THE CYP4F2*3 VARIANT POLYMORPHISM AND RISK FACTORS FOR STROKE AT THAI NGUYEN NATIONAL HOSPITAL

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ABSTRACT

Background: The incidence of stroke in young people is high, but most of the research done has focused on complications of stroke and stroke therapies, but there are no studies looking for genes and mutations of these genes related to stroke in Vietnam. **Objective:** This study aims to identify of CYP4F2*3 variant polymorphism and risk factors in stroke patients at ThaiNguyen National Hospital. **Methods:** A cross-sectional descriptive study was conducted on 105 patients (>18 years old) diagnosed with stroke at the Stroke Center, Thai Nguyen National Hospital between October 2020 and May 2021. **Result:** The most common risk factors of stroke are hypertension accounting for 76.2%, lipid metabolism disorders 60.0%, obesity 24.8%, diabetes mellitus is 15.2%, the lowest was smoking 11.4% and alcoholism 6.7%. The genotype of the CYP4F2*3 homozygous AA SNP accounted for 7.6% and 16.2% of patients with rabies homozygous GA heterozygous genotype accounted for the highest rate of 76.2%. The allele distribution frequency of SNP CYP4F2*3: allele A (0.16) was very low compared to allele G (0.84).

Keywords: CYP4F2*3; Stroke

INTRODUCTION

In recent years, the incidence of stroke among young people has increased by 25%, representing an almost 50% increase over the past 12 years[1]. The risk factors for stroke can be mentioned in two categories: the type of risk factors for stroke can be changed such as smoking, drinking, physical inactivity, obesity and risks that cannot be changed such as age, race, genetics. Among the risk factors for stroke that have been studied, genetic risk is of

great concern to doctors and researchers because nearly 50% of stroke cases have an unexplained cause[2]. However, studying genetic factors and gene mutations in stroke is also very challenging, especially in identifying the involved genes, as different genes tend to cause different types of stroke. In Vietnam, although the rate of stroke in young people is increasing, most of the research has focused on stroke complications and stroke therapies, but there have been no studies investigating genes and mutations related to stroke in Vietnam. This study aims to investigate the CYP4F2*3 variant polymorphism and assess risk factors for stroke at Thai Nguyen National Hospital.

METHODS

Subject, place and time of study

Study subject

Stroke patients over the age of 18 at the Stroke Center, Thai Nguyen National Hospital.

Criteria for selecting patients: Clinical criteria: Based on the definition of stroke of the World Health Organization; Subclinical criteria: CT-Scanner or MRI scan of the brain confirms presence of cerebral infarction lesions or acute cerebral haemorrhage.

Exclusion criteria: patients with brain bleeding, brain tumor, traumatic brain injury, encephalitis; with a transient ischemic attack (TIA); have a history or current diagnosis of cardiovascular diseases such as: heart failure, valvular heart disease, atrial fibrillation, dilated cardiomyopathy, myocardial infarction; not agreeing to participate in the study.

Place and time of study

Duration: from 10/2020 to 5/2021

Study location: Stroke Center, Department of Immunology – Molecular Genetics, Thai Nguyen National Hospital

Methods

Study design

Study sample size: Convenient. In this study, we collected 105 patients who were eligible to participate in the study.

Research indicators

Clinical criteria: Age, gender, BMI, cardiovascular diseases, diabetes mellitus, hypertension, ...

Subclinical criteria: Hematology, biochemistry, cerebral angiography test results.

Results of gene analysis: CYP4F2*3 breakdown genes.

Data collection methods

Stroke patients treated at Thai Nguyen National Hospital's Stroke Center, agreed to participate in the study. Collect clinical and subclinical information into research records. Especially information about thrombotic complications, hemorrhage when participating in the study. At the same time, the patient will have 2 ml of venous blood collected in an EDTA anticoagulant tube stored at -20°C until genetic analysis is carried out.

Evaluate the data obtained against standards

Obesity assessment: obesity assessment according to the BMI (Body Mass Index) of the Asian diabetes association:

BMI = Weight (kg) / Height (m2): BMI < 18.5: Lean; 18.5 ≤ BMI < 23: Normal; 23 ≤ BMI < 25: Overweight; 25 ≤ BMI < 30: Degree obesity I; 30 ≤ BMI < 35: Degree obesity II; BMI ≥ 35: Degree obesity III.

