Endothelial Nitric Oxide Synthase (eNOS) Gene Polymorphisms and Risk of Aneurysmal Subarachnoid Hemorrhage: An Updated Meta-Analysis

N. Manabesh[1], M. Shubham[1], K. Amit[2], S. Rakhee[2], K. Pradeep[1*]
[1]Department of Neurology, All India Institute of Medical Sciences, New Delhi, India
[2]Department of Paediatrics, Army Hospital Research and Referral, New Delhi, India

Corresponding author: Pradeep Kumar
E-mail: pradeepguptaneuro@gmail.com
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ABSTRACT.

Previously published studies have examined whether the polymorphisms (G894T, 4b/a, and T786C) in the Endothelial Nitric Oxide Synthase (eNOS) gene are associated with risk of Aneurysmal Subarachnoid Hemorrhage (aSAH), have reported conflicting results. Thus, we performed a meta-analysis to examine the potential association between these three single nucleotide gene polymorphisms (SNPs) of eNOS gene and aSAH risk. A literature search was carried out for eligible candidate gene studies published before April 30, 2020 in the PubMed, EMBASE, Google Scholar and Trip databases. The strength of association between eNOS and G894T, 4b/a, and T786C gene polymorphisms was calculated by pooled Odds Ratios (ORs) with 95% Confidence Intervals (95%CIs) using either a fixed-effect model (I2<50%) or a random-effect model (I2>50%). Heterogeneity between studies was examined using influence diagnostics analysis and publication bias was evaluated using Begg's funnel plots. All the statistical analyses were assessed using R version 3.6.2 software.13 case-control studies involving 2131 aSAH cases and 2223 controls were analyzed in our meta-analysis. In the overall analysis, no evidence of significant association for G894T (OR= 1.04; 95% CI: 0.89 to 1.23; p=0.52), T786C (OR= 1.16; 95% CI: 0.97 to 1.40, p=0.14) and 4b/a (OR= 1.17; 95% CI: 0.81 to 1.68, p<0.01) eNOS gene polymorphisms and

risk of aSAH was observed. Our comprehensive meta-analysis ascertains that G894T, T786C and 4b/polymorphisms of eNOS gene may not be associated with the risk of aSAH. Further prospective large epidemiological studies are required to substantiate these findings.

Keywords: Endothelial Nitric Oxide Synthase; Gene Polymorphism; Hemorrhagic Stroke; Subarachnoid Hemorrhage; Meta-Analysis

INTRODUCTION

Aneurysmal Subarachnoid Hemorrhage (aSAH) remains a disturbing condition where only 25% of victims are believed to be living an independent life (Van Gijn et al. 2007). Approximately, 85% of non-traumatic SAH cases are caused by rupture of an Intracranial Aneurysm (IA). Despite the development in the intensive care and neurosurgical therapy, high mortality and morbidity of aSAH has become a major health concern (Karamanakos et al. 2012; Malmivaara et al. 2012). Although the molecular mechanisms underlying aSAH remain unclear, genetic and environmental factors may play a crucial role in the pathogenesis of SAH. Recent advancement in genetics suggests that Endothelial Nitric Oxide Synthase (eNOS) gene polymorphisms are among the genetic factors known to be associated with IA.

Nitric Oxide (NO) is mostly synthesized by catalysing action of 3 Nitric Oxide Synthase (NOS) family enzyme via the conversion of L-arginine. It is a multifunctional molecule which participates in a large number of biological activities including vasodilation, platelet monocytes adhesion, and maintenance of vessel wall geometry and relaxation of vascular smooth muscle (Knowles et al. 1989; Kubes et al. 1991; Napoli et al. 2013; Palmer et al. 1987; Rudic et al. 1998; Wolf et al. 1997). It is hypothesized that NO may participate in the mechanism of IA formation and down regulation of NO level has been reported to be associated with several vascular diseases (Oemar et al. 1998). NO is generated by eNOS gene, located on chromosome 7q35-36 which consists of 26 exons and extends upto 21 kb of the genome (Marsden et al. 1993).

