CYLD* gene was inactivated, the effect on reducing the number of Annexin V^+/PI^- positive cells and the accumulation of ROS was no longer clear.

Thus, the expression of *A20* gene is associated with the proliferation and apoptosis of K562 cells, while the expression of *CYLD* gene may also be related to these processes, but this relationship needs to be further studied and evaluated.

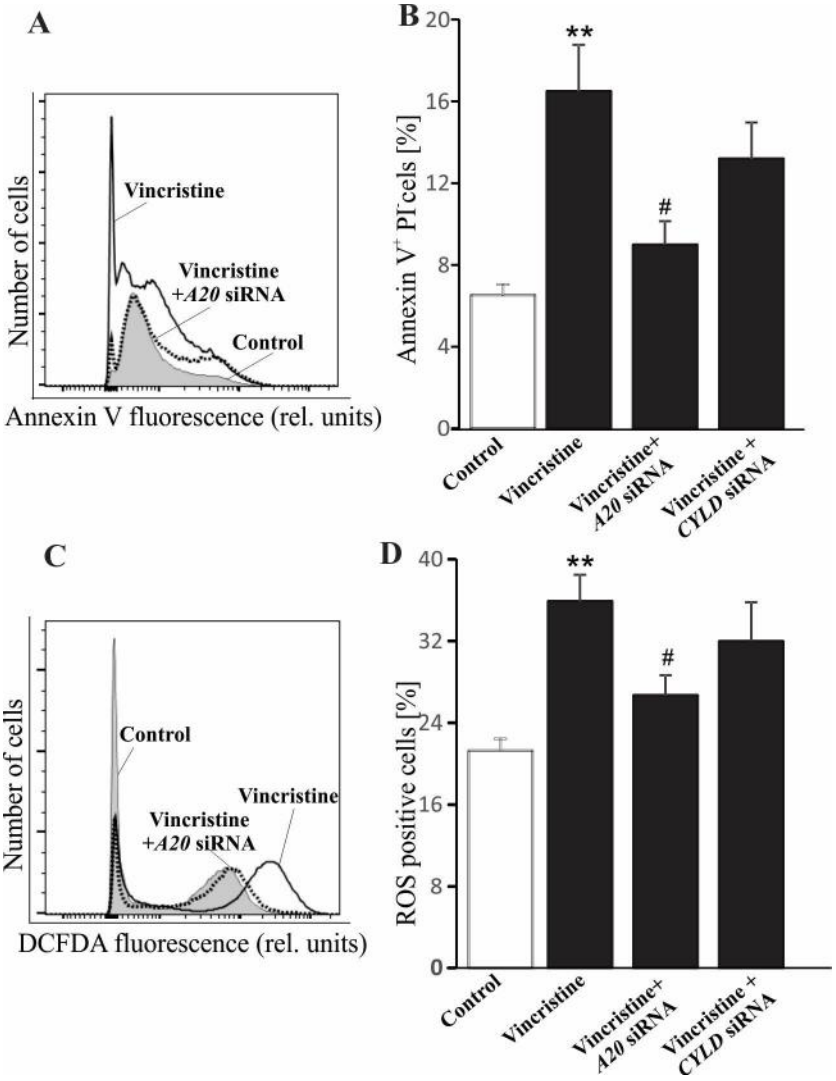


Figure 3.12. Effect of A20, CYLD on the viability of K562 cells

*** (p<0.01) indicates a statistically significant difference between vincristine-treated and untreated K562 cells; # (p<0.05) indicates a statistically significant difference between vincristine-treated K562 cells in the inactivation and non-inactivation of A20, CYLD*

3.6. Determining the role of *CYLD* gene in M-CSF-differentiated macrophages through STAT1 signaling

3.6.1. The role of CYLD in M-CSF-differentiated macrophages

Cells after being differentiated from PBMCs were inactivated *CYLD* gene by siRNA interference method to block the expression of *CYLD* gene with high specificity and efficiency, thereby evaluating the effect of *CYLD* gene to the expression of other signaling genes.

