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RESEARCH ARTICLE

Potential Biomarkers in Serum for Ischemic Stroke

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ABSTRACT

Early detection of ischemic stroke is essential for initiating treatment before irreversible brain injury occurs. This study aimed to identify novel serum biomarkers capable of detecting ischemic stroke in early stages, prior to the development of severe brain damage caused by cerebral artery occlusion. Male Sprague-Dawley rats (9 weeks old; *n*= 3 per group) underwent middle cerebral artery occlusion for 0 minutes (sham), 30 minutes (0.5 hour), 1 hour, 2 hours, or 3 hours. Next-generation sequencing of brain tissue identified integrin subunit alpha M (*Itgam* ; CD11B), C-C motif chemokine ligand 2 (*Ccl2*), and alpha-2-macroglobulin (*A2m*) as candidate biomarkers. Western blot validation analysis revealed that CD11B and CCL2 levels progressively increased in both brain tissue and serum, correlating with occlusion duration and the extent of brain damage. In contrast, A2M expression decreased in brain tissue but increased in serum with longer occlusion times. Notably, in the 0.5 hour occlusion group, no visible brain damage was observed by 2,3,5-triphenyltetrazolium chloride staining; however, serum levels of CCL2 and A2M were significantly elevated compared to the sham group. These findings suggest that CD11B, CCL2, and A2M are promising early biomarkers for ischemic stroke. Increases in their serum concentrations occur at the earliest stages of ischemia, potentially enabling early diagnosis and therapeutic intervention before irreversible brain damage becomes detectable by conventional imaging techniques.

Keywords: Ischemic stroke, biomarker, CD11B, CCL2, A2M

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Introduction

Stroke is a major cerebrovascular disorder characterized by impaired cerebral blood flow resulting from either arterial occlusion (ischemic stroke) or vessel rupture (hemorrhagic stroke), leading to focal or global neurological deficits (Paul & Candelario-Jalil, 2022). It is the second leading cause of mortality worldwide and a significant contributor to long-term disability (Campbell et al., 2019). Over 85% of stroke-related deaths occur in low- and middle-income countries, which also account for 81% of global disability-adjusted life years lost to stroke (Feigin, 2007; Kalkonde et al., 2019). The global annual incidence of stroke is estimated to exceed 35

million cases, ranking it as the second most common cause of death after ischemic heart disease and the third leading cause of disability worldwide (Kuriakose & Xiao, 2020).

Of the two primary types, ischemic stroke is more prevalent, representing for approximately 80–85% of all cases. It occurs when a cerebral artery is blocked, triggering a cascade of pathophysiological events such as hypoxia, inflammation, and neuronal death, ultimately resulting in focal brain injury (Majumder, 2024; Qiu et al., 2021). Neuronal and tissue damage may occur rapidly after vascular occlusion, underscoring the urgent need for prompt diagnosis and intervention (Saludeen et al., 2024) (Fig. 1).

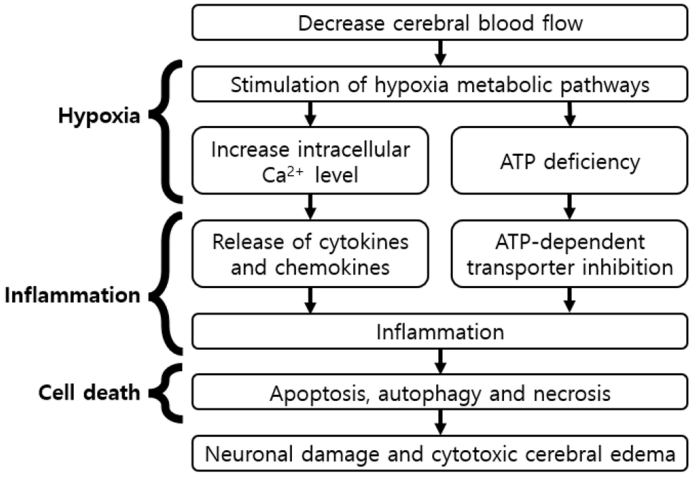

Fig. 1. The pathophysiological mechanisms of ischemic stroke. Brain injury in ischemic stroke results from a cascade of biological processes started by reduced cerebral blood flow, leading to hypoxia, inflammatory responses, and subsequent neuronal cell death.

