Abstract. Cerebral vasospasm following subarachnoid hemorrhage (SAH) has been studied in terms of a contraction of the major cerebral arteries, but the effect of cerebrum tissue in SAH is not yet well understood. To gain insight into the biology of SAH-expressing cerebrum, we employed oligonucleotide microarrays to characterize the gene expression profiles of cerebrum tissue at the early stage of SAH. Functional gene expression in the cerebrum was analyzed 2 h following stage 1-hemorrhage in Sprague-Dawley rats. mRNA was investigated by performing microarray and quantitative real-time PCR analyses, and protein expression was determined by Western blot analysis. In this study, 18 upregulated and 18 downregulated genes displayed at least a 1.5-fold change. Five genes were verified by real-time PCR, including three upregulated genes [prostaglandin E synthase (PGES), CD14 antigen, and tissue inhibitor of metalloproteinase 1 (TIMP1)] as well as two downregulated genes [KRAB-zinc finger protein-2 (KZF-2) and γ-aminobutyric acid B receptor 1 (GABA B receptor)]. Notably, there were functional implications for the three upregulated genes involved in the inflammatory SAH process. However, the mechanisms leading to decreased KZF-2 and GABA B receptor expression in SAH have never been characterized. We conclude that oligonucleotide microarrays have the potential for use as a method to identify candidate genes associated with SAH and to provide novel investigational targets, including genes involved in the immune and inflammatory response. Furthermore, understanding the regulation of MMP9/TIMP1 during the early stages of SAH may elucidate the pathophysiological mechanisms in SAH rats.

Introduction

Subarachnoid hemorrhage (SAH) is a complex disease associated with high mortality and high morbidity and has no effective therapy. SAH can be caused by a ruptured aneurysm, leaking arteriovenous malformation or head trauma. Brain cells in this area may be irritated and damaged as blood spills into the subarachnoid space. Accumulating evidence supports the role of free radical formation and perturbations in nitric oxide, endothelin-1 and nicotinamide adenine dinucleotide (NADPH) oxidase activities in the cerebral basal artery, which are mediators of vasoconstriction in vaso- spasm (1-3). However, pathophysiological studies evaluating other brain tissues and their hemorrhagic molecular mechanisms of acute SAH-induced injury are rare.

Recently, complementary DNA (cDNA) or oligonucleotide microarray hybridization has made possible a broad survey of all the functional categories of genes, revealing their differential expression patterns in pathological tissue from various brain diseases, including the human middle cerebral artery following cerebral ischemia (4), brain tissue in intracerebral hemorrhage (5) and cerebral arteries following subarachnoid hemorrhage (6).

Currently, one key homeostatic mechanism of the brain in tissue repair is maintained through the production of tissue inhibitor of metalloproteinases (TIMPs) (7,8). An imbalance of matrix metalloproteinase (MMP)/TIMP regulation has been implicated in a number of inflammatory diseases of the central nervous system (9,10). Increased expression of
MMP9 is intimately involved in the mechanisms of SAH (11). Therefore, we hypothesized that MMP9 and TIMP1 are likely to play significant roles in the repair mechanisms of cerebrum-SAH, particularly during acute phases of SAH.

In this study, we demonstrate the potential of oligonucleotide microarray as a method to identify candidate genes associated with SAH, and to provide novel investigational targets including genes involved in the immune and inflammatory response. Targeting these mediators of systemic inflammation associated with SAH is a promising therapeutic strategy for the future.

