RESEARCH ARTICLE

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The Effect of *Caspase 8, 9* Gene Polymorphisms on Non-Hodgkin Lymphoma Susceptibility and Clinical/Pathological Features

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Abstract

Background: Caspases (CASPs) are the main executors of the apoptotic process. Studies to date have shown the role of caspase-8 (*CASP8*) and caspase-9 (*CASP9*) in carcinogenesis. Therefore, the aim of this study was to investigate the associations between *CASP9*-rs4233532, *CASP9*-rs4646018, and *CASP8*- rs1045485 gene polymorphisms and non-Hodgkin lymphoma (NHL) susceptibility in an Iranian population-based study. Moreover, it was examined whether such the genotype of these polymorphisms is related with clinicopathological characteristics of NHL. **Methods:** 175 patients with NHL and 175 age- and sex-matched controls were enrolled in this study. We determined the genotypes of single nucleotide polymorphisms (SNPs) from CASP genes with Tetra ARMS-PCR (Amplification refractory mutation system) method. **Results:** Statistically significant association were observed between *CASP9*-rs4646018 and increased risk of NHL under codominant CC, codominant TC, and dominant TC+CC genetic models. Our results showed that the A allele of *CASP8*-rs1045485 was a protective factor for NHL and GArs1045485 genotype significantly reduced risk of NHL. In contrast, *CASP9*- rs4233532 was not linked to NHL susceptibility. No relationship was detected between *CASP8*-rs1045485 and *CASP9*-rs4233532 and NHL clinicopathological characteristics, however genetic variation in *CASP9*-rs4646018 was associated with histology, treatment and radio therapy of NHL. **Conclusions:** Our study presented that the *CASP8*- rs1045485 and *CASP9*-rs4646018 polymorphisms could affect the risk of NHL in Iranian populations which was the first report to show the significant relationship between rs1045485, rs4646018 polymorphisms and NHL susceptibility. Replication large-scale case-control studies in different ethnicities are warranted to verify these findings.

Keywords: Caspase-Non-Hodgkin lymphoma- polymorphism- apoptosis- cancer

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Introduction

Non-Hodgkin lymphoma (NHL) is a neoplasm of the lymphoid hematopoietic system in the world, menacing children's health (Zhu et al., 2021). This disorder mostly implicates lymph nodes, spleen, and other immune organs, causes to decline of immune function in patients, and decreases the protection against such unfavorable foreign stimuli including pathogens. NHL comprises of various subtypes with different etiologies and response to therapy which divided into B, T and NK cell types. The development to NHL is related with multi-level factors(Liu et al., 2020). Therefore, finding the NHL predisposing factors is notably effective in the prevention, recognition, and therapy of NHL (Mei et al., 2020; Sargazi et al., 2021). Gene polymorphism is defined as the individual variations in alleles or genotypes at a specific locus within a population, and such variations may be associated to the development of some diseases (Teimoori et al., 2019; Salimi et al., 2020). It has been approved that single nucleotide polymorphism (SNP), the most prevalent form of gene polymorphism, affect the susceptibility of lymphoid hematopoietic system diseases.(Ten et al., 2017; Yaghmaei et al., 2020).

Apoptosis can be determined as the principal controlled biological procedure in cell death. Moreover, it has substantial role in cell growth and development, tumor suppression, immunity, tissue homoeostasis, and elimination of defective and abnormal cells(Heidari et al., 2020). Dysregulation of apoptosis leads to many human

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disorders such as cancers. In addition, it is an essential mechanism to decrease proliferation of a cancerous cell (Wiman and Zhivotovsky, 2017). Caspases (CASPs) are member of the cysteine-aspartic acid proteases and play a central role in apoptosis. Based on their proapoptotic functions, CASPs can be classified into two classes and are either initiators or effectors. The initiator CASPs convey apoptotic signals and *CASP8*, 9, and 10 belong to this class. The effector CASPs such as CASP3, 6, and 7 accomplish the final cell death process (McIlwain et al., 2015).

Caspase-9 is encoded by the *CASP9* gene located on chromosome 1p36.1. *CASP9*, has critical role in the mitochondrial death pathway. Apoptic signals lead to mitochondrial release of cytochrome c, which binds and facilitates formation of the heptameric apoptosome that recruits and activates *CASP9* (Yilmaz et al., 2017). The extrinsic and intrinsic pathways of apoptosis in humans utilize the caspase enzyme cas¬cade; the *CASP8* and CASP10 are used in extrinsic pathway, while *CASP9* is employed in the intrinsic pathway (is also known as mitochondrial apoptosis). Active *CASP9* causes the initiation of apoptosis through activation of effector CASPs(McIlwain et al., 2015).

