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Comparison of Polymorphism rs3813865 Cytochrome P450 Family 2 Subfamily E Polypeptide 1 (Cyp2e1) Gene in Various Clinical Stage of Undifferentiated Type Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma is caused by interaction of Epstein-Barr virus chronic infection, environtment, and genetic factors. Epstein-Barr virus (EBV) infect nasopharyngeal ephitelial cell in latent period. This infection will cause mutation and further causing malignancy. This is a cross-sectional study in undifferentiated type NPC patients after hystopatological examination and were examined in RSUP Sanglah Denpasar from January 2017 to December 2018. This study is using 62 subjects who meets inclusion criteria. Univariate analysis was done to show subject characteristics which include age, gender, occupation, clinical stage, gene allele and rs3813865 polymorphism CYP2E1 gene. Mean age of subject is 48.05 years with standard deviation of 10.86 years. The youngest is 17 years old and the oldest is 73 years old. The most are men as many as 47 subjects (75.8%), and the most occupation are government employee as many as 17 subjects (27.4%). The most clinical stage of undifferentiated type NPC are stage II as many as 7 subjects (11.3%). Based on TNM, the most are T4 as many as 32 subjects (51.6%), N3 as many as 21 subjects (33.9%), and M0 as many as 60 subjects (96.8%).

Keywords: Nasopharyngeal carcinoma; polymorphism rs3813865 CYP2E1 gene; RSUP Sanglah.

Nasopharyngeal carcinoma (NPC) is the most common head and neck malignancy in the world.[1] The incidence of NPC is 1.2/100,000 person per year, with 2-3 times higher incidence in men than women. Incidence of NPC in Indonesia (2012) is 5.6/100,000 person per year, which included in top three of South East Asia.[2,3] Nasopharyngeal carcinoma is caused by interaction of Epstein-Barr virus chronic infection, environtment, and genetic factors. Epstein-Barr virus (EBV) infect nasopharyngeal ephitelial cell in latent period. This infection will cause mutation and further causing malignancy.[4]

Non-viral exposure related to NPC is salted fish consumption. Salted fish is traditional food in some endemic area of NPC which carcinogenic potential of salted fish was shown in mice.[5] Salted fish contain mutagene bacterial, genotoxin, and EBV activator substance.[6] One of the genetic factor is gene polymorphism. Gene polymorphism related to carcinogen metabolism is the risk factor of NPC, such as cytochrome P450 family 2 subfamily E polipeptida 1 (CYP2E1) gene which coding CYP2E1 gene to activate procarcinogene.[7] Most od carcinogene metabolism

pathway in human body are mediated by enzime derived from cytochrome P450 which is coded by cytochrome P450 gene (CYP).[8]

Cytochrome P450 gene (CYP) have some single nucleotide polimorphisms (SNP) variations which statistically related to NPC risk, such as rs2031920 and rs3813865 in the CYP gene family 2 subfamily E polypeptide 1 (CYP2E1).[9] The existence of mutant homozigot genotype of rs 38813865 could change regulation of CYP2E1 gene transcryption which lead to carcinogene activation shift.[10]

The role of genetic factors in NPC patogenesis could be detected by using ARMS-PCR (Amplification Refractory Mutation System Polymerase Chain Reaction) method. There are many study about cytochrome P450 family 2 subfamily E polypeptide 1 (CYP2E1) gene polymorphims in China, however they are still limited in Indonesia. CYP2E1 gene polymorphism as one of biomarker in NPC by using ARMS-PCR technique is interesting to study, because there is strong correlation between CYP2E1 with NPC.

METHOD

Research Design

This is a cross-sectional study in undifferentiated type NPC patients after hystopatological examination.

Sample Research

Sampling is done by consecutive sampling, every patient who fulfill the inclusion criteria of the research is included as the research sample. Inclusion criteria in this study were patients diagnosed with NPC after anatomy-patological examination in nasopharyngeal biopsy preparation, already known clinical stage, and were examined in RSUP Sanglah Denpasar from January 2017 to December 2018. Exclusion criteria is unreadable biopsy preparation.

Data Collection Method

Data was taken from parafin block of nasopharyngeal biopsy preparations of NPC patients which are taken in Patologiy Anatomy Udayana University, Sanglah General Hospital Denpasar. The examination results are recorded in the data collection sheets for further analysis.

Data processing

The results are analyzed by univariate and bivariate. The result then presented in tables. The data were analized using chi-square test with 95% confident interval and p value 0.05. all the data were analized using SPSS ver. 22.0 for windows.

RESULTS

This study is using 62 samples who meets inclusion criteria. Univariate analysis was done to show subject characteristics which include age, gender, occupation, clinical stage, gene allele and polymorphism rs3813865 CYP2E1 gene.

