NAZARBAYEV UNIVERSITY

GENETIC MARKER OF THE RISK OF THE DEVELOPING BRAIN ARTERIOVENOUS MALFORMATIONS AMONG KAZAKHSTAN POPULATION

BY

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THESIS

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis. This thesis has also not been submitted for any degree in any university previously.

_____Seksenbayeva Nurgul_____
24 April 2024

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Abstract

Brain arteriovenous malformations (brain AVMs) are a relatively rare but life-threatening neurovascular disease. It is characterized by the presence of aberrant blood vessel tangles, or "bags of worms" of various shapes and sizes, formed as a result of the random interweaving of pathologically altered vessels, while direct drainage of blood from the arterial system to the venous system occurs bypassing capillary network. As a result of the pressure in the vessels, veins increase and lead to their detrimental consequences such as intracranial hemorrhage as a result of rupture, stroke, seizures, long-term effects depending on the location of the tangles on the human brain part, even fatality.

Recently brain AVM is the controversial theme in neurology, since pathogenesis of brain AVM debate over whether a disease is congenital or acquired which arise as a result of genetic predisposition. There are several hypotheses regarding the occurrence of brain AVM. AVMs belong to a heterogeneous group of dysembryogenetic formations of angiomatous structure and considered as congenital disease, while mean age of patients with brain AVM which vary between 20-40 ages verify that brain AVM is arised at a postembryonic period. De novo study of Brain AVM suggests that genesis of brain AVM formation is explained by processes such as angiogenesis, vasculogenesis, and inflammatory response since mutations can be modified during listed processes. However, the gap around the brain AVM is directly associated with the pathogenesis of disease since it is still unclear and not fully understood. Since brain AVM is asymptomatic and arises among the young population, it’s significant to find the exact genetic marker and explain its causative mechanism and further investigate the preventing or treating options among Kazakhstan population. Therefore the main purpose of this study is to find the susceptible gene of developing brain AVM. To find out the

genotyping by Real-Time PCR will be conducted on 200 people with mean age 39±12.6, whose samples were collected on the basis of the National Center of Neurosurgery, Astana in the period from 2021-2022. As a result, genotype(χ^2=1.51, p=0.47) and allelic frequencies(χ^2=3,47, p=0.062) were in Hardy-Weinberg equilibrium. In addition, genotype frequency of rs1333040 was statistically significantly associated with brain AVM in dominant(p=0.0316), recessive(p=0.0211) and trend (p=3.5*e^{-09}) models. Also, it was verified with the result of logistic regression analysis, where OR(1.94 (95% CI: 1.1-3.43, p=1.11*e^{-05}) illustrated significant strong positive association between exposure and outcome, where it is SNP rs1333040 of gene CDKN2B-AS1 and brain AVM.

In conclusion, it was identified that SNP rs1333040 of gene CDKN2B-AS1is genetic marker of risk of developing brain arteriovenous malformations among population of Kazakhstan. Hypotheses were accepted as a result of conducted research study.

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ABBREVIATIONS

ISSVA	International Society for the Study of Vascular Abnormalities
AVM	arteriovenous malformation
AVF	arteriovenous fistula
dAVF	dural arteriovenous fistula
CCF	cavernous carotid fistula
VOG	vein of Galen
HHT	Hereditary Hemorrhagic Telangiectasia
CM-AVM	Capillary Malformation -Arteriovenous Malformation
TGFβ	transforming growth factor β
ENG	endoglin
ALK1	Anaplastic Lymphoma Kinase1
ACVRL1	activin receptor-like kinase 1
SMAD4	Mothers Against Decapentaplegic Homolog 4
EPHB4	ephrin receptor type B
RAS	reticular activating system
RASA 1	RAS protein activator 1
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-Activated Protein Kinase
ERK	Extracellular-Signal-Regulated Kinase
BRAF	B-Rat Fibro sarcoma
PI3K	phosphoinositide 3-kinase
mTOR	mammalian target of rapamycin
BMP 9/10	Bone morphogenetic proteins 9 and 10
PTEN	Phosphatase and tensin homolog
EC	endothelial cells
MEK	methyl ethyl ketanone
pERK	Protein Kinase RNA-Like ER Kinase
SNP	single nucleotide polymorphism
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B
PCR	polymerase chain reaction
MMP	Matrix metalloproteinases
IL	interleukin
MAF	minor allele frequency
OR	odd ratio
CI	confidence interval

I. INTRODUCTION

1.1 Classification of vascular abnormalities. What are the vascular abnormalities and arteriovenous malformations?

Vascular abnormalities are the result of errors in vascular morphogenesis. Recent genetic research has shown that a large number of vascular abnormalities are caused by postzygotic or genomic genetic variations that trigger growth and cancerous pathways (5). Vascular abnormalities are typically detectable from birth, however they are sometimes imperceptible at first and become more noticeable as the child gets older. U. Hunter was the first to describe a vascular abnormality in 1757. Only a century later, the results of in-depth morphological studies of this pathology were presented by H. Luschka and R. Virchow in 1854 and 1863, respectively (4). As a result numerous research, origin, characteristics and classification of vascular abnormalities were formulated.

International Society for the Study of Vascular Abnormalities (ISSVA) divided vascular abnormalities into two categories such as vascular neoplasms and vascular malformations (1). Vascular neoplasm or vascular tumor arises as a result of raised endothelial cell turnover, while vascular malformations are abnormalities of the blood vessels and can affect any area of an artery, vein, or capillary, among other blood vessel types (2). The Hamburg classification system defined vascular malformations as arterial, venous, arteriovenous, lymphatic, or combined depending on morphological appearance, arteriovenous shunting, hemodynamics, and contrast angiography (3, 4, 5) (Table 1).

Table 1. Hamburg classification system of vascular malformations

Predominant type	**Lesion form**
Arterial malformation	Lesions with high blood flow which are likely to show warmth and palpable agitation. They are distinguished by radiology or physical examination.
Venous malformation (VM)	Majority of VMs are unifocal and sporadic, for example TIE2 gene somatic mutations are linked to nearly half of these lesions.
Lymphatic malformation(LM)	Lymphatic malformations (LMs) are made up of cysts or dilated lymphatic channels lined with lymphatic endothelium. Common LMs can appear anywhere on the

	body, but they are most common in regions with many lymphatic vessels, like the head and neck, etc.
Arteriovenous malformation(AVM)	Type of vascular malformation where blood flows directly from artery to vein without capillary system because of "nidus" or "tangles" and causing stroke, hemorrhage and other serious consequences.
Combined/mixed malformation	Combined vascular malformations are defined as two or more distinct vascular malformations within a single lesion. In order to correctly diagnose combined vascular malformations, radiographic and/or histological evidence may be needed for these diseases, as they may conceal deeper components beneath more outwardly visible superficial deformities.

1.2 Brain arteriovenous malformation is rare but life-threatening neurological disease

Among these 5 groups types of vascular malformations, the arteriovenous malfunction is the most serious types which is characterized by an abnormal leash of vessels allowing for direct arteriovenous shunting without intervening capillary bed. They can occur anywhere in the body but are most common in the brain (5, 6).