Despite extensive research into the molecular and cellular mechanisms underlying ischemic stroke (Chen et al., 2023; Zhao et al., 2022), our understanding remains incomplete, and effective pharmacological therapies remain unavailable (Crowe et al., 2016). Addressing key clinical challenges including the narrow therapeutic time window and the limited efficacy of existing interventions is critical to improving outcomes across a broad patient population. Neuroprotective strategies that enhance tissue regeneration and repair have shown potential to reduce neuronal damage and partially restore function, thereby improving prognosis (Brown, 2021).

In this context, identifying reliable serum biomarkers for early ischemic stroke detection is paramount (Herpich & Rincon, 2020). Although several candidate molecules have been proposed as prognostic indicators or therapeutic targets (Kamtchum-Tatuene & Jickling, 2019), none have yet demonstrated adequate sensitivity, specificity, or cost-effectiveness for routine clinical implementation. Further research is required to validate and develop biomarkers with translational potential for clinical practice (Qin et al., 2022).

In the present study, we investigated the expression profiles of serum proteins associated with ischemic stroke pathophysiology to evaluate their potential as early diagnostic biomarkers. Transcriptomic analysis identified three inflammation-related genes as candidate markers: C-C motif chemokine ligand 2 *(Ccl2*), integrin subunit alpha M (*Itgam*; encoding CD11B), and alpha-2-macroglobulin (*A2m*). CCL2, a chemokine involved in monocyte recruitment,

is significantly upregulated in both brain and blood following ischemic injury and is associated with stroke risk and acute brain damage (Angiolillo et al., 2004; Stonesifer et al., 2017). CD11B, a surface marker of activated myeloid cells, is upregulated in vascular endothelium during inflammation and contributes to intravascular plaque formation and microcirculatory dysfunction (Yao et al., 2015). A2M, a broad-spectrum protease inhibitor, plays a role in coagulation and inflammatory regulation; elevated serum levels have been linked to endothelial dysfunction and white matter lesion severity in stroke patients (Varma et al., 2017). Alterations in the circulating levels of CCL2, CD11B, and A2M may thus serve as early biomarkers of ischemic stroke and represent promising targets for timely diagnosis and therapeutic intervention.

Materials and Methods

Animals and Middle Cerebral Artery Occlusion (MCAO) Model

Nine-week-old male Sprague-Dawley rats (*n*= 3 per group) were obtained from Daehan Biolink (Eumseong, Korea). All animals were housed under environmentally controlled conditions: a constant temperature (23 ± 3 °C), relative humidity (50 ± 10%), and a 12-hour light/dark cycle. Rats had ad libitum access to standard rodent chow and purified water (Yoon et al., 2022). After a one-week acclimatization period, ten-week-old rats underwent MCAO following established protocols. Anesthesia was induced using isoflurane (Hana Pharm. Co. Ltd., Hwaseong, Korea), and rats were placed in a supine position. After a midline cervical incision, a nylon suture was inserted through the external carotid artery (ECA) and advanced into the internal carotid artery to occlude the origin of the middle cerebral artery, inducing cerebral infarction (Sommer, 2017; Li & Zhang, 2021). For transient MCAO, the rats were re-anesthetized at 0.5 to 3 hours post-occlusion, and the suture was removed via the ECA stump. All procedures were performed in accordance with the guidelines from the Laboratory Animal Center at Chungbuk National University (CBNU), and all experimental procedures were approved by the Institutional Animal Care and Use Committee of CBNU (IACUC; Approval No. CBNUA-1527-21-02).

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

Brains were collected from MCAO rats, frozen, and coronally sectioned into 2 mm-thick slices using a stainless-steel brain matrix (Roboz Surgical Instrument Co. Inc., MD, USA). The sections were incubated in 2% of TTC (Sigma-Aldrich, Seoul, Korea) at room temperature for 20 minutes to assess infarcted regions (Aronowski et al., 1999).

Next-generation Sequencing and Bioinformatics Analysis

NGS data were analyzed to identify differentially expressed genes (DEGs) significantly upregulated or downregulated in the 0.5 hour and 3 hours MCAO models. DEGs were functionally annotated using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to identify enriched biological processes and pathways. Genes associated with the pathophysiological mechanisms of ischemic stroke and conserved in humans were further

screened. Protein-protein interaction (PPI) networks were constructed using STRING and visualized in Cytoscape software to identify candidate hub genes.

