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A Study to Investigate the Prevalence of Single Nucleotide Polymorphisms (Snps) in, Cyp2c19, Hdac9, Apoe, Pmf1 and Pitx2 in Patients with Hemorrhagic or Ischemic Stroke

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Abstract

In present study, 46% of recurrent stroke patients had clopidogrel resistance, our data is comparable to western data where the prevalence rate is 5 -44%. This data shows that it is imperative to check the prevalence of clopidogrel resistance in new onset stroke, recurrent stroke as well as those patients who are on dual antiplatelet drug therapy for cardiac disease, but develop atherothrombotic stroke. The results of the study are in congruence with the current FDA guidelines which recommend routine testing of clopidogrel resistance. The present study focuses on HDAC9 gene in all patients and found that three genotype polymorphisms are present, namely CC, CT, TT. Studies have shown HDAC9 gene to be significantly associated with progressive atherosclerosis and higher incidence of strokes. HDAC9 gene was compared to MRA study with mild, moderate and severe disease. The CC genome polymorphism showed high rates of atherosclerosis disease on MRA when compared to CT/ TT polymorphism groups. From this study we infer that CC genotype is more prevalent, and CC group genotype has higher prevalence of risk factors.

Keywords: Cerebrovascular disease (CVD), stroke, atherosclerosis, antiplatelet, polymorphism, TOAST and metabolizer

1. Introduction

Cerebrovascular disease (CVD) is the most common disease group among all neurological diseases. Stroke -induced mortality is the third most common cause of death in developed countries. Disabilities related to stroke lead to a serious individual and socioeconomic burden. The incidence of

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stroke recurrence is high despite of developments in primary and secondary preventive treatment. The stroke incidence in India is much higher than western industrialized countries. In 2013 approximately globally 6.9 million people had an ischemic stroke and 3.4 million people had a hemorrhagic stroke1. In 2015 there were about 42.4 million people who had previously had a stroke and were still alive 1 . Between 1990 and 2010 the incidence of stroke decreased by approximately 10% in the developed world and increased by 10% in the developing world 2. In 2015, stroke was the second most frequent cause of death after coronary artery disease, accounting for 6.3 million deaths (11% of the total) 3. About 3.0 million deaths resulted from ischemic stroke while 3.3 million deaths resulted from hemorrhagic stroke 5. Overall, two thirds of strokes occurred in those over 65 years old 4.

The cumulative incidence of stroke recurrence was 14.5% by the end of 5 years, with the highest incidence during first year of stroke(5.6%).case fatality at 30 days after first recurrence stroke was 43.2% 5. The recurrence risk varies depending on CVD type, Large vessel intracranial atherosclerosis is the commonest cause of ischemic stroke in India . The standard approach in preventing recurrence is by determining etiology and treating patients using pharmacological methods, such as antithrombotic and anticoagulant medications, and non - pharmacological methods, such as carotid endarterectomy and stenting procedures for occlusive vascular lesions.

Risk factors for stroke-

Risk factors for stroke categorized as modifiable and non modifiable.

Non modifiable - age, sex, race, ethnicity

Modifiable - hypertension, hyperlipidemia, diabetes, diet, smoking, alcoholic consumption physical inactivity, cardiovascular disease, atrial fibrillation, atherosclerotic disease (extra cranial and intracranial disease) .

Genetic Risk Factors - Heredity is generally considered a non modifiable risk factor, although some may be modifiable like sickle cell anaemia in which treatment with exchange transfusion reduces stroke risk . Gene environmental interactions which leads to genetic mutation may also be modifiable.

Genetic causes of stroke 6

1, single gene disorders that primarily cause stroke - CADASIL -AD & AR types familial amyloid angiopathy, collagen 4 (COL4A1) mutations

2, genetic disorders that include stroke as manifestation

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- Ehlers danlos type 4
- Fabry disease
- Marfan syndrome
- Mitochondrial encephalopathy with lactic acidosis and stroke like episodes
- Sickle cell disease

3, common genetic variants - Today's clinical molecular research is focused on SNP analysis

SNP -SINGLE NUCLEOTIDE POLYMORPHISM - smallest molecular changes are confined to variation of one single

base pair where a nucleotide has been exchanged for another.

