Aus der Universitätsklinik für Neurochirurgie Tübingen

Ärztlicher Direktor: Professor Dr. M. Tatagiba

Analysis of Single Nucleotide Polymorphisms in the first exon of TGFB1 in patients that have Moyamoya Disease

Inaugural-Dissertation

zur Erlangung des Doktorgrades

der Medizin

der Medizinischen Fakultät

der Eberhard Karls Universität

zu Tübingen

vorgelegt von

Chao Liu

aus

Changchun/China

2013

Dekan:	Professor Dr. med. I. B. Autenrieth
1.Berichterstatter:	Professor Dr. med. B. Krischek
2.Berichterstatter:	Frau Professor Dr. D. Berg

Meinen Eltern

List of contents

Abbreviation

MMD: Moyamoya disease

DSA: Digital Subtract Angiography

CT: computerized tomography

CTA: computer tomography angiography

MR: magnetic resonance

MRA: magnetic resonance angiography

STA-MCA: superficial temporal artery-middle cerebral artery

SMCs: smooth muscle cells

CSF: cerebrospinal fluid

VEGF: vascular endothelial growth factor

bFGF: basic fibroblast growth factor

HGF: hepatocyte growth factor

TGFB1: transforming growth factor B 1

PDGF: platelet-derived growth factor

HIF-1α: hypoxia-inducing factor-1 alpha

NF1: neurofibromatosis type 1

TAAD: thoracic aortic aneurysm and dissections

DNA: Deoxyribonucleic acid

Chr: chromosome

EST: Expressed Sequence Tag

HLA: human leukocyte antigens

RNF213: ring finger protein 213

OR: odds ratio

CI: confidence intervals

SNP: Single Nucleotide Polymorphisms

LAP: latency-associated protein

LTBP: Latent TGFB binding Protein

EDTA: Ethylene Diamine Tetraacetic Acid

TE: tris EDTA

PCR: Polymerase Chain Reaction

rpm: Revolutions per minute

bps: base pairs

dNTP: deoxy-ribonucleoside triphosphate

HWE: Hardy-Weinberg equilibrium

RT-PCR: reverse transcription polymerase chain reaction

mRNA: messenger Ribonucleic Acid

MLS: Maximum LOD score

A: Adenine

C: Cytosine

G: Guanine

T: Thymine

1 Introduction

1.1 Introduction of Moyamoya disease

History and background

Moyamoya disease (MMD) is a progressive disease affecting cerebral vasculature. One of the characteristics of this disease is the progressive narrowing or stenosis of the intracranial arteries, beginning from the terminal portions of the bilateral internal carotid arteries and progressing to the proximal portions of anterior cerebral arteries and middle cerebral arteries [12,21,24]. The formation of an abnormal extensive collateral circulation, including the neo-vessel networks also known as 'Moyamoya vessels', is another special feature of this disease [24]. The formation of the vascular network is thought to compensate for the cerebral ischemia due to the primary internal carotid artery stenosis. This disease was first reported in 1957 by Japanese researchers [1], and then named after the Japanese description as the puffing of smoke for its distinguishing features in angiography [2]. The occlusive changes of the cerebral vessels can cause transient ischemia attacks or cerebral infarction, and severe intracranial hemorrhage can result from the rupture of the fragile collateral vessels, both of the two clinical presentations can cause severe neurologic deficits [27-29,59-61]. While the prevalence of Moyamoya disease is most in Japanese population, it has been reported that this disease occurs in various populations around the world [4-8]. Children under 10 years of age account for nearly half of all Moyamoya cases [4]. Most Moyamoya cases are reported to be sporadic, but about 10% cases have family history [5]. Some pathological findings in vivo or in vitro, including some important cytokines for cellular regulation or morphological changes of the endovascular thickening, have been proposed in the progress of the vascular occlusion and in the development of the new compensating vessels, but till now no animal model of

this disease is established [3,13-16,26,27,36]. These historical achievements in pathological findings provide essential information about the pathogenesis of Moyamoya disease. Previous infection in the head was reported to precede this disease, but the hypothesis of infection involvement in the pathogenesis is not supported by strong evidence and still remains controversial [21,38]. The fact that some cases of Moyamoya disease occur in association with diseases of known genetic origins, indicates the possibility of a genetic role in this disease [9,61-63]. Genetic study for Moyamoya disease is traced back to 1990s, and some genetic abnormalities have been revealed as candidates for Moyamoya disease genes by linkage or association studies [63-69,102,106-109], however, it appears that till now no single gene or no single locus alone can explain the pathogenesis of this disease. Therefore, it's believed that Moyamoya disease results from multiple reasons. Although various techniques and procedures have been applied to the diagnosis and treatment of the disease in the past half century, the diagnosis is still dependent on angiography and no causative treatment that prevents the disease or blocks the progression of this disease has been found [27-29,59-61,104]. Breakthroughs in genetic research are needed to elucidate the complex mechanism underlying Moyamoya disease, and some key genetic findings may achieve the goal of cardinal treatment.

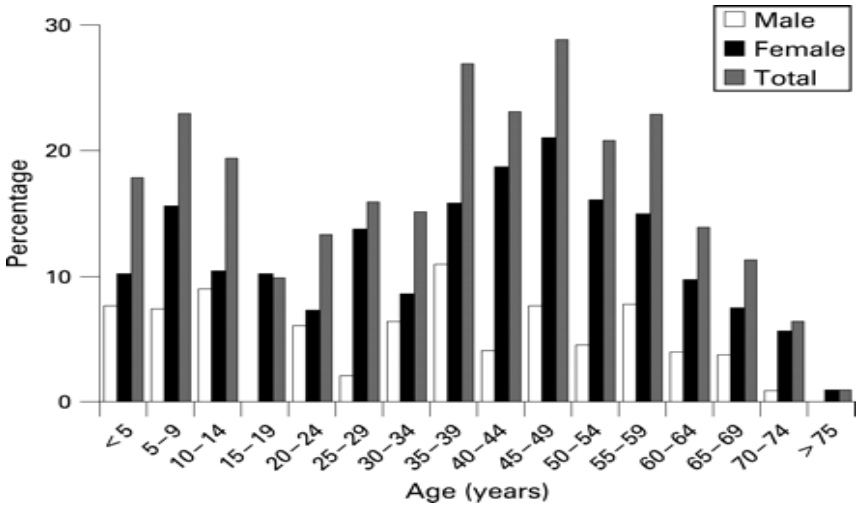
Epidemiology

Regional and racial differences of Moyamoya disease between Japanese and Caucasian populations are discribed in epidemiological reports [112]. According to the previous surveys of Moyamoya patients, an annual incidence of 0.35 per 100,000 persons was reported in Japan [111], as compared to the significantly lower incidence of 0.086 per 100,000 in US [11], and one-tenth of the Japanese incidence was reported in Europe [10]. The incidence of Moyamoya disease in Europe is reported to have increased in recent years,

which mainly reflects an increase in the awareness of the occurrence of stroke in children in the general population and the medical society [119,120]. Both in the Japanese and the Caucasian population, a tendency of occurrence in the younger generation was noteworthy, and female cases were reported to be nearly twice as many as male cases. Interestingly in a nationwide all-inclusive epidemiological study in 2008 on Japanese patients, different results from previous data were reported [4]. Other than the previous surveys on epidemiological features of Moyamoya disease conducted by using questionnaires, a new sampling method was adopted in this study, which included not only large hospitals and university hospitals, but numerous neurosurgical, neurological or pediatric specialized small hospitals or clinics in Japan. The disease incidence in this study was 0.94 per 100,000 persons; the ratio of patients under 10 years of age to the patients aged 10 years and older at onset was 1:6.18. Double peak of age at onset was observed according to this report, one was seen between 45 and 49 years of age, and the second was between the age of5 and 9 years. There were more female patients than male patients (**Figure1**). The increase of incidence in Japan was similar to that reported in another study [8]. The higher incidence (0.94 /100,000) than previously described (0.35 /100,000) might be due to the availability of diagnostic procedures and skills and the brain checkup procedure that is available in Japan [4,8]. Familial occurrence was seen in about 10% of all the cases [5,62].

Such regional and ethnic difference in susceptibility and familial occurrence strongly suggest a possible role of a genetic predisposition in the etiolgy of Moyamoya disease.

Fig.1: Age distribution of Moyamoya disease at onset in the Japanese population. (Baba T, et al., 2008. Reference # 4)



Symptoms

The Japanese Ministry of Health and Welfare defines four types of Moyamoya symptoms (ischemic, hemorrhagic, epileptic and other type), with the ischemia being the most common type and hemorrhage being the second most frequent type [4-5]. Evidence of age and sex distribution in different symptoms of Moyamoya disease (**Figure 2-3**) reveals that child patients are more likely to present ischemia than hemorrhagic events, which is more common in adults. Both in familial cases and in sporadic cases, some patients appear as asymptomatic and can be found only incidentally by angiography screening, suggesting that the affected persons may have different levels of disease severity and they respond differently to the pathological changes of Moyamoya disease [8]. Due to the continuous decrease of the intracranial blood perfusion, Moyamoya children present with retardation in mental state, seizures, TIAs, or headache before the disease develops into a severe stroke, but recognition of these atypical symptoms is usually delayed due to the children's poor complaining ability [39-42]. Cerebral infarction caused by the progressive occlusion of intracranial arteries can disable the patients' speech or mobility and may result in a high mortality. Symptoms due to hemorrhage are more

commonly seen in adult patients than in adolescents, but the onset at this time is always on a catastrophic stage of neurological deficits due to intracranial hemorrhage, subarochnoid hemorrhage or intraventricular hemorrhage [43].

Fig.2: Types of symptoms with ages, showing that the ischemia type is most frequent in children and adults alike, but the hemorrhage is more frequent in adult patients than in children. (Baba T, et al., 2008. Reference # 4)

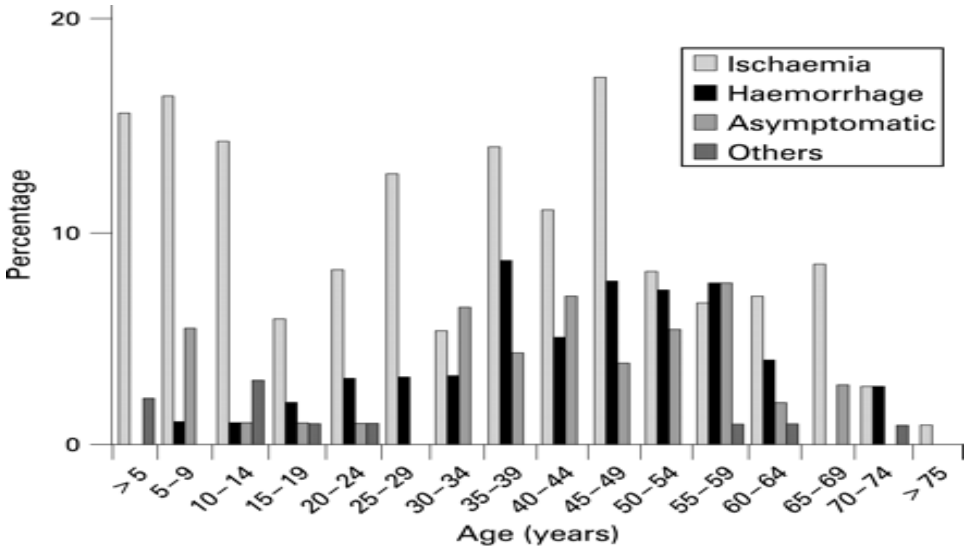
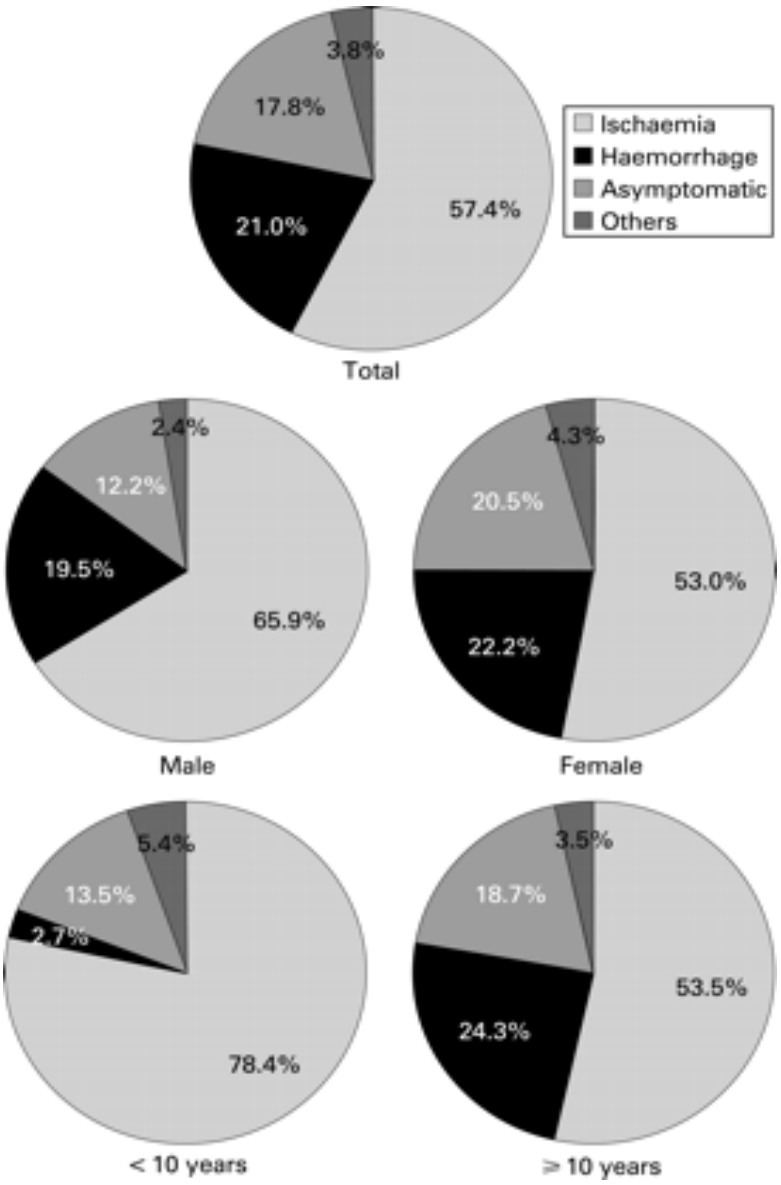


Fig.3: Clinical findings with age distribution at onset. (Baba T, et al., 2008. Reference # 4)



Diagnosis

Diagnosis of Moyamoya disease is based on the clinical presentation of various stroke and radiographic findings. Digital Subtract Angiography (DSA) is regarded as the golden-standard tool to diagnose Moyamoya disease [5, 42,103], this method can demonstrate the severity and localization of the occlusive intracranial vessels, and the ‘smoke puffing’ collaterals are also detected (**Figure 4**). Normal computerized tomography of brain can show the

typical low density of cerebral infarction or the high density of hematoma, and magnetic resonance is a more sensitive tool than CT for the detection of small lacunar infarction of the basal ganglia and thalamus [44-46]. Because of the non-invasive advantage, magnetic resonance angiography (**Figure 5**) or computer tomography angiography is more acceptable to detect asymptomatic patients with a family history of Moyamoya disease, and with the development of technology, more sophisticated apparatus can give better and more sensitive images. Both of the two diagnostic methods (CTA and MRA) are recommended to be performed on suspected Moyamoya patients when DSA is not available [47-49,104]. In recent years, much attention has been paid to the study on the cerebral hemodynamics of Moyamoya disease, and the image methods are progressing with the development of sophisticated technology, including positron emission tomography (PET) [51-52] and SPECT [50]. Xenon-enhanced CT, dynamic perfusion CT, Doppler ultrasonography, and MR imaging with dynamic susceptibility contrast and with arterial spin labeling are used at some medical centers but they are not used widely yet [133].

Fig.4: The characteristic collateral vessels of the left internal carotid artery of a Moyamoya patient demonstrated in the anteroposterior and lateral digital subtraction angiograms. (Weinberg DG, et al., 2011. Reference # 103)

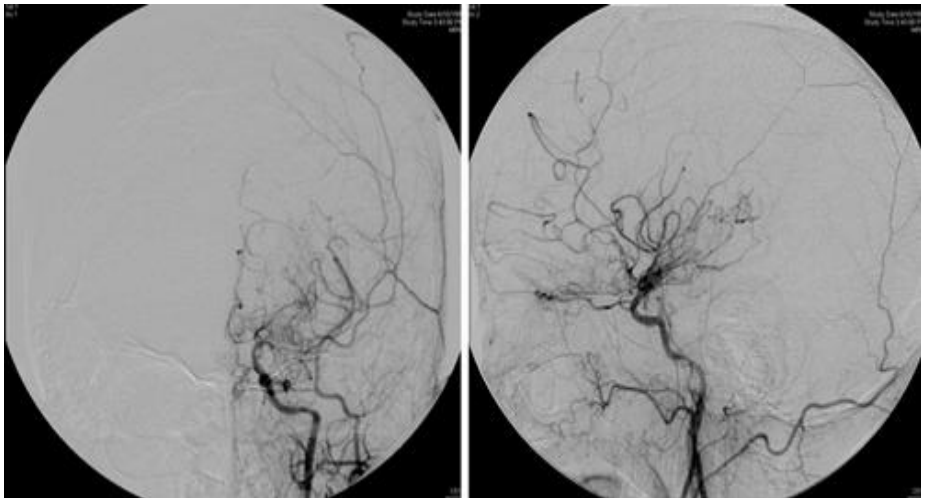
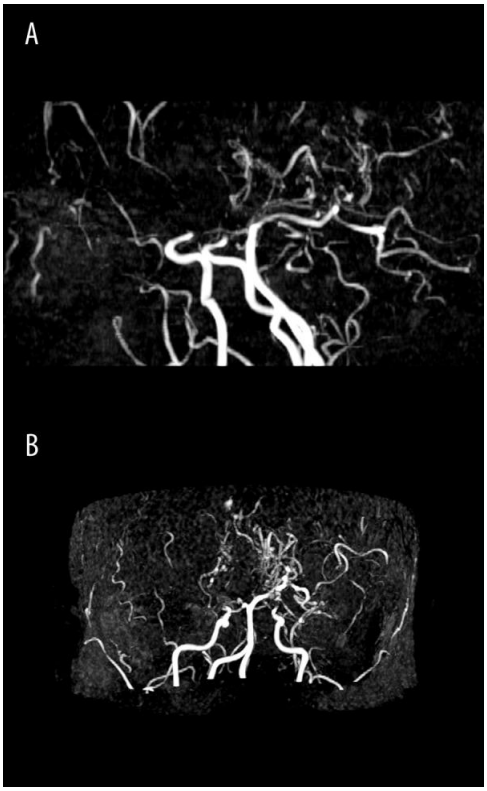


Fig.5: Magnetic resonance angiography of a Moyamoya patient. (Tarasów E, et al., 2011. Reference #104)

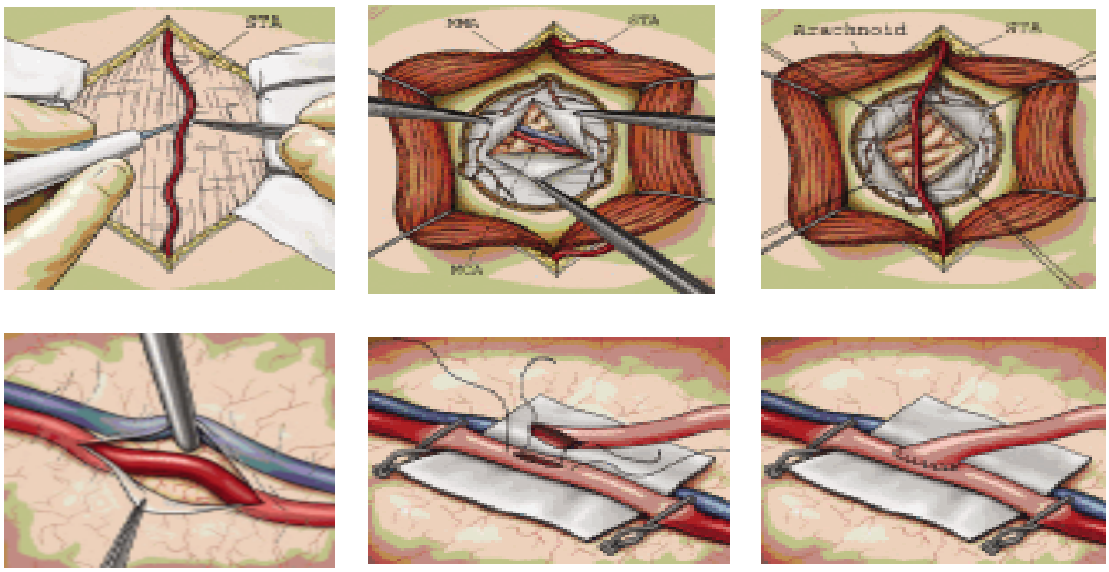


Treatments

Surgical intervention should be considered once the diagnosis of Moyamoya disease is confirmed, because much evidence suggests a potential benefit with surgery in the treatment for reversing the inadequate cerebral blood perfusion [54]. The first operative technique to handle cerebral ischemia was performed by Yasagil in 1967 using direct extracranial-to-intracranial revascularization, later in 1973, Kikuchi and Karasawa pioneered the anastomosis surgery for Moyamoya patients with the superficial temporal artery-middle cerebral artery (STA-MCA) procedure [53-54,105]. Interestingly, a positive outcome with a decreased rate of re-bleeding of the revascularization method was reported for the hemorrhagic Moyamoya disease, but this result has not been obtained in other medical centers so this suggestion is controversial [55-56]. Direct bypass surgery to anastomose a

cortical artery with a branch of the external carotid artery is regarded as the standard procedure for revascularization (**Figure 6**), but this approach can be challenging. Morbidity and mortality of the surgery are not easily assessed because a variety of procedures are performed at different centers and the operative outcome is always closely dependent on the surgeons' experience [57-58,119]. Antiplatelet agents, anticoagulants or calcium-channel blockers are routinely used in Moyamoya patients to relieve the ischemic symptoms or in the hope of reducing the severity of ischemia, although there is no positive and significant evidence to advocate medication to improve the outcome, and the adverse effects of the medication to surgery should be taken into consideration [59-60].

