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ORIGINAL RESEARCH

Downregulation of IL-20RA in Cerebrospinal Fluid Associated with the Risk of Moyamoya Disease: A Molecular Signatures Analysis with an Inflammation Proteomics Landscape

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Purpose: Moyamoya disease (MMD) is a cerebrovascular disorder with diverse clinical manifestations. Surgical revascularization is currently the optimal choice in the treatment of MMD; however, it could not prevent the progression of the disease. Inflammation and immunity factors have been reported to play the pivotal role in the pathogenesis of MMD, but there were still limited studies concerning the inflammatory landscape. Here, we aimed to investigate the molecular signatures of MMD to outline the inflammatory feature of MMD.

Patients and Methods: A total of 89 MMD patients and 93 healthy subjects were recruited for this study. We then divided all patients into screening cohort (15 MMD patients and 21 healthy subjects) and validation cohort (74 MMD patients and 72 healthy subjects). Proteomic analysis of the cerebrospinal fluid (CSF) was performed in the screening cohort, which contained 363 inflammation-related molecules. Then we used ELISA assay to validation of molecules of differential expression.

Results: We screened 192 inflammation-related proteins that were differentially expressed in the CSF of MMD patients. Among that, 191 proteins were upregulated, while IL-20RA were downregulated (p=0.042). The bioinformatic analysis found a potential inflammatory landscape of MMD, offering clues for pathogenetic and therapeutic targets in the mechanistic study. We then validated that downregulation of IL-20RA in CSF was associated with the risk of MMD in the validation cohort.

Conclusion: This study provided molecular signatures of MMD with a large-scale proteomic analysis of CSF. IL-20RA might be a key element in the pathogenesis of MMD.

Keywords: moyamoya disease, proteomics, IL-20RA, inflammation, angiogenesis

Introduction

Moyamoya disease (MMD) is an idiopathic progressive cerebrovacular disorder characterized by occlusion and stenosis of internal carotid artery (ICA) and the intracranial section of its proximal branches.[1,2] The subsequent formation of small and net-like compensatory collaterals usually leads to ischemic or hemorrhagic stroke.[3] The epidemiological study showed that the annual incidence of MMD was about 1.14 per 100000 inhabitants in China with the male-to-female ratio of 1:2.[4,5] Surgical revascularization is the optimal choice in treatment of MMD; however, the current treatment methods can not prevent the progression of vascular stenosis. Although it has been reported that the heritage plays a crucial role in MMD onset, especially in the Asian patients, the pathogenesis of MMD concerning the molecules is still unclear.[6] Therefore, research focusing on the potential therapeutics-targeting molecules of MMD is urgently needed.

In either hypothesis of the pathogenesis of MMD onset, inflammation and immunity have been reported to be essential pathophysiological markers in the occurrence and development of MMD.[7,8] The histopathology evidence of MMD showed the smooth muscle hyperplasia accompanied by infiltration of macrophages and T cells.[9] Some studies showed that inflammatory

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biomarkers were upregulated in the circulation, such as monocyte chemoattractant protein 1, interleukin 1 beta (IL-1β), and stromal cell-derived factor 1α.[10,11] Furthermore, in the vascular injury hypothesis, the angiopathy of MMD had similarities with that of diabetes and arteriosclerosis, and inflammation was the key factor.[12] Therefore, identifying biomarkers in MMD patients can promote our understanding of the disease's pathogenesis and progression.

Compared with the peripheral circulation, the environment in cerebrospinal fluid (CSF) can better reflect the micro-environmental condition of the disorders in central nervous system. So far, some studies, focusing on protein, microRNA and metabolites, have investigated the associations of biomarkers in CSF with the risk of MMD. Ota et al[13] analyzed extracellular vesicle-derived miRNAs in the CSF of MMD patients using next generation sequencing, and found that 153 upregulated miRNAs as well as 98 downregulated miRNAs were associated with MMD. Yu et al[14] utilized a liquid chromatography coupled with mass spectrometry approach to screen the metabolite levels in CSF and found 129 differentially expressed biomarkers. As for the inflammation-related biomarkers, Abhinav et al[3] examined the expression of 62 secretory biomarkers in the CSF of MMD patients, and the results showed that some cytokines and chemokines were associated with MMD. However, since not all of their biomarkers were related to inflammation or immunity, it is more specific and targeted to detect inflammatory and immune molecules in the CSF of MMD patients using a proteomics panel that includes as many inflammatory and immune molecules as possible.