- SNP RELATED TO ISCHEMIC STROKE - TSPAN2, FOXF2, ABO, HDAC9 , causing atherosclerosis of cerebral vessels (large vessels and small vessels) leading to ischemic stroke.
- PITX2 , ZFHX3 - causing atrial fibrillation leading to cardio embolic and ischemic stroke.
- SNP RELATED TO HAEMORRHAGIC STROKE RISK - APOE GENE variations especially ε2 or ε4 alleles, COL4A1 gene variation were related to increased risk of sporadic Intra cerebral haemorrhage.114 PMF1 /SLC25A44,KCNK17 gene variation was also related to ICH risk 13-16.

In the recent era, recurrence of stroke in Asian population is more common than Western population due to resistance to antiplatelets drugs clopidogrel and aspirin .In this study we are performing genetic evaluation to identify the responders and non-responders of clopidogrel by testing the SNPs in the CYP2C19 gene.

Resistance to anti platelet drugs is considered as one of the major reason for recurrent events in CVA patients. Clopidogrel is an inhibitor of the ADP receptor P2Y12 which is a platelet aggregator. It is widely used in the management of severe vascular events such as stroke. Clopidogrel Resistance is a phenomena that has recently emerged in everyday medical practice, there is no clear definition for this phenomena. A widely accepted description is the persistent activity of clopidogrel target, i.e,

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P2Y12receptor of the platelet, despite an adequate antiplatelet regime.

High platelet reactivity due to clopidogrel resistance was associated with adverse thrombotic events. The primary reason is suboptimal generation of active metabolites due to individual variability in intestinal absorption, drug interaction and polymorphism in CYP isoenzymes. Clopidogrel non-responsiveness is reported to vary between 4%-44% among different populations. The five CYP forms contributing to formation of active metabol ites includeCYP3A, CYP2B6,CYP1A2, CYP2C19, and CYP2C97 (Kazui et al., 2010). CYP450 enzymes (CYP2C19, CYP3A4, CYP3A5) enzymes play a role in generation of active metabolite of clopidogrel, and hence variation in t he production of enzymes responsible for clopidogrel resistance.

2. *MATERIALS AND METHODS*

Study design

The study is a hospital based study.

Study Place

The study will be conducted at Sree Balaji Medical College and Hospital, Chennai over a period of two years on patient admitted with stroke of 3 groups with ischemic stroke, lobar and non lobar ICH, lacunar infarcts in Sree Balaji Medical College and Hospital.

Sample Size

100 Patients with stroke of 3 groups with ischemic stroke , lobar and non lobar ICH, lacunar infarcts attending inpatient and outpatient Department of Neurology of Sree Balaji Medical College and Hospital, Chennai will be taken for the present study.

Inclusion Criteria:

- Consenting Patients
- Patients in all age groups will be included for the study.
- Patients with known diabetes, hypertension, asthma, CAD, IHD
- Patients with Alcoholic and Smoking History
- Patients with history of Clopidogrel drug treatment.

Exclusion Criteria :

- Non consenting patients

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- Patients with Familial History of Stroke.

Methodology:

All patients who meet the inclusion and exclusion criteria will be taken into the study after taking an informed and written consent from the patient/attenders. Detailed history will be taken. A thorough neurological examination will be done. All the patients will be investigated with MRI, Cerebral Angiogram and carotid and vertebral Dopler. 5ml blood sample is collected from each patient which is sent for genetic study and Clopidogrel resistance.

Patients stroke proforma will be filled according to TOAST classification.

The **TOAST (trial of ORG 10172 in acute stroke treatment)classification** denotes five sub types of ischemic stroke.

