Studying on single nucleotide polymorphisms of *A20* gene in non-Hodgkin lymphoma patients

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Summary

Objective: To identify single nucleotide polymorphisms (SNPs) in exon 7 of *A20* gene in non-Hodgkin lymphoma (NHL) patients and evaluate the association between these SNPs and some prognostic indicators in NHL patients. *Subject and method:* 83 patients were diagnosed with NHL and 83 healthy controls. Sanger sequencing technique was used to analyze SNPs in exon 7 of *A20*. *Result:* Seven SNPs were identified in the patient sample including *A20*: c.1308 A>C (p.K337Q), c.1341 C>T (p.L348F), c.1356 T>C (p.Y353H), rs2114496684 C>T, rs1776324713 A>T, c.1378 G>C (p.S360T) and rs2114496220 T>A. In which, two genotypes CT and GC of SNP rs2114496684C>T and c.1378G>C respectively had higher frequencies in the NHL group compared to the control group with a statistically significant ($p<0.05$). Four SNPs: c.1308A>C (p.K337Q), c.1341C>T (p. L348F), c.1356T>C (p.Y353H) and rs2114496684C>T (p.Q357*) were predicted to be associated with a high risk of NHL using Mutation Taster, CADD and Polyphen_2 bioinformatics software. There was no statistically significant difference in the genotype distribution of these SNPs with some prognostic indicators such as stage, progression, LDH and beta2-microglobulin index in NHL patients. *Conclusion:* Seven SNPs were identified in exon 7 of *A20* in the NHL patient group, of which two SNPs with heterozygous genotypes appeared with a higher frequency in the patient group compared to the control group with statistical significance, and four SNPs were predicted to be associated with hight risk of NHL. There was no statistically significant association between the genotype distribution of these SNPs and some prognostic indicators in NHL patients.

Keywords: Non-Hodgkin lymphoma, SNPs in exon 7 of *A20* gene.

I. BACKGROUND

Non-Hodgkin lymphoma (NHL) is a neoplasm of the lymphoid tissues originating from B cell precursors, mature B cells, T cell precursors and mature T cells. NHL comprises various subtypes, each with different epidemiologies, etiologies, immunophenotypic, genetic, clinical features, and response to therapy. It can be divided into two groups, 'indolent' and 'aggressive', based on the disease's prognosis[1]. According to GLOBOCAN 2020, NHL ranks 13th both incidence and mortality rates in Vietnam among all cancers[2]. There are many methods for treating NHL including: Chemotherapy, radiotherapy, targeted therapy and stem cell transplantation... However, there are also cases that respond poorly or do not respond to these treatments. Therefore, understanding the molecular mechanism of NHL plays important role in assessing the prognosis of the disease as well as targeting treatment in intracellular signaling pathways to improve clinical outcomes and overcome resistance.

A20 is a deubiquitinase (DUB) gene that inhibits activation of the NF-κB pathway, thereby functioning as a tumor suppressor gene[3]. Mutations that inactivate *A20* lead to activation of the NF-κB

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pathway, which is one of the mechanisms of carcinogenesis. Based on the structure of *A20* gene, exon 7 plays an important role in encoding the ZF4 region of protein A20, which promotes the polyubiquitination of RIP1 by lysin48 linkage, leading to the activation of RIP1 degradation at the proteasome and thus inhibiting NF-κB activation. In particular, recent studies have shown that variants in exon 7 of *A20* are associated with a number of malignancies, including hematological malignancies. Until now, the etiology and pathogenesis of NHL are unclear, so determining the role of SNPs in *A20* gene is important to clarify the pathogenesis towards applying targeted treatments to improve the effectiveness of disease treatment. Therefore, this study was conducted: *To identify SNPs in exon 7 of A20 gene in NHL patients and evaluate the relationship between these SNPs and some prognostic factors in NHL patients.*

II. SUBJECT AND METHOD

2.1. Subjects

Total peripheral blood samples from 126 participants, divided into two groups: NHL group and healthy group.

Inclusion criteria: NHL patients' group: Including 83 patients with newly diagnosed by histopathology and immunohistochemistry and untreated. No history of chemotherapy or radiation therapy before; Healthy group: 83 volunteer blood donors without clinical symptoms or history of NHL and autoimmune diseases.

Exclusion criteria: Patients with autoimmune diseases: Systemic lupus erythematosus, scleroderma, rheumatoid arthritis, concomitant cancer or without testing for the *A20* polymorphic gene were not included in this study.

