

Association of Toll-Like Receptor-3 Polymorphism with Nasopharyngeal Carcinoma in The Minangkabau Ethnic Group

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy in the head and neck region that has a unique geographic distribution and is closely related to Epstein-Barr Virus (EBV) infection. The growth of malignancy due to viruses is largely determined by the host immune response. Toll-Like Receptors (TLRs) emerged as important components of the innate immune system playing an important role in sensitizing, controlling, destroying pathogens and responding to viral infections. TLR3 single nucleotide polymorphisms (SNPs) are thought to affect individual susceptibility to cancer. This inherited polymorphism has an impact on the heterogeneity of the immune system response in the form of changes in antiviral function and effects. This comparative analytic study used a cross sectional comparative study design on 23 Minangkabau ethnic NPC patients and 23 healthy Minangkabau ethnicity patients as controls. Respondents were examined for TLR3 gene polymorphism by polymerase chain reaction sequence (PCR) sequencing method. Data were analyzed statistically with a computer program and declared significant if $p < 0.05$. TLR3 polymorphisms (rs3775291) were found in both the NPC and control groups in the form of homozygous mutants and heterozygous mutants. Homozygous mutants were found in the NPC group as much as 21.7%, while in the control group it was 30.4%. Heterozygous mutants were found in the NPC group as much as 39.1%, while in the control group as much as 34.8%. Statistically there was no significant relationship between TLR3 polymorphism (rs3775291) and the incidence of NPC with $p > 0.05$. There is no relationship between TLR3 polymorphism (rs3775291) and the incidence of NPC in the Minangkabau ethnic group.

Keywords: Nasopharyngeal Carcinoma, Toll-Like Receptor-3, Epstein-Barr virus, Minangkabau.

1. INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy in the head and neck region that has a unique geographic distribution and is closely related to Epstein-Barr Virus (EBV) infection. Most of NPC is found in South China, Southeast Asia and North Africa [1-3]. Based on data from Global Burden Cancer in

2012, there were 87,000 new cases of NPC every year (61,000 new cases in men and 26,000 new cases in women) with 51,000 deaths due to NPC (36,000 in men and 15,000 in women) [4]. This has increased in 2018, there were 129,079 new cases (93,416 new cases in men and 35,663 new cases in women) with 72,987 deaths due to NPC (54,280 deaths in men and 18,707 deaths in women). NPC is mainly found in men of productive age

(60% aged between 25-60 years) [5]. In Indonesia, NPC is the 4th most common cancer [4].

Until now, the cause of NPC cannot be known with certainty, but it is estimated that interaction of multiple factors such as EBV infection, genetic susceptibility, carcinogenic diet and environmental and health factors were contribute to the development of NPC [1-3], [6-9]. EBV, a ubiquitous virus that infects more than 90% of adults worldwide, is an important risk factor for the development of NPC. However, NPC occurs in a small percentage of the EBV-infected population. The absence of NPC in most EBV carriers raises the suspicion that EBV infection alone is not sufficient to cause NPC. This has been reported to be associated with T cell- mediated control of immunity [3], [10-12].

The growth of malignancy due to viruses is largely determined by the host immune response. Toll-like receptors (TLRs) emerged as important components of the innate immune system that can recognize a wide variety of microbial structures known as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) of viruses, bacteria, protozoa and fungi [3], [13-16]. TLRs have an important role in resistance to infection by initiating the immune system during the infection process through various host defense mechanisms [3,17]. The facts show that TLRs control the ability of dendritic cells to sensitize naive T cells and initiate the adaptive immune system. Due to the ability of TLRs to modulate the adaptive immune system, TLRs become one of the promising target strategies for treatment in conditions where the adaptive immune system does not work optimally such as sepsis, auto-immune disease, cancer and allergies [3].

The most varied toll-like receptor-3 (TLR3) SNPs were rs3775291 and rs3775290 in Asian populations [14]. A meta-analysis of African ethnicity studies conducted in Morocco and Tunisia found that rs3775291 was significantly associated with the risk of developing NPC [17]. Another study in North Africa also found SNPs rs3775391 had a strong relationship with the risk of NPC events [18].

Research on ethnic-based malignancies is important for the future because the cancer approach is not only based on the population but will shift to individual genetic profiles as the basis for determining cancer prevention, early detection and therapy strategies [19]. The close relationship between viral infection, exposure to carcinogenic chemicals and the genetic susceptibility of host cells that underlie the pathogenesis of NPC

makes NPC an object of research that aims to gain a better understanding of the interrelationships between various factors that influence each other in the development of NPC [20]. Besides, the absence of data regarding the relationship of TLR3 polymorphisms in NPC patients in the Minangkabau ethnic group is being the reason for the need for this research.