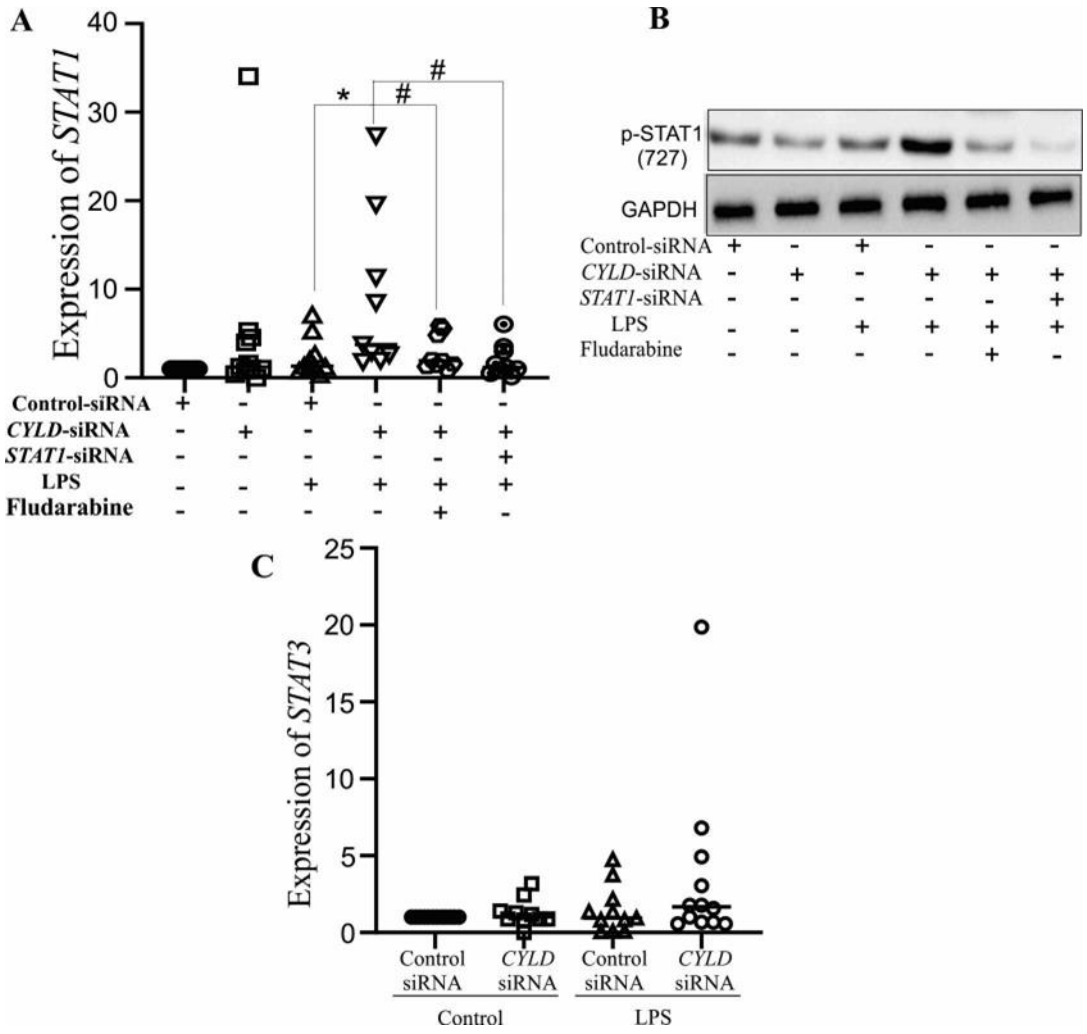


Figure 3.13. Effect of CYLD on STAT1 signaling on macrophages (M-HP)

** ($p < 0.05$) indicates a statistically significant difference between inactivated and non-CYLD-inactivated M-HPs; # ($p<0.05$) indicates a statistically significant difference between CYLD-inactivated or non-CYLD-inactivated M-HPs compared with these cells when activated by LPS*

The results shown in Figure 3.13A-B also showed that *CYLD* siRNA-treated macrophages significantly increased mRNA expression of *STAT1* and STAT1 phosphorylation when activated by LPS but did not affect the expression levels of *STAT3* (Figure 3.13C). However, when macrophages were treated with fludarabine, the effect of increasing the mRNA expression levels of *STAT1* decreased. Thus, expression of *CYLD* contributed significantly to the inhibition of STAT1 signaling activation in macrophages.

