Circular RNAs and neutrophils: Key factors in tackling asymptomatic moyamoya disease

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Abstract:

Moyamoya disease (MMD) represents a rare steno-occlusive disorder affecting the terminal ends of the internal carotid artery and promoting the development of a poor, abnormal vascular network at the brain's base. Primarily affecting East Asian countries over Western populations, MMD can be further divided into symptomatic and asymptomatic subtypes. The current knowledge of the underlying mechanisms and potential management strategies for asymptomatic cases of MMD are largely lacking and thus warrant investigation to elucidate the pathology of this rare disorder. Here, we assess research examining the expression profile of circular RNAs (circRNAs) of neutrophil transcriptome in asymptomatic MMD patients. These findings conclude that 123 differentially expressed circRNAs significantly contributed to metabolism, angiogenesis, and immune response. The hypoxia-inducing factor-1α signaling pathway was also revealed to be crucial in angiogenesis. We also evaluate current therapeutic options demonstrating the potential for MMD patients, such as EC-IC bypass and ischemic pre- and post-conditioning. These approaches combined with recent findings on the circRNA expression profile suggest a crucial role of anti-inflammatory and angiogenic-related mechanisms underlying MMD. Investigating the role of circRNAs and neutrophils in the asymptomatic MMD subtype may provide insight into its elusive pathology and direct future approaches to combat the progression of this rare disease.

Keywords:

Angiogenesis, anti-inflammation, asymptomatic, circular RNAs, hypoxia-inducing factor-1α, ischemic conditioning, microarray, moyamoya disease, neutrophils, stroke

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Involvement of Neutrophils and Circular RNAs in Moyamoya Disease

Moyamoya disease (MMD) is an uncommon cerebrovascular disorder characterized by stenosis of the terminal ends of the bilateral internal carotid arteries and development of an atypical, fragile vascular network at the base of the brain.[1,2] The distinctive characteristic of MMD is the progressive narrowing of the intima with the propagation of smooth muscle cells, excluding any indication of inflammation or atherosclerosis.[3] MMD can be further

classified as symptomatic or asymptomatic. Symptomatic patients endure ischemic attack, hemorrhage, or infarction, whereas the asymptomatic subtype can be diagnosed by high-resolution magnetic resonance imaging (HR MRI) without such indications. Commonly affecting East Asian populations,[4] the incidence of this rare disease is a bimodal distribution, peaking at 35–45 years of age and 5–9 years of age.[5]

While the exact mechanism of MMD remains unknown, our current grasp on its complex pathology involves genetic and acquired elements.[6] Genetic factors, specifically mutations in genes RNG213, ACTA2, HLA, TIMP, and MMP, have been involved in instances of MMD. In addition, MMD has been linked with factors beyond genetic

background, such as vessel wall damage, angiogenesis, immune responses, and thrombogenic factors.[7] Moreover, the role of noncoding RNAs (ncRNAs) (microRNAs and circular RNAs [circRNAs]) has been implicated in the development of MMD.[8-10] The expression profile of circRNAs has been explored in symptomatic MMD cases, revealing abnormal expression of at least 146 specific circRNAs.[10] However, the analysis of asymptomatic patients is less explored.[1]

Recent studies on circRNAs have revealed its emerging role in a variety of disorders.[11] Unlike other ncRNAs, a unique property of circRNAs is the back-splicing of linear host genes and covalent bonding to form a circular secondary structure. This covalent bonding also contributes to the increased stability of circRNAs by preventing exoribonucleases from degrading exposed ends.[12] In addition, these ncRNAs are highly abundant and conserved in the mammalian brain.[13] circRNAs are involved in transcription regulation, functioning as miRNA sponges as well as by binding RNA polymerase II.[11,14,15] In addition to the regulatory role of circRNAs in transcription, their dynamic and elevated expression during aging suggests the involvement of circRNAs in brain development.[12]

Neutrophils play a major role in the incidence of ischemic stroke as precursors and employ complex functions soon after stroke onset.[16] After recruitment to the lesion, activated neutrophils transmigrate into the tissue and secrete inflammatory agents including cytokines, ROS, and proteases to facilitate the disruption of blood–brain barrier (BBB).[17] Targeting the elaborate function of neutrophils in stroke progression represents a promising therapy to promote anti-inflammatory and defensive mechanisms.[18] Investigating the involvement of circRNAs and neutrophils in MMD may provide insight into its poorly understood pathology and potential course of treatments.