There are many functional polymorphisms in different regions of eNOS gene and various studies have shown the influence of three common polymorphisms in the eNOS gene that have been widely studied including the G894T polymorphism in exon 7, 4b/a polymorphism in intron 4, and T786C polymorphism in the promoter region respectively. T786C (rs2070744) is an important point mutation of thymine to cytosine at codon-786 in the 5'-flanking region of the eNOS gene, which can significantly reduce eNOS gene promoter activity and serum NO level; G894T (Glu298Asp, rs1799983) corresponds to a Glu-Asp change at nucleotide 298 in exon 7 that demonstrates a trend for a reduced eNOS enzyme activity, and 27-bp-variable number of tandem repeats (VNTRs, 27 bp) in intron 4 influences the basal plasma NO generation (Nakayama et al. 1999; Wang et al. 1997, 2000). Studies on these three polymorphisms in the eNOS gene have shown conflicting results with the risk of aSAH (Akagawa et al. 2005; Khurana et al. 2003, 2004; Kim et al. 2011; Koshy et al. 2008; Krex et al. 2006; Krischek et al. 2006; Ozüm et al. 2008; Song et al. 2006; Staalsø et al. 2014). Therefore, in this study, we planned to conduct a meta-analysis to clarify the association between these three SNPs of eNOS gene polymorphisms and aSAH risk.

MATERIALS AND METHODS

Identification of Relevant Studies

A literature search was carried out for eligible candidate gene studies published before April 30, 2020 in the PubMed, EMBASE, Google Scholar and Trip databases. The following combinations of main keywords were used: (endothelial nitric oxide synthase) or (eNOS) and (G894T, 4b/a, and T786C) and (polymorphism) or (polymorphisms) and (Hemorrhagic stroke or 'HS') and ('aneurysmal subarachnoid hemorrhage or 'aSAH') and ('genetic polymorphism' or 'single nucleotide polymorphisms' or 'SNP'). Only those studies were selected whose full-text was available in English language.

Inclusion and Exclusion Criteria

For inclusion in the meta-analysis, the following criteria were set: (1) It should be a case-control study investigating the association between G894T or 4b/a or T786C gene polymorphisms of eNOS gene and risk of aSAH; (2) Cases should meet the diagnostic criteria for SAH; (3) studies should have sufficient genotypic frequency to calculate ORs with corresponding 95% CIs. The major reasons for excluding studies were: (1) not a case-control study; (2) presence of duplicate publications with overlapping subjects from the same study; and (3) no genotypic data reported. This meta-analysis was conducted in accordance with Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Moher et al. 2009). In order to reduce the risk of retrieval bias, none of the authors were contacted regarding the missing data that was required for the meta-analysis.

Data Extraction

Each full-text article was checked for eligibility by two authors (PK and SM) independently as per the PRISMA guidelines and the following data was extracted from the eligible studies: first author surname, publication year, country, ethnicity, number of cases and controls, genotyping method, age, sex, genotypic and allelic frequencies etc. Disagreements among authors were resolved by discussion until a consensus was reached.

Quality Assessment

We also examined the methodological quality of every study which is included in our meta-analysis using a quality assessment scale developed for genetic association studies (Attia et al. 2003) which was modified by us to increase the relevance of our study. Both the traditional epidemiological considerations and genetic issues were taken into consideration by using this scale. The scores ranged from 0 (worst) to 16 (best). Contents of the quality scale are mentioned. The quality of the included studies was independently assessed by two authors (PK and SM). Discrepancies over the quality scores were resolved by discussion among all the authors and subsequent consensus was reached. Publication bias was assessed by Begg's funnel plot (Begg and Mazumdar 1994) analysis using the Egger's regression test (Egger et al. 1997) and counter-enhanced funnel plot analysis (Peters et al. 2008).

Statistical Analysis

The strength of association between aSAH and G894T, 4b/a, and T786C polymorphisms of eNOS gene was calculated by pooled Odds Ratios (ORs) with 95% confidence intervals (95% CIs) under three genetic models, including dominant, recessive and co-dominant model. Heterogeneity between studies was examined by using Cochran's Q statistic and I2 metric (Higgins et al. 2003). 19In our study, the I2 values exceeding 50% was considered as an indicator of significant heterogeneity. We used a fixed-effect model to estimate the pooled ORs with 95% CIs when no heterogeneity was found with I2<50%. Otherwise, a random-effect model was used when I2>50% which depicted significant heterogeneity. The heterogeneity between studies was assessed by using meta-regression analysis of the included studies using the measured effect size and quality score of the studies. Furthermore, influence diagnostics analysis was also conducted to evaluate the heterogeneity arising out of the studies. All the statistical analyses were done using R version 3.6.2 software and a p-value<0.05 was considered statistically significant.