Western Blot Analysis

Western blot analysis was performed on both brain and serum samples derived from normal (wild-type, WT) and MCAO model rats. Samples were mixed with an equal volume of 2X Laemmli sample buffer (Bio-Rad Laboratories, Inc., CA, USA), diluted with distilled water (Crowe et al., 2016), and denatured in sodium dodecyl sulfate (SDS) buffer. Proteins were separated on 5–10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were probed with specific primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (Fig. 2). A list of primary and secondary antibodies used is provided in Table 1. Band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized to actin.

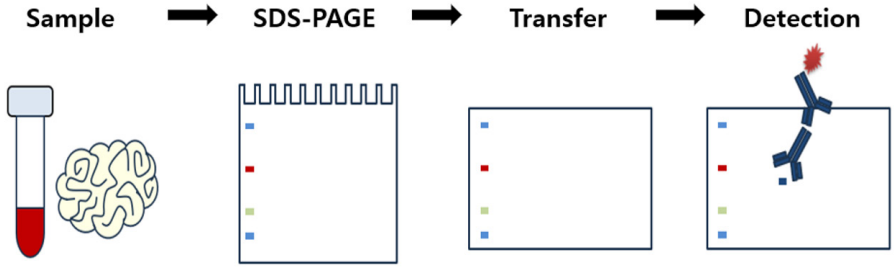

Fig. 2. Western blotting of undepleted brain and serum samples. Proteins were denatured in SDS buffer, separated by SDS-PAGE (5-10%) electrophoresis gels, and transferred to PVDF membranes. Target proteins were detected using primary antibodies and HRP-conjugated secondary antibodies.

Table 1. List of antibodies used in the current study

Epitope	Company	Dilution	2nd Ab (IgG)
A2M	Bioss	1:1000	Rabbit
MAP2	Cell Signalling Technology	1:1000	Rabbit
CEP55	Cell Signalling Technology	1:1000	Rabbit
CD45	Abcam	1:500	Rabbit
CD11B	Abcam	1:1000	Rabbit
MCP1	Abcam	1:1000	Rabbit
MAP1A	Abcam	1:1000	Rabbit
Actin	Cell signaling Technology	1:1000	HRP-conjugation
Goat anti-rabbit IgG	Jackson ImmonoResearch	1:1000	-

Statistical Analysis

The normality and homogeneity of variance were assessed for all datasets before statistical testing. Group comparisons were conducted using one-way analysis of variance (ANOVA). Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± standard error of the mean (SEM), and a p-value < 0.05 was considered statistically significant.

Results

Occlusion Time-Dependent Progression of Infarct Volume

The infarcted area of the brain was assessed using TTC staining (Sanchez-Bezanilla et al., 2019; Schiffner et al., 2017). Infarcted regions appeared white, while viable tissue was stained red. In the MCAO models, infarcts were observed in the cortex and dorsolateral striatum at various time points following occlusion (0.5 hour, 1 hour, 2 hours, 3 hours) (Ferrer et al., 2003). At 0.5 hour post-occlusion, infarction was restricted to the striatum; however, with occlusion durations of 1 hour or longer, infarcts areas appeared in both the striatum and cortex (Chiang et al., 2011) (Fig. 3A). Notably, in the 3 hours MCAO group, the infarct volume accounted for approximately 75 ± 3% of the ipsilateral hemisphere (Fig. 3B).