The *CASP8* gene is located on chromosome 2q33–2q34 and translated to a cysteine-aspartic acid protease 8 protein which can directly commence the death pathway. *CASP8* is an initiator of either extrinsic or intrinsic apoptotic pathways after being activated by Fas cell surface death receptor (FAS) and Fas-associated death domain (FADD). Activated *CASP8* may act lonely or in collaboration with other initiator CASPs, leading to activation of the downstream effector CASPs, including CASP3 to terminate the apoptotic pathway (Tummers and Green, 2017).

There are numerous published documents that caspase family proteases, and genes that encode and regulate their transcription are principal in lymphomagenesis. Somatic mutations in caspase genes, such as CASP3, 7, 8, and 10, have been studied in a multitude of cancers including NHL (Shin et al., 2002; Soung et al., 2004). In addition, previous reports have shown various expression of caspase genes among the NHL subtypes (Lan et al., 2009; Sargazi et al., 2020). Previous studies have shown that genetic variants may alter expression or function of apoptotic proteins within the tumor (Xie et al., 2016; Harati-Sadegh et al., 2021b). Moreover, the evidence proposed that genetic variation is involved in etiology of NHL (Rendleman et al., 2014).

Accumulating studies try to detect genetic variation related to cancer risk with the hope that SNPs genotyping can be utilized to improve clinical management of cancers. While it is recognized that mutations in CASPs genes markedly increase risk of developing different kind of cancers (Xu et al., 2012), the association between CASP 9, 8 gene polymorphisms and NHL has not been known yet. In this study, therefore, the gene polymorphisms at Caspase 9 gene loci rs4233532, rs4646018, and Caspase 8 gene locus rs1045485 were explored in NHL patients and healthy people so as to explore the effect of *CASP9* and 8 polymorphisms on susceptibility to NHL.

Materials and Methods

Subjects

Patients A total of 175 NHL patients (110 males and 65 females, aged 14–90 years, mean age: 45.36±15.69) and 175 healthy people (94 males and 81 females, aged 21–75 years, mean age: 42.51±12.26) were collected as the objects of study. There were no statistically significant differences in such general data as age and gender between the two groups (P=0.059 and P=0.104, respectively). The protocol of the current study was verified by the Ethics Committee of Zahedan Medical Sciences University (Zahedan, Iran), and signed written informed consents were obtained from all participants before the study.

Sample Collection and Genotyping

To genotype the subjects for examination the correlation between *CASP8*/*9* polymorphism and NHL, a total of 5 mL of peripheral blood was collected from all participants in this study and DNA was extracted using standard salting out method. The sequences of specific designed primers for each variant are shown in Table 1. The genomic sequence of three studied polymorphisms in *CASP8* and *CASP9* genes was amplified using allele-specific amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method according to the condition which are listed in Table 2. Each PCR reaction was 15 µL, including 8 µL master mix (AmpliqonTaq 2x mastermix, Denmark), 0.5 µL of each primer (10 ng/mL), 0.5 µL genomic DNA (~1 ng/µl), and dH2O. The amplified fragments were then electrophoresed in 3% agarose gel and analyzed as follows: rs1045485 (G:225 bp, A:134 bp), rs4646018 (C:157 bp, T:224 bp), rs4233532 (T: 136 bp, C:319 bp). The amplicons for each genotype are displayed in Figure 1.

Statistical Analysis

The statistical analysis was conducted using SPSS software (IBM Corp., USA) version 20. Enumeration data were compared using χ2-test, and the Hardy-Weinberg equilibrium test was performed. The strength of link between variants and NHL using 95% confidence intervals (CI) and odds ratio (OR) was measured by the logistic regression analyzes. P<0.05 considered the statistically significant difference.