2.2. Method

Study design: The study was conducted using a cross-sectional descriptive method and case-control study. Convenience samples were used to recruit participants.

Time and place: The NHL patients were collected at Military Hospital 103, Vietnam Military Medical University and the National institute of Hematology and Blood transfusion. The healthy group was collected at the Hematology-Transfusion Center, Military Hospital 103 from March 2021 to June 2022.

Research process:

All eligible patients were carefully examined for medical history, clinical symptoms and laboratory tests for disease diagnosis, disease stage and type of histopathology. To analyse the polymorphism in exon 7 of *A20,* peripheral blood samples of the study subjects was collected and performed on following these steps:

Step 1: Genomic DNA was isolated from 4mL peripheral blood samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Step 2: The target gene segment was amplified by the polymerase chain reaction (PCR) technique using the following primer pair:

A20-forward: 5'-TGAGCTAATGATGTAAAATCTTGTG-3'

A20-reverse: 5'-AGGAGGCCTCTGCTGTAGTC-3'

The PCR product was electrophoresed in 1.2% agarose gel in a tank containing 1X TAE solution for 30 minutes, then stained with ethidium bromide and visualized under UV light. PCR products were purified using Thermo's GeneJET kit according to the manufacturer's instructions.

- Step 3: The PCR products were sequenced by the Sanger method. BioEdit software was used for the initial sequence analysis. This sequence was compared with the reference sequence published in the NCBI databases, thereby determining whether the single nucleotide polymorphisms (SNPs) were novel or previously published variants.

The pathogenicity of candidate variants was further evaluated by using combined annotation dependent depletion (CADD), Mutation Taster and the PolyPhen- 2.

The entire process from DNA isolation to gene sequencing is performed at Institute of Genome

Research, Vietnam Academy of Science and Technology.

LDH, beta2-microglobulin were analyzed by the AU5800 testing machine at the Biochemistry Department of the National Institute of Hematology and Military Hospital 103.

Indicators	Unit	Reference values
LDH	U/L	0 - 247
Beta2-microglobulin	mg/L	0,8 – 2,4

2.3. Statistical analysis

Using SPSS 20.0 software. Statistical analysis was carried out using frequency, percentage, mean and comparison of categorical variables using the $\chi 2$ test or Fisher's exact test. The percentage was rounded to one decimal place. Differences were considered statistically significant when $p < 0.05$.

2.4. Ethics statement

All patients and volunteers provided written informed consent to participate in this study. Person care and experimental procedures were performed according to Vietnamese law for the welfare of humans and approved by the Ethical Committee of Military hospital 103, No. 14/2021/CNChT-HĐĐĐ, 30/ 02/ 2021.

III. RESULT

3.1. Characteristics of NHL patients

The mean age of NHL patients in the study was 56.4 ± 16.1 years, the oldest age was 86 years, the youngest was 17 years. male 63.9%, female 36.1%.

Table 1. Characteristics of stage of the NHL patients

Stage	n	Percent (%)
Stage I	20	24,1
Stage II	14	16,9
Stage III	22	26,5
Stage IV	27	32,5

Comments: 41% patients in stage I, II and stage III, IV accounted for 59%, of which patients in stage IV had the highest rate (32.5%).

A20 polymorphism and association of SNPs with some prognostic factors of NHL patients

The results of the *A20* exon 7 sequencing were compared with the *A20* sequence with the code NM_001270508.2 on GenBank. Seven single nucleotide polymorphisms were identified in the patient sample included *A20:* c.1308A>C (p.K337Q), c.1341C>T (p.L348F), c.1356T>C (p.Y353H), rs2114496684C>T, rs1776324713A>T, c.1378G>C (p.S360T) and rs2114496220 T>A (Figure 1).

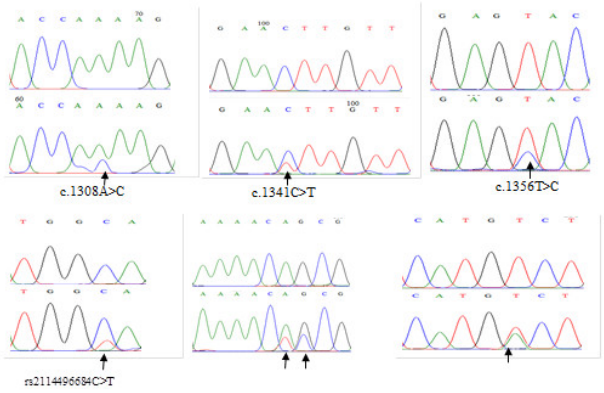

Figure 1. Seven SNPs in exon 7 of A20.

