Involvement of PTEN Promoter Methylation in Cerebral Cavernous Malformations

Yuan Zhu, PhD; Andreas Wloch, MSc; Qun Wu, MSc; Christian Peters, MSc; Axel Pagenstecher, MD; Helmut Bertalanffy, MD; Ulrich Sure, MD

**Background and Purpose**—Cerebral cavernous malformations (CCMs) are prevalent cerebral vascular lesions involving aberrant angiogenesis. However, the underlying mechanism is poorly understood. Phosphatase and tension homolog deleted on chromosome 10 (PTEN), a tumor suppressor, is frequently deficient in various pathologies due to mutation or epigenetic alterations. PTEN promoter hypermethylation is a major epigenetic silencing mechanism leading to activation of angiogenesis in tumors. The present study aimed to investigate whether PTEN promoter methylation was involved in CCMs.

**Methods**—PTEN promoter methylation was detected in surgical specimens of CCMs (n=69) by methylation-specific polymerase chain reaction. The methylation status was correlated to the clinical manifestations and to PTEN expression, which was analyzed by both Western blot and immunohistochemistry. To investigate the endothelial proliferation and the potential signaling pathways affected by PTEN methylation, proliferating cell nuclear antigen as well as phosphor-Akt and phosphor-Erk1,2 were detected by immunofluorescence and Western blot, respectively, in CCM specimens.

**Results**—Methylation-specific polymerase chain reaction revealed PTEN promoter methylation in 15.9% CCMs. Strikingly, 5 of 6 familial CCMs showed PTEN promoter methylation (83.3%), which was significantly higher than in sporadic cases (9.4%; \( P<0.001 \)). In addition, PTEN promoter methylation appeared more frequently in multiple CCMs, including familial cases (46.7%), than that in single-lesioned CCMs (11.8%; \( P<0.05 \)). Immunostaining and Western blot revealed a more significant PTEN downregulation in PTEN-methylated CCMs in comparison to PTEN-unmethylated CCMs. Reduced PTEN expression was inversely correlated to the expression of proliferating cell nuclear antigen and to the activation of Erk1,2, but not of Akt.

**Conclusion**—We reported here for the first time the involvement of PTEN promoter methylation in CCMs, particularly in familial CCMs, suggesting this epigenetic alteration as a potential pathomechanism of CCMs. The identification of Erk1,2 as triggered signaling in the lesions may be valuable for the development of effective therapy for this disease. (Stroke. 2009;40:00-00.)

**Key Words:** cerebral cavernous malformation ■ endothelial proliferation ■ Erk1,2 activation ■ PTEN downregulation ■ PTEN promoter methylation

Cerebral cavernous malformation (CCM) is a prevailing type of vascular lesions in the central nervous system, which affects approximately 0.5% of the population. Patients with CCM often clinically manifest with headache, epilepsy, or hemorrhagic stroke during the second to fourth decades of life. The location and number of lesions determine the severity of this disorder. The lesion can change in size and number over time, indicating that CCMs are dynamic rather than static lesions. Pathologically, the lesion is characterized by closely packed, enlarged vessels without intervening brain parenchyma. These vessels are thin-walled and lined by a single layer of endothelial cells lacking smooth muscle cells. Ultrastructural analysis revealed the absence of the tight junctions between the endothelial cells, which may account for the repetitive hemorrhage in brain.1 CCMs appear as sporadic (50% to 80%) or hereditary (familial) forms. The understanding of the pathogenesis of this disease has been advanced by the identification of distinct genes, including CCM1, CCM2, and CCM3, that predispose individuals to familial manifestations of the lesions.2,3 Mutations in CCM1 are thought to account for 56% of familial CCMs, and CCM2 and CCM3 mutations are estimated to be responsible for 33% and 6% of familial cases, respectively.4,5 These findings revealed the importance of CCM genes on one hand and suggested an involvement of other pathogenesis mechanisms in CCMs on the other hand.
Phosphatase and tension homolog deleted on chromosome 10 (PTEN) is a tumor suppressor that regulates multiple cellular functions, including cell growth and survival, differentiation and proliferation, apoptosis, focal adhesion, invasion, migration as well as angiogenesis. These biological functions are mediated by both lipid phosphatase and the dual-specificity protein phosphatase property of PTEN. Phosphatidylinositol 3-kinase (PI3K)/Akt signaling is one of the best characterized pathways targeted by PTEN through its lipid phosphatase activity. PTEN deficit may cause an accumulation of phosphatidylinositol 3,4,5-triphosphate, a lipid product of PI3K, which in turn activates the survival-promoting factor Akt. Downregulation or silencing of PTEN allows constitutive activation of the PI3K/Akt signaling pathway, resulting in uncontrolled cellular processes. In addition, PTEN is capable of inhibiting integrin/FAK and Ras/MAPK/Erk1/2 signaling pathways under certain circumstances through its protein tyrosine phosphatase activity, thereby negatively regulating cell cycling, proliferation, focal adhesion, and cell migration.

