Lack of association between seropositivity of vasculopathy-related viruses and moyamoya disease

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Short Title: No relation between onset of MMD and viral infection

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Keywords: moyamoya disease, viral infection, titer, IgG antibody, *RNF213*, familial

Abstract

Objectives: Although the association between genetic factors, such as *RNF213* mutations, and moyamoya disease (MMD) has been well investigated, environmental factors are largely undetermined. Thus, we aimed to examine whether viral infection increases the risk of MMD.

Materials and Methods: To eliminate the effect of presence or absence of the *RNF213* p.R4810K mutation, the entire study population was positive for this mutation. We collected whole blood from 111 patients with MMD (45 familial and 66 sporadic cases) and 67 healthy volunteers, and we measured the immunoglobulin G titer of 11 viruses (cytomegalovirus, varicella-zoster virus, measles virus, rubella virus, herpes simplex virus, mumps virus, Epstein–Barr virus, human parvovirus B19, human herpesvirus 6 [HHV6], human herpesvirus 8, and John Cunningham virus) that were presumed to be associated with vasculopathy using the enzyme-linked immunosorbent assay. Positivity for past viral infection was determined by cut-off values obtained from previous reports and the manufacturer's instructions, and the positive rate was compared between cases and age- and sex-matched controls. We performed familial case-specific and sporadic case-specific analyses, as well as a case–control analysis.

Results: There was no significant difference in the positive rate between the case group and the control group in any of the analyses. A significant difference was only observed in the combined case–control analysis for HHV6 ($p = 0.046$), but the viral antibody-positive rate in control individuals was higher than in MMD cases.

Conclusions: Our cross-sectional study suggest that the investigated 11 viruses including HHV6 are unlikely to have an impact on MMD development.

Introduction

Moyamoya disease (MMD) is a progressive occlusive vasculopathy that affects the terminal portion of the internal carotid arteries.[1] Abnormal vascular networks, termed moyamoya vessels, are formed at the base of the brain as collaterals that compensate for insufficient cerebral blood flow. Patients with MMD are at a high risk of both ischemic and hemorrhagic stroke.

Through a pedigree analysis of familial MMD,[2] *RNF213* has been identified as a susceptibility gene,[3] and the p.R4810K founder mutation has been reported to increase the risk of MMD by >300 times.[3,4] However, the mutation has low penetrance, and only 1% of mutation carriers develop MMD. It is estimated that there are more than 15 million asymptomatic p.R4810K carriers in East Asia, which equates to 2%–3% of the general population.[5] Therefore, it is thought that some environmental factors could trigger MMD by acting on this genetic predisposition.

Considering the environmental risk of MMD, it is important to consider that around half of patients suffer from the disease under the age of 10 years,[1] whereas lifestyle factors are unlikely to play a major role. It is thought that some factor other than lifestyle is involved in the onset of MMD as an environmental factor. In childhood, cerebrovascular disease around the unilateral terminal portion of the internal carotid artery is called focal cerebral arteriopathy (FCA), and it is mainly associated with transient cerebral arteriopathy (TCA), MMD, and dissection. Because a large proportion of cases of TCA are caused by the varicella-zoster virus (VZV; post-varicella arteriopathy)[6] and some patients with infectious arteriopathy develop MMD,[7] viral infection has been postulated as a risk factor for MMD. Echizenya et al. reported that patients with the p.R4810K mutation developed TCA 2–3 weeks after having hand, foot, and mouth disease, which is usually caused by group A Coxsackie virus or Enterovirus 71.[8] In addition, expression of *RNF213* in endothelial cells is upregulated by interferon-β and interferon-γ, both of which are induced by viral infection.[9] *RNF213* is also associated with mortality from the Rift Valley fever virus,[10] further supporting the pathological link between *RNF213* and viral infection as a cause of MMD.

Previously, associations between both cytomegalovirus (CMV) and Epstein–Barr virus (EBV) and MMD have been reported.[11] However, no replication study has been conducted, and comprehensive research with inclusion of other types of virus is required. To verify the association between the history of viral infection and the prevalence of MMD, we evaluated antibody titers of 11 viruses that are assumed to cause inflammatory vascular disease, including CMV,[12] VZV,[13] measles virus (MeV),[14] rubella virus (RuV),[15] herpes simplex virus (HSV),[4] mumps virus (MuV),[16] EBV,[4] human parvovirus B19 (PVB19),[17] human herpesvirus 6 (HHV6),[4] human herpesvirus 8 (HHV8),[4] and John Cunningham virus

(JCV).[18] To eliminate the risk of genetic risk factor confounding, the study population was restricted to individuals with the p.R4810K mutation.

