Eight middle cerebral artery (MCA) specimens were obtained from six patients [16], [17]. In addition, the expression of various angiogenesis-related genes proliferation, the media layer thickness is thinner [2], [10], and some lipid deposits [3], [7], [8], [9]. Despite there is an intimal intimal thickening with a multilayered elastic lamina and have demonstrated that the main vascular lesion in MMD particularly polymorphism in familial cases suggest the involvement of a genetic factor, MMD in Japanese population and high proportion of changes in the brain [1], [2], [3]. The high incidence of vessels) is formed as a result of progressive ischemic changes in the brain [1], [2], [3]. The high incidence of MMD in Japanese population and high proportion of familial cases suggest the involvement of a genetic factor, particularly polymorphism in RNF213 gene [4], [5], [6].

Pathological evaluations on autopsy subjects have demonstrated that the main vascular lesion in MMD is steno-occlusion of distal ICA caused by a fibrocellular intimal thickening with a multilayered elastic lamina and some lipid deposits [3], [7], [8], [9]. Despite there is an intimal proliferation, the media layer thickness is thinner [2], [10], and the smooth muscle cells undergo apoptosis [11].

Previous studies have revealed an abnormal expression of various angiogenesis-related genes and their products in MMD [2], [12], [13], [14], [15]. Of note, those studies mainly focused on a single or few interrelated genes or products at a time. Several studies reported that mutant RNF213 mice could not recapitulate MMD pathology in vivo [16], [17]. In addition, the phenotype also could manifest as secondary to pre-existing diseases [18], [19], [20], [21].

Hence, pathobiology of MMD might involve multiple pathways rather than single pathway. In this study, we collected tiny pieces of middle cerebral artery (MCA) walls from patients with MMD and analyzed global gene expression using RNA microarray to explore the novel mechanism of remodeling of the arterial walls in the MMD patients.

Materials and Methods

Patients' population

This study was performed under our institution guidelines provided by the ethics committee
in our center as being described in our previous reports [3], [10], [11], [22]. Six patients underwent superficial temporal artery to MCA (STA-MCA) bypass procedures for the treatment of MMD at our center. In addition, we included two patients with large cerebral artery aneurysms that underwent bypass procedure as control. Clinical data of the patients were summarized in Table 1.

**Sample collection**

During STA-MCA bypass surgery, a 10 or 11–0 nylon monofilament was passed around the wall of the recipient artery. Then, vessel was pulled up by lifting the monofilament with forceps, and the operators (YT, SM) performed arteriotomy with micro-scissors (Figure 1). Then, collected tissues were rinsed with cold phosphate-buffered saline and immediately placed into RNAlater (Ambion, Austin, TX, USA) and stored at −80°C until RNA extraction. The control specimens were obtained in a similar manner as being described in our previous reports [3], [10], [11], [22].

**RNA preparation**

Total RNA was isolated separately from 6 MMD and 2 control MCA specimens using RNAqueous-Micro Kit (Ambion, Austin, TX, USA). The concentration and quality of RNA were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Wilmington, DE, USA). The extracted RNA was used for further procedures.

**Microarray analysis**

Microarray analysis was conducted by DNA Chip Research Inc. (Yokohama, Japan). RNA amplification was performed with Ovation® Pico-WTA (NuGEN Technologies, Inc., San Carlos, CA, USA). In brief, 500 pg of total RNA was reversely transcribed with random and oligo-dT primer. Then, the resultant first-strand complementary DNA (cDNA) was used to make the second strand. The purified cDNA was processed with SPIA® primer (NuGEN Technologies, San Carlos, CA, USA) and DNA polymerase to make a sufficient dose of cDNA. Purified cDNA was labeled with cyanin3, then hybridized to the Agilent Whole Human Genome DNA microarray 4x44K ver. 2.0 (Agilent Technologies, Inc., Wilmington, DE, USA). After washing, the microarray chip was scanned with DNA MicroArray Scanner (Agilent Tech.).

**Data analysis**

The data were digitalized with Feature Extraction ver. 9.5.3 (Agilent Technologies, Inc., Wilmington, DE, USA) as the manufacturer’s protocol. The digitalized data were imported into GeneSpring GX 11 (Agilent Technologies, Inc., Wilmington, DE, USA) and processed with the 75th percentile shift and followed by quantile normalization. Normalization also performed among the genes with adjusted baseline to median of all sample. Each spot data were flagged in accordance with the feature and cut off if the feature is saturated, not uniform, population outlier, not positive, not significant, or not above background.