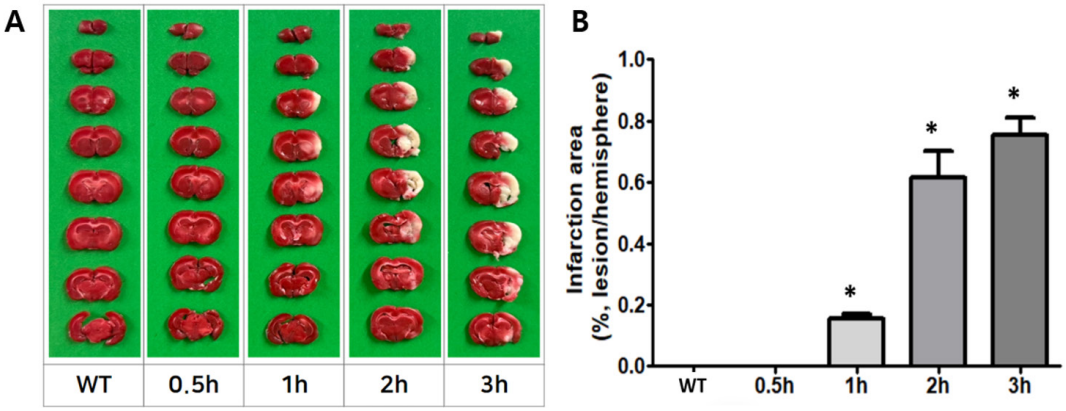

Fig. 3. Correlation between occlusion time and infarct area. The infarct area increased with occlusion time. (A) Representative images of TTC-stained brain slices from normal (WT) and transient MCAO models at 0.5, 1, 2, and 3 hours. White indicates infarction tissue; red indicates viable tissue. (B) Quantification of infarct area. Data are presented as mean ± standard error of the mean (SEM). * Significantly different from normal control ($p < 0.05$).

Next-Generation Sequencing Data Analysis

To identify potential biomarkers of ischemic stroke, we conducted a comprehensive gene expression analysis comparing brain samples from normal and MCAO models at two time points (0.5 hour and 3 hours). The analysis was carried out in a sequential workflow consisting of NGS, DEGs, comparative DEG analysis across time points, functional annotation and initial candidate filtering, PPI network construction, hub gene identification, and final selection of candidate biomarkers.

NGS was performed to generate transcriptomic data from the brain tissues of normal and MCAO rats. DEGs were identified by comparing each MCAO group to the normal group. A total of 473 DEGs were detected, including 410 upregulated and 63 downregulated genes in the MCAO models relative to normal (Fig. 4A). To assess temporal expression dynamics, Venn diagrams were generated to visualize overlapping DEGs between the 0.5 hour and 3 hours MCAO groups (Fig. 4B). GO and KEGG pathway enrichment analyses revealed associations between the DEGs and key ischemic stroke-related processes such as hypoxia, inflammation, and apoptosis. To narrow the list of potential biomarkers, we cross-referenced the identified genes with existing literature and selected only those whose protein products are known to be detectable in human serum. This filtering step resulted in 42 genes that were consistently

regulated at both time points and associated with ischemic stroke pathophysiology.

These 42 genes were then subjected to PPI network analysis using the STRING database. The network was visualized in Cytoscape, and CytoHubba algorithms were applied to identify hub genes based on connectivity and centrality within the network (Fig. 4C). By integrating biological relevance to ischemic stroke, detectability in human serum, and network centrality, six candidate biomarkers were selected. Among them, four genes—protein tyrosine phosphatase receptor type C (*Ptprc*; CD45), C-C motif chemokine ligand 2 (*Ccl2*), integrin subunit alpha M (*Itgam*; CD11B), and alpha-2-macroglobulin (*A2m*)—were associated with inflammatory responses. The remaining two, centrosomal protein 55 (*Cep55*) and microtubule-associated protein 1A (*Map1a*), were linked to cell death-related mechanisms.

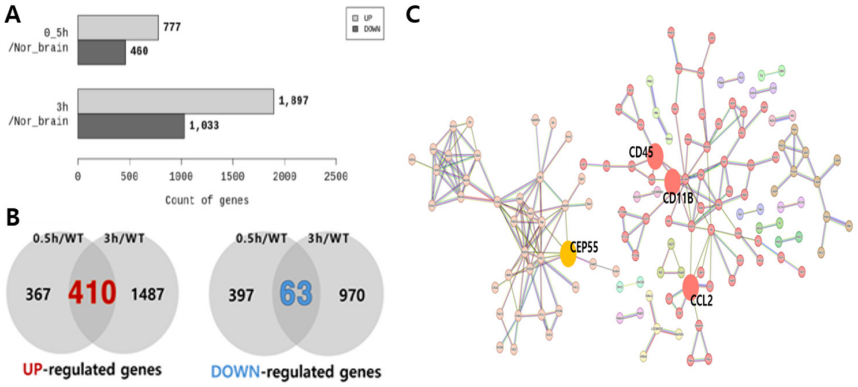

Fig. 4. NGS data analysis. NGS data were analyzed to identify candidate genes for the early diagnosis of ischemic stroke. (A) Number of upregulated or downregulated DEGs in 0.5 hour and 3 hours MCAO groups compared to normal. (B) Venn diagram showing overlapping DEGs between 0.5 hour and 3 hours and normal. (C) PPI networks and Hub-genes among DEGs were visualized in Cytoscape. Genes in the red cluster are associated with inflammation; genes in the yellow cluster are related to cell death.