Fig.6: Procedures for STA-MCA bypass. (Zipfel GJ, et al., 2005. Reference #105)



Pathologic findings

Pathologic studies in vascular lesions of Moyamoya patients reveal that the vascular stenosis usually involves the distal portions of internal carotid arteries

and also occurs in the proximal portions of anterior and middle cerebral arteries [12]. The carotid fork is the most affected site, and the affected vessels are usually bilateral. The severity of the vascular lesions in Moyamoya disease is similar to that found in patients who have arteriosclerotic disease, although the characteristics of the stenotic changes are quite different. The typical arteriosclerotic or inflammatory changes leading to occlusion, such as the lipid pool or inflammatory cell or macrophage invasion to the intimal layer, are not commonly exhibited in the affected vessels of Moyamoya patients [12-14,21,22]. The pathological changes of the occlusive lesion in Moyamoya disease are characterized by the fibrocellular thickening of the intima with abundant extracellular matrixes which cause the diameter of the vascular lumen to decrease, and the abnormal waving form without rupture in the intimal elastic lamina [14]. The vessel occlusion in Moyamoya disease is thought to be caused by the hyperplasia of smooth-muscle cells, and the media is always attenuated with irregular elastic lamina [3,25,27]. The typical vascular changes of Willis of Moyamoya patients displayed the lumen obstruction, uncommon deformation of the internal elastic lamina, fibrocellular thickening of the intima, and the attenuation of media (**Figure 7**), and the vessel is narrowed or obstructed by eccentric proliferation of the endothelium, which is composed of several laminated elastic lamina intermingled with abundant fibrosis [13,14,25]. In a study to find the differences of elastin gene transcripts and elastin synthesis between Moyamoya patients and control subjects, the arterial smooth muscle cells (SMCs) obtained from patients and controls were cultured, and the result showed that the levels of elastin mRNA and protein were higher in all smooth muscle cell samples from Moyamoya patients than in the control samples, indicating a possible role of elastin accumulation in the thickening of vascular intima in Moyamoya disease [37].These classic findings in pathological studies indicate that the mechanism of stenotic changes seen in Moyamoya disease may be triggered by a non-arteriosclerotic reason, but no clear hints are obtained to elucidate the true

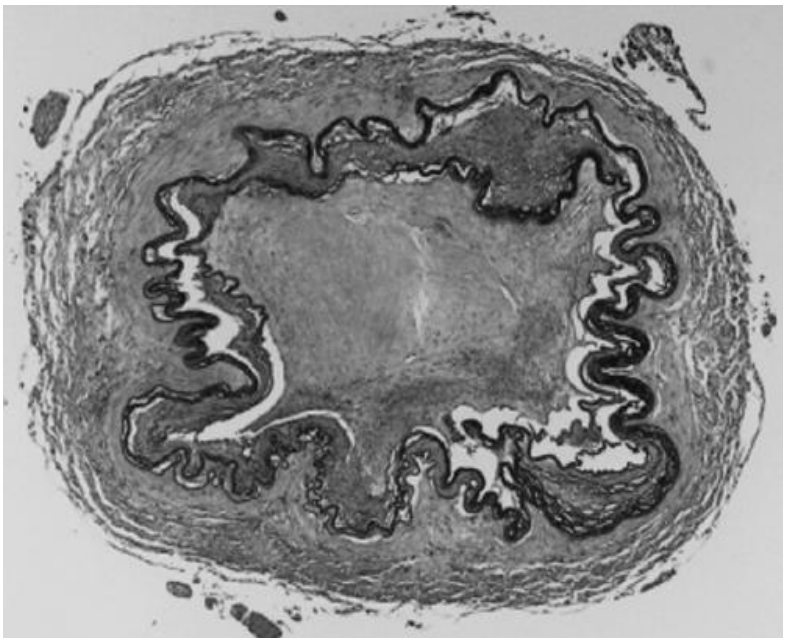
pathogenesis of Moyamoya disease [15-16]. A series of studies with the superficial temporal arteries (STA) of Moyamoya patients were conducted to explore the mechanisms of the neo-intimal formation [17-19,118]. Similar pathological changes in the intracranial arteries, such as the fibrocellular thickening of the intima with abundant elastin deposits, were observed in the superficial temporal arteries of Moyamoya patients, while in the intima of control samples the elastic fibers were weakly stained [19]. The result suggests the contribution of the vascular cells to the development of intimal thickening in Moyamoya disease. In Moyamoya patients, significant intimal thickening leading to vascular stenosis also occurred in some extracranial arteries such as renal artery and pulmonary artery, indicating that this disease should be recognized as a part of systemic vascular disorders [21-24]. The contributory role of prothrombotic disorders in the pathological changes of Moyamoya disease was hypothesized, and according to a series of studies, lupus anticoagulant, anticardiolipin antibodies and inherited protein S deficiency were detected in about 40% of the Moyamoya syndrome children, and prothrombotic disorders were also detected in these patients [36]. Thus, the possible contribution of hemostatic abnormalities is also considered in the pathogenesis of Moyamoya disease. Involvement of inflammatory responses was suggested in the vascular stenosis of Moyamoya disease, and because adhesion molecules are known to mediate inflammation process during cerebral ischemia, a study was conducted by measuring the levels of soluble isoforms of the endothelial adhesion molecules in serum and cerebrospinal fluid (CSF) samples from Moyamoya syndrome children [35]. Although there was no significant difference of the soluble vascular cell adhesion molecule Type 1, intercellular adhesion molecule Type 1 and E-selectin in the serum , significantly elevated CSF levels of the three soluble adhesion molecules were all detected. A significantly higher level of the albumin index for Moyamoya patients than that for the controls was also found. These findings suggested the possibility that the blood-brain barrier is impaired slightly by the ongoing

central nervous system inflammation in the process of vascular changes in Moyamoya syndrome.

Another special feature of Moyamoya disease is the formation of collateral vessel network, compensatory for the ischemia caused by the occlusion of intracranial vessels. The neo-vessel network also known as ‘Moyamoya vessels’, are generally dilated perforating arteries, and their formation is believed to result from the preexisting vessels combined with the newly developed vessels [25-27]. As compared to other ischemic neurovascular diseases, only Moyamoya disease develops compensatory collaterals in the formation of network, indicating a special etiology of angiogenesis for this disease. The pathological changes of these collaterals, such as fragmented elastic lamina, attenuated media and the presence of micro-aneurysms, indicate the vascular wall bears the stress caused by the increase of the blood flow [12,28]. These pathological findings help explain why some patients present with hemorrhage, which is the major cause of death in Moyamoya patients [12]. Two of the main possible causes of hemorrhage are the rupture of micro-aneurysms and the rupture of the fragile ‘Moyamoya vessels’ [28-29].

The typical pathological changes seen in Moyamoya patients, the fibrocellular intimal thickening and the formation of collateral vascular network, indicate a special etiology, which is still unknown. The limitations of current pathological approaches in the study of Moyamoya disease are the difficulty of obtaining the specimens of the terminal portion of the internal carotid artery from Moyamoya patients and the lack of best matched animal models.

Fig.7: Microscopic findings in the left middle cerebral artery of a Moyamoya patient. Elastica H&E, original magnification x 40. (Takekawa Y, et al., 2004. Reference # 3)



Proteins

In the past two decades, there have been many successful studies on the detection of cytokine abnormalities in the specimens obtained from Moyamoya patients, and these results provide important information to elucidate the etiology of Moyamoya disease. Levels of various enzymes, growth factors, and other proteins have been revealed to be increased in association with Moyamoya disease. Most studies are based on the measurement of individual cytokines in cerebrospinal fluid (CSF), dura, cultured smooth muscle cells and vessels.

#Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein of 36-46 kDa that is actively involved in the pathological process in various

intracranial lesions [121]. The central role of VEGF in human pathology is to induce and enhance not only angiogenesis, vascular permeability, but also endothelial cell proliferation, migration and adhesion of leukocytes [30]. Upregulated expression of VEGF has been reported to play an important role in development of angiogenesis and tumor progression in animal experiments, and in acute ischemic stroke the angiogenic properties of VEGF is suggested to be protective for neural and glial cells [103,121]. In a study of the involvement of VEGF in patients with and without Moyamoya disease, a significant increase of meningeal cellularity and VEGF expression in the dura mater of Moyamoya patients was found as compared to the control groups, and also a hypothesis was given that the hypoxic environment induced VEGF to stimulate vessel formation in a sprouting mode [30]. The measurement of this cytokine in patients' cerebrospinal fluid was not significantly elevated, indicating that VEGF might be only a response and not the cause of Moyamoya disease [103]. Although its over-expression in Moyamoya patients is significantly demonstrated, the clear mechanism of VEGF in the pathogenesis of Moyamoya disease remains undefined.

#Basic fibroblast growth factor (bFGF)

Basic fibroblast growth factor (bFGF) has attracted much attention in the study for the pathological changes seen in Moyamoya disease because it normally stimulates the proliferation of neuroectodermal cells and induces the growth of vascular smooth cells [16,31]. In a study of angiogenic factors in Moyamoya patients, a high level of bFGF in the cerebrospinal fluid taken from Moyamoya patients was found to be disease specific since it was significantly higher than in other types of cerebral ischemic diseases, so a high level of bFGF is suggested to be one of the major factors for the formation of collateral circulation and to be involved in the pathogenesis of intracranial arterial stenosis [31]. A possible role of being a potiential biomarker for Moyamoya

disease was also suggested in this study. Furthermore, it has been reported that bFGF was partly responsible for the abnormal thickening of tunica media of intracranial vessels of Moyamoya patients [31,103]. In immunohistochemical examinations of specimens in the circle of Willis from Moyamya patients and cadavers with atherosclerotic stenosis of the intracranial carotid arteries, staining of bFGF was found only in the endothelium of Moyamoya patient specimens and was not present in control samples, indicating the suppressive effect of bFGF in the process of SMCs apoptosis in the intima [122].

#Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) is a stroma-derived mediator that stimulates motility, proliferation of epithelial cells, angiogenesis and morphogenesis in various organs not only in liver [123]. Thus, its participation in the pathological changes in Moyamoya disease is under close investigation. In a study to investigate the role of HGF in the development of Moyamoya disease, both the levels of HGF in cerebrospinal fluid and its distribution in the carotid bifurcation were analyzed between patients with Moyamoya disease and control patients with other diseases, including cervical spondylosis and internal carotid artery occlusion [32]. The level of HGF in the cerebrospinal fluid assessed with the method of enzyme-linked immunosorbent assay technique was significantly higher in Moyamoya patients than in control patients, and the result of immunohistochemistry showed wide distribution of the HGF-positive cells in the media and sparse distribution of this cytokine in the intima of the intracranial arteries obtained from Moyamoya patients, however, in the specimens of control patients only a much smaller number of the HGF-positive cells could be found [32]. This study revealed the markedly elevated expression of HGF in the carotid bifurcation and the increased level of HGF in cerebrospinal fluid of Moyamoya patients, suggesting that HGF may play an

important role in the development of Moyamoya disease.

#Transforming growth factor B 1 (TGFB1)

Transforming growth factor B 1 (TGFB1) is an important polypeptide for cell growth and angiogenesis, and it has received additional attention in an attempt to elucidate the various processes of human diseases, including Moyamoya disease. In a later section, this factor will be discussed in detail.

#Platelet-derived growth factor (PDGF)

Platelet-derived growth factor (PDGF) is a growth factor that promotes and regulates cell migration, proliferation, and differentiation. PDGF is revealed to be one of the important cytokines in the development of various intracranial disorders. Therefore, a possible role of PDGF in the pathological process of intimal fibrocellular proliferation in Moyamoya disease is given. The pathological findings of the superficial temporal arteries (STA) obtained from Moyamoya patients resembled the changes in the intracranial arteries, and the development of intimal thickening STA was found to be started significantly earlier in Moyamoya patients than the control subjects [19]. In a related study, the response of the cultured smooth muscle cells (SMCs) of the superficial temporal arteries to platelet derived growth factor (PDGF) was found to be significantly less in Moyamoya patients than in control patients [118]. In the examination of the production of inflammatory cells in smooth muscle cells derived from arteries of Moyamoya patients and control subjects, the cell migration and DNA synthesis of SMCs were markedly stimulated by PDGF in control group but DNA synthesis was not changed with PDGF stimulus in Moyamoya patients [124]. A reduced number of PDGF receptors in smooth muscle cells in Moyamoya patients were thought to explain the different responses to PDGF, however, an involvement of this cytokine in the pathogenesis of intimal thickening was indicated to lead to the different

responses found in Moyamoya disease.

#Hypoxia inducible factor- 1 alfa (HIF-1α) and Endoglin

In a study that analyzes the role of different factors in Moyamoya disease, the middle cerebral artery (MCA) was obtained during surgical procedures from Moyamoya patients and control patients, and the specimens were analyzed by immune-histochemical methods [34]. The result of the immune-reactivity indicated that expressions of hypoxia-inducing factor-1 alpha and endoglin were higher in the endothelium and intima of Moyamoya specimens than control specimens, and mainly in the endothelium of Moyamoya specimens, transforming growth factor B 3 was detected to be co-localized with HIF-1α and endoglin [34]. Thus, the association of HIF-1α and endoglin with Moyamoya disease was indicated in this study. In almost all nucleus mammalian cells, hypoxia-inducible factors (HIFs) mediate the primary transcriptional response to hypoxic stress, and HIF-1α is one of the most important subunits that functions as a master regulator for the fundamental process adapted to cellular oxygen alteration [125]. Endoglin is an accessory receptor for transforming growth factor beta (TGFB) and its expression is up-regulated in actively proliferating endothelial cells [93]. The clear role of the two factors in the pathogenesis of Moyamoya disease is still unknown, but the increased expression was thought to be secondary due to response to hypoxia.

In conclusion, various cytokines responsible for the cell proliferation and angiogenesis have been considered in the etiology of Moyamoya disease, but no clear mechanism has been defined to elucidate the disease process. In the serum and STA specimens of Moyamoya patients, high expression of bFGF, TGFB1, HGF and HIF-1α are found to be specific, and in the cerebrospinal fluids of Moyamoya patients, only bFGF and HGF display significantly higher

levels. It is well known that in other hypoxic and ischemic circumstances than Moyamoya disease, levels of these proteins in the specimens of patients are also observed to be elevated accordingly. These proteomic findings might simply reflect the response of the proteins to hypoxic and ischemic processes in Moyamoya disease, but cannot provide positive evidence to support their direct causative role in the pathological process of Moyamoya disease. A genetic association between the related proteins and Moyamoya disease may be of great interest. It may suggest that underlying genetic defects lead to coding regulatory proteins, which in turn have the potential to initiate the typical changes in the intracranial vessels of susceptible individuals.

Associated conditions

Moyamoya disease is recognized as a disease in an isolated inheritance (familial Moyamoya disease) or in an associated pattern with other genetic syndromes. Some disorders of certain genetic origins are reported to occur with associated pathological changes which are seen in Moyamoya disease, and these conditions are known as Moyamoya syndromes [24,61-63,103,115], such as neurofibromatosis type 1, sickle cell disease, Down syndrome, familial thoracic aortic aneurysm and dissections (TAAD) and Marfan syndrome. They are distinct from Moyamoya disease but the manifestation of angiopathy found in these syndromes is similar to the pathological changes in Moyamoya disease. Studies with the associated diseases of a known genetic cause may not be directly elucidative for Moyamoya disease, but the genetic mutations or pathological findings of these diseases may give us important clues that lead to further exploration of the pathogenesis of Moyamoya disease.

#Neurofibromatosis type 1 (NF1)

Neurofibromatosis type 1 is one of the most common genetic diseases in

human, which is caused by the mutations of NF1 gene located on chromosome 17q11.2, and the inheritance mode is known t be autosomal dominant [113]. The NF1 patients develop small pigmented skin lesions and small soft fleshy growths called neurofibromata [113]. Occasionally the characteristic pathological changes of Moyamoya disease are also seen in NF1 patients (with or without intracranial tumor involvement), so the hypothesis that a linkage of Moyamoya disease to a particular chromosomal region near the NF1 gene was aroused [65]. Although it was not revealed that the NF1 gene participated directly in the occurrence of Moyamoya disease, a susceptive locus on Chromosome 17q25 was found.

#Sickle cell disease

Sickle cell disease is the most common hemoglobinopathy, which is caused by a point mutation resulting in the substitution of glutamine with valine at codon 6 of the ß globin polypeptide. The erythrocytes are deformed due to this mutation and adopt a sickle shape. The removal of the abnormal erythrocytes from circulation by the spleen causes serious anemia and occlusion of small vessels by the sickle cells can cause ischemia [114,115]. In some cases of sickle cell disease, patients can develop Moymoya-like changes around the circle of Willis, presenting with the occlusion of the distal internal carotid arteries and proximal middle arteries [115]. The genetic variants in sickle cell disease may be also contributors to the pathogenesis of Moyamoya disease.

#Down syndrome

Down syndrome, also known as trisomy 21, is caused by the presence of all or part of a third copy of chromosome 21, and it is the most common chromosomal abnormality in humans. There are several cases of association between Down syndrome and Moyamoya disease, and there is evidence of a

higher incidence of Moyamoya disease in Down syndrome children than in other children [15,116,117]. Cerebral angiography of some Down syndrome patients who developed ischemic stroke demonstrated the similar features to the radiographic findings in Moyamoya disease, such as the occlusion of bilateral internal carotid arteries and the formation of compensatory collateral circulations. Thus, it is hypothesized that the intracranial vascular dysplasia important for the development of Moyamoya disease might be associated with the chromosome abnormalities in Down syndrome [116].

#Thoracic aortic aneurysm and dissection (TAAD)

Heterozygous ACTA2 mutation is a major cause for familial thoracic aortic aneurysm and dissections (TAAD), and linkage and association studies revealed that ACTA2 mutation carriers are predisposed a diversity of vascular disorders, including Moyamoya disease [108]. Thus, this mutation is suggested to be partly responsible for the occlusive vascular lesions seen in Moyamoya disease.

#Marfan syndrome

Marfan syndrome is a genetic disorder of fibrous-connective tissues, which is characterized by extracellular-matrix proteins defects, the responsible genes of this syndrome are mapped to chromosome 3p, and this region is also suspected for the pathogenesis of Moyamoya disease [64]. The neurovascular complications are not rare in some Marfan syndrome cases, so the gene products of this chromosome region are considered to be important to maintain the homeostasis of vascular wall.

By definition Moyamoya disease is an idiopathic disorder and the affected intracranial vascular lesions are normally bilateral, although the severity is

different between sides [1,2]. As described above, characteristic angiopathy which is commonly seen in Moyamoya disease is similar to that in some other diseases and these conditions are known as Moyamoya syndrome. Once there are unilateral angiopathic changes, the diagnosis of Moyamoya syndrome is also considered whether or not there are associated conditions, and about 40% of these patients eventually develop contralateral disease [21,42, 132]. Bypass surgery is also applied to relieve the ischemic symptoms caused by the reduction of cerebral perfusion in Moyamoya syndrome patients [120]. Although different pathogenesis may underlie Moyamoya syndrome and Moyamoya disease, studies of these syndromes have given some meaningful information on the etiology of Moyamoya disease [131].