- large-artery atherosclerosis (embolus / thrombosis)*
- cardioembolism (high-risk / medium-risk)*
- small-vessel occlusion (lacune)*
- stroke of other determined aetiology *
- stroke of undetermined aetiology

Sample collection: Name, age, gender, race, region / place of birth, nature of work (occupation), habitual factors (tobacco chewing / smoking) and medical history of each patient will be recorded. A total of 100 patients (inclusive of both ICH and ischemic stroke) will be included in the study after obtaining informed consent. A prototype of the consent form is attached. 2ml of blood sample will be collected in EDTA sample collection vials and stored at 4°C. Samples will be transported on ice to laboratory for DNA processing and SNP analysis.

METHODS:

Genomic DNA extraction: 0.1ml of peripheral blood was lysed in 100µl of cell lysis buffer containing 36% to 50% guanidine hydrochloride (Cat# 740951.50, Nucleospin Blood DNA Kit, Machery Nagel, Germany) and incubated at 57°C for 2 hours to enable complete lysis of leucocytes. Following lysis, an equal volume of 100% ethanol was added to precipitate the genomic DNA. Subsequently, the entire content was transferred to DNA spin columns containing silica membrane and centrifuged at 8000 rpm for 1min at room temperature. The precipitated DNA gets captured in the silica membrane during this step. Following DNA capt ure, the silica columns were washed twice with wash

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buffer (supplied by the manufacturer Machery Nagel). Degraded proteins and membrane lipid particles get washed off during the wash steps. After the two wash steps, the captured DNA from the silica membrane was eluted with 50µl of elution buffer (supplied by the manufacturer Machery Nagel).

Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism (PCR-RFLP) to detect CYP2C19*2 and CYP2C19*3 polymorphisms. The following set of primers were used to amplify the CYP2C19*2 (rs4244285) region that causes G681A transition in exon Forward: accagagcttggcatattgtatct, Reverse: gattcttggtgttcttttactttct. To amplify the CYP2C19*3 (rs4986893) region that causes G636A transition in exon 4 the following set of primers were used. Forward: tttcatcctgggctgtgctc, Reverse: tgtacttcagggcttggtcaat. Both primers were used independently on 50ng of DNA samples and were subjected to amplification under the following conditions. Initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, primer extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The CYP2C19*2 primers amplified a 321bp fragment, while the CYP2C19*3 primers amplified a 234bp fragment. To detect CYP2C19*2 and CYP2C19*3 polymorphisms, the PCR fragments was subjected to restriction digestion with Sma1 enzyme (Cat#1085A, Clonetech Takara, Japan) and BamH1 enzyme (Cat#1010A, Clonetech Takara, Japan) respectively. Following digestion, 10µl aliquots of digested PCR products were analyzed by running them in a 1.7% agarose gel at 100V for 15 minutes with 1X TAE (Tris Acetate EDTA) buffer. The DNA bands were visualized by staining the gel with ethidium bromide (a DNA intercalating agent that fluoresces when excited by UV in the range of 302nm to 364nm), and images were captured with gel documentation unit.

Data interpretation

For CYP2C19*2 polymorphism: When G allele was present in homozygous condition, the 321bp fragment was completely digested into 211bp and 110bp fragments, which migrated as two bands during electrophoresis. These samples were designated as CYP2C19*1/*1 (normal metabolizer). When A allele was present in homozygous condition, the 321bp fragment remained undigested and migrated as a single band during electrophoresis. These samples were designated as CYP2C19*2/*2 (poor metabolizer). When both G and A alleles were present in heterozygous condition, the 321bp fragment carrying G allele was digested into 211bp and 110bp fragments, while the other part carrying A allele remained undigested. As a result three bands were observed during electrophoresis. These samples were designated as CYP2C19*1/*2 (intermediate metabolizer).