Increasing evidence indicates a crucial role for PTEN in regulating angiogenesis in tumor and in normal vascular development. PTEN deletion was correlated to increased tumor microvessel density and to the mortality of patients with tumors. Furthermore, Huang et al demonstrated a role for PTEN in regulating angiogenesis through directly modulating the endothelial function. The mechanism underlying this effect involves the regulation of angiogenesis factors like vascular endothelial growth factor. Therefore, the PTEN pathway has been recognized to govern normal vascular and tumor angiogenesis.

PTEN is frequently deficient in human cancers due to germline or somatic mutations or through an epigenetic gene silencing mechanism, e.g., promoter methylation. However, few studies have investigated this in noncancerous disease. Tam et al reported PTEN promoter methylation in benign ovarian tumors. Increased DNA methylation was also induced by in vitro and in vivo models of ischemia. These data indicate possible involvement of DNA methylation in the pathogenesis of certain noncancerous diseases.

CCMs are clearly related to abnormal vascular assembly or maintenance. We and others have characterized the bioactivity of angiogenesis in the lesion of CCMs, supporting the notion that aberrant angiogenesis is involved in this disease. Based on the frequent involvement of epigenetic alterations of PTEN in aberrant angiogenesis, the aim of the present study was to evaluate whether PTEN promoter methylation occurs in patients with CCM. To address this issue, we analyzed PTEN promoter methylation in surgical specimens of CCMs. The methylation status of PTEN was correlated to the clinical manifestations, to the PTEN expression, and to the proliferation status of endothelial cells. The potential signaling pathways that could be affected by PTEN methylation were also investigated.

Materials and Methods

Patient Collection

The diagnosis of CCM was based on the specific characteristics of MRI and histopathologic criteria as described in our previous review. Patients commonly had one or more clinical presentations such as seizures, cerebral hemorrhages, and focal neurological deficits. Subjects enrolled in the study provided informed consent. The experiment protocol was approved by the ethic committee of University Hospital of Marburg (Approval Number AZ67/07).

Sixty-nine CCM cases who received surgical treatment at our department between 2000 and 2006 were consecutively collected. One patient had 2 lesions when diagnosed by MRI in 2003. One lesion was immediately operated in 2003, and another was removed in 2006 due to its growth and repeated hemorrhage. Seventy surgical specimens were finally used for PTEN methylation study. We handled all samples in an identical process, i.e., the freshly resected specimens were immediately frozen in liquid nitrogen in an operation station and then transported to our laboratory. The specimens were stored at −80°C until experiments.

In the present study, a patient with CCM matched with one of the following items was considered as familial CCM: (1) the patient presents multiple lesions (diagnosed by the gradient echo MRI) with CCM1, CCM2, or CCM3 gene mutation; or (2) more than one familial member had CCM. Among 69 CCM cases, 6 patients had familial CCM with CCM1 gene mutations, and one patient received radiation therapy 9 years before the operation due to non-CCM-related disease. The patients included 31 female and 38 male individuals with a mean age 37±16 years (range, 2 to 65 years).

To study PTEN expression, the frozen surgical specimens (n=25) and the paraffin sections (n=50) were randomly selected from PTEN-unmethylated CCMs and from all PTEN-methylated cases for Western blot analysis and for immunohistochemistry, respectively. Surgical specimens from patients who underwent anterior temporal lobe resection due to temporal lobe epilepsy without vascular lesions served as controls.