According to Thomas et al. (2016), the arteriovenous malformations in the brain (brain AVMs) are relatively rare but life-threatening neurovascular disease. It is characterized by the presence of aberrant blood vessel tangles, or nidus, or "bags of worms" in various shapes/sizes between the arterial and venous circulations. They are formed as a result of the random interweaving of pathologically altered vessels while direct drainage of blood from arterioles to venules occurs (7) (example schematic illustration of AVMs in Fig. 1).

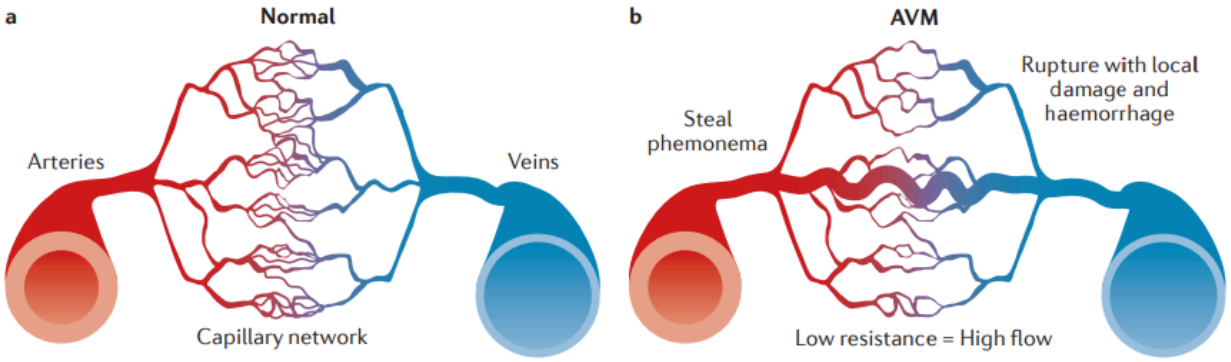

Figure 1. Schematic representation of normal vasculature and an arteriovenous malformation (AVMs). a) Normal connection of blood vessels; b) Abnormal connection of blood vessels with

AVMs (Sources: Thomas, J. M., Surendran, S., Abraham, M., Rajavelu, A., & Kartha, C. C. (2016). Genetic and epigenetic mechanisms in the development of arteriovenous malformations in the brain. Clinical epigenetics, 8, 78. https://doi.org/10.1186/s13148-016-0248-8

1.2.1 Clinical characteristics of Brain AVM

According to clinical characteristics, brain AVMs are found on average in one person out of 100 000 population per year. In most cases, they are clinically captured between the ages of 20 and 40, in some cases in people over 50 years of age (8,9, 24). Even if it's a rare neurological disease, it has dangerous consequences - the possibility of their rupture, since the arteries that form AVMs have thinned walls with an underdeveloped muscle layer. In the presence of brain AVM, the risk of its rupture is estimated at about 2-4% per year (10). In addition, brain AVMs account for 1.5-4.0% of all intracranial volume formations, cause 8.6% of non-traumatic subarachnoid hemorrhages, 1% of cerebral strokes. If the hemorrhage has already taken place, then the probability of its recurrence is 6-18% (15). These detrimental effects of brain AVMs, including intracranial hemorrhage, stroke, and seizures, lead to brain damage or long-term consequences, depending on the location of the tangles in the part of the person's brain. Listed consequences of brain AVM can be distinguished as symptoms of brain AVM since brain AVM is mostly characterized as asymptomatic and is often an accidental finding. Hence, mortality in intracranial hemorrhage from AVM is observed in 10% of cases, and in 48% of cases the disease leads to deep disability (8, 11-14, 24).

1.2.2 Classification of brain AVM

There are two common types of classification of brain arteriovenous malformations; depending on the features of the structure of the brain AVM formation (Table 2) and depending on the size of brain AVM lesions (6, 16, 18) (Table 3).

Table 2. Classification of brain AVM

Depending on the anatomy of brain AVM	Angiomatous or classic arteriovenous malformations(cAVM)	It is an abnormal collection of tortuous blood vessels in the brain or spinal cord. It is a result of a poor network of interconnected vessels, which include feeding arteries, nidus, and draining veins.
	Arteriovenous fistulous(AVF)	The artery passes directly into a vein with a high flow without the formation of a vascular tangle. These include dural arteriovenous fistula (dAVF), cavernous carotid

		fistula (CCF) and Galena vein malformation (VOG).
	Mixed	Combines angiomatous and fistulous forms.

AVMs and AVFs are anomalous direct connections that obstruct the typical capillary bed between arteries and veins. An AVM is commonly used to describe a focus or “tangle” of abnormal vessels, whereas an AVF implies a single, direct, high-flow connection (6). As a result of rapid blood flow through these lesions, AVMs and AVFs typically produce a palpable sensation of fluttering and warmth when examined compared to the surrounding tissue. The differentiation of AVMs and AVFs from other vascular abnormalities is aided by these clinical characteristics as well as distinctive ultrasonography findings.

Table 3. Classification of brain AVM depending on the size of lesions.

	Types	**Treatment options**
Depending on the size of brian AVM lesions	Small - diameter does not exceed 3 cm;	Surgery
	Medium - 3-6 cm in diameter;	Multidisciplinary treatment; surgery, radiosurgery, endovascular
	Large - size 6 cm or more.	Conservative management

Overall, depending on the size, location and drainage, brain AVMs are divided into 5 gradations according to the Spetzler-Martin scale (19). According to Spetzler-Martin scale Grade I-II is operable and it has a low morbidity and mortality rate, however as gradation is increased the possibility of surgery and chance of survival rate is declined. That’s why, Grade III can be managed by various treatment options such as endovascular, radiosurgery or surgery, while Grade IV-V is not operable and treatment option is conservative management (17,19,20) (Table 3).

Evaluation of brain AVM with the help of Spetzler-Martin grading scale done after diagnostics with tool inspections, which allow to make a conclusion about treatment options (21,22):

- Ultrasound and ultrasound (duplex scanning of blood vessels). It allows you to detect a pathological focus, assess the speed and direction of blood movement, as well as distinguish

AVM from other volumetric formations (tumors).

- Angiography. The introduction of a contrast agent into the vessels allows them to be displayed on a series of X-rays and to visualize the AVM.
- CT and MRI. They are carried out with insufficient information content of other techniques, requiring contrasting vessels.

1.3 Pathogenesis of Brain arteriovenous malformation

There are several hypotheses regarding the occurrence of brain AVM. AVMs belong to a heterogeneous group of embryogenetic formations of angiomatous structure and considered as congenital disease (25). The embryogenesis of brain AVM has not yet been definitively studied. However, Mullan and collaborators based on embryological evidence of normal cerebral angiogenesis hypothesize that AVMs originate from fistulas created by transverse veins that flow at right angles to developing longitudinal arteries during embryo development(31). Hence damaging effect on the processes of angiogenesis, leading to dysplastic metamorphosis and the formation of an aberrant section of the vascular network, can occur only up to 6 weeks of intrauterine development, until the differentiation of primary capillaries into arteries and veins has occurred (26). It is established that the process of AVM formation itself occurs between the 7th and 12th week of development. That's why such diseases are diagnosed using advanced prenatal ultrasound imaging at 10-14 weeks of pregnancy (27).

