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# Claudin 5 (CLDN5) gene polymorphism in Leukoaraiosis in a South Korea Population

**Binod Kumar Yadav** [1,2*] | **Renu Yadav** [3] | **Byoung-Soo Shin** [4,5]

1. Department of Biochemistry, Maharajgunj Medical Campus, Institute of Medicine, Tribhuvan University, Kathmandu, Nepal

2. Madhesh Institute of Health Sciences, Janakpurdham, Nepal

3. Department of Dietetics, Tribhuvan University Teaching Hospital, Kathmandu, Nepal

4. Research Institute of Clinical Medicine of Chonbuk National University-Biomedical Research Institute of Chonbuk National University Hospital, Jeonju, South Korea

5. Department of Neurology, Chonbuk National University Medical School, Jeonju, South Korea

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***Correspondence**:
binod3aug@gmail.com (BKY)

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**Abstract**:

**Introduction**: Claudin-5(CLDN-5) is one of the principal proteins predominantly expressed at blood-brain barrier (BBB) and plays an important role in tight junction (TJ) formation and maintaining the integrity of BBB. Thus, this study was designed to study the association of polymorphism rs1548359, rs10314, and rs739371 at CLDN-5 gene and leukoaraiosis in the Korean population.

**Materials and Methods**: A real-time PCR, a LightSNiP typing assay (coupled with primer and probe, TIB-MolBiol, Berlin, Roche Applied Science, Germany) was used to detect the genotypes of CLDN-5. Logistic regression analyses and stratification analyses with adjustment for traditional risk factors (age, sex, diabetes and hypertension) were used to find the genetic effect.

**Results**: We did not find any significant association for overall leukoaraiosis or any subtype analyses. Analyses of both leukoaraiosis subtypes (PVWM and DWM) did not show any significant associations (p = 0.29-0.59).

**Conclusions**: CLDN-5 polymorphisms may not play a significant role in the development of leukoaraiosis in the studied population. However, further replications in different ethnic groups and larger populations are recommended.

**Keywords**
Blood-brain barrier, Claudin 5, gene polymorphism, Leukoaraiosis

## INTRODUCTION

Leukoaraiosis (LA), a white matter disease, is described as the abnormal appearance of the subcortical brain white matter which appears as hypertense periventricular caps or rims or halos or multiple punctuate or patchy, partially confluent or confluent area on CT or fluid-attenuated inversion recovery (FLAIR) MRI(1). Around 70% of ischemic stroke patients had leukoaraiosis and is also common in demented and aged individual[2, 3]. Smith EE had concluded that there is strong association of LA with stroke outcome, as well as stroke incidence [4] and is independent of other stroke risk factors(5). Previous study has documented that increasing age and LA are associated with the increasing permeability of blood –brain barrier (BBB) [6, 7].

BBB is a highly selective semipermeable membrane that separates the circulation blood from the brain extracellular fluid in the central nervous system (CNS). BBB is composed of brain microvascular endothelium cells (BMVEC), astrocytes, basement membrane, and pericytes and neurons that are in physical proximity to the endothelium. Under normal physiological condition, transport of nutrients across BBB is controlled by both physical (tight junctions) and metabolic barriers enzymes (enzymes and diverse transport system). BMVEC is the major structural component of BBB which include the tight junction, adherent junction and junctional adhesion molecules [8, 9]. TJ proteins occludin, claudins, junctional adhesion molecules (JAMs), zonula occludens (ZO1, ZO2, ZO3), cingulin, AF6, 7H6 are the main component of TJ which are responsible for controlling the permeation of polar solutes between the endothelial cells from the blood plasma to the brain extracellular fluid [10, 11]. Claudins (20-24kDa proteins) are the principal BBB- forming proteins (with four transmembrane domains) which include a multigene family consisting of 24 members in mammals and one of the most important structural components of TJ strands[12]. The hemophilic and heterophilic interaction between adjacent claudin molecules ensures the tight contact of the monolayers at TJ [13].