Analysis was performed using the base-2 logarithm transform of the normalized signal data with Office Excel 2010 (Microsoft, WA, USA). Welch’s t-test was used to identify the differentially expressed genes in MMD compare to control, and p < 0.05 was considered statistically significant. The genes were ranked according to their fold change and selected by criteria of fold change >2.0 for further pathway analysis with the online database PANTHER [23].

**Results**

As difficulty is expected, before the RNA preparation of these rare human surgical samples, we optimized our protocol of RNA extraction through

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**Table 1: Clinical features of 2 Control and 6 patients with MMD**

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Onset symptom</th>
<th>Surgery</th>
<th>Concomitant vascular risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>F</td>
<td>Cerebral aneurysm</td>
<td>Incident</td>
<td>STA-MCA bypass &amp; clipping</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
<tr>
<td>52</td>
<td>F</td>
<td>Cerebral aneurysm</td>
<td>SAH</td>
<td>ECA-MCA bypass &amp; trapping</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>MMD</td>
<td>Adult/Ischemia</td>
<td>STA-MCA bypass</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
<tr>
<td>43</td>
<td>M</td>
<td>MMD</td>
<td>Adult/Ischemia</td>
<td>STA-MCA bypass</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>MMD (Unilateral)</td>
<td>Adult/Ischemia</td>
<td>STA-MCA bypass</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
<tr>
<td>42</td>
<td>F</td>
<td>MMD (Unilateral)</td>
<td>Adult/Ischemia</td>
<td>STA-MCA bypass</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
<tr>
<td>44</td>
<td>F</td>
<td>MMD</td>
<td>Adult/Ischemia</td>
<td>STA-MCA bypass</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
<tr>
<td>41</td>
<td>F</td>
<td>MMD (Unilateral)</td>
<td>Adult/Ischemia</td>
<td>STA-MCA bypass</td>
<td>HTN (-), Dyslipidemia (-), DM (-), Smoking (-)</td>
</tr>
</tbody>
</table>
animal experimentation in mice. We found that the manufacture’s protocol was effective for RNA extraction from a small piece of mice blood vessels. However, the quality of extracted RNA from surgical specimens was not entirely satisfactory. RNA integrity number below ideal value for microarray analysis ($r < 7$). We decided to accomplish the microarray as the screening work with the current available method.

Figure 2: Normalized gene expression of distal MCA from MMD patients with $>2.0$ fold change. A total of 921 genes were distinctly regulated, 499 genes were up-regulated, and 422 genes were down-regulated.

In this study, 42,405 gene probes remained after filtering for flags. Then, we selected the gene expression based on fold change $>2.0$ and $p < 0.05$; as a result, 921 genes were found distinctly regulated between MCA of MMD and control. MCA of MMD was found to up-regulate 499 genes expression and down-regulate 422 genes expression compare to control (Figure 2).

Figure 3: Normalized gene expression of distal MCA from MMD patients in angiogenesis pathway with $>2.0$ fold change. A total of 12 genes were distinctly regulated, 10 genes were up-regulated, and 2 genes were down-regulated.

Among them, for example, SOS1 (Fold change: 6.01 detected with each probe), AKT2 (5.71), OCLN (5.40), CD34 (4.38), HEY1 (3.68), ITGB2 (3.63), CLDN5 (3.40), SELP (2.86), NES (2.39), BAMBI (2.38), IL1B (2.31), STAT1 (2.27), APOE (1.95), RB1 (1.94), ELN (1.93), NOTCH4 (1.86), ENG (1.48) and TLR4 (1.47) are up-regulated, and ACTN2 (2.72), IGFBP4 (2.61), SOX4 (2.27), DES (1.92), JunD (1.89), ETV6 (1.80), AKT1 (1.27), and XBPI (1.09) are down-regulated. In this study, major angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), or RNF213, did not show a significant change in this study. On the other hand, Endoglin, previously reported as highly expressed in the endothelium of cerebral arterial wall of MMD patients, also up-regulated in this study.

Figure 4: Normalized gene expression of distal MCA from MMD patients in inflammatory pathway with $>2.0$ fold change. A total of 22 genes were distinctly regulated, 16 genes were up-regulated and 6 genes were down-regulated.