Hypertension: Patients with a history of a diagnosis of hypertension, are on antihypertensive medication, or have recently been diagnosed with hypertension upon admission. Diagnosis of hypertension according to JNC VII criteria: when systolic (140 mmHg and/or diastolic (90 mmHg and increase BP according to JNC VII.

Dyslipidemia: patients with a history of dyslipidemia or laboratory disorders with metabolic disorders of at least one of the blood lipid components as recommended by the Vietnam Heart Association as follows:

- Cholesterol > 5.2 mmol/l (>200 mg/dl)
- Triglycerides > 2.3 mmol/l (>200 mg/dl)
- LDL-C > 3.2 mmol/l (>125 mg/dl)
- HDL-C < 0.9 mmol/l (<35 mg/dl)

Glucose: Normal between 3.9 and 6.4 mmol/l (fasting), increased when blood glucose ≥ 7.0 mmol/l; HbA1C: Normal

blood levels are about 4-6%, assessed to increase when the amount of HbA1C ≥ 7%

Alcoholism:

- According to WHO (1996), men drink ≥ 60 grams of alcohol / day corresponding to 1200 ml of beer at 5% concentration or 180ml of spirits.

- Women drink ≥ 20 grams of wine / day, equivalent to 250ml of wine or 60ml of spirits continuously for many years.

Tobacco addiction: according to WHO 1996, smoking more than 10 cigarettes / day, continuously for 2 years is called smoking addiction

Techniques used in research

PCR-RFLP restriction enzyme method identifies gene polymorphism.

To determine the allele frequency, genotype of CYP4F2*3 weselected PCR-RFLP restriction enzyme method with the advantages of accuracy, low cost and fast results. The primer pairs we used to isomorphize the CYP4F2*3 gene polymorphism were:

F: 5'ATCAACCCGTTCCCACCT3',

R: 5'ACATTGTGCTCCCAGACG3'.

To amplify the gene fragment with the above primer sequence, we standardized the process with primer mounting temperature, primer concentration, DNA concentration. The results of reaction composition and thermal cycle are presented in the table below:

Table 1. Composition and thermal cycle of PCR reaction

Reaction composition	Thermal cycle
dNTP Mix: 0.2mM;	- Denaturation: 95°C-3 minutes.
Gol Taq: 0.02u/µl;	- 35 Cycle:
Down-bait, back-bait of each type: 0.5uM;	+ 95°C-50 second;
DNA: 50ng/µl	+ 59°C-30 second;
	+ 72°C-30 second
	- Finish: 72°C in 5 minutes.

The PCR product tested by electrophoresis on this 2%.

SNP agarose gel has a specific shear site with PvuII restriction enzyme. The product after PCR will be purified and diluted or concentrated to reach a concentration of 40-50 ng/µl and RFLP will be carried out according to the following process:

Table 2. CYP4F2*3 SNP analysis procedure according to RFLP

No	Ingredient	Volume
1	PCR Products	10µl
2	Cushion 10x	1.5µl
3	Water	2.5µl
4	Enzym	1µl
5	Total volume	15µl

The mixture is incubated at 37°C for 18-22 hours then electrophoretic for testing on 2% agarose gel. Genotypic results are read based on electrophoresis images.

Use 25 µl of PCR product to multiply the gene region containing SNP CYP4F2*3 on a specific electrophoresis tape, with a minimum concentration of 20 ng/µl, purification and sequencing.

DNA purification

PCR products of SNPs after quality control by electrophoresis method on agarose gel if qualified for quality (clear clear tape, correct theoretical size, no breakage, smear) will be purified by the method using GeneJET PCR purification kit before sequencing is performed.

The sequencing results were opened using BioEdit software version 7.1.9, based on the resulting sequencing image, we determined the genotype of SNP CYP4F2*3. Sequencing results showed match with enzymatic results.

Data processing methods

The following data is entered into the machine and processed by SPSS 20.0 software.

Research ethics

The study was accepted through the Medical Council of Thai Nguyen National Hospital.

RESULTS

Table 3. The distribution by gender, age of the study group

Gender	n	%	Median age
Male	60	57.1	63.1 ± 12.3
Female	45	42.9	64.3 ± 12.9
General	105	100	63.6 ± 12.6

Our study results noted a higher prevalence of stroke among men (57.1%) than women (42.9%). The average age of the study group was 70.2 ± 13.2 years, with the average age of females (64.3 ± 12.9) higher than that of males (63.1 ± 12.3).