Claudins 1, 3, 5 and 12 are present at the BBB and more importantly, claudins 3 and 5 are predominantly expressed by brain endothelial cells at BBB[14-16]. Previous study has reported that clauidn-3 and claudin-5 play a significant role in TJ formation and integrity at the BBB. Depletion of claudin-5 demonstrated the disruption of the BBB in cultured human brain endothelial cell and genetically-altered mice causing early death due to size-selective loosening of the BBB for molecules with MW<800 Da[14, 15], whereas exogenous expression of claudin-5 strengthens the barrier properties in cultured rat brain endothelial cells. Thus, it appears that claudins are known to form the backbone of the TJ of BBB and the presence of the Claudin-1, claudin-3, and claudin-5 were reported and identified as major component of the tight junction strands at the blood brain barrier and disappearance of either claudin-3 or claudin-5 from the tight junctional complexes can result in a compromised BBB [17, 18] which play a major role in LA development. Infact, the study of Topakian R et al [7] had also showed the association between BBB permeability and leukoaraiosis. Some Previous research findings have demonstrated that association of claudin loss with stroke [19], brain metastasis[20], colorectal cancer [21], and breast cancer [22].

Several studies have reported the association of different SNPs with leukoaraosis and stroke [23-26] and some of them were also regulator of homeostasis of the BBB [27]. We hypothesize that disruption of the genes responsible for regulation (structural and functional) of the tight junction at BBB may enhance the development of leukoaraiosis. As claudin -1, caludin-3 and claudin-5 are the major structural component of tight junction and one of our previous studies [28] have already showed significant association of claudin-1 genetic polymorphism with leukoaraiosis insisted us to design this study to investigate the association of genetic polymorphism of next tight junction gene (claudin-5) with leukoaraiosis.

## Materials and Methods:

### *Patients and control selection*

The study subjects enrolled in this study were only in part the same as included in our previous study [24]. In this study, we grouped the patients and control separately which leads to different number of patients and control in two groups of LA. The selection of patients controls and their clinical check-up procedures were the same as earlier [24]. Briefly, we included the patients who had definite diagnosis of leukoaraiosis which is diagnosed as per the scale of Fazekas et al [29]. According to this scale, leukoaraiosispatients were grouped into two classes: periventricular white matter (PVWM) and deep white matter (DWM). Class LA-PVWM includes the patients with irregular periventricular hyperintensities of grade 2 and 3 in T2-weighted MRI scan, while class LA-DWM consists the patients having deep white matter hyperintensive signals of grade 2and 3. The individual bearing “punctuate (0)” and “cap (1)” lesion in periventricular and deep white matter lesion of their brain MRI and have no any definite clinical symptoms of stroke or leukoaraiosis were included as control in this study. With this categorization scales of leukoaraiosis, class 1(LA-PVWM) consists of total 183 patients and 202 controls, and class-II (LA-DWM) consists of 156 patients and 229 control. Any individuals with less than 40 years of age and any significant neurological disorder (an intracerebral hemorrhage, a subarachnoid hemorrhage,

an intracranial infection, a malignant tumor, toxic encephalopathy, Parkinson's disease, ischemic heart disease, multiple sclerosis, or hydrocephalus) were not included in this study.

***Demographic and clinical characteristics***

At the time of patients and control evaluation at neurology clinic of Chonbuk National University, Jeonju, South Korea, a standardized questionnaire sheet was used to collect the information about age, sex, cerebrovascular risk factors such as systemic hypertension blood pressure (≥140/90 mm Hg or by previous antihypertensive medication use before ), diabetes mellitus ( blood sugar >126 mg/dl in fasting and >200 mg after 2 hour of meal) , dyslipidemia (total serum cholesterol ≥ 200 mg/dL or LDL ≥ 100 mg/dL or HDL ≥ 50 mg/dL or triglycerides ≥150 mg/dL or on lipid lowering medication).