In this study, we used a proteomic analysis method that contained 363 inflammatory biomarkers to detect the association between the molecules and the risk of MMD. Additionally, the aim of study may be also to provide pathogenic clues and potential therapeutic targets of MMD.

Materials and Methods

Patients and Sample Collection

In this study, a total of 89 MMD patients and 93 control subjects were randomly recruited. We included 15 MMD patients (including 6 male and 9 female, age: 47.60 ± 16.40) and 21 healthy subjects (including 9 male and 12 female, age: 47.95 ± 11.56) in the screening cohort, as well as 74 MMD (including 29 male and 45 female, age: 44.34 ± 16.31) patients and 72 healthy subjects (including 29 male and 43 female, age: 45.03 ± 16.78) in the validation cohort. The diagnosis of MMD was confirmed by two senior neurosurgeons with supporting evidence from magnetic resonance imaging. CSF samples were obtained from each patient via cistern drainage or lumbar puncture operations. The CSF samples were extracted from the populations who had secondary hydrocephalus. And the time interval between secondary hydrocephalus and primary neurological disorders was beyond 3 months. We defined these cohort as control subjects. The exclusion criteria were: 1) patients with other types of stroke (eg, subarachnoid hemorrhage, spontaneous intracerebral hemorrhage, arteriovenous malformation, and traumatic-induced intracranial hemorrhage); 2) inadequate amount or quality of CSF samples available for the study (eg, severely hemolyzed or contaminated sample during transportation or storage; 3) patients who comorbid with other neurological diseases or CNS infectious diseases; 4) patients who had systemic inflammatory diseases or malignant tumors. All samples were stored at −80°C. The demographic and clinicopathological data are summarized at Table 1.

Table 1 Demographic and Clinicopathological Data of Participants. There Are No Significant Differences of Age and Gender Between Moyamoya Disease (MMD) and Control Subjects

		Screening Cohort		Validation Cohort	
		MMD (n=15)	Control Subject (n=21)	MMD (n=74)	Control Subject (n=72)
Age		47.60 ± 16.40	47.95 ± 11.56	44.34 ± 16.31	45.03 ± 16.78
Gender					
	Male	6	9	29	29
	Female	9	12	45	43
Suzuki stage (II/III/IV/V)		5/5/2/3	–	27/21/17/9	–
Type					
	Hemorrhagic	15	–	47	–
	Ischemic	0	–	27	–

Proximity Extension Assay

The CSF samples in screening cohort were tested using the proteomics method. An inflammation-related panel containing 363 molecules were measured in the CSF of all participants using Proximity Extension Assay (PEA) technology on the Olink® Proteomics Multiplex Assay platform.[15] The assays were performed by Sinotech Genomics Co. Ltd. (Shanghai, China). Briefly, antibodies that specifically recognized target proteins were designed by conjugating DNA tags at the end. DNA tags hybridize to form paired double strands when the antibodies correctly match the protein. The DNA tag sequences were amplified by qPCR for quantitative detection. Results were expressed as Normalization Protein eXpression (NPX) values on log2-scale. The molecules contained in the platform were summarized at Supplementary Table 1.

Enzyme-Linked Immuno Sorbent Assay (ELISA) in Validation Cohort

The CSF samples in validation cohort were tested using ELISA assay. The CSF samples were obtained after centrifugation, and a standard solution was prepared according to the manufacturer's instructions. The Human IL-20RA ELISA Kit (cat. no. SEE768Hu, USCN Business Co., Ltd.) were obtained to perform the assays. The procedure of ELISA was basically according to the manufacturer's protocol; blank sample wells were used in the procedure.

Statistical Analysis

All statistical analyses were performed in R script (v. 4.0.3) with the primary analysis focusing on the CSF results. All continuous data were depicted as the mean±standard deviation (mean±SD) and then analyzed with Student's *t*-test. Binary data were analyzed using the chi-squared test. In the correlation analysis among the molecules in CSF, Pearson's correlation coefficients were assessed in each molecule. Receiver operating characteristic (ROC) curve analysis was conducted to assess the diagnostic efficacy of molecules in PNBM via the index of area under the curve (AUC). $P<0.05$ was considered to indicate statistical significance.