3.6.2. Role of CYLD in macrophage functions through STAT1 signaling

The study results shown in Figure 3.14A-B show that, normally, the percentage of macrophages expressing $CD40^+$ and $CD86^+$ were 3.9% and

4.22%, respectively, but when activated by LPS, the percentage of cells expressing these biomarkers increased. More specifically, when there was no expression of *CYLD*, this rate was even higher and reached about 22.1% ($CD40^+$), 26% ($CD86^+$) respectively. However, $CD40^+$ and $CD86^+$ expression levels of mature macrophages with *CYLD* inactivation were significantly reduced when treated with fludarabine.

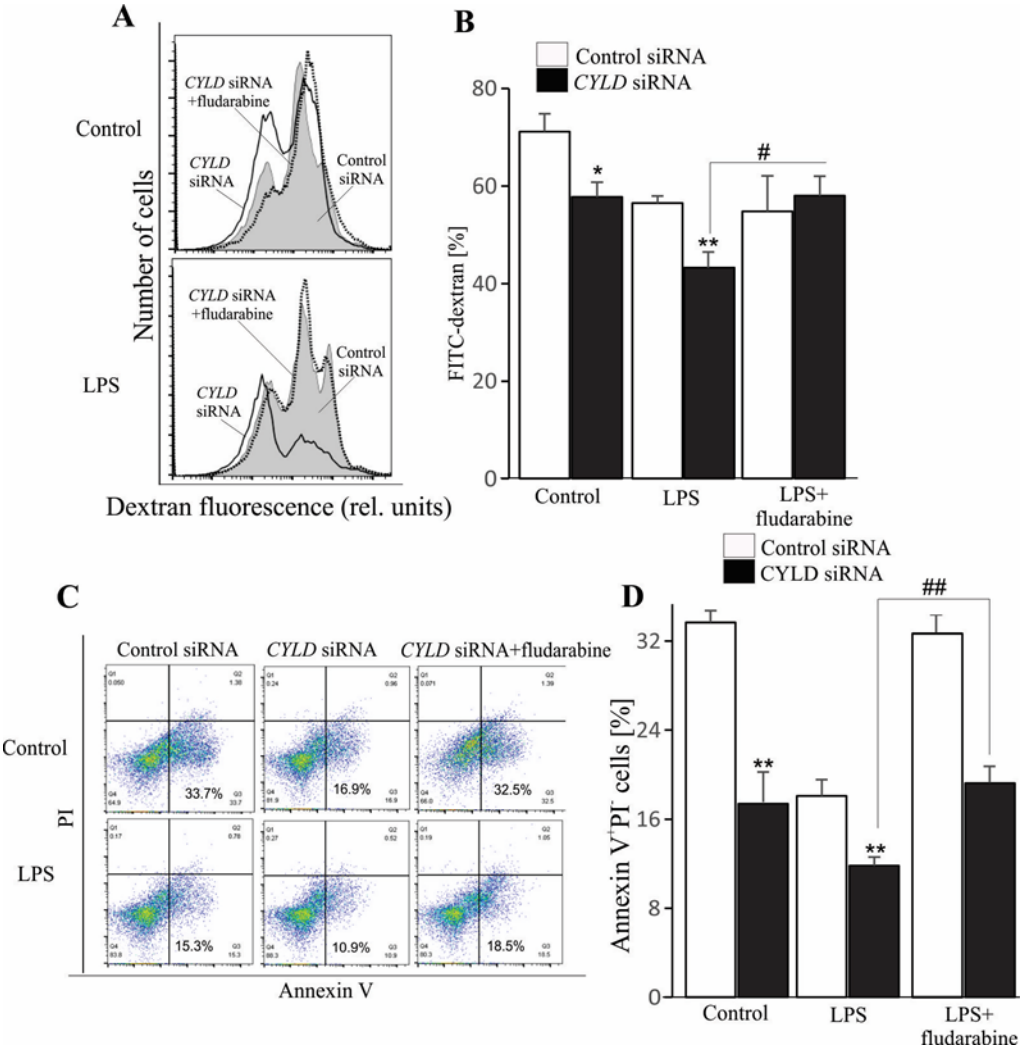


Figure 3.16. Effect of CYLD on the function of M-HP through STAT1 signaling

**(p<0.05), ** (p<0.01) indicates a statistically significant difference between the proportion of inactivated and non-inactivated M-HP macrophages that are positive for FITC- dextran and Annexin V+/PI-; #(p<0.05) and ##(p<0.01) indicate a statistically significant difference between LPS-activated M-HP and CYLD-inactivated in case of treatment or no treatment. with fludarabine positive for FITC-dextran and Annexin V^+/PI^-*

Similarly, when activated by LPS, macrophages were both able to release IL-6, and this cytokine concentration was even higher when macrophages were inactivated with *CYLD* gene compared with cells that

did not. treated with *CYLD* siRNA. Meanwhile, there was no difference in IL-6 concentration between gene-inactivated and non-*CYLD*-inactivated macrophages when fludarabine was present in cell culture (Figure 3.15).