***Blood collection, DNA isolation, biochemical analysis and genotyping:***

Peripheral venous blood (10 ml) in minimum 8-hour fasting was collected from all study subjects in two vials, 5ml in EDTA tube and 5 ml in gel tube. Gel tube blood was centrifuged and serum was separated and collected in plain tube and stored at -80$^0$ C until biochemical parameter analysis. Genomic DNA isolation and its purification were done from EDTA blood with Quick Gene DNA whole blood kit S (Life Science, Japan), according to the manufacturer's instruction. DNA quantification was done spectrophotometrically at 280/260 and DNA stored at -80$^0$ C till PCR was performed. The SNPs {rs1548359 (SNP1), rs10314 (SNP2) and rs739371 (SNP3)} in the clauidn-5 gene were selected via searching dbSNP database(http://www.ncbi.nlm.nih.gov/projects/SNP/) search, SNPs more than 0.15 minor allele frequency (MAF) and previous finding [30-32. In order to detect the genotypes of SNPs, a LightSNiP typing assay (coupled with primer and probe, TIB-MolBiol, Berlin, Germany) on a LightCycler 2.0 Real-Time PCR system (Roche Applied Science, Mannheim, Germany) was used. Briefly, the amplifications were performed as the recommendation of the manufacturer. Shortly, the thermal cycling profile was initial denaturation at 95 $^0$C for 10 min, then amplification for 45cycles by denaturing at 95 $^0$C for 10 s, annealing at 60 $^0$C for 10 s, and extension at 72 $^0$C for 15 s. After amplification, PCR products were denatured at 95 $^0$C for 20s and cooled to 40 $^0$C for 20s to form double-strand DNA. Then the melting curve analyses were performed by gradually increasing the temperature to 85 $^0$C at a rate of 0.2 $^0$C/s. Obtained data were analyzed using Gene Scanning software (Roche Diagnostics, Germany). PCR samples showing clear melting curves were only included in this study. Double distilled sterilize water (PCR grade) was used as negative control to confirm the accuracy of the PCR process. The representative melting curve and melting peaks each SNPs PCR are shown in **Figure 1**.

***Data management and statistical analysis:***

SPSS 16.0, GraphPad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA) and MedCalc (version 7.4 for windows; Frank. Schoolnjans, Belgium) were used for the evaluation of the differences of all parameters between controls and patients, and risk confined by them. Fisher's exact test was used to compare the categorical variable of demographic characteristic, vascular risk factors and biochemical risk factors between controls and patients, while Chi-square ($\chi 2$) test was used for categorical variables of the same. Odd ratio (OR) and 95% confidence interval (CI) were calculated by logistic regression model to find the association between claudin-5 genetic polymorphism and risk of LA. A p-value <0.05 was considered statistically significant.