1.2 Genetic research of Moyamoya disease

The genetic contribution to Moyamoya disease is strongly suggested by the evidence of geographic distribution feature and the fact of high incidence in concordant monozygotic twins and the family cases [61-62]. The high incidence in the migrants of familial Moyamoya patients from Japan to North America also indicates that the genetic role is more important for the disease than environmental factors [5,10,63]. The genetic involvement in this disease is also suggested by the association of Moyamoya disease with syndromes of a known genetic cause. Three methods most widely used in genetic studies are performed to identify specific loci or the causative genes for the disease, the linkage analysis based on family cases such as parametric linkage analysis or sibling pair analysis, the case-control strategy for association studies in sporadic cases research, and the combination of both methods. In the past two decades, several suspected genes have been found and many important findings enlighten the possible genetic contribution to the disease. These achievements have given evidence suggesting the genetic involvement in Moyamoya disease. The discoveries of these suspected genes or loci yield the possibilities of early diagnosis and new therapeutic modality to arrest or reverse the progression of Moyamoya disease.

3p24.2-p26

In 1999, Ikeda et al conducted a study of familial Moyamoya disease with 16 Japanese families, and a linkage of this disease to the genetic markers located at 3p24.2-26 was found in this study. This is the first genetic locus reported to be related with familial Moyamoya disease [64]. In this study, 371 polymorphic microsatellite markers that spanned the 22 autosomes in 77 persons of the 16 families (both affected and unaffected individuals were analyzed) were genotyped, and because inheritance of either autosomal dominant or

autosomal recessive mode was assumed, calculation of two-point LOD scores was made. Nonparametric analysis was also performed for multipoint calculations. By the nonparametric analysis, a linkage of Moyamoya disease to the microsatellite polymorphism D3S3050 was strongly suggested with an NPL score of 3.46 and this marker is mapped to chromosome 3p24.2-p26. An associated condition with Moyamoya disease, Marfan syndrome, is also caused by the mutations of genes that are close to the linked region on chromosome 3p.

17q25

Since the pathological changes of Moyamoya disease are occasionally observed in some cases of neurofibromatosis type 1 disease and the NF1 gene is mapped to chromosome 17q11.2, Yamauchi and colleagues conducted a microsatellite linkage analysis with 56 individuals of Moyamoya disease from 24 families in 2000 [65]. With the methods of two-point and multipoint linkage analyses, a gene for familial Moyamoya disease was localized on chromosome 17q25. Two-point maximum LOD score of 3.1 for the microsatellite polymorphism marker D17S939, and maximum LOD score of 4.58 for the markers within the 9-cM region between D17S785 and D17S836, implied the possibility of the regions near chromosome 17q25 to accommodate genes for familial Moyamoya disease.

In a similar approach in 2005, Nanba and coworkers identified several candidate genes for Moyamoya disease within a 9cM region on chromosome 17q25 [66]. Their sequence analysis and bioinformatics analysis with 9 individuals from one family including 4 patients provided the possibilities of 9 candidate genes in the 9cM region within 17q25 and 26753 EST with significant similarity to the sequence of 17q25.

Although Nanba did not give positive results in their finding, Mineharu and coworkers continued the sequence analysis in 2008, on the purpose of finding the possible involvement of chromosome 17 in Moyamoya disease [67]. In a genome-wide analysis with 103 individuals from 15 affected families including 55 patients, they genotyped 382 markers on 22 autosomes and 18 markers on X-chromosome, and the maximum LOD score of 8.07 and 6.57 for the marker D17S704 offered positive results that the possible genes encoding Moyamoya disease might be located at chromosome 17q25.3. At this region there is a candidate gene, BAIAP2, which encodes proteins to induce angiogenesis, so it renders the possibility of this locus to be responsible for the pathogenesis of Moyamoya disease.

These genetic findings reinforced the important involvement of chromosome 17q25 in the pathogenesis of Moyamoya disease. It should be noted that further studies are required to elucidate the biochemical function and pathological role of the candidate genes in this region.

Chr.6

Inoue and coworkers reported previously the association between Moyamoya disease and several genes for human leukocyte antigens (HLA), which are located at chromosome 6. To further investigate the genetic involvement of chromosome 6 in Moyamoya disease, they performed a linkage study of 20 affected sibling pairs in 2000 [68]. They studied 15 microsatellite markers of chromosome 6. An allele with possible linkage was identified. The marker, D6S441, was shared among 16 of 19 families (82%). The results indicated the linkage of chromosome 6 to Moyamoya disease. This finding reinforced the possible role of immune response in the occurrence of Moyamoya disease.

HLA alleles

To assess the association of Moyamoya disease with HLA alleles in Korea, Han and coworkers investigated genes of HLA class Ⅰ and class Ⅱ in 28 patients with Moyamoya disease and 198 control subjects [109]. HLA-B35 allele frequency in Moyamoya patients was significantly increased compared to the controls with the result of 32.1% vs 10.1% (OR 4.2, $p<0.008$). Stratificated study of this allele on sex and age at onset also detected significantly higher frequency in late-onset and female groups, and no associations of HLA alleles previously reported by Japanese were found in this study, suggesting that the association of HLA alleles may differ between ethnic groups and this genetic marker particularly offers hints for suspected female Moyamoya patients of late onset in Korea. Association of HLA-B35 with autoimmune and infectious disease indicated direct influences of this allele on the pathological changes of Moyamoya disease following head radiation or bacterial infection.

ACTA2

In an association and linkage study for heterozygous ACTA2 mutations of 127 members in 20 families with clear history of thoracic aortic aneurysms and dissection (TAAD) in 2009, Guo and coworkers reported the assumption that ACTA2 carriers are predisposed to a variety of occlusive vascular disorders, including Moyamoya disease [108]. Five of the 127 patients of ACTA2 mutations in this study suffered early-onset ischemic stroke secondary to Moyamoya disease, and seven members with premature stroke shared the common p.R258C/H mutations. These results aroused the hypothesis that the smooth muscle cell proliferation of internal carotid artery in Moyamoya disease was dependent on the ACTA2 mutations. This cohort of Moyamoya disease was of Northern European descent.

8q22.3 and 12p12

In a genome-wide study with 12 Moyamoya families with 428 microsatellite markers in 2004, Sakurai and coworkers reported the significant evidence of the linkage of chromosome 8q23 to Moyamoya disease and the suggestive evidence for the linkage of 12p12 [69]. A linkage of the region near D8S546 on 8q23.1 was strongly suggested by an MLS of 3.6 and a possible linkage of marker D12S1690 on 12p12 was considered by an MLS of 2.3. One of the candidate Moyamoya genes, TIEG, is located in 8q22.3 and some genes important for the vascular development during embryogenesis or angiogenesis, such as ANGPT1, EBAG9 and DD5, are also located in the 8q region, so a possible involvement of this region is indicated.

HLA-DRB1 and HLA–DQB1

To clarify the association between Moyamoya disease and HLA alleles, Hong and coworkers performed a case-control study with 10 children of familial Moyamoya disease, 54 children with non-familial Moyamoya disease and 207 normal controls in 2009 [85]. The frequencies of the allele HLA-DRB1*1302 showed a significant increase in familial Moyamoya patients as compared to both the non-familial Moyamoya patients and the normal controls, with the results of OR of 12.76 for familial MMD vs non MMD and OR of 13.42 for familial vs non-familial MMD. The results of OR of 14.67 for familial Moyamoya disease vs non Moyamoya disease and OR of 35.33 for familial vs non-familial Moyamoya disease with HLA-DQB1*0609 also showed significant differences. The two alleles were noteworthy for the pathogenesis in familial Moyamoya diseases. And no association of HLA-DRB1 and HLA–DQB1 variants with sporadic cases was found in this study.

TGFB1 Exon1 and PDGFRB

The first genetic study for European Moyamoya patients was reported by Roder and our colleagues in 2010 [107]. Genotyping was conducted with thirteen single nucleotide polymorphisms in or upstream to four genes (bFDF,

CRABP1, PDGFRB and TGFB1) to analyze their involvement in the pathological changes of Moyamoya disease, and 40 Moyamoya patients and 68 control subjects were enrolled to this study. Two SNPs were found associated with the disease, rs382861 in the promoting region of PDGFRB with the result of OR of 1.81 and p=0.0373, and rs1800471 in the first exon of TGFB1 with the result of OR of 7.65 and p=0.0345. PDGFRB and TGFB1 are considered to play a role in the vascular changes and angiogenesis in Moyamoya disease. This study indicated the possible genetic risk factors for the pathogenesis of Moyamoya disease in Europe.

RNF213

A series of recent studies give positive results that RNF213 is a causative gene for Moyamoya disease, and it improves our understanding of the genetic background and promises the possible early detection of the disease. Kamada and coworkers performed a genome-wide association of 785720 SNPs with 72 Japanese Moyamoya patients and 45 Japanese controls in 2011, and the results provided strong evidence that chromosome 17q25-ter region was associated with Moyamoya disease and RNF213 was identified as the first Moyamoya gene [106]. Because a definite result was not obtained for any of the previously reported candidate loci in 20 familial cases in Japan, they hypothesized a founder mutation might exist. The consequent locus-specific association study was performed with 384 SNPs markers within chromosome 17q25-ter region and a high association of p.R4859K was seen within the RNF213 region, and the linkage disequilibrium block analysis indicated a possible existence of founder mutation in the region of RNF213. Around the same time, Liu and coworders employed a combination of several methods with patients from Japan, China, Korea, Germany and Czechia, with assumption that Moyamoya disease being inherited by the pattern of autosomal dominant mode with incomplete penetrance [102]. p.R4810k in the

region of RNF213 was demonstrated to be strongly associated with Moyamoya disease by an odds ratio of 111.8. In their study, the involvement of RNF213 was strongly suggested in Moyamoya disease.

Xq28 deletion

In a study in France with three unrelated Moyamoya syndrome families whose inheritance pattern was X-linkage, Miskinyte and coworkers reported the association of BRCC3 loss with the typical abnormal angiogenesis of Moyamoya syndrome [131]. Xq28 deletion leading to BRCC3 loss and angiogenesis defects was detected in their study, and the rescue of BRCC3 morphant zebrafish caused by endothelium-specific expression of BRCC3 strongly suggested a novel important role of the deubiquitinating enzyme in angiogenesis.

Maternal predominance or mother-to-daughter transmission is usually seen in familial Moyamoya cases and female cases are always more than male cases, so the sex determination is considered to be associated with Moyamoya disease, but the evidence of X-linkage to Moyamoya disease or susceptible mitochondrial genomic mutations was not found [4-5,87,103]. A possible pattern of autosomal dominant inheritance of incomplete penetrance was indicated in a study with 15 highly aggregated Japanese families [87]. Most of the genetic studies of Moyamoya disease are conducted with the cohort of Asian cases, particularly Japanese patients of familial Moyamoya disease. But only about 10% Moyamoya patients have a family history, and in Caucasian population, the incidence of this disease is reported to be increasing recently, so different mechanism and different loci should be considered to cause this disease [4-5,7,10]. Important genetic findings of Moyamoya disease are summarized in **Table 1**.

Table 1 : Summary of important genetic findings

Author (year)	Method	Ethnicity	Coverage	markers	Findings
Ikeda 1999	Non-parametric linkage analysis	Japanese	autosomes	371 microsatellites	3p24.2-p26
Yamauchi 2000	Parametric non-parametric linkage analysis	Japanese	Chr.17	22 microsatellites	17q25
Nanba 2005	Sequence analysis bioinformatics analysis	Japanese	17q25	26753 EST	no variation
Mineharu 2008	Genome-wide linkage mutation analysis	Japanese	autosomes Chr.X	382 SNPs 18 SNPs	17q25.3
Inoue 2000	Non-parametric linkage analysis	Japanese	Chr.6 HLA alleles	15 microsatellites	6q25
Han 2003	HLA genotyping	Korean	HLA alleles 6p21.3	HLA-B35	HLA-B35

Guo 2009	Genome-wide parametric linkage analysis	US European	genome	ACTA2 SNPs	ACTA2
Sakurai 2004	Non-parametric linkage analysis	Japanese	genome	428 microsatellites	8q23 12p12
Hong 2009	HLA Genotyping	Korean	HLA-DRB1 HLA-DQB1	SNPs	HLA-DRB1 HLA-DQB1
Roder 2010	Genotyping	European	bFGF, TGFB1	13 SNPs	rs382861 rs1800471
Kamada 2011	Genome-wide locus-specific association	Japanese	genome, 17q25	785720 SNPs, 335 SNPs	p.R4859K, RNF213
Liu 2011	Genome-wide linkage association study, functional analysis	East Asian	autosomes Chr.X	382 SNPs 18 SNPs	p.R4810K, RNF213
Miskinyte 2011	Parametric linkage analysis	French	Chr.X	19 microsatellites 6 microsatellites	Xq28 BRCC3

1.3 TGFB1 and its correlation with Moyamoya disease

Transforming growth factor B 1 (TGFB1) is a central regulator that plays diverse roles in human cell proliferation and differentiation, adhesion, angiogenesis, apoptosis, immunity and extracellular matrix formation [71,72,78,33]. In humans the gene encoding TGFB1 is located at the long arm of chromosome 19 and this cytokine consists of molecules of 25,000 Daltons [70]. It is found in different cell types in humans, but its highest concentration is in platelets, suggesting its function in wound healing [70-71]. TGFB1 belongs to a family of secreted polypeptide growth factors, including its three mammalian isoforms (TGFB1, TGFB2, and TGFB3), activins, inhibins and some other ligands, among which TGFB1 is the most widely studied [73]. Accumulating evidence has revealed the important role of this cytokine both in normal or abnormal biological processes, including maturation, development and diseases [72, 75,110]. In normal human environments, TGFB1 plays a pivotal role in controlling the sequencing of cell growth and inhibits the epithelial proliferation; furthermore, this cytokine also enhances the maturation of fibroblasts into fibrocytes leading to increased production of fibrous tissues, promotes angiogenesis and inhibits the breakdown of extracellular matrix [78,79,89,95,110]. In addition to being widely produced throughout the body, TGFB1 can also be measured in blood. Increased plasma TGFB1 levels have been found in patients of many diseases, including various types of cancers. In the initial stages of primary tumor, TGFB1 is an antiproliferative factor that has tumor-suppressive effects [72]. But with the oncogenic development and within the tumor microenvironment, most malignant cells are resistant to the growth inhibitory effects of TGFB1 and lose the control of their growth and proliferation, and in these circumstances this cytokine also promotes the local growth of tumor and enhances metastasis [81,101,110]. A variety of different mechanisms may underlie the complexity of processes in which TGFB1

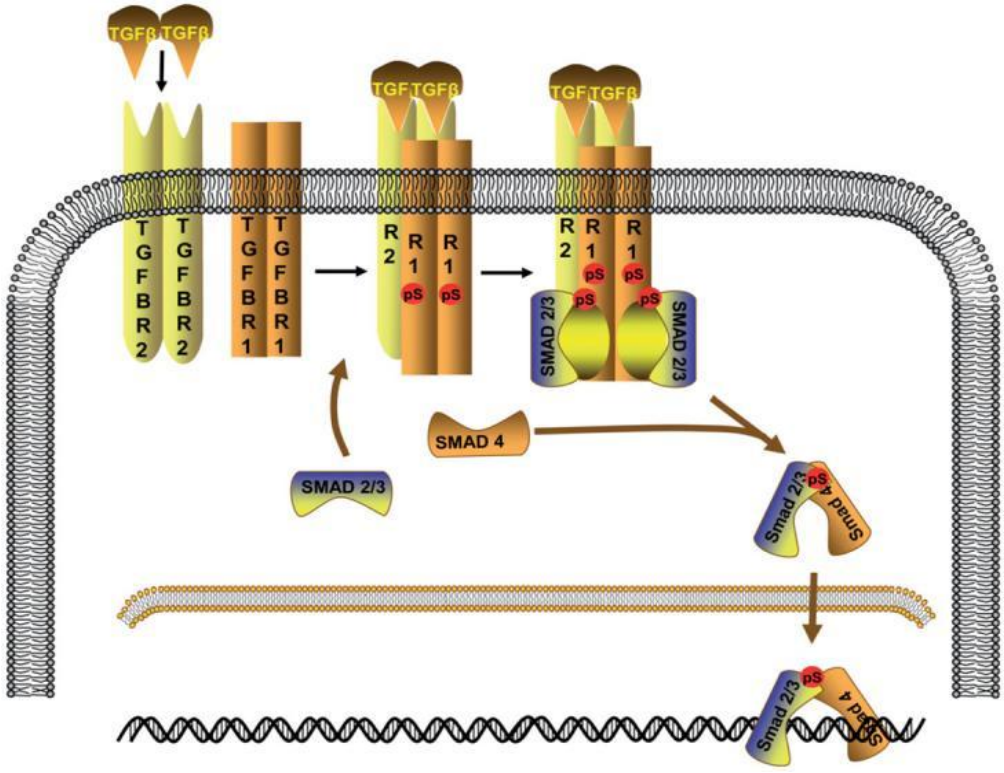
participates. In vitro experiments with induction of exogenous TGFB1, the cell cycle was stopped to enter S phase from G1 phase and epithelial proliferation was induced to arrest [78,86]. Over production of TGFB1 was found in various disorders resulting from abundant cell proliferation or cell differentiation, including cancer and pulmonary fibrosis, but mixed hypotheses are aroused that the tumor would be the source of TGFB1 or the production of TGFB1 might be response to the tissue injury [89-91].

Genetic studies revealed a role of TGFB1 and its receptors in the establishment and maintenance of vessel wall integrity, suggesting the importance of this cytokine for vascular architecture and regulation of angiogenesis [93]. The regulative role of TGFB1 for vascular formation is another promoting activity for tumor growth [110]. VEGF and TGFB1 both induce the formation of new vessels but they have opposite effects on endothelial cells. In in-vitro experiments, it was found that the induction of angiogenesis by TGFB1 required an apoptosis-like effect which was transient and rapid, and the mediation of VEGF and VEGFR2 was important in this process [80,83]. Knockout mice missing a component of TGFB1 gene were viable, however, the vascular wall integrity and haematopoiesis appeared severely defective, indicating that TGFB1 is required for angiogenesis and loss of TGFB1 expression by genetic engineering is a lethal mutation [77]. Regulation of epithelial proliferation by TGFB1 was suggested to be accountable for the vascular wall development via interaction with other cytokines [76,79,92-95]. An angiogenesis array in hypoxic circumstances suggested that epithelial apoptosis induced by TGFB1 may be relevant to VEGF-dependent vascular formation, and the results of some other studies indicated the importance of the interaction of bFGF and VEGF with TGFB1 for the vascular formation [80-82,92-94]. These controversies still need elucidating but the role of TGFB1 for regulation and angiogenesis is believed to contribute to the pathogenesis of many human diseases, including cancers

and intracranial lesions.

TGFB1 is secreted as precursor molecules in an inert form with bandage to a latency-associated protein (LAP), which is secreted from cells and can be activated by several methods, and then this small complex may also form a large latent complex in covalent bondage with another protein called Latent TGFB binding Protein (LTBP), and this latent complex is sequestered in a innate form containing both LTBP and LAP in the extracellular matrix until it is activated by dissociation from latency associated peptides [72-74]. Many proteins interact with the transmembrane receptors for TGFB1 and signaling proteins (Smads) within the cytoplasm, affecting the signaling crosstalk and protein transcription [72,88,100,101]. The dominant pathway is to involve the binding of TGFB1 to its receptor TGFBR Ⅱ, which provides a potential target to interfere with the binding of TGFB1 to its receptors, thus preventing the initiation of the potential downstream interactions (**Figure 8**) [72,88,96,100]. In vitro experiments with animal models, the result showed that TGFB was inhibitory for the vascular sprouting through ligation with type Ⅰ receptors, indicating that interaction with different receptors leads to the different functions of this cytokine [33]. There is increasing evidence that TGFB1 signaling via Smads-independent pathways may stimulate overproduction of fibrous tissue, or stimulates several critical responses that are important for the production of extracellular matrix [77,95,129,130]. Disruption of the TGFB signaling pathway which results in failure to respond to growth inhibitory factors is possibly involved in various diseases. These findings have improved our understanding of the molecular mechanisms of TGFB1 in different disease processes.

Fig.8: TGFB signaling pathway: after bondage of TGFB to TGFBR Ⅱ and TGFBR Ⅰ, TGFBR Ⅰ kinase is activated to phosphorylate SMAD2 and SMAD3, and SMAD4 is associated with them. It has active effect after moving to nucleus. (Xu Y, et al., 2007. Reference # 110).