Results

Genotypes and allele frequencies of *CASP8* rs1045485 and *CASP9* rs4646018 and rs4233532 polymorphisms are presented in Table 3. The data indicated that A allele of rs1045485 is a protective factor for NHL, and its frequency was higher in healthy subjects in comparison with NHL cases (OR = 0.030 95% CI=0.015-0.059, P <0.001). GA genotype of rs1045485 in the heterozygous codominant model was strongly associated with reduced risk of NHL (OR=0.003 95%CI=0.001-0.008, P<0.001). Moreover, the C allele of rs4646018 in either heterozygous/homozygous codominant or dominant models significantly enhanced NHL susceptibility (OR = 4.77 95%CI = 2.03-11.24, P<0.001 / OR = 4.66 95%CI = 1.32-16.44, P=0.017, and

Table 1. The Primers Used for Detection of *caspase 8* (rs1045485 G/C, A, T), *caspase 9* (rs4646018 T/C, rs4233532 C/T) Polymorphisms

Polymorphism	PCR primers (5'→3')	Fragment, bp
Caspase 8 (rs1045485)	FO: TCCAGCTGTGGTCTGTGAATTAC	314
	RO: GGGTTTTCCAGCAAGGGAAG	
	FI: CATTTTGAGATCAAGCCCCTCG	225 (Allele G)
	RI: GATTTGCTCTACTGTGCAGTCCTT	134 (All A)
Caspase 9 (rs4646018)	FO: AGGGGACAAGAAAGAGAATGAG	157 (All C)
	RI: ATACAGATGGGGAAATCACAG	
	FI: GAGCTCAGAGTCTCTGGTCT	224 (All T)
	RO: AGTACACACACTGCACTCATAC	
Caspase 9 (rs4233532)	FO: GAAACCCCTGAGGCACGATTCC	136 (All T)
	RI: TGAGCGGGCAAGGATGCTCT	
	FI: AAGGGCGCAGGCCTCCTTGCTGC	319 (All C)
	RO: CCGAGCCAGATGCCACCCCGTTC	

F, Forward; R, Reverse; I, inner; O, outer

OR = 4.77 95%CI = 2.03-11.21, P<0.001, respectively). No significant difference was found between the NHL cases and control group regarding the allele frequency of rs4646018 (P= 0.096). We did not detect any significant correlation between studied groups under different genetic models for rs4233532, however, we observed that this SNP

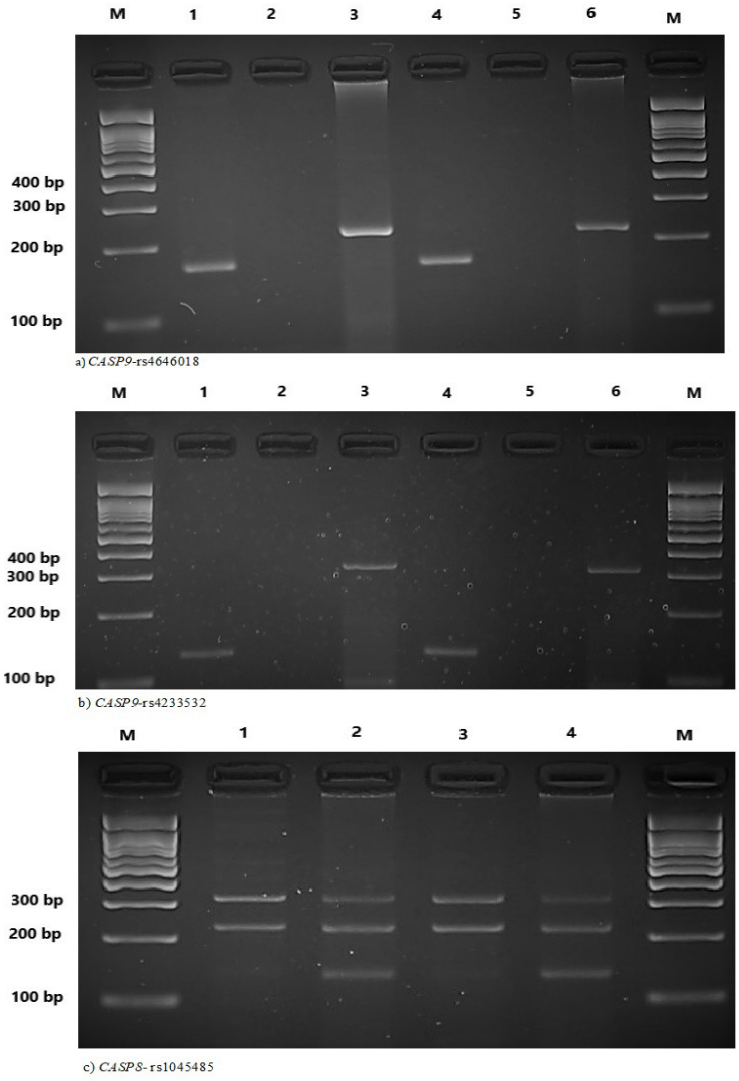

Figure 1. Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) results for a) *CASP9*-rs4646018, b) *CASP9*-rs4233532, and c) CASP8- rs1045485 gene polymorphisms, M, Marker. rs1045485 (G:225 bp, A:134 bp), rs4646018 (C:157 bp, T:224 bp), rs4233532 (T: 136 bp, C:319 bp).

