Immunoexpression of MMP-8 and MMP-9 in chronic subdural hematoma

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To determine the possible role of matrix metallopeptidase (MMP)-8 and MMP-9 in the development of chronic subdural hematoma (CSDH), we investigated their expression in CSDH. In our previous study, we analyzed hematoma fluid and peripheral blood of 83 patients with CSDH, including 17 postoperative patients. Based on these results, we included 50 people in the normal group and analyzed 20 markers in the peripheral blood of each person. In order to identify representative markers, it was assessed by using overall differential gene expression. The concentration of MMP-8 was significantly higher in the normal group than that in the preoperative and postoperative groups. The concentration of MMP-9 was significantly lower in the normal group than in both preoperative and postoperative groups. Immunohistochemistry confirmed the expression of MMP-8 and MMP-9 in CSDH membranes. In conclusion, our results provide evidence of the expression of MMP-8 and MMP-9 in CSDH. In addition, the expression of MMP-8 and MMP-9 suggests angiogenesis in CSDH formation.

KEYWORDS
chronic subdural hematoma, matrix metallopeptidase, immunoexpression, differential gene expression, disease development

Introduction

As a common neurosurgical disease, chronic subdural hematoma (CSDH) involves the collection of blood in the subdural space (1). The incidence rates of CSDH have been rising on account of the aging population and the increasing use of anticoagulants and antplatelet medications (2). However, the development of CSDH is still poorly understood (3, 4). Previous research has investigated the mechanisms underlying CSDH, including angiogenesis, fibrinolysis, and inflammation (5).

In a rat model, inflammation and angiogenesis were found to play important roles in the formation of CSDH (6, 7). Numerous biomarkers have been identified in patients with CSDH, including interleukin (IL)-1, IL-6, IL-8, IL-10, vascular endothelial growth factor (VEGF), matrix metallopeptidase (MMP)-2, and MMP-9 (5, 8–10). In our previous study, we found that MMP-8 and MMP-9 may play a key role in the pathophysiology of CSDH (11).
MMPs are a family of zinc-dependent proteolytic enzymes that contribute to pathological inflammation and endothelial dysfunction (12). This appears to be related to CSDH formation. However, there have been few studies on the expression and activity of MMPs in CSDH. Based on our previous study, we used immunohistochemistry to analyze CSDH membranes to determine the possible role of MMP-8 and MMP-9 in the development of CSDH (11).

Materials and methods

Patients and tissue samples

We recruited 50 individuals to the normal group based on our previous study (11). In addition, the membranes of CSDHs were obtained from 10 patients (eight males and two females) who underwent surgeries at the Shenzhen Second People’s Hospital. The patients ranged from 35 to 84 years of age (mean: 65.3 years) and had not been previously treated for CSDH. This clinical study was approved by the Ethics Committee of the Shenzhen Second People’s Hospital (20200422003-XZ2021-XZ2021). Informed consent was obtained from all the participants involved in this study.

Cytokine measurements

Peripheral blood samples were collected from 50 patients in the control group. All samples were collected in tubes containing a coagulator and immediately centrifuged at 2,000 rpm for 15 min. After centrifugation, the supernatants were stored in sealed polypropylene tubes at $-80^\circ$C until further analysis.

We analyzed the hematoma fluid and preoperative and postoperative peripheral blood samples using the 20-plex human panel A system (R&D Systems, Minneapolis, MN, USA), Lumixx system (Lumixx, Austin, TX, USA), and Bioplex software (BioRad, Hercules, CA, USA). We evaluated IL-1α, IL-6, IL-10, Angiopoietin-2, platelet-derived growth factor-BB (PDGF-BB), MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, D-dimer, epidermal growth factor (EGF), C-C motif chemokine ligand 2 (CCL2), tumor necrosis factor (TNF-α), hepatocyte growth factor (HGF), VEGF, insulin-like growth factor binding protein-3 (IGFBP-3), prolactin, and VEGF receptor-2 (VEGFR2) levels.

Differentially expressed gene screening

We searched the genes corresponding to cytokines through PubMed. The overall differential gene expression was evaluated by using the ggplot2 package in the R statistical software. Furthermore, the LIMMA package was used to select DEGs.

### Table 1: Age and sex in preoperative chronic subdural hematoma (CSDH), postoperative CSDH, and normal groups.

| Group               | Age (years) | Gender |
|---------------------|-------------|--------|
|                     | Mean ± SD   | Male   | Female |
| Preoperative CSDH   | 66.73 ± 15.14 | 76     | 7      |
| Postoperative CSDH  | 62.63 ± 15.17 | 17     | 0      |
| Normal group        | 67.16 ± 4.13  | 34     | 16     |

We used the empirical Bayes moderated t-test to calculate the $p$-values for each cytokine. The adjusted $p$-values were calculated based on the false discovery rate. Only genes with a log$_2$ fold change $>2$ and false discovery rate $<0.01$ were considered DEGs.