In this study, transfecting *CYLD* siRNA into macrophages significantly reduced FITC-dextran uptake (Figure 3.16) and partially suppressed phagocytosis of AML cells (Figure 3.17) also further confirmed the phagocytosis of mature macrophages. However, in the presence of fludarabine, the role of CYLD on FITC-dextran uptake by macrophages was also eliminated (Figure 3.16).

By flow cytometry, it is possible to determine the percentage of cells that are positive for Annexin V^+/PI^- and thereby knew the viability of these cells. Specifically, *CYLD* siRNA-transfected macrophages significantly reduced the number of Annexin V^+/PI^- positive cells, but this effect was abrogated in the presence of fludarabine (Figure 3.16C-D). Thus, CYLD promotes FITC-dextran uptake and apoptosis of macrophage in the presence of STAT1 signaling.

3.6.3. Determining the role of CYLD in macrophage phagocytosis in AML

When M-AML macrophages treated with fludarabine were shown, the expression levels of *CYLD* were significantly increased (Figure 3.17A) and in the case of *STAT1* gene inactivated, the number of macrophages differentiated from AML subtypes, the expression of $CD11b^+CD68^+CD86^+$ and $CD11b^+CD68^+CD40^+$ is unchanged. Likewise, the levels of IL-6 and TNF-α secreted by M-AML by the subtypes were unchanged in the case of fludarabine-treated cells. This information confirmed that there is no relationship between *CYLD* expression and inflammatory responses in AML patients. Furthermore, while FITC-dextran uptake by M-AML was comparable to that of M-HP (Figure 3.17B), phagocytosis induced by M-AML was significantly lower than that of M-HP (Figure 3.17C-D). These data suggest that the phagocytic activity of M-AML was affected by the pathogenesis of the disease. Therefore, given information on the function of STAT1, as well as the relationship with CYLD, does inactivation of *STAT1* improve M-AML activity in the case of fludarabine-treated?

The results shown in Figure 3.18A showed that the FITC-dextran absorption of M-AML (M4/M5b form) was significantly increased compared with that of M-HP when treated with fludarabine, however this effect was eliminated when macrophages were transfected with *CYLD*

siRNA (Figure 3.18A). In addition, treatment with fludarabine also stimulated M-AML to eliminate white blood cells a lot for the M5b subtype and only partially for the M4 subtype (Figure 3.18B-C). Thus, CYLD plays an important role in the phagocytosis of M-AML (M4/M5b).

