



The LRG-TGF- β -Alk-1/TGF β RII-Smads as Predictive Biomarkers of Chronic Hydrocephalus after Aneurysmal Subarachnoid Hemorrhage

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Abstract

Background Chronic hydrocephalus is a common complication of aneurysmal subarachnoid hemorrhage (aSAH); however, the risk factors and the mechanisms underlying its occurrence have yet to be fully elucidated. The purpose of this study was to identify biomarkers that could be used to predict chronic hydrocephalus after aSAH and to investigate the relationships.

Methods We analyzed cerebrospinal fluid (CSF) samples from 19 patients with chronic hydrocephalus after aSAH and 44 controls without hydrocephalus after aSAH. Enzyme-linked immunosorbent assay was used to determine the levels of leucine-rich alpha-2-glycoprotein (LRG), transforming growth factor- β (TGF- β), Smad1, Smad4, Smad5, Smad8, activin receptor-like kinase 1 (Alk-1), activin receptor-like kinase 5 (Alk-5), P38, and TGF- β type II receptor (TGF β RII) in CSF samples.

Results In the CSF of patients with chronic hydrocephalus after aSAH, the levels of LRG, TGF- β , Alk-1, Smad5, and TGF β RII were significantly increased ($p < 0.05$) and the levels of Smad1, Smad4, and Smad8 were significantly decreased ($p < 0.05$). There were no significant differences between the two groups concerning the levels of P38 and Alk-5 ($p > 0.05$). The analysis also identified significant correlations between specific biomarkers: LRG and Smad1, LRG and Smad5, TGF- β and Alk-1, and Alk-1 and Smad4 ($p < 0.05$); the Pearson's correlation coefficients for these relationships were -0.341 , 0.257 , 0.256 , and -0.424 , respectively.

Conclusion The levels of LRG, TGF- β , Alk-1, TGF β RII, Smad1/5/8, and Smad4 in the CSF are potentially helpful as predictive biomarkers of chronic hydrocephalus after aSAH. Moreover, the LRG-TGF- β -Alk-1/TGF β RII-Smad1/5/8-Smad4 signaling pathway is highly likely to be involved in the pathogenic process of chronic hydrocephalus after aSAH.

Keywords

- ▶ chronic hydrocephalus
- ▶ subarachnoid hemorrhage
- ▶ cerebrospinal fluid
- ▶ leucine-rich alpha-2-glycoprotein
- ▶ transforming growth factor- β

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Introduction

Chronic hydrocephalus is a common clinical disease with several clinical manifestations, including gait disorders, memory decline, nausea, and vomiting. This disease can be subclassified into secondary chronic hydrocephalus and idiopathic chronic hydrocephalus according to whether there is a clear cause. Secondary chronic hydrocephalus is a severe complication of aneurysmal subarachnoid hemorrhage (aSAH), with a reported incidence of approximately 20%.¹ However, the risk factors and mechanisms underlying the occurrence of chronic hydrocephalus remain unclear. The current factors considered for the clinical prediction of chronic hydrocephalus include blood clot obstruction, barrier injury, transforming growth factor- β (TGF- β) levels, and blood component stimulation.² However, these factors are of limited value when formulating treatment plans for patients with aSAH and create a significant economic burden for patients. If predictive diagnostic indicators for chronic hydrocephalus after aSAH can be identified, this would make the clinical management of patients much more efficient.

In our previous studies, we demonstrated that the levels of leucine-rich alpha-2-glycoprotein (LRG), TGF- β 1, and TGF- β 2 were significantly increased in the cerebrospinal fluid (CSF) of patients with idiopathic normal pressure hydrocephalus (INPH)³ and that the TGF- β family of proteins and activation of the Smad signaling pathway played essential roles in stem-cell self-renewal, differentiation, and fiberization.^{4,5} Collectively, our previous data verified that the TGF- β system and the LRG are associated with the occurrence and formation of hydrocephalus. Other research has shown that the LRG levels are closely related to the expression of TGF- β ^{6,7} and TGF- β is a potent fibrogenic factor implicated in a wide range of fibrotic diseases⁸ and can contribute to subarachnoid space fibrosis via activation of TGF- β /Smad/CTGF axis.^{9,10} TGF- β signals mainly through TGF- β type I receptor (TGF β RI), also known as activin receptor-like kinase 1 (Alk-1) and activin receptor-like kinase 5 (Alk-5),¹¹ and TGF β RII. The family of Smad proteins is widely regarded as the primary effector of TGF- β signaling.^{12,13} It has also been established that TGF- β ligands signal via a heteromeric receptor complex consisting of type II and type I receptors, which phosphorylates downstream effectors, such as Smad1/5/8 and Smad2/3.^{11,14} Subsequently, phosphorylated Smad proteins form a dimer with the coactivator Smad4,¹⁵ which can be directly phosphorylated by Alk-1.¹⁶