The characteristics of subject are shown in table 1.

Mean age of subject is 48.05 years with standard deviation of 10.86 years. The youngest is 17 years old and the oldest is 73 years old. The most are men as many as 47 subjects (75.8%), and the most occupation are government employee as many as 17 subjects (27.4%).

The most clinical stage of undifferentiated type NPC are stage II as many as 7 subjects (11.3%). Based on TNM, the most are T4 as many as 32 subjects (51.6%), N3 as many as 21 subjects (33.9%), and M0 as many as 60 subjects (96.8%). The distribution of polymorphism rs3813865 CYP2E1 gene are shown in table 3.

Bivariate analysis is done to asses the relation of CYP2E1 gene allele polymorphism with clinical stage of undifferentiated type NPC as shown in table 4.

Table 4 shows polymorphism and mutation of rs3813865 CYP2E1 gene as many

Table 1. Characteristics of Subject

Characteristic	n = 62
Age (year)	
Mean ± SD	48.05 ± 10.86
Min-Max	17-73
Gender	
Man	47 (75.8%)
Woman	15 (24.2%)
Occupation	
Goverment employee	17 (27.4%)
Private employee	12 (19.4%)
Enterpreneur	12 (19.4%)
Housewife	10 (16.1%)
Farmer	9 (14.5%)
Retired	1 (1.6%)
Unemployed	1 (1.6%)

as 18 subjects (42.9%) and as many as 3 subjects (15.0%) in below stage IV. In stage IV as many as 24 subjects (57.1%) and as many as 17 subjects below stage IV whose having polymorphism and mutation of rs3813865 CYP2E1 gene (wild type). Total subjects who shows mutation of rs3813865 CYP2E1 gene as many as 21 subjects in both group. To asses the relation between polymorphism of rs3813865 CYP2E1 gene with clinical stage of undifferentiated type NPC using chi square test with p value of 0.030 ($p<0.05$), statistically significant. The chance of having polymorphism of rs3813865 CYP2E1 gene in undifferentiated type NPC is 4.25 times greater in stage IV subjects.

Table 2. Clinical Stage of Undifferentiated type Nasopharyngeal Carcinoma

Variable	(n=62) n (%)
Clinical Stage of undifferentiated NPC	
Stage I	0 (0%)
Stage II	7 (11.3%)
Stage III	13 (21.0%)
Stage IV A	18 (29.0%)
Stage IV B	22 (35.5%)
Stage IV C	2 (3.2%)
TNM (Tumor)	
1	1 (1.6%)
2	12 (19.4%)
3	17 (27.4%)
4	32 (51.6%)
TNM (Nodul)	
0	10 (16.1%)
1	16 (25.8%)
2	15 (24.2%)
3	21 (33.9%)
TNM (Metastasis)	
0	60 (96.8%)
1	2 (3.2%)

DISCUSSION

As many as 62 subjects was included in this study with age ranging from 17 to 73 years old with mean age 48.05 ± 10.86 years old. This was in accordance with study in Turkey, which shows most of NPC occur in age group 40 -50 years old.11 Cao *et al* (2011) reports that in malignancy need carcinogene exposure since early age. Cancer cells arise from normal cells which underwent transformation to malignancy because of spontaneous mutation as well as carcinogene induction. Long induction time is needed from contact with carcinogene to malignant cell emerge which are 12-30 years.12 DNA repair mechanism is not functioning properly and there is a decrease in endurance at the age of more than 40 years. DNA repair mechanisms are needed to improve the sequence of amino acids in the mutated DNA genetic code. If the mechanism of DNA repair fails in carrying out its function, the DNA gene

Table 3. Polymorphismrs3813865 CYP2E1 gene

Variable	(n=62) n (%)
rs3813865*CYP2E1* gene	
Allele GG	41 (66.1%)
Allele CC	15 (24.2%)
Allele GC	6 (9.7%)
Mutation and Non-mutation	
Mutation	21 (33.9%)
Non-mutation	41 (66.1%)

Table 4. Bivariate analysis result of the relation of CYP2E1 gene allele polymorphism with clinical stage of undifferentiated type NPC