For its promoting role in fibrosis and angiogenesis, Transforming growth factor B 1 is proposed to be involved in both of the two pathological changes seen in Moyamoya disease, the intimal fibrosis of the intracranial vascular wall and the abundant collateral vascular formation. The first study of association between TGFB1 and Moyamoya disease was conducted with cultured smooth muscle cells (SMCs) obtained from arterial specimens of Moyamoya patients to assess the elastin gene transcripts and elastin synthesis by Yamamoto and coworkers in 1997 [37], and a significantly greater response of elastin synthesis and elastin gene transcripts to exogenous TGB1 was seen in Moyamoya specimens than in control specimens, suggesting an abnormal

regulative role of TGFB1 in the extracellular matrix leading to intimal thickening. In a study with cultured smooth muscle cells obtained from the superficial temporal arteries (STAs) of Moyamoya patients [20], a significantly higher expression level of TGFB1 was found in specimens of Moyamoya patients than in specimens of patients with arteriosclerotic cerebrovascular disease and in this study a significantly higher level of TGFB1 was also seen in spasm of Moyamoya patients than control subjects. The result suggested the possible association between TGFB1 and the abundant neovascularization, but TGFB1 could not be recognized as a direct causative factor by these findings. Besides, in the cerebrospinal fluids of Moyamoya patients, the content of TGFB1 was not significantly higher and a possible indirect way of its involvement in Moyamoya disease was considered [31]. In a study with case-control method using picked SNPs, Roder and colleagues of our research team found a possible association between a variant of TGFB1 exon 1 and Moyamoya disease [107]. But until now, no genetic studies focusing on TGFB1 signaling pathways had been conducted to elucidate the pathogenesis of Moymoya disease.

2 Study Objective

In a previous study of our research group with 13 picked single nucleotide polymorphisms (SNPs) of four genes, two SNPs located in the first exon of TGFB1, rs1800471 and rs1800470, showed a significant association and a tendency toward significance with Moyamoya disease respectively, suggesting that TGFB1 mutation was a possible genetic risk factor for the pathogenesis of Moyamoya disease. The aim of this doctoral study was focused on exon 1 of TGFB1 and its possible associations with Moyamoya patients. Analysis of the single nucleotide polymorphisms (SNPs) of the interested sequence was conducted in a cohort of 41 Moyamoya patients to test the hypothesis that exon 1 of TGFB1 might play an important role in the pathogenesis of Moyamoya disease. This is a case-control study.

3 Materials and methods

3.1 Patients and controls

Between 2008 and 2009, forty-one Moyamoya patients mainly from central Europe, including one female of Chinese origin who was adopted after her birth and grew up in Germany, were recruited at the University of Tübingen , as well as sixty-eight healthy German individuals that were the control team (**Table 2**). This study was approved by the Ethics committee of the University of Tübingen. Written consents were obtained from all patients and controls, and the parent or guardian would consent instead if the participant was unable to give consent because of too young age or disability. The diagnosis of Moyamoya disease was confirmed according to the guidelines established by the Research Committee on Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare of Japan. Peripheral blood samples were obtained from all the participants and stored in EDTA tubes at -80℃ for further study. Each tube was labeled with number and letter for clear identification of the participant, with K representing the control participant and M representing the case participant.

Patient group

The mean age at onset was 27.2, ranging from 4 to 66. The sex ratio of females vs males was 28:13. The characteristics of age and sex distribution were consistent with the previous published epidemiological study. The symptoms at onset were various and the patients were not from families of obvious history of Moyamoya disease. Patients of other known inherited diseases were excluded from this study.

Control group

The mean age of controls was 27.6, ranging from 15 to 65, which was matching the age of patients' group. The sex ratio of females vs males was 43:25. All the participants were healthy by their medical record and had no record of familial stroke or other neurovascular diseases by questionnaire.

Table 2: The information of patients and controls.

Ethnicity	Patients	Controls
Germany	26	68
Switzerland	7	
Italy	2	
Holland	2	
Czech	1	
Sweden	1	
Austria	1	
China	1	
Mean age	27.2	27.6
Sex ratio (F:M)	28:13	43:25

3.2 Solutions, equipments and web resources

Solutions

10%SDS-buffer	
Proteinase K	
TE buffer	
SE buffer	
Erylysis buffer	Leipzig
Primers	metabion international AG
BigDye Terminator V3.1 cycle sequencing	Applied Biosystems
5xPCR-Puffer GoTaq	Promega

Equipment

Refrigerator	Bosch
Vortexer	Bender & Hobein AG
Abi Prism 3100 Genetic Analyzer	Applied Biosystems
NanodropSpectrophotometerND1000	Peqlab Biotechnologie GmbH
Centrifuge 5810R	Eppendorf
Electrophoresis chamber	Peqlab Biotechnologie GmbH
Tube-Strips 0,2 ml and cover	Peqlab Biotechnologie GmbH
Thermocycler DNA-Engine	DYAD Bio-Rad
Gene Scan-120 LIZ Size Standard	Applied Biosystems

Web resources

International HapMap Project: *http://hapmap.ncbi.nlm.nih.gov/*

NCBI BLAST: *http://blast.ncbi.nlm.nih.gov/Blast.cgi/*

NCBI Entrez Gene: *http://www.ncbi.nlm.nih.gov/gene/*

NCBI Single Nucleotide Polymorphism Database:

http://www.ncbi.nlm.nih.gov/snp/

UCSC Genome Browser: *http://genome.ucsc.edu/*

Primer-Design-Software Primer3: *http://primer3.sourceforge.net/*

3.3 DNA extraction

We extracted genomic DNA from peripheral venous blood leukocytes with the standard method of phenol-chloroform procedures. The measurement of the concentration of DNA products was performed on Nanodrop system.

DNA extraction procedures

Step 1

The frozen sample stored in EDTA tubes frozen at -80℃ was thawed and put into a 50ml Falcon tube. To each 10 ml sample, 40 ml of 1X Erylysis buffer was added to lyse the red blood cells, and the tube was inverted. The mixtures at Falcon tubes were centrifuged for 20 minutes at 3400 rpm at 4℃. The supernatant was removed and discard into disinfectant. The tube was refilled with 30 ml of 1X Erylysis buffer and vortexed. The mixture was centrifuged as mentioned above, and all of the supernatant was removed.

Step 2

5 ml of SE buffer was added to each pellet and the tube was vortexed briefly. Then 500 ul of 10% SDS and 5 ul of Proteinase K (20 mg/ml H2O) were added to the tube, and the tube was vortexed briefly. The tube was incubated at 37℃ overnight.

Step 3

2.5ml of phenol-chloroform alcohol was added to the tube and the tube was vortexed for 30 seconds. The mixture was centrifuged for 10 minutes at 4℃ at 2700 rpm in a microcentrifuge tube. The aqueous layer was carefully removed to a new 1.5 ml microcentrifuge tube. 15 ml of cold 100% ethanol was filled to it and mixed.

Step 4

The supernatant was discarded. Then the pellet was washed twice with 10 ml of 70% ethanol. The pellet was stored in DNA-tube without cover and dry at room temperature overnight.

Step 5

The pellet was dissolved by adding 400 ul of 1x TE buffer (10mM Tris HCl, 0.1 mM EDTA, pH 8.0) and incubated overnight at room temperature. The pellet was vortexed periodically to dissolve the genomic DNA.

Step 6

DNA concentration was measured by Nanodrop system .The purified samples were stored at -20℃.

3.4 PCR preparation

Sequence of TGFB1

The TGFB1 exon 1 comprises of 1237 bps (**Table 3**), and the information of reference sequence was obtained from GenBank (*http://genome.ucsc.edu/*).

Table 3: The sequence of exon1 of TGFB1, length=1237 bps

ccccgccgcc gccgcccttc gcgccctggg ccatctccct cccacctccc
tccgcggagc agccagacag cgagggcccc ggccgggggc agggggacg
ccccgtccgg ggcacccccc cggctctgag ccgcccgcgg ggccggcctc
ggcccggagc ggaggaagga gtcgccgagg agcagcctga ggccccagag
tctgagacga gccgccgccg cccccgccac tgcggggagg agggggagga
ggagcgggag gagggacgag ctggtcggga gaagaggaaa aaaacttttg
agacttttcc gttgccgctg ggagccggag gcgcggggac ctcttggcgc
gacgctgccc cgcgaggagg caggacttgg ggaccccaga ccgcctccct
ttgccgccgg ggacgcttgc tccctccctg ccccctacac ggcgtccctc
aggcgccccc attccggacc agccctcggg agtcgccgac ccggcctccc
gcaaagactt ttccccagac ctcgggcgca ccccctgcac gccgccttca
tccccggcct gtctcctgag cccccgcgca tcctagaccc tttctcctcc
aggagacgga tctctctccg acctgccaca gatcccctat tcaagaccac
ccaccttctg gtaccagatc gcgcccatct aggttatttc cgtgggatac
tgagacaccc ccggtccaag cctcccctcc accactgcgc ccttctccct
gaggacctca gctttccctc gaggccctcc taccttttgc cgggagaccc
ccagcccctg caggggcggg gcctccccac cacaccagcc ctgttcgcgc
tctcggcagt gccgggggcc gccgcctccc cc***atgccgcc ctccgggctg***
cggctgctgc cgctgctgct accgctgctg tggctactgg tgctgacgcc
tggccggccg gccgcgggac tatccacctg caagactatc gacatggagc
tggtgaagcg gaagcgcatc gaggccatcc gcggccagat cctgtccaag
ctgcggctcg ccagcccccc gagccagggg gaggtgccgc ccggcccgct
gcccgaggcc gtgctcgccc tgtacaacag cacccgcgac cgggtggccg
gggagagtgc agaaccggag cccgagcctg aggccgacta ctacgccaag
gaggtcaccc gcgtgctaat ggtggaaacc cacaacg

The coding sequence is emphasized in red.

Sequence of primers for exon 1

Primers (**Table 4**) used for the amplification of exon 1 of TGFB1 in this study was designed with the assistance of Primer-Design-Software Primer3 (*http://primer3.sourceforge.net/*). The primers were designed to screen the coding regions of the first exon, including the flanking regions, and the amplified sequence products contained 495 base pairs. The primers were purchased from metabion international AG and were diluted to 10ug/nl. Genomic DNA samples were also diluted to 10ng/ul.

Table.4: sequence of the primers.	
primer 5'→3'	PCR product length (bps)
F:ACCACACCAGCCCTGTTC R: CTGCCAGTCACTTCCTACCC	495
F: forward. R: reverse.	

3.5 Amplification of exon1 of TGFB1

For further study of DNA sequencing, it is demanded to have a large amount of copies of the interested region. In this study, the first exon of TGFB1 was amplified by polymerase chain reaction (PCR) from the genomic DNA.

PCR contents

PCRs were performed in a 50ul reaction mixture and the contents were composed of the following reagents (**Table 5**).

Table 5: PCR reaction mixture of exon1.

Reagents	Volume (uL)
dNTPs(10mM for each)	10
Templates (genomic DNA products)	2
Primer F (10uM)	0.5
Primer R (10uM)	0.5
Tag polymerase 2U/uL）	0.25
ddH_2O	31.75
10xPCR Buffer	5
Total	50

PCR buffer was purchased from a commercial company, Promega.

The Tag polymerase was purchased from the Invitrogen Cooperation.

PCR cycle profile

Amplification was carried out in a DNA Thermal Cycler using the following conditions (**Table 6**).

Table 6: PCR profile			
PCR	Time	Temperature (℃)	Cycle
Denaturation	5minutes	94	1x
Initiating denaturation	30 seconds	94	35x
Annealing	30seconds	65	
Elongation	30seconds	72	
Final elongation	10 minutes	72	1x

At the final elongation step, dNTP was added to terminate the reaction chain.

3.6 Preparing the sequencing samples

Polymerase chain reaction-sequencing reaction

The sequencing PCR was performed in 10ul reaction mixture (**Table 7**). In the reaction system content, the forward primer of exon 1 was used for DNA elongation. BigDye terminator enzymes and sequencing buffers were purchased from commercial company, Applied Biosystems.

Table 7: Sequencing mixture.	
Reagents	Volume (uL)
Templates (PCR products,10uM)	5
Primer F (10uM)	1
5x BigDye Terminator	1.5
ddH_2O	1.5
BigDye SEQ Bufferr	1
Total	10

Cycle sequencing was performed in the following profile (**Table 8**).

Table 8 : Sequencing profile			
Cycle sequencing	Time	Temperature (℃)	Cycle
Denaturation	2 minutes	94	1x
Initiating denaturation	1 minute	94	30x
Annealing	10 seconds	54	
Elongation	4 minutes	60	

Gel electrophoresis

Macromolecules can be separated on gel electrophoresis according to their size and charges. DNA runs in a gel as a function of the logarithm of its molecular weight. In our study, the PCR products were checked on gel electrophoresis, and a DNA ladder of 500 bps was set to identify each band.

Step.1 Preparing a Gel

Four grams of ararose in 100ml buffer were prepared to make 4% gel. All the powdered agarose was melted when the microwave was switched on. After boiling, the contents were cooled to approx 50℃ and poured into the gel apparatus. A comb was inserted into the apparatus and the gel was left to be solidified. When the gel hardened, the comb was carefully removed and the solidified gel was placed into the running apparatus.

Step.2 Running a Gel

DNA samples were loaded into the wells of the gel. DNA was negatively charged and run towards the positive electrode. The electrical cords were connected to the power supply and the power was set to 100V. The gel ran for about 2 hours and then the power was turned off. The electrodes were disconnected off the apparatus.

Step.3 Visualization

The gel was transferred to a staining tray and covered with stain solution. The tray was placed on a shaker for 20 minutes, and then the gel was removed from the tray and was rinsed with water to remove the excess stain. The gel was visualized on the UV light box.

3.7 DNA sequencing

Purifying the samples

Before sequencing, the samples were purified by the following methods. The entire sequencing reaction samples were pipeted into 1.5 ml microcentrifuge tubes and added with 2.5 volume of EtOH/NaAc (100% Ethanol +3M sodium acetate, pH4.8 in ratio of 24:1). The mixture was vortexed briefly and left at room temperature for 15 minutes. The tubes were centrifuged at 4000 rpm for 45 minutes in a microcentrifuge. The supernatants were discarded completely with a separate pipet tip for each sample, and the tube was added with 100ul of 70% Ethanol. The tube was centrifuged at 4000rpm for 15 minutes. The supernatants were discarded. Another 100ul of 70%Ethanol was added and the tubes were centrifuged at 4000rpm for 15 minutes. The supernatants were discarded and the tubes were centrifuged at 1727rpm for 1 minute with the bottom upside down to dry the samples. 15 ul of sterile water was added to each tube and stored at 4℃ until ready for electrophoresis.

DNA sequencing

During preparation for sequencing, the DNA fragments must be chemically labeled with fluorescent ideoxynuclotides (ddNTPs) at the 3’ end of the strand to facilitate the detection and identification. To detect mutations of the amplified gene fragments, direct genotyping was performed by using the automated sequencer, the ABI 3100 genetic analyzer (Applied Biosystems, USA) according to the manufacture’s protocol. The samples were separated on 96-well plates. The plates were placed in the instruments and the sequencing process began automatically. From the samples molecules were injected into the capillaries electrophoretically. Once all the capillaries were applied with a voltage, all samples began to run on electrophoresis. The fragments of DNA molecules moved from one end to the other of the capillaries, and the laser

beam would inspire the dye to fluoresce in the detection cell during the migration of DNA molecules through the path. Consequently the fluorescence signals were translated into electronic information, and the data were transferred to the computer workstation. Then the data processed by the 3100 Data Collection software was stored in the database and displayed as an electropherogram. Each peak was recognized as a single fragment in the electropherogram. The extraction and analysis of these data were processed automatically. The base sequence was determined by the shapes and positions of the peaks in electropherogram.

Sequence analysis

Sequencing analysis software, Staden package software (Roger Staden, Cambridge, UK), was used to analyze the sequencing results.

3.8 Statistical analysis

Genotype distributions for Hardy-Weinberg equilibrium and allelic frequencies for risk association were tested by Chi-square test. Fisher's exact test was used to analyze the small allele frequencies of re1800471, rs56281462 and IVS+13. A p value <0.05 was regarded as statistically significant. Odds ratios (OR) was calculated with 95% confidence intervals (CI).

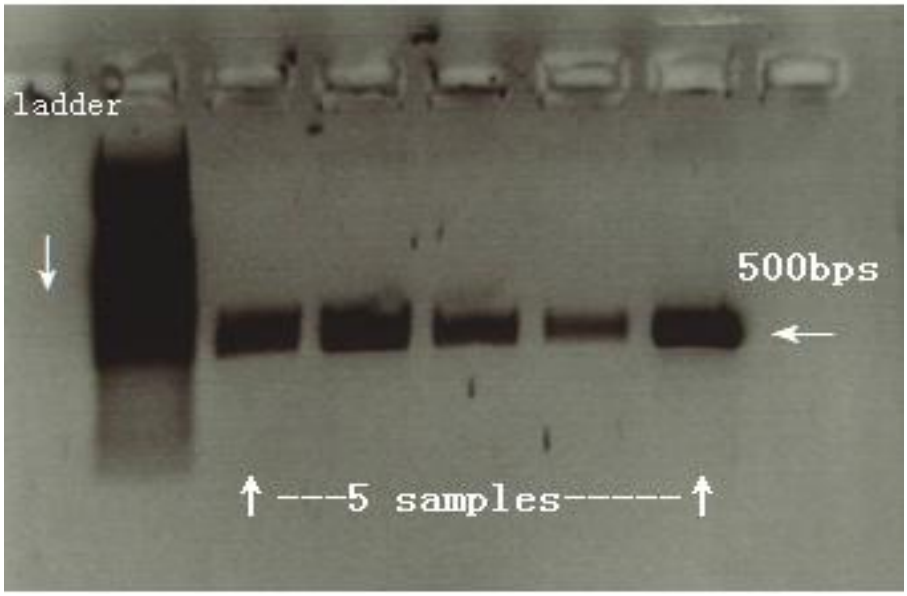
4. Result

4.1 PCR products

Genomic DNA products were optimized at 260nm.

The bands on the gel were identified as parallel to the ladder of 500 bps (**Figure 9**). This result confirmed the amplification of the sequencing DNA.

Fig.9: Visualization of the DNA samples run on gel.



4.2 Single nucleotide polymorphisms

Sequencing PCR products were genotyped and mutations were identified by direct base calling. Endpoint fluorescence readings were marked by four different colors (**Table 9**), which reflected the fluorescence emission spectrum of the labeled ddNTPs inspired by a laser beam.

The individual electropherogram on the array view displayed a single fragment,

the shape and the peaks represented the intensity of the fluorescence with time-lapse, therefore SNPs could be genotyped by direct sequencing. A double peak indicated a heterozygote mutation. To distinguish the homozygous mutant type sequence, control DNA was mixed with the samples that showed a single peak, and a double peak revealed the presence of the homozygote mutant sequence significantly. In our study four SNPs were identified (**Table 10**). Three of the SNPs were included in the HapMap data base (NCBI dbSNP build 137), and were seen in Moyamoya patients and controls as well (**Figure 10-12**). A novel SNP in the non-coding region intron 1, at the nucleotide position +13 downstream of codon 1, was found in one control participant (**Figure 12**).

Table 9: The color marked for ddNTPs

ddNTP	color
A	green
G	black
C	blue
T	red

Table 10: SNPs genotyped in our study.