DNA Extraction and Bisulfite Modification

Genomic DNA was extracted from surgical tissues using the QIAamp DNA mini kit (Qiagen, Frankfurt, Germany). DNA (1 μg) was denatured with 3 mol/L NaOH at 50°C for 10 minutes followed by incubation with 3.3 mol/L sodium bisulfite and 10 mmol/L hydroquinone at 55°C for 16 hours, which converts all unmethylated cytosine residues to uracil. The modified DNA was purified by using a NucleoSpin Extract II kit (Macherey-nagel, Dueren, Germany). The eluted DNA was suspended in 25 μL H2O and stored at −80°C until polymerase chain reaction.

Using a positive control template of genomic DNA extracted (as described previously) from blood of a healthy human subject was treated with 20 U of SssI methylases (New England Biolabs, Frankfurt, Germany) at 37°C for 4 hours, generating DNA completely methylated at all CpG sites followed by the bisulfite modification as described previously.

Methylation-Specific Polymerase Chain Reaction

Two primer sets were used to amplify the promoter region of the PTEN gene that incorporated a number of CpG sites, one specific for the methylated sequence (PTEN-M; forward: 5′-GGTTGGGGA-TTTTTTITTGC-3′; reverse: 5′-AACCCCTCTTACTGCGCCG-3′) and the other for the unmethylated sequence (PTEN-UM; forward: 5′-TATTAGATTGGGTATTITTTTGT-3′; reverse: 5′-CCAAACCCCTCTACACCCACA-3′). The primers used in the present study detect specifically the promoter sequence of the PTEN gene rather than that of the PTEN pseudogene. The polymerase chain reaction for PTEN-UM and PTEN-M were carried out in a 50 μL volume containing 1× polymerase chain reaction buffer (15 mmol/L MgCl2), 2.5 mmol/L mixture of dNTPs, 10 pM of each primer, 4 U HotStart Taq DNA polymerase (Qiagen, Frankfurt, Germany), and 25 to 50 ng of bisulfite-modified DNA. Amplification was performed in a thermocycler with the following conditions: 95°C for 15 minutes, cycled at 94°C for 30 seconds, 60°C for 1
minute, and 72°C for 30 seconds (40 cycles) followed by extension at 75°C for 5 minutes.

**Western Blot**

The surgical specimens were sonicated in lysis buffer containing 10% glycerol, 3% sodium dodecyl sulfate, 0.05 mol/L Tris (pH 6.8), and 0.01% protease inhibitor cocktail. The protein assay was carried out using a BCA kit (PerbioScience, Bonn, Germany). Samples containing an equal amount of total protein were loaded on 12.5% sodium dodecyl sulfate–polyacrylamide gels. After electrophoresis, protein was transferred onto a nitrocellulose membrane. Unspecific binding was blocked by a buffer containing 0.1% Tween-20, 2% BSA, and 5% nonfat dry milk in Tris-buffered saline. The blots were then incubated with primary antibodies from mouse anti-PTEN (1:1000; Cell Signaling Technology), rabbit antiphosphor-Akt (P-Akt, 1:1000; Cell Signaling Technology), and rabbit antiphosphor-Erk1,2 (P-Erk1,2, 1:1000; Cell Signaling Technology) diluted in blocking buffer overnight at 4°C. Actin (mouse antiactin, 1:1000; Sigma) was detected for control of equal protein loading. After the secondary antibody reaction, the signal was produced by ECL detection reagents (Amersham Bioscience, Freiburg, Germany).

For semiquantification of the blot, integrative optical density of the bands on the blots was measured by the National Institutes of Health image software. The expression of target protein was calculated by the integrative optical density ratio of the target protein to the housekeeping protein actin and normalized as a percentage of the control.