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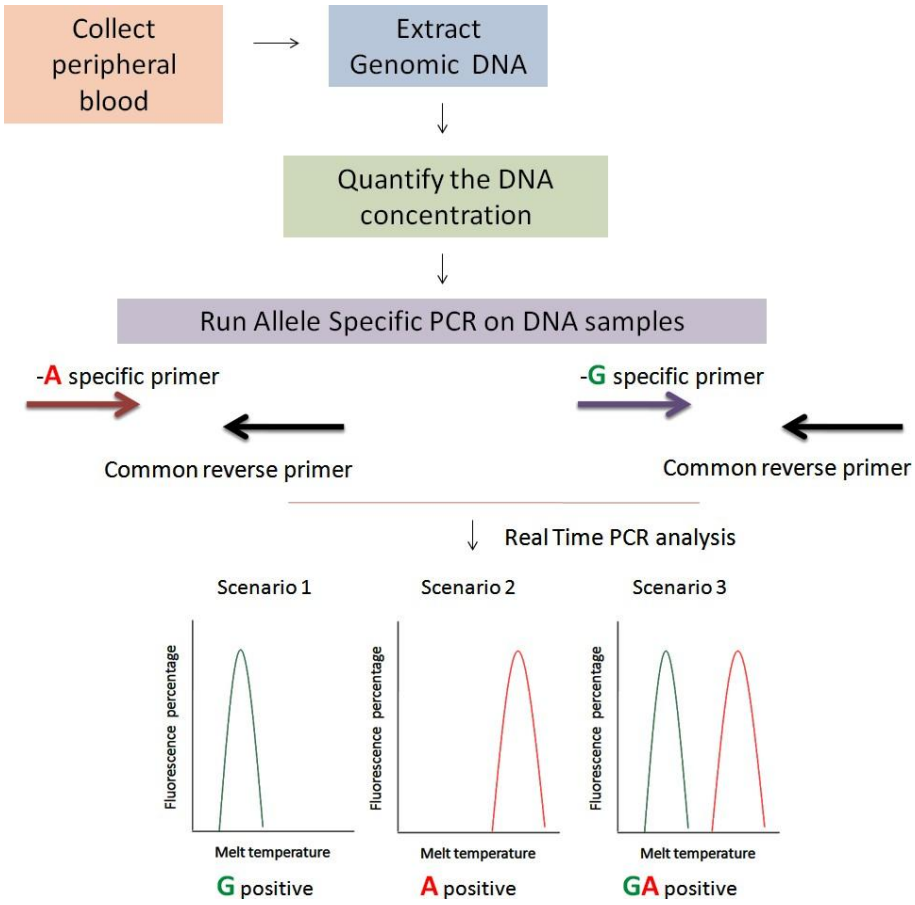
A schematic of the protocol is shown below:



Statistical Analysis: The collected data was analyzed with IBM SPSS statistics software 23.0 Version. Percentage analysis of the data was used to analyze descriptive statistics frequency. To find the significance in categorical data Chi-Square test was used similarly. If the expected cell frequency was less than 5 in 2×2 tables then the Fisher's Exact was used. In both the above statistical tools the probability value of 0.05 was considered as significant and less than 0.01 as highly significant.

3. Results

The study was undertaken to investigate the association of polymorphisms in CYP2C19 gene – CYP2C19*2 and CYP2C19*3, HDAC9 gene - (rs2107595, C□ T), APOE gene - rs429358 (C□ T), PMF1 gene - rs2984613 (C□ T) and PITX2 gene- rs6843082 (A□ G) in relation to stroke and co-morbid conditions. In order to identify the polymorphisms, the polymorphic region present in each of the above five genes were analyzed as follows: For CYP2C19*2 and CYP2C19*3: Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism (PCR-RFLP). The PCR-RFLP analysis resulted in successful determination of the CYP2C19 genotypes in the analyzed sample, as is evident from (figure 1). For HDAC9: Allele Specific Real Time Polymerase Chain Reaction followed by Melt Curve Analysis (ASP-PCR-MC). The ASP-PCR-MC analysis resulted in successful determination of the HDAC9 genotypes in the analyzed sample, as is evident from (figure 2). For APOE, PMF1 and PITX2:

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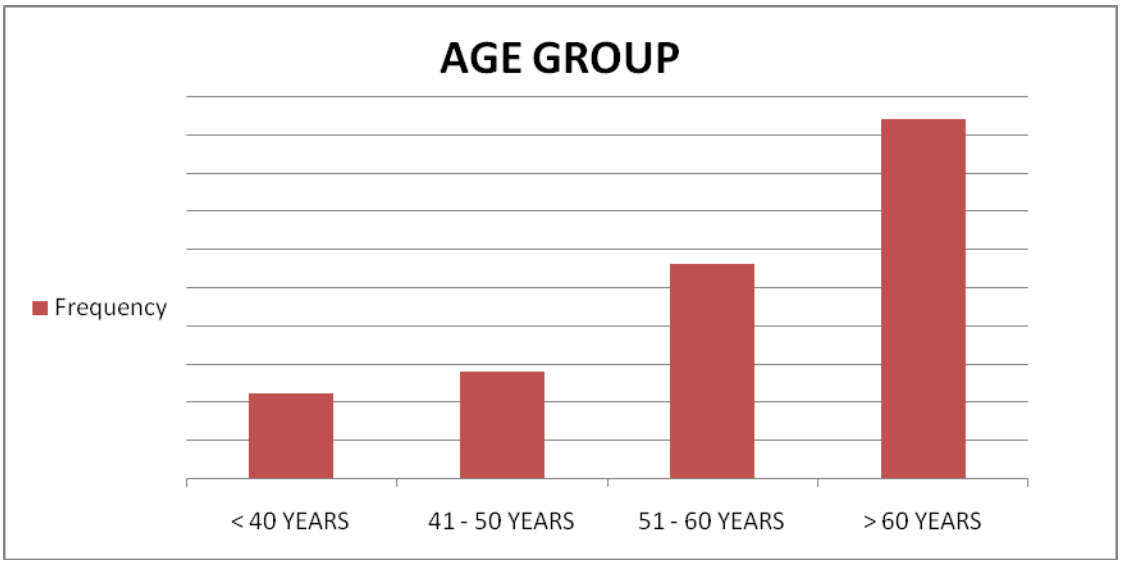
Real Time Polymerase Chain Reaction followed by High Resolution Melt Curve Analysis (PCR-HRM).The PCR-HRM analysis did not detect the associate / specific genotypes in the above three analyzed genes – APOE, PMF1 and PITX2 efficiently. The genotypes were detected in few samples, while in the rest of them the amplification pattern was indistinguishable due to background noise. This indicated that there could be a novel polymorphism at the primer binding site, which impaired effective amplification of the samples during real time PCR, so melt curve analysis could not be performed. Further analysis on these genes was not performed as it was beyond the scope of the present study. Data of APOE, PMF1 and PITX2 samples failed to show expected melt curves are presented in (figure 3).

AGE AND SEX DISTRIBUTION

TABLE 1 AGE GROUP

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	< 40 YEARS	11	11.0	11.0	11.0
	41 - 50 YEARS	14	14.0	14.0	25.0
	51 - 60 YEARS	28	28.0	28.0	53.0
	> 60 YEARS	47	47.0	47.0	100.0
	Total	100	100.0	100.0	

Chart 1



Total of 100 patients were recruited in the study, 81 males (81%) and 19

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females (19%) respectively. 11% of patients were <40 years, 14% were < 41 -50 years, 28% (28) were <51-60, 47 % (47) were >60 years.