Microarray Reveals Differential Circular RNA Expression Profile of Neutrophils in Asymptomatic Moyamoya Disease

A study by Ma *et al.* examined the circRNA expression profile of neutrophil transcriptome in asymptomatic cases of MMD.[19] The profile was determined by comparative circRNA microarray analysis of neutrophil samples between asymptomatic MMD patients and healthy controls. Quantitative reverse-transcription polymerase chain reaction verified the microarray results. The study utilized Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses (KEGG) to predict the roles of variably expressed circRNAs. This microarray-based circRNA profiling revealed 123 circRNAs that were differentially expressed between the asymptomatic MMD patients and healthy controls. Fifty-four of these were upregulated, whereas 69 were downregulated compared to controls (fold change >2.0 and $P < 0.05$). The aberrantly expressed circRNAs were found by the GO and KEGG analyses to be primarily contributing to immune responses, metabolism, and angiogenesis in the asymptomatic subtype. Moreover, the results suggested a critical involvement of the hypoxia-inducing factor-1α (HIF-1α) signaling pathway in angiogenesis.

Aberrantly Expressed Circular RNAs in Neutrophil Transcriptome Provide Future Direction for Pathological Studies of Moyamoya Disease

MMD is a rare cerebrovascular disease primarily affecting the distal ends of the internal carotid artery, and subsequently the anterior cerebral artery and the start of the middle cerebral artery.[7] Hyperplasia and the progressive stenosis of the intima of the internal carotid artery, elastic lamina fluctuation, and middle membrane destruction are the main characteristics defining MMD pathology.[3] A previous study used microarray to investigate the circRNA expression profile in symptomatic cases of this disease and discovered 146 differentiation-expressed circRNAs contributing to the development of MMD.[10] The study by Ma *et al.* revealed 123 differentially expressed circRNAs among asymptomatic MMD patients, which may be involved in the disease pathogenesis.[19]

Previous studies investigating circRNAs determined a high abundance in the brain compared to other mammalian tissues, which suggests that their derivative from host genes such as NTRK2, HOMER1, and RTN4 is involved in the regulation of various brain functions.[20] In addition, circRNA levels of expression significantly differ between fetal and adult brains and between adult rhesus brains and aged brains.[21] These results suggest the involvement of circRNAs in brain development and aging processes.

In the study by Ma *et al.*, the analysis of circRNA expression of neutrophil transcriptome in blood remained consistent with previous findings in the field of MMD. Results of this research revealed the involvement of the aberrantly expressed circRNAs in angiogenesis and metabolic functions in symptomatic MMD patients. Furthermore, the contribution of blood vessel endothelial cell propagation in angiogenesis remained in line with the natural disease pathology of MMD.[19] Recent studies have examined the involvement of MMP-9, vascular endothelial growth factor (VEGF), Cav-1, and Ang-2 to gain understanding of the causal mechanism of the abnormal vessels.[22-24] Moreover, a current study

determined an upregulation of VEGF in the dura mater of patients with MMD and suggested a correlation to abnormal collateral vessel formation in pediatric MMD patients. In addition, VEGF signaling factors were revealed to be highly expressed in asymptomatic MMD cases compared to healthy controls.[25] Ma *et al.* discovered 16 specific circRNAs genes markedly expressed in the VEGF signaling pathway and the VEGF–Ras–ERK axis, suggesting a potential contribution to endothelial proliferation.[19] These findings provide a possible explanation for the activation of angiogenesis during the natural course of MMD.

It is generally accepted that the elevation of angiogenesis-related cytokines within the ischemic tissue region promotes puff neovessels. HIF-1α was discovered to be overly expressed within the thicker intima of MMD patients and localized together with transforming growth factor-β3 in the endothelium.[26] KEGG analysis in the study by Ma *et al.* underscored the term "HIF-1 signaling pathway," which accordingly activates HIF-1α/β and upregulates VEGF to enhance angiogenesis during hypoxic conditions.[27-29] The transcription factor HIF-1 contributes significantly to oxygen homeostasis.[30] The HIF-1α subunit faces hydroxylation under normoxia milieu, resulting in rapid ubiquitination and proteasomal degeneration. However, under hypoxia, HIF-1α maintains stability and cooperates with other activators to regulate transcriptional processes. HIF-1 functions upstream to regulate numerous genes contributing to hypoxia. In this regard, genes targeting HIF-1 produce proteins that function to attenuate oxygen deprivation in MMD. This suggests an RTK/HIF-1/VEGF mechanism involved in angiogenesis, contributing to our knowledge of asymptomatic MMD to direct future pathological studies.