Results

Differential Expression of Inflammation-Related Protein Biomarkers in CSF of MMD in the Screening Cohort

In the screening cohort that contained 15 MMD patients and 21 healthy controls, we used a proteomic analysis with 363 inflammation-related molecules. First, we performed quality control for all samples. Figure 1A showed the distribution of NPX of all samples, the results indicated no outlier samples. Moreover, we performed principal component analysis (PCA), and the results indicated the two cohorts can be generally distinguished (Figure 1B). The overall proteomic expression was presented at Figure 1C. The results showed that 192 (52.89%) proteins were differentially expressed between the two cohorts ($p < 0.05$) (Figure 1C).

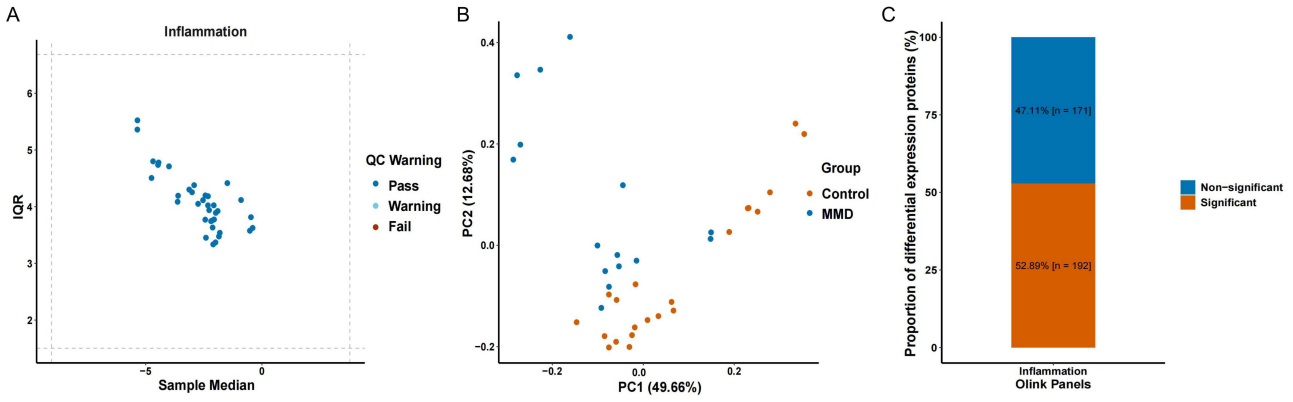

Figure 1 Quality control and general results for 36 samples in the proteomic analysis. (**A**) Distribution scatter plot of sample quality control with Normalization Protein eXpression (NPX) analysis. (**B**) Principal component analysis (PCA). (**C**) Proportion of differentially expressed proteins.

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By drawing heat maps of the NPX values of samples in different groups and performing hierarchical clustering on rows and columns, a global overview was provided for all samples (Figure 2A and B). Among all the differentially expressed (DE) molecules, 191 proteins were upregulated, while IL-20RA were downregulated (p = 0.042, Figure 2C).

Bioinformatic Analysis of DE Proteins Revealed Potential Pathogenic Pathways of MMD

We used multiple bioinformatic tools to predict subcellular locations and functional pathways that might provide potential information on the pathogenesis of MMD. We performed Gene Ontology (GO; Figure 3A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (Figure 3B) analyses to show the potential biological functions and pathways. The Benjamini-Hochberg correction method was applied to correct for multiple comparisons (false discovery rate < 0.05). The enriched KEGG signaling pathways were analyzed using the ClusterProfiler package in