RESULTS

A total of 78 published articles were identified by using the pre-specified search strategy. Figure 1 depicts a flow chart of both the retrieved and excluded studies with their reasons for exclusion. Out of the 78 retrieved articles, twenty-nine were excluded due to its irreverence to our interest, nine studies were excluded due to unavailability of the article text in English language and fifteen studies were excluded as they were in review articles. Keeping the inclusion criteria in mind, 25 case-control studies were assessed for their eligibility and 13 were included in our meta-analysis consisting of 2131 aSAH cases and 2223 control subjects. Eleven studies represented the association between aSAH and T786C polymorphism of eNOS gene while the association of aSAH

with G894T and 4b/a polymorphisms of eNOS gene were depicted in nine and seven studies respectively. Studies were carried out in two major ethnic populations; eight studies were in Asian (Akagawa et al. 2005; Kim et al. 2011; Konar et al. 2019; Koshy et al. 2008; Krischek et al. 2006; Song et al. 2006; Zhe and Bo 2019) while five studies were in Caucasian (Khurana et al. 2003, 2004; Krex et al. 2006; Özüm et al. 2008; Staalsø et al. 2014) population.

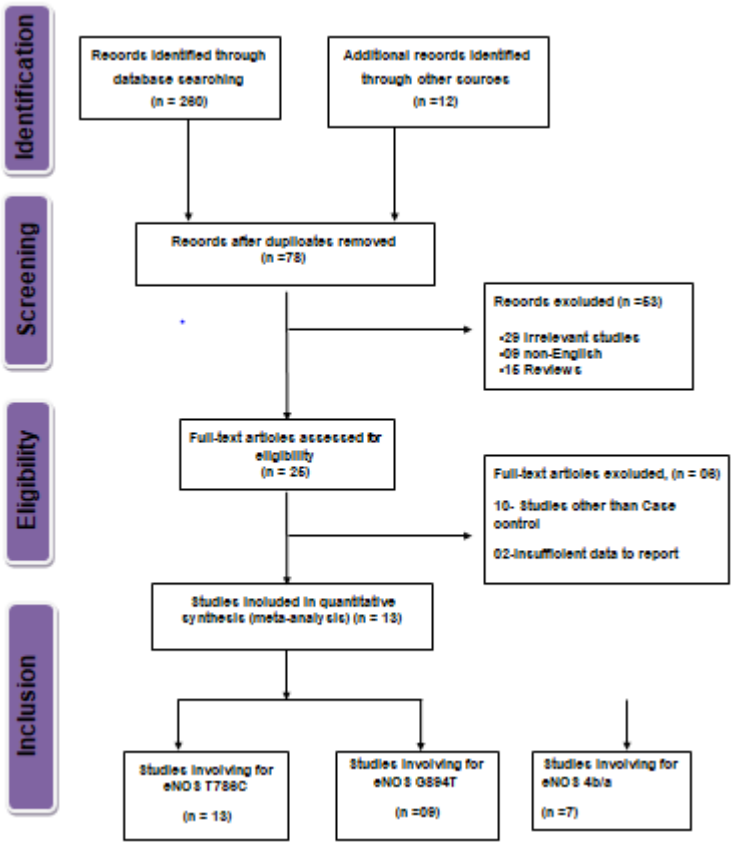

Figure 1. Flow diagram for the selection of studies and specific reasons for exclusion from the present meta-analysis.

The publication years of the included studies ranged from 2003 to 2019. Twelve studies in this meta-analysis had controls in Hardy-Weinberg Equilibrium (HWE). The quality scores of all the included studies were moderately high and ranged from eight to twelve. Out of 13 studies, seven studies had hospital-based and six studies had population-based source of controls.