Existing evidence suggests that during the formation of chronic hydrocephalus after aSAH, it is likely that LRG induces the phosphorylation of the family of Smad proteins via the TGF β RII/Alk-1/Alk-5 pathway, which acts on TGF- β ; these events then influence the dimerization of Smad1/5/8 and Smad4, leading to the formation of heteromeric complexes.¹⁷ In addition, P38 is required for TGF- β -induced apoptosis¹⁸; we suspected that the formation of chronic hydrocephalus after aSAH was related to apoptosis of the TGF- β signal induced by P38. In the present study, we focused on the TGF- β /Smad system and 10 different biomarkers: LRG, TGF- β , Smad1, Smad4, Smad5, Smad8, P38, Alk-1, Alk-5, and

TGF β RII. Our overall aim was to determine the levels of these biomarkers in patients and demonstrate how these might be associated with chronic hydrocephalus after aSAH. Finally, we attempted to identify the specific signaling pathways involved in forming chronic hydrocephalus after aSAH.

Methods

Patients and Samples

This study included 63 patients (recruited between 2019 and 2021) with aSAH; the mean age was 59.2 years (range, 48–77), and the male-to-female ratio was 1.52:1. The primary selection and exclusion criterion was Fisher grades; the patients we selected were Fisher grades III–IV. Hunt and Hess (H&H) grades were II–V, and we did not use H&H grade as a selection and exclusion criterion. This study did not include patients with minor bleeding and apparent signs of death. We grouped patients according to whether they had the following after 1 month: (1) whether they showed the classic triad, dementia, dyskinesia, and urinary incontinence; (2) according to the latest international guidelines published in 2012,¹⁹ assessment of ventricle morphology using computed tomography (CT) or magnetic resonance imaging (MRI), patients with Evan's index greater than 0.3 were considered to have secondary hydrocephalus; (3) furthermore, the significant relief of symptoms after ventriculoperitoneal drainage was considered a definitive diagnosis of chronic hydrocephalus.

Forty-five patients had follow-up examinations in our hospital 1 month later. For the other 18 patients who did not return to our hospital, we followed up with them by telephone and asked them about their current symptoms—whether they had done shunt surgery or imaging studies at other hospitals and whether their consciousness was clear, urination was uncontrolled, and activities were convenient. According to the feedback and the above-mentioned diagnostic criteria, 19 of the 63 patients developed chronic hydrocephalus about a month later; their Evan's index was greater than 0.3. All of these 19 patients had CSF shunt surgery, which showed significant clinical improvement. These patients formed the experimental group; the mean age was 60.5 years and the male-to-female ratio was 2.25:1 (**►Table 1**). Thirty-four patients had none of this classic triad, and 10 patients who were followed up by telephone had only atypical symptoms; we rechecked their head CT in our hospital and find no ventriculomegaly. Evan's index of these 44 patients was less than 0.3. Therefore, they were considered the control group; the mean age was 58.6 years and the male-to-female ratio was 0.78:1 (**►Table 1**).

Samples of CSF were harvested by lumbar puncture from all patients of aSAH, and all of the samples were red or dark red. According to the condition of the patients, all the samples were collected on the second or third day after SAH. CSF samples were centrifuged and stored in polypropylene tubes at -80°C to await analysis.

This study is approved by the Institutional Research Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) (ethical code: YJSKY2022-147). All procedures were performed according to the

Table 1 Clinical and imaging data of the experimental and control groups

	Experimental group	Control group
Number of patients	19	44
Mean age, y	60.5	58.6
Male-to-female ratio	2.25:1	0.78:1
Fischer grade III	8	21
Fischer grade IV	11	23
H&H scale II	4	10
H&H scale III	11	25
H&H scale IV	3	7
H&H scale V	1	2
Evan's index	>0.3	<0.3

Abbreviation: H&H, Hunt and Hess.