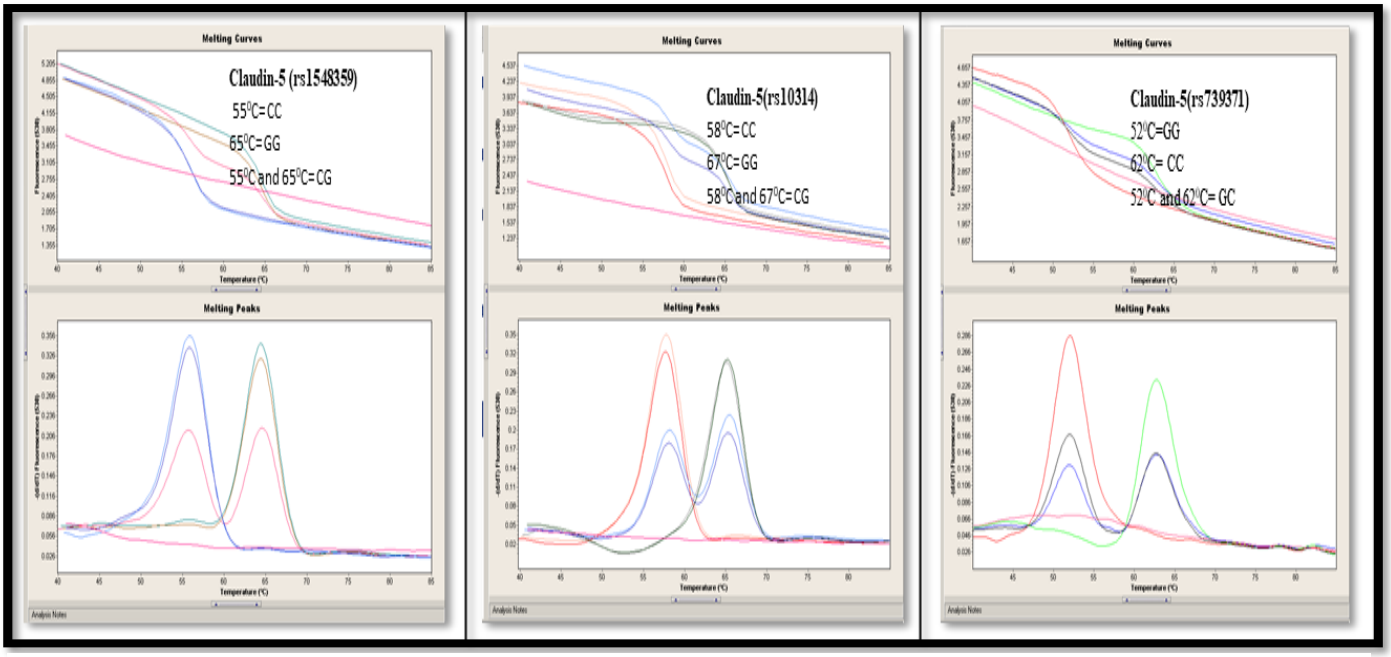


**Figure 1|Representative diagram of melting curves and melting peaks of each SNPs PCR**

## RESULTS

The demographic characteristics and clinical features of the study population are shown in Table 1. Male covered for around 48.0% among controls and 62.0% among the leukoaraiosis (LA-PVWM and LA-DWM) patients ($p<0.05$). Both LA cases are elderly with hypertension and diabetes than the control group ($p<0.05$).

Among the biochemical parameters, total plasma homocysteine level was found marginally increased in LA-PVWM ($p=0.08$) and LA-DWM ($p=0.06$). The genotypic frequency of claudin-5 between controls and LA-PVWM and LA-DWM are presented in Table 2a and 2b, respectively.

**Table 1| Demographic and clinical characteristics in control and patients with LA**

| Characteristics | Control | LA-PVWM | *p* | Control | LA-DWM | *p* |
|---|---|---|---|---|---|---|
| Age | 61.66±13.04 | 74.46±7.76 | 0.000 | 63.87±13.28 | 73.44±8.87 | 0.000 |
| Gender(male),n(%) | 127(62.9%) | 91(49.7%) | 0.009 | 143(62.4%) | 75(48.1%) | 0.006 |
| Hypertension, n (%) | 111(55.2%) | 134(73.2%) | 0.000 | 132(57.9%) | 113(72.4%) | 0.004 |
| Diabetes,n (%) | 57(28.4%) | 65(35.5%) | 0.132 | 70(30.7%) | 52(33.3%) | 0.586 |
| Cholesterol(mg%) | 165.84±43.41 | 169.21±41.13 | 0.435 | 166.40±43.07 | 168.97±41.25 | 0.560 |
| Trigylcerid(mg%) | 146.94±130.89 | 131.62±104.08 | 0.208 | 148.65±130.78 | 126.46±98.13 | 0.072 |
| HDL-Chol(mg%) | 40.43±10.89 | 41.26±12.02 | 0.477 | 40.46±10.76 | 41.36±12.37 | 0.451 |
| LDL-Chol(mg%) | 98.12±34.68 | 97.92±31.90 | 0.954 | 97.80±34.47 | 98.36±31.74 | 0.872 |
| Chol/HDL | 4.27±1.20 | 4.311.28 | 0.732 | 4.28±1.23 | 4.30±1.26 | 0.896 |
| LDL/HDL | 2.53±0.94 | 2.51±0.93 | 0.834 | 2.52±0.94 | 2.51±0.93 | 0.973 |
| ApoA1(mg%) | 1.23±0.78 | 1.1905±0.26 | 0.552 | 1.21±0.73 | 1.20±0.27 | 0.805 |
| ApoB(mg%) | 0.968±0.29 | 0.93±0.25 | 0.345 | 0.96±0.28 | 0.92±0.26 | 0.261 |
| ApoB/ApoA1 | 0.84±0.31 | 0.81±0.27 | 0.414 | 0.85±0.30 | 0.79±0.27 | 0.072 |
| Lp(a)(mg%) | 30.41±26.17 | 28.23±26.13 | 0.463 | 31.12±28.39 | 26.65±21.86 | 0.141 |
| Glucose(mg%) | 144.78±66.08 | 141.78±81.76 | 0.696 | 145.26±65.41 | 140.51±85.08 | 0.543 |
| HbA1C(%) | 6.35±3.53 | 6.21±1.38 | 0.613 | 6.34±3.34 | 6.19±1.40 | 0.593 |
| tHCy(µmol/L) | 13.26±6.30 | 14.34±5.78 | 0.081 | 13.29±6.30 | 14.48±5.67 | 0.062 |