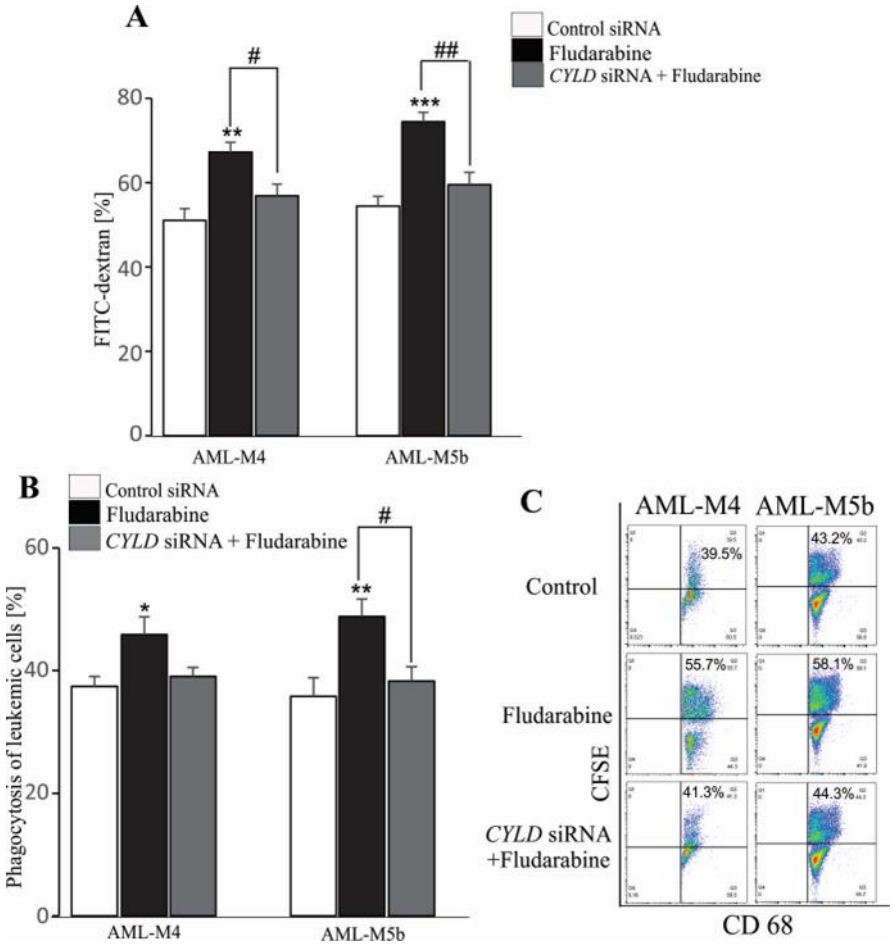


Figure 3.18. Effect of CYLD on the phagocytosis of M-AML

(p<0.05), **(p<0.01) and * (p<0.001) indicate a statistically significant difference between M-AML macrophages in FITC-dextran uptake and leukocyte phagocytosis when treated and untreated with fludarabine. #(p<0.05) and ## (p<0.01) indicated a statistically significant difference between inactivated and non-CYLD-inactivated M-AML in FITC-dextran uptake and white blood cell phagocytosis when treated by fuldarabine*

CHAPTER 4: DISCUSION

4.1. Evaluation of polymorphism, gene expression of *A20, CYLD* genes in myeloid leukemia patients

4.1.1. Evaluation of polymorphisms of A20/CYLD genes

Proteins A20, CYLD are known to be two DUB enzymes that play important roles in cleavage of polyubiquitin chains in target proteins in order to inhibit the activity of NF-κB signaling pathways in response to

stimulation signaling, thereby promoting cell growth, proliferation and apoptosis. Therefore, research to identify abnormalities in *A20* and *CYLD* genes in diseases, especially cancer, is done to find out the pathogenesis of the disease, and at the same time to develop effective treatment measures.

Several studies of *A20* in rheumatoid arthritis have demonstrated an association between several SNPs and disease risk. Whole-genome studies have shown that SNPs of *A20* gene are correlated with susceptibility to inflammatory and autoimmune diseases in humans. Because of the strong anti-inflammatory function of A20, the SNPs of this gene are both disease-related and reduce the expression or function of *A20*. A single nucleotide polymorphism (SNP) on *A20* gene reduces the binding of A20 to NF-κB subunits, which in turn leads to decreased functional expression of *A20* and leads to the pathogenesis of autoimmune disease and cancer.

In 2002, Gutierrez, after analyzing mutations based on leukocyte DNA, as well as tumor DNA and RNA from a German family of four generations, revealed frameshift mutations in *CYLD* gene (2253delG). Studies on *CYLD* gene have shown that *CYLD* is highly expressed during mitosis and is involved in the cell cycle. These findings set the stage for Yunfan Yang et al (2015) to explore the potential role of CYLD in regulating leukemic cell sensitivity to microtubule-targeting drugs. Dysregulation of CYLD has been shown to be one of the factors promoting the progression of acute and chronic lymphocytic leukemia. The function of CYLD in preventing lymphocytes from tumor formation is through the regulation of apoptosis, and cell necrosis.