Materials and Methods

Study subjects

Patients with MMD were recruited from Kyoto University Hospital and collaborating hospitals. Healthy controls were selected from the general population in Japan, as previously reported.[5, 19, 20] The diagnosis of MMD was based on the diagnostic criteria of the Japanese Research Committee on moyamoya disease of the Ministry of Health, Welfare and Labour, Japan.[21] Information on family history, sex, age at onset, symptoms at onset, and unilateral or bilateral MMD was obtained either by interview or clinical chart review, as previously reported.[22, 23]

To rule out the effect of the p.R4810K mutation on MMD onset, only individuals with the p.R4810K mutation were included in the analysis. A total of 111 patients with MMD and 67 control subjects were confirmed to have the p.R4810K mutation, and they were further analyzed for viral antibody titers. Among the 111 patients, 45 were unrelated cases with a family history of MMD and 67 were sporadic cases. To conduct familial case- and sporadic case-specific analyses, age- and sex-matched controls were selected from 67 control individuals. For age matching, we chose a control subject who was in the same age group as the case subject. The age groups were 10–29 years, 30–49 years, 50–69 years, and 70–89 years at blood collection. Due to a paucity of control individuals with the p.R4810K mutation, some of the control individuals were used in both familial case- and sporadic case-specific analyses.

Measurement of viral antibody titers

We measured the IgG titers of CMV, VZV, MeV, RuV, HSV, MuV, EBV, PVB19, HHV6, HHV8, and JCV using the enzyme-linked immunosorbent assay (ELISA) method. The ELISA IgG kit manufactured by Denka Seiken (Tokyo, Japan) was used to measure CMV, VZV, MeV, RuV, HSV, MuV, EBV, and PVB19; the ELISA IgG kit manufactured by MyBioSource (San Diego, United States) was used to measure HHV6 and HHV8; and the ELISA-VIDITEST anti-JCV IgG kit manufactured by VIDIA (Prague, Czech Republic) was used to measure JCV. Antibody titer is usually measured in serum or plasma, but most of our stored samples were whole blood. Thus, a verification experiment was conducted in advance to compare the IgG antibody titer of whole blood with that of serum by measuring the IgG titers of whole blood/serum paired samples from the same healthy individuals. Healthy individuals who donated both whole blood and serum were randomly selected, and the antibody titer in whole blood was approximately 0.6-times that of serum. This is consistent with the fact that human hematocrit is

approximately 40%, and the amount of antibody in erythrocytes can be ignored. The serum-equivalent IgG titer was calculated by multiplying the IgG titer of whole blood by 1.66. To estimate viral infection history positivity, cut-off values for serum IgG titer were obtained from past reports and procedure manuals. The cut-off value was set to 3 for CMV, VZV, MeV, RuV, HSV, MuV, and EBV; to the weak positive control for PVB19; to the IgG titer that corresponded to the absorbance of the negative control after adding 0.10 on the calibration curve for HHV6; to 10 (IgG titer with a 2% positive rate in the control group) for HHV8; and to the IgG titer that corresponded to the absorbance obtained by multiplying the average absorbance of the calibrator on the calibration line by 0.28 for JCV.

Genotyping

Peripheral blood (2–10 ml) was collected from all subjects. Genomic DNA was extracted from the blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer's instructions. The quality and concentration of the extracted DNA were measured using an Infinite M200 PRO (TECAN, Kanagawa, Japan). The DNA was stored in a freezer at −30°C until analysis. Genotyping of the p.R4810K mutation was conducted for all participants using TaqMan probes (Custom TaqMan SNP Genotyping Assays; Applied Biosystems, Foster City, CA, USA) and a 7300/7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis

The positive rate of each viral antibody was compared between cases and controls using Fisher's exact test in Easy R software,[24] version 1.54, which is based on R software. We also performed familial case-specific and sporadic case-specific analyses. A p value of <0.05 was considered statistically significant.