Association between G894T polymorphism of eNOS gene and aSAH

The eNOS G894T gene polymorphism was assessed in 9 case-control studies with a total of 1416 aSAH cases and 1615 controls. Overall no significant association was observed under the dominant (GT+TT *vs* GG: OR= 1.04; 95% CI: 0.89 to 1.23; p=0.52), recessive (TT *vs* GT+GG: OR=0.93; 95% CI: 0.61 to 1.43; p=0.10) and co-dominant model (GT *vs* TT+GG: OR=1.07; 95% CI: 0.88 to 1.29; p=0.32) of G894T polymorphism of eNOS gene with the risk of aSAH. No statistically significant association was observed between the eNOS gene polymorphism (G894T) and risk of aSAH as well when sub-group analysis was done for the different ethnicities of the included studies using the different genetic models. No significant heterogeneity was observed for all the nine studies on eNOS gene G894T polymorphism in any of the genetic models (Dominant model: I2=0%, Recessive model: I2=42%, and Co-dominant model: I2=14%) (Figure 2).

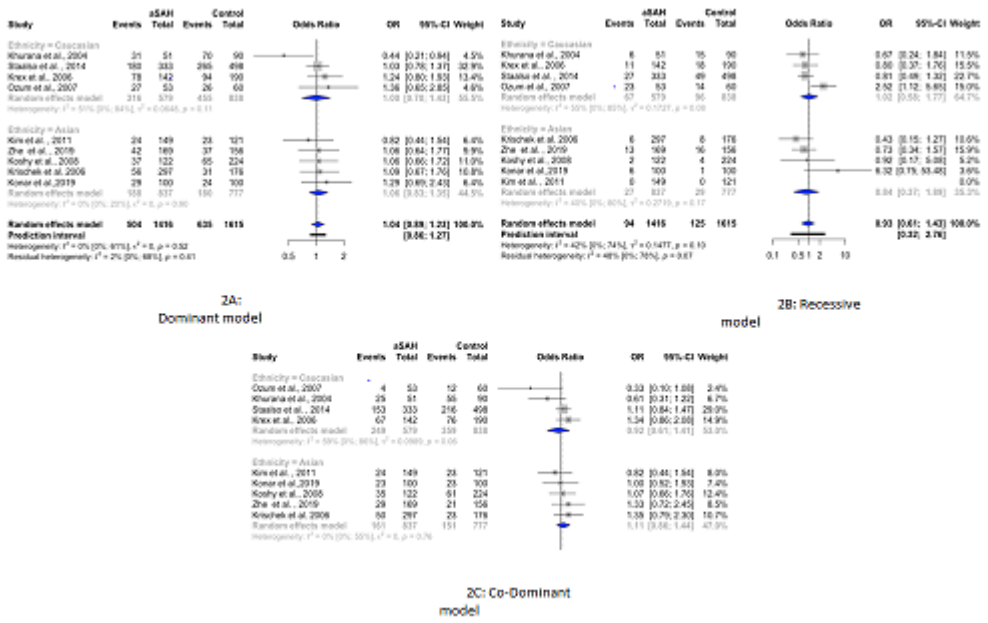

Figure 2. (A-C): Forest plots for association between eNOS G894T gene polymorphism and aSAH risk in (A) Dominant model (GT+TT *vs*. GG); (B) Recessive model (TT *vs*. GT+GG); (C) Co-Dominant model.

Association between T786C polymorphism of eNOS gene and aSAH

The eNOS T786C gene polymorphism was assessed in 11 case-control studies with a total of 2027 aSAH patients and 2073 control subjects. Overall no significant association was observed under the dominant (CT+CC *vs* TT: OR=1.16; 95% CI: 0.97 to 1.40, p=0.14), recessive (CC *vs* CT+TT: OR=0.92, 95% CI: 0.66 to 1.28, p=0. 32) and co-dominant model (CT *vs* CC+TT: OR=1.21, 95% CI: 0.96 to 1.54, p<0.01) of T786C polymorphism of eNOS gene with risk of aSAH. Sub-group analysis with Asian and Caucasian ethnicities of the studies included in our meta-analysis revealed no significant association with the risk of aSAH in recessive and co-dominant models. However, the Asian population under dominant model showed association between eNOS T786C gene polymorphism and risk of aSAH (OR=1.26; 95%CI: 1.03 to 1.54) but the association was not statistically significant (p=0.30>0.05). No significant heterogeneity was observed in the dominant (I2=33%) and recessive models (I2=13%) but the co-dominant model had some heterogeneity (I2=57%) (Figure 3).