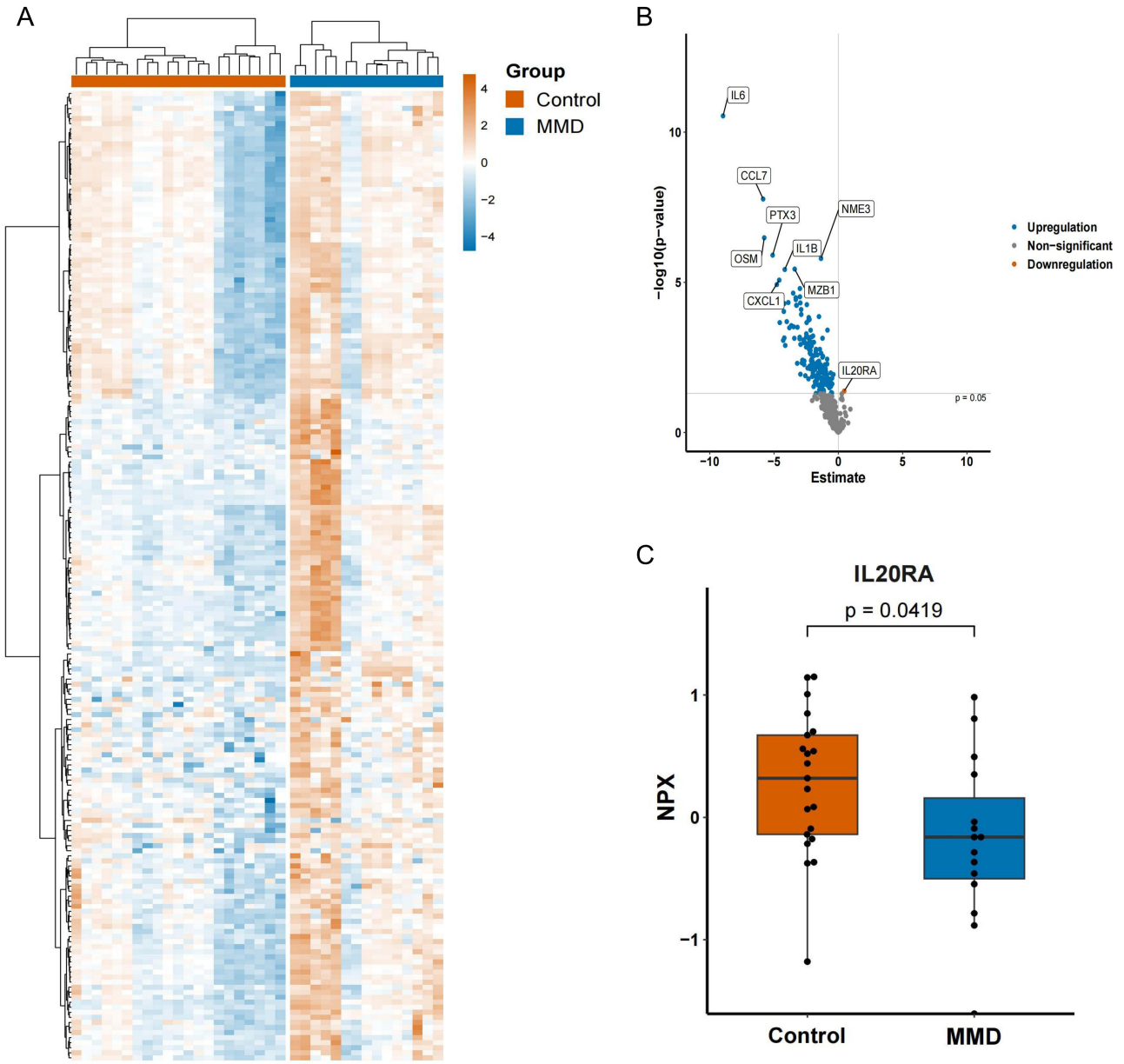

Figure 2 Protein expression profiles of CSF between the MMD and control group. (**A**) A heatmap of 363 proteins in all samples. (**B**) A Volcano plot of the proteins with differentially expressed. (**C**) NPX analysis of IL-20RA expression in CSF between the groups.