Materials and methods

SAH rat model. A total of 31 male Sprague-Dawley rats (300-400 g) were divided into a normal saline-operated group (S), an SAH group (H) and a normal group (C). We used 8 normal control rats [two for the microarray experiment, three for the reverse transcription polymerase chain reaction (RT-PCR) and three for the Western blot analyses], 11 sham-operated rats [two for the microarray experiment, three for the real-time PCR, three for the RT-PCR and three for the Western blot analyses], and 12 rats for the SAH model [three for the microarray experiments, three for the real-time PCR, three for the RT-PCR and three for the Western blot analyses]. All rats were anesthetized with ketamine [100 mg/kg intraperitoneally (i.p.)] and xylazine (10 mg/kg i.p.) and the animals were allowed to breathe spontaneously. Following anesthesia, the femoral artery was exposed and catheterized with a PE-50 catheter to allow measurement of blood gas values, blood pH and continuous recording of mean arterial blood pressure. The physiological values immediately following SAH were as follows: 36.5±0.6°C body temperature, 7.33±0.05 pH, 143.2±19.3 PaO₂ and 40.3±7.6 PaCO₂ (values are the mean ± SD, n=5). Aided by a surgical microscope, the atlantooccipital membrane was tapped carefully into the cisterna magna with a 27-gauge needle. After 0.3 ml of cerebrospinal fluid was aspirated, an equal amount of autologous blood was infused, followed by 4% paraformaldehyde exceeding 3 min. The rats were then placed in a head-down prone position at a 30-degree angle for 30 min to hold the blood in the basal cisterns. Sham-control rats were injected with 0.9% sterile NaCl solution instead of blood. Rats were sacrificed at 2 or 24 h following SAH, all rats from each group were anesthetized with ketamine [100 mg/kg i.p.] and xylazine (10 mg/kg i.p.). The chest was opened and the aorta was cannulated with a blunted 23-gauge needle through the left ventricle. An incision was made in the right atrium to allow for outflow of perfusion solutions. A total of 20 ml of normal saline was infused, followed by 4% paraformaldehyde for 10 min. All perfusates were delivered at 60 to 80 mm Hg. The cerebrum was then harvested and stored in a refrigerator at -70°C.

RNA isolation and microarray analysis. RNA preparation and analysis were performed according to Affymetrix's protocol. The precipitated products were treated with DNase (Promega, Madison, WI, USA) and further purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA from each sample was collected into a total of 10 µg of RNA. The integrity of the RNA from the pooled samples was determined using Spectra Max Plus (Molecular Devices) and the ratio of A260/A280 was from 1.9 to 2.1. mRNA was then extracted from the total RNA samples using the Oligotex mRNA Kit (Qiagen, Valencia, CA, USA). The RNA from each sample using the GeneChip Oligo Microarray 3' Amplification Reagents Two-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900432), with the exception that the primer used for the reverse transcription reaction was a modified T7 primer with 24 thymidines. Following this, labeled cRNA was synthesized from the cDNA using the GeneChip Expression 3' Amplification Reagents Two-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900182) according to the manufacturer's instructions. Fragmentation of biotinylated cRNA (15 µg) by GeneChip Sample Cleanup Module (Affymetrix, P/N 900371), protocols and reagents for hybridization, washing, and staining were all performed following instructions provided by Affymetrix (http://www. affymetrix.com/support/technical/manuals.affx). Prior to hybridization of the Affymetrix Human Genome U133A Array, labeled cRNA was hybridized to the Affymetrix GeneChip Test 3 Array to verify quality.

Data analysis and clustering algorithm. The microarray data were analyzed using the GeneSpring® 6.2 software (Silicon
Sequences on Affymetrix chips were generally represented by an average of 16 perfect matched (PM) probe sets and corresponding mismatch (MM) sequences as false-positive control and nonspecific hybridization. Absolute intensity statistics were based on feature intensities, and corresponding standard deviations were provided in the microarray chip-generated CEL files. To evaluate gene expression patterns, hierarchical clustering using the Pearson’s correlation metric and average linkage was performed. Data sets from two normal (C), two saline-operated (S) and three SAH (H) rats were used for this analysis. Genes showing fold change (FC) values >1.5 or <1.5 and unpaired t-test p-values of <0.05 between H specimens and S specimens were defined as denoting a significant FC. Two additional criteria were performed on the list. i) The significantly differentially expressed genes (p<0.05) between C and S specimens were removed. ii) Of the genes that were differentially expressed >25%, two S genes were removed.