Congenital anomalies are present at birth and manifest later in life. According to clinical characteristics of brain AVM, the mean age varies between the ages of 20 to 40 (8). Consequently, a novel hypothesis about the mechanism behind the emergence of AVM de novo has surfaced. Recent advances of pathogenesis of brain AVM postulated that 95% of cases are sporadic which arise as a result of somatic mutation, while approximately 3-5 % of cases are familial form as a result of germline mutation (25, 26). According to Mouchtouris et al. (2015) pathophysiology of brain AVM formation is explained by processes such as angiogenesis, vasculogenesis, and inflammatory response since mutations can be modified during listed processes (27). In addition, there is growing evidence that the localized nature of the brain AVM lesion suggests the role of additional factors contributing to the development of the lesion. Kim et al. (2011) explained it by "two-hit mechanism", wherein an environmental trigger (such as trauma, ischemia, infection, inflammation, or angiogenic factors) or a random somatic mutation coincide with an inherited mutation in one copy of a gene to cause the pathology of the disease(35)(Figure 2).

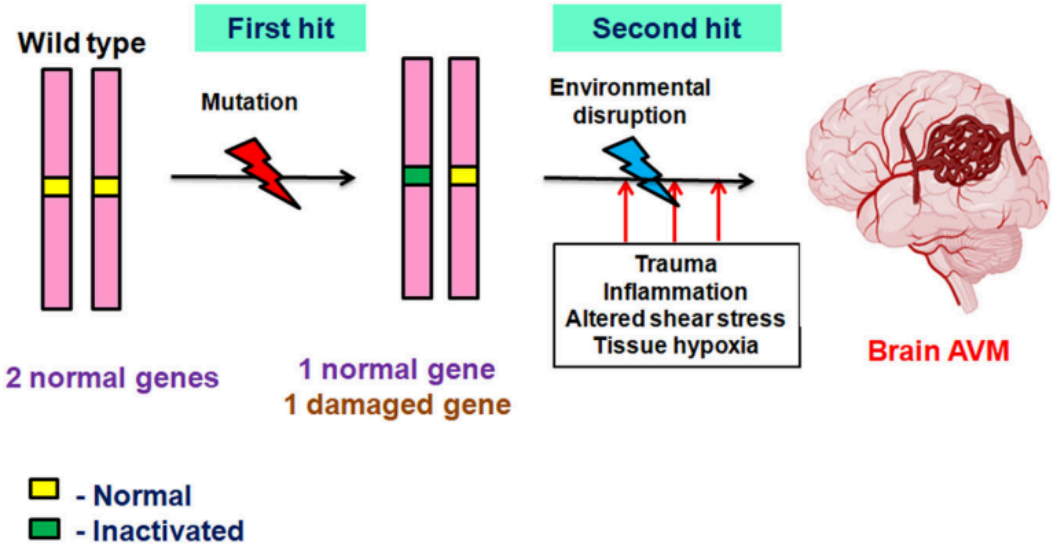

Figure 2. Schematic representation of the "two-hit" mechanism. (Sources: Kim, H., Su, H., Weinsheimer, S., Pawlikowska, L., & Young, W. L. (2011). Brain arteriovenous malformation pathogenesis: A response‐to‐ injury paradigm. Acta Neurochirurgica Supplement, 111, 83–92. https://doi.org/10.1007/978-3-7091-0693-8_14)

Since the specific genetic mechanism that explains the brain AVM development is still unclear and Figure 2 explains that brain AVM is the result of coincidence of genetic and environmental factors, consequently brain AVMs are multifactorial disease. Nevertheless, all genetic and molecular factors that are involved in the pathophysiology of cerebral arteriovenous malformations are involved in cerebral angiogenesis, and their dysregulation creates an environment with increased angiogenesis, leading to abnormal vascular growth and impaired arteriovenous remodeling. Also, despite the angiogenesis, causative genes of brain AVM involved in vasculogenesis, inflammation plays a role in etiology of brian AVM.

1.3.1 Signaling pathways of familial brain AVMs

Familial form of brain AVM is considered 5 % of brain AVM (26). As a genetic disorder 90% of brain AVM linked with Hereditary Hemorrhagic Telangiectasia (HHT), Rendu-Osler-Weber Syndrome, also recently described that autosomal dominant disease Capillary Malformation -Arteriovenous Malformation (CM-AVM) syndrome (32, 33).

Causative genes of HHT are members of transforming growth factor β (TGFβ) signaling pathway, such as endoglin(ENG), activin receptor-like kinase 1 (ALK1 or ACVLR1) and Mothers Against Decapentaplegic Homolog 4 (SMAD4) (34).

The causative genes of CM-AVM syndrome are ephrin receptor type B (EPHB4) or RAS p21 activator protein 1 (RASA1) since they are also a marker for diagnosing this autosomal dominant disease(33). Regarding Eerola et al. (2003) about 70% of patients with RASA1-CM-AVM syndrome and about 80% of children with EPHB4 CM-AVM syndrome have affected parents (33).

1.3.2 Signaling pathways of sporadic brain AVMs

About 95% of brain AVM are sporadic. Somatic mutations are found on several signaling pathways. Majority of sporadic brain AVMs arise as a result of somatic mutations of KRAS via *Rat Sarcoma (RAS)/Mitogen-Activated Protein Kinase (MAPK)/Extracellular-Signal-Regulated Kinase (ERK)* pathway, also excessive KRAS activity may have an impact on other several downstream signal pathways. Nikolaev et al.(2018) identified activating KRAS mutations on 45 out of 72 brain AVM patients (36). Regarding Oka et al.(2019) more than half of bAVM tissue samples consisted of activating KRAS mutations, which may imply the pathogenic function of these KRAS mutations (37). Changes in endothelial cell morphology and size, ectopic sprouting, increased vascular lumen diameter, and direct connections between arteries and veins have all been linked to active KRAS signaling.

Other somatic mutations of brain AVM is B-Rat Fibro sarcoma (BRAF). Hong and collaborators indicate the prevalence of KRAS and BRAF mutations in brain AVM and spinal cord AVM, which are equal to 81.0% in brain AVM and 100% in spinal cord AVMs (38). Whereas BRAF p.V600E was uncommon and was only detected in one brain AVM patient and one spinal cord AVM patient, KRAS p.G12D and p.G12V were mutation centers in both spinal cord AVMs and brain AVMs, with a prevalence of 30.0% and 30.0% in spinal cord AVMs and 52.4% and 19.0% in brain AVMs, respectively(38).