Chart 2

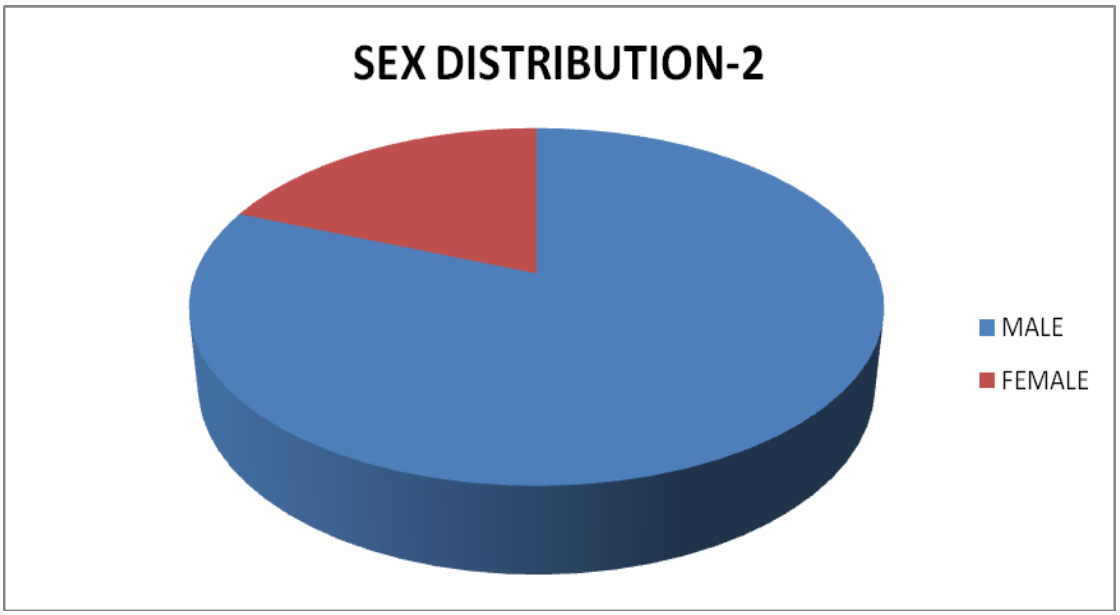

TABLE 2. CYP2C19 GENOTYPE

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	*1/*1	41	41.0	41.0	41.0
	*1/*2	36	36.0	36.0	77.0
	*2/*2	21	21.0	21.0	98.0
	*2/*3	2	2.0	2.0	100.0
	Total	100	100.0	100.0	

41 of 100 patients were normal metabolizers indicating normal response to clopidogrel with CYP2C19 1*/1* genotyping, 36 patients (36%) were intermediate metabolizer with CYP2C19 1*/2* genotyping, and 23% patients were poor metabolizer with CYP2C19 2*/2* (21/23) and 2*/3* (2/23) genotyping.

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TABLE 3 CYP2C19 ACTIVITY

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	NORMAL	41	41.0	41.0	41.0
	LOW	36	36.0	36.0	77.0
	NO ACTIVITY	23	23.0	23.0	100.0
	Total	100	100.0	100.0	

TABLE 4A: CYP2C19 GENOTYPE *RECURRENT STROKE

			RECURRENT STROKE		
			YES	NO	Total
CYP2C19 Genotype	*1/*1	Count	11	30	41
		% within CYP2C19 Genotype	26.8%	73.2%	100.0%
	*1/*2	Count	14	22	36
		% within CYP2C19 Genotype	38.9%	61.1%	100.0%
	*2/*2	Count	19	2	21
		% within CYP2C19 Genotype	90.5%	9.5%	100.0%
	*2/*3	Count	2	0	2
		% within CYP2C19 Genotype	100.0%	.0%	100.0%
Total		Count	46	54	100
		% within CYP2C19 Genotype	46.0%	54.0%	100.0%

$\chi 2 = 25.870$, $p = 0.001$ *** significant association of CYP2C19 genotype and recurrence of stroke .