**Table 2a|Comparison of genetic frequency of Claudin-5 polymorphisms in the control and LA-PVWM**

| Genotypes | Control | LA-PVWM | OR (95%CI), P |
|---|---|---|---|
| **rs1548359** (SNP1) | n=202(%) | n=183(%) | p=0.590 |
| CC | 137(67.8%) | 124(67.8%) | 1.0(Reference) |
| CG | 59(29.2%) | 50(27.3%) | 0.93(0.59-1.46),0.81 |
| GG | 6(3.0%) | 9(4.9%) | 1.65(0.57-0.479),0.42 |
| Dominant | | | |
| CC vs CG+GG | 137/65 | 124/59 | 1.00(0.65-1.53),1.00 |
| **Recessive** | | | |
| CC+CG vs GG | 196/6 | 174/9 | 1.69(0.58-4.84),0.43 |
| Genotypes | Control | LA-PVWM | OR (95%CI),P |
| **rs10314(SNP2)** | n=202(%) | n=183(%) | 0.294 |
| CC | 29(14.4%) | 17(9.3%) | 1.0(Reference) |
| CG | 103(51.0%) | 96(52.5%) | 1.59(0.82-3.077),0.19 |
| GG | 70(34.7%) | 70(38.3%) | 1.70(0.86-3.38),0.13 |
| **Dominant** | | | |
| CC vs CG+GG | 29/173 | 17/116 | 1.14(0.60-2.11),0.74 |
| Recessive | | | |
| CC+CG vs GG | 132/70 | 113/70 | 1.16(0.77-1.77),0.52 |
| **Genotypes** | Control | LA-PVWM | OR (95%CI),P |
| **rs739371 (SNP3)** | n=202(%) | n=183(%) | p=0.838 |
| CC | 63(31.2%) | 52(28.4%) | 1.0(Reference) |
| CG | 100(49.5%) | 94(51.4%) | 1.13(0.71-1.80),0.63 |
| GG | 39(19.3%) | 37(20.2%) | 1.14(0.64-2.05),0.65 |
| **Dominant** | | | |
| CC vs CG+GG | 63/139 | 52/137 | 1.19(0.77-1.84),0.439 |
| **Recessive** | | | |
| CC+CG vs GG | 163/39 | 146/37 | 1.05(0.64-1.75),0.89 |

**Table 2b| Comparison of genetic frequency of Claudin-5 polymorphisms in the control and LA-DWM**

| Genotypes | Control | LA-DWM | OR(95%CI),P |
|---|---|---|---|
| **rs1548359 (SNP1)** | n=229(%) | n=156(%) | p=0.406 |
| CC | 157(68.6%) | 104(66.7%) | 1.0(Reference) |
| CG | 61(26.6%) | 48(30.8%) | 1.18(0.75-1.86),0.48 |
| GG | 11(4.8%) | 4(2.6%) | 0.54(0.17-1.77),0.418 |
| **Dominant** | | | |
| CC vs CG+GG | 157/72 | 104/52 | 1.09(0.70-1.68),0.73 |
| **Recessive** | | | |
| CC+CG vs GG | 218/11 | 152/4 | 0.52(0.16-1.66),0.29 |
| Genotypes | Control | LA-DWM | OR(95%CI),P |
| **rs10314 (SNP2)** | n=229(%) | n=156(%) | p=0.817 |
| CC | 29(12.7%) | 17(10.9%) | 1.0(Reference) |
| CG | 119(52.0%) | 80(51.3%) | 1.14(0.59-2.24),0.74 |
| GG | 81(35.4%) | 59(37.8%) | 1.24(0.62-2.46),0.60 |
| **Dominant** | | | |
| CC vs CG+GG | 29/190 | 17/139 | 1.24(0.65-2.36),0.52 |
| **Recessive** | | | |
| CC+CG vs GG | 148/81 | 97/59 | 1.11(0.72-1.69),0.66 |
| **Genotypes** | Control | LA-DWM | OR(95%CI),P |
| **rs739371 SNP3** | n=229(%) | n=156(%) | 0.653 |
| CC | 65(28.4%) | 50(32.1%) | 1.0(Reference) |
| CG | 116(50.7%) | 78(50.0%) | 0.87(0.54-1.39),0.633 |
| GG | 48(21.0%) | 28(17.9%) | 0.75(0.41-1.37),0.37 |
| **Dominant** | | | |
| CC vs CG+GG | 65/164 | 50/106 | 0.84(0.53-1.30),0.49 |
| **Recessive** | | | |
| CC+CG vs GG | 181/48 | 128/28 | 0.82(0.49-1.38),0.51 |