Table 2. The PCR Condition for Studied Polymorphism

Gene polymorphism	Denaturation		Annealing		Extension		Cycles
	Time	Temp	Time	Temp	Time	Temp	
Caspase8 rs1045485	30 s	95 °C	30 s	56 °C	30 s	72 °C	30
Caspase9 rs4646018 (FO-RI)	30 s	95 °C	30 s	54 °C	35 s	72 °C	35
Caspase9 rs4646018 (FI-RO)	30 s	95 °C	30 s	56 °C	30 s	72 °C	30
Caspase9 rs4233532 (F0-RI)	30 s	95 °C	30 s	64 °C	40 s	72 °C	35
Caspase9 rs4233532 (FI-RO)	30 s	95 °C	30 s	67 °C	30 s	72 °C	30

Caspase 8 SNP genotyping was performed using Tetra-Primers ARMS PCR and Caspase 9 SNPs genotyping was performed using two-tube ARMS-PCR with different condition; Temp, Temperature; S, second; F, Forward; R, Reverse; I, inner; O, outer

Table 3. Association of *Caspase 8* (rs1045485 G/C D302H), *Caspase 9* (rs4646018, rs4233532), Polymorphisms and the Risk of NHL.

Polymorphism	Case n (%)	Control n (%)	OR (95%CI)	p-value
Caspase 8 (rs1045485)				
G/G	166 (94.9)	10 (5.7)	1	-
G/A	9 (5.1)	165 (94.3)	0.003 (0.001-0.008)	<0.001
A/A	0 (0.0)	0 (0.0)	-	-
Allele				
G	341 (97.4)	185 (52.9)	1	-
A	9 (2.6)	165 (47.1)	0.030 (0.015-0.059)	<0.001
Caspase 9 (rs4646018)				
Codominant				
T/T	7 (4.0)	29 (16.6)	1	-
T/C	159 (90.9)	138 (78.9)	4.77 (2.03-11.24)	<0.001
C/C	9 (5.1)	8 (4.5)	4.66 (1.32-16.44)	0.017
Dominant				
T/T	7 (4.0)	29 (16.6)	1	-
T/C+C/C	168 (96.0)	146 (83.4)	4.77 (2.03-11.21)	<0.001
Recessive				
T/T+T/C	166 (94.9)	167 (95.5)	1	-
C/C	9 (5.1)	8 (4.5)	1.13 (0.43-3.00)	0.803
Allele				
T	173 (49.4)	196 (56.0)	1	-
C	177 (50.6)	154 (44.0)	1.30 (0.97-1.75)	0.096
Caspase 9 (rs4233532)				
Codominant				
C/C	18 (10.3)	9 (5.1)	1	-
C/T	150 (85.7)	165 (94.3)	0.45 (0.20-1.04)	0.089
T/T	7 (4.0)	1 (0.6)	3.50 (0.37-32.97)	0.483
Dominant				
C/C	18 (10.3)	9 (5.1)	1	-
C/T+T/T	157 (89.7)	166 (94.9)	0.47 (0.21-1.08)	0.109
Recessive				
C/C+C/T	168 (96.0)	174 (99.4)	1	-
T/T	7 (4.0)	1 (0.6)	7.25 (0.88- 59.56)	0.073
Allele				
C	186 (53.1)	183 (52.3)	1	-
T	164 (46.9)	167 (47.7)	0.97 (0.72- 1.30)	0.88

Table 4. Association of *Caspase 8* (rs1045485), *Caspase 9* (rs4646018) and *Caspase 9* (rs4233532) Polymorphisms with Clinicopathological Characteristics of NHL Patients