Mutations of vascular malformations are related with mutations that are discovered in cancer. For example, mutations on phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) and the RAS-MAPK-ERK pathway in vascular malformations including venous, lymphatic malformations and brain AVM (36, 39, 40). There are also brain AVMs that arise without KRAS, but with increased levels of phosphorylated ERK1/2 (40). Hence, it indicates that RAS–MAPK–ERK pathway activation is a hallmark of all brain AVMs.

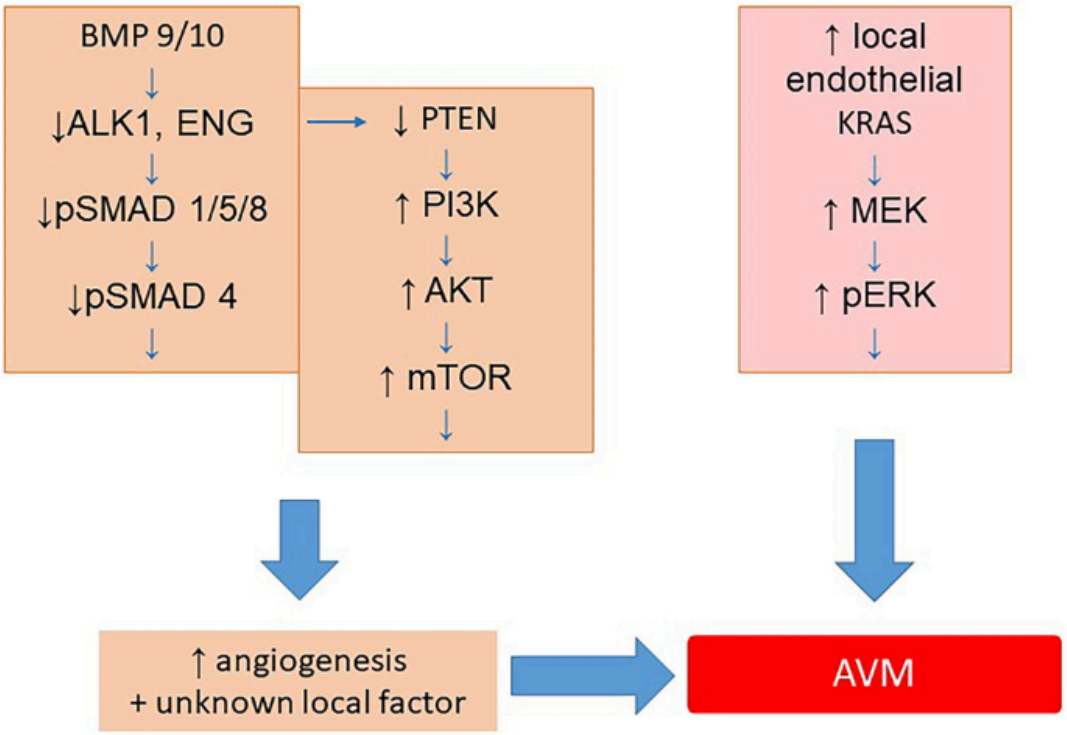

Figure 3. Cell signaling cascades engaged in brain AVM development. Left column: Genes and cell signaling pathways involved in HHT, which are germline mutations of brain AVM development. Right column: Cell signaling pathway during somatic mutations of brain AVM development (Sources: Steiger, HJ. Recent progress understanding pathophysiology and genesis of brain AVM-a narrative review. *Neurosurg Rev* **44**, 3165–3175 (2021). https://doi.org/10.1007/s10143-021-01526-0).

Overall, angiogenesis is regulated by TGFβ1 family members , when Bone morphogenetic proteins 9 and 10 (BMP9/10) bind to ALK1/ENG and SMAD4 is phosphorylated. Hence, PTEN activity is increased and PI3K signaling is decreased. Here, it's important to mention that the PI3K signaling pathway is one of the important regulators of the angiogenic process that governs endothelial cells(EC) survival, migration, and proliferation (34). That's why familial mutation of HHT genes as ALK1/ENG reduces pSMAD and PTEN activity in order to increase the PI3K activity or pERK level, resulting in an increased angiogenesis process. During somatic mutation of brain AVM, activating KRAS/BRAF or MAP2K1 gene mutations enhance the MEK and pERK, resulting in brain AVM development (32).

1.4 SNPs association with Brain AVM

Genome-wide association studies (GWAS) have revolutionized the search for genetic influences on complex traits, where single-nucleotide polymorphisms (SNPs) are tested for

association with disease in humans (41). Over the past five years, genome-wide association studies have identified SNPs located at hundreds of loci (i.e., specific locations of the genome) that are highly reproducible with respect to common traits, including brain AVMs. National Human Genome Research Institute (2024) describes a single nucleotide polymorphism (abbreviated as SNP, pronounced snip) as a point mutation where one nitrogenous base is replaced by another (A, T, G, C) (42).

The discovery of single nucleotide polymorphisms (SNPs) linked to arteriovenous brain malformations can aid in risk-based stratification, the development of customized treatment plans, and better understanding of the molecular mechanisms involved in the formation and progression of this illness. As a result of investigation of candidate genes for brain AVM formation Leonardo and coworkers (2014) identified 860 genes susceptible for brain AVM, where 300 were upregulated and 560 were downregulated genes (28).

Studies of patients with brain AVMs have revealed two main families of polymorphisms associated with genes involved in angiogenesis and inflammatory cascades. A cross-section study by Jiang et al. (2023) summarized the SNPs engaged in angiogenic pathways and determined SNPs such as *ANGPT2*, *FLT4*, *KDR*, *TIE2*, *EPHB4*, *VEGFA*, *TGFβ1*, *TGFβR2*, *NOTCH4, GPR124* on promoting and coding regions of angiogenic genes. Also, it was determined SNPs in expression of nine genes such as *IL6*, *IL10*, *TNF, APOE*, *IL1B*, *IL17A*, *MMP9* involved in inflammatory cascades increased in presence of brain AVM compared with normal tissue (43). Also, in accordance with that, Mukhtarova et al.(2022) reported genetic associations of genes susceptible for brain AVM linked to the inflammatory pathway (TGFß and IL6), angio- or vasculogenesis (ENG, ALK1, and ACVRL1), and other genes such as CDKN2B and etc (29).

Identification of SNPs associated with susceptibility to AVM in the brain allows us to understand the mechanism based on the pathogenesis of the disease and to stratify and predict patients based on risk and further select optimal treatment methods.

To sum up, taking into account recent advances in pathophysiology and natural history of brain AVM, it concluded that brain AVM is a multifactorial disease. Genetic, molecular and environmental factors that are involved in the pathophysiology of brain arteriovenous malformations by regulating angiogenesis, vasculogenesis and inflammatory cascades. Nevertheless, exact pathogenetic mechanisms of brain AVM development and its rupture is still unknown and not fully understood. On the other hand, GWAS studies determined SNPs as a new trend to define brain AVM formation as much as earlier before detrimental consequences such as hemorrhage, stroke, deep disability or mortality.