Immunohistochemistry

CSDH membrane tissues were placed in 4% paraformaldehyde for 20 h at $4^\circ$C. The tissue was embedded in paraffin, and 4 µm serial sections were cut. The sections were deparaffinized in xylene and dehydrated using descending dilutions of ethanol (100% and 95%). The primary antibodies (Goat monoclonal, Anti-MMP-8, No: AF908-SP, 1:50, R&D Systems; Rabbit monoclonal, Anti-MMP-9, No: ab76003, 1:50, Abcam, Cambridge, UK) were incubated for 30 min at 25 $^\circ$C and overnight at $4^\circ$C and stained using the avidin-biotin peroxidase method and 3, 3'-diaminobenzidine as a chromogen. Finally, the sections were counterstained with Mayer's hematoxylin and mounted using an aqueous mounting medium.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney U-test. Data are presented as mean ± standard deviation. All analyses were performed using the SPSS software. Statistical significance was set at $p < 0.05$.

Results

Patient characteristics

The mean age in the preoperative CSDH group was 67.16 ± 4.13 years. The general characteristics of each group are presented in Table 1.
Identification of DEGs

All peripheral blood samples obtained from the preoperative CSDH and normal groups were analyzed. An overview of the differential gene expression is presented in Table 2. MMP-2, PDGF-BB, IL-10, EGF, IL-1α, CCL2, Angiopoietin-2, IL-6, MMP-8, VEGF, VEGFR2, HGF, MMP-9, IGFBP-3, TNF-α, and Prolactin were identified as DEGs between the preoperative and normal groups. MMP-1, MMP-3, and D-dimer were not significantly different between the preoperative and normal groups. All peripheral blood samples obtained from the postoperative and normal groups were analyzed. An overview of the differential gene expression is presented in Table 3. CCL2, EGF, IL-10, IL-1α, Angiopoietin-2, IL-6, MMP-8, HGF, TNF-α, MMP-9, IL-8, VEGFR2, VEGF, Prolactin, D-dimer, IGFBP-3, MMP-1, and PDGF-BB were identified as DEGs between postoperative and normal groups. MMP-2 and MMP-3 were not significantly different between the postoperative and normal groups.

Data analysis

The concentrations of MMP-8 and MMP-9 in the control (normal) group are shown in Table 4 (p < 0.01). Based on our previous study (11), the concentration of MMP-8 was significantly higher in the normal group compared to that in both preoperative and postoperative CSDH groups, while that of MMP-9 was significantly lower. All CSDH membranes were immunostained for MMP-8 and MMP-9 in all samples. These proteins were positively immunostained in vascular endothelial cells. The results for the immunostained membranes are shown in Figures 1-2.

Discussion

Based on our previous study, MMP-8 and MMP-9 may contribute to CSDH pathogenesis in different ways (11). We found that the MMP-8 concentration was significantly higher in the normal group compared to that in preoperative and postoperative groups, whereas the MMP-9 concentration was significantly lower. Simultaneously, we observed that both MMP-8 and MMP-9 were expressed in vascular endothelial cells. Nowadays, an increasing number of studies consider that CSDH formation may be related to the growth of new vessels and angiogenesis (14). In addition, MMPs significantly contribute to blood vessel formation, remodeling, and angiogenesis by regulating the functions or behaviors of stem/progenitor and vascular cells (14).

Although CSDH is the most common neurosurgical disease, a few questions remain to be answered. Tamura et al. (15) indicated that the split dural border cell layer produced a dural hemoma by forming the inner and outer membranes in CSDH. The outer membranes drive inflammation and angiogenesis. After studying the role of MMPs in the development of CSDH, Nakagawa et al. (16) found that MMPs degrade the integrity of the outer membranes.
TABLE 3  Differential expression of all cytokines in peripheral blood samples between the postoperative CSDH and normal groups.