**Immunohistochemistry**

The paraffin-embedded tissue blocks from CCMs were used after routine analysis by the Department of Neuropathology at our hospital. The sections were deparaffinized using graded ethanol. The sections were then treated with 3% H2O2 for 20 minutes to block endogenous peroxidase activity followed by blocking nonspecific binding using a blocking buffer containing 2% BSA and 10% goat serum in phosphate-buffered saline. The sections were then incubated with mouse anti-PTEN (1:50) overnight at 4°C. The negative control sections were incubated with nonimmune mouse or rabbit IgG. After washing, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Hamburg, Germany). The substrate HistoGreen (LINARIS Biologische Produkte GmbH, Werheim-Bettingen, Germany) solution was applied to the sections according to the manufacturer’s protocol. The sections were analyzed by a Leica DFC320 microscope and the Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). The positive cells were counted by a blinded observer. Using a Biotin Blocking System (DakoCytomation), biotin-conjugated antimouse IgG was added to the sections followed by the incubation with fluorescein avidin D (Vector Laboratories, Peterborough, UK). Thereafter, a Texas red-conjugated antirabbit IgG was applied to the sections. The sections were analyzed by a confocal laser scanning microscope (Zeiss, Jena, Germany). The specific signal was obtained by setting scanning conditions with respect to laser energy, signal detection (gain, offset), and pinhole size in a way that no signal was detectable in the negative staining controls (IgG-incubated section). Immunoreactivity of PTEN was analyzed under a background-subtracted condition.

**Statistics**

Statistical analysis was performed using the WinSTAT program. The probability of the differences of PTEN methylation between 2 categorical variables was analyzed by Fisher exact test. In immunohistochemistry study, the difference of PTEN loss score between the groups was analyzed by a nonparametric method Wilcoxon test. Western blot data were analyzed by analysis of variance followed by Fisher test. \( P < 0.05 \) was considered a significant difference.

**Results**

**PTEN Promoter Methylation in Cerebral Cavernous Malformations: Correlation With Clinical Manifestations**

The results from methylation-specific polymerase chain reaction (MSP) study of PTEN promoter methylation in 69 CCMs were summarized in Table 1 and illustrated in Figure 1. PTEN promoter methylation was detected in 15.9% CCM cases. The mean age of PTEN-methylated cases was 25±16.9, which was significantly younger than that of PTEN-unmethylated CCMs (38.7±14.6; \( P = 0.0130 \)). Analysis of the correlation of PTEN promoter methylation status with the clinical manifestations revealed striking features: (1) PTEN methylation was detected in 83.3% familial CCM, which was significantly higher than that in sporadic cases (9.5%; \( P = 0.0002 \)); (2) the PTEN-methylated familial cases had a mean age of 13.8 years old, which was significantly younger than that of PTEN-methylated sporadic cases (34.3 years old).

**Table 1. Summary of the Profile of PTEN Promoter Methylation in CCMs**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total No. of Cases</th>
<th>PTEN Methylation-Positive</th>
<th>Percentage of Positive</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child (&lt;18 years)</td>
<td>9</td>
<td>3</td>
<td>33.3</td>
<td>0.1798</td>
</tr>
<tr>
<td>Adult (&gt;18 years)</td>
<td>60</td>
<td>8</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td>Inheritance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporadic</td>
<td>63</td>
<td>6</td>
<td>9.5</td>
<td>0.0002</td>
</tr>
<tr>
<td>Familial</td>
<td>6</td>
<td>5</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>32</td>
<td>6</td>
<td>18.7</td>
<td>0.7450</td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>5</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td>Multiplicity*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single lesion</td>
<td>34</td>
<td>4</td>
<td>11.8</td>
<td>0.0215</td>
</tr>
<tr>
<td>Multiple lesion</td>
<td>15</td>
<td>7</td>
<td>46.7</td>
<td></td>
</tr>
<tr>
<td>Location*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial</td>
<td>28</td>
<td>2</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>Deep</td>
<td>34</td>
<td>8</td>
<td>23.5</td>
<td>0.0972</td>
</tr>
<tr>
<td>Spinal</td>
<td>4</td>
<td>1</td>
<td>25.0</td>
<td>0.6120</td>
</tr>
<tr>
<td>Lesion size*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small lesion</td>
<td>19</td>
<td>3</td>
<td>15.8</td>
<td>1.0000</td>
</tr>
<tr>
<td>(Ø≤10 mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large lesion</td>
<td>42</td>
<td>8</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>(Ø&gt;10 mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