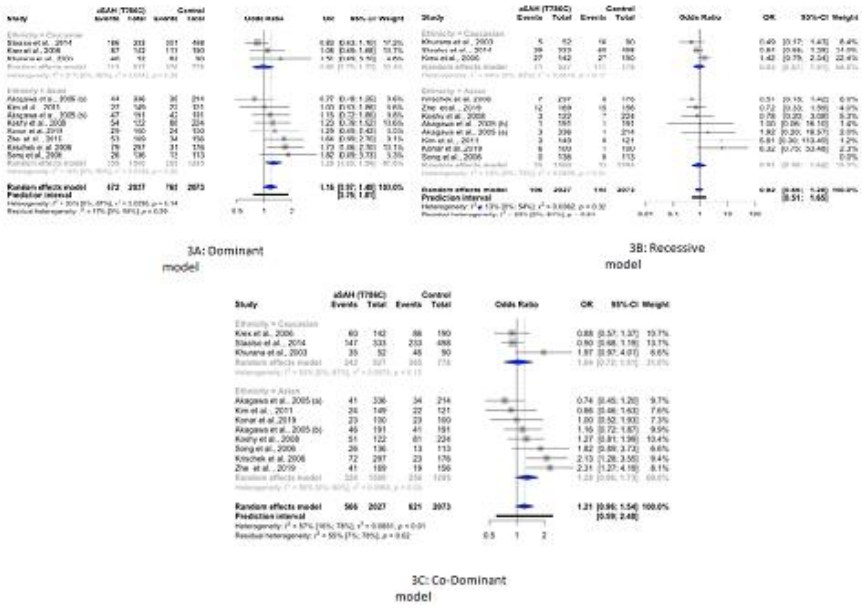

Figure 3. (A-C): Forest plots for association between eNOS T786C gene polymorphism and aSAH risk in (A) Dominant model (CT+CC *vs* TT); (B) Recessive model (CC *vs*. CT+TT); (C) Co-Dominant model (TC *vs* TT).

Association between 4b/a polymorphism of eNOS gene and aSAH

The eNOS 4b/a gene polymorphism was assessed in 7 case-control studies with a total of 1194 aSAH patients and 1399 controls. Overall, no significant association was observed under the dominant (aa+ab *vs* bb: OR=1.17; 95% CI: 0.81 to 1.68, p<0.01), recessive (aa *vs* ab+bb: OR=1.30, 95% CI: 0.76 to 2.23, p=0.51) and co-dominant model (ab *vs* aa+bb: OR=1.15, 95% CI: 0.77 to 1.70, p<0.01) with the risk of aSAH and eNOS gene 4b/a polymorphism. No significant association was observed in subgroup analysis using ethnicities of the population (Asian and Caucasian) as a factor in the dominant and co-dominant models. However, an association of 4b/a polymorphism of eNOS gene and risk of aSAH was observed in the recessive model of Asian population (OR=2.17; 95% CI: 1.01 to 4.63) despite being statistically non-significant (p=0.91>0.05). Significant heterogeneity was observed in the dominant (I2=74%) and co-dominant model (I2=76%) but not in the recessive model (I2=0%) (Figure 4).