Neutrophils are critical in their role as inducers of angiogenesis.[31] Inflammatory signals (e.g., VEGF-A, interleukin-8, or CXCL12) initiate the recruitment of neutrophils, which then secrete growth factors (VEGF-A and Bv8 [prokineticin 2]), chemokines, and MMP-9 to recruit other leukocytes to the damaged tissue.[32,33] In the current study, circRNA expression was found in the neutrophils of patient's blood. Varying expression has been demonstrated in the brain between circRNAs derived from astrocytes, glial cells, and neurons.[13] Compromised brain tissue suffers oxygen and glucose deprivation, triggering the release of cytokines and transmigration across the BBB. The neutrophils subsequently infiltrate the damaged tissue and exert their elaborate functions of the inflammatory response. The present study suggests that varying neutrophil phenotypes facilitate an inherent autoimmune state within asymptomatic MMD patients, as well as crucially promote angiogenesis. Furthermore, this study

was modeled utilizing a standard immune response microenvironment. A previous study investigating the pluripotency of stem cells and their key regulatory pathways in this inflammatory state may suggest the potential of a combination therapy involving stem cells and neutrophils. Specifically, amnion-derived stem cells were used to suggest therapeutic potential by harnessing the cells' multipotent differentiation and anti-inflammatory capabilities.[34,35] This growing knowledge of the role of neutrophils in the inflammatory environment may apply to future direction aiming to target mature neutrophils *in vivo*.

The further subtypes of symptomatic MMD represent an ongoing area of study among numerous populations to better understand the potential clinical therapies to prevent ischemic attack and attenuate deficits. However, current knowledge of the pathology of this rare disease remains elusive, and a therapeutic strategy has yet to be found for approaching asymptomatic cases. An Asymptomatic Moyamoya Registry study has been established in Japan to elucidate the prognosis and treatment strategies of asymptomatic MMD.[36] The knowledge gained from the present study exploring the circRNA expression profile in asymptomatic MMD patients may support future direction in the ongoing study of this rare disorder.

Current Status and Therapies for Moyamoya Disease: Revascularization, Ischemic Conditioning, and Underlying Anti-Inflammatory or Angiogenetic-Related Factors

Although several factors have been postulated to contribute to the pathology of MMD, including inflammatory, angiogenic, and genetic influences, the mechanisms underlying MMD remain poorly understood. Moreover, representative *in vitro* or *in vivo* model systems have yet to be confirmed. A review by Hamauchi *et al.* explores the immune and inflammatory-based models in the field of MMD, yet cites several drawbacks limiting their current application, such as an absence of intima wall thickening and vascular lesions. Of these, endothelial progenitor cells (EPCs) have gained attention as a potential *in vitro* focus.[37] Several studies have demonstrated abnormal levels of circulating EPCs in cases of MMD and further suggest their defective angiogenic-related function which may contribute to the disease pathogenesis.[4,38] The study by Ma *et al.* also investigated factors underlying the angiogenetic component of MMD, implicating differential neutrophil phenotypes.

While circulating EPCs gained attention for their role in MMD, the following studies identifying the ring

finger protein 213 (RNF213) gene R4810K variant as a susceptible locus dramatically changed the direction of research toward the genetic components contributing to the development of MMD. A recent study by Xue *et al.* demonstrated an association between the RNF 213 R4810K variant and increased risk of intracranial major artery stenosis/occlusion (ICASO) among 114 ICASO patients and 268 healthy controls among the Han Chinese population. Interestingly, HR MRI revealed that all female patients possessing the RNF213 variant displayed characteristics of MMD, yet only one male patient presented signs of an atherosclerotic disorder, but not MMD.[39] Marking the relevance of the study by Ma *et al.* in the development of MMD, future studies should explore the potential comparison of circRNA expression profile of neutrophil transcriptome among female and male populations.

With regard to the current therapeutic strategies for MMD, a very recent study examined the trends of utilization and outcomes of extracranial–intracranial (EC–IC) bypass in symptomatic steno-occlusive diseases.[40] The investigators analyzed 346 patients from two vastly diverse states, New York and Florida, across a 10-year period. Data obtained from this cohort analysis indicated a significant drop in the use of EC–IC bypass beginning in the year 2011. A possible explanation for this decrease may be the 2011 publication of the Carotid Occlusion Surgery Study, which concluded that EC–IC bypass did not provide any advantage for symptomatic patients. Even so, the study by Saber *et al.* reported that 30-day rates of complication, including instances of stroke, hemorrhage, or death, decreased in EC–IC bypass patients over the 10-year period.[40] However, an indirect bypass procedure such as encephalo-myo-synangiosis (EMS) is often instead performed in instances of pediatric MMD rather than direct surgical revascularization. Although simple, EMS can provide inadequate collateral flow. A study by Nishihiro *et al.* found that EMS combined with high-mobility group box-1 injection enhanced

angiogenesis via VEGF-dependent mechanisms, increasing cerebral blood flow (CBF).[41] These symptomatic cases are relevant to strategies targeting MMD, yet the asymptomatic subtype still remains less explored.