Gene Expression as Potential Biomarkers for Ischemic Stroke in Samples

In brain tissue, MAP1A, CEP55, and CD45 expression levels were elevated in the 0.5 hour group compared to normal (n= 3 per group). As occlusion time increased, CD11B and CCL2 expression levels progressively increased. In contrast, A2M expression decreased (Fig. 5A). Quantification of protein bands confirmed that the expression levels of these potential biomarkers changed in a time-dependent manner (Fig. 5B). CD11B (R^2= 0.9125) and CCL2 (R^2= 0.9247) showed strong linear increases with occlusion time (R^2> 0.9, p< 0.05). In contrast, A2M exhibited a significant inverse correlation with occlusion time (R^2= 0.6054, p< 0.05). MAP1A (R^2= 0.6101), CEP55 (R^2= 0.4179), and CD45 (R^2= 0.5804) showed moderate correlations with occlusion time (Fig. 5C).

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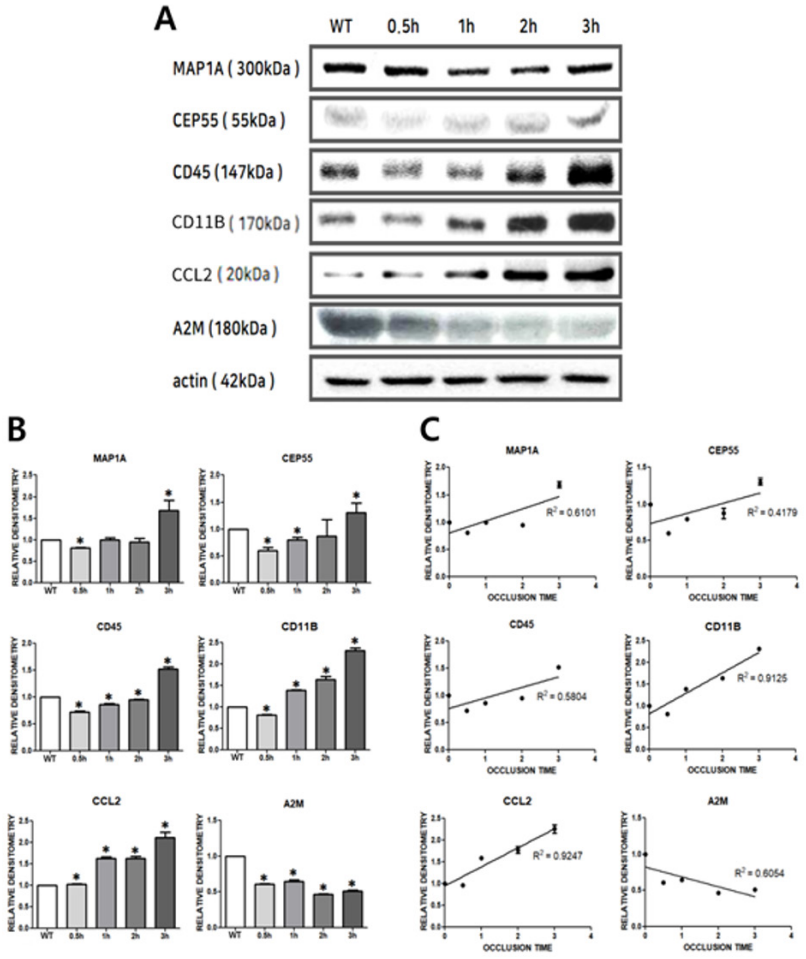

Fig. 5. Expression of proteins associated with ischemic stroke pathogenesis in brain tissue . (A) Western blot analysis of protein levels in normal (WT) and MCAO groups from 0.5 to 3 hours post-occlusion. Band intensities were normalized to actin and quantified using densitometry. (B) Densitometric analysis of western blot signals expressed relative to normal levels. (C) Linear regression analysis of biomarker expression levels versus occlusion time. Data are presented as mean ± standard error of the mean (SEM). * Significantly different from normal control ($p < 0.05$).