**Real-time PCR.** A total of 10 genes were selected for quantitative expression analysis, and 2 µg total RNA was reverse transcribed into single-strand cDNA using random primers. cDNA products were amplified using gene-specific primers (Table I) and real-time PCR was performed with an ABI Prism 7300 Sequence Detection System and 2X SYBR Green PCR Master mix (Applied Biosystems, CA, USA). Cycling parameters were as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and then 60°C for 1 min (40 cycles). Each sample was analyzed three times, each time in triplicate and each experiment was performed three times using independent rat total RNA. β-actin was used as an internal control.

**RT-PCR.** Total RNA was prepared using TRIzol (Qiagen). An aliquot (1 µg) of RNA was reverse-transcribed by MMLV-RT using oligo-dT as the primer (Qiagen). The reaction was carried out at 42°C for 60 min in a 20-µl volume. The product was diluted to 100 µl with water and an aliquot (1 µl) was used as the template for PCR. A final volume of 20 µl containing 1X amplification buffer, 200 µM each deoxynucleobonucleotide triphosphate (dNTP) and 0.5 unit of Taq DNA polymerase, 2 µM of each primer and 1 µl of cDNA solution was performed. Temperatures and time schedules were: initial denaturation for 5 min at 95°C, 30 cycles with denaturation at 95°C for 60 sec, annealing at 59-64°C for 60-120 sec, extension at 72°C for 120 sec and final extension at 72°C for 7 min. The primer used for amplification included MMP2 (436 bp), F: CCACAT TCTGGCCTGAGCCTCCC; R: GATTTGTAGCTTCCAAA CTCCA and MMP9 (193 bp), F: AGGCTACAGCTTTGCT G CCCC; R: GCTGCTTCTGAAGCTACAGCA. The primers used for amplifying TIMP1 were the same as those used in real-time PCR. PCR products were run on a 2% agarose gel and stained with ethidium bromide.

**Protein extraction and Western blot analysis.** Total proteins were extracted from cerebrum tissues and homogenized in buffer [10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mg/ml leupeptin, 1 mg/ml aprotinin and 1 mg/ml antipain]. The homogenates were centrifuged at 1500 x g for 10 min at 4°C, followed by a second centrifugation of the supernatant at 12,000 x g for 10 min. The supernatant was then used for the analysis. Protein samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond-ECL membranes (Amersham). These were saturated with 5% nonfat milk in PBS and 0.1% Tween-20 for 1 h at room temperature and then incubated with a mouse anti-human TIMP1 monoclonal antibody (dilution 1:300) (Chemicon; Temecula, CA) in the same solution for 2 h. The membranes were rinsed in phosphate buffered saline (PBS) containing 0.1% Tween-20 and incubated for 1 h in the milk buffer with a peroxidase-coupled sheep antimouse IgG (dilution 1:3,000) (Amersham). Immunoreaction signals were visualized with enhanced chemiluminescence (ECL-PLUS, Amersham). β-actin (dilution 1:2,000) (rabbit β-actin antibody, Sigma) was used to normalize the immunoreactivity of TIMP1 in each sample.

**Results**

**Differential expression of genes in the SAH cerebrum.** To investigate genes involving SAH, we used Affymetrix oligonucleotide microarray to analyze the differential expression pattern of 19,174 genes between two normal (C), two saline-operated control (S) and three SAH (H) samples (Fig. 1A). Based on the selection criteria, 36 out of 19,174 genes were considered to be deregulated genes, and were separated into three clusters, C, H and S (Fig. 1B). Those in cluster H were all upregulated compared to all of the genes in cluster S and were also downregulated, indicating that our microarray analysis was an effective approach to identify those putatively deregulated genes for their possible involvement in this SAH. The 18 genes that were significantly upregulated in SAH cerebrum including prostaglandin E synthase (PGES), CD14 antigen, TIMP1 and TNF superfamily 2a (Table II), while 18 genes were significantly downregulated in SAH cerebrum (Table III). The ontology of the 36 genes was further used to classify categories including immune and inflammatory factors, cell surface molecules, synaptic transmission proteins, metabolism and unknown functions. Notably, the expression of genes involved in the regulation of the immune and inflammatory response (PGES, CD14 antigen, TIMP1, TNF receptor superfamily member 12a, TIS11 complete cds and fibrinogen-like 2) were increased and the expression of the genes for synaptic transmission (γ-aminobutyric acid B receptor 1), chloride homeostasis (chloride channel 3) and protein metabolism/turnover (KRAB-zinc finger protein KZF-2 and cytochrome P450-like protein) were reduced.