Weighing the need for surgical revascularization measures to the risk of ischemic attack remains a challenge in asymptomatic stenosis patients. As such, it is critical to establish suitable prognostic measurements. A recent study suggests the potential of plaque morphology and cerebral hemodynamics as reliable measurements of high-stroke risk in asymptomatic carotid artery stenosis,[42] which may have implications in MMD. Moreover, ischemic conditioning to attenuate the risk of stroke in asymptomatic patients with intracranial atherosclerotic arterial stenosis represents a potential management strategy.[43] Ischemic preconditioning (IPC) involves inducing moderate ischemia to exert protective functions against following severe ischemic events. Epigenetics has gained attention for its role in modulating the pathology and outcome of stroke. Recent studies reveal a unique miRNA expression following IPC; miRNA profiling 3 h after IPC discovered an upregulation of two miRNA families (miR-200 and miR-182) which were involved with neuroprotective effects among the prolyl hydroxylase 2 and HIF-1 pathways.[44,45] KEGG analysis performed in the study by Ma *et al.* of the circRNA profile highlighted this HIF-1 signaling pathway in upregulating VEGF and promoting angiogenesis.[19] These findings along with previous discoveries in the field further suggest a potential HIF-1/VEGF mechanism involved in angiogenesis.

Moreover, IPC has been found to promote anti-inflammatory mechanisms by modifying the expression of cytokines during ischemic insults, suggesting a critical role of the vasculature and endothelial cells during ischemic conditioning stimuli.[46] Recent studies also suggest the capacity of conditioning
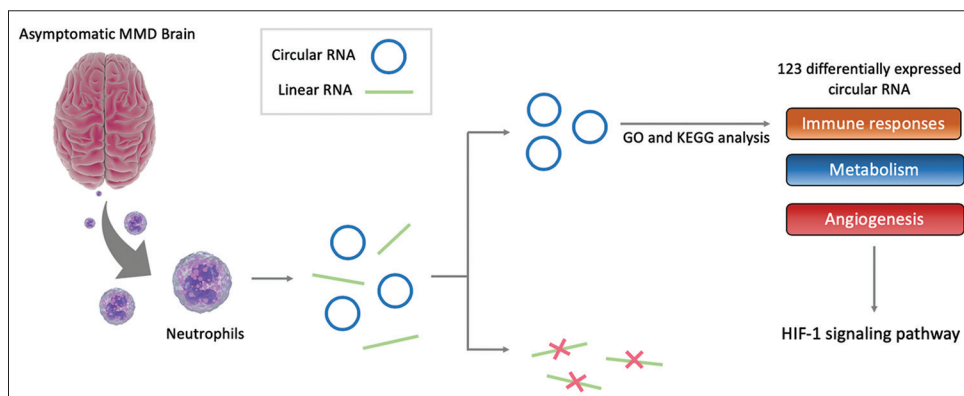

Figure 1: New data reveal that circular RNA expression profile suggests a crucial role of anti-inflammatory and angiogenic-related mechanisms underlying moyamoya disease, which may provide novel insights into the pathology of moyamoya disease

to attenuate endothelial dysfunction following acute myocardial infarction.[47] Ischemic postconditioning also represents a promising neuroprotective strategy against ischemic insult by the way of anti-inflammatory, anti-apoptotic, and CBF-based mechanisms.[48,49] The study by Ma *et al.* thus has significant relevance into strategies to approach MMD by suggesting the critical role of anti-inflammatory mechanisms, specifically involving neutrophils, in combatting the progression of this disease [Figure 1]. Future investigation into the potential effects of approaches such as conditioning stimuli on cases of MMD is thus warranted to further shed light on the pathology and therapeutic avenues of this rare disorder.

Conclusion

The study by Ma *et al.* reveals a critical function of circRNAs and neutrophils in differentiating asymptomatic MMD patients, suggesting the underlying anti-inflammatory and angiogenic factors. These findings suggest that targeting the complex role of neutrophils in the progression of MMD may be a potential approach to better understand this elusive disease.

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Conflicts of interest
There are no conflicts of interest.

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