| Gene          | logFC  | AveExpr | t     | p-value       | adj.p.Val | B     |
|---------------|--------|---------|-------|---------------|-----------|-------|
| CCL2          | 988.5303 | 1,032.101 | 21.16245 | $7.22 \times 10^{-20}$ | $1.44 \times 10^{-20}$ | $-4.03779$ |
| EGF           | 762.2283 | 682.9133 | 20.75541 | $2.06 \times 10^{-20}$ | $2.06 \times 10^{-20}$ | $-4.04051$ |
| IL-10         | 79.30262 | 63.22905 | 16.70569 | $1.71 \times 10^{-24}$ | $1.14 \times 10^{-25}$ | $-4.07664$ |
| IL-1α         | 118.1598 | 98.51254 | 15.5764 | $5.56 \times 10^{-23}$ | $2.78 \times 10^{-22}$ | $-4.09068$ |
| Angiopoietin-2| 5,129.373 | 4,748.264 | 15.33773 | $1.19 \times 10^{-22}$ | $4.74 \times 10^{-22}$ | $-4.09395$ |
| IL-6          | 80.18971 | 67.46492 | 14.74762 | $7.92 \times 10^{-22}$ | $2.64 \times 10^{-21}$ | $-4.1025$ |
| MMP-8         | 23,775.32 | 20,468.85 | 14.63503 | $1.14 \times 10^{-21}$ | $3.10 \times 10^{-21}$ | $-4.10422$ |
| HGF           | 902.3919 | 832.87 | 14.61023 | $1.24 \times 10^{-21}$ | $3.10 \times 10^{-21}$ | $-4.1046$ |
| TNF-α         | 98.88158 | 87.63968 | 14.45219 | $2.08 \times 10^{-21}$ | $4.63 \times 10^{-21}$ | $-4.10706$ |
| MMP-9         | -306.457 | 88.102.92 | -14.0502 | $7.92 \times 10^{-21}$ | $1.58 \times 10^{-20}$ | $-4.11359$ |
| IL-8          | 113.0108 | 97.32476 | 10.75424 | $9.67 \times 10^{-16}$ | $1.76 \times 10^{-15}$ | $-4.18463$ |
| VEGFR2        | 13,529.04 | 19,157.61 | 10.10128 | $1.15 \times 10^{-14}$ | $1.92 \times 10^{-14}$ | $-4.20335$ |
| VEGF          | 355.4574 | 358.676 | 10.06804 | $1.35 \times 10^{-14}$ | $2.07 \times 10^{-14}$ | $-4.20459$ |
| Prolactin     | 40,781.43 | 38,473.71 | 3.968871 | $0.000192$ | $0.000274$ | $-4.47445$ |
| D-dimer       | 1,788,130 | 3,265,902 | 3.009334 | $0.003797$ | $0.005062$ | $-4.52305$ |
| MMP-1         | 2,094.374 | 8,114.673 | 2.457844 | $0.01682$ | $0.024301$ | $-4.55248$ |
| PIGF-BB       | 902.3919 | 832.87 | 14.61023 | $1.24 \times 10^{-21}$ | $3.10 \times 10^{-21}$ | $-4.1046$ |
| MMP-8         | 98.88158 | 87.63968 | 14.45219 | $2.08 \times 10^{-21}$ | $4.63 \times 10^{-21}$ | $-4.10706$ |
| MMP-9         | -306.457 | 88.102.92 | -14.0502 | $7.92 \times 10^{-21}$ | $1.58 \times 10^{-20}$ | $-4.11359$ |
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MMP, Matrix metalloproteinases; PIGF-BB, platelet-derived growth factor-BB; IL, interleukin; VEGF, vascular endothelial growth factor; CCL2, C-C motif chemokine ligand 2; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; HGF, hepatocyte growth factor; IGFBP-3, insulin-like growth factor binding protein-3; TNF-α, tumor necrosis factor-alpha. logFC, differential expression multiple; AveExpr, mean expression level; t, T value (paired-samples t-test); adj.p.Val, adjusted p-value; B, logarithm of the standard deviation after Bayes analysis.

TABLE 4 Concentration of MMP-8 and MMP-9 in the normal, preoperative, and postoperative groups ($p < 0.01$).

| Factor            | MMP-8 (ng/mL) | MMP-9 (ng/mL) |
|-------------------|---------------|---------------|
| Preoperative serum| 9.18 ± 9.78   | 217.19 ± 155.02 |
| Normal serum      | 25.89 ± 6.09  | 15.178 ± 2.47  |
| Postoperative serum| 2.97 ± 3.80  | 354.13 ± 231.55 |

MMP, Matrix metalloproteinases.
Anti-MMP-8 antibody immunostaining: (A) ×40, (B) ×100, (C) ×400 (arrows).

Anti-MMP-9 antibody immunostaining: (A) ×40, (B) ×100, (C) ×400 (arrows).

Conclusions

Our study provides evidence of MMP-8 and MMP-9 expression in CSDH. In addition, the expression of MMP-8 and MMP-9 is indicative of angiogenesis in CSDH formation. Identification of the biomolecules involved in CSDH formation may be helpful in the development of new clinical therapies. However, further studies are necessary to clarify the association between MMP-8 and MMP-9 expression during the transformation and progression of CSDH.

There were some limitations in this study. Firstly, from our limited number of cases, it is difficult to represent the wide spectrum of patients with CSDH. Further studies including a larger sample of patients will be needed to clarify this point. Secondly, the experimental analysis of the CSDH membrane was relatively limited, and so the results may not be completely convincing. More approaches are needed to verify the expression and role of MMP-8 and MMP-9 on the membrane.
Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Ethics Committee of Shenzhen Second People’s Hospital. Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

Author contributions

Conceptualization, methodology, and project administration: G-JS, DZ, and X-JH. Validation, resources, and investigation: G-JS and DZ. Software: J-NW, C-WW, and X-JZ. Formal analysis: Y-HD, C-WW, and X-JZ. Data curation, visualization, and writing—original draft preparation: G-JS. Writing—review and editing and supervision: X-JH. Funding acquisition: X-JH and X-JZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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