Table 2. Prediction deleterious non-synonymous SNPs in exon 7 of *A20* by bioinformatics analyses

SNPs	Amino acid change	Variant type	Mutation Taster		CADD		Polyphen_2	
			Score	Pre-diction	Score	Pre-diction	Score	Pre-diction
c.1308 A>C	p.K337Q	Missense	1	Deleterious	22,7	Quite likely deleterious	0,707	Possibly damaging
c.1341 C>T	p. L348F	Missense	1	Deleterious	27,5	Likely deleterious	0,999	Damaging

SNPs	Amino acid change	Variant type	Mutation Taster		CADD		Polyphen_2	
			Score	Pre-diction	Score	Pre-diction	Score	Pre-diction
c.1356 T>C	p.Y353H	Missense	1	Deleterious	25,7	Likely deleterious	1,000	Damaging
rs2114496684 C>T	p.Q357*	Stop gained	1	Deleterious	39,0	Damaging	-	-
rs1776324713 A>T	p.S360C	Missense	0,59	Polymorphism	21,9	Quite likely deleterious	0,124	Benign
c.1378 G>C	p.S360T	Missense	1	Deleterious	0,033	Likely benign	0,000	Benign
rs2114496220 T>A	p.S397T	Missense	0.52	Polymorphism	17	Likely benign	-	-

Comments: There were six missense variants, leading to the replacement of old amino acids with new amino acids. One variant rs2114496684 C>T (p.Q357*) was a nonsense variant. To determine susceptibility to NHL by evaluating the deleterious effects of the SNPs in the *A20* gene by using bioinformatics: Mutation Taster, CADD and Polyphen_2. The results showed that c.1308A>C was quite likely deleterious, c.1341C>T and c.1356T>C were likely deleterious, the rs2114496684 SNP was predicted to be deleterious.

Table 3. Comparing genotype distribution of SNPs in exon 7 of *A20* gene in NHL patients and controls

SNPs	Genotype	NHL group (n= 83)	Control group (n = 83)	p	p (HWE)
c.1308A>C	AC	4 (4.8%)	0 (0%)	0.12	0.98
	AA	7 (96.83%)	83 (100%)		
c.1341C>T	CT	4 (4.8%)	0 (0%)	0.12	0.98
	CC	79 (96.83%)	83 (100%)		
c.1356T>C	TC	4 (4.8%)	0 (0%)	0.12	0.98
	TT	79 (96.83%)	83 (100%)		
rs2114496684C>T	CT	11 (9.52%)	0 (0%)	0.001	0.90
	CC	72 (90.48%)	83 (100%)		
rs1776324713A>T	AT	4 (4.8%)	0 (0%)	0,12	0,98
	AA	79 (96.83%)	83 (100%)		
c.1378G>C	GC	6 (7.2%)	0 (0%)	0.013	0.97
	GG	77 (92.8%)	83 (100%)		
rs2114496220T>A	TA	2 (2.4%)	4 (4.8%)	0.68	0.97
	TT	81 (97.6%)	79 (95.2%)		

p: Fisher's Exact test

Comments: The heterozygous CT and GC genotypes of SNP rs2114496684C>T and c.1378G>C, respectively, were significantly higher in NHL patients than in healthy controls (p<0.05). The genotype distribution of the seven SNPs, was in good agreement with Hardy-Weinberg equilibrium (HWE, p>0.05).

Table 4. The relationship between the genotype distribution of SNPs in exon 7 of *A20* and stage in NHL patients (n = 83)

