Human Cytomegalovirus Increases the Risk of Future Hemorrhagic But Not Ischemic Stroke
– A Nested Case-Control Study –

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Background: The cause-and-effect relationship between human cytomegalovirus (HCMV) and stroke has not been widely elucidated. We aimed to determine if HCMV infection has an increased risk of future stroke in hypertensive patients in rural areas of China.

Methods and Results: This was a nested case-control study from a prospective cohort study. A total of 300 newly diagnosed stroke cases with a median follow-up period of 8.4 years and 300 matched controls were selected for the present analysis. Adjusted odds ratio (OR) for stroke associated with HCMV DNA seropositivity was calculated by conditional logistic regression. HCMV DNA was detected in 38 of 300 samples from stroke patients and in 17 of 300 control samples (12.7% vs. 5.7%; P=0.023). Seropositivity for HCMV DNA increased the risk of incident stroke (unadjusted OR, 1.437; 95% confidence interval (CI), 1.023–2.020, P=0.037) and adjustment for other potential cardiovascular confounders only slightly changed the OR (1.464; 95% CI, 1.003–2.137, P=0.048). After controlling for potential cardiovascular confounders, the OR for hemorrhagic stroke associated with HCMV DNA was 1.718 (95% CI, 1.042–2.832), whereas the OR for ischemic stroke was 0.450 (95% CI, 0.142–1.428).

Conclusions: Seropositivity for HCMV DNA was positively associated with total and hemorrhagic but not ischemic stroke, which persisted after controlling for other cardiovascular factors. 

Key Words: Human cytomegalovirus; Hypertension; Nested case-control study; Stroke

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troke is the second commonest cause of death and the leading cause of adult disability worldwide. Results from a large standardized case-control study (INTERSTROKE) indicate that traditional major risk factors for stroke include hypertension, current smoking, abdominal obesity, diet, and physical inactivity. However, these risk factors cannot account for all of the global risk of all strokes. How to identify novel risk factors for stroke is still an urgent issue.

More recently, inflammation has been associated with an increased risk of cardiovascular events, including stroke. Atherosclerosis is regarded as a chronic low-grade inflammation, initiated and deteriorated by a combination of classical risk factors and novel risk factors, such as viruses. Human cytomegalovirus (HCMV) belongs to the β-herpes virus subfamily, which after primary infection persists for the lifespan of the host. In the past decade, several cross-sectional and retrospective studies have reported an increased risk of stroke and coronary artery disease (CAD) in association with HCMV infection. Guech-Ongey et al and Potena et al reported a positive association between HCMV infection and cardiovascular disease (CVD), and indicated that HCMV infection is one of the main causes in cerebral infarction patients. In a recent case-control study, Huang et al demonstrated that HCMV seropositivity was higher in the stroke group than in controls (55.0% vs. 23.5%, P<0.05) and the presence of HCMV DNA increased the risk of stroke. However, there are also a few epidemiological studies that have found different results.

In a prospective study over a 12-year follow-up period, Ridker et al found that IgG antibodies directed against CMV do not appear to be a marker for increased risk of future stroke (hazard ratio: 0.72, 95% confidence interval (CI), 0.6–0.9). In summary, the association between HCMV and risk of
stroke is still not well-defined and prospective studies are sparse. Thus, whether HCMV infection has an etiologic role in the development of stroke remains uncertain. We therefore tested whether HCMV is a marker of risk for future stroke in a nested case-control study from a prospective cohort study within a large cohort of hypertensive patients who were followed over an 8-year period.