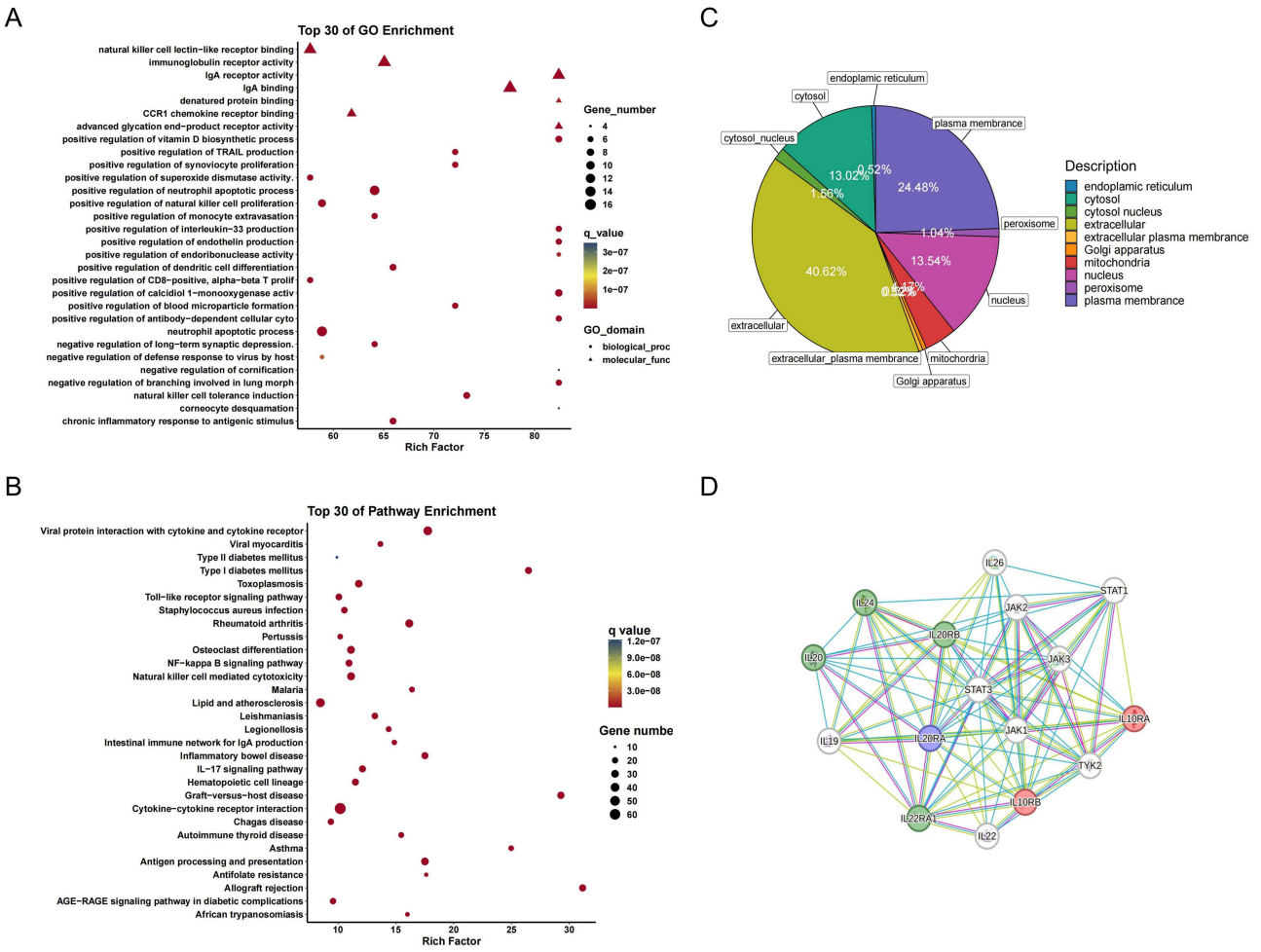

Figure 3 Bioinformatic analysis of DE proteins in MMD. (**A**) GO enrichment. (**B**) KEGG pathway enrichment. (**C**) Subcellular locations. (**D**) Protein–protein interactions. Node in purple: downregulated; node in red: upregulated; node in green: not significant; node in white: not tested.

R software (version 3.18.0) to demonstrate the biological actions of DE proteins in the patients with MMD and healthy control (HC). In the KEGG analysis, we found that IL-20RA involved 3 pathways: viral protein interaction with cytokine and cytokine receptor, cytokine–cytokine receptor interaction, and JAK-STAT signaling pathway. Additionally, the subcellular location analysis showed that the DE proteins mostly located in extracellular region (40.62%), plasma membrane (24.48%), nucleus (13.54%) and cytosol (13.02%) (Figure 3C).

IL-20RA Network with Protein–Protein Interaction (PPI) Analysis

Given that IL-20RA was the core molecule of this study, we performed the PPI network analysis not using DE molecules. Alternatively, we input IL-20RA as the core molecules and predicted the functional partners using the online database (https://cn.string-db.org). The results showed in Figure 3D that IL-20RA had significant interactions with JAK1, JAK2, JAK3, STAT1 and STAT3, indicating its involvement in the JAK-STAT signaling pathway. Furthermore, we also found that in other molecules that we tested in the proteomic analysis, IL-10RA and IL-10RB were upregulated in CSF of MMD patients, while IL-20, IL-20RB, IL-22RA1 and IL-24 had no statistical differences.