SNPs	Genotype	Stage I, II (n = 34)	Stage III, IV (n = 49)	p	OR (95%CI)
c.1308A>C	AC	3 (8.8%)	1 (2.0%)	0,3	4.65 (0.46- 46.69)
	AA	31 (91.2%)	48 (98.0%)		
c.1341C>T	CT	3 (8.8%)	1 (2.0%)	0,3	4,65 (0.46- 46.69)
	CC	31 (91.2%)	48 (98.0%)		
c.1356T>C	TC	3 (8.8%)	1 (2.0%)	0,3	4.65 (0.46- 46.69)
	TT	31 (91.2%)	48 (98.0%)		
rs2114496684C>T	CT	5 (14.7%)	6 (12.2%)	0,7	1.24 (0.35- 4.43)
	CC	29 (85.3%)	43 (87.8%)		
rs1776324713A>T	AT	3 (8.8%)	1 (2.0%)	0,3	4.65 (0.46- 46.69)
	AA	31 (91.2%)	48 (98.0%)		
c.1378G>C	GC	5 (14.7%)	1 (2.0%)	0,08	8.27 (0.92- 74.39)
	GG	29 (85.3%)	48 (98%)		
rs2114496220T>A	TA	1 (2.4%)	1 (4.8%)	1,0	1.46 (0.09- 24.09)
	TT	33 (97.6%)	48 (95.2%)		

p: Fisher's exact test

Comments: There were no statistically significant differences between the genotype distribution of SNPs in exon 7 of *A20* and stages in NHL patients.

Table 5. The relationship between the genotype distribution of SNPs in exon 7 of *A20* and the disease's advance in NHL patients (n = 83)

SNPs	Genotype	Aggressive (n = 70)	Indolent (n = 13)	p
c.1308A>C	AC	4 (5.7%)	0 (0%)	1.0
	AA	66 (94.3%)	13 (100%)	
c.1341C>T	CT	4 (5.7%)	0 (0%)	1.0
	CC	66 (94.3%)	13 (100%)	
c.1356T>C	TC	4 (5.7%)	0 (0%)	1.0
	TT	66 (94.3%)	13 (100%)	
rs2114496684C>T	CT	10 (14.3%)	1 (7.7%)	1.0
	CC	60 (85.7%)	12 (92.3%)	
rs1776324713A>T	AT	4 (5.7%)	0 (0%)	1.0
	AA	66 (94.3%)	13 (100%)	

SNPs	Genotype	Aggressive (n = 70)	Indolent (n = 13)	p
c.1378G>C	GC	6 (8.6%)	0 (0%)	0.58
	GG	64 (91.4%)	13 (100%)	
rs2114496220T>A	TA	2 (2.9%)	0 (0%)	1.0
	TT	68 (97.1%)	13 (100%)	

p: Fisher's Exact test

Comments: The genotype distribution of SNPs in exon 7 of *A20* in the two groups indolent and aggressive NHL had no statistically significant difference.

Table 6. The relationship between the genotype distribution of SNPs in exon 7 of *A20* and the subclinical symptoms in NHL patients (n = 83)

SNPs	Genotype	LDH (U/L) *Trung vị (25%- 75%)*	p	Beta2-microglobulin (mg/L) *Trung vị (25%- 75%)*	p
c.1308A>C	AC	814 (432-1688)	0,093	1.58 (1.22-4.39)	0.52
	AA	419 (303-598)		2.08 (1.56-3.58)	
rs2114496684C>T	CT	472 (332- 852)	0,45	1.87 (1.20- 3.18)	0.38
	CC	423 (303- 598)		2.04 (1.58- 3.77)	
c.1378G>C	GC	500 (401-1101)	0,20	1.58 (1.17-3.69)	0.39
	GG	419 (303-598)		2.08 (1.59-3.64)	
rs2114496220T>A	TA	338 (146-530)	0,42	3.61 (1.87-5.35)	0.35
	TT	427 (319-599		2.01 (1.54-3.25)	

p: Mann-Whitney test.

Comments: There were no statistically significant differences between the genotype distribution of SNPs in exon 7 of *A20* and LDH, beta2-microglobulin concentration in NHL patients.

IV. DISCUSSION

The results of sequencing exon 7 of *A20* gene identified seven SNPs, of which two SNPs including rs2114496684C>T, c.1378G>C with heterozygous CT, GC genotypes had frequency at 9.52% and 7.2% respectively in the NHL group, but did not appear in the control group, this difference was statistically significant with p<0.05 (Table 3). To determine the susceptibility of *A20* gene SNPs to NHL, through bioinformatics softwave, predicted four harmful SNPs were identified including c.1308A>C (p.K337Q), c.1341C>T (p. L348F), c.1356T>C (p.Y353H) and rs2114496684C>T (p.Q357*).