SNP ID	nucleotide change	amino acid change
rs1800470	c.29 C>T	p.P10L
rs1800471	c.74 G>C	p.R25P
rs56281462	c.348 C>T	p.T116T
Novel SNP	IVS1+13 G>A	

rs1800470 (Figure.10)

Ref SNP Alleles: C/T

Ancestral Allele: C

Function: missense

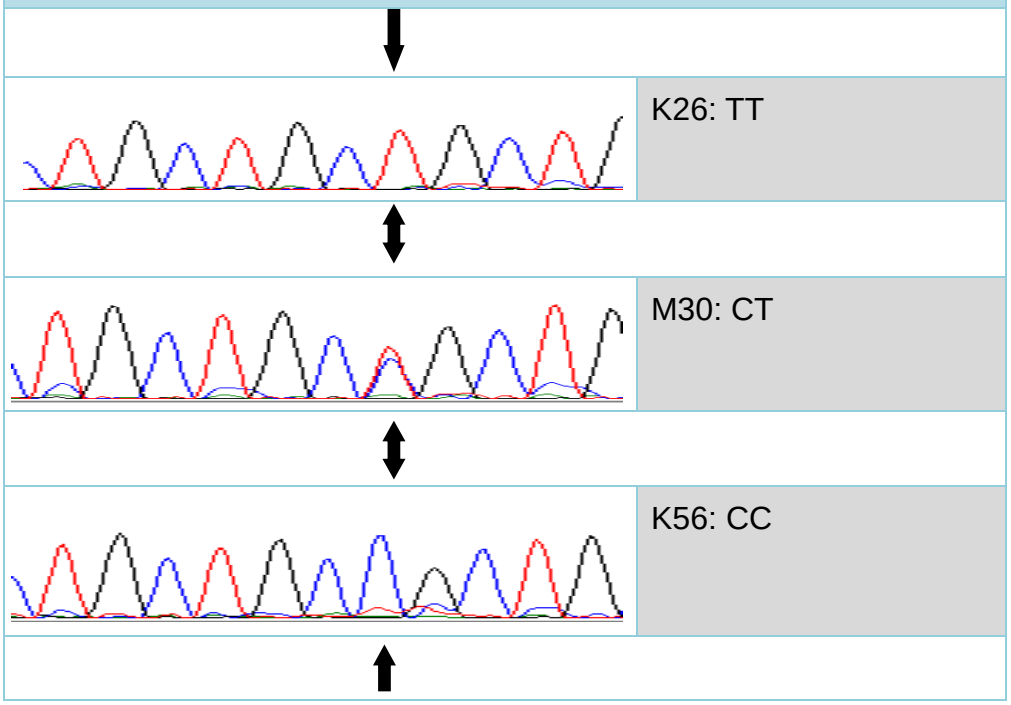
Residue change: P[Pro] →L[Leu]

Clinical signifigance: none

Sequence (NCBI build 137)

GGTACCAGAT CGCGCCCATC TAGGTTATTT CCGTGGGATA CTGAGACACC
CCCGGTCCAA GCCTCCCCTC CACCACTGCG CCCTTCTCCC TGAGGACCTC
AGCTTTCCCT CGAGGCCCTC CTACCTTTTG CCGGGAGACC CCCAGCCCCT
GCAGGGGCGG GGCCTCCCCA CCACACCAGC CCTGTTCGCG CTCTCGGCAG
TGCCGGGGGG CGCCGCCTCC CCCATGCCGC CCTCCGGGCT GCGGCTGCTG
C
[T]
GCTGCTGCTA CCGCTGCTGT GGCTACTGGT GCTGACGCCT GGCCGGCCGG
CCGCGGGACT ATCCACCTGC AAGACTATCG ACATGGAGCT GGTGAAGCGG
AAGCGCATCG AGGCCATCCG CGGCCAGATC CTGTCCAAGC TGCGGCTCGC
CAGCCCCCCG AGCCAGGGGG AGGTGCCGCC CGGCCCGCTG CCCGAGGCCG
TGCTCGCCCT GTACAACAGC ACCCGCGACC GGGTGGCCGG GGAGAGTGCA
G

Fig.10: sequencing of rs1800470 of 3 samples



rs1800471 (Figure.11)

Ref SNP Alleles: C/G Ancestral Allele: G

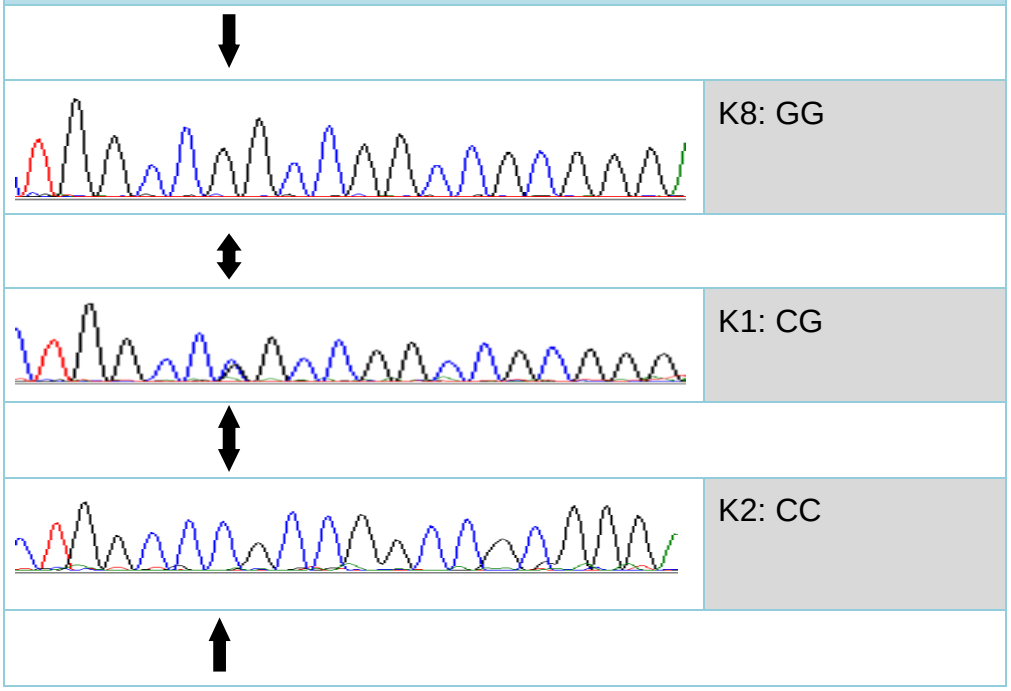
Function: missense Residue change: R[Arg]→P[Pro]

Clinical signifigance: NA

Sequence (NCBI build 137)

ACACCCCCGG TCCAAGCCTC CCCTCCACCA CTGCGCCCTT CTCCCTGAGG
ACCTCAGCTT TCCCTCGAGG CCCTCCTACC TTTTGCCGGG AGACCCCCAG
CCCCTGCAGG GGCGGGGCCT CCCCACCACA CCAGCCCTGT TCGCGCTCTC
GGCAGTGCCG GGGGGCGCCG CCTCCCCCAT GCCGCCCTCC GGGCTGCGGC
TGCTGCCGCT GCTGCTACCG CTGCTGTGGC TACTGGTGCT GACGCCTGGC
C
[G]
GCCGGCCGCG GGACTATCCA CCTGCAAGAC TATCGACATG GAGCTGGTGA
AGCGGAAGCG CATCGAGGCC ATCCGCGGCC AGATCCTGTC CAAGCTGCGG
CTCGCCAGCC CCCCGAGCCA GGGGGAGGTG CCGCCCGGCC CGCTGCCCGA
GGCCGTGCTC GCCCTGTACA ACAGCACCCG CGACCGGGTG GCCGGGGAGA
GTGCAGAACC GGAGCCCGAG CCTGAGGCCG ACTACTACGC CAAGGAGGTC
A

Fig.11: sequencing of rs1800471 of 3 samples



rs56281462 (Figure.12)

Ref SNP Alleles: N/G Ancestral Allele: G

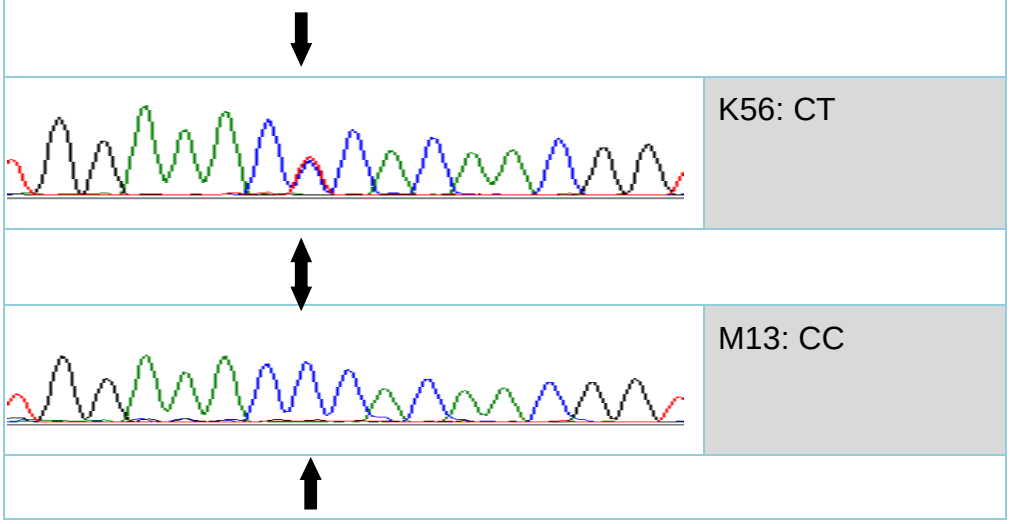
Function: cds→synon Residue change: T[Thr]→T[Thr]

Clinical signifigance: NA

Sequence (NCBI build 137)

GTCGCACTCT AGAAGCGGTC CACTTCGCTA TCTCCTCCTC TCCAAGACCA
GACACCTGGG TGGTAGGGGG CTCAGTGCCA TCCTCTTTCG GACACCCCCC
TCCCACCATC ACACGTTCCC TTTGCCCCGG GGTGTCCTCT TCCTCCAGCC
AGTTTCTTCT GCCAGTCACT TCCTACCCGT GGCCCCGGCA CTCCGGCGCC
CCCTGGGGGC CCCCCTCCCG GCTCCCCTGC CCCTCCGAGC TCACCGTTGT
G
R
GTTTCCACCA TTAGCACGCG GGTGACCTCC TTGGCGTAGT AGTCGGCCTC
AGGCTCGGGC TCCGGTTCTG CACTCTCCCC GGCCACCCGG TCGCGGGTGC
TGTTGTACAG GGCGAGCACG GCCTCGGGCA GCGGGCCGGG CGGCACCTCC
CCCTGGCTCG GGGGGCTGGC GAGCCGCAGC TTGGACAGGA TCTGGCCGCG
GATGGCCTCG ATGCGCTTCC GCTTCACCAG CTCCATGTCG ATAGTCTTGC
A

Fig.12: sequencing of rs56281462 of 2 samples.



IVS1+13 G>A (Figure.13)

A novel single nucleotide polymorphism in intron1, at the nucleotide position +13 downstream of exon1 (IVS+13bp G>A) was found in one control individual as a heterozygous variant. This variant was not included in HapMap data base and so we named it according to the standard nomenclature system.

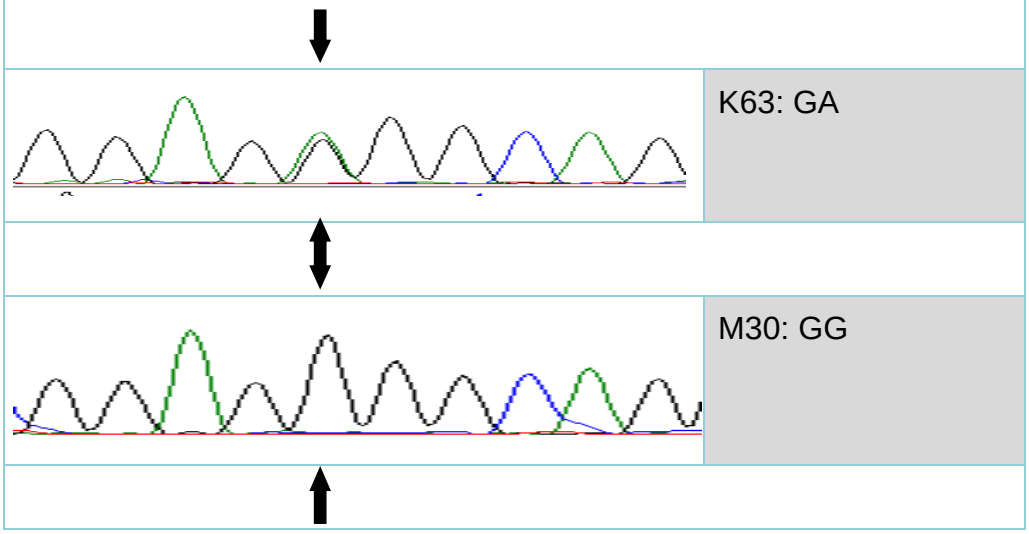
Sequence

ggatactgag acacccccgg tccaagcctc ccctccacca ctgcgccctt ctccctgagg acctcagctt tccctcgagg ccctcctacc ttttgccggg agacccccag cccctgcagg ggcggggcct ccccaccaca ccagccctgt tcgcgctctc ggcagtgccg gggggcgccg cctcccccat gccgccctcc g

a

ggctgcggct gctgccgctg ctgctaccgc tgctgtggct actggtgctg acgcctggcc ggccggccgc gggactatcc acctgcaaga ctatcgacat ggagctggtg aagcggaagc gcatcgaggc catccgcggc cagatcctgt ccaagctgcg gctcgccagc cccccgagcc aggggggaggt gccgcccggc c

Fig.13: sequencing of IVS1+13 G>A of 2 samples.



4.3Statistic analysis

Genotype analysis (Table 11)

The genotype distributions of the control samples of three alleles (rs1800470 *p=0.34,* rs56281462 *p*=0.90, IVS1+13 *p*=0.95) were consistent with Hardy-Weinberg equilibrium by Chi-square test. The genotype distribution of the control samples of rs1800471 was deviated from HWE (*p*=0.011). The heterozygous type of CT and homozygous type of TT for rs1800470 were more frequent than the homozygous type CC both in the case and control samples, and the frequencies of the both genotypes were more in case sample than in control samples. For rs1800471, homozygous genotype of GG was obviously more common than the other genotypes both in case and control samples. No homozygous genotype of TT for rs56281462 was seen both in case and control samples, and for this SNP, homozygous genotype of CC appeared most frequent in both case and control samples. In the novel SNP IVS1+13, only in one case sample was a heterozygous genotype of GA was seen, and in the other samples, the genotypes were all GG.

Table 11: Analysis of the genotypic frequencies.				
variant	genotype	controls (n=68)	cases (n=41)	HWE(controls)
rs1800470	CC	13 (19, 12%)	4 (9, 76%)	0.34
	CT	29 (42, 65%)	19 (46, 34%)	
	TT	26 (38, 24%)	18 (43, 90%)	
rs1800471	GG	59 (86, 76%)	39 (95, 12%)	0.011
	CG	7 (10, 29%)	2 (4, 88%)	
	CC	2 (2, 94%)	0 (0%)	
rs56281462	CC	66 (97, 06%)	40 (97, 56%)	0.90
	CT	2 (2, 94%)	1 (2, 44%)	
	TT	0 (0%)	0 (0%)	
IVS1+13 G>A	GG	67 (98, 53%)	41 (100%)	0.95
	GA	1 (1, 47%)	0 (0%)	
	AA	0 (0%)	0 (0%)	

Allelic analysis (Table 12)

For rs1800470, the frequency of allele T showed no significant difference between the case and control samples (p=0.27, OR=1.38), so the possible association of this variant with Moyamoya disease was excluded. The allelic frequency of G for rs1800471 was more in case samples than in control samples, but the possibility of this allele being a risk allele was rejected by statistic analysis (p=0.14, OR=0.28). Almost the most frequent allele for rs56281462 was allele C, as compared with allele T, both in case and control samples, but this allele also showed no significant difference (p=1, OR=0.83). All the three SNPs were included in the HapMap data base (NCBI dbSNP build 137), and the alleles could cause an amio-acid change, which would possibly change the function of TGFB1 consequently, but in this study this hypothesis was rejected with negative statistic data. Only in one control sample, did the A allele appear once as a heterozygous genotype GA, and both in case and control samples, the G allele was the most frequent, so the possibility of this variant being a risk factor for Moyamoya disease was improbable.

Table 12: Analysis of the allelic frequencies.

variant	allele	controls	cases	*p* value	OR (95%CI)
rs1800470	C	55 (40.44%)	27 (32.93%)	0,27	1,38 (0.78-2.46)
	T	81 (59.56%)	55 (67.07%)		
rs1800471	G	125 (91, 91%)	80 (97, 56%)	0,14*	0,28 (0.06-1.32)
	C	11 (8, 09%)	2 (2, 44%)		
rs56281462	C	134 (98, 53%)	81 (98, 78%)	1*	0,83 (0.07-9.27)
	T	2 (1, 47%)	1 (1, 22%)		
IVS1+13 G>A	G	135 (99, 23%)	82 (100%)	1*	N/A
	A	1 (0, 74%)	0 (0%)		

N/A: not applicable. * Fisher’s exact test was utilized for accuracy.

5. Discussion

Study purpose and design

A previous study with individually selected SNPs using a case-control method indicated a significant association between single nucleotide polymorphism rs1800471 (p=0.0345, OR=07.65) in the first exon of TGFB1 and Caucasian patients that had Moyamoya disease [107]. In this doctoral study we also performed a case-control association study to analyze genetic variants of TGFB1 exon 1, in the hope of finding more significantly associated SNPs and elucidating the possible role of TGFB1 in Moyamoya disease. We genotyped the complete sequence of exon 1 including the flanking regions from all the participants, and mutations were detected by direct base calling. Different automated genetic sequence analyzer and analysis software were used in the two studies (3100 ABI and Roger Staden in our study, 3130 ABI and GENETYX-Mac version 15.0.1 in the previous). Forty Caucasian Moyamoya patients as reported previously and one additional Chinese female patient were enrolled as the case group in this study, and 68 healthy volunteers were enrolled as controls. The patients and the control participants were similar in age and sex ratio. The single nucleotide variants found in our results showed no significant association with Moyamoya disease, including the previously published rs1800471 (p=0.14, OR=0.28 in our study). Discrepancy between the two studies may be explained by the enrollment of a Chinese patient, which significantly changed the statistical data. In the previous study, there was only one patient sample that was the heterozygous type CG in SNP rs1800471, while in our study when one female Chinese patient participated, two patient samples of the heterozygous type CG in SNP rs1800471 were detected (the other genotypes were not changed), so her participation changed the frequency of all the genotypes and alleles. In a study of small

cohorts, a small change can lead to a significantly different result. Furthermore, there is no study focusing on the genetic role of TGFB1 in Chinese patients who have Moyamoya disease, we cannot judge whether or not this variant is associated with Chinese patients. The significant result was not drawn in our study did not disprove the result in the previous study with Caucasian patients. Genetic changes for disease predisposition may be due to population stratification, so a different mechanism of Moyamoya disease in Chinese patients should be considered in the study for the pathogenesis in European patients [10]. The fact that only 41 sporadic Moyamoya patients were taken as our study subjects, weakened the power of our results, but to our knowledge this is the largest DNA sample number of Moyamoya disease patients in Europe.

Evaluation of the results

(1) Genetic polymorphisms of TGFB1 and Moyamoya disease

Single nucleotide polymorphisms are the most frequent variants in human genome, providing a large amount of DNA markers for genetic studies. The detection of the responsible mutations for a rare disease is not easily achievable as a result of limitations imposed by the current technology of gene sequencing, but high throughput sequencing of SNPs facilitates the finding of variants and can provide meaningful information on etiology, particularly when the function of the gene products is altered by only one base change [97,98]. The four SNPs found in our study did not reach significance. Because we genotyped the whole length of exon 1, including the flanking region by direct base calling, our results do not indicate a possible role of TGFB1 exon 1 to be a genetic factor for Moyamoya disease. The rare but important variants which may be disease-causing in Moyamoya disease have been detected using large cohorts in familial cases and with multiple methods, such as the detection of RNF213 and chromosome 17q25. Some important variants may

have been missed in our study because of the relatively small sample size of sporadic Moyamoya patients. Furthermore, SNPs rs1800470, rs1800471 and IVS+13 are adjacent markers, so they may possibly be part of a combination of alleles (haplotype) that would cause a greater impact on phenotypes than that is caused by individual alleles. Thus, family-based transmission analysis is needed to test the possible linkage disequilibrium of the haplotype and its association with Moyamoya disease. Moyamoya disease is a chronic and progressive disease, the process of vascular stenosis and the formation of new collateral vessels do not occur at the same time and the onset severity is also various according to the affected lesions, so different subtypes of symptoms in the disease process, such as ischemia or hemorrhage, may be due to different mechanisms and different factors. Our subjects were not divided into subgroups according to their symptoms, which may represent the processes of vascular stenosis or vascular formation. Since the age distribution at onset and the sex distribution are special features of Moyamoya disease, a possible risk factor may influence different aspects of Moyamoya disease in patients of different ages and sexes. Due to the small study cohorts, we did not analyze the distributions of the variants according to age and sex. So studies with different Moyamoya symptoms and different patient age and sex are required to analyze the possible role of genetic variants of TGFB1 on the different aspects of Moyamoya disease.