Therefore the research question of the study will be “What is the genetic marker of the brain arteriovenous malformations?”. Hence, this study was devoted to SNPs that are associated with brain arteriovenous malformations and proposed to identify the genetic marker of developing brain AVM among the Kazakhstan population. Since brain AVM is asymptomatic and arises among the young population, it’s significant to find the exact genetic marker and explain its causative mechanism and further investigate the preventing or treating managements among Kazakhstan population.

II MATERIALS AND METHODS

2.1. Experimental Plan of the research process.

1. Understanding the basic concepts of brain AVM requires literary research, which includes searching for articles, reviews and meta-analyses related to epidemiology, etiology, pathophysiology, pathogenesis, diagnosis and therapy of brain AVM.
2. Focusing on the pathophysiology of brain AVM by looking up academic publications to comprehend the origins of brain AVM and its relationship to SNPs.
3. Compile all of the brain AVM SNPs, create a database, and then choose the most vulnerable SNP for the brain AVM based on the database.
4. Performing genotyping for the chosen SNP.
5. Performing a statistical investigation.

2.2. Literature Search.

We searched PubMed and Google Scholar from 2013 to 2023 for research on SNPs associated with the risk of brain AVM formation. Various phrases that we used in our search were "brain", "arteriovenous malformation", "genetics", "SNP", and "polymorphism". 105 SNPs collectively are detected across 60 resources (2013–2023). A database was created from gathered SNPs that are vulnerable to brain AVM, containing details on the gene's location, the type of mutation, and the group that is most likely to experience brain AVM.

2.3. Sample preparation.

Blood samples of patients were collected at the National Center of Neurosurgery in the period from 2021-2022(NCN, Astana, Kazakhstan). All participants provided informed and voluntary consent for the use of their biological materials. Experimental work was carried out on the basis of the National Center of Biotechnology of the Ministry of Health of the Republic of Kazakhstan and approved by the Ethics Committee of the National Center for Biotechnology according to the principles set out in the Helsinki Declaration.

Study was conducted on 200 people in 2 groups: 100 people in a case group for patients with Brain AVM and 100 people in a healthy control group. The presence of arteriovenous malformations in the case group was confirmed using selective cerebral angiography and CT/MRI imaging. According to the collected data, a database of patients with cerebral arteriovenous malformation (AVM) has been created. This database contains detailed information about each patient's smoking habits, location and size of the AVM, venous drainage, and any other relevant problems, such as

bleeding, seizures, epilepsy, and aneurysms.

2.4. DNA isolation.

Using a commercial MaqMAXTM DNA Multi-Sample kit from Thermo Fisher Scientific, genomic DNA from whole blood was isolated in accordance with the manufacturer's instructions. The exact DNA concentration and frequencies of DNA samples was measured on a NanoDrop 1000 spectrophotometer by measuring the absorption at 260 nm.

2.5. Genotyping by Real-time polymerase chain reaction(PCR).

Genotyping was performed using real-time PCR, CFX-96 amplifier (Bio-Rad, Hercules, CA,USA). Real-time PCR was established using a set of Taqman SNP Genotyping Assays in 96-well plates and in 384-well plates. Thermal cycling conditions of Real-time PCR were as follows:

- Polymerase activation for 1 cycle at 95°C for 10 minutes;
- Denaturation process in 39 cycles at 95°C for 15 sec;
- Annealing process at 60°C for 1 minute.

Data analysis was studied using the program "CFX Maestro Software for CFX Real-Time PCR Instruments".

2.6. Statistical analysis.

Descriptive statistics in order to illustrate the demographic, clinical characteristics were processed in the R studio. Statistical significance for categorical variables was determined by χ^2 test and for continuous variables used Student t-test. Differences between groups were significant at P-value<0.05. Differences of allelic, genotype frequencies between groups were evaluated using Hardy-Weinberg equilibrium($p>0.05$) using χ^2 test. To evaluate association of SNP and disease used multivariate logistic regression analysis.

All statistical analysis was conducted using following programs:

- For data cleaning used Microsoft Excel;
- R 4.3.0 (Foundation for Statistical Computing, Vienna, Austria);

III AIMS OF THE PROJECT

Research gap of study is directly associated with the pathogenesis of disease, because exact causative mechanism, its genesis still unknown. The main overall goal of this study is primarily directed to find the susceptible gene of developing brain AVM. Recent advances suggest that the cause of brain AVM is multifactorial and it is associated with genetic predisposition and environmental factors (26, 35). Furthermore, in recent times, there has been a shift in genetic research towards examining the connection between single-nucleotide polymorphisms, or variations in single nucleotides between paired chromosomes in humans or between genomes within biological species, and sporadic arteriovenous malformations. These variations can impact a person's natural history and determine their susceptibility to complex diseases leading up to short-term and long-term effects of brain AVM. Numerous modifications in two distinct gene families have been linked to an increased risk of bleeding and an increased susceptibility to brain AVM. These genes are primarily involved in the neurovascular inflammatory response and the regulation of angiogenesis (30). Regarding to the recent progress of understanding the pathogenesis of brain AVM single nucleotide polymorphisms (SNPs) of candidate gene which is susceptible for brain AVM is significantly associated with developing brain AVMs and they are best marker to identify patients who are at risk of brain AVM. Hence, the hypothesis of this study is that rs1333040 of the CDKN2B-AS1 gene which is located on chromosome 9p21 will be significantly associated with brain AVM and will be a genetic marker on identifying risk of developing brain AVM.

To test the hypothesis, I will pursue the following Specific Aims:

Specific Aim 1: to identify the candidate genes by making a database which are susceptible to brain AVM involved in a) inflammation b) in angiogenesis in order to understand the relationship between SNPs and pathophysiology of brain AVM.

Specific Aim 2: to set the study groups of research and examine the demographic, clinical, angiographic characteristics of participants.

Specific Aim 3: to proceed with the experimental part of research study by genotyping rs1333040 for case and control groups.

Specific Aim 4: to investigate the relationship between rs1333040 and brain AVM and explain it in terms of pathogenesis of brain AVM.

IV RESULTS

4.1 Selection of single nucleotide polymorphism for genotyping

SNP selection was started from a literature review. The major goal of the literature review was to gather data on SNPs, including their location on genes and chromosomes, odd ratios with significance levels, and the populations in which they were discovered.

As a result of literature reviews following points were concluded (Table1):

- Overall 105 SNPs are found among 60 resources (between 2013-2023 years)
- 90% of founded SNPs are sporadic
- Detected SNPs were located on genes such as VEGFA, IL-1a, IL-1B, IL-17, ALK1, CDKN2A/B on chromosomes 6, 8, 9, 12 and etc.
- Majority of patients with brain AVM are found in Caucasian races (Italy, Brazil, Netherlands, Scotland, Germany, Dutch patients) and in the Chinese population.

Table 4. SNPs association with brain AVM.