In this study, a number of polymorphisms were found on *A20, CYLD* genes in myeloid leukemia. Specifically, there are 4 polymorphisms on *CYLD* gene in AML (p.E723E/c.G2411A; p.E731H/c.G2435C; p.E735H/c.G2445A and p.E747K/c.G2481A) and 2 polymorphisms on this gene in CML (p.V725V/c.T2417G; p.Q731H/c.G2435C); 4 polymorphisms on *A20* gene in AML (p.L335S/c.T1303C; p.K337Q/c.A1308C; p.K354N/c.G1361T; p.S376T/c.T1425A) and 2 polymorphisms on this gene in CML (rs37471883/p.G456V; rs200878487/p.S466R). In which, SNP rs200878487/p.S466R was identified by Yuli (2016) located at exon 7 on *A20* gene and is associated with the risk of oral squamous cell carcinoma; SNP rs374721883/p.G456V has also been found and documented as a mutation in *A20* gene in patients with mantle cell

lymphoma (International Patent, WO number 2016/071770); SNPs p.E723E/c/G2411A; p.E731H/c.G2435C and p.E735K/c.G2445A were also confirmed by Do Thi Trang et al (2022) to be mutations in *CYLD* gene in patients with idiopathic erythrocytosis; The remaining SNPs (p.V725V/c.T2417G; p.L335S/c.T1303C; p.K337Q/c.A1308C; p.K354N/c.G1361T; p.S376T/c.T1425A) are SNPs. new. Among these polymorphisms, only p.Q731H polymorphism on *CYLD* gene had a significant difference in GC genotype between myeloid leukemia patients and healthy subjects when compared with the GG genotype. Moreover, the frequency of C allele is also associated with a reduced risk of myeloid leukemia. Besides, based on the results of analysis by PolyPhen-2 software to predict the influence of polymorphism/mutations on protein structure and function, it was shown that at the point of polymorphism c.G2435C did. change the amino acid Glutamine (Q) at position 731 to Histidine (H) in the protein chain, simultaneously. This position has a relatively high influence on protein structure and function with the HumDiv and HumVar reliability scores reaching 0.989 and 0.638, respectively. These results initially provide useful information to promote disease detection and even contribute to finding more effective disease therapies in the future.

4.1.2. Gene expression levels in myeloid leukemia patients

In B cells, A20 limits the activation of signaling pathways by physiological inducers involved in the entry of pathogens into the body. In aged mice, when *A20* is inactivated in B cells, it can induce an inflammatory syndrome with autoimmune manifestations such as chronic chronic inflammation, elevated IL-6 concentration, significantly increased plasma cell count and presence of specific autoantibodies. Thus, when B cells are more responsive to environmental stimuli due to reduced A20 function, it has been shown that there is an association between inherited gene mutations or polymorphisms in *A20* and different autoimmune diseases in humans.

CYLD is the first DUB enzyme found in benign cylindrical tumors, but it has recently been recognized that CYLD has a role in regulating signaling pathways, involved in many physiological and pathological processes. It induces apoptosis and inhibits tumor formation. In addition, *CYLD* expression is down-regulated in lung cancers, breast cancers, colon cancers, hepatocellular carcinomas and other solid tumors. Currently, studies on

CYLD in hematological malignancies are still very limited. The absence of CYLD may be a prognostic marker of the status of some hematological malignancies. To date, the regulatory mechanism of CYLD on cellular function is still unclear and requires further investigation. Elucidating the mechanisms that regulate their signaling pathways may help develop molecularly targeted therapies for hematological malignancies.

Thus, in studies on patients, especially cancer patients, the expression levels of *A20* and *CYLD* genes is often reduced. In this study, *A20, CYLD* gene expression was also significantly reduced in myeloid leukemia patients.

Genes of the *STAT* family are extensively studied in a variety of diseases and their expression is often increased in many cancer patients. *STAT1* gene has a tyrosine phosphorylation-dependent expression levels and, when expressed, inhibits tumor growth and promotes cell death. In STAT1-deficient mice, tumor growth was stronger than that of healthy mice, and at the same time, mice would also be more susceptible to viral diseases and not respond to harmful effects of virus, even death while this amount of virus does not affect the normal body. Meanwhile, STAT3 and STAT5 are two important proteins involved in cancer development. Continuous STAT3/5 activation promotes chronic inflammation, increasing the susceptibility of healthy cells to carcinogens. Besides, according to Giang et al (2021), the expression levels of *STAT1* gene was significantly higher than that of the control group and this difference was statistically significant ($p < 0.05$). However, in this study, the expression of *STAT1*, *STAT3* genes in myeloid leukemia patients did not change significantly compared with the healthy group.