Results

The distributions of age and sex in the study population are shown in Table. 1. Age and sex were matched between cases and controls. The proportion of females was 71.1% for familial cases and 63.6% for sporadic cases. For cases, the age at onset and the age at blood collection were not necessarily the same; the age at onset was on average around 10 years earlier than the age at blood collection. For familial cases, the age at onset was 39.7 ± 18.7 years and the age at blood collection

was 50.2 ± 14.8 years. For sporadic cases, the age at onset was 47.9 ± 13.9 years and the age at blood collection was 54.7 ± 12.7 years.

The seroprevalence of each virus was compared between patients with familial MMD and control subjects (Table. 2). No significant association was observed with any virus tested. The largest difference was observed for PVB19, for which the seroprevalence was 84.4% in case subjects and 68.9% in control subjects. However, the *p* value did not reach statistical significance ($p = 0.13$). We also compared the seroprevalence between patients with sporadic MMD and control subjects (Table. 3). Again, there was no significant difference in the seroprevalence between cases and controls. The *p* value for HHV6 showed a trend ($p = 0.058$) toward a positive association, but the seroprevalence was higher in controls than in cases (100% vs. 92.4%, respectively).

Although there was no significant difference in the seroprevalence of each virus when we analyzed familial and sporadic cases separately, the *p* value was similar for CMV, RuV, HHV6, and HHV8. Then, we conducted a further analysis by combining familial and sporadic cases together (Table. 4). There was a significant difference in the seroprevalence of HHV6 between combined cases (n = 111) and controls (n = 67) ($p = 0.046$). However, the seroprevalence of HHV6 in the case group (93.7%) was lower than in the control group (100%). A similar trend was also observed for CMV, where the seroprevalence was lower in cases than in controls (86.5% vs. 95.5%, respectively).

Discussion/Conclusion

We tested the association between MMD and 11 viruses that are assumed to cause vascular inflammation. We compared the seroprevalence of these viruses between patients with MMD and age- and sex-matched healthy control subjects. We assumed that viruses spread more easily within families; thus, we performed a familial case-specific analysis. However, none of the viruses were associated with familial MMD. Absence of an association was also confirmed in the sporadic case-specific analysis. When we conducted the combined analysis of familial and sporadic cases, the seroprevalence of HHV6 showed a significant difference between cases and controls. However, the seroprevalence was higher in controls than in cases, suggesting that HHV6 is unlikely to be involved in MMD development. It is difficult to interpret biologically and clinically that the seroprevalence of HHV6 is higher in controls than in cases. This might have occurred by chance due to multiple comparisons.

In the present study, the seroprevalence was high (approximately 70%) in most cases, and it was 100% for VZV and MeV. This is likely because CMV, VZV, HSV, EBV, and HHV6 are in the herpes family of viruses, and MuV, PVB19, and JCV are pathogens that are naturally transmitted to the majority of

people in infancy. MeV and RuV naturally infect the majority of people before the start of the monovalent vaccinations for measles and rubella. Because the average age at sample collection was greater than 50 years, it is reasonable that the average seroprevalence of these viruses was high. On the other hand, the seroprevalence of HHV8 was low (<10%), which was consistent with the fact that the main infection route of HHV8 is sexual transmission.

Given that viral infection was not associated with MMD, another possibility would be that other pathogens are involved in the development of this disease. According to a review by Mikami et al., such pathogens include *Leptospira*, *Propionibacterium acnes*, *Streptococcus pneumoniae*, group A beta-hemolytic *Streptococcus*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae*.[25] The microbiota may also be a risk factor, since it accelerates MMD onset. It is already known that metabolic factors, such as hyperlipidemia, high homocysteine concentration, low high-density lipoprotein concentration, and daily alcohol consumption, increase the risk of MMD development or progression.[26,27] Thus, another possible scenario would be that microorganisms affect metabolic function in patients with MMD. Interestingly, recent reports have demonstrated that *RNF213* has both antibacterial[28] and metabolic[29, 30] functions.

This study has some strengths and limitations that should be noted. One of the strengths is that we targeted only people with the *RNF213* p.R4810K mutation. By doing so, the effect of presence or absence of this mutation on MMD onset was ruled out. Moreover, we performed a familial case-specific analysis because it is assumed that pathogens are more easily spread between family members; thus, we fully investigated the association between viral infection and MMD.