**Validation of 10 deregulated genes in SAH cerebrum specimens by real-time PCR.** Eight potentially upregulated genes including, PGES, CD14 antigen, protein tyrosine phosphatase receptor type J, TIMP1, TNF receptor superfamily member 12a, TIS11 complete cds, cortistatin and fibrinogen-like 2, and two downregulated genes, KRAB-zinc finger protein KZF-2 (KZF-2) and GABA B receptor, were selected for confirmation. Consistent with the Affymetrix oligonucleotide microarray analysis, the upregulation of PGES, CD14 antigen and TIMP1, and downregulation of KZF-2 and GABA B receptor were also observed in SAH cerebrum specimens,
and for all five of these genes the fold changes obtained by microarray analysis. It was found that protein tyrosine phosphatase receptor type J, TNF receptor superfamily...
Table III. Differentially expressed downregulated genes in the subarachnoid hemorrhage cerebrum.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Gene description</th>
<th>Fold change</th>
</tr>
</thead>
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<tr>
<td>Other functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF312319</td>
<td>γ-aminobutyric acid (GABA) B receptor, 1 (neurotransmitter)</td>
<td>-2.7</td>
</tr>
<tr>
<td>A1170346</td>
<td>Cytochrome P450-like protein (protein metabolism/turnover)</td>
<td>-1.9</td>
</tr>
<tr>
<td>U67083</td>
<td>KRAB-zinc finger protein (KZF)-2 (transcriptional regulator)</td>
<td>-1.8</td>
</tr>
<tr>
<td>A1763523</td>
<td>Chloride channel 3 (chloride homeostasis)</td>
<td>-1.6</td>
</tr>
<tr>
<td>Unknown functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1070489</td>
<td>Transcribed sequences</td>
<td>-3.1</td>
</tr>
<tr>
<td>BI275261</td>
<td>UI-R-CX0-bwr-d-11-0-UI.s1 UI-R-CX0 Rattus norvegicus cDNA clone</td>
<td>-2.6</td>
</tr>
<tr>
<td>A1029637</td>
<td>UI-R-CO-jg-a-09-0-UI.s1 UI-R-CO Rattus norvegicus cDNA clone</td>
<td>-2.2</td>
</tr>
<tr>
<td>BF409997</td>
<td>Transcribed sequences</td>
<td>-2.2</td>
</tr>
<tr>
<td>A1763870</td>
<td>Transcribed sequences</td>
<td>-1.9</td>
</tr>
<tr>
<td>A1176360</td>
<td>Similar to palmadelphin (LOC310811), mRNA</td>
<td>-1.9</td>
</tr>
<tr>
<td>A1105067</td>
<td>Similar to ADP-ribosylhydrolase like 1; -1.9 ADP-ribosylhydrolase 2 (LOC290880), mRNA</td>
<td>-1.9</td>
</tr>
<tr>
<td>A1406369</td>
<td>Similar to protein kinase nek 1 (EC 2.7.1.-) - mouse (LOC290705), mRNA 9</td>
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</tr>
<tr>
<td>BE096504</td>
<td>Similar to hypothetical protein dj465n24.2.1 (LOC362626), mRNA</td>
<td>-1.8</td>
</tr>
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<td>AW524454</td>
<td>Similar to epithelial stromal interaction 1, isoform a (LOC364433), mRNA</td>
<td>-1.8</td>
</tr>
<tr>
<td>AA899216</td>
<td>Transcribed sequences</td>
<td>-1.7</td>
</tr>
<tr>
<td>BI294801</td>
<td>Similar to SR rich protein (LOC297942), mRNA</td>
<td>-1.7</td>
</tr>
<tr>
<td>BF401042</td>
<td>Transcribed sequences</td>
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<tr>
<td>BF397669</td>
<td>UI-R-BS2-bed-c-02-UI.s1 UI-R-BS2 Rattus norvegicus cDNA clone</td>
<td>-1.6</td>
</tr>
<tr>
<td></td>
<td>UI-R-BS2-bed-c-02-UI 3’, mRNA sequence</td>
<td></td>
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Verification of altered expression of TIMP1, MMP2 and MMP9 by RT-PCR and Western blot analysis. We further investigated the roles of TIMP1 and MMP regulation in cerebrum pathophysiology of SAH. RT-PCR data revealed the altered expression of MMP2 and MMP9 (Fig. 3). The expression patterns of both TIMP-1 gene and protein were well matched with the expression profile obtained from the microarray analysis, supporting the validity of the data obtained from the microarray analysis. The quantitative data revealed that mRNA levels of only MMP9, and not MMP2, were differentially regulated in the SAH specimens. Therefore, MMP9/TIMP1 expression is differentially regulated in acute activation of SAH.