Declaration of Helsinki. All patients or their relatives gave informed written consent.

Enzyme-Linked Immunosorbent Assay

We measured the levels of 10 biomarkers (LRG, TGF-β, Smad1, Smad4, Smad5, Smad8, P38, Alk-1, Alk-5, and TGFβRII) in each CSF sample using specific enzyme-linked immunosorbent assay (ELISA) kits as described previously and in accordance with the manufacturer's guidelines (Ruixinbio, Quanzhou, China). Absorbance was measured at 450 nm.

Statistical Analysis

All data arising from ELISA were used to determine mean values with standard deviations (means ± SDs). Significant differences between the hydrocephalus group and the controls were identified by applying the *t*-test for independent

samples. The bivariate Pearson's test assessed correlations between the 10 biomarkers. Statistical analyses were performed using SPSS version 26.0 for Windows (SPSS China Inc.). Differences were considered significant at *p* < 0.05. First, we selected statistically significant biomarkers; then, we selected the pairs of biomarkers that were significantly correlated. Finally, we attempted to identify a new signaling pathway based on the significant relationships identified.

Results

Differences in the Levels of 10 Different Biomarkers between the Hydrocephalus Group and the Nonhydrocephalus Group

The levels of the 10 biomarkers were measured in all CSF samples in triplicate, and the mean values are presented in ▶Table 2. We successfully detected the 10 biomarkers in CSF samples from all cases. The analysis demonstrated that CSF samples from the post-SAH chronic hydrocephalus group showed a significant increase in the levels of LRG, TGF-β, Smad5, Alk-1, and TGFβRII when compared with control samples (*p* < 0.05) (▶Fig. 1; ▶Table 2). In contrast, the levels of Smad1, Smad4, and Smad8 were significantly lower in the post-SAH chronic hydrocephalus group than in the controls (*p* < 0.05) (▶Fig. 1; ▶Table 2). There were no significant differences between the two groups concerning the levels of P38 and Alk-5 (*p* > 0.05) (▶Table 2).

Associations between Biomarkers

Application of the bivariate Pearson's test identified several essential correlations. LRG levels in the CSF of both controls and patients with chronic hydrocephalus after aSAH were significantly correlated with Smad1 levels (▶Fig. 2A; ▶Table 3) (*p* < 0.05). Alk-1 levels in the CSF of both controls and patients with chronic hydrocephalus after aSAH were significantly correlated with Smad4 levels (▶Fig. 2B; ▶Table 3) (*p* < 0.05).

Table 2 ELISA results for LRG, TGF-β, Smad1,4,5,8, Alk-1, Alk-5, P38, and TGFβRII levels in the CSF of controls and patients with hydrocephalus after aneurysmal subarachnoid hemorrhage

	Controls (ng/mL)		Hydrocephalus (ng/mL)		<i>p</i> -Value
	Mean ± SD	Range	Mean ± SD	Range	
LRG	151.6 ± 24.7	110.1–187.4	179.8 ± 21.6	134.7–207.4	0.043*
TGF-β	252.9 ± 34.3	192.2–315.2	279.7 ± 45.2	197.5–347.7	0.026*
Smad1	13.7 ± 2.6	9.5–18.0	10.5 ± 2.4	7.0–14.3	0.046*
Smad4	13.1 ± 2.2	9.3–16.8	10.4 ± 2.4	6.6–13.7	0.044*
Smad5	13.5 ± 2.8	9.7–18.5	14.2 ± 2.6	11.9–18.5	0.009*
Smad8	6.5 ± 1.1	4.7–8.2	6.4 ± 1.2	4.6–8.4	0.002*
Alk-1	7.5 ± 0.9	5.8–9.0	8.1 ± 1.0	6.2–9.5	0.022*
TGFβRII	38.0 ± 5.6	28.2–46.8	41.9 ± 6.6	33.3–51.7	0.024*
Alk-5	12.8 ± 2.7	8.8–17.6	13.3 ± 2.3	8.9–17.5	0.051
P38	168.6 ± 24.6	131.9–213.3	202.2 ± 23.7	160.4–234.0	0.069

Abbreviations: Alk-1, activin receptor-like kinase 1; Alk-5, activin receptor-like kinase 5; Hyd, patients with hydrocephalus; LRG, leucine-rich alpha-2-glycoprotein; SD, standard deviation; TGFβRII, TGF-β type II receptor; TGF-β, transforming growth factor-β.