Inflammation is a process that promotes tumor growth through direct or indirect effects of cytokines, chemochines, and growth factors on tumor cells. In normal blood cells, activation of cell surface receptors by cytokines, chemokines, and growth factors can modulate signal transduction pathway activity. However, several reports have confirmed that abnormalities in signaling pathways can induce leukemia and that cytokines are considered to have profound effects on the progression of malignancies such as malignancies. myeloid leukocytes. While inflammatory mediators such as IL-1β, TNF-α, and IL-6 tend to be elevated in AML patients, anti-

inflammatory mediators such as TGF-β and IL-10 appear to impede the progression of the disease. development of AML.

According to Beatriz *et al*., the levels of proinflammatory cytokines such as IL-6 and TNF-α or the anti-inflammatory cytokine IL-10 were elevated in AML patients in both groups of patients over 65 years old and under 65 years old. Low IL-6 and high IL-10 concentration are considered to be favorable prognostic factors for survival in AML patients. However, the interaction between proinflammatory and anti-inflammatory cytokines in AML may also stimulate the proliferation, survival, and drug resistance of leukemic cells. In addition, Nievergall *et al.* have also shown that high serum TNF-α and IL-6 concentration are considered biomarkers in the diagnosis of CML disease. Serum IL-2 and IL-6 were significantly increased in CML compared with the control group. Similarly, in this study, IL-6 and TNF-α concentration were found to be significantly elevated in myeloid leukemia patients compared with healthy subjects. This proves that in these patients the inflammatory process is quite severe, which can weaken the patient's immune system.

Thus, the results obtained on polymorphisms of A20, CYLD genes, expression levels of *A20, CYLD* genes all decreased in myeloid leukemia patients compared with healthy people, as well as IL-6 cytokine levels. and elevated TNF-α in these patients are necessary information for further analysis of the association between them, thereby determining the pathogenesis of myeloid leukemia.

4.1.3. Association between SNPs, expression of *A20, CYLD genes, expression of STAT1* and STAT3 genes, and cytokine levels in myeloid leukemia patients

A20, CYLD is a DUB protein that plays a role in regulating various signaling pathways that regulate physiological processes within the body, from immune and inflammatory responses to cell cycle progression, spermatogenesis and bone formation. Specifically, A20, CYLD downregulates NF-κB signaling. In it, the family of transcription factors mediates the expression of a large number of genes in the inflammatory response. Many studies show that the expression levels of *A20* and *CYLD* genes are often low in some cancer patients, and when these genes are inactivated, they promote the proliferation of cancer cells.

Analysis of the relationship between *A20, CYLD* gene polymorphisms, the expression of *A20, CYLD* genes and *STAT1* signaling gene, as well as the levels of cytokines IL-6 and TNF-α secreted in myeloid leukemia patients showed that, only found a close relationship between polymorphisms at the p.Q731H position on *CYLD* gene and the levels of cytokine IL-6 secreted by patients with AML. Despite this, *A20, CYLD* gene is still a potential target for the study and assessment of disease progression, and the results of this evaluation can provide useful information for studies on the role of the role of A20, CYLD in myeloid leukemia; From there, it is possible to find ways to assess the condition of the disease, as well as orient effective treatment measures.

4.2. Determining the role of A20, CYLD in proliferation and apoptosis of K562 cells

Vincristine is a substance extracted from periwinkle (Cantharanthus roseus) that has an effective inhibitory effect on some cancers such as mononucleosis, breast cancer, liver cancer, ovarian cancer, and cancer. head and neck cancer, testicular cancer, and lymphoid leukemia. The mechanism of action of vincristine was determined to be similar to that of cell cycle-specific antineoplastic agents. During treatment, vincristine is often toxic to the nervous system, which then releases proinflammatory mediators such as IL-6 and TNF-α when the cells are activated. By activating the NF-κB/STAT signaling pathway, vincristine is implicated as a partial regulator of antitumor activity. Several studies have also shown that vincristine can reduce peripheral blood leukocytes and marrow failure, and can significantly prolong the life of mouse lymphoid leukemia transplant models. According to Paloma *et al*. (2011), Vincristine can significantly reduce viability of K562 cells by blocking cell cycle progression.

In this study, an inhibitory role of vincristine on K562 proliferation and survival was determined. When co-treated with vincristine, the mRNA expression levels of *A20, CYLD* increased significantly in K562 cells, while in PBMCs the mRNA expression of these two genes increased only slightly. In case *A20* gene is inactivated, the inhibitory effect on the proliferation and survival of K562 is no longer there, while only the proliferation of K562 is inhibited in the presence of the CYLD gene when this cell is processed by vincristine. Compared with A20, CYLD had no significant effect on K562

apoptosis. As such, vincristine inhibits K562 proliferation and survival through enhancing *A20* expression.