Discussion

The present study was designed to gain insight into the early phase of SAH in a well-established 1-hemorrhage rat model of brain injury. The 2-h time point that was selected was the clear choice to capture dynamic changes in mRNA regulation preceding the more pronounced functional changes that occur 24 h post-SAH (1,12). This time point also matches the time...
Table IV. Comparison of microarray and real-time PCR expression for 10 selected genes in the subarachnoid hemorrhage specimens vs. the sham-control specimens.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Gene description</th>
<th>Fold change (Microarray)</th>
<th>Fold change (Real-time PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF280967</td>
<td>Prostaglandin E synthase</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>NM_021744</td>
<td>CD14 antigen</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>NM_053819</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>2.6</td>
<td>1.9</td>
</tr>
<tr>
<td>BI303379</td>
<td>Tumor necrosis factor superfamily, member 12a</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>AB025017</td>
<td><em>Rattus norvegicus</em> gene for TIS11, complete cds</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td>NM_012835</td>
<td>Cortistatin</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>AF323608</td>
<td>Fibrinogen-like 2</td>
<td>2.0</td>
<td>1.2</td>
</tr>
<tr>
<td>NM_017269</td>
<td>Protein tyrosine phosphatase, receptor type J</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U67083</td>
<td>KRAB-zinc finger protein (KZF)-2</td>
<td>-1.8</td>
<td>-1.8</td>
</tr>
<tr>
<td>AF312319</td>
<td>γ-aminobutyric acid (GABA) B receptor, 1</td>
<td>-2.7</td>
<td>-1.9</td>
</tr>
</tbody>
</table>

Data shown are fold changes in SAH (H) relative to the saline-operated (S) specimens from three determinations. Real-time PCR was standardized with β-actin expression.

Figure 3. Expression of TIMP1, MMP2 and MMP9 in rat cerebrum following 1-hemorrhage. (A) Gene expression of TIMP1, MMP2 and MMP9 was analyzed by in SAH (H) compared with saline-operated controls (S) and normal (C) specimens using RT-PCR. Relative mRNA amount was expressed as the ratio between TIMP-1, MMP9, MMP2 and β-actin. (B) Protein expression of TIMP1 was analyzed using Western blot analyses. Relative protein amount was expressed as the ratio between TIMP-1 and β-actin. Data were expressed as the mean ± SEM (n=3). *P<0.05 versus S (Student's t-test).
course of an immediate global decrease in cerebral blood flow following SAH induction (13). In addition, in the experimental SAH rats we observed that the injected blood accumulated and compressed the whole brain tissue. Our microarray identification of 19,174 genes revealed 18 upregulated and 18 downregulated genes. None of the early genes, including jun and fos, known to be upregulated in stress conditions were identified in the microarray analysis. Notably, a little cluster of inflammation genes, including PGES, CD14 and TIMP1 were upregulated in the early phase of SAH. These results coincide with results of SAH-related inflammatory reactions in numerous studies reported in the literature (14,15).