Methods

Study Population
This study was designed as a nested case-control study from a prospective cohort study. From 2004 to 2006, a multistage, random cluster sampling process was performed to select a representative cohort of the rural population with hypertension aged at least 35 years from 50 rural villages in Liaoning Province. The detailed methodology on data collection and physical examination is described elsewhere.15,16

All study participants were invited to return for follow-up: from January to June 2008; from July to October 2010; and from August to October 2014. Of the 6,412 participants with hypertension at baseline, 634 were lost to follow-up because of unavailability of contact information. Overall, 5,778 participants aged ≥35 years at baseline examination were eligible to participate in the follow-up study. From this population, a total of 5,474 study participants (94.7%) (or their guardians) were identified and subsequently agreed to be interviewed as part of the follow-up study. For the purposes of this study, hypertensive patients with prior stroke (n=294), and CAD (angina, myocardial infarction, and arrhythmia) (n=73) were excluded, leaving 5,107 hypertensive patients free from CVD for prospective analysis.

During a median follow-up period of 8.4 years, we identified 510 incident cases of newly diagnosed stroke. From this group, 300 incident stroke cases were randomly selected for the present analysis. Using risk-set sampling,17 we then randomly selected controls from among the hypertensive patients who remained free from stroke and matched them to the case patients in a 1:1 ratio, according to age (within 1 year), sex, duration of follow-up (within 1 month), and stage of hypertension (1, <160/100 mmHg; 2, 160–179/100–109 mmHg; and 3, ≥180/110 mmHg). On the basis of these criteria, 300 stroke patients and 300 matched controls were selected from the hypertensive cohort. The research protocol was approved by the Shengjing Hospital of China Medical University Research Ethics Committee, and written informed consent was formally obtained from all the participants or their guardians.

Stroke Assessment
According to the WHO Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) criteria,18,19 stroke events were defined as rapidly developing signs of focal (or global) disturbance of cerebral function lasting ≥24 h (unless interrupted by surgery or death) with no apparent nonvascular cause. The definition included patients presenting with clinical signs and symptoms of subarachnoid hemorrhage, intracerebral hemorrhage, thrombosis and embolism. Hemorrhagic stroke was defined as stroke event with signs of focal (or global) disturbance of cerebral function lasting ≥24 h (unless interrupted by surgery or death) with no apparent nonvascular cause. The definition included patients presenting with clinical signs and symptoms of subarachnoid hemorrhage, intracerebral hemorrhage, thrombosis and embolism. Hemorrhagic stroke was defined as stroke event with a diagnosis of subarachnoid hemorrhage or intracerebral hemorrhage, and ischemic stroke was defined as stroke event with a diagnosis of thrombosis or embolism. Transient ischemic attacks and silent brain infarctions (cases without clinical symptoms or signs) were not included, neither were events associated with trauma, hematologic disorders, or malignancy. All stroke cases were diagnosed by computed tomography, magnetic resonance imaging (including diffusion image), magnetic resonance angiography of the brain, and carotid duplex imaging. The relevant information was obtained by direct reference to medical records by a single investigator. All materials were independently reviewed by the end-point assessment committee, whose members were all blinded to the study participants’ baseline risk factor information.

Laboratory Procedures
Blood samples at baseline were collected in EDTA vacutainer tubes and stored at −80°C. Matched case and control specimens were handled identically and assayed in random order, with each pair in the same analytical run for each cohort. Laboratory personnel were unaware of the case-control status of the samples throughout the analytical process.

Q-polymerase chain (PCR) was performed with a commercially available TaqMan system (Q-CMV Real-Time System, Da An Co, China). HCMV AD169 UL123 genomic region (Immediately Early 1) was used as the target amplification region. DNA was purified from 400 μl of blood using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Thermal cycling conditions were as follows: 93°C for 2 min, 10 cycles of 93°C for 45 s and 55°C for 1 min, 30 cycles of 93°C for 30 s and 55°C for 45 s. Fluorescence signals were recorded using the optimal excitation/emission wave lengths. An internal quantification standard curve was constructed with dilutions, ranging from 102 to 107 copies/reaction of a plasmid carrying the HCMV UL123 gene. All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or incorrect product size. PCR analyses were conducted in triplicate for each sample. Results were then expressed as copies/ml of plasma. A negative result meant no DNA was detected. Any positive PCR result was regarded as seropositive for HCMV irrespective of viral load.