In terms of the limitations of this study, the number of individuals under the age of 30 years was small. This is because younger people do not usually undergo health check-ups, and it is difficult to identify control subjects without comorbidities who have the p.R4810K mutation. Second, we did not account for the timing of viral infection. There was a difference between the age at onset and the age at blood collection; hence, the seroprevalence values in this study may not reflect those at the time of onset. Even so, the seropositivity should be biased to inflation in patients, and the seroprevalence cannot be higher in cases than in controls. Therefore, our conclusion of lack of an association between viral infection and MMD onset remains unchanged. Third, we did not test the association between viral infection and MMD onset in subjects without the p.R4810K mutation. There remains a possibility that individuals without this mutation will demonstrate an association between viral infection and MMD onset. However, in Japan, more than 80% of patients with MMD have the p.R4810K mutation, and even if viral infection increases the susceptibility of patients without the mutation to MMD, its contribution is considered to be low. Taken together, our data

show that viral infection has no impact on the onset of MMD in Japan, but prospective studies on populations comprising different ethnicities, subjects without the p.R4810K mutation, and younger age groups should be performed.

In conclusion, our cross-sectional study demonstrates no correlation between MMD and history of infection with the 11 targeted viruses. However, it is important to draw a conclusion on the association between MMD and viral infection when considering the relationship with other environmental factors. In the future, the association between inflammatory environmental factors other than viral infection, such as bacterial infection and autoimmunity, and MMD onset should be investigated.

Statements

Acknowledgement

This research was performed using control samples acquired from the Kyoto Human Specimen Bank. We would like to thank those who cooperated in collecting samples.

Statement of Ethics

This study was conducted in accordance with the World Medical Association Declaration of Helsinki. This study protocol was approved by the Ethics Committee of Kyoto University School of Medicine, Kyoto University, Kyoto, Japan (approval numbers: G138, G342, and G1109; approval dates: October 18, 2004; December 25, 2009; and February 9, 2018).

All subjects provided written informed consent, or for those considered too young to consent, informed consent was obtained from their parent or guardian.

Conflict of Interest Statement

Akio Koizumi holds a patent for *RNF213* (JPWO2011049207A1), “Moyamoya disease-related genes and their use.” The other authors report no conflicts of interest.

Author Contributions

The work was conceptualized and designed by K.H.H, and A.K. Study subjects were recruited by Y.M., T.K., T.F., S.M. and A.K. Serological analysis was performed by Y.N. Y.N., Y.M. and K.H.H. wrote the manuscript text and analyzed results. S.M. and A.K. supervised this study. All authors reviewed the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material file. Further enquiries can be directed to the corresponding author.

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Table Legends

Table. 1. Age and sex of the study population.

Table. 2. Seroprevalence of each viral antibody in patients with familial MMD and age- and sex-matched controls

Table. 3. Seroprevalence of each viral antibody in patients with sporadic MMD and age- and sex-matched controls

Table. 4. Seroprevalence of each viral antibody in patients with MMD (familial and sporadic) and controls (entire control group)

Table 1: Age and sex of the study population.

		10–29 yrs.	30–49 yrs.	50–69 yrs.	70–89 yrs.	Total
Familial cases (n=45)	**Male**	1	7	5	0	13
	Female	1	12	16	3	32
Controls for familial cases (n=45)	**Male**	1	7	5	0	13
	Female	1	12	16	3	32
Sporadic cases (n=66)	**Male**	1	9	12	2	24
	Female	1	12	23	6	42
***Controls for sporadic cases (n=65)**	**Male**	1	8	12	2	23
	Female	1	12	23	6	42

All individuals had the p.R4810K mutation.

*Since the number of control subjects with the p.R4810K mutation was considerably lower than the number of cases with the p.R4810K mutation, some of the control subjects that were used for comparison with familial cases were also used for comparison with sporadic cases.

Table 2: Seroprevalence of each viral antibody in patients with familial MMD and age- and sex-matched controls.