TIMP1 expression was differentially regulated in a marked similar pattern to that of MMP9 in acute activation following 2 h of 1-hemorrhage cerebrum SAH, suggesting that an imbalance of MMP9/TIMP1 regulation is implicated in the acute phase of SAH. The elevated expression of TIMP1 along with MMP9 following acute SAH injury may reflect the fact that MMP inhibitors play a role in matrix remodeling at specific time points following brain injury. Recently, one study reported a model for astrocyte TIMP1 regulation in acute inflammation injury, where acute activation of neural cells (astrocytes, microglia and neurons) by pro-inflammatory cytokines may lead to enhanced levels of TIMP1 in the tissue microenvironment and may elicit a typical repair response early in injury (8). Our results appear to corroborate the hypothesis that in the early phase of SAH conditions, acute immune activation of the cerebrum increases TIMP1, which participates in a protective response and elicits repair as a balance mechanism. Of note, TIMP1 is induced in response to a number of pro-inflammatory stimuli, including cytokines activated following brain injury such as tumor necrosis factor (TNF)-α, IL-1 or lipopolysaccharide (LPS) (16-18). Further studies are required to demonstrate whether the expression of these cytokines is concomitant with the upregulation of TIMP1 following inflammatory SAH injury.

Prostaglandins have been implicated in normal cellular processes and pathophysiological conditions, including inflammation, edema, bronchoconstriction and fever (19). PGES is a rate-limiting enzyme in prostanoid synthesis. The activity of mPGES-1 and COX-2, or coupling of COX-2 with PGES is a rate-limiting enzyme in prostanoid synthesis. The release of prostaglandins is part of the inflammatory response and may mediate tissue inflammation or anti-inflammatory effects (21). COX-2 expression was detected in basilar arterial tissue in both acute and chronic stages following SAH (22). Therefore, PGES may, in turn, produce sufficient quantities of eicosanoids to affect hemodynamics following SAH. Clearly, more research is required to elucidate the mechanisms by which the early immediate response genes promote the inflammatory response by the rapid and excessive production of prostaglandins, initiating an inflammatory cascade that is critical for survival following acute brain injury following SAH. According to the present data array, CD14 expression may contribute to prime and/or maintain microglial activation within the cerebrum and yield an exaggerated immune response that could be potentially active for certain cytokines, including TNF-α and their receptors, such as TNF receptor superfamily member 12a, one of the founding genes of this present data array. However, the role of CD14 within the brain cells following SAH still needs be investigated.

In our series, two downregulated genes, KZF-2 and GABA B receptor, were verified and related to the SAH process. Members of the C2H2 zinc finger transcription factor family, such as KZF-2, play key roles in the regulation of cell proliferation, differentiation and apoptosis in response to a variety of stimuli (23,24). The metabolotropic GABA B receptors are coupled to G proteins and modulate synaptic transmission through intracellular effector systems of the mammalian central nervous system (25). GABA B receptors function by inhibiting presynaptic transmitter release or by increasing the potassium conductance responsible for long-lasting inhibitory postsynaptic potentials (26,27). However, the mechanisms leading to decreased KZF-2 and GABA B receptor expression in SAH have not yet been characterized.

In conclusion, our microarray evidence reveals that the activation of inflammatory cascades following SAH is a complex systemic response. Further studies should verify whether the novel gene expression profiles of MMP9/TIMP1 co-regulate PGES induction of the inflammation pathway through CD14. Targeting these inflammatory mediators of systemic inflammation associated with SAH could be a promising therapeutic strategy.

Acknowledgements

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