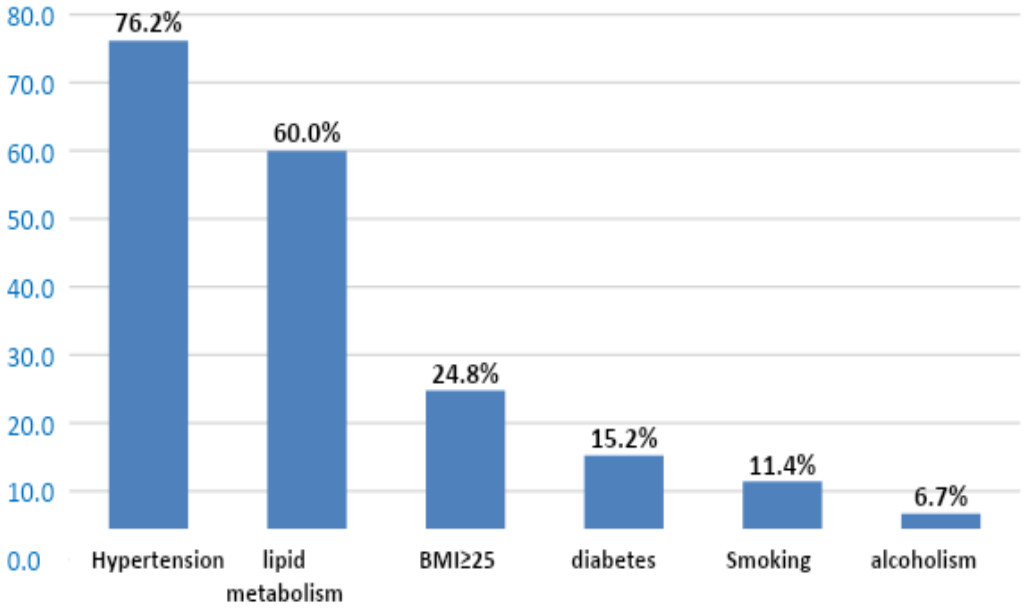

Figure 1. Some risk factors for stroke of the research group

The most common risk factor in our study was increased BP at 76.2%, followed by lipid metabolism at 60.0%, obesity at 24.8%, diabetes at 15.2%, smoking at 11.4% and alcoholism at 6.7%.

*Determination of genetic polymorphism, allele frequency, and genotypic frequency of CYP4F2*3 by PCR –RFLP technique*

PCR products are electrophoretic tested on 2% agarose gel

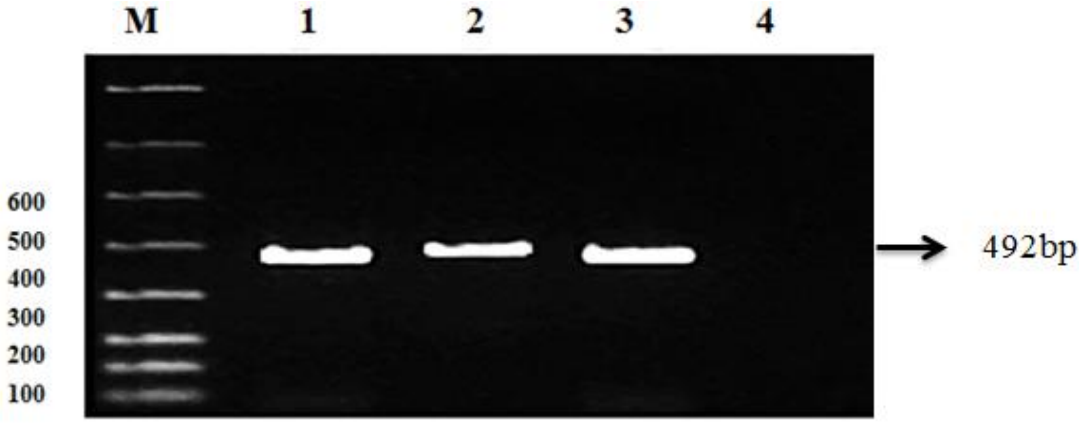

Figure 2. Electrophoresis results of PCR product amplification of gene fragments rather than SNP CYP4F2*3

Lane 4 is the 100bp scale marker, lane 1,2,3 is the patient samples, lane 4 is the negative control.