As three genes—*Itgam*, *Ccl2*, and *A2m* —showed significant dysregulation in brain tissue, they were prioritized for protein-level analysis, with MAP2 included as a control. In serum samples, expression levels of CD11B, CCL2, A2M, and MAP2 increased with prolonged occlusion time (Fig. 6A). Quantification of protein bands confirmed time-dependent changes in expression (Fig. 6B). All three biomarkers exhibited upregulation in serum, consistent with trends observed in brain tissue. CD11B showed a strong linear correlation with occlusion time ($R^2 = 0.7351$, $p < 0.05$), while CCL2 ($R^2 = 0.6627$) and A2M ($R^2 = 0.5811$) showed moderate correlations. MAP2 demonstrated a near-perfect correlation ($R^2 = 0.9842$), further supporting assay specificity (Fig. 6C).

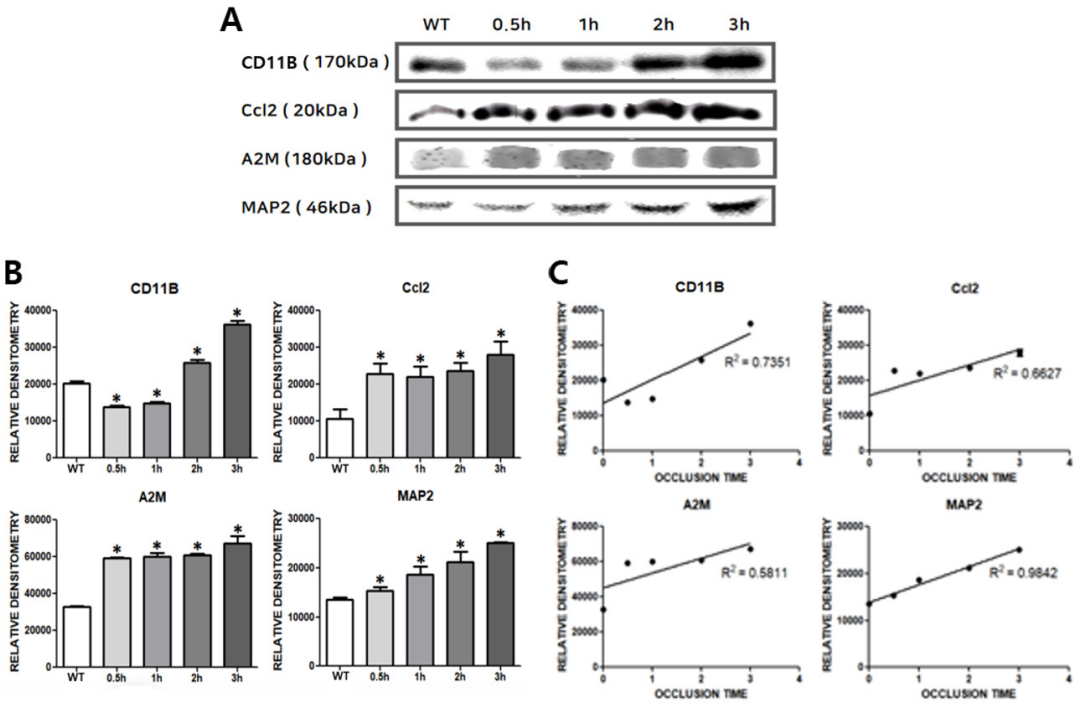

Fig. 6. Expression of proteins associated with ischemic stroke pathogenesis in serum. (A) Western blotting analysis of protein levels in normal (WT) and MCAO groups from 0.5 to 3 hours post-occlusion. Band intensities were normalized to actin and quantified using densitometry. (B) Densitometric analysis of western blot signal expressed relative to normal levels. (C) Linear regression analysis of biomarker expression levels versus occlusion time. Data are presented as mean ± standard error of the mean (SEM). * Significantly different from normal control ($p < 0.05$).