4.3. Determining the role of the CYLD gene in M-CSF-differentiated macrophages through STAT1 signaling

CYLD inhibits maturation and cytokine secretion as well as induces phagocytosis and apoptosis of macrophages through STAT1 signaling. In the absence of CYLD, the expression of CD40 and CD86 molecules, as well as the release of IL-6 by macrophages were significantly enhanced. Meanwhile, the uptake of FITC-dextran and Annexin V/PI of these macrophages is less than that of macrophages without *CYLD* gene inactivation. Therefore, it could be seen that CYLD promoted macrophage phagocytosis, however in the presence of fludarabine, this effect of CYLD on macrophages was abolished. Thereby, it is confirmed that the inhibitory effect of CYLD on macrophage function depends on the activation of STAT1 signaling for the first time in this study. Unlike M-HP, when M-AML (M4/M5b) is treated with fludarabine, elevated *CYLD* expression activates FITC-dextran uptake and phagocytosis of M-AML. In addition, CYLD performs a pro-phagocytic role of macrophages from the M4/M5b subtype through the STAT1 pathway.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

** Association between polymorphisms/mutations in A20, CYLD genes, signal gene expression, and cytokine levels in myeloid leukemia*

- 5 polymorphisms have been identified on *CYLD* gene; 6 polymorphisms on the A20 gene in myeloid leukemia patients. In particular, *CYLD* gene polymorphism at the p.Q731H position in AML and CML disease has a significant difference in GC genotype compared with GG genotype when compared with healthy group.

- It has been determined that the expression levels of *A20, CYLD* are significantly reduced in myeloid leukemia patients compared with healthy people; Meanwhile, the levels of IL-6, TNF-α were noted to be increased in these patients.

- A close association has been determined between *CYLD* gene polymorphism at the p.Q371H position and the IL-6 cytokine levels in myeloid leukemia patients.

* *The role of A20, CYLD genes in cell function*

- The role of *A20* and *CYLD* genes has been identified in inhibiting the proliferation and promoting the apoptosis of vincristine-treated K562 cells.

- The role of *CYLD* gene has been identified in the activation of macrophages differentiated from healthy human PBMCs (M-HP) in the presence of *STAT1* expression, while *CYLD* expression enhances the activation of PBMCs. activity of M-AML macrophages when treated with fludarabine.

5.2 Recommendations

Continue to evaluate the effectiveness of using M-CSF and vincristine-differentiated cells in developing a therapeutic approach for myeloid leukemia patients in combination with target *A20, CYLD* gene therapy or target cell therapy related to *A20, CYLD.*

NEW FINDINGS OF THE THESIS

1. Some polymorphisms of *A20* and *CYLD* genes have been detected, and also an association has been identified between polymorphism at position p.Q371H and IL-6 levels in myeloid leukemia patients.

2. The role of *A20* and *CYLD* genes in the proliferation and apoptosis promotion of K562 cells has been determined, as well as, the role of CYLD has been clarified in activating macrophages differentiated from PBMCs of AML patients treated with fludarabine.

LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

1. **Nguyen Thanh Huyen**, Nguyen Thy Ngoc, Nguyen Hoang Giang, Do Thi Trang, Ha Hong Hanh, Vu Duc Binh, Nguyen Van Giang, Nguyen Xuan Canh, Nguyen Thi Xuan (2023). *CYLD stimulates macrophages phagocytosis of leukemic cells through STAT1 signaling in acute myeloid leukemia*. PLOS ONE 18(8):e0283586.

2. Nguyen Huy Hoang, **Nguyen Thanh Huyen**, Do Thi Trang, Nguyen Xuan Canh, Can Van Mao, Mentor Sopjani, Nguyen Ba Vuong, Nguyen Thi Xuan (2022). *Effects of Vinblastine and Vincristine on the function of chronic myeloid leukemic cells through expression of A20 and CYLD*. Cellular and Molecular Biology. 68(10): 47-53.

3. **Nguyen Thanh Huyen**, Nguyen Hoang Giang, Nguyen Thi Xuan. *Expression of deubiquitinase genes and inflammatory response in myeloid leukemia.* Vietnam Journal of Biotechnology. 20(3): 401-408.

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