On the electrophoretic gel, DNA samples form a single, clear tape, without an extra band, without smear. The PCR product amplifies the gene fragment containing SNP CYP4F2*3 electrophoresis for tape size: 492 bp. The results showed that the primer pairs used for the gene fragment amplification reaction containing the SNPs in the study were specific, and other necessary elements of the gene fragment amplification reaction containing the SNPs in the study were well optimized. The PCR products of the SNPs in the study are eligible for use in the RFLP process and subsequent sequencing.

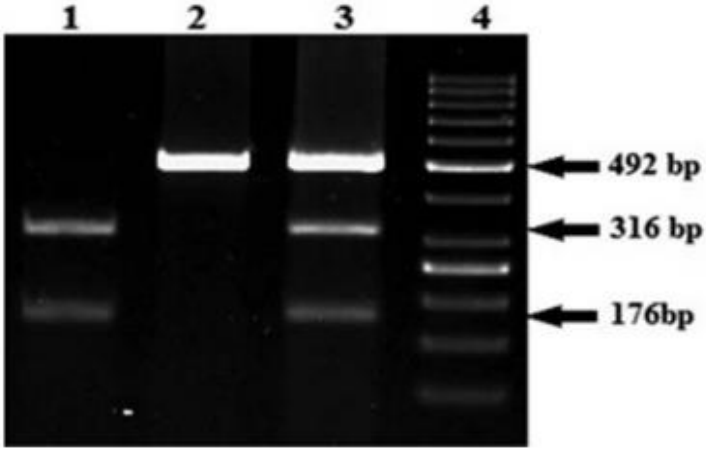

Figure 3. RFLP Product Electrophoresis Results

Lane 4 is a 50bp scale marker, lanes 1,2,3 are patient samples

The RFLP results of SNP CYP4F2*3 image clear, specific electrophoresis tapes. The electrophoresis results showed that the shear enzyme used for the allele was specific, and the elements of the shear reaction were well optimized. RFLP results for 3 genotypes:

- Homozygous genotype (AA) for a single electrophoresis tape size: 492 bp.

- Electrophoretic homozygous (GG) genotype for two bands in size: 176 and 316 bp.

- Heterozygous genotype (AG) for three electrophoretic bands of size: 176; 316 and 492 bp SNP CYP4F2*3 were sequenced to test the accuracy of the enzyme technique limited to 10% of patient samples (10 samples).

Use 25 µl of PCR product to multiply the gene region containing SNP CYP4F2*3 on a specific electrophoresis tape, with a minimum concentration of 20 ng/µl, purification and sequencing.

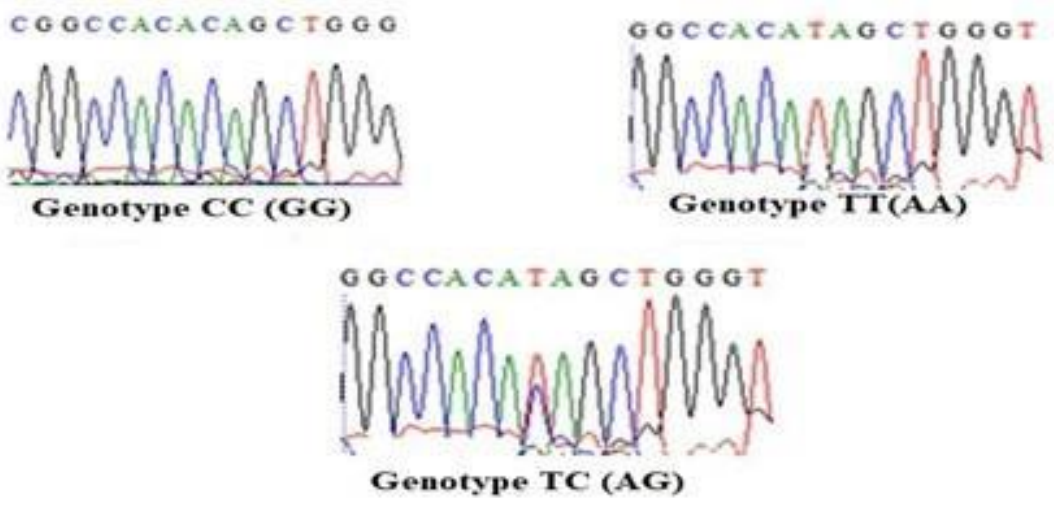

Figure 4. Gene sequencing images identify CYP4F2*3 gene polymorphisms

The gene sequencing results identified three genotypes: mutant homozygous genotype: AA, heterozygous genotype: AG, rabid homozygous genotype: GG consistent with the results of gene analysis using cutting enzyme techniques.