SNP	location in gene	chromosome posit	form of mutati	odd ratio	population
rs 1333040	ANRIL in the INK4 locus	9p21	sporadic	1.93(1.28-2.92 p<0.01) for 1 "at risk" genotype	Italian(A. Gemelli University Hospital of Ro
		9p21.3	sporadic	1.27 (1.01-1.58, p=0.04)	Caucasian (University of California, San Fr
	CDKN2B-AS1	chr8	sporadic	0.79 (0.63 to 0.99)p= 0.04	Caucasian(Netherlands, Germany, Italy an
				1.03 (0.81 to 1.32) p=0.80	Caucasian(Netherlands, Germany, Italy an
				0.89 (0.76 to 1.05) p=0.18	Caucasian(Netherlands, Germany, Italy an
				0.93 (0.80 to 1.08) p=0.336	Caucasian(Netherlands, Germany, Italy an
rs 7865618	CDKN2A/B	9p21	sporadic	2.97 (0.61-14.5 p=0.15) for 2 "at risk" genotypes	Italian(A. Gemelli University Hospital of Ro
rs 10757278	CDKN2A/B	9p21.3	sporadic	1.23 (0.99-1.53, p=0.0064)	Caucasian (University of California, San Fr
rs 9298506	SOX-17	8q11.23	sporadic	1.35 (1.02-1.79, p=0.35)	168 Dutch and 338 American
				2.16 (1.20–3.88, p = .01) for AVM with IA vs. cont	168 Dutch and 338 American
rs 10958409	SOX-17	8q11.23	sporadic	1.08 (0.77 – 1.50, p= 0.660) AVMpatients vs contr	In Dutch cohort
rs 11082043	RBBP8	18q11.2	sporadic	1.31(1.06-1.61, p=0.01)	in American cohort
rs 11672433	ANGPTL 4	9p21	sporadic	1.73 (1.15–2.61, p = .046)	Caucasian (University of California, San Fr
				1.24 (0.91 to 1.70, p=0.18)	Caucasian patients, Netherlands
				0.82 (0.57–1.17, p = .27)	Russian population
rs3087465	TGFR-B2	chr 3	sporadic	(p < 0.05)	Han Chinese from South China
rs2275913	IL-17	chr 6	sporadic	(p > 0.05)	Han Chinese from South China
rs443198	NOTCH 4	chr6:32222629	sporadic	1.31(0.89-2.15, p=0.465)	white population
rs915895	NOTCH 4	chr6:32222440	sporadic	1.43(0.94-2.19, p=0.657)	white population
rs1109771	NOTCH 4	chr 6	sporadic	p>0.05(not specified)	white population
rs16930129	ALK1 (ACVRL1) + ENG	chr12	familial	2.68(1.64–4.39, p<0.001)	Caucasian race
				1.06(0.61–1.82)	Dutch patients
				1.20(0.72–2.01)	Dutch patients
rs10987759	ALK1 (ACVRL1) + ENG	chr12	familial	1.00(0.57–1.76)	Dutch patients

The following 3 SNPs were selected with high prevalence among all collected SNPs of brain AVM based on published scientific literature, meta-analysis and reviews:

- Rs522616 from MMP-3 gene located on chromosome 11q22
- Rs1333040 from CDKN2B-AS1 gene located on chromosome 9p21
- Rs1800587 from IL1A gene located on chromosome 7.

According to GWAS, chromosome 11q22 regulates sense of smell and chromosome 7 is

responsible for regulating cell growth and division, while chromosome 9p21 is one of the strongest genetic marker of cardiovascular diseases, coronary arterial disease(CAD) related events as stroke, aneurysms, peripheral artery disease and etc(44).

Rs1333040 located on chromosome 9p21 locus which contains noncoding RNA named ANRIL overlapping at its 5' end with CDKN2B-AS1(45). Among other SNPs of CDKN2B-AS1 gene, rs1333040 C>T polymorphism is strongly associated with intracranial and extracranial vascular diseases and become an independent predictor of intracranial aneurysms (46).

As a result of analysis of 3 SNPs, rs1333040 C>T polymorphism from CDKN2B-AS1 gene which is located on chromosome 9p21 was chosen to test the possible association with sporadic brain AVMs.

4.2 Demographic and clinical characteristics of study groups.

The demographic characteristics of brain AVM cases and healthy control groups shown in Table 5. The mean age of the studied case group is equal to 39±12.6, while in the control group it is equal to 40.7±6.93. Hence, there is no statistically significant difference in mean ages between the two populations ($p>0.05$). For sex ratio of participants, the results show no statistical difference in the gender distribution between the brain AVM cases group and the control group($p>0.05$).

Table 5. Demographic characteristics of case and control groups.

	Brain AVM cases	Control group	p-value
Total number of participants, n	100	100	
Mean age ± SD, years	39±12.6	40.7±6.93	0.239
Sex **Women** **Men**	 59 41	 50 50	0.256
Height, cm	166.8±8.74	N/A	
Weight, kg	68.7±14.2	N/A	
BMI, kg/m	24.76±4.85	N/A	

N/A= not applicable; P-value: for continuous variable (mean size±standard deviation) used two-tailed t-test, significant at $p<0.05$; for categorical variable(sex ratio) χ^2 test, significant at $p<0.05$

Among populations with brain AVM cases, 47% had ruptured AVMs and 39% had deep venous drainage. Also, it was determined that 80% of patients with brain AVMs did not have relatives with AVM, in the same way 61% patients with brain AVM did not have Intracranial

hemorrhage in relatives. Additional information about clinical characteristics of patients with brain AVM explained on Appendix 1.

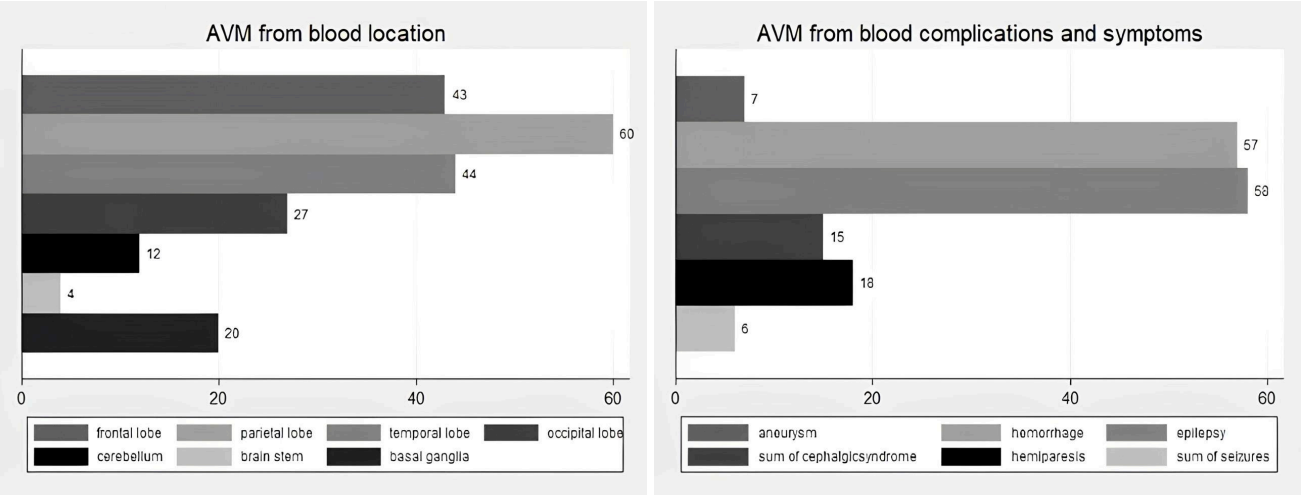

Figure 4. Clinical characteristics of case group. a) Location of AVMs in the brain. b) Long-term complications of brain AVMs.