*Information was not available with some cases enrolled in the present study.
years old; \( P = 0.0339 \); (3) of note, PTEN methylation only in one among 4 blood cell samples from familial CCMs, which was PTEN methylation-positive in surgical specimens (a blood sample was not available from another PTEN-methylated familial case), suggesting a potential tissue-specific PTEN methylation for familial CCM (Figure 1B); and (4) a significant higher frequency of methylation was seen in CCMs with multiple lesions (46.7\%) in comparison with the single lesion cases (11.8\%; \( P = 0.0215 \)). A tendency for higher numbers of PTEN methylation was also observed in pediatric CCMs (33.3\%) as compared with adult cases (13.1\%). The frequency of PTEN methylation did not differ between female and male, small lesions and large lesions as well as between deep lesions and superficial lesions.

For the patient who received surgeries twice within a 3-year interval, we confirmed that both specimens (separately removed in 2003 and in 2006) were PTEN methylation-negative. This result was not surprising because the specimen from the secondary operation was not a de novo-formed lesion.

**Correlation Between Promoter Methylation and the Expression of PTEN**

To further evaluate the correlation of PTEN promoter methylation status and PTEN level, PTEN expression was detected in the surgical specimens of PTEN-methylated (\( n = 11 \)) and -unmethylated (\( n = 14 \)) CCMs as well as in the control brain tissue (\( n = 8 \)) by Western blot. As illustrated in Figure 2A, PTEN expression was downregulated in both PTEN-M and -UM CCMs in comparison with the control. Semiquantification of the integrate optical density ratio of the target protein (PTEN) to actin revealed a 2-fold and 4- to 5-fold lower levels of PTEN in PTEN-unmethylated (CCM-UM, \( P < 0.05 \)) and in methylated CCMs (CCM-M, \( P < 0.01 \)), respectively, in comparison with the controls. Furthermore, PTEN expression in CCM-M was significantly lower than that in CCM-UM (\( P < 0.05 \); Figure 2B).

To confirm this finding, immunohistochemistry detection of PTEN was performed in all PTEN methylation-positive cases (\( n = 11 \)) and in randomly selected PTEN-unmethylated cases (\( n = 39 \)). PTEN immunoreactivity was detected in the neurons of the positive control section from epilepsy resection (Figure 3A-d) but absent in the negative control (Figure 3A-c). Due to the enriched capillary-like vessels lined by a single layer of endothelial cells in the lesions, we focused on the PTEN expression in the vascular endothelial layer. The immunostaining data were summarized in Table 2 and illustrated in Figure 3A. PTEN immunoreactivity was detected both in the cytoplasm and in the nuclei of endothelial cells (Figure 3A-a). Reduced PTEN expression was frequently detected in the vessels (Figure 3A-b) of PTEN-methylated cases as well as in some vessels of PTEN-unmethylated CCMs. Analysis of PTEN immunostaining scores indicated a significantly higher frequency of PTEN downregulation in PTEN-methylated cases (\( P = 0.0059 \)).

To study the possible association of PTEN expression and the status of cell proliferation, double immunofluorescence of PTEN and proliferating cell nuclear antigen (PCNA), a proliferation marker, was performed in 20 randomly selected CCM sections. We found that PTEN expression was inversely correlated with the expression of PCNA in the majority of endothelial layers of vessels of all sections (Figure 3B–C). This result suggested a potential role of PTEN in the regulation of endothelial proliferation.
Correlation of PTEN Expression With the Levels of P-Akt and P-Erk1,2

To study the potential signaling pathways affected by PTEN downregulation, the levels of P-Akt and P-Erk1,2 were detected by Western blot in 25 surgical specimens of CCMs and in 8 control specimens as illustrated in Figure 2A. Semiquantification of the blots indicated a 3- and 4-fold increased expression of P-Erk1,2 in CCM-UM (P < 0.001) and in CCM-M (P < 0.001), respectively, in comparison with the controls. Of note, the increase in the levels of P-Erk1,2 was more pronounced in CCM-M than that in CCM-UM (P < 0.05), which was inversely correlated with the expression of PTEN. However, no significant difference of P-Akt expression was observed among CCM-UM, CCM-M, and controls (Figure 2B).