After analyzing the genotype of SNP CYP4F2*3 by RFLP method and gene sequencing, we determined the allele frequency results, genotype in the research group as follows:

Table 4. Allele frequency and genotype of the CYP4F2*3 gene

CYP4F2*3	n	%
GG	80	76.2
GA	17	16.2
AA	8	7.6
A	33	16
G	177	84

The results of identification and genotypic analysis of SNP CYP4F2*3 (with n=105) showed that AA variant homozygous genotype accounted for 7.6% and 16.2% of patients with rabies homozygous GA heterozygous genotype accounted for the highest rate of 76.2%. Allele distribution frequency of SNP CYP4F2*3: A allele (0.16) was very low compared to G allele (0.84).

DISCUSSION

Our results indicated that the prevalence of stroke was higher among men (57.1%) than among women (42.9%). The average age of the study group was 70.2 ± 13.2 years, with females having a higher average age (64.3 ± 12.9) than males (63.1 ± 12.3). Numerous domestic and foreign studies demonstrate that stroke incidence increases with age. The older the age, the more vascular pathologies, of which atherosclerosis is predominant. Additionally, the accumulation of risk factors escalates with age. Stroke skyrockets from 50 years old and older[3]. The most common risk factor in our study was increased diastolic at 76.2%, followed by lipid metabolism at 60.0%, obesity at 24.8%, diabetes at 15.2%, smoking at 11.4% and alcoholism at 6.7%. In fact, a patient may have a combination of risk factors, which in turn increases the incidence of stroke.

Hypertension: Hypertension is considered a leading risk factor in the pathogenesis of stroke. In the medical literature and many studies, it has been shown that the higher the blood pressure

index, the greater the risk of stroke and this change varies depending on race. In the REGARDS (Reasons for Geographic and Racial Differences in Stroke) study, a 10 mmHg increase in diastolic resulted in an approximate 8% increase in the risk of stroke in whites. However, the risk of stroke among African Americans increased by 24%, more than 3 times that of whites. Many studies show that treatment that lowers blood pressure readings also reduces stroke rates by a significant amount. In our study, hypertension was a high prevalent risk factor. Hypertension in the cerebral infarction groups was 31.2%, brain hemorrhage was 54.5%. According to research by Nguyen Dinh Toan, hypertension in cerebral infarction patients accounts for 68.75%[4.] A study of 77 patients with acute cerebral infarction Pham Van Tu recorded hypertension accounting for 72.7%[5]. This results in a higher proportion of hypertension patients in our study. In a study of 245 cerebral infarction patients, Zhang, J.L recorded a history of hypertension accounting for 71.8%[6]. This result is close to our study. In a study of 480 brain hemorrhage patients, Ngo Thi Kim Trinh et al recorded patients with a history of hypertension accounting for 73.8%[7]. Thus, in this study, hypertension was a high risk factor in both cerebral infarction and brain hemorrhage groups. This is similar to some of the studies stated.

Dyslipidemia: It can be defined as an abnormal increase in cholesterol and/or triglycerides in the blood, and/or a decrease in HDL-cholesterol. According to Stamler J et al., when cholesterol-LDL increased by 10%, cardiovascular risk increased by 20% through atherosclerosis. The decrease in cholesterol – HDL also increases the risk of cardiovascular diseases, including NMN.

Obesity (BMI>25): In the past, it was thought that fat was inert. However, scientists have now proven that fat, especially intra-abdominal fat, significantly affects the body's metabolism. On the other hand, it increases blood lipids and interferes with the action of the hormone insulin. Increased BMI often entails increased blood pressure, increased "bad cholesterol," triglycerides, blood sugar levels, and inflammatory reactions. According to Raphae S Barlas (2016), excessive weight gain of 30% increases the risk of stroke. The relative risk of stroke in the group with a high body mass index was 2.33 compared to the group with a low body mass

index in the study of 28.634 men[8].