(2) Exon1 and TGFB1

TGFB1 gene is very large and the coding sequence is spread over seven exons with a total length of 2346 base pairs, each of which may have to be sequenced individually. The first exon 1 consists of 1237 base pairs and is the most widely investigated for its important promoting role in transcription and mutations of this region [90-91, 98-99,126]. Allelic frequency data of our result suggested that no variants of exon 1 were significantly associated with

Moyamoya disease, thus, sequencing the whole length of all TGFB1 exons in a larger cohort could be recommended for later studies. Performing reverse transcription polymerase chain reaction (RT-PCT) to convert mRNA molecules into cDNA from tissue sample is a useful approach to sequence all the exons of an interested gene, but this procedure is not always possible to conduct in genetic studies for Moyamoya disease because affected vessels of patients are not easily obtained [18-20,118].

(3) Flanking regions and TGFB1

Exons are the coding segments of genes and they are usually separated by the sequences that do not contain useful information called introns. These flanking regions are usually much longer than exons and account for the majority of the sequence of the gene. Before the genetic information of a gene is translated to synthesize a protein, the introns must be spliced from RNA molecules to leave the exons and the coding information continuous. If variants of the introns cause a lethal change in the gene sequence, which leads to a significant different reading frame, such as converting non-coding regions to a start codon initializing transcription, these mutations in the introns are possible disease-causing factors. Intron mutations are rarely found but polymorphisms are more frequent, and some intron-specific variants are also useful as molecular markers and helpful for elucidating the disease's pathogenesis [127,128]. In the TGFB1 gene, the non-coding regions consist of much more base pairs than the seven exons, so the importance of these sequences should not be neglected. In our study no variants were proven to be responsible for Moyamoya disease, but it is worth notice that because of the methodology adopted in our study some possible variants lying outside of the screened regions could not be detected. A novel variant at intron 1 was found in our study and was not confirmed to be significantly associated with Moyamoya disease, however, this result gives some hints that the non-coding

sequences would also possibly contribute to offer some useful information. Thus, a total gene sequencing of TGFB1 including all the flanking regions is required to elucidate the molecular mechanisms underlying the disease pathogenesis.

(4) TGFB1 signaling pathways and Moyamoya disease

Candidate genes are not easily selected for Moyamoya disease, because the etiology is still unknown, and multiple reasons may underlie the pathogenesis. Prominent pathological findings of Moyamoya disease indicate the possible role of the involvement of TGFB1. The high expression of TGFB1 in tissue samples or blood of Moyamoya patients raises the hypothesis that TGFB1 might account for the typical changes, though a secondary response to hypoxia should be considered. Therefore, the TGFB1 gene is one of the possible candidates that play a role in Moyamoya disease. TGFB1 is a growth factor that not only regulates cell differentiation and proliferation, but also plays an important role in new vessel formation. It may be regulated by a complex signaling pathway, including the ligation of TGFB1 to its different receptors [79,83,100]. The promoting role of TGFB1 in cell proliferation and differentiation plays a role in the process of wound healing, cancer and liver diseases. Much evidence has revealed that variants of the signaling pathway of TGFB1 have important effects upon the pathological changes in these processes, such as the mutations of its receptors that would block the ligation to initiate the regulating function of TGFB1 [96-98,110,130]. Also in intracranial diseases, such as meningioma or aneurysm, some variants in the signaling pathway have been found to be significantly associated [126]. Until now, no studies on TGFB1 and its signaling pathway have been conducted with Moyamoya patients [88,96].

Outlooks

Despite these results, TGFB1 may still play a role in the pathogenesis of Moyamoya disease. Larger cohorts are needed to look for possible variants within the gene. In those larger cohorts, a substratification of patients with Moyamoya disease may lead to significant association of variants.

6. Conclusion

In this doctoral research, we conducted a case-control study to analyze the possible association between the single nucleotide polymorphisms of exon 1 of transforming growth factor B 1 and Moyamoya disease. Forty Caucasian patients and one female Chinese patient were enrolled in our study, and sixty-eight healthy German individuals participated as controls. The first exon of TGFB1 was sequenced from genome DNA of patients and control volunteers by direct base calling. Three single nucleotide polymorphisms in exon 1 which were registered in the HapMap data base (NCBI dbSNP build 137) were detected in both cases and controls, including SNP rs1800471 which showed significant association with Moyamoya disease in a previously published study with Caucasian patients. In this study the significant result of this variant could not be replicated. The possible reason for this discrepancy may be due to the statistical difference caused by a small change in our study cohort which enrolled one more Chinese patient than the previous cohort of 40 Caucasian cases, and the possibility that the association of genetic risk factors with Moyamoya disease may differ between ethnic groups would also change the distribution of the alleles. Of the three detected SNPs, a novel SNP in intron 1 was detected in one of the control participants. A limitation of our study is the relatively small size; therefore a substratification was also not possible. In conclusion, in this study there was no detection of an association of single nucleotide polymorphisms in exon 1 of TGFB1 with Moyamoya disease. Larger patient cohorts are required to detect possible further variants associated with Moyamoya disease. A later substratification of patients may lead to more individual results.

7. Summary

Moyamoya disease is a progressive cerebral vascular disorder, characterized by the typical pathological changes of intracranial vascular intimal fibrosis and new vessels formation. Ischemic stroke and hemorrhage are the severest symptoms, which can lead to catastrophic neurological deficits. Its incidence has increased in recent years. Regional and racial distribution of Moyamoya disease strongly indicates a possible role of genetic factors for the pathogenesis of this disease. Transforming growth factor B 1 (TGFB1) is an important regulatory cytokine and has been shown highly expressed in tissue samples and plasma of Moyamoya patients. Therefore TGFB1 is suspected to play an important role in Moyamoya disease, and a significantly associated SNP (rs1800471) of this cytokine was found in a previous study with Caucasian patients. In this case-control study, 41 sporadic Moyamoya patients including 40 Caucasian patients and one female Chinese patient were enrolled, and 68 healthy German individuals participated as controls. Genomic DNA was extracted from blood samples. The first exon of TGFB1 was amplified by PCRs, and sequenced by direct base calling. Three variants in exon 1 in patient samples and one variant in non-coding intronic region in one control sample were found but there was no association. In this study the hypothesis that TGFB1 exon1 may contain a genetic variant that plays a role in Moyamoya disease could not be confirmed.

8. Zusammenfassung

Die Moyamoya Erkrankung ist eine fortschreitende zerebrale Gefäßerkrankung, die durch typische pathologische Veränderungen wie Fibrose der Intima und Bildung neuer Gefäße gekennzeichnet ist. Der ischämische Schlaganfall und intrazerebrale Blutungen zählen zu den folgenreichsten Konsequenzen, die wiederum zu katastrophalen neurologischen Defiziten führen können. Die Inzidenz hat sich in den vergangenen Jahren stark erhöht. Eine regionale und ethnische Verteilung der Moyamoya Erkrankung weist auf eine mögliche Rolle genetischer Faktoren bezüglich der Pathogenese dieser Krankheit. Transforming growth factor B 1 (TGFB1) ist eine wichtiges regulatorisches Zytokin und wurde erhöht in Gewebeproben und im Plasma von Patienten die an der Moyamoya Krankheit leiden nachgewiesen. Es wird daher vermutet, dass TGFB1 eine wichtige Rolle bei der Moyamoya Erkrankung spielt. Ein signifikant assoziiertes SNP (rs1800471) des Gens dieses Zytokins wurde in einer früheren Studie bei kaukasischen Patienten nachgewiesen. In der vorliegenden Fall-Kontroll-Studie wurden nun 41 Patienten mit der sporadischen Form der Moyamoya Erkrankung untersucht, darunter 40 kaukasische Patienten und eine weibliche chinesische Patientin, sowie 68 gesunde Individuen deutscher Herkunft, die als Kontrolle dienten. Es wurde DNA aus Blutproben extrahiert. Das erste Exon des TGFB1 wurde mittels PCR amplifiziert und durch “Direct Base Calling” sequenziert. Es wurden drei unterschiedliche Varianten im Exon 1 in den Patientenproben und eine Variante in einer nichtkodierenden intronischen Region in einer Kontrollprobe nachgewiesen, wobei keine signifikante Assoziation zu der Erkrankung festzustellen war. Durch diese Studie konnte die Hypothese, dass das Exon 1 von TGFB1 möglicherweise eine genetische Variante enthält, die bei der Erkrankung eine Rolle spielt, nicht bestätigt werden.

9. List of figures

10. List of Tables

11. List of References

1. Suzuki J, Takaku A. Cerebrovascular" moyamoya" disease. Disease showing abnormal net-like vessels in base of brain. Arch Neurol. 1969; 20(3): 288-99.

2. Takeuchi K, Shimizu K. Hypoplasia of the bilateral internal carotid arteries. Brain Nerve. 1957; 9: 37-43.

3. Takekawa Y, Umezawa T, Ueno Y, Sawada T, Kobayashi M. Pathological and immunohistochemical findings of an autopsy case of adult moyamoya disease. Neuropathology. 2004; 24(3): 236-42.

4. Baba T, Houkin K, Kuroda S, Novel epidemiological features of moyamoya disease. British Medical Journal. 2008; 79(8): 900-4.

5. Fukui M. Current state of study on moyamoya disease in Japan. Surgical Neurology. 1997; 47(2): 138-43.

6. Harish A, George T, Kurian VM, Mullasari Ajit S. Arteriovenous malformation after transradial percutaneous coronary intervention. Indian Heart J. 2008; 60(1): 64.

7. Hallemeier CL, Rich KM, Grubb RL Jr, Chicoine MR, Moran CJ, Cross DT 3rd, Zipfel GJ, Dacey RG Jr, Derdeyn CP. Clinical features and outcome in North American adults with moyamoya phenomenon. Stroke. 2006; 37(6): 1490-6.

8. Kuriyama S, Kusaka Y, Fujimura M, Wakai K, Tamakoshi A, Hashimoto S, Tsuji I, Inaba Y, Yoshimoto T. Prevalence and clinicoepidemiological features of moyamoya disease in Japan: findings from a nationwide epidemiological survey. Stroke. 2008; 39(1): 42-7.

9. Nagaraja D, Verma A, Taly AB, Kumar MV, Jayakumar PN. Cerebrovascular disease in children. Acta Neurol Scand. 1994; 90(4): 251-5.

10. Yonekawa Y, Ogata N, Kaku Y, Taub E, Imhof HG. Moyamoya disease in Europe, past and present status. Clin Neurol Neurosurg. 1997; 99 Suppl 2: S58-60.

11. Uchino K, Johnston SC, Becker KJ, Tirschwell DL. Moyamoya disease in Washington state and California. Neurology. 2005; 65(6): 956-8.

12. Fukui M, Kono S, Sueishi K, Ikezaki K. Moyamoya disease. Neuropathology. 2000; 20 Suppl: S61-4.

13. Coakham HM, Duchen LW, Scaravilli F. Moya-moya disease: clinical and pathological report of a case with associated myopathy. J Neurol Neurosurg Psychiatry. 1979; 42(4): 289-97.

14. Masuda J, Ogata J, Yutani C. Smooth muscle cell proliferation and localization of macrophages and T cells in the occlusive intracranial major arteries in moyamoya disease. Stroke. 1993; 24(12): 1960-7.

15. Nagasaka T, Shiozawa Z, Kobayashi M, Shindo K, Tsunoda S, Amino A.Autopsy findings in Down's syndrome with cerebrovascular disorder. Clin Neuropathol. 1996; 15(3): 145-9.

16. Araki Y, Yoshikawa K, Okamoto S, Sumitomo M, Maruwaka M, Wakabayashi T. Identification of novel biomarker candidates by proteomic analysis of cerebrospinal fluid from patients with moyamoya disease using SELDI-TOF-MS. BMC Neurol. 2010; 8(10): 112.

17. Aoyagi M, Fukai N, Matsushima Y, Yamamoto M, Yamamoto K. Kinetics of 125I-PDGF binding and down-regulation of PDGF receptor in arterial smooth muscle cells derived from patients with moyamoya disease. J Cell Physiol. 1993; 154(2): 281-8.

18. Aoyagi M, Fukai N, Sakamoto H, Shinkai T, Matsushima Y, Yamamoto M, Yamamoto K. Altered cellular responses to serum mitogens, including platelet-derived growth factor, in cultured smooth muscle cells derived from arteries of patients with moyamoya disease. Journal of cellular physiology. 1991; 147(2): 191-8.

19. Aoyagi M, Fukai N, Yamamoto M, Nakagawa K, Matsushima Y, Yamamoto K. Early development of intimal thickening in superficial temporal arteries in patients with Moyamoya disease. Stroke. 1996; 27(10): 1750-4.

20. Hojo M, Hoshimaru M, Miyamoto S, Taki W, Nagata I, Asahi M, Matsuura N, Ishizaki R, Kikuchi H, Hashimoto N. Role of transforming growth factor-beta1 in the pathogenesis of moyamoya disease. J Neurosurg. 1998; 89(4): 623-9.

21. Haltia M, Iivanainen M, Majuri H, Puranen M. Spontaneous occlusion of the circle of Willis (moyamoya syndrome). Clin Neuropathol. 1982; 1(1): 11-22.

22. Ellison PH, Largent JA, Popp AJ. Moya-moya disease associated with renal artery stenosis. Arch Neurol. 1981; 38(7): 467.

23. Halley SE, White WB, Ramsby GR, Voytovich AE. Renovascular hypertension in moyamoya syndrome. Therapeutic response to percutaneous transluminal angioplasty. Am J Hypertens. 1988; 1(4 Pt 1): 348-52.

24. Ikeda E. Systemic vascular changes in spontaneous occlusion of the circle of Willis. Stroke. 1991; 22(11): 1358-62.

25. Takagi Y, Kikuta K, Nozaki K, Hashimoto N. Histological features of middle cerebral arteries from patients treated for Moyamoya disease. Neurologia medico-chirurgica. 2007; 47(1): 1-4.

26. Kono S, Oka K, Sueishi K. Histopathologic and morphometric studies of leptomeningeal vessels in moyamoya disease. Stroke. 1990; 21(7): 1044-50.

27. Rao M, Zhang H, Liu Q, Zhang S, Hu L, Deng F. Clinical and experimental pathology of Moyamoya disease. Chin Med J (Engl). 2003; 116(12): 1845-9.

28. Yamashita M, Tanaka K, Matsuo T, Yokoyama K, Fujii T, Sakamoto H. Cerebral dissecting aneurysms in patients with moyamoya disease. Report of two cases. J Neurosurg. 1983; 58(1): 120-5.

29. Oka K, Yamashita M, Sadoshima S, Tanaka K. Cerebral haemorrhage in Moyamoya disease at autopsy. Virchows Archiv. 1981; 392(3): 247-61.

30. Sakamoto S, Kiura Y, Yamasaki F, Shibukawa M, Ohba S, Shrestha P, Sugiyama K, Kurisu K. Expression of vascular endothelial growth factor in dura mater of patients with moyamoya disease. Neurosurg Rev. 2008; 31(1): 77-81.

31. Yoshimoto T, Houkin K, Takahashi A, Abe H. Angiogenic factors in moyamoya disease. Stroke. 1996; 27(12): 2160-5.

32. Nanba R, Kuroda S, Ishikawa T, Houkin K, Iwasaki Y. Increased expression of hepatocyte growth factor in cerebrospinal fluid and intracranial artery in moyamoya disease. Stroke. 2004; 35(12): 2837-42.

33. Ito C, Akimoto T, Ioka T, Kobayashi T, Kusano E. TGF-beta inhibits vascular sprouting through TGF-beta type I receptor in the mouse embryonic aorta. Tohoku J Exp Med. 2009; 218(1): 63-71.

34. Takagi Y, Kikuta K, Nozaki K, Fujimoto M, Hayashi J, Imamura H, Hashimoto N. Expression of hypoxia-inducing factor-1 alpha and endoglin in intimal hyperplasia of the middle cerebral artery of patients with Moyamoya disease. Neurosurgery. 2007; 60(2): 338-45.

35. Soriano SG, Cowan DB, Proctor MR, Scott RM. Levels of soluble adhesion molecules are elevated in the cerebrospinal fluid of children with moyamoya syndrome. Neurosurgery. 2002; 50(3): 544-9.

36. Bonduel M, Hepner M, Sciuccati G, Torres AF, Tenembaum S. Prothrombotic disorders in children with moyamoya syndrome. Stroke. 2001; 32(8): 1786-92.

37. Yamamoto M, Aoyagi M, Tajima S, Wachi H, Fukai N, Matsushima Y, Yamamoto K. Increase in elastin gene expression and protein synthesis in arterial smooth muscle cells derived from patients with Moyamoya disease. Stroke. 1997; 28(9): 1733-8.

38. Yamada H, Deguchi K, Tanigawara T, Takenaka K, Nishimura Y, Shinoda J, Hattori T, Andoh T, Sakai N. The relationship between moyamoya disease and bacterial infection. Clinical Neurology and Neurosurgery. 1997; 99: S221-4.

39. Scott RM, Smith JL, Robertson RL, Madsen JR, Soriano SG, Rockoff MA. Long-term outcome in children with moyamoya syndrome after cranial revascularization by pial synangiosis. J Neurosurg (Pediatrics 2). 2004; 100: 142-9.

40. Seol HJ, Wang KC, Kim SK, Hwang YS, Kim KJ, Cho BK. Headache in pediatric moyamoya disease: review of 204 consecutive cases. J Neurosurg. 2005; 103(5 Suppl): 439-42.

41. Yamada M, Fujii K, Fukui M. Clinical features and outcomes in patients with asymptomatic moyamoya disease--from the results of nation-wide questionnaire survey. No Shinkei Geka. 2005; 33(4): 337-42.

42. Kuroda S, Ishikawa T, Houkin K, Nanba R, Hokari M, Iwasaki Y. Incidence and clinical features of disease progression in adult moyamoya disease. Stroke. 2005; 36(10): 2148-53.

43. Iwama T, Morimoto M, Hashimoto N, Goto Y, Todaka T, Sawada M. Mechanism of intracranial rebleeding in moyamoya disease. Clin Neurol Neurosurg. 1997; 99 Suppl 2: S187-90.

44. Handa J, Nakano Y, Okuno T, Komuro H, Hojyo H, Handa H. Computerized tomography in Moyamoya syndrome. Surg Neurol. 1977; 7(5): 315-9.

45. Takahashi M, Miyauchi T, Kowada M. Computed tomography of Moyamoya disease: demonstration of occluded arteries and collateral vessels as important diagnostic signs. Radiology. 1980; 134(3): 671-6.

46. Fujiwara H, Momoshima S, Kuribayashi S. Leptomeningeal high signal intensity (ivy sign) on fluid-attenuated inversion-recovery (FLAIR) MR images in moyamoya disease. Eur J Radiol. 2005; 55(2): 224-30.

47. Murai Y, Takagi R, Ikeda Y, Yamamoto Y, Teramoto A. Three-dimensional computerized tomography angiography in patients with hyperacute intracerebral hemorrhage. J Neurosurg. 1999; 91(3): 424-31.

48. Yamada I, Nakagawa T, Matsushima Y, Shibuya H. High-resolution turbo magnetic resonance angiography for diagnosis of Moyamoya disease. Stroke. 2001; 32(8): 1825-31.

49. Yamada I, Suzuki S, Matsushima Y. Moyainoya Disease: Comparison of Assessment with MR Angiography and MR Imaging versus Conventional Angiography. Radiology. 1995; 196: 211-8.

50. Hoshi H, Ohnishi T, Jinnouchi S, Futami S, Nagamachi S, Kodama T, Watanabe K, Ueda T, Wakisaka S. Cerebral blood flow study in patients with moyamoya disease evaluated by IMP SPECT. J Nucl Med. 1994; 35(1): 44-50.

51. Kuwabara Y, Ichiya Y, Sasaki M, Yoshida T, Masuda K, Matsushima T, Fukui M. Response to hypercapnia in moyamoya disease. Cerebrovascular response to hypercapnia in pediatric and adult patients with moyamoya disease. Stroke. 1997; 28(4): 701-7.

52. Nariai T, Matsushima Y, Imae S, Tanaka Y, Ishii K, Senda M, Ohno K. Severe haemodynamic stress in selected subtypes of patients with moyamoya disease: a positron emission tomography study. J Neurol Neurosurg Psychiatry. 2005; 76(5): 663-9.

53. Sainte-Rose C, Oliveira R, Puget S, Beni-Adani L, Boddaert N, Thorne J, Wray A, Zerah M, Bourgeois M. Multiple bur hole surgery for the treatment of moyamoya disease in children. J Neurosurg. 2006; 105(6 Suppl): 437-43.

54. Vilela MD, Newell DW. Superficial temporal artery to middle cerebral artery bypass: past, present, and future. Neurosurg Focus. 2008; 24(2): E2.