Validation of IL-20RA Expression in CSF of MMD Patients

We validated the IL-20RA expressions in CSF among 74 MMD patients and 72 healthy subjects using ELISA tests. The results showed that the concentration of IL-20RA of CSF in MMD patients (22.95 ± 7.16 pg/mL) was significantly lower than that in healthy subjects (31.59 ± 10.83 pg/mL) ($p < 0.001$) (Figure 4A). The result of validation set was in

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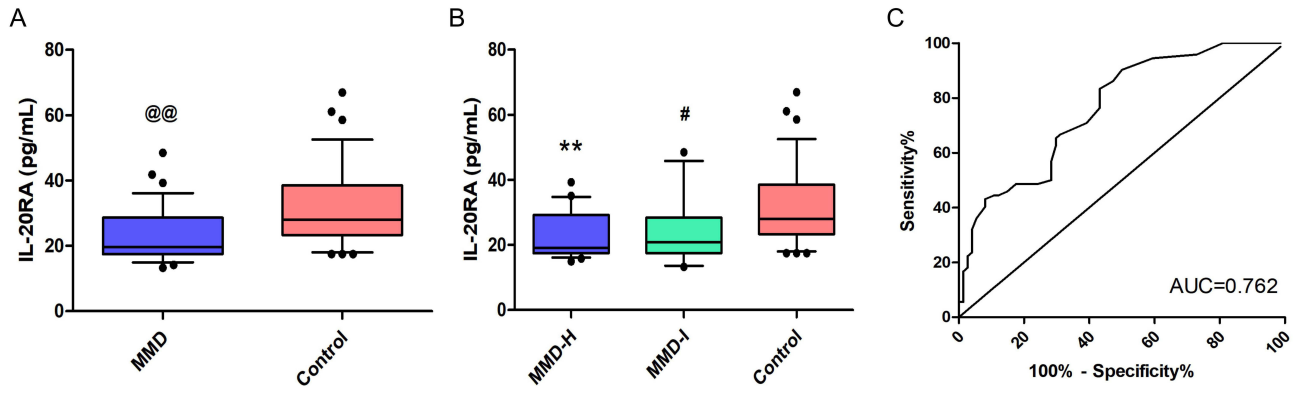

Figure 4 CSF levels of IL-20RA in validation cohort. (**A**) IL-20RA expression in CSF between general MMD patients and control cohort. (**B**) Sub-cohort analysis (MMD-H: moyamoya disease with hemorrhagic phenotype; MMD-I: moyamoya disease with ischemic phenotype). (**C**) ROC analysis. @@ indicates p<0.001 as compared to the control group; ** indicates p<0.001 to the control group; # indicates p<0.05 to the control group.

accordance with that of screening set. However, there was no statistical difference between MMD-hemorrhagic patients and MMD-ischemic patients (p = 0.616) (Figure 4B). Furthermore, we performed a ROC analysis of IL-20RA between the two cohorts. The result found a mild-to-moderate value (AUC = 0.762) of IL-20RA in diagnosis of MMD (Figure 4C). In regard to the clinical subtypes of MMD, we found the CSF levels of IL-20RA in both MMD-hemorrhagic patients (p < 0.001) and MMD-ischemic patients (p = 0.001) were significantly lower than that in healthy controls.

Discussion

In this study, we performed a proteomic analysis of CSF to investigate the association between inflammatory signatures and risk of MMD. The results showed that 192 inflammatory proteins were differentially expressed in the CSF of MMD patients in comparison with those of control subjects. Among that, we validated in another cohort that the downregulation of IL-20RA was significantly associated with MMD of both hemorrhagic and ischemic patients.

Increasing evidence showed that the pathogenesis of MMD stemmed from the combined effects of genetics and the environmental factors. In previous report, He et al performed a proteomic analysis that contained 14726 peptides and 1555 proteins by mass spectrometry method to detect the differentially expressed molecules in the serum of MMD patients. In combining with the results of in vitro experiments, they found the upregulation of FLNA and ZYX proteins were related to the pathology of cerebrovascular intimal hyperplasia in MMD.[16] Additionally, they also observed the upregulation of SAA2 protein in the serum of MMD patients and further demonstrated that it promoted phenotypic changes in vascular smooth muscle cells, indicating its relationship to cerebrovascular intimal thickening in MMD.[17] These data provided molecular and pathway information related to the pathogenesis of MMD from different perspectives, offering important references for the diagnosis and treatment of MMD.