We did not find any significant association between any one of the claudin-5 SNPs studied with leukoaraiosis. The frequencies of the CC, CG and GG genotypes of all the three SNPs were also not significantly difference

**Table 3a.Haplotype frequency of Claudin-5 in control and LA**

| Genotype | Control | LA-PVWM | OR(95%CI) | p-value |
|---|---|---|---|---|
| C-C-C | 0.3802 | 0.3374 | 0.82(0.61-1.10) | 0.2 |
| C-C-G | 0.0098 | 0.01352 | 1.38(0.36-5.19) | 0.74 |
| C-G-C | 0.1685 | 0.1962 | 1.21(0.83-1.74) | 0.34 |
| C-G-G | 0.2658 | 0.2674 | 1.01(0.73-1.39) | 0.93 |
| G-C-C | 0 | 0 | ……. | … |
| G-C-G | 0.0086 | 0.0046 | 0.73(0.122-4.42) | 1.00 |
| G-G-C | 0.0107 | 0.0073 | 0.82(0.18-3.71) | 1 |
| G-G-G | 0.1565 | 0.1739 | 1.14(0.78-1.67) | 0.49 |

**Table 3b|Haplotype frequency of Claudin-5 in control and LA**

| Genotype | Control | LA-DWM | OR(95%CI) | p-value |
|---|---|---|---|---|
| C-C-C | 0.3681 | 0.3488 | 0.91(0.68-1.24) | 0.59 |
| C-C-G | 0.0078 | 0.0166 | 1.84(0.49-6.94) | 0.49 |
| C-G-C | 0.1592 | 0.2135 | 1.44(0.99-2.08) | 0.057 |
| C-G-G | 0.2837 | 0.2416 | 0.78(0.57-1.11) | 0.188 |
| G-C-C | 0 | 0 | 0 | ……. |
| G-C-G | 0.0105 | 0 | 0.13(0.007-2.39) | 0.08 |
| G-G-C | 0.0098 | 0.0082 | 1.10(0.24-4.96) | 1.00 |
| G-G-G | 0.1608 | 0.172 | 1.08(0.73-1.59) | 0.69 |

between control and leukoaraiosis. Compared to the wild genotypes group, variant genotypes (CG or GG or CG+GG) genotype group had no increased risk for LA. We also investigated two different inheritance models including dominant model (homozygous wild type versus variant-containing genotype) and recessive model (wild-type-containing genotype versus homozygous variant genotype), but did not find any relation of these SNPs in leukoaraiosis susceptibility. We further investigated the combination effect among the three SNPs on LA which also did not show any significant effect for LA development (data not shown here). To simplify synergistic allele combinations associated with LA development, we constructed haplotype analysis with each SNP. Of eight individual haplotypes, the haplotypes C-G-C (rs1548359/rs10314/rs739371) only showed borderline significant association with LA-DWM, Table 3b. The risk of LA-DWM is increased by 1.44 times in the individual having this haplotype. Furthermore, we sought to determine whether beta-catenin polymorphisms were associated with environmental risk factors like hypertension, diabetes, total homocysteine and other biochemical parameter and stratified analyses were performed for the same. None of the SNPs able to show any level of association with LA susceptibility in combination of any one of the environmental factors.