**p* < 0.05.

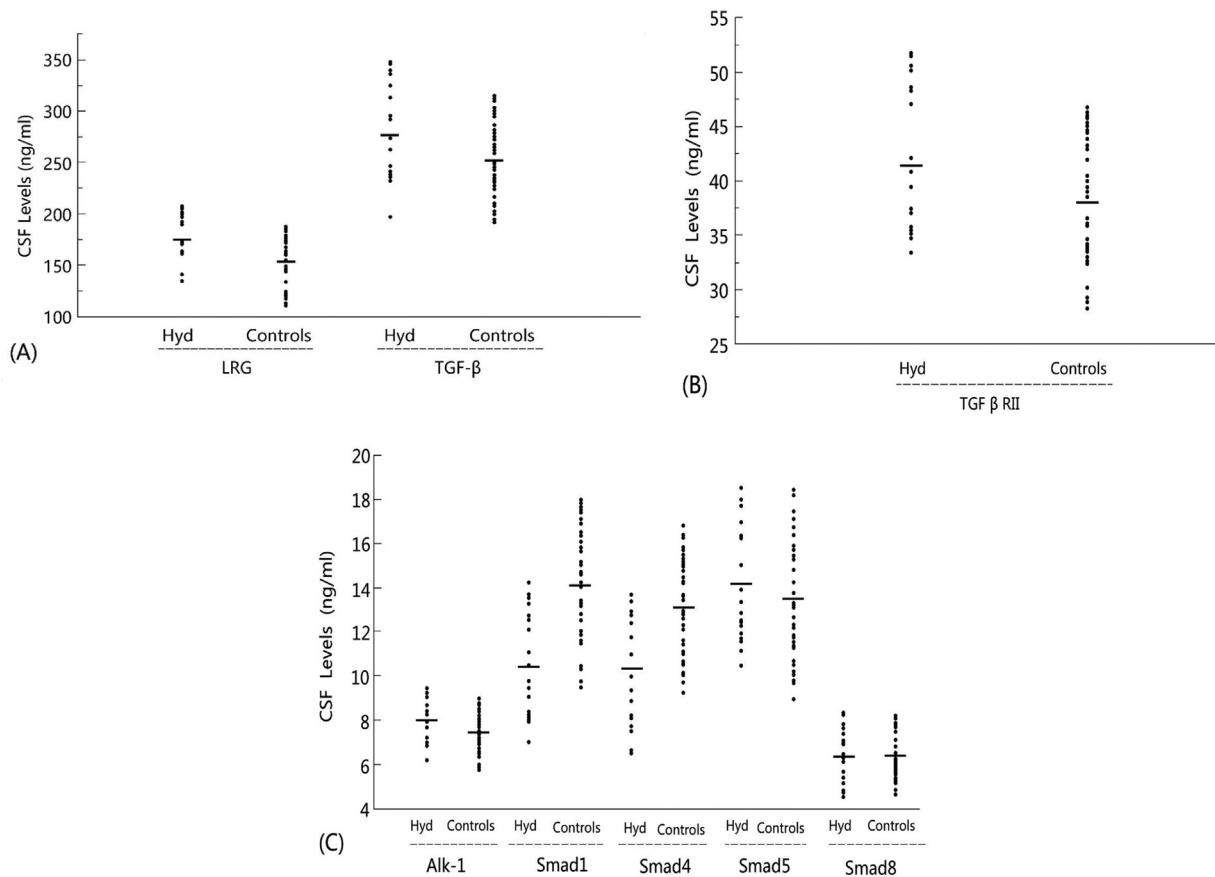


Fig. 1 Levels of leucine-rich alpha-2-glycoprotein (LRG), transforming growth factor- β (TGF- β), Smad1, Smad4, Smad5, Smad8, activin receptor-like kinase 1 (Alk-1), and transforming growth factor- β type II receptor (TGF β RII) (ng/mL) in the cerebrospinal fluid of patients with hydrocephalus after aneurysmal hemorrhage and controls. Each dot corresponds to one patient. The horizontal bar indicates the mean value in each group. (A) Levels of LRG and TGF- β , (B) levels of TGF β RII, and (C) levels of Alk-1, Smad1, Smad4, Smad5, and Smad8.

TGF- β levels