Inflammation, infection and immunity were recognized as risk factors for MMD. Kim et al[18] found the autoantibodies in the CSF samples of MMD patients. Among that, CD40-mediated inflammation was reported to be a key factor of vascular wall-induced stenosis.[19] Furthermore, in another aspect, Liu et al combined machine learning with external validation methods to investigate the roles of genes related to necrotic inflammation and necrotic apoptosis in patients with moyamoya disease.[20] Additionally, some studies also found that the occurrence and development of MMD may have significant correlations with the imbalanced ratio of initial B cells, initial CD4 cells, resting natural killer cells, and regulatory T cells.[19,21,22] Furthermore, the unmutated pathogenic gene of MMD, RNF213, can protect cells from endoplasmic reticulum stress and inflammation that are induced by lipotoxicity, indicating a pivotal role of inflammation in the pathogenesis of MMD.[23] As far as we know, it's the largest panel of inflammatory factors in the association analysis of biomarkers in CSF with MMD. In reviewing of previous studies, we found that just 10 out of 192 inflammatory proteins in serum/CSF, including interleukin 1 beta (IL-1β), IL-6, IL-10, IL-18, (interstitial collagenase) MMP-1, interferon gamma (IFN-γ), C-X-C motif chemokine 1 (CXCL1), CXCL9, hepatocyte growth factor (HGP) and

tumor necrosis factor (TNF).[3,10,24–27] Meanwhile we also found 3 molecules, including C-C motif chemokine 4 (CCL4),[28] CCL20[29] as well as T-cell surface glycoprotein CD4 (CD4)[30] were significantly associated with MMD in transcriptome level, and tumor necrosis factor receptor superfamily member 5 (CD40),[19] pyruvate kinase (PKLR)[31] and transforming growth factor beta-1 proprotein (TGF-β1)[32] were correlated to MMD in genetic level. Furthermore, a study reported the involvement of tumor necrosis factor receptor superfamily member 11A (TNFRSF11A) in the mechanistic pathogenesis of MMD.[33] While other 175 proteins have not been investigated. These molecules provided information about the inflammatory microenvironment of CSF for the patients with MMD, which could help the understanding of the pathogenesis of MMD in the aspects of inflammation and immunity. Besides, given that the balance of anti- and pro-inflammatory reactions plays an important role in the development of MMD, the inflammatory profiles would also offer potential therapeutic targets. Further mechanism studies that were based on the DE biomarkers and bioinformatics-related pathways should be performed. Among all the DE molecules in CSF of MMD patients, some molecules, such as IL-1β, IL-6, and IL-10, have been studied in clinical and basic research on various types of stroke, because they were reported to be the inflammatory biomarkers in secondary brain injury after stroke. As the only down-regulated protein, IL-20RA, after reviewing relevant literature, we found that this protein has not been reported in MMD-related studies. However, IL-20RA has been previously found to be related to autoimmune diseases, neuroinflammation, and neuroimmunity. These data suggest that it may play an important role in the pathogenesis of MMD, and we speculate that IL-20RA and its related pathways may be important factors affecting the pathogenesis of MMD. Therefore, we chose to detect it in an expanded sample. Therefore, we validated its expression in an augmented cohort with another platform. The results showed that the downregulation of IL-20RA might be significantly associated with both hemorrhagic and ischemic phenotypes of MMD. IL-20RA gene, locating at 6q23, is one of the member of IL-10 family.[34] The heterodimer of IL-20RA and IL-20RB constitutes IL-20 receptor type 1, and could specifically binds to IL-20R cytokines, such as IL-19, IL-20, and IL-24 and thus generate intracellular signals, consequently involving in inflammatory responses and tissue repairs.[35–37] However, there are still limited mechanistic studies of IL-20RA in CNS disorders. Some reports demonstrated that the IL-20RA/RB complex activated JAK-STAT signaling pathway, inducing downstream cascade reaction and regulating the expression of various genes involved in immune responses and cell growth in autoimmune diseases and tumors.[38,39] Other studies also showed that IL-20 cytokines may also impact neuroinflammatory and neurodegenerative processes within the CNS.[40,41] IL-20 family cytokines impacts microglial activation, the immune response initiated by microglia, the CNS's resident immune cells, which release pro-inflammatory cytokines when reacting to injury or infection.[42] On the other hand, Li et al[43] performed a Mendelian Randomization analysis and found the association of IL-20RA with diabetic microangiopathy. Recently, Dayton et al[44] found the high expression of IL-20RA in human brain microvascular endothelial cells, and it could produce signaling in blood-brain barrier via IL-20RB, thus activating neuroinflammatory reactions. In our study, we found the downregulation of IL-20RA in the CSF samples of MMD, indicating either a loss of anti-inflammatory signaling or resolution of inflammation, that is, an anti-inflammatory response. However, considering the expression results of other molecules in Figure 3D, where the levels of IL-20, IL-24, IL-20RB, and IL-22RA1 did not change significantly, while the levels of IL-10RA and IL-10RB increased, we believe that the neuroinflammation in MMD is a complex change and cannot be directly defined as the activation of the anti-inflammatory effect of the IL-10 family. A further validation should be performed to explore the detailed mechanism of IL-20RA-related signaling pathway in the pathogenesis of MMD.