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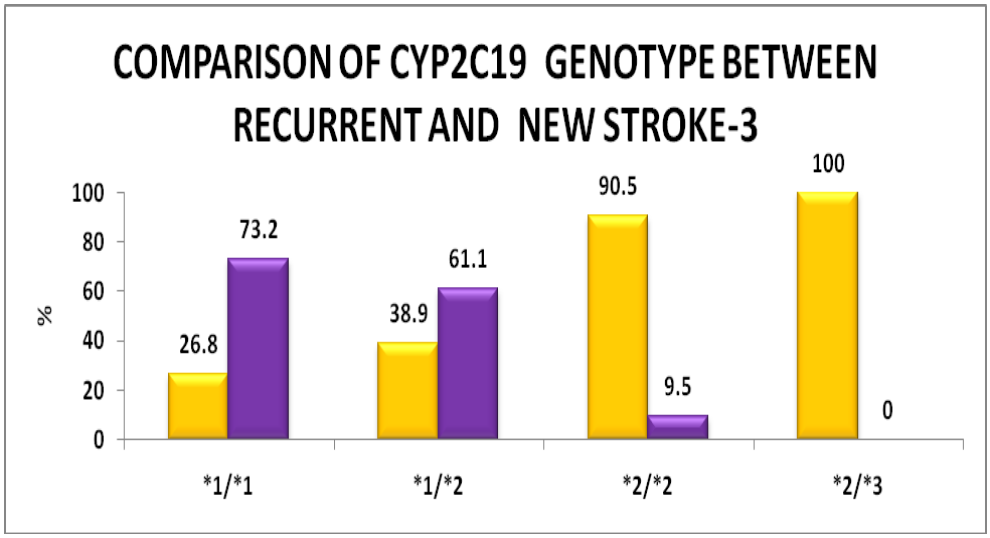
Chart 3



Discussion

The present study titled "A study to investigate the prevalence of single nucleotide polymorphism in CYP2C19, HDAC9, APOE, PMF1 & PITX2 in patients with haemorrhagic or ischemic stroke" was done in the department of Neurology, Sree Balaji Medical College and Hospital, Chennai over a period of two years from March 2017 to February 2019. This is a hospital based study done in 100 stroke patients who were attending stroke unit in our hospital. The aim of our study was to identify the prevalence of SNPs in CYP2C19gene and to identify responders and non responders to clopidogrel, an antiplatelet drug in patients with ischemic stroke and also prevalence of SNPs of APOE, PMF1 gene for hemorrhagic stroke and HDAC9, PITX2 for ischemic stroke in cerebrovascular accident patients. Stroke is defined by the world health organisation, rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin" 8. In Caucasian population, 80% of all strokes are ischemic, 10 to 15% are due to intracerebral hemorrhage, 5% subarachnoid hemorrhagic and the rest is due to other cause of stroke.

Age is the most important nonmodifiable risk factor for strokes and also significant independent predictors for recurrence strokes 17,18,19. After the age of 55, for each successive 10 years the risk of stroke increases twice in men than women18, the mean age of > 75 years was important factor for stroke recurrence 18, 167,168 In our study, the mean age was 69 ±10 years, and sex distribution wise 81 % were male and 19% were females.

In our study the most common etiology was large artery arterosclerosis (60%), cardioembolism (23%), small vessel disease (3%) and undetermined origin divided according to TOAST classification. Large artery atheroscleosis also increases the risk of stroke recurrences. In RESQUE study that evaluated 889 patients with stroke, small vessel disease is the most common etiologica l factor followed

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by large vessel atherosclerosis and cardioembolic stroke. In a study by Petty et al cardioembolic strokes were most common169. Study by kolominsky et al in Europe shows cardioembolic stroke mainly 170 followed by large vessel atherosclerosis. We conclude that differences in etiology of stroke are a result of varied prevalences of risk factors in our study population, quality of preventive treatments and methological variations between studies.