Variable	Clinical Stage		OR 95% CI	P value
	Stage IV (n=42)	Stage < IV (n=20)		
Polymorphism				
Present	18 (42.9%)	3 (15.0%)	4.25	0.03
None	24 (57.1%)	17 (85.0%)	(1.079-16.744)	
rs3813865				
Mutation	18 (42.9%)	3 (15.0%)	4.25	0.03
Non-mutation	24 (57.1%)	17 (85.0%)	(1.079-16.744)	

mutation that has already occurred will cause uncontrolled cell growth.13This study shown male predominance of 3.13 : 1. Cao *et al* (2011) shows the same result with ratio of 2-3:1 in accordance with Adham *et al* with ratio of 2.4:1.11,14 The higher incidence in male are suspected because of occupation and lifestyle which more exposed to carcinogene.15 The most exposed occupation in this study is government employee (27.4%). NPC is thought to arise due to interactions of VEB infection, environment and genetic sensitivity. Risk factors in the work environment that are suspected to play roles in the occurrence of NPC include exposure to vapors, smoke, dust and chemical gases in the workplace which increases the risk of NPC 2-6 times while formaldehyde exposure increases risk 2-4 times.[15,16]

The most of research subjects based on clinical stage of undifferentiated type NPC was clinical stage IV B as many 22 patients (35.5%). Hasibuan *et al* (2014) found 62.5% of patients with stage IV NPC and there were no patients with stage I.17 Cheng *et al* (2014) found 39.1% of patients with early stage NPC and 60.9% of patients with advanced stage.18 Early diagnosis of NPC is difficult because the nasopharynx stops at the base of the skull. The position of the nasopharynx is related to many important areas in the skull and neck. In addition, NPC often shows minimal or unspecified symptoms found at an advanced stadium with enlarged lymph nodes.

Optimization of ARMS-PCR (Amplification Refractory Mutation System Polymerase Chain Reaction) primary tetra rs3813865 is carried out with several concentrations of outer and inner primers (1: 1, 1: 2, 1: 8, and 1:10) each amplified with annealing temperature of 540C , 560C and 580C. The uppermost DNA band is the control fragment (499 bp) and the two DNA bands below it are specific to the allele (303 bp for C allele and 236 bp for G allele). The ratio of outer and inner primary concentrations of 1: 2 and annealing temperature of 540C are optimal conditions for distinguishing alleles from rs3813865 shown by three clearly visible DNA bands (Figure 1).

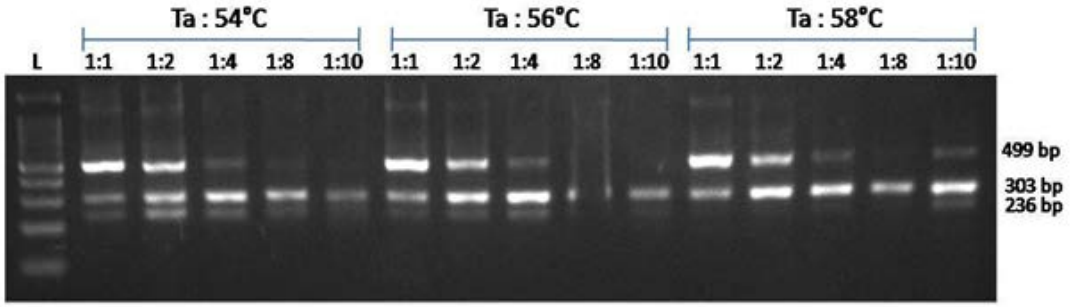

Fig. 1. Three DNA bands on the ARMS-PCR examination to detect alleles from rs3813865[19]

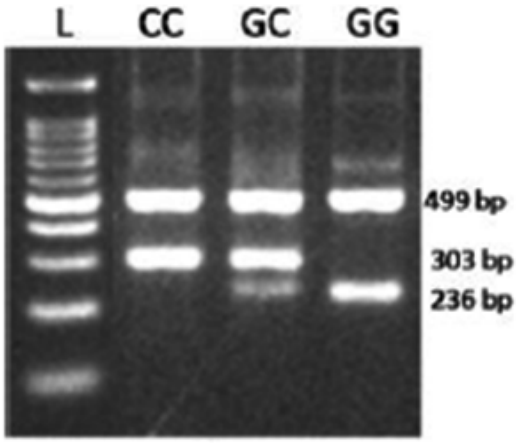

Fig. 2. ARMS-PCR primary tetra results for detecting the rs3813865 allele in this study

In this study the ARMS-PCR primary tetra protocol was performed with a PCR master mix reagent. This causes the concentration of each component in the master mix can not be modified, for example the concentration of MgCl2 and polymerase enzymes. Previous studies have shown that modification of MgCl2 concentration can improve PCR results, although there are studies that do not prove significant improvement after the modification of PCR reagents.[19,20]

The proportion based on the polymorphism of the rs3813865 CYP2E1 gene in undifferentiated

type NPC subjects in this study was 41 in GG alleles (66.1%), CC alleles as many as 15 subjects (24.2%), and GC alleles as many as 6 subjects (9.7 %). Comparison of the proportion of polymorphisms in the rs3813865 CYP2E1 gene in undifferentiated type NPC subjects in this study were mutations (CC and GC alleles) of 21 patients (33.9%) and non-mutations / wild types (GG alleles) of 41 patients (66, 1%).