55. Fujii K, Ikezaki K, Irikura K, Miyasaka Y, Fukui M. The efficacy of bypass surgery for the patients with hemorrhagic moyamoya disease. Clin Neurol Neurosurg. 1997; 99 Suppl 2: S194-5.

56. Yoshida Y, Yoshimoto T, Shirane R, Sakurai Y. Clinical course, surgical management, and long-term outcome of moyamoya patients with rebleeding after an episode of intracerebral hemorrhage: An extensive follow-Up study. Stroke. 1999; 30(11): 2272-6.

57. Fung LW, Thompson D, Ganesan V. Revascularisation surgery for paediatric moyamoya: a review of the literature. Childs Nerv Syst. 2005; 21(5): 358-64.

58. Huang AP, Liu HM, Lai DM, Yang CC, Tsai YH, Wang KC, Yang SH, Kuo MF, Tu YK. Clinical significance of posterior circulation changes after revascularization in patients with moyamoya disease. Cerebrovasc Dis. 2009; 28(3): 247-57.

59. Ikezaki, K. Rational approach to treatment of moyamoya disease in childhood. J Child Neurol. 2000; 15(5): 350-6.

60. Wanifuchi H, Takeshita M, Izawa M, Aoki N, Kagawa M. Management of adult moyamoya disease. Neurol Med Chir (Tokyo). 1993; 33(5): 300-5.

61. Nanba R, Kuroda S, Tada M, Ishikawa T, Houkin K, Iwasaki Y. Clinical features of familial moyamoya disease. Childs Nerv Syst. 2006; 22(3): 258-62.

62. Yamauchi T, Houkin K, Tada M, Abe H. Familial occurrence of Moyamoya disease. Clinical Neurology and Neurosurgery. 1997; 99 Suppl 2: S162-7.

63. Fukuyama S, Kanai M, Osawa M. Clinical genetic analysis on the moyamoya disease. Annual report. 1990; 53–9.

64. Ikeda H, Sasaki T, Yoshimoto T, Fukui M, Arinami T. Mapping of a familial Moyamoya disease gene to chromosome 3p24. 2-p26. The American Journal of Human Genetics. 1999; 64(2): 533-7.

65. Yamauchi T, Tada M, Houkin K, Tanaka T, Nakamura Y, Kuroda S, Abe H, Inoue T, Ikezaki K, Matsushima T, Fukui M. Linkage of familial Moyamoya disease (Spontaneous Occlusion of the Circle of Willis) to Chromosome 17q25. Stroke. 2000; 31(4): 930-5.

66. Nanba R, Tada M, Kuroda S, Houkin K, Iwasaki Y. Sequence analysis and bioinformatics analysis of chromosome 17q25 in familial moyamoya disease. Child's Nervous System. 2005; 21(1): 62-8.

67. Mineharu Y, Liu W, Inoue K, Matsuura N, Inoue S, Takenaka K, Ikeda H, Houkin K, Takagi Y, Kikuta K, Nozaki K, Hashimoto N, Koizumi A. Autosomal dominant moyamoya disease maps to chromosome 17q25.3. Neurology. 2008; 70(24 Part 2): 2357-63.

68. Inoue TK, Ikezaki K, Sasazuki T, Matsushima T, Fukui M. Linkage Analysis of moyamoya disease on chromosome 6. J Child Neurol. 2000; 15(3): 179-82.

69. Sakurai K, Horiuchi Y, Ikeda H, Ikezaki K, Yoshimoto T, Fukui M, Arinami T. A novel susceptibility locus for moyamoya disease on chromosome 8q23. J Hum Genet. 2004; 49(5): 278-81.

70. Fujii D, Brissenden JE, Derynck R, Francke U. Transforming growth factor beta gene maps to human chromosome 19 long arm and to mouse chromosome 7. Somat Cell Mol Genet. 1986; 12(3): 281-8.

71. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem. 1983; 258(11): 7155-60.

72. Massagué J, Blain SW, Lo RS.TGFbeta signaling in growth control, cancer, and heritable disorders. Cell. 2000; 103(2): 295-309.

73. Olofsson A, Miyazono K, Kanzaki T, Colosetti P, Engström U, Heldin CH. Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. J Biol Chem. 1992; 267(27): 19482-8.

74. Saharinen J, Taipale J, Monni O, Keski-Oja J. Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4. J Biol Chem. 1998; 273(29): 18459-69.

75. Brown TL, Patil S, Howe PH. Analysis of TGF-beta-inducible apoptosis. Methods Mol Biol. 2000; 142: 149-67.

76. Choi ME, Ballermann BJ. Inhibition of capillary morphogenesis and associated apoptosis by dominant negative mutant transforming growth factor-beta receptors. J Biol Chem. 1995; 270(36): 21144-50.

77. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development. 1995; 121(6): 1845-54.

78. Pollman MJ, Naumovski L, Gibbons GH. Vascular cell apoptosis: cell type-specific modulation by transforming growth factor-beta1 in endothelial cells versus smooth muscle cells. Circulation. 1999; 99(15): 2019-26.

79. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A. 1986; 83(12): 4167-71.

80. Ferrari G, Pintucci G, Seghezzi G, Hyman K, Galloway AC, Mignatti P. VEGF, a prosurvival factor, acts in concert with TGF-beta1 to induce endothelial cell apoptosis. Proc Natl Acad Sci U S A. 2006; 103(46): 17260-5.

81. Pardali K, Moustakas A. Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta. 2007; 1775(1): 21-62.

82. Segura I, Serrano A, De Buitrago GG, González MA, Abad JL, Clavería C, Gómez L, Bernad A, Martínez-A C, Riese HH. Inhibition of programmed cell death impairs in vitro vascular-like structure formation and reduces in vivo angiogenesis. FASEB J. 2002; 16(8): 833-41.

83. Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P. Transforming growth factor-beta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. J Cell Physiol. 2009; 219(2): 449-58.

84. Shah R, Hurley CK, Posch PE. A molecular mechanism for the differential regulation of TGF-beta1 expression due to the common SNP -509C-T (c. -1347C > T). Hum Genet. 2006; 120(4): 461-9.

85. Hong SH, Wang KC, Kim SK, Cho BK, Park MH. Association of HLA-DR and -DQ Genes with Familial Moyamoya Disease in Koreans. J Korean Neurosurg Soc. 2009; 46(6): 558-63.

86. Miano JM, Berk BC, Retinoids: versatile biological response modifiers of vascular smooth muscle phenotype. Circ Res. 2000; 87(5): 355-62.

87. Mineharu Y, Takenaka K, Yamakawa H, Inoue K, Ikeda H, Kikuta KI, Takagi Y, Nozaki K, Hashimoto N, Koizumi A. Inheritance pattern of familial moyamoya disease: autosomal dominant mode and genomic imprinting. J Neurol Neurosurg Psychiatry. 2006; 77(9): 1025-9.

88. Massagué J, Chen YG. Controlling TGF-beta signaling. Genes Dev. 2000; 14(6): 627-44.

89. Tarantal AF, Chen H, Shi TT, Lu CH, Fang AB, Buckley S, Kolb M, Gauldie J, Warburton D, Shi W. Overexpression of transforming growth factor-beta1 in fetal monkey lung results in prenatal pulmonary fibrosis. Eur Respir J. 2010; 36(4): 907-14.

90. Awad MR, El-Gamel A, Hasleton P, Turner DM, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor-beta1 gene: association with transforming growth factor-beta1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. Transplantation. 1998; 66(8): 1014-20.

91. Dunning AM, Ellis PD, McBride S, Kirschenlohr HL, Healey CS, Kemp PR, Luben RN, Chang-Claude J, Mannermaa A, Kataja V, Pharoah PD, Easton DF, Ponder BA, Metcalfe JC. A transforming growth factorbeta1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. Cancer Res. 2003; 63(10): 2610-5.

92. Mallet C, Vittet D, Feige JJ, Bailly S. TGFbeta1 induces vasculogenesis and inhibits angiogenic sprouting in an embryonic stem cell differentiation model: respective contribution of ALK1 and ALK5. Stem Cells. 2006; 24(11): 2420-7.

93. Pepper M.S. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev. 1997; 8(1): 21-43.

94. Zheng W, Seftor EA, Meininger CJ, Hendrix MJ, Tomanek RJ. Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-beta. Am J Physiol Heart Circ Physiol. 2001; 280(2): H909-17.

95. Yang EY, Moses HL. Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. J Cell Biol. 1990; 111(2): 731-41.

96. Nagaraj NS, Datta PK. Targeting the transforming growth factor-beta signaling pathway in human cancer. Expert Opin Investig Drugs. 2010; 19(1): 77-91.

97. Haukim N, Bidwell JL, Smith AJ, Keen LJ, Gallagher G, Kimberly R, Huizinga T, McDermott MF, Oksenberg J, McNicholl J, Pociot F, Hardt C, D'Alfonso S. Cytokine gene polymorphism in human disease: on-line databases, supplement 2. Genes Immun. 2002; 3(6): 313-30.

98. Cambien F, Ricard S, Troesch A, Mallet C, Générénaz L, Evans A, Arveiler D, Luc G, Ruidavets JB, Poirier O. Polymorphisms of the transforming growth factor-beta 1 gene in relation to myocardial infarction and blood pressure. The Etude Cas-Temoin de l'Infarctus du Myocarde (ECTIM) Study. Hypertension. 1996; 28(5): 881-7.

99. Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC, Carter ND, Spector TD. Genetic control of the circulating concentration of transforming growth factor type beta1. Hum Mol Genet. 1999; 8(1): 93-7.

100. Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol Lett. 2002; 82(1-2): 85-91.

101. Margadant C, Sonnenberg A. Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. EMBO Rep. 2010; 11(2): 97-105.

102. Liu W, Morito D, Takashima S, Mineharu Y, Kobayashi H, Hitomi T, Hashikata H, Matsuura N, Yamazaki S, Toyoda A, Kikuta K, Takagi Y, Harada KH, Fujiyama A, Herzig R, Krischek B, Zou L, Kim JE, Kitakaze M, Miyamoto S, Nagata K, Hashimoto N, Koizumi A. Identification of RNF213 as a susceptibility gene for moyamoya disease and its possible role in vascular development. PLoS One. 2011; 6(7): e22542.

103. Weinberg DG, Arnaout OM, Rahme RJ, Aoun SG, Batjer HH, Bendok BR. Moyamoya disease: a review of histopathology, biochemistry, and genetics. Neurosurg Focus. 2011; 30(6): E20.

104. Tarasów E, Kułakowska A, Lukasiewicz A, Kapica-Topczewska K, Korneluk-Sadzyńska A, Brzozowska J, Drozdowski W. Moyamoya disease: Diagnostic imaging. Pol J Radiol. 2011; 76(1): 73–9.

105. Zipfel GJ, Fox DJ Jr, Rivet DJ. Moyamoya disease in adults: the role of cerebral revascularization. Skull Base. 2005;15(1): 27-41.

106. Kamada F, Aoki Y, Narisawa A, Abe Y, Komatsuzaki S, Kikuchi A, Kanno J, Niihori T, Ono M, Ishii N, Owada Y, Fujimura M, Mashimo Y, Suzuki Y, Hata A, Tsuchiya S, Tominaga T, Matsubara Y, Kure S. A genome-wide association study identifies RNF213 as the first Moyamoya disease gene. Hum Genet. 2011; 56(1): 34-40.

107. Roder C, Peters V, Kasuya H, Nishizawa T, Takehara Y, Berg D, Schulte C, Khan N, Tatagiba M, Krischek B. Polymorphisms in TGFB1 and PDGFRB are associated with Moyamoya disease in European patients. Acta Neurochir (Wien). 2010; 152(12): 2153-60.

108. Guo DC, Papke CL, Tran-Fadulu V, Regalado ES, Avidan N, Johnson RJ, Kim DH, Pannu H, Willing MC, Sparks E, Pyeritz RE, Singh MN, Dalman RL, Grotta JC, Marian AJ, Boerwinkle EA, Frazier LQ, LeMaire SA, Coselli JS, Estrera AL, Safi HJ, Veeraraghavan S, Muzny DM, Wheeler DA, Willerson JT, Yu RK, Shete SS, Scherer SE, Raman CS, Buja LM, Milewicz DM. Mutations in smooth muscle alpha-actin (ACTA2) cause coronary artery disease, stroke, and moyamoya disease, along with thoracic aortic disease. Am.J.Hum.Genet. 2009; 84(15): 617-27.

109. Han H, Pyo CW, Yoo DS, Huh PW, Cho KS, Kim DS. Association of Moyamoya patients with HLA class Ⅰ and class Ⅱ alleles in the Korean population. J.Korean. Med.Sci. 2003; 18(6): 876-80.

110. Xu Y, Pasche B. TGF-β signaling alterations and susceptibility to colorectal cancer. Hum Mol Genet. 2007; 16 Spec No 1:R14-20.

111. Wakai K, Tamakoshi A, Ikezaki K, Fukui M, Kawamura T, Aoki R, Kojima M, Lin Y, Ohno Y. Epidemiological features of moyamoya disease in Japan:findings from a nationwide survey. Clin Neurol Neurosurg. 1997; 99(Suppl): S1-5.

112. Goto Y, Yonekawa Y. Worldwide distribution of moyamoya disease. Neurol Med Chir (Tokyo). 1992; 32: 883-6.

113. O'Connell P, Leach RJ, Ledbetter DH, Cawthon RM, Culver M, Eldridge JR, Frej AK, Holm TR, Wolff E, Thayer MJ, Schafer AJ, Fountain JW, Wallace MR, Collins FS, Skolnick MH, Rich DC, Fournier REK, Baty BJ, Carey JC, Leppert MF, Lathrop GM, Lalouel JM, White R. Fine structure DNA mapping studies of the chromosomal region harboring the genetic defect in neurofibromatosis type I. Am J Hum Genet. 1989; 44(1): 51-7.

114. Kohne E. Hemoglobinopathies: clinical manifestations, diagnosis, and treatment. Dtsch Arztebl Int. 2011; 108(31-32): 532-40.

115. Francis J, Raghunathan S, Khanna P. The role of genetics in stroke. Postgrad Med J. 2007; 83(983): 590-5.

116. Mito T, Becker LE. Vascular dysplasia in Down syndrome: a possible relationship to moyamoya disease. Brain Dev. 1992;14(4): 248-51.

117. Fukuyama Y, Osawa M, Kanai N. Moyamoya disease (syndrome) and the Down syndrome. Brain Dev. 1992 Jul;14(4): 254-6.

118. Aoyagi M, Fukai N, Yamamoto M, Matsushima Y, Yamamoto K. Development of intimal thickening in superficial temporal arteries in patients with moyamoya disease. Clin Neurol Neurosurg. 1997; 99 Suppl 2: S213-7.

119. Khan N, Yonekawa Y. Moyamoya angiopathy in Europe: the beginnings in Zurich, practical lessons learned, increasing awareness and future perspectives. Acta Neurochir Suppl. 2008; 103: 127-30.

120. Khan N, Yonekawa Y. Moyamoya angiopathy in Europe. Acta Neurochir Suppl. 2005; 94: 149-52.

121. Mărgăritescu O, Pirici D, Mărgăritescu C. VEGF expression in human brain tissue after acute ischemic stroke. Rom J Morphol Embryol. 2011; 52(4): 1283-92.

122. Houkin K, Yoshimoto T, Abe H, Nagashima K, Nagashima M, Takeda M, Isu T. Role of basic fibroblast growth factor in the pathogenesis of moyamoya disease. Neurosurg Focus. 1998; 5(5): e2.

123. Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. Proc Jpn Acad Ser B Phys Biol Sci. 2010; 86(6): 588-610.

124. Yamamoto M, Aoyagi M, Fukai N, Matsushima Y, Yamamoto K. Differences in cellular responses to mitogens in arterial smooth muscle

cells derived from patients with moyamoya disease. Stroke. 1998; 29(6): 1188-93.

125. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell. 2010; 40(2): 294-309

126. Santiago-Sim T, Mathew-Joseph S, Pannu H, Milewicz DM, Seidman CE, Seidman JG, Kim DH. Sequencing of TGF-beta pathway genes in familial cases of intracranial aneurysm. Stroke. 2009; 40(5): 1604-11.

127. Jha P, Jha P, Pathak P, Chosdol K, Suri V, Sharma MC, Kumar G, Singh M, Mahapatra AK, Sarkar C. TP53 polymorphisms in gliomas from Indian patients: Study of codon 72 genotype, rs1642785, rs1800370 and 16 base pair insertion in intron-3. Exp Mol Pathol. 2011; 90(2): 167-72.

128. Vos M, Adams CH, Victor TC, van Helden PD. Polymorphisms and mutations found in the regions flanking exons 5 to 8 of the TP53 gene in a population at high risk for esophageal cancer in South Africa. Cancer Genet Cytogenet. 2003;140(1): 23-30.

129. Anscher MS. Targeting the TGF-beta1 pathway to prevent normal tissue injury after cancer therapy. Oncologist. 2010; 15(4): 350-9.

130. Dooley S, ten Dijke P. TGF-β in progression of liver disease. Cell Tissue Res. 2012; 347(1): 245-56.

131. Miskinyte S, Butler MG, Hervé D, Sarret C, Nicolino M, Petralia JD, Bergametti F, Arnould M, Pham VN, Gore AV, Spengos K, Gazal S, Woimant F, Steinberg GK, Weinstein BM, Tournier-Lasserve E. Loss of BRCC3 Deubiquitinating Enzyme Leads to Abnormal Angiogenesis and Is Associated with Syndromic Moyamoya. Am. J. Hum. Genet. 2011; 88(6):718-28.

132. Scott RM, Smith ER. Moyamoya disease and moyamoya syndrome. N Engl J Med. 2009; 360(12): 1226-37.

133. Lee M, Zaharchuk G, Guzman R, Achrol A, Bell-Stephens T, Steinberg GK. Quantitative hemodynamic studies in moyamoya disease: a review. Neurosurg Focus. 2009;26(4):E5.

12. Original article

European Journal of Medical Genetics

journal homepage: http://www.elsevier.com/locate/ejmg

Original article

Analysis of TGFB1 in European and Japanese Moyamoya disease patients

Chao Liu [a, b,1], Constantin Roder [a,1], Claudia Schulte [c], Hidetoshi Kasuya [d], Hiroyuki Akagawa [e], Tsutomu Nishizawa [f], Taku Yoneyama [d], Yoshikazu Okada [d], Nadia Khan [g], Marcos Tatagiba [a], Daniela Berg [h], Boris Krischek [a, *]

[a] Department of Neurosurgery, University of Tübingen, Tübingen, Germany
[b] Department of Neurosurgery, 2nd Hospital of Jilin University, Changchun, China
[c] The Hertie-Institute for Clinical Brain Research, Department of Neurodegeneration and German Center for Neurodegenerative Diseases, University of Tübingen, Tübingen, Germany
[d] Department of Neurosurgery, Medical Center East, Tokyo Women's Medical University, Tokyo, Japan
[e] Tokyo Women's Medical University, Institute for Integrated Medical Sciences (TIIMS), Tokyo, Japan
[f] Division of Virology, Department of Infection and Immunity, Jichi Medical School, Shimotsuke, Japan
[g] Moyamoya Clinic, Children's University Hospital Zürich, Zürich, Switzerland
[h] Center of Neurology, Department of Neurodegeneration, University of Tübingen, Tübingen, Germany

article info

Article history:
Received 29 September 2011
Received in revised form
5 May 2012
Accepted 5 May 2012
Available online xxx

Keywords:
Genetics
Growth factors
Moyamoya disease
Single nucleotide polymorphism
TGFB1

abstract

Background: Despite large efforts in researching the genesis of Moyamoya disease (MMD), the etiology of this rare disease remains widely unknown. In a previous publication we described two genetic variants in the first exon of transforming growth factor beta 1 (TGFB1) which were associated and showed a tendency toward significance, respectively. In this study we performed a follow-up analysis of TGFB1 by sequencing the complete exon 1 in European and by genotyping previously described positively associated single nucleotide polymorphisms (SNPs) in Japanese patients with MMD.Methods: The complete first exon of TGFB1 was genotyped in 40 MMD patients and 68 healthy controlsfrom central Europe. For verification, genotyping of the previously described SNPs rs1800470 and rs1800471 was performed in 45 Japanese MMD patients and 79 healthy controls. Analysis was performed by capillary sequencing with custom made primers. Results: Sequencing of the first exon of TGFB1 in the European cohort did not reveal any new disease- associated nor other genetic variations. The previously described disease association of rs1800471 and tendency toward significance of rs1800470 could not be replicated in the Japanese cohort. Conclusions: As no new genetic variants were uncovered in this study of the first exon of TGFB1 in European MMD patients and because of the negative association of rs1800470 and rs1800471 in Japanese MMD patients, a role of this exon of TGFB1 in the genesis of MMD is unlikely. Further analyses with even larger cohorts may be necessary to detect causal genetic factors that contribute to the genesis of this disease.