	Familial cases n=45	**Control subjects n=45**	**Odds ratio**	**95% CI**	***p* value**
CMV	39 (86.7%)	42 (93.3%)	0.47	0.071–2.4	0.49
VZV	45 (100%)	45 (100%)	N/A	N/A	1
MeV	45 (100%)	45 (100%)	N/A	N/A	1
RuV	42 (93.3%)	39 (86.7%)	2.1	0.42–14	0.49
HSV	32 (71.1%)	31 (68.9%)	1.1	0.41–3.0	1
MuV	37 (82.2%)	42 (93.3%)	0.33	0.053–1.5	0.20
EBV	44 (97.8%)	45 (100%)	N/A	N/A	1
PVB19	38 (84.4%)	31 (68.9%)	2.4	0.80–8.0	0.13
HHV6	43 (95.6%)	45 (100%)	N/A	N/A	0.49
HHV8	2 (4.4%)	0 (0%)	N/A	N/A	0.49
JCV	35 (77.8%)	36 (80.0%)	0.88	0.28–2.7	1

CMV: cytomegalovirus; VZV: varicella-zoster virus; MeV: measles virus; RuV: rubella virus; HSV: herpes simplex virus; MuV: mumps virus; EBV: Epstein–Barr virus; PVB19: human parvovirus B19; HHV6: human herpesvirus 6; HHV8: human herpesvirus 8; JCV: John Cunningham virus; N/A: not applicable; MMD: moyamoya disease; CI: confidence interval.

Table 3: Seroprevalence of each viral antibody in patients with sporadic MMD and age- and sex-matched controls.

	Sporadic cases n=66	**Control subjects n=65**	**Odds ratio**	**95% CI**	***p* value**
CMV	57 (86.4%)	62 (95.4%)	0.31	0.051–1.3	0.13
VZV	66 (100%)	65 (100%)	N/A	N/A	1
MeV	66 (100%)	65 (100%)	N/A	N/A	1
RuV	64 (97.0%)	59 (90.8%)	3.2	0.55–34	0.16
HSV	46 (69.7%)	48 (73.8%)	0.82	0.35–1.9	0.70
MuV	59 (89.4%)	60 (92.3%)	0.70	0.17–2.7	0.76
EBV	66 (100%)	65 (100%)	N/A	N/A	1
PVB19	46 (69.7%)	48 (73.8%)	0.82	0.35–1.9	0.70
HHV6	61 (92.4%)	65 (100%)	N/A	N/A	0.058
HHV8	4 (6.1%)	1 (1.5%)	4.1	0.39–210	0.37
JCV	54 (81.8%)	51 (78.5%)	1.2	0.48–3.2	0.67

CMV: cytomegalovirus; VZV: varicella-zoster virus; MeV: measles virus; RuV: rubella virus; HSV: herpes simplex virus; MuV: mumps virus; EBV: Epstein–Barr virus; PVB19: human parvovirus B19; HHV6: human herpesvirus 6; HHV8: human herpesvirus 8; JCV: John Cunningham virus; N/A: not applicable; MMD: moyamoya disease; CI: confidence interval.

Table 4: Seroprevalence of each viral antibody in patients with MMD (familial and sporadic) and controls (entire control group).

	Cases **n = 111**	**Controls** **n = 67**	**Odds ratio**	**95% CI**	***p* value**
CMV	96 (86.5%)	64 (95.5%)	0.30	0.054–1.1	0.072
VZV	111 (100%)	67 (100%)	N/A	N/A	1
MeV	111 (100%)	67 (100%)	N/A	N/A	1
RuV	106 (95.5%)	61 (91.0%)	2.1	0.50–9.0	0.34
HSV	78 (70.3%)	50 (74.6%)	0.80	0.38–1.7	0.61
MuV	96 (86.5%)	62 (92.5%)	0.52	0.14–1.6	0.33
EBV	110 (99.1%)	67 (100%)	N/A	N/A	1
PVB19	84 (75.7%)	49 (73.1%)	1.1	0.53–2.4	0.73
HHV6	104 (93.7%)	67 (100%)	N/A	N/A	0.046
HHV8	6 (5.4%)	1 (1.5%)	3.7	0.44–180	0.26
JCV	89 (80.2%)	53 (79.1%)	1.1	0.46–2.4	0.85

CMV: cytomegalovirus; VZV: varicella-zoster virus; MeV: measles virus; RuV: rubella virus; HSV: herpes simplex virus; MuV: mumps virus; EBV: Epstein–Barr virus; PVB19: human parvovirus B19; HHV6: human herpesvirus 6; HHV8: human herpesvirus 8; JCV: John Cunningham virus; N/A: not applicable; MMD: moyamoya disease; CI: confidence interval.