In our study, a total of 100 samples were analyzed to identify the association of CYP2C19 genotypes with clinical conditions such as type 2 diabetes, hypertension, dyslipidemia and coronary artery diseases (CAD) of the patients. Stratification of these conditions with respective CYP2C19 metabolizer genotypes was done by cross- tabulation followed by data analysis to identify significance. Statistical analysis indicated significant association of poor metabolizer genotype with recurrent stroke in patients with type 2 diabetes, hypertension, dyslipidemia and CAD (Table 14A, 14B, 14C, 14D). Analysis also indicated significant association of poor metabolizer genotype with recurrent stroke when patients with type 2 diabetes and hypertension were grouped together, relative to patients with acute stroke. Hypertension is the most prevalent and important modifiable risk factor among all stroke types and it also increases the stroke recurrence by 4 fold. 11,12,13. In our study, out of 100 patients, 54 patients had a single acute stroke of which 31 had hypertension and 46 patients had recurrent stroke of which 34 had hypertension. Out of 31patients in the single acute stroke group with hypertension, only 2 were poor metabolizers and 15 were intermediate metabolizers, where as in the recurrent stroke group 10 were intermediate metabolizers, 8 were normal metabolizers and 16 were poor metabolizers, which was stastically significant . This suggests that identification of metabolizer pattern is essential to prevent recurrent strokes.

Statistical analysis (p value< 0.001) clearly indicates that CYP2C19 poor metabolizer genotypes were associated with increased incidence of recurrent stroke. This data when extrapolated is similar to a meta-analysis of seven RCT PATS (indapamide, a diuretic), HOPE(ramipril), an d PROGRESS (perindopril, with or without indapamide) studies which showed antihypertensive drugs reduce stroke recurrence.(RR 0.76; 95% CI 0.63 –0.92)174,175,176,177,178. In another study an effective antihypertensive treatment showed to reduce stroke recurrence by 50% 181. The proportion of patients with recurrent stroke who received insufficient antihypertensive medication was found to be 39% in the study by Laloux et al 18. Stroke incidence increases 2-fold in the presence of Diabetes Mellitus (DM) in patients with atherosclerotic disease13. DM was the fifth most common disease with a rate of 24% in the RESQUE trial 18. It was also determined that 59% of the patients had insufficient medication10. An insufficient medication rate was also high in our patients, which depended on a mean HgbA1C level of 6.80±1.59% in all patients and 7.94±1.85% in the diabetic group. In our study, out of 54 acute stroke patients, 32 patients had diabetes and out of 46 with recurrent stroke, 31 had diabetes. In pts with

acute stroke, 11pts were intermediate metabolizers, 19 were normal metabolizers and 2 were poor metabolizers. In recurrent stroke patients 16 were identified as poor metabolizers. Statistical analysis shows significant p value < 0.005.

Similar to CAD, total cholesterol/HDL cholesterol levels have a linear relationship with stroke 184. HL was the second ranked etiological factor for recurrent stroke with a rate of 56% in the RESQUE trial 18. It was found in the study by Laloux et al. 12 that HL (high lipids) is the second most common disease (43%) and that treatment was insufficient in 42% of the patients. In our study Dyslipidemia was present in 55 patients, out of 54 acute stroke patients 24 had dyslipidemia and out of 46 recurrent stroke patients 31 had dyslipidemia. Incidence of dyslipidemia is high in poor metabolizer and they presented with recurrent stroke, this shows significant association of dyslipidemia with p value =0.009, in pts with r ecurrent stroke who were CYP2C19 poor metabolizers.

CONCLUSION

In our study 46% of patients had Clopidogrel resistance. Poor metabolizers genotype is more prevalent in recurrent stroke group. The relationship of co morbid risk factors such as Hypertension, Diabetes, Dyslipidemia, CAD with clopidogrel resistance is evident in our study. This suggests that identification of metabolic pattern is essential to prevent recurrent strokes. It is imperative to check the prevalence of clopidogrel resistance in new onset stroke, recurrent stroke as well as those patients who are on dual antiplatelet drug therapy for cardiac disease, but develop atherothrombotic stroke. Our study shows that HDAC9 gene with CC phenotype is associated with increased atherosclerosis.

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Ethical approval: The study was approved by the Institutional Ethics Committee

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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