## Discussion

A number of studies have documented that alteration in BBB structure and function may contribute to the pathogenesis of leukoaraiosis [7, 17, 18]. Given that Claudin-5, important component of TJ in BBB, plays a significant role in the in the homeostasis of BBB [14, 15]. In last decade, several genetic variant have been discovered as an aggravating factor for leukoaraiosis susceptibility[4, 33, 34]. In past, we have also identified several SNPs (AQP-4 and claudin-1, β-catenin, cadherin) significantly associated with leukoaraiosis [24, 35] and these gene are involved in the regulation of BBB homeostasis. We further hypothesize that claudin-1 networking genes which involve regulation and disruption of BBB might promote the development of leukoaraiosis. Claudin-5 is one of the family members of clauidn -1, which is involved in the tight regulation of BBB through maintaining the TJ structure and function [12-16]. It is well understood now that claudin-5 is playing a significant role in blocking exogenous pathogenic material entering the blood and brain. If claudin-5 is damaged or defective by any cause, the brain would be endangered to the harmful pathogens and exogenous materials which can damage brain ultimately(35). Some previous studies have demonstrated the claudin-5 genetic polymorphism is likely to be associated with one of the brain disorder, schizophrenia and most of them are from a single ethnic population, China and still the results were not consistent as some showed strong association, some reported weak association and while other showed no any association [30,32,37-40]. These findings highlighted the role of claudin-5 in brain related disorder and unavailability of this study in leukoaraiosis , a BBB disorder, insisted us to designe this study to confirm the role of genetic polymorphism of claudin-5 with leukoaraiosis susceptibility. Till date, there are no any single study has been performed in any population to check the function of the clauidn-5 SNPs (rs1548359, rs10314 and rs739371) in leukoaraiosis susceptibility. To our best knowledge, this is the first hospital-based genotype study of claudin-5 with leukoaraiosis susceptibility. We investigated 3 SNPs (rs10314, rs1548359 and rs739371) in leukoaraiosis patients and control subjects in South Korea population. The SNP rs10314 lies in the 3'-untranslated region of the clauidn-5 locus, while the SNP rs1548359 in the CDC45L locus centromeric of rs10314 and the 73971 is situated in 5'UTR of claudin-5[30]. Our study demonstrated neither the heterozygous nor heterozygous genotypes of any SNPs showed the significant association with the leukoaraiosis susceptibility. In consistent of our finding, Yamamoto et al has also reported the lack of association SNP rs1548359 with schizophrenia [32]. However, one haplotypes C-G-C (rs1548359/rs10314/rs739371) showed a borderline significant association with LA-DWM susceptibility. Similar finding was reported in Chinese population but in schizophrenia [32,38].

We analyzed our data with different strategies (Logistic regression with AOR, combination effect of different genotypes with LA, stratified effect between different genotypes and environmental factors, haplotype analysis), but none of our attempts showed any further evidence of significant relation between clauidn-5 and leukoaraiosis. The lack of association of these SNPs with leukoaraiosis may be due to a small number of sample and single centered hospital-based study in Korean ethnic only. Since claudin protein plays a significant role in maintaining the BBB homeostasis and our previous study also found significant association of clauidn-1 with leukoaraiosis, we still believe that there may be the great association of claudin-5 with leukoaraiosis too, therefore, it is highly recommended to conduct the similar study in larger and ethnically different population to confirm the our finding and other too to conclude its real status for the prediction of leukoaraiosis susceptibility.

## CONCLUSION

Claudin-5 SNPs (rs1548359, rs10314, and rs739371) on chromosome 22q11 may not be significantly associated with leukoaraiosis susceptibility in Korean population. However, some studies showed the weak association between claudin-5 and other brain disorder (schizophrenia) with conflicting results, further studies to confirm these finding are warranted.

## ADDITIONAL INFORMATION AND DECLARATIONS

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**Conflict of interest**: None to declare.

**Author Contributions:** Concept and design: **BKY** and **BSS**; data collection, lab work and statistical analysis: **BKY** and **RY**; writing, editing and preparation of draft of the manuscript: **BKY**, **RY** and **BSS**; monitoring and supervising the research finalizing the manuscript: **BKY** and **BSS**. All authors have read and agreed with the contents of the final manuscript towards publication.

**Availability of data and materials**: The datasets used and analyzed for the study are available from the corresponding author upon reasonable request

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