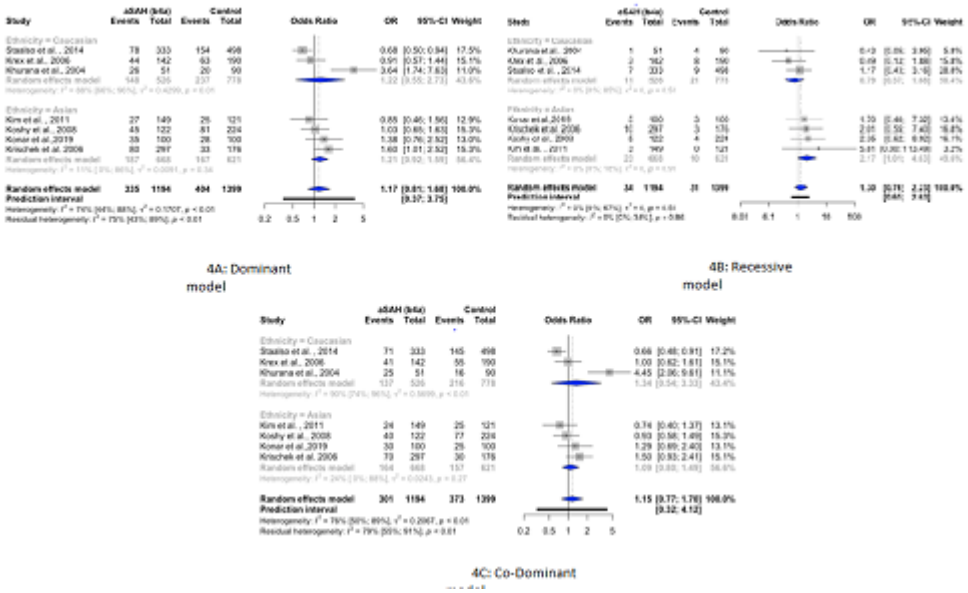

Figure 4. (A-C): Forest plots for association between eNOS 4b/a gene polymorphism and aSAH risk in (A) Dominant model (aa+ab *vs*. bb); (B) Recessive model (aa *vs*. ab+bb); (C) Co-Dominant model.

Publication Bias

Begg's funnel plot and counter-enhanced funnel plot was used to test for the presence of any publication bias in the studies included in our meta-analysis. There was no observable asymmetry observed in the funnel plots for G894T and T876C polymorphisms of eNOS gene that ascertained the absence of publication bias in these studies. However, there was some asymmetry present in the funnel plots of 4b/a polymorphism of eNOS gene, which suggested the presence of publication bias (Figures 5 and 6). These results were also confirmed by Egger's test for assessing publication bias.

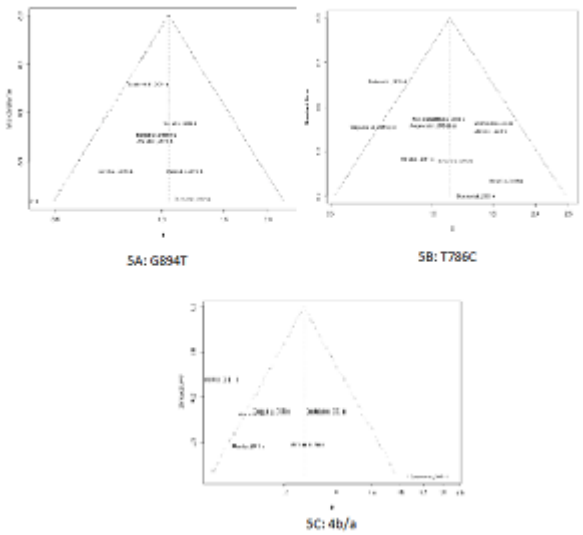

Figure 5. (A-C): Begg's Funnel Plot to assess publication bias for association between eNOS gene polymorphism and aSAH risk in (A) G894T gene polymorphism (B) T786C gene polymorphism (C) 4b/a gene polymorphism.

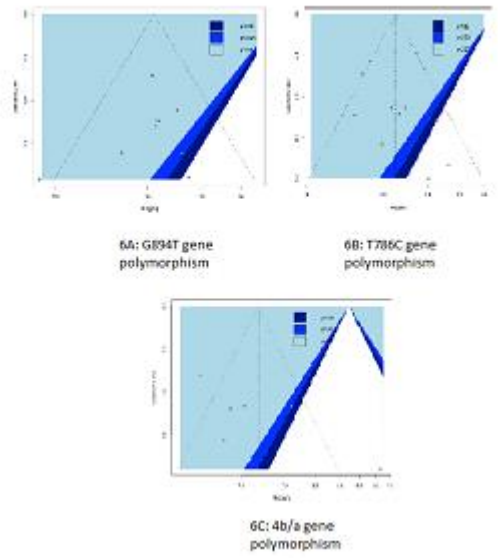

Figure 6. (A-C): Counter Enhanced Funnel Plot for publication bias for association between eNOS gene polymorphism and aSAH risk in (A) G894T gene polymorphism (B) T786C gene polymorphism (C) 4b/a gene polymorphism.