Diabetes: Diabetes increases the incidence of stroke in all[9]. Diabetes mellitus increases the incidence of stroke by 2–6.5 times, increasing mortality by 2 times[10]. The ACCORD (Action to Control Cardiovascular Risk in Diabetes) study found that in patients with type 2 diabetes, a target diastolic of < 120 mmHg did not reduce the incidence of cardiovascular events compared with those with a diastolic < 140 mmHg, except for a stroke outcome, as tight BP therapy reduced the risk of stroke (HR = 0.59; 95% CI, 0.39 - 0.89) and no death from stroke (HR = 0.63; 95% CI, 0.41 - 0.96)[11]. In our study, diabetes accounted for 15.2% of both patients with brain hemorrhage and cerebral infarction, especially diabetes in combination with other risk factors. According to research by Ngo Thi Kim Trinh on 480 social patients, the proportion of diabetic patients accounts for 8.1%[7]. According to author Pham Van Tu, the proportion of diabetic patients accounted for 45.5% in the study of 77 acute cerebral infarction patients[5].

Cigarette smoking: People who are current smokers have a 2-4 times increased risk of stroke compared to those who have not smoked or quit for more than 10 years. Cigarette smoking increases the risk of cerebral infarction and subarachnoid cavity hemorrhage[9]. In a large Danish cohort study, people with atrial fibrillation and smoking were associated with a higher risk of brain hemorrhage, embolism, and death, even after adjusting for other known risk factors[12]. In our study, cigarette smoking in the cerebral infarction group was 2.1%, not reported in the cerebral haemorrhage group. Especially smoking in combination with other risk factors such as hypertension, alcohol consumption, ... The study of patients with Pham Van Tu noted that the risk factor for smoking was 46.8%[5]. In Nguyen Hoang Hai's study, cerebral infarction patients smoked cigarettes accounted for 52.9%[13]. In a study of 480 brain hemorrhage patients, the authors noted a history of smoking accounting for 31%[7]. In a study of 245 cerebral infarction patients, Zhang, J.L. noted a history of smoking accounting for 29%[6]. Cigarette smoking increases the risk of stroke, according to results from many studies and according to many authors because smoking causes temporary

THA associated with atherosclerosis[14]. In summary, smoking is an important risk in stroke patients.

Alcoholism: Whether alcohol reduces or increases the risk of stroke depending on the level of alcohol consumption and the body. Alcohol abuse increases blood pressure, hypertriglyceridemia, paroxysmal atrial fibrillation attacks, cardiomyopathy and is associated with an increased risk of stroke, particularly brain hemorrhage, and death from stroke. Analysis of prospective and controlled studies identified the risk of stroke in alcoholics compared with those who quit drinking[15,16]. In our study, alcohol consumption accounted for 6.7%. Similar to smoking, drinking alcohol is often coordinated with other risk factors. The study of Ngo Thi Kim Trinh et al. in brain hemorrhage patients who consumed alcohol accounted for 38.5%[7]. According to Nguyen Dinh Toan, in cerebral infarction patients, patients with a history of alcohol consumption accounted for 10%[4]. In another study in cerebral infarction patients, the authors noted patients with a history of alcohol consumption accounted for 15.3%[1]. Thus, a history of alcohol abuse is a common risk in stroke patients.

Gene polymorphism CYP4F2*3: The results of identification and genotyping analysis of SNP CYP4F2*3 (with n=105) showed that the AA variant homozygous genotype accounted for 7.6% and 16.2% of patients with rabies homozygous GA heterozygous genotype accounted for the highest rate of 76.2%. The allele distribution frequency of SNP CYP4F2*3: allele A (0.16) is very low compared to allele G (0.84). This study result is lower than the study of some authors in the world such as Shumin Denga et al. in 2010 on Han Chinese ethnicity showing that rabies homozygous genotype accounts for 70%, heterozygous 20% and mutant homozygous is 10%, the proportion of variant alleles is 19.5%, the rabies-type allele is 80.5%[17]. The study by Kalpana et al on the Indian population in 2019 showed that the frequency of GG homozygous genotypes was 12.64%, heterozygous was 45.97%, variant homozygous was 40.4%. The variant allele is 0.36, the variant allele is 0.64. Such differences are due to differences in geographic race[18].

CONCLUSION

The most common risk factors were hypertension accounting for 76.2%, lipid metabolism disorders 60.0%, obesity 24.8%, diabetes mellitus 15.2%, the lowest being smoking 11.4% and alcoholism 6.7%.

The genotype of the CYP4F2*3 homozygous AA SNP accounted for 7.6% and 16.2% of patients with rabies homozygous GA heterozygous genotype accounted for the highest rate of 76.2%. Allele distribution frequency of SNP CYP4F2*3: A allele (0.16) is very low compared to G allele (0.84).

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