To date, variants in exon 7 of *A20* gene have mainly reported in autoimmune diseases and some hematological malignancies, there have been few studies in the NHL. In a study on polycythemia vera patients, *A20* gene sequencing, Do Thi Trang identified three SNPs in exon 7, including rs776591390 G>T, rs141376366 G>A and rs745670694 G>A, the heterozygous TG genotype of SNP rs776591390 genotypes had frequency at 1.29% in the disease but did not appear in the control group, while the GA and AG genotypes of the two SNPs rs141376366 and rs745670694 respectively only appeared in the control group but did not appear in the disease group, however, the genotype distribution did not have a statistically significant

difference. To predict the susceptibility of these SNPs to the disease using the Polyphen-2 bioinformatics software, the author has not recorded any correlation on the influence of these SNPs on polycythemia vera[4].

Nguyen Thanh Huyen (2023) studied on 92 acute myeloid leukaemia (AML) and 50 chronic myeloid leukemia (CML) patients, the results showed that in AML patients four nucleotides 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) were changed and were all non-synonymous SNPs that caused changes in amino acids and polymorphisms at p.L335S, p.K337Q, p.K354N, p.S376T, genotypes: TC, AC, GT and TA had statistically significant differences when compared with genotypes TT, AA, GG and TT respectively ($p<0.05$). In addition, 2 nucleotides in exon 7 (rs374721883/p.G456V; rs200878487/p.S466G) were changed and were both non-synonymous SNPs in CML patients[5].

According to Chang Y.C three mutations/polymorphisms c.1081 G>A (p.E361K), c.1398 C>G (p.S466R) (rs200878487), and c.1760 C>T (p.P587L) (rs 150056192) found in patients with oral squamous cell carcinoma are all located in exon 7[6]. Similarly, in a study of T-Acute Lymphoblastic Leukemia (T-ALL) patients, three polymorphisms g.3033 C>T, g.3910 G>A and g.3904 A>G were found in exon 7 of the *A20* gene. Of these, g.3033 C>T was the most common SNP in T-ALL, accounting for 60%. These mutations all caused amino acid changes[7].

A20 (*TNFAIP3*- tumor necrosis factor alpha-induced protein 3) encodes the protein A20, an essential negative regulator of NF-κB - mediated inflammation. *A20* is a potent inhibitor of NF-κB signaling in response to TNF-α, numerous other proinflammatory cytokines, and innate immune receptor activation via its ubiquitin-editing domains. *A20* dysfunction is associated with a number of other autoimmune and autoinflammatory diseases as well as lymphomas and other cancers[8]. In addition, several studies have demonstrated that *A20* is a tumor suppressor. When reexpressed in a lymphoma derived cell line in which *A20* is functionally lost, it resulted in cell growth inhibition and apoptosis induction accompanied by downregulation of NF-κB activity. Dysregulated NF-κB signaling due to loss of *A20* function has been implicated in the pathogenesis of B-cell NHL by overactivating NF-κB[9]. Recent studies have shown that both synonymous and non-synonymous mutations affect mRNA stability, mRNA processing, and mRNA maturation, which consequently affect allelic mRNA expression, protein abundance and function[10].

Research on the association between polymorphism of *A20* gene and clinical characteristics was published such as: Shaat RM studied on diffuse large B-cell NHL (DLBCL), the results: GA genotype of *A20* in DLBCL patients did not have a statistically significant difference according to disease stage, IPI prognostic index as well as group B symptoms. It is noteworthy in the study that this mutation of *A20* gene is related to the prognosis of patient survival time, specifically, patients with GA genotype have shorter disease free survival time and overall survival time compared to those with GG genotype[11]. Another study by Elbaz O showed different results on the association of *A20* gene SNP with clinical features in DLBCL patients: CT genotype of SNP rs387907272 was more common in late stages, this difference was statistically significant with $p<0.001$[12]. In our study, no statistically significant differences were found in the genotype distribution of SNPs in exon 7 of *A20* in disease stages, advanced of NHL, as well as LDH and beta2-microglobulin concentrations. This may be due to the small sample size of our study, while NHL has various subtypes, so further research with larger sample sizes is needed.

V. CONCLUSION

Seven SNPs were identified in exon 7 of *A20* gene in the NHL patient group, of which two SNPs with heterozygous genotypes appeared with a higher frequency in the patient group compared to the control group with statistical significance, and

four SNPs were predicted to be associated with an increased risk of NHL susceptibility.

No statistically significant association was found between the genotype distribution of these SNPs and some prognostic indicators in NHL patients.

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