Additionally, in the pathway enrichment analysis, the autoimmunity-related disorders, such as type I diabetes, asthma, allograft rejection, and graft-versus-host disease, emerged as top-ranked pathways. In clinical practice, the proportion of patients with Moyamoya disease who also have some kind of autoimmune disease seemed to be higher than that in the general population.[45] This coexistence suggests that there might be some connection between the two types of disease, but the specific mechanism remains unclear. Ge et al found the dysfunction of circulating immune cell and T-cell abnormalitiesin MMD patients, including a reduction in effector T cells, an increase in Tregs, diminished natural killer cells and dendritic cells, and an dysregulation in the ratio of fragile and stable Tregs.[46] Furthermore, Li et al used the immune infiltration analysis and observed the different abundances of the eosinophils, natural killer T cells, Th2 cells in the middle cerebral artery of MMD.[45] Additionally, Mineharu et al demonstrated that the expression of GATA2 in peripheral circulation increased the penetrance of the RNF213 p.R4810K variant.[47] These results indicated that

autoimmunity, such as type 17 inflammation and type 2 inflammation, may act as a trigger or accelerator, especially in individuals with genetic susceptibility.

Some limitations for our study should be carefully concerned. First, although we used a two-step method to investigate the association between IL-20RA level in CSF and risk of MMD, the overall number of patients was still relatively small. Considering that the samples originated from a single center, another validation in an augmented population and from multi-centers should be also performed. Furthermore, in this study, we only utilized proteomics screening and ELISA verification methods to investigate the association between IL-20RA and MMD, without further verification in the in vitro experiments. Systematic experiments are still needed to explore how IL-20RA and its related molecules and signaling pathways affect angiogenesis through inflammation and immunity. Additionally, the control group population included in our study was not an absolutely healthy population, but those with secondary hydrocephalus. Although the time interval of secondary hydrocephalus and primary neurological disorders was beyond 3 months, we found that in the reports on IL-10 and idiopathic normal pressure hydrocephalus (iNPH), two studies showed that the level of IL-10 in the CSF of iNPH patients did not change significantly.[48,49] Additionally, the level of IL-6 in the CSF of iNPH patients was significantly increased.[49] Based on the above information, we proposed in the limitation section that since our control group samples were not completely healthy patients, the results should be considered carefully, to avoid false positives.

Conclusion

In conclusion, we performed a proteomic analysis to investigate the molecular signatures of CSF, offering potential inflammatory landscapes of MMD. Furthermore, the downregulation of IL-20RA in CSF was validated to be associated with the risk of MMD.

Data Sharing Statement

The original contributions presented in the study are included in the article/Supplementary Material further inquiries can be directed to the corresponding author (Lei Ye).

Ethics Approval and Informed Consent

This study involving human participants was conducted according to the Declaration of Helsinki and was approved by the Institutional Ethics Board of the First Affiliated Hospital of Anhui Medical University. Written informed consents were obtained from all participants.

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Disclosure

The authors declared no conflicts of interest in this work.

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