Characteristic of patients	*Caspase 8* (rs1045485)			P value	*Caspase 9* (rs4646018)				*Caspase 9* (rs4233532)			P value
	GG	GA	AA		TT	TC	CC		CC	CT	TT	
Age, years				0.535				0.932				0.404
≤50	109	5	0		5	103	6		11	100	3	
>50	57	4	0		2	56	3		7	50	4	
Histology				0.679				**0.022**				0.093
DLBCL	80	5	0		7	74	4		5	75	5	
Others	85	4	0		0	84	5		13	74	2	
Stage				0.656				0.401				0.545
I	91	4	0		6	83	6		8	83	4	
II	25	1	0		0	24	2		3	22	1	
III	13	0	0		1	12	0		0	12	1	
IV	35	3	0		0	37	1		7	3	1	
Treatment				0.599				**0.027**				0.591
R-CHOP	88	5	0		7	80	6		8	82	3	
Others	78	3	0		0	78	3		10	67	4	
Radio Therapy				0.37				**0.048**				0.347
No	146	7	0		4	141	8		15	133	5	
Yes	20	2	0		3	18	1		3	17	2	
Kind				0.329				0.275				0.104
Primary	150	9	0		7	145	7		14	139	6	
Recurrent	16	0	0		0	14	2		4	11	1	

may increase risk of NHL in recessive model (marginally insignificant, P=0.073)

Table 4 represents the data regarding the association of *CASP8*-rs1045485, *CASP9*-rs4646018 and *CASP9*-rs4233532 polymorphisms with clinicopathological characteristics of NHL patients. No relationship was identified between *CASP8*-rs1045485 and *CASP9*-rs4233532 and NHL clinicopathological characteristics. The data showed *CASP9*-rs4646018 was associated with histology (P=0.022), treatment (0.027) and radio therapy (0.048) of NHL.

Discussion

Apoptosis is a vital cellular defense mechanism against cancer development. The both extrinsic and intrinsic apoptosis pathways are controlled by genes which detected in different human malignancies (Lin et al., 2012; Sargazi et al., 2019a). Apoptotic deficiency allows altered cells evade destruction and so carcinogenesis, but are also related to deregulated cell proliferation, differentiation and angiogenesis (Hassan et al., 2014). Therefore, all proteins involving in apoptosis pathways are appropriate candidates to examine in pathogenesis and treatment of cancer (Sargazi et al., 2022). The altered levels of caspases in cancerous tissues have been reported in several studies and are linked to clinical outcomes (Li et al., 2017). Single-nucleotide polymorphisms are the most common human genetic variation and may contribute to an individual's susceptibility to cancer (Harati-Sadegh et al., 2021a). A large number of reports have represented the effect of some variants on the proteins expression and activity which therefore leads to development of cancer (Sargazi et al., 2019b; Barani et al., 2021; Wiggins et al., 2021). Mutations within caspase family proteases are not unusual in cancers (Ghavami et al., 2009). The current research investigated the potential link between Caspase 9 and Caspase 8 and NHL risk.

The efficiency of apoptosis possibly associated with polymorphisms in any gene regulating or carrying out apoptosis (Olsson and Zhivotovsky, 2011; Su et al., 2015). Many candidate polymorphisms within the *CASP9* and 8 genes have been disseminated in public databases (http://www.ncbi.nlm.nih.gov/SNP). Although the functional effects of these variants have not been clarified, it has been suggested that some of these SNPs, can affect gene expression or activity, thereby modulating cancer development. In order to examine the possible association between Caspase 9 rs4233532, rs4646018 and Caspase 8 rs1045485 and NHL risk we performed a case–control study in an Iranian population.

The rs4646018 and rs4233532 are intronic polymorphisms on *CASP9* gene. Kelly et al. carried out a comprehensive assessment of 36 apoptosis pathway genes including 7 SNPs of *CASP9* gene with risk of lymphoma. Their results showed the significant role of 3 intronic polymorphisms on susceptibility of NHL. However, they reported marginally insignificant association between rs4646018 and NHL risk (OR: 0.84, 95%CI: 0.69-1.02, P=0.078), the data showed significant link of rs4646018 SNP on susceptibility of chronic lymphocytic leukemia/small lymphocytic

lymphoma (OR: 0.65, 95%CI:0.49-0.88, P=0.0049) (Kelly et al., 2010). The lack of relationship between *CASP9*-rs4646018 and stomach cancer(Ni et al., 2011) and colorectal cancer(Liu et al., 2013) risk was reported previously. The data of our study provided evidence for the association of the *CASP9*-rs4646018 polymorphism and NHL risk. In the present study we found that the carriers of CC and TC genotypes of rs4646018 had an increased risk of NHL. Moreover, we observed the significant association of *CASP9*-rs4646018 with clinicopathological characteristics of NHL including histology, treatment and radio therapy. Numerous studies have shown the link of gene polymorphisms and clinical features of cancers(Xu et al., 2021; Su et al., 2022); our finding, however, is the first report demonstrating this correlation among NHL cases and *CASP9*-rs4646018 SNP.