2. METHODS

This research is an observational analytic study with a cross sectional comparative study design using 23 samples of 300 µl whole blood of control and NPC patients. This research was granted the ethical clearance by the ethical committee board Faculty of Medicine Andalas University. Samples was patients from the Minangkabau ethnic group from three generations who were diagnosed with NPC based on anatomical pathology examination of the nasopharyngeal biopsy results. The control was non-nasopharyngeal carcinoma matched by sex and age (± 3 years) and had no family relationship with the patient. Then, the entire sample will be processed according to the PCR sequencing process at Department of Biochemical Science, Faculty of Medicine Andalas University, Padang, Indonesia and 1stBASE, Malaysia.

Genotyping of the TLR3 gene was carried out using the polymerase chain reaction sequence (PCR) sequencing method. The primers are used for TLR3 amplification. Primers to identify TLR3 gene polymorphisms (rs3775291) were constructed using Geneious 11.1.2 software. The primers used for TLR3 amplification (rs3775291) were forward primer: 5'TGTCCACCACCAGCAATACAAC-3' and for reverse primer: 5'CTCAAGACCTCCAACATGTCATC-3' which was used to amplify DNA with a size of 812bp.

PCR reactions of a total volume of 25 l consisting of 12.5 l GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 l each of the forward and reverse primers, 1 l of genomic DNA and 9.5 l of nuclease-free water. The amplification steps were carried out as follows: initial denaturation of 95°C for 1 minute, followed by further denaturation of 35 cycles of 95°C for 15 seconds, annealing at 65°C for 15 seconds, 72°C elongation for 10 seconds and final elongation of 72°C for 5 minutes. A total of 5µl of PCR products were examined by electrophoresis using 1.5% agarose gel, 120 volts for 60 minutes, then the agarose gel was observed with GelDoc. The purified 812bp PCR product sample was sequenced at 1stBASE, Malaysia.

The results of the sequencing data were then processed using Genious 11.1.2 software. In individuals with TLR3 genotype (rs3775291) who did not undergo polymorphism, no CC base changes were found, which is referred to as wild type. Meanwhile, individuals who undergo a CC to CT base change are called heterozygous mutants and those who undergo a change from CC to TT are referred to as homozygous mutants. The results of the sequencing were then aligned with the reference gene (NG_007278.1). Spearman correlation test is done to know the correlation of polymorphism and NPC with the significance level $<0,05$.

3. RESULTS AND DISCUSSION

3.1 Results

Demographic and clinical characteristics of study subjects by sex and age can be seen that the incidence of NPC is more common in men, namely 15 people (65.2%) compared to 8 women (34.8%) with a ratio of 1.9:1. The average age of occurrence of NPC is 46.7 ± 12.9 years and the highest age group is in the 40-49 years age group, which is 39.1%. The youngest respondent who suffered from NPC was 20 years old while the oldest was 69 years old. Based on the results of anatomical histopathology examination, non-keratinizing undifferentiated carcinoma (WHO III) was the most common type of anatomical histopathology, which was 65.2%, followed by non-keratinizing differentiated carcinoma (WHO II) as much as 34.8% and no keratinizing squamous cells were found. carcinoma (WHO I). Based on the NPC stage, most of the NPC respondents in this study were in stage IV, which was 78.3%, followed by stage III as much as 13% and stage II as much as 8.7%. There are no NPC respondent patients who come for treatment at stage I.

TLR3 DNA Isolation PCR (rs3775291)

The results of the PCR sequencing can be seen in Figure 1. Based on Figure 1, it can be seen that the success of PCR, shows the amplified band with an amplicon size of ± 812 bp.

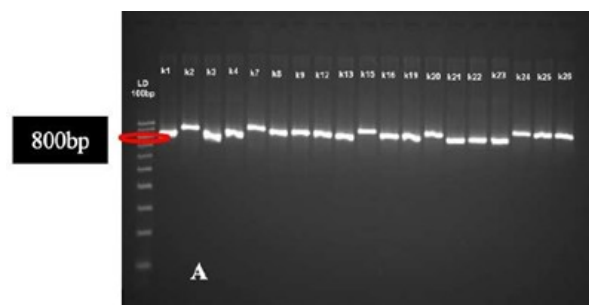


Figure 1. Electrophotogram of TLR3 (rs3775291) PCR results with a length of 812bp in several samples of NPC cases (A) and controls (B)

TLR3 DNA Sequencing PCR (rs3775291)

Figure 2 shows the heterozygous mutant TLR3 (rs3775291) gene polymorphism which is characterized by a change in base from CC to CT and a homozygous mutant characterized by a change in base from CC to TT. In the case of NPC, there were heterozygous mutations in nine samples, consecutively in samples K2, K4, K8, K9, K14, K16, K19, K21 and K22. Homozygous mutations were found in five samples, consecutively in K3, K7, K11, K17 and K26. In the control sample, heterozygous mutations were present in eight samples, sequentially in S2, S4, S7, S13, S16, S19, S22 and S25. Homozygous mutations were found in seven samples, sequentially in S1, S6, S10, S14, S18, S21 and S24 as shown in Figure 3.