This is in accordance with the results of several previous studies which found that GC and CC allele polymorphisms in rs3813865 CYP2E1 gene. Jia *et al*21 reported on a case control study in China by comparing several specific gene rs as risk factors for nasopharyngeal carcinoma events. There was only one rs that was statistically significant for the emergence of nasopharyngeal cancer, namely rs3813865, where in the sample of cases there were 326 subjects who experienced polymorphism of rs3813865 out of a total of 755 patients (GC and CC alleles), while in the control sample there were 287 subjects experiencing rs3813865 polymorphisms of a total of 755 controls.

Tang *et al*7 comparing NPC patients in several cities namely Shantou, Shanghai, Shenyang, Xian, and China. In each city several specific gene rs were compared as risk factors for nasopharyngeal carcinoma. The subjects was 100 NPC patients in each of these cities, which consisted of 50 male subjects and 50 female subjects, aged 18-53 years. A comparison of the proportion of polymorphisms rs3813865 in each city is as follows: Shantou by 18.7%; Shanghai 14%; Shenyang 23.4%; Xian 22.4% and China 19.7%. This result is higher than the proportion of polymorphism rs3813865 in European and American countries by 3.7% but lower than the population in Asian countries which is 16.1%.

Case control study by Hildesheim *et al* (2007) in Taiwan where 364 people with NPC as a case and 320 people as a control, showed that CC allele polymorphism was in 30 subjects in case group and 14 subjects in control group, statistically these results were not significant but CC allele polymorphism increases the risk of nasopharyngeal carcinoma by 1.9 times. The CC allele polymorphism of CYP2E1 gene in Asian races is 20-25% while Caucasian races are 10%.

In stage IV there were 24 subjects (57.1%) and in stages under IV there were 17 subjects (85.0%) who did not experience polymorphism and mutations of rs3813865 CYP2E1 gene (wild type). The total number of subjects who experienced gene allele mutations were 21 subjects both in stage IV and below IV. To assess the relationship between polymorphism of rs3813865 CYP2E1 gene with clinical stage of undifferentiated type NPC with Chi square type test, with p value 0.030 ($p < 0.05$) there was a significant relationship between polymorphism of rs3813865 CYP2E1 gene with the clinical stage of undifferentiated type NPC. The chance of the polymorphism rs3813865 CYP2E1 gene in undifferentiated type NPC is 4.25 times greater in stage IV subjects. Examination of the polymorphism rs3813865 CYP2E1 gene can be used as a supporting modality in assessing tumor growth, progression, and prognosis of NPC. This is in accordance with previous studies which found polymorphism of the rs3813865 CYP2E1 gene was significantly associated with metastases to the lymph nodes and clinical stage.[7,21,22]

The study of Jia *et al*21 regarding the polymorphism rs3813865 of NPC patients in China, there is statistically significant results with a p value of 0.050 ($p < 0.05$) and the probability of the occurrence of GC and CC allele polymorphisms of the CYP2E1 gene in undifferentiated type NPC was 1.25 times greater in patients with advanced stages. Tang *et al*.7 compared the proportion of GC and CC allele polymorphisms rs3813865 CYP2E1 gene in undifferentiated type NPC patients in several countries including Finland by 1.2%, Europe 1%, Africa 4%, and Asia 16.1%. A case control study by Hildesheim *et al*22 in Taiwan, found the chance of CC allele polymorphism rs3813865 CYP2E1 gene in undifferentiated type NPC was 2.6 times greater in patients with advanced stage with 95% CI values ??of 1.2 – 5.7.

An increase proportion of the polymorphism rs3813865 CYP2E1 gene was associated with worse overall survival (OS) and disease free survival (DFS) for 5 years, so the polymorphism rs3813865 CYP2E1 gene could be used as a prognostic factor for NPC.21 Gene polymorphisms related to carcinogenic metabolism are one of the risk factors for NPC. One of them is the cytochrome P450 family 2 subfamily E polypeptide 1 (CYP2E1) gene which codes for the CYP2E1 enzyme for activation of procarcinogens.[7,23]

CONCLUSIONS

In this study, it was found that the proportion of the polymorphism rs3813865 CYP2E1 gene based on the level of clinical stage undifferentiated type NPC in the primary tetra examination of ARMS-PCR (Amplification Refractory Mutation System Polymerase Chain Reaction) was statistically significant, it could be concluded that the polymorphism of rs3813865 CYP2E1 gene was associated with clinical stage of undifferentiated type NPC.

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