Meta regression analysis

Meta-regression analysis was evaluated based on the quality scores of the included studies for determining the association of eNOS gene polymorphisms (G894C, T876C and 4b/a) and the risk of aSAH did not cause any significant deviation from the original pooled measured effect. However, the analysis identified Khurana et al. 2004 (Khurana et al. 2004) as a potential outlier study for G894T and 4b/a polymorphisms of eNOS gene (Figure 7).

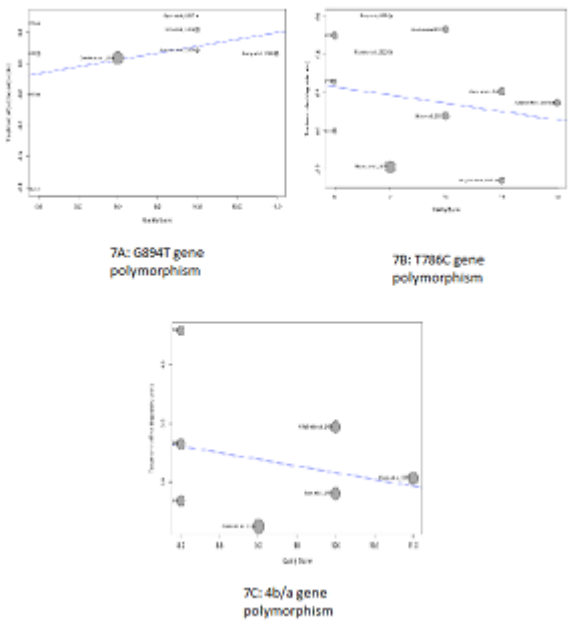

Figure 7. (A-C): Meta regression plot for association between eNOS gene polymorphism and aSAH risk in (A) G894T gene polymorphism (B) T786C gene polymorphism (C) 4b/a gene polymorphism.

Influence diagnostics analysis

The between-study heterogeneity that was observed in some of the results in our analysis was explained further using influence diagnostics tools that generated four plots: 1) Baujat plots, 2) Influence analysis plots, 3) Leave-one-out analysis sorted by heterogeneity and 4) Leave-one-out analysis sorted by effect size. For the association between G894T polymorphism of eNOS gene and risk of aSAH, the Baujat and influence analysis plots did not identify any outlier study, which could be due to homogeneity ($I2<50\%$) observed in the measurement of pooled effect size. During the leave-one-out analysis sorted by both heterogeneity and effect size, it was observed that there was no significant change in the effect size when any of the studies were omitted.

In case of association between T786C polymorphism of eNOS gene and risk of aSAH, the Baujat and influence analysis plots revealed that Staalso et al. 2014 (Staalsø et al. 2014) was a potential outlier study which could be a contributing factor for the heterogeneity observed between studies in the co-dominant model. After omitting this study in the leave-one-out analysis sorted in terms of heterogeneity and effect size, it was observed that the effect size changed significantly (OR=1.24; 95% CI: 1.05 to 1.47) for the overall observation.

For 4b/a polymorphism of eNOS gene and its association with the risk of aSAH, the Baujat and influence analysis plots found that Khurana et al. 2004 (Khurana et al. 2004) was a potential outlier for the between-study heterogeneity observed for the dominant and co-dominant models of the association studies. When this study was omitted to undergo the leave-one-out analysis ordered by both heterogeneity and effect size, we observed no significant change in the overall measured effect size (Figure 8).

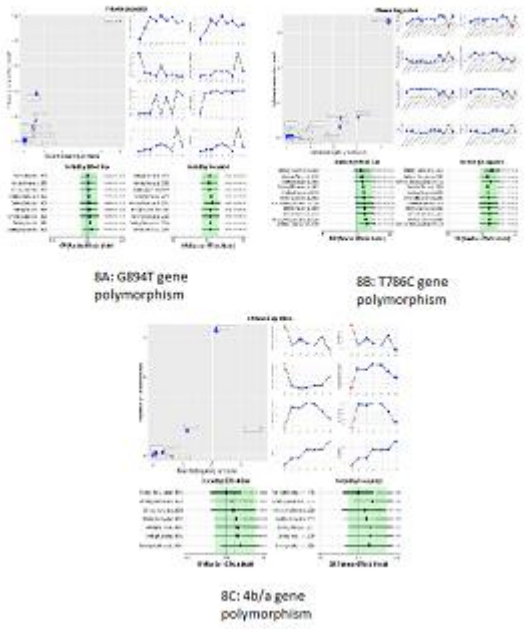

Figure 8. (A-C): Influence Diagnostic analysis for association between eNOS gene polymorphism and aSAH risk in (A) G894T gene polymorphism (B) T786C gene polymorphism (C) 4b/a gene polymorphism.