Association of TLR3 Polymorphism (rs3775291) with NPC

Association of TLR3 Polymorphism (rs3775291) with NPC can be seen in the Table 1. Table 1 shows that the TLR3 polymorphism (rs3775291) was found in both group (NPC and the control group). Homozygous mutants were found in 21.7% of the NPC group, while 30.4% in the control group. Heterozygous mutants were found in 39.1% NPC group, while 34.8% in the control group. Statistically, there was no significant relationship between TLR3 polymorphism (rs3775291) and the incidence of NPC with $p > 0.05$.

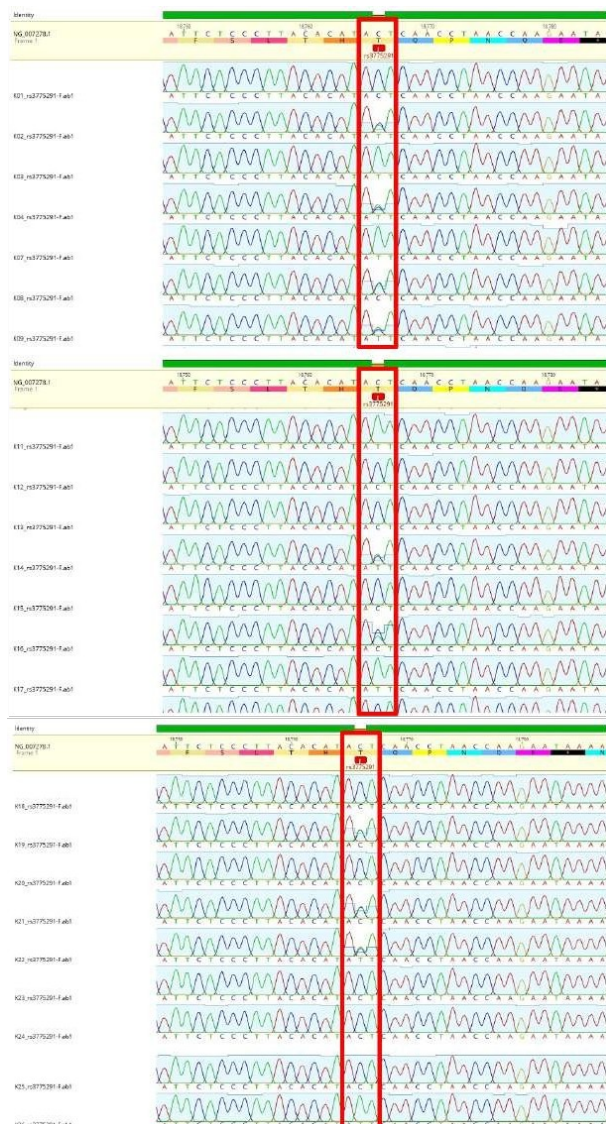


Figure 2. Alignment of sequencing results of NPC cases with the TLR3 reference gene



Figure 3. Alignment of sequencing results of control cases with the TLR3 reference gene

3.2 Discussion

In this study, patients with NPC were more common in men (65.2%) compared to women (34.8) with a ratio of 1:1.87. Various studies have consistently reported that NPC is more common in men, such as data reported by Mahdavifar et al who reported 71.02% of 68,272 NPC patients were male [21]. Wu et al also reported 69% of 3,880 NPC patients were male [22]. Global Burden Cancer in 2018 also reported new cases of NPC, mainly found in men by 72.37% of 129,079 new cases of NPC [5].