Discussion

In this study, we investigated alterations in protein expression levels associated with the pathological mechanisms of ischemic stroke. Six genes were identified as potential early diagnostic biomarkers. Among them, four genes—protein tyrosine phosphatase receptor type C (*Ptprc*; CD45), C-C motif chemokine ligand 2 (*Ccl2*), integrin subunit alpha M (*Itgam*; CD11B), and alpha-2-macroglobulin (*A2m*)—are involved in inflammatory responses, while the remaining two—centrosomal protein 55 (*Cep55*) and microtubule-associated protein 1A (*Map1a*)—are associated with cell death. Each of these genes is functionally linked to key pathophysiological processes in ischemic stroke.

Among the identified candidates, several genes are directly implicated in the inflammatory and degenerative pathways associated with ischemic stroke. For instance, CD45 may contribute to blood-brain barrier (BBB) disruption by activating innate immune responses and promoting inflammation, exacerbating brain injury (Shen et al., 2023). Similarly, CCL2 plays a dual role in promoting neuroinflammation, aiding recovery, or driving secondary damage post-stroke (Stonesifer et al., 2017). Complementing this, CD11B is strongly upregulated in response to vascular occlusion, amplifying inflammatory and immune responses (Wilden et al., 2022). In contrast, A2M, a protease inhibitor, has been closely linked to ischemic stroke pathology; its dysfunction is predictive of neuronal and tissue damage when dysfunctional (Varma et al., 2017). While the previous markers are linked to inflammation, CEP55 has been implicated in neuroinflammation and neuronal apoptosis, making it relevant to central nervous system diseases such as spinal cord

injury (Shi et al., 2021).

Changes in circulating levels of these proteins suggest their utility as early serum biomarkers and potential therapeutic targets (Lyu et al., 2024). Among the six candidates, CD11B, CCL2, and A2M showed significant time-dependent expression changes correlating with occlusion duration. In brain tissue, CD11B and CCL2 exhibited strong linear correlations with occlusion time (R^2= 0.9125 and 0.9247, respectively), with similar trends observed in serum (R^2= 0.7351 and 0.6627, respectively), supporting their involvement in early inflammatory responses. A2M showed an inverse correlation in brain tissue (R^2 = 0.6054), likely reflecting leakage into the bloodstream, as supported by its time-dependent increase in serum (R^2= 0.5811).

Both CD11B and CCL2 are associated with neutrophil infiltration and BBB disruption, consistent with their acute-phase upregulation in both brain and serum. In contrast, the opposing expression pattern of A2M—decreasing in the brain but increasing in circulation—may reflect its redistribution from the central nervous system to the periphery in response to oxidative stress and inflammation. Collectively, our results highlight CD11B, CCL2, and A2M as promising early-stage biomarkers detectable in serum samples for ischemic stroke, capable of identifying ischemic events before they are detectable by conventional imaging techniques such as CT or MRI.

Reduction in cerebral blood flow due to thrombosis induces hypoxic conditions, which subsequently trigger inflammatory responses. These inflammatory processes upregulate the expression of A2M, leading to the inhibition of the NF-κB signaling pathway. Suppression of NF-κB modulates the expression of CD11B, resulting in the increased secretion of pro-inflammatory cytokines such as CCL2. This cascade amplifies neuroinflammation and promotes cell death, ultimately accelerating the progression of stroke. Based on these findings, we propose CD11B, CCL2, and A2M as potential biomarkers for the early diagnosis of ischemic stroke. Given their involvement in neuroinflammatory responses to disrupted cerebral blood flow, these proteins represent promising targets for clinical application and therapeutic intervention. (Fig.7).