DISCUSSION

This comprehensive meta-analysis was conducted on three well-characterized genetic polymorphisms of the eNOS gene and the studies were not associated with the risk of aSAH. eNOS gene catalyses the synthesis of nitric oxide which is a regulator of cerebral blood flow as well as smooth muscle cell proliferation in the arterial endothelium. Although the precise molecular effects of polymorphisms in eNOS gene have not been established yet, still some biochemical evidence exists for the decreased eNOS gene promoter activation, reduced protein expression and reduced enzyme activation. It is possible that these polymorphisms will prevent the development and rupture of IA. The previous meta-analysis published by McColgan et al. 2010 (McColgan et al. 2010) showed a significant association between T786C polymorphism and SAH; however the study was limited to five studies. We included 13 studies in our meta-analysis and found no significant association between T786C polymorphism and aSAH. However, our findings for G894T polymorphism of eNOS gene are in accordance with the study published by McColgan et al. 2010 showing no significant association with aSAH. For 4b/a gene polymorphism of eNOS gene, Khurana et al (Khurana et al. 2004) reported an association between the allele and SAH. They found the b/b genotype to be more frequent in patients with unruptured aneurysms as compared to those with ruptured aneurysms (80% *vs*. 50%). Our findings involving seven studies suggest that 4b/a gene polymorphism is not significantly associated with risk of aSAH. Another meta-analysis showed a significant association of T786C gene polymorphism with the risk of IA among the Asian population, whereas G894T and 4b/a gene polymorphisms might have no influence on the susceptibility of IA (Yang et al. 2015). Our present meta-analysis shows a strong evidence for no association of all the three gene polymorphisms in eNOS gene. However, we did observe an association in the dominant model of T786C polymorphism of eNOS gene and risk of aSAH but the resultant effect size was not statistically significant.

A more recent meta-analysis in 2018 found significant association between T786C polymorphism of eNOS gene with the risk of ruptured/unruptured IA and aSAH which is contradictory to the findings in our meta-analysis (Paschoal et al. 2018). However, the analysis included a total of 9 studies out of which the association of T786C polymorphism involved 7 studies with IA and 4 studies with aSAH and the significance of association was obtained in dominant model only. We analysed 11 studies involving T876C polymorphism and found no association with risk of aSAH in any of the genetic models. The Begg's funnel plot and counter-enhanced funnel plot for the G894T and T786C gene polymorphisms of eNOS gene suggested no publication bias in the analysis. However, the funnel plots for 4b/a gene polymorphism of eNOS gene suggested the presence of significant publication bias in the

analysis. The circled dots represent the studies and if all the studies are present inside the funnel then it is representative of no publication bias whereas studies lying outside the funnel plot are suggestive of significant publication bias in the analysis. Homogeneity was found in case of studies involving G894T polymorphism of eNOS gene but between-study heterogeneity was observed in studies involving T876C and 4b/a gene polymorphisms. This was countered using meta-regression and influence diagnostic analysis, which identified the potential outlier studies that affected the heterogeneity in the analysis.

LIMITATIONS

The present meta-analysis needs to be interpreted with caution because of certain limitations. First, the studies included in the meta-analysis varied in ethnicity, age and environmental factors. Second, the use of different methodologies for genotyping method, selection of controls and matching criteria may have lead to heterogeneity. Third, publication bias was observed in certain models that could also affect the desired outcome of effect size. Therefore, more evidences that are credible are required to illustrate solid conclusions on the association between eNOS gene polymorphisms and risk of aSAH.

CONCLUSION

Our comprehensive meta-analysis infers that G894T, T786C and 4b/a polymorphisms of eNOS gene may not be associated with the risk of aSAH. Further prospective large-scale epidemiological studies should be conducted for gathering evidence that could ascertain the possible role of eNOS gene polymorphisms in developing IA and subsequent aSAH.

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