According to angiographic characteristics of the case group, 60% of AVM occur in the parietal lobe of brain and least of brain AVM, which counts 4% of patients occur at basal ganglia of the brain (Figure 4a). In addition, patients with brain AVM had several long-term effects of disease such as aneurysm, hemorrhage, epilepsy, cephalgic syndrome, hemiparesis and seizures (Figure 4b).

4.3 Genotyping results

Genotypes and alleles frequencies are shown in Table 6. According to Table 6 genotype frequencies of case group and control group were in Hardy-Weinberg equilibrium (χ^2=1.51, p=0.47). Also, allelic frequencies of case and control groups were consistent with Hardy-Weinberg equilibrium(χ^2=3,47, p=0.062)

Table 6. Genotype and allele frequencies of rs1333040 for case and control groups.

Gene(SNP)	Location	Study groups	Genotypes			χ^2	HWE p*
			TT	TC	CC		
CDKN2B-AS1 rs1333040	chr9	Case	50	45	5	4.53	0.104
		Control	34	50	16	3.92	0.141
			Allele				

			T	C	χ^2	HWE p*
		Case	0.725	0.275	3.47	0.063
		Control	0.59	0.41		

HWE p - the level of statistical significance of the correspondence of genotype frequencies to the Hardy Weinberg distribution law (significant at p>0.05)

Distribution of genotype frequencies of rs1333040 (TC, TT, CC) was statistically significantly different between case and control groups (p=0.0107, Table 7). The TT genotype was significantly associated with brain AVM in the dominant model(p=0.0316), as well as in the recessive model(p=0.0211). In addition, T allele was also significantly associated with brain AVM in the additive (trend) model ($3.5*e^{-09}$).

Table 7. Genotype frequencies of rs1333040 between case and control groups

SNP	Test	Genotype	Case group	Control group	p-value
rs1333040	Genotypes Dominant Recessive Trend	TT/TC/CC TT+TC/CC TT/TC+CC T/C	50/45/5 95/5 50/50 145/55	34/50/16 84/16 34/66 118/82	0.0107 0.0316 0.0211 $3.5*e^{-09}$

P-value calculated by Student t-test, significant p<0.05.

Based on the results of genotype frequency of rs1333040, it was that TT genotype was significantly associated with brain AVM. Multivariable logistic regression analysis used the compute odd ratio in order to evaluate and quantify the strength between rs1333040 and brain AVM.

Table 8. Association of rs1333040 of CDKN2B-AS1 gene with the risk of developing a brain AVM

Gene	SNP	Additive (trend) model	MAF		OR(95% CI)	p-value
			Case (n=100)	Control (n=100)		
CDKN2B-AS1	rs1333040	T/C	0.275	0.41	1.94 (1.1-3.43)	$1.11*e^{-05}$

According to Table 8, minor allele frequency(MAF) is equal for case group MAF=0.275, for control group MAF=0.41. It indicates that for case group population in 100 individual minor allele(C allele) distributed 27.5 % and for control group among 100 individuals C allele distributed 41%. If

comparing the obtained MAF results with a base of dbGAP from ALFA allelic frequencies similar with Asian population from HapMap study (C allele=0.268) and with Siberian population (C allele=0.27). Furthermore, multivariate logistic regression analysis results is equal to 1.94 (1.1-3.43), p-value = $1.11*e^{-05}$. It means that there is statistically significant association of rs1333040 in the presence of T allele and brain AVM among Kazakhstan population (Table 8).

V DISCUSSION

The controversy surrounding the pathogenesis of brain arteriovenous malformation continues, since the specific mechanism of formation of brain arteriovenous malformation is still unknown. There are several theories that claim that the disease is the result of sporadic or hereditary genetic disorders that affect both the brain and other organs in the cases of Osler-Weber-Rendu syndrome, Wyburn–Mason syndrome and Sturge–Weber syndrome(47, 48). Kim et al. (2011) argue that disease is a combination of genetic and environmental factors that makes brain AVM a multifactorial disease(35). Regarding to the recent advances of brain AVM study discovery of single nucleotide polymorphisms (SNPs) associated with arteriovenous brain malformations may aid in risk-based stratification, development of individualized treatment plans, and better understanding of the molecular mechanisms involved in the formation and progression of this disease.

In this study, it was investigated that SNP rs1333040 of the CDKN2B-AS 1 gene which is located on short arm chromosome 9 (band p213.3) is one of the strongest genetic markers of cardiovascular diseases, coronary arterial disease(CAD) related events such as stroke, aneurysms, peripheral artery disease and mainly brain AVM(44). CDKN2A/2B are anti-oncogenes and related to cyclin-dependent kinase inhibitor gene families. It contains 4 exons such as 1α, 1β, 2, 3 and they are coding P16INK4a (P16) and p14ARF (P14)(45). Polymorphism rs133304 C/T located on antisense RNA in INK4 locus(ANRIL), which is also known as CDKN2B-AS1 located in the intron of the CDKN2A/2B gene, where the cytosine(C) nucleotide is replaced by the thymine (T) nucleotide(55)(Figure 5).