Statistical Analysis
Continuous variables are presented as the mean and standard deviation (SD) and were compared with Student’s t-test. Categorical variables are expressed as frequencies and Pearson’s χ2 tests for independent proportions. Because the incidence-density sampling method was used to match controls to case patients on the basis of the cohort person-time,15 we evaluated the odds ratios (OR) and corresponding 95% CI for stroke by using univariate and multivariate analyses of conditional logistic regression models. Before the multivariate conditional logistic regression analysis was conducted, all the preliminary variables were included in an OLS (ordinary least squares) model to test for multicollinearity and the variance inflation factor (VIF) was used to check for problem of multicollinearity among the independent variables.20 All variables not excluded for multicollinearity (VIF <5) were included in the multivariate conditional logistic regression model for incident stroke. Multivariate models were adjusted by pulse rate, body mass index (BMI), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, fasting glucose, smoking, drinking, antihypertensive medication, statins, antiplatelet agents, and anticoagulants. Initial analyses were performed on the entire study group; patients were then stratified into the 2 major stroke subtypes: ischemic and hemorrhagic. All analyses were performed with SPSS statistical software version 13.0 (SPSS Inc, Chicago, IL, USA) and SAS statistical software version 9.2 (SAS Institute Inc, Carey, NC, USA). A P value less than 0.05 was accepted as indicating statistical significance.
HCMV and Risk of Stroke

Results

As expected, participants who developed stroke during follow-up (cases) were more likely than their counterparts (controls) to have a higher levels of average systolic blood pressure, LDL-C, HDL-C, and triglycerides (all P<0.05). There was no significant difference for diastolic blood pressure, pulse rate, BMI, fasting plasma glucose, and proportions of current smoking and drinking status between stroke cases and controls (all P>0.05). A total of 37.7% stroke cases and 22.7% controls were receiving antihypertensive medication at baseline (P<0.001). As a result of the 1:1 case-control matching criteria, the parameters for age, sex and stage of hypertension were identical in the 2 groups (Table 1). Table 1 also shows the baseline parameters for ischemic (n=207) and hemorrhagic (n=93) stroke patients.

Using quantitative real-time PCR assays, HCMV DNA was detected in 38 of 300 plasma samples obtained from the stroke group, and in 17 of 300 samples obtained from the control group (12.7% vs. 5.7%, P=0.023). Seropositivity for HCMV DNA increased the risk of incident stroke (unadjusted OR, 1.437; 95% CI, 1.023–2.020, P=0.037), and adjustment for other potential confounding cardiovascular factors only slightly changed the OR (adjusted OR, 1.464; 95% CI, 1.003–2.137, P=0.048) (Table 2). Considering the fact that hypertensive patients under antihypertensive medication showed a higher risk for stroke than patients with similar blood pressure levels without medication,21 we also conducted a sensitivity analysis using hypertensive patients with antihypertensive medication (yes/no) as a matching variable. The sensitivity analysis (177 stroke cases and 177 controls) indicated that the adjusted OR of HCMV DNA for incident stroke was 1.947 (95% CI: 1.121–3.381) after adjustment for other potential confounders. Table 3 shows the OR of other factors associated with stroke. Increasing triglycerides level (OR and 95% CI: 1.047, 1.001–1.096) and antihypertensive medication (OR and 95% CI: 1.364, 1.070–1.739) were also significantly associated with incident stroke.

Next, we analyzed the association between HCMV DNA and the subtypes of stroke. After controlling for other potential confounding cardiovascular factors, the adjusted OR of hemorrhagic stroke associated with HCMV was 1.718 (95% CI, 1.437; 95% CI, 1.023–2.020, P=0.037), and adjustment for other potential confounding cardiovascular factors only slightly changed the OR (adjusted OR, 1.464; 95% CI, 1.003–2.137, P=0.048) (Table 2). Considering the fact that hypertensive patients under antihypertensive medication showed a higher risk for stroke than patients with similar blood pressure levels without medication,21 we also conducted a sensitivity analysis using hypertensive patients with antihypertensive medication (yes/no) as a matching variable. The sensitivity analysis (177 stroke cases and 177 controls) indicated that the adjusted OR of HCMV DNA for incident stroke was 1.947 (95% CI: 1.121–3.381) after adjustment for other potential confounders. Table 3 shows the OR of other factors associated with stroke. Increasing triglycerides level (OR and 95% CI: 1.047, 1.001–1.096) and antihypertensive medication (OR and 95% CI: 1.364, 1.070–1.739) were also significantly associated with incident stroke.