In Indonesia, reported that the most NPC characteristics were in men at 71.92% at Saiful Anwar Hospital, Malang [23]. This is in line with previous

reported Bali, Bandung, Banda Aceh, Palembang, Lombok, and Medan who found NPC were dominant consecutively 82%, 64,7% 67,6%, 72,7%, 72,73%, 65,9% and 68,2% in male patients [24-29]. Research reports that men are 2-3x more likely to develop NPC than women [2]. This may because of the role of estrogen which has a protective effect on the development of NPC in women [1,25]. In addition, lifestyle differences between men and women such as smoking habits were also reported to be correlated with the incidence of NPC [2,8,27]. Heavy and chronic smokers (more than 15 cigarettes/day) had a significantly high risk for the occurrence of NPC.8 In individuals without family history of NPC, the risk of NPC is 1.7 times in smokers compared to non-smokers. The risk is 9 times higher in smokers with a family history of NPC [7]. In addition, men have a greater risk of NPC due to exposure to chemicals in the work environment [27,29].

Table 1 Relationship of TLR3 Polymorphism (rs3775291) with NPC

Polymorphism	NPC (%)	Group Control (%)	p
Wild type (CC)	9 (39,1)	8 (34,8)	0,798
Heterozygous Mutant (CT)	9 (39,1)	8 (34,8)	
Homozygous Mutant (TT)	5 (21,7)	7 (30,4)	
Total	23 (100)	23 (100)	

There are mixed reports regarding the peak age of the incidence of NPC, but in areas of high incidence, NPC has a similar pattern. Lin et al., reported differences in the peak age of the occurrence of NPC in high and low risk areas. In high-risk areas, the peak of NPC occurs at the age of 40-55 years and in low risk areas occurs at the age of 15-24 years and 65-79 years [8]. Wu et al., reported the similar result, the cases peak in endemic areas occurred at the age of 45-59 years and in low-risk areas there is a bimodal age distribution, namely at the age of 15-19 years and 65-79 years [22]. According to research conducted by Shield et al, the age pattern of NPC describes a different etiology, for instance, in adolescence and early adulthood (15-34 years) may be due to the changes in genes that increase the risk of NPC whereas at older ages (50 years and over) it may be due to alcohol and tobacco consumption [30].

Poh et al., obtained from a serological survey that primary EBV infection in NPC endemic areas occurred at an early age, for example in the age under 5 years and most cases occurred during pregnancy in the South China area. This condition also occurs in Eskimos in Greenland and other high-risk populations with the postulated presence of the same genetic polymorphism due to an increase in chronic EBV infection [31]. Other studies have also reported that the development of NPC in childhood is closely associated with EBV infection or is an inherited genetic predisposition in the black population [22].

This study found that most of NPC patients (91.3%) were at an advanced stage, accordingly stage III (13%) and stage IV (78.3%). Almost all studies on NPC report that most NPC patients present at an advanced stage. The torus tubarius is more prominent posteriorly and superiorly sometimes obstructing a direct view of the Rosenmueller fossa [32]. This is thought to be due to the anatomically hidden location of the nasopharynx behind the nose and atypical symptoms in the early growth of the tumor itself.

This study obtained the similar results with the studies in endemic areas of NPC. The most common type found that 65.2% was non-keratinizing undifferentiated carcinoma (WHO III). According to histopathology type, various studies have reported that in high-risk areas, non-keratinizing carcinoma (WHO II and III) is the most common type. It's consisting of more than 95% of cases. On the other hand, in low-risk areas is only about 75% of cases and the rest are keratinized carcinomas [33]. Correspondingly, Wang et al also reported that 94.3% of cases in high-risk areas were non-keratinizing carcinoma [34]. Adham M et al reported that 85% of cases were non-keratinizing undifferentiated carcinoma [35]. Non-keratinizing undifferentiated carcinoma. (WHO III) is closely associated with EBV infection [36].

This study found that the TLR3 gene polymorphism rs3775291 was found in the NPC and control groups. The polymorphisms found were homozygous (TT) and heterozygous (CT) mutants. Homozygous mutant polymorphisms were slightly more common in the control group and slightly more heterozygous mutants were found in the case group, but there was no significant difference between the two groups.

Studies in North Africa with a high incidence of NPC in Moroccan (Kasablanka and Rabat) and Tunisian

(Tunis and Sousse) showed a strong association between SNP rs3775291 and the risk of NPC occurrence [18]. TLR3 SNP rs3775291 will cause substitution the amino acid Leu412Phe which is located on the ligand surface for receptor activation which further causes conformational changes and dimerization of the TLR receptor [18, 37, 38]. The substitution of the Leu412Phe amino acid affects residues in the ECD core TLR3 [39]. In addition, the TLR3 SNP allele polymorphism rs3775291 can interfere with NF- κ B-mediated poly(I:C) binding, interferon activity and NK cells that affect TLR3 surface expression. Inherited gene polymorphisms in sensors against viral cells are potential determinants of immune response heterogeneity that can influence immune responses by altering antiviral function and effects. Genetic variants in this gene have been implicated as important regulators of immunity and host responses to infection and malignancy. Therefore, the TLR3 allele polymorphism rs3775291 will affect the recognition of EBER which can cause apoptosis inhibition or EBV avoidance of the immune system which increases the risk of NPC [22].