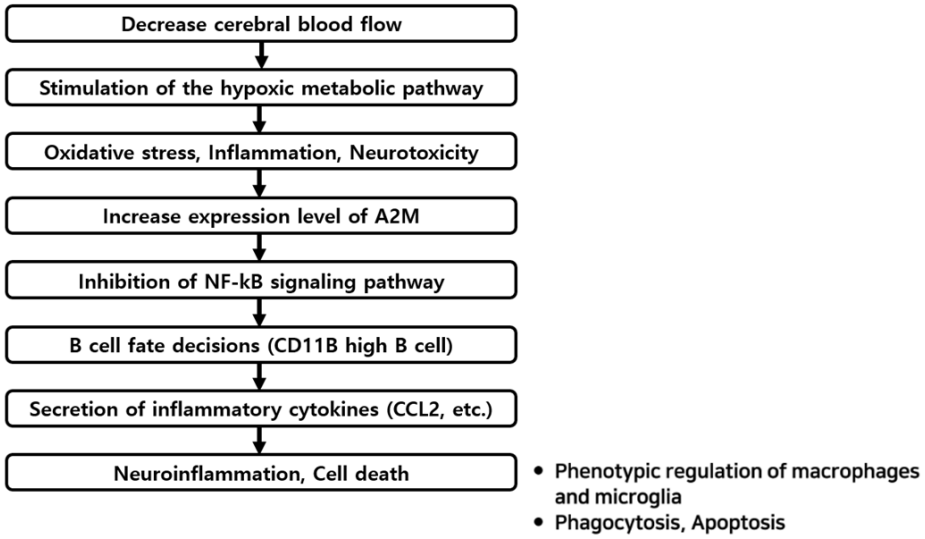

Fig. 7. Pathological mechanisms of ischemic stroke. The candidate biomarkers identified in this study—CD11B, CCL2, and A2M—are involved in inflammatory and apoptotic responses triggered by cerebral vascular occlusion.

Conclusions and Implications

In the MCAO rat model, the expression of A2M increases in response to inflammation and oxidative stress caused by impaired cerebral blood flow. Elevated A2M levels may inhibit the NF-κB signaling pathway, which regulates the differentiation of B cells that express high levels of CD11B on their surface. These $CD11B^{high}$ B cells secrete large amounts of pro-inflammatory cytokines, including CCL2, promoting leukocyte infiltration. This inflammatory cascade amplifies the neuroinflammation and contributes to subsequent brain injury.

The three candidate biomarkers—A2M, CD11B, and CCL2—are closely associated with the pathogenesis of ischemic stroke and exhibit time-dependent upregulation in serum samples as the vascular occlusion duration increases. Notably, changes in their expression were detected as early as 0.5 hour post-occlusion, even in the absence of visible infarction on TTC staining. The capacity to detect these expression changes in circulating blood shortly after vessel occlusion highlights their utility in identifying ischemic events prior to confirmation by conventional imaging modalities such as CT or MRI (Fig. 8).

Thus, A2M, CD11B, and CCL2 may serve as promising serum biomarkers for ischemic stroke, enabling timely diagnosis and therapeutic intervention before irreversible brain damage occurs.

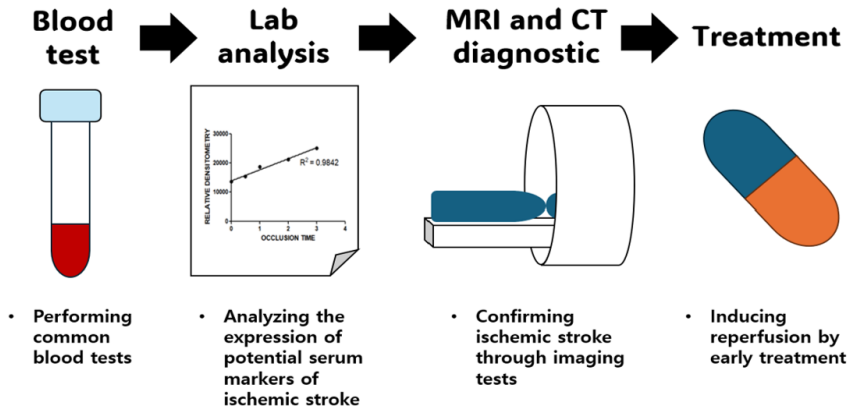

Fig. 8. Implications of this study for early diagnosis and treatment of ischemic stroke. Serum expression levels of potential biomarkers (CD11B, CCL2, and A2M) of ischemic stroke can be detected through blood tests. Based on significant changes in the expression of markers, imaging tests such as MRI and CT are performed to confirm the outbreak. Their early upregulation may prompt early diagnosis and treatment, thereby reducing the incidence of disability and mortality associated with ischemic stroke.

Conflicts of Interest

The author(s) declared no conflicts of interest.

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