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Firstly, the enrolled study populations were different. For differences in study results might be attributed to several reasons. The adjusted OR for stroke was 1.464 for HCMV. The difference in stroke incidence (total and hemorrhagic), after adjustment for other potential cardiovascular factors.

In the past decade, the association between HCMV and stroke has been revealed by several retrospective observational studies. Yi et al detected the presence of HCMV DNA and its antigens in the internal carotid arteries obtained from 35 patients with ischemic stroke. The levels of HCMV IE gene/protein were significantly higher in the stroke group than in the control group detected by 3 different methods (immunohistochemistry 34.3% vs. 10.0%; hybridization in situ 40.0% vs. 10.0%; PCR 60.0% vs. 30.0%). Huang et al revealed that the presence of HCMV DNA increased the risk of stroke (unadjusted OR, 3.98; 95% CI, 2.59–6.11), and the association remained after adjustment for age, sex and other cardiovascular factors. However, the cause-effect relationship between HCMV and stroke is yet to be confirmed by prospective studies. In a large cohort of apparently healthy middle-aged American men followed over a 12-year period, Ridker et al also found no evidence of a positive correlation between baseline IgG antibodies directed against CMV and the development of future thrombembolic stroke. However, they also believed that these data alone were not sufficient to exclude a potential role for HCMV infection in the development of CVD. A cohort study of elderly participants from the Framingham Heart Study with an average follow-up of 10 years revealed that CMV IgG antibody seropositivity was not associated with an increased risk of incident stroke. In contrast to the aforementioned prospective studies, the present prospective study confirmed a correlation between HCMV and stroke, whereby the adjusted OR for stroke was 1.464 for HCMV. The differences in study results might be attributed to several reasons. Firstly, the enrolled study populations were different. For example, in contrast to apparent healthy men and a general population, the current study subjects were hypertensive patients from rural areas of China who were at a higher risk of incident stroke. Secondly, the differences might be partly related to the fact that the number of stroke cases in our study was more than in the other studies, thus improving the statistical power of our analysis. Finally, we concentrated on HCMV DNA detection because it can reveal both productive and latent infections, which is inconsistent with the anti-HCMV antibodies (IgG) detected in previous studies.

In addition, few studies have simultaneously reported the association between HCMV infection and subtypes of stroke in the same study. Yi et al indicated that HCMV infection was associated with ischemic stroke, but their study did not deal with hemorrhagic stroke.

Results from another case-control study demonstrated that HCMV DNA was related to both ischemic and hemorrhagic stroke. The present study revealed that HCMV infection was independently associated with the risk of total and hemorrhagic stroke, but not with ischemic stroke, in hypertensive patients from rural areas of China. Despite the negative association between HCMV infection and ischemic stroke, we believe that these data alone cannot exclude a potential role for HCMV infection in the development of ischemic stroke. It is likely that chance may explain the negative association between HCMV and ischemic stroke.

The pathophysiological mechanisms underlying the association between HCMV infection and stroke have not yet been clearly elucidated. It is well known that low-grade inflammation is associated with an increased risk of stroke, and HCMV infection may have a role as a chronic inflammatory stimulant contributing to stroke. HCMV infection exerts an inhibitory effect on endothelial nitric oxide synthase activation and thus reduces nitric oxide production, thereby promoting thrombosis. HCMV-infected cells also secrete a number of cytokines, chemokines, growth factors, and adhesion molecules, some of which play important roles in the migration and proliferation of smooth muscle cells and vascular endothelial cells. These findings may provide a pathophysiological basis for the association between HCMV infection and vascular diseases, including stroke. In addition, HCMV infection might be a marker of low socioeconomic status, which is closely related to higher risk of intraparenchymal hemorrhage caused by high salt and low fat intake. Further studies concerning the mechanisms of HCMV infection with stroke are encouraged.