1. Introduction

Moyamoya disease (MMD) is defined as a bilateral stenosis of the terminal ortions of the internal carotid arteries (ICAs) accompanied by diffuse abnormal vessels which bypass the stenosis. The term "Moyamoya" is Japanese and describes a "puff of smoke" resembling the angiographic findings of diffuse fine collaterals. Clinical presentation of patients varies from symptoms such as headaches, transient ischemic attacks, epileptic seizures or disturbances of speech and cognition to severe neurological conditions with ischemic or hemorrhagic stroke. Epidemiological data show two peaks of clinical manifestation at 5e10 and 30e40 years of age, as well as a much higher incidence in Asian coun- tries (mainly in Japan and Korea with approximately 1 per 100.000 in Japan) than in non-Asian countries (approximately 0.1 per 100.000). Although the first description of this disease entity was more than 50 years ago, the etiology of MMD is still widely unknown [13, 15]. Major efforts have been put into the research of histopathological changes, variations in biological messenger molecules as well as genetic analysis [1, 13]. In a recent study we combined the above mentioned techniques and analyzed single nucleotide polymorphisms (SNPs) in potential candidate genes that

* Corresponding author. Division of Neurosurgery, Toronto Western Hospital, University of Toronto, 399 Bathurst Street, Toronto, ON M5T 2S8, Canada .
Tel.: þ1 416 301 9927; fax: þ1 416 913 1131.
E-mail address: krischek@gmail.com (B. Krischek).
1Authors contributed equally to this work.

Please cite this article in press as: C. Liu, et al., Analysis of TGFB1 in European and Japanese Moyamoya disease patients, European Journal of Medical Genetics (2012), http://dx.doi.org/10.1016/j.ejmg.2012.05.002

were hand-picked by reviewing the literature on histopathological and signal-peptide related variations in patients with MMD [14]. We found an association for rs1800471 (p ¼ 0.0345, OR ¼ 7.65, 95% CI ¼ 0.97e59.95) and a tendency for overrepresentation of the risk allele T for rs1800470, both SNPs located in the first exon of transforming growth factor beta 1 (TGFB1) and both capable of inducing an amino acid change. Our findings were underlined by reports by Hojo et al. [5] who showed higher concentrations of TGFB1 in smooth muscle cells (SMCs) of superficial temporal arteries (STAs) and in the peripheral blood of patients with MMD. Also Mohren et al. [12] showed that certain allele combinations (which were more frequent in our MMD cohort than in the control group) in the first exon of TGFB1 result in higher TGFB1 concentrations in vitro [14]. These findings gave us reason to further investigate the possible role of exon1 of TGFB1 in patients with MMD. In this study we present our comparison of the sequence of exon1 of 40 MMD patients with that of 68 controls from central Europe. Additionally the SNPs rs1800470 and rs1800471 were genotyped in a Japanese cohort of 45 patients with MMD as well as in 79 healthy controls.

2. Methods and material

2.1. Patients

40 EDTA blood samples of patients from central Europe with definite MMD (diagnostic criteria based on the recommendations of the Research Committee on the Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare, Japan [2]), as well as 68 healthy controls from Germany were collected and analyzed as described previously [14]. All patients had undergone four vessel angiography and had filled out a standardized ques- tionnaire asking about their medical history. The healthy controls had no history of cerebrovascular disease. The Ethics committee of the University of Tübingen approved the study protocol and all participants or their legal guardians gave written informed consent.

45 EDTA blood samples of patients with definite MMD (diag-nostic criteria based on the recommendations of the Research Committee on the Spontaneous Occlusion of the Circle of Willis of the Ministry of Health and Welfare, Japan [2]), as well as 79 healthy controls from Japan were collected. In 9 patients a familial history of MMD was described. The Ethics committee of the Tokyo Women's Medical University approved the study protocol and allparticipants or their legal guardians gave written informed consent.

2.2. DNA analysis

DNA was isolated from EDTA whole blood and stored at À20 C. Sequencing was performed using a Genetic Analyzer (3130xl for the Japanese samples, 3100 for the European samples, Applied Biosystems, USA) and GENETYX-Mac version 15.0.1 (GENETYX Corporation, Japan) analysis software for Japanese samples, Staden package software (Roger Staden, Cambridge, UK) for European samples. Comparison of SNPs between the groups was calculated with the Chi-squared test. Because of the small number of samples, Fisher's exact test was used for rs1800471. A value of $p < 0.05$ was considered statistically significant. The HardyeWeinberg equilibrium was tested by the Chi-squared method.

The sequence of TGFB1 was obtained from http://genome.ucsc.edu/. The following primers were used for the sequencing of exon1 in the European cohort:

Forward: 50 -ACCACACCAGCCCTGTTC-30 .

Reverse: 50 -ctgccagtcacttcctaccc-30 .

Genotyping of the SNPs in the Japanese cohort was performed with the following primers:

rs1800470:

Forward: 50 -CGCCCTTCTCCCTGAGGACC-30 .

Reverse: 50 -TCCGCTTCACCAGCTCCATG-30 .

rs1800471:

Forward: 50 -GCAGCTTGGACAGGATCTGG-30 .

Reverse: 50 -GTTCGCGCTCTCGGCAGT-30 .

3. Results

3.1. Clinical characteristics of the European cohort

Exon 1 of TGFB1 was sequenced in 40 patients with MMD from central Europe and 68 healthy controls from Germany:

Clinical characteristics of the European cohort were described in detail in our previous publication [14].

The female to male case ratio was 2.1:1, that of the controls' was 1.7:1. The mean age of the participants was 25.5 years for the cases and 27.6 years for the controls.

3.2. Clinical characteristics of the Japanese cohort

rs1800470 and rs1800471 were genotyped in 45 patients with MMD as well as 79 controls free of vascular disease from Japan:

The patients' mean age was 37 (1e68yo) years, the female to male ratio was 2.2:1.

The controls' mean age was 47.5 (16e79yo) years, the female to male ratio was 1:1.

The examined SNPs were consistent with the HardyeWeinberg equilibrium (HWE) ($p > 0.05$) for cases and controls, the allele frequency of the control samples of rs1800471 in the European cohort deviated from HWE (p ¼ 0.027) [14].

3.3. Sequencing of exon1 of TGFB1 in European MMD patients and controls

One new heterozygous variant was found at intron1 þ13bp G > R in one healthy control.

Other than that, no new sequence variations or mutations were found in patients with MMD or healthy controls.

Table 1a
Analysis of rs1800470 and rs1800471 in the European cohort. Source [14].

Name	Controls			Cases			Genotypic p value	Allelic p value	Odds ratio (95% CI)
rs1800470 [C/T]	n ¼ 68			n ¼ 40			0.2580	0.2444	1.41(0.79e2.52)
	CC13	19.11%	AF:	CC3	7.50%	AF:			
	CT29	42.65%	C ¼ 0.404	CT20	50.00%	C ¼ 0.325			
	TT26	38.24%	T ¼ 0.596	TT17	42.50%	T ¼ 0.675			
rs1800471 [C/G]	n ¼ 68			n ¼ 40			0.1232	0.0345a	7.65(0.97e59.95)
	CC2	2.94%	AF:	CC0	0.00%	AF:			
	CG8	11.77%	C ¼ 0.090	CG1	2.50%	C ¼ 0.012			
	GG58	85.29%	G ¼ 0.910	GG39	97.50%	G ¼ 0.988			

Table 1b
Analysis of rs1800470 and rs1800471 in the Japanese cohort.

Name	Controls			Cases			Genotypic p value	Allelic p value	Odds ratio (95% CI)
rs1800470 [C/T]	n ¼ 79			n ¼ 45			0.9085	0.7013	0.90(0.54e1.52)
	CC20	25.3%	AF:	CC12	26.7%	AF:			
	CT35	44.3%	C ¼ 0.47	CT21	46.6%	C ¼ 0.5			
	TT24	30.4%	T ¼ 0.53	TT12	26.7%	T ¼ 0.5			
rs1800471 [C/G]	n ¼ 79			n ¼ 45			N/A	N/A	N/A
	CC0	0%	AF:	CC0	0%	AF:			
	CG0	0%	C¼0	CG0	0%	C¼0			
	GG79	100%	G¼1	GG49	100%	G¼1			

AF allele frequency. CI Confidence interval. P values were calculated by using the Chi-squared test. The odds ratio is according to the allelic test. N/A not applicable.
aResults were calculated using the Fisher's exact test for higher accuracy.

3.4. Analysis of rs1800470 and rs1800471 in European and Japanese MMD patients (Tables 1a and 1b)

The analysis of rs1800470 and rs1800471 in the European cohort was published previously [14]:

In the European cohort the SNP rs1800471 showed a significant frequency difference between cases and controls with a p value of 0.0345, OR ¼ 7.65, 95% CI ¼ 0.97e59.95 (allele test). Although no significant difference was found for rs1800470, a tendency toward a risk allele T was described. (Table 1a).

In the Japanese cohort rs1800470 did not show any significant differences between cases and controls (allele test): 12 (26.7%) vs. 20 (25.3%) were homozygous CC, 21 (46.6%) vs 35 (44.3%) were heterozygous CT, 12 (26.7%) vs. 24 (30.4%) were homozygous TT (p ¼ 0.7013, OR ¼ 0.90, 95% CI ¼ 0.54e1.52).

SNP rs1800471 was homozygous GG in all Japanese cases and controls (Table 1b).

Subgroup-analysis of familial and sporadic cases with MMD in the Japanese cohort did not show any significant differences (data not shown).

4. Discussion

TGFB1 is known to regulate various biological processes including cell proliferation, cell survival, cell differentiation, cell migration, synthesis of extracellular matrix (ECM) proteins, immune response, wound healing, or angiogenesis [6]. These attributes would make TGFB1 a possible candidate regarding the genesis of MMD, a disease in which neurovascular stenosis and neovascularization (collaterals) are key issues [5]. Previous studies on TGFB1 in patients with MMD did not show significant correlation of concentrations in the cerebro-spinal fluid (CSF), but Hojo et al. were able to show increased levels in smooth muscle cells (SMCs) of superficial temporal arteries (STAs) and in the blood serum of patients with MMD [5, 16]. Based on this knowledge we had genotyped SNPs in the TGFB1 gene and found association for rs1800471 and a tendency toward association for rs1800470 [14]. Both SNPs are located in the coding region of exon1 of TGFB1 and both are capable of causing an amino acid change (rs1800470 [C/T ¼ Pro10Leu], rs1800471 [C/G ¼ Arg25Pro]). Mohren et al. [12] analyzed differences in expression and secretion of TGFB1 caused by these sequence variations in vitro and described allele/amino acid combinations resulting in significantly increased expression of TGFB1. We combined the results of Mohren's study with the results of the genotyping of rs1800470 and rs1800471 and found that the allele combination with the highest TGFB1 expression (T allele in rs1800470 and the G allele in rs1800471) was much more common in patients with MMD than in healthy controls [14]. These results strongly encouraged us to further elucidate the role of TGFB1 by fully sequencing exon 1 in European patients with MMD and by trying to replicate our previous findings by genotyping rs1800470 and rs1800471 in Japanese case and control cohorts.

The sequencing of exon1 in the European cohort did not reveal any new disease-associated genomic variations nor could we replicate our previous findings on rs1800470 and rs1800471 in the Japanese cohort.

The negative genotyping results of both SNPs in the Japanese cohort weaken the theory of an involvement of TGFB1 in the genesis of MMD. However differences between the Asian and Caucasian form of MMD, such as the timing of the disease's onset or variable rates of hemorrhage should be considered as these might be a hintfor different disease-causing mechanisms and therefore different genetic changes [8,9]. Also genetic variability between Asians and Caucasians might contribute to the difficulties in identifying common disease-causing genetic factors [1]. Recent publications described multisystem familial disorders such as Thoracic Aortic Aneurysms and Dissection (TAAD) along with various smooth muscle cell dysfunctions and other dysmorphisms accompanied by MMD [3,4,10,11]. TGFB1 has been described to be associated with various diseases including myocardial infarction among others [7]. We think that the combined analysis of genetic findings of other cardiovascular diseases and MMD might provide further insight into the diseases' etiology and might also elucidate the role of TGFB1 which only has been analyzed partly in our study. The TGFB1 gene consists of seven exons with a total length of 2.346 base pairs (bp) spread over more than 23.000 bp genomic sequence. Our study only covered a small part of this gene. Further sequencing of the entire sequence of TGFB1, the exons, harboring intronic regions and especially the promotor region as well as SNPs within the sequence might provide additional information on its role in the genesis of MMD.

We are aware that the small number of samples caused by the rareness of this disease might be the strongest limitation in finding replicable results. Therefore, it has to be kept in mind that negative results might be caused by a lack of statistical power. However, we think that it is important to keep the focus on the development of rare diseases like MMD and possibly enable larger studies in collaboration with other groups as each increase in the number of samples might increase the statistical power of new findings.

To our knowledge, this is the largest MMD cohort examined in Europe.

5. Conclusions

In conclusion, we performed a follow-up study on a previously published association between MMD and SNPs located in exon 1 of TGFB1. Exon 1 of TGFB1 was sequenced in a European cohort of 40 MMD patients and in 68 healthy controls. The previously described SNPs rs1800470 and rs1800471 were genotyped in a Japanese cohort with 45 MMD patients and in 79 healthy controls. The results of the sequencing did not reveal any new disease-associated sequence variations in exon 1. The genetic association of rs1800471 and overrepresentation of certain allele combinations of rs1800470 in the European cohort could not be replicated in the Japanese

samples. A broader examination of this gene, including other exons, intronic parts and the promotor region, as well as other studies with larger cohorts including different ethnicities are needed to elucidate the genetic background of Moyamoya disease Replicable results may lead to improvement of early detection of patients at risk of developing MMD and subsequently to future preventive therapies.

Conflicts of interest/disclosures

None.

Acknowledgments

This study was partly supported by a grant-in-aid for scientific research (C) to Hidetoshi Kasuya from the grant Encouraging Development of Strategic Research Centers, Special Coordination Funds for Promoting Science and Technology, Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] A.S. Achrol, R. Guzman, M. Lee, G.K. Steinberg, Pathophysiology and genetic factors in Moyamoya disease, Neurosurg. Focus 26 (2009) E4.
[2] M. Fukui, Guidelines for the diagnosis and treatment of spontaneous occlusion of the circle of Willis (Moyamoya disease), Clin. Neurol. Neurosurg. 99 (Suppl 2) (1997) S238eS240.
[3] D.-C. Guo, C.L. Papke, V. Tran-Fadulu, E.S. Regalado, N. Avidan, R.J. Johnson, D.H. Kim, H. Pannu, M.C. Willing, E. Sparks, R.E. Pyeritz, M.N. Singh, R.L. Dalman, J.C. Grotta, A.J. Marian, E.A. Boerwinkle, L.Q. Frazier, S.A. LeMaire, J.S. Coselli, A.L. Estrera, H.J. Safi, S. Veeraraghavan, D.M. Muzny, D.A. Wheeler, J.T. Willerson, R.K. Yu, S.S. Shete, S.E. Scherer, C.S. Raman, L.M. Buja, D.M. Milewicz, Mutations in smooth muscle alpha-actin (ACTA2) cause coronary artery disease, stroke, and Moyamoya disease, along with thoracic aortic disease, Am. J. Hum Genet. 84 (2009) 617e627.
[4] D. Hervé, P. Touraine, A. Verloes, S. Miskinyte, V. Krivosic, D. Logeart, N. Alili, J.D. Laredo, A. Gaudric, E. Houdart, J.P. Metzger, E. Tournier-Lasserve, F. Woimant, A hereditary Moyamoya syndrome with multisystemic manifestations, Neurology 75 (2010) 259e264.
[5] M. Hojo, M. Hoshimaru, S. Miyamoto, W. Taki, I. Nagata, M. Asahi, N. Matsuura, R. Ishizaki, H. Kikuchi, N. Hashimoto, Role of transforming rowth factor-ß1 in the pathogenesis of Moyamoya disease, J. Neurosurg. 89 (1998) 623e629.
[6] K. Janssens, P. ten Dijke, S. Janssens, W. van Hul, Transforming growth factor- ß1 to the bone, Endocr. Rev. 26 (2005) 743e774.
[7] W. Koch, P. Hoppmann, J.C. Mueller, A. Schomig, A. Kastrati, Association of transforming growth factor-ß1 gene polymorphisms with myocardial infarction in patients with angiographically proven coronary heart disease, Arte rioscler. Thromb. Vasc. Biol. 26 (2006) 1114e1119.
[8] M. Kraemer, W. Heienbrok, P. Berlit, Moyamoya disease in Europeans, Stroke 39 (2008) 3193e3200.
[9] B. Krischek, H. Kasuya, N. Khan, M. Tatagiba, C. Roder, M. Kraemer, Genetic and clinical characteristics of Moyamoya disease in Europeans, Trends Neurovasc. Surg. Acta Neurochir. 112 (Suppl) (2011) 31e34.
[10] D.M. Milewicz, C.S. Kwartler, C.L. Papke, E.S. Regalado, J. Cao, A.J. Reid, Genetic variants promoting smooth muscle cell proliferation can result in diffuse and diverse vascular diseases: evidence for a hyperplastic vasculomyopathy, Genet. Med. 12 (2010 196e203.
[11] D.M. Milewicz, J.R. Østergaard, L.M. Ala-Kokko, N. Khan, D.K. Grange, R. Mendoza-Londono, T.J. Bradley, A.H. Olney, L. Adès, J.F. Maher, D. Guo, L.M. Buja, D. Kim, J.C. Hyland, E.S. Regalado, De novo ACTA2 mutation causes a novel syndrome of multisystemic smooth muscle dysfunction, Am. J. Med. Genet. 152A (2010) 2437e2443.
[12] S. Mohren, R. Weiskirchen, Non-synonymous gene polymorphisms in the secretory signal peptide of human TGF-ß1 affect cellular synthesis but not secretion of TGF-ß1, Biochem. Biophys. Res. Commun. 379 (2009) 1015e1020.
[13] C. Roder, N.R. Nayak, N. Khan, M. Tatagiba, I. Inoue, B. Krischek, Genetics of Moyamoya disease, J. Hum. Gen. 55 (2010) 711e716.
[14] C. Roder, V. Peters, H. Kasuya, T. Nishizawa, Y. Takehara, D. Berg, C. Schulte, N. Khan, M. Tatagiba, B. Krischek, plymorphisms in TGFB1 and PDGFRB are associated with Moyamoya disease in European patients, Acta Neurochir. 152 (2010) 2153e2160.
[15] R.M. Scott, E.R. Smith, Moyamoya disease and Moyamoya syndrome, N. Engl. J. Med. 360 (2009) 1226e1237.
[16] T. Yoshimoto, K. Houkin, A. Takahashi, H. Abe, Angiogenic factors in Moyamoya disease, Stroke 27 (1996) 2160e2165.

13. Danksagung

Ich möchte allen Personen danken, die mir bei der Anfertigung dieser Arbeit hilfreich zur Seite standen:

Meinem Doktorvater, Herrn Prof. Dr. med. Boris Krischek, für die Möglichkeit zur Durchführung dieser Promotionsarbeit, auch für das Verständniss und seine Hilfe.

Herrn Prof. Marcos Tatagiba für die Einladung an die Universität Tübingen.

Frau Claudia Schulte und Herrn Dr. med. Constantin Roder, für die freundliche Hilfe im Labor.

Den Ärztinnen und Ärzten in der Klinik für Neurochirurgie am UKT, die Zusammenarbeit mit ihnen freute mich sehr.

14. Curriculum Vitae

Persönliche Information

Name: Chao Liu

Geschlecht: männlich

Geburtstag und Geburtsort: 1979-01-07, Changchun, China

Staatsangehörigkeit:China

Schulbildung

1997.9-2004.6

Diplom Studium an Medizinische Fakultät, Jilin Universität, China

Thema: Mini-invasive surgical treatment for intracerebral hemotoma

2009.10-2010.10

Doktorarbeit an Medizinische Fakultät, Tübingen Universität, Deutschland

Dissertation: Analysis of single nucleotide polymorphysms in the first exon of TGFB1 in patients that have Moyamoya disease

Doktorvater: Prof.Dr.med. Boris Krischek

Arbeitserfahrung

2004.7-2006.6 Assistentarzt, Neurochirurgie, Jilin Universität, China

2006.6-2006.10 Gastwissenschaftler, Neurochirurgie, UKT

2006.11-2009.9 Assistentarzt, Neurochirurgie, Jilin Universität, China

2010.11-jetzt Facharzt, Neurochirurgie, Jilin Universität, China