To investigate gene interaction, we clustered the gene probes according to signaling pathway with PANTHER database. In this study, at least five pathways were distinctly regulated in MMD: Angiogenesis (Figure 3), inflammation (Figure 4), integrin (Figure 5), WNT (Figure 6), and platelet-derived growth factor (PDGF) (Figure 7). SOS1 was shown to involve in all pathways, but WNT pathway. AKT2 was shown to involve in angiogenesis, inflammation, and PDGF pathway. Therefore, suggesting the importance of SOS1 and AKT2 in MMD.

Discussion

MMD is a steno-occlusive disease, primarily affects for brain vessels, or at least affects predominantly in cerebral arteries, and the etiology of this unique pathological condition remains elusive [1], [2], [24]. Recently, RNF213 was identified as a susceptibility gene for MMD. RNF213 is located in chromosome 17q25.3 and c.14576 G>A SNP increased the risk of having MMD, particularly in Asian population [4], [5], [6], [25].
Deletion of RNF213 in zebrafish results in abnormal sprouting of intracranial vessels and inter-segmental arteries [4]. The over-expression of RNF213 in endothelial cells results in poor tube formation and decreased of proliferation rate [26]. However, mice with RNF213 mutation failed to recapitulate MMD phenotype [16], [17]. The pathology of MMD artery is characterized by intimal thickening with fibromuscular hyperplasia and media layer thinning, remains inexplicable through previous RNF213 studies. Hence, another approach is necessary to elucidate MMD pathobiology.

AKT2 is a member of the protein kinase B family and acts as a downstream of RAS pathway, has been implicated in several cancer [28]. SOS1 is a member of guanosine nucleotide exchange factors that increases nucleotide exchange rate of GDP for GTP, resulting in RAS activation. Clinically related syndrome to SOS1 mutation is Noonan syndrome, a member of RASopathies [29]. Noonan syndrome is a congenital malformation characterized by variable congenital heart defects, short stature, skeletal malformations, and distinct facial anomalies [30]. In line with our results, moyamoya vasculopathy has been reported in patients with Noonan like-disorders [31], [32], [33], [34], [35], [36]. Moyamoya vasculopathy is also likely to occur in patients with other forms of RASopathies. Other forms of RASopathies: Cardiofaciocutaneous syndrome and Von Recklinghausen disease also reported to have a higher risk of having moyamoya vasculopathy [37], [38].

Nevertheless, our study has a major limitation due to the rarity of the specimens, we could not confirm the relevant gene expression on tissue samples. In this study, we also obtained a low RNA integrity number, suggesting some RNA has been degraded before extraction. RNA is susceptible for hydrolysis and rapidly degenerates, many manipulations and long duration for tissue harvesting, and probably the surgical field and tools were not RNAse free, all those factors may contribute to low RNA integrity number. We recommend for harvesting larger specimens and utilize RNAse free
surgical tools to minimize enzymatic degradation of RNA.

Nevertheless, this investigation is useful as a biomolecular screening of abnormal pathways with the current available method. In line with our previous study, the distal MCA of patients with MMD involves in MMD pathology and has a distinct molecular expression in comparison to control distal MCA. In this study, RAS activation was observed in the distal MCA of patients with MMD, presumably contributes to intimal proliferation and stenosis of ICA, and thus plays a role in MMD pathology. Further investigation is needed to confirm the role of RAS pathway in MMD.

Conclusions

Through microarray analysis, we obtained that gene expression of distal MCA of patients with MMD was distinctly regulated in comparison with control. At least 5 pathways were altered in MMD: Angiogenesis, inflammation, integrin, PDGF, and WNT. Furthermore, SOS1 and AKT2 were the most altered genes in those aforementioned pathways. Nevertheless, our understanding about MMD pathobiology is still immature, further study is necessary.

References


PMid:6823668


PMid:8969773


PMid:9761057


PMid:42. https://doi.org/10.1111/j.1440-1789.2004.00550.x

PMid:15484702

PMid:2040653

PMid:1528455

PMid:26315378

PMid:26126547

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PMid:8969773

PMid:9761057

PMid:1528455

PMid:26315378

PMid:26126547

PMid:10.1161/01.str.14.1.50
Moyamoya syndrome associated with neurofibromatosis