No data exist regarding *CASP9*-rs4233532 polymorphism and the risk of NHL, however the role of this SNP on different diseases have assessed before. Liu et al. considered the variant C allele of rs4233532 as a risk allele for susceptibility to colorectal cancer. Their data showed that CC genotype (OR: 1.667, 95% CI: 0.967–2.876) and CT genotype (OR:1.435, 95% CI: 0.998–2.063) of rs4233532 was correlated with significantly increase risk of colorectal cancer(Liu et al., 2013). Moreover, Yao et al. observed that heterozygous statistical model (T/C) of rs4233532 was associated with psoriasis susceptibility(Yao et al., 2019). However, we did not detect significant association between *CASP9*-rs4233532 polymorphism and NHL risk, in consistent to previous studies, we observed that C/T carriers of rs4333532 SNP had marginally significant association with risk of NHL.

Albeit, the role of *CASP9* in activation via the apoptosome driven intrinsic pathway is obvious, the influence of the polymorphisms as well as their functional significance in the *CASP9* activation through the apoptosome complex and in the proficiency of effector caspase activation by *CASP9* is mostly ambiguous. It can be speculated that the *CASP9* rs4646018 and rs423532 SNPs may alter either the stability and capability binding of the apoptosome or the *CASP9*-induced activation of other caspases, so as to affect negatively or positively the intrinsic apoptotic pathway. Due to the biological role of *CASP9*, the functional alterations, whether to activate or inhibit apoptosis, may also be expressed in an inherently variable, promoting or prohibitive, tumor microenvironment.

Caspase8-rs1045485, also known as D302H, is located in exon 10 of the *CASP8* gene. The findings of a recent meta-analysis (pooled analysis of 41 studies) by Hashemi et al. showed that rs1045485 variant meaningfully reduced cancer susceptibility and stratified analysis also showed the reduced breast cancer risk in Caucasians, population- and hospital-based studies(Hashemi et al., 2020). Enjuanes et al. observed a strong correlation between rs1045485 SNP and decreased risk of chronic lymphocytic leukemia in all statistical models including codominant, dominant and recessive. Their presented data proposed that the C variant of rs1045485, which leads in an aspartic acid-to-histidine substitution, is linked with a reduced susceptibility to chronic lymphocytic leukemia (OR, 0.56; 95% CI, 0.44–0.72)(Enjuanes et al., 2008). However, the functional consequences of this missense variant have not known yet, the result of the present study in agreement to previous reports showed a strong correlation of rs1045484 variant with decreased risk of NHL. Although there is not any published report regarding the association of *CASP8*-rs1045484 and NHL, Lan et al. provided an evidence that *CASP8* polymorphisms were related to development of all major NHL subtypes(Lan et al., 2009). *CASP8* is one of the main initiator caspases which determined as a death receptor-mediated apoptosis pathway. Moreover, extensive roles of *CASP8* are known so far, including lymphocyte homeostasis regulation, monocyte differentiation and NF-κB activation which all may related to etiology of NHL(Lan et al., 2007; Mashhadi et al., 2021). Moreover, it has been reported that activation of natural killer cells, B/T cells as well as lymphocyte apoptosis had decreased in people with *CASP8* mutations(Chun et al., 2002).

The relatively small sample size was a limitation of the present study. Detecting a low-penetrance risk allele is a complex effort in molecular epidemiological studies, therefore enlargement of sample size and adjustment of all possible confounding factors are efficient procedures to increase the output. Nevertheless, it is effortful to gather large sample and recognize all possible confounding factors. Therefore, further studies in larger populations and other ethnicities need to perform for verifying our findings.

In conclusion, our study provides the first document to show the significant role of *CASP8*-rs1045485, CASP9-rs4646018 to development of NHL as well as its related clinicopathological characteristics. Due to possible differences in genet variants in diverse ethnic groups, further researches in other populations as well as functional tests are needed to elucidate the effect of caspase gene polymorphisms on risk of NHL.

Author Contribution Statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [FB], [GB], [AN], [SMH], [MT] and [MH]. The first draft of the manuscript was written by [FB and GB] and all authors commented on previous versions of the manuscript. All authors read and approved the final attest that all listed authors meet the authorship criteria and that no other authors meeting the criteria have been omitted.

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the manuscript.

Conflicts of interests

The authors declare that they have no conflict of interest.

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