In this study, there was no significant difference between the two groups of NPC cases and controls. The possibility of a silent or synonymous mutation or a neutral mutation that has not changed the amino acid composition is not fulfilled considering that the ACT and ATT codons have different amino acid groups, namely threonine and isoleucine [40]. Meta-analytical studies reported the presence of TLR3 polymorphisms in other genes, such as SNP rs3775290, rs5743312 and rs5743305 which are also associated with the risk of cancer incidence in Asian and African ethnicities [17]. Data show that the ability of certain individuals to respond appropriately to TLR ligands can be impaired by polymorphisms (SNPs) located in the TLR gene that can increase susceptibility to infection, inflammation and cancer [3,17]. Variations in the TLR3 gene not only affect the anti-cancer immune system but also changes in the apoptotic process that result in increased survival of tumor cells [22]. Ruuskanen M et al stated that viruses or tumors associated with certain viruses will elicit different immunological reactions, but the host response may vary for no known cause. This may partly explain the reasons for the differing efficacy of oncology therapy. However, it is based on the hypothesis proposed by Wee et al as cited by Ruuskanen M et al that TLRs gene polymorphisms may influence the innate immune response and make certain

populations or individuals more susceptible to cancer-associated infections. In NPC, changes in the function of TLRs allow the virus to enter the nasopharyngeal mucosa and cause persistent infection that leads to the carcinogenesis process itself. In addition, activation of TLRs at abnormal sites may be associated with carcinogenesis [13].

TLR3 has an important role in initiating host defense mechanisms against bacteria, viruses, protozoa and fungi through the recognition of PAMPs and DAMPs which further stimulates intracellular signals to secrete inflammatory cytokines [3], [13-16]. Furthermore, studies have shown that neoplastic processes will interfere with the TLR signaling pathway thereby accelerating the neoplastic process. Cancer stems from the accumulation of gene changes due to errors in the innate immune system as part of the multigenetic risk of cancer [3,17].

The complex interactions between tumor cells, immune cells and PAMPs and DAMPs in the tumor microenvironment will cause tumor cell progression (tumorigenic effects). The stimulation of TLRs that can act as anti- or pro-tumorigenesis depends on the function of TLRs receptors and the type of cancer itself [13]. The immune system mediated by TLRs can be activated by various immunoadjuvants, thus enabling the hypothesis of anti-tumor immunity through increased activity of TLRs. Strengthening the activity of TLRs can prevent the process of carcinogenesis, while impaired function of TLRs will cause tumor cells to avoid the introduction of the immune system and ultimately cannot be eliminated. At the same time, activation of TLRs can also lead to carcinogenesis, creating a pro-inflammatory microenvironment that is favorable for tumor progression, chemoresistance and immunosuppression. Therefore, this condition is referred to as double-edged sword [16, 41, 42]. Given the above conditions, it should be there is a balance between high and low TLRs activities [41].

The meta-analysis research conducted by Cheng D et al., reported that heterogeneity is a term in meta-analysis to see the homogeneity of the sample. This may be because of different cancers have a different carcinogenesis mechanism at the initiation and progression phases of cancer. Furthermore, different TLR3 polymorphisms have an important role [17]. Soo et al., stated that TLR3 polymorphisms can cause differences in the function of stimulating the immune

system that also differ between individuals [43]. Inherited polymorphisms in viral cellular gene sensors are potential determinants of immune response heterogeneity that can affect immune responses by altering the function and antiviral effects of proteins. appropriate. Genetic variants in this gene are implicated as important regulators of immunity and host responses to infection and malignancy [18]. In addition, hormone levels, chronic inflammation, viral infections, smoking and family history can cause various types of cancer [17].

Meanwhile, different genetic backgrounds and different environmental factors in different populations are also the main factors for heterogeneity [17]. Ethnic background has an important role in genetic susceptibility and ethnic differences with different lifestyles will have different environmental factors [14]. Furthermore, an advance researchs is needed to explore more detailed about this issue

4. CONCLUSION

TLR3 gene polymorphism rs3775291 was found in the NPC and control groups. The polymorphisms found were homozygous (TT) and heterozygous (CT) mutants. Homozygous mutant polymorphisms were slightly more common in the control group. On the other hand, slightly more heterozygous mutants were found in the case group, although there was no significant difference between both groups.

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