**Table 3. Other Factors Associated With Incident Stroke From Conditional Logistic Regression Model†**

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse rate (beats/min)</td>
<td>0.996 (0.985–1.006)</td>
<td>0.397</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.004 (0.971–1.038)</td>
<td>0.822</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>1.163 (0.972–1.390)</td>
<td>0.096</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.072 (0.716–1.803)</td>
<td>0.737</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.037 (1.001–1.046)</td>
<td>0.046</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>1.005 (0.956–1.058)</td>
<td>0.834</td>
</tr>
<tr>
<td>Current smoking (yes vs. no)</td>
<td>1.082 (0.819–1.430)</td>
<td>0.578</td>
</tr>
<tr>
<td>Current drinking (yes vs. no)</td>
<td>1.072 (0.795–1.445)</td>
<td>0.649</td>
</tr>
<tr>
<td>Antihypertensive medication (yes vs. no)</td>
<td>1.364 (1.070–1.739)</td>
<td>0.012</td>
</tr>
<tr>
<td>Statins (yes vs. no)</td>
<td>0.890 (0.406–1.950)</td>
<td>0.770</td>
</tr>
<tr>
<td>Antiplatelet agents (yes vs. no)</td>
<td>0.864 (0.471–1.586)</td>
<td>0.637</td>
</tr>
<tr>
<td>Anticoagulants (yes vs. no)</td>
<td>1.076 (0.308–3.758)</td>
<td>0.909</td>
</tr>
</tbody>
</table>

†Included variables: pulse rate, BMI, LDL-C, HDL-C, triglycerides, fasting glucose, smoking, drinking, antihypertensive medication, statins, antiplatelet agents, anticoagulants, and seropositivity for HCMV DNA. Abbreviations as in Tables 1, 2.
Some of the strengths of this study include its prospective design and the long follow-up period. These, as well as the fact that all study participants were derived from a socioeconomically homogeneous study population, all reduce the possibility of bias and confounding factors in our data. Furthermore, in addition to the 1:1 case-control matching criteria for age, sex and stage of hypertension, a wide variety of other potential confounding cardiovascular factors were controlled for in our analysis. Thus, the potential for residual confounding factors in our study is at a bare minimum. Finally, the relatively large sample size, and large number of stroke cases accrued, increased the statistical power of our analysis. Some limitations should also be acknowledged in the light of these findings. Firstly, our study cohort consisted of hypertensive patients selected exclusively from rural areas of China, which may be limited in diversity. For these results to be extrapolated to the general population, we encourage prudent validation in other populations with greater diversity. Secondly, we found HCMV infection to be associated with total and hemorrhagic stroke, but not with ischemic stroke. This observation deviates from the findings of other retrospective studies. Further studies focusing on HCMV infection and subtypes of stroke are encouraged in the future to clarify this issue. Thirdly, there were no data for high-sensitivity C-reactive protein, interleukin, or cytokines, which might help us to explain the underlying inflammatory mechanisms for the association between HCMV infection and incident stroke. Finally, we detected HCMV DNA based only on the primary infection, and did not test whether ongoing HCMV infection is related to stroke.

In conclusion, we have provided direct evidence for a cause-effect relationship between HCMV infection and incident stroke (total and hemorrhagic), and demonstrated that the association remains even after adjustment for other stroke risk factors. This study will provide important additional insights into the pathophysiological mechanisms of stroke, and uncover potential therapeutic targets for the prevention and clinical management of stroke.

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Disclosures

The authors declare no conflicts of interest.

References


HCMV and Risk of Stroke