Discussion

In the present study, we investigated whether PTEN promoter methylation was involved in CCMs. The methylation status of the PTEN promoter was correlated to the clinical manifestations as well as to the expression pattern of PTEN and to the potential targeted downstream molecules and signaling pathways. We reported here for the first time an implication of PTEN promoter methylation in CCMs with a significant higher frequency in familial CCMs and in multiple lesions. Furthermore, PTEN methylation was associated with a marked PTEN downregulation, indicating the involvement of this epigenetic mechanism in the deficits of this protein in CCMs. PTEN expression was inversely correlated with an increased immunoreactivity of PCNA and to the activation of Erk1,2, but not of Akt. These findings suggested PTEN as a novel molecule involved in the pathogenesis of CCMs and furthermore as a potential molecular biomarker for familial CCMs.

Table 2. Summary of PTEN Loss Detected by Immunohistochemistry in CCMs

<table>
<thead>
<tr>
<th>Groups</th>
<th>PTEN Loss Score*</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>UM-CCM, % of cases (n=39)</td>
<td>44</td>
<td>15</td>
</tr>
<tr>
<td>M-CCM, % of cases (n=11)</td>
<td>27</td>
<td>18</td>
</tr>
</tbody>
</table>

*PTEN expression was scored as 0 to 4 representing the 0% to 20%, 21% to 40%, 41% to 60%, 61% to 80%, and 81% to 100% of vessels showing PTEN deficiency, respectively.
†The difference of PTEN loss score between UM-CCM and M-CCM was analyzed by Wilcoxon test.
UM-CCM indicates PTEN promoter unmethylated CCM cases; M-CCM, PTEN promoter methylated CCM cases.
counts for PTEN silencing in numerous neoplasms. An interestingly, PTEN methylation was found in benign tumor. An increased DNA methylation was also observed in vitro and in vivo models of ischemia. These data indicate possible involvement of DNA methylation in the pathogenesis of certain noncancerous diseases. We demonstrated the involvement of PTEN promoter methylation in CCMs with particular high impact for familial CCMs. Because all familial CCMs enrolled in the present study were multiple lesion cases, a significantly higher frequency of PTEN methylation was thus also observed in multiple CCMs in comparison to the single CCMs. It was noted that sporadic CCMs with multiple lesions showed a higher tendency of PTEN methylation than CCMs with a single lesion. Stahl et al suggested that these probands with multiple CCMs are most seemingly familial due to an asymptomatic parent. Thus, PTEN promoter methylation in these sporadic cases with multiple lesions may be indicative of a potential risk of familial CCMs.

Although CCMs usually become clinically manifest in the second and fourth decades of life, one fourth of CCMs occur in children. In the present study, MSP revealed a clear tendency of a higher frequency of PTEN methylation in the pediatric group (33.3%) as compared with adults (13.3%); however, the difference between these 2 groups did not show statistical significance due to the low number of children enrolled in the study. The study of PTEN methylation in a larger series of pediatric CCMs may clarify whether PTEN promoter methylation serves as a biomarker for an earlier statistical significance due to the low number of children enrolled in the study. The study of PTEN methylation in a larger series of pediatric CCMs may clarify whether PTEN promoter methylation serves as a biomarker for an earlier prediction of a potential development of CCM, particularly for those children with a familial tendency of CCM.

Increasing evidence has pointed to an association of PTEN promoter methylation with the tumor progression as well as poor outcome and short survival of patients with tumors. Whether PTEN promoter methylation plays a causative role in CCMs remains unclear. In the present study, PTEN promoter methylation occurred in 5 among 6 familial CCMs, and strikingly, all these 6 cases exhibited CCMI mutations according to the genetic diagnosis. Plummer et al recently reported typical vascular defects, mimicking CCM lesions in Ccm1<sup>-/-</sup> and p53<sup>-/-</sup> double mutant mice, but not in Ccm1<sup>-/-</sup>- mice, indicating that loss of p53 sensitized mice to the development of CCMs. Our finding raises a question whether PTEN promoter methylation in CCMs functions synergistically with CCMI gene mutations contributing to the pathogenesis of familial form of CCM. Further study is needed to prove this issue.