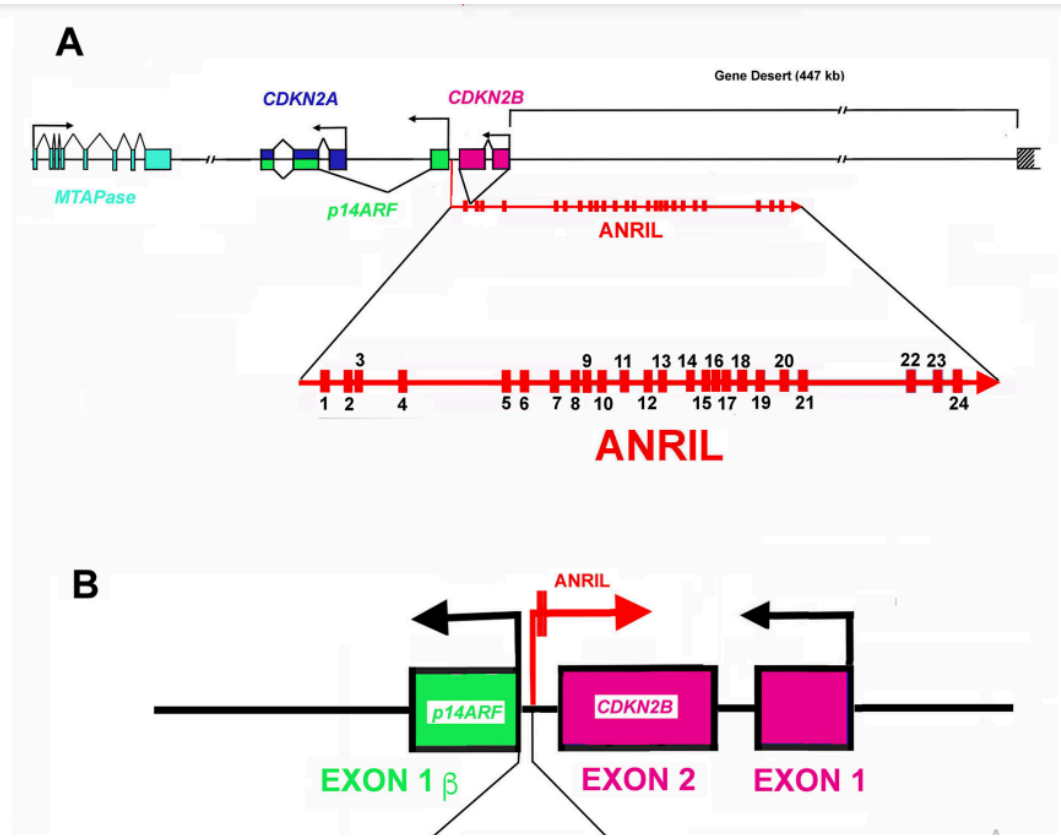

Figure 5. Schematic representation of CDKN2A/CDKN2B locus. a) Organization of CDKN2A/CDKN2B, p14(ARF) and MTAPase gene. b) Location of ANRIL, where rs1333040 is located. (Source: Bencivenga, D., Stampone, E., Vastante, A., Barahmeh, M., Della Ragione, F., & Borriello, A. (2022). An Unanticipated Modulation of Cyclin-Dependent Kinase Inhibitors: The Role of Long Non-Coding RNAs. *Cells*, *11*(8), 1346. https://doi.org/10.3390/cells11081346)

Various studies identify chr 9 as a strongest genetic marker of cardiovascular disease. Furthermore, CDKN2B-AS1 is also identified as a genetic marker for cerebrovascular diseases, brain malignant tumors(50,51). Pasmant et al.(2011) ANRIL is expressed by vascular endothelial cells, monocytes and macrophages, smooth muscle cells of coronary arteries and atheromatous vessels (atherosclerosis) and perform transcriptional regulation of gene expression CDKN2A and CDKN2B(52).

Among other SNPs of CDKN2B-AS1 gene, rs1333040 C>T polymorphism is strongly associated with intracranial and extracranial vascular diseases and become an independent predictor of intracranial aneurysms and brain AVM (46, 49). That's why the current study examined distribution of rs1333040 of chromosome 9p21 and revealed that TT genotype of rs1333040 was statistically significantly associated with brain AVM (p=0.0316). Strength of association between rs1333040 and brain AVM were tested via OR, which was equivalent to OR= 1.94 (95% CI:

1.1-3.43, p=1.11*e^{-05}). It means that there is a statistically significant positive association between the rs1333040 and brain AVM.

Results of current research study could be proven with other studies where authors suggest the association of rs1333040 and brain AVM. On the genome-wide study Wild et al.(2011) searched for association of 950 SNP variants with cardiovascular lesions and association of these polymorphic genes variants with the risk of developing vascular diseases, where results shows statistically significance associations with OR=1.22 (1.117-1.25, 95% of CI,), p-value=0.0169. Sturilae and coworkers were the first who studied the association of SNP in chr9p21 region and brain AVM and identified that pathogenic mechanisms of cardiovascular and cerebrovascular diseases such as intracranial aneurysms, stroke may be common with pathogenesis of brain AVM. Also, they were who identified first the association of brain AVM and rs1333040, it was confirmed that rs1333040 is candidate genetic marker of brain AVM with statistically significant association of TT genotype(in dominant model p=0.009 and recessive model p=0.006) and T allele(p=0.01)(54). One year later, in 2014 Sturilae and coauthors upgraded their research project and studied rs1333040 and rs7865618 SNPs association with brain AVM among 206 patients with brain AVM and 171 healthy controls. Both SNPs shows significant association in dominant, recessive and additive model genotypes (p=0.013, p=0.012, p=0.002) with brain AVM and concluded that chromosome 9p21 is one of the strongest marker for not only for stroke, intracranial aneurysms, coronary heart diseases, also it can be genetic marker of brain AVM(49).

VI LIMITATIONS

This study had limitations. Demographic statistics about brain AVM and other cerebrovascular diseases were unavailable to compare the obtained results. First of all, a small population for case and control groups. Because even if association of SNP rs1333040 and brain AVM is relieved, it cannot be concluded that this polymorphic gene is a marker for the entire population of Kazakhstan. It's necessary to confirm such a hypothesis with large prospective studies with other ethnic groups who live in Kazakhstan.

VI CONCLUSION AND PERSPECTIVES

To sum up, it was identified that SNP rs1333040 of gene CDKN2B-AS1is genetic marker of risk of developing brain arteriovenous malformations among population of Kazakhstan. Hypotheses were accepted as a result of conducted research study.

It was confirmed with statistically significant association of TT genotype in dominant, recessive and additive (trend) models and significant association of T allele with brain AVM. Also, it was verified with the result of logistic regression analysis, where OR illustrated significant strong positive association between exposure and outcome, where it is SNP rs1333040 of gene CDKN2B-AS1 and brain AVM.

Identification of SNPs associated with susceptibility to AVM in the brain allows us to understand the mechanism based on the pathogenesis of the disease and to stratify and predict patients based on risk and further select optimal treatment methods. Considering the fact that pathogenesis of brain AVM is still unclear, it was supposed that such research studies will contribute to the development of brain AVMs and study of its pathogenic mechanism.

Further perspectives of study include further study of development of brain AVM, its association with SNPs which are part of inflammation, angiogenesis and vasculogenesis. Also, it's interesting to study brain AVM in the case of neuroinflammation and in terms of neurology.

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IX APPENDIXES

Appendix 1. Clinical characteristics of case group.

Clinical characteristics	Total (n=100)
Smoking status	Smoking - 26 Not smoking - 74
Arterial hypertension	Yes - 16 No - 86
AVM in relatives	Yes - 3 No - 87 Do not know - 10
Intracranial hemorrhage	hemorrhage - 47 No hemorrhage - 53
Intracranial hemorrhage in relatives	Yes - 16 No - 61 Do not know - 23
Size of AVM	0-3 cm - 28 3-6 cm - 59 >6 cm - 13
Spetzler-Martin score	Grade I - 6 Grade II - 24 Grade III - 31 Grade IV - 30 Grade V - 9
Vein drainage	Deep vein drainage - 39 Surface vein drainage - 57 Both - 4

Appendix 2. Mean age of case and control group based on gender.