The Association of PTEN Promoter Methylation With Downregulation of PTEN in Cerebral Cavernous Malformations

An aberrant promoter DNA methylation is a key epigenetic mechanism mediating gene silencing and function loss in cancer. We demonstrated a significant reduction of PTEN expression in PTEN-methylated CCMs in comparison with that in PTEN-unmethylated CCMs by Western blot (Figure 2) and by immunostaining (Table 2), respectively, suggesting that PTEN promoter methylation resulted in PTEN downregulation in the lesion. It is noted that PTEN expression in PTEN-unmethylated CCMs was also significantly lower than that in control brain tissue as revealed by Western blot (Figure 2). This may be at least partially explained by the presence of the neuron-sourced PTEN in the lysis of the control brain tissue. On the other hand, besides PTEN promoter methylation, other mechanisms like PTEN mutations, LOH at the PTEN locus on chromosome 10q23 may also lead to PTEN loss. Activation of the transcriptional factor nuclear factor kB was shown to suppress PTEN expression. In addition, PTEN expression can be influenced at the posttranslational levels. The mechanism of PTEN downregulation in PTEN-unmethylated CCMs needs to be further studied.

It has been shown that loss of PTEN function results in cell proliferation, increased cell viability, and tumor invasion. A frequent PTEN insufficiency observed in the vascular endothelial cells of CCMs raised our interest to check the proliferation status of these cells. The expression of the PCNA in endothelial cells has been considered as an indication of angiogenetic bioactivity of a vessel. Double immunostaining revealed an increased expression of PCNA in the endothelial cells where PTEN was missing or downregulated, suggesting a potential input of PTEN insufficiency to the activated angiogenesis in the lesions.

Possible Signaling Pathways Affected by PTEN Downregulation in Cerebral Cavernous Malformations

PI3K/Akt signaling is one of best characterized downstream pathway of PTEN through its lipid phosphatase activity. As a dual-specificity protein phosphatase, PTEN is able to activate the Ras/MAPK/Erk1,2 signaling pathway. In the present study, we tested whether downregulated PTEN expression influenced PI3K/Akt and MAPK/Erk1,2 pathways in CCMs. Interestingly, the levels of P-Akt were not significantly different between CCMs and the controls, suggesting that PI3K/Akt signaling was not markedly altered by PTEN downregulation in CCMs. The disassociation of PTEN expression and Akt activation was also reported by others. Indeed, Lue et al and Dey et al showed a cell growth suppression effect of nuclear PTEN and the inhibition of glioma cell migration by PTEN, respectively, through Akt signaling-independent mechanisms. Considering the significant input of the constitutively activated Akt signaling to tumor formation and progression, our findings may explain why CCM is a dominantly noncancerous vascular disease although PTEN was significantly downregulated in the lesions and support the notion that PTEN deficiency activates distinct downstream signaling in a tissue- and disease-specific manner.

MAPK/Erk signaling is commonly activated by growth factors to transmit signals from the receptors to regulate target gene expression. Furthermore, activation of MAPK/Erk signaling can in turn induce growth factors. Many of these factors like vascular endothelial growth factor and basic fibroblast growth factor are also angiogenetic factors. We and others have previously reported an increased expression of angiogenetic factors in CCMs. The increase in P-Erk1,2 levels in CCMs suggested a possible negative regulatory function of PTEN on MAPK/Erk1,2 signaling. A marked activation of MAPK/Erk1,2 signaling in CCMs may establish an
autocrine loop, which results in the continuous stimulation of endothelial cell growth, proliferation, migration, and finally an aberrant angiogenesis.

In summary, PTEN promoter methylation characterized in CCMs suggested an implication of this epigenetic mechanism in the pathogenesis of this disease. PTEN methylation, synergistically with CCM gene mutations, may potentially contribute to the familial CCMs. Identification of an apparent activation of Erk1,2, but not of the Akt pathway in CCMs, contribute to the familial CCMs. Identification of an apparent

Acknowledgments

We thank Ms Sabine Motzny for technical assistance.

Source of Funding

This study was financially supported by research funds from University Hospital of Marburg.

Disclosures

None.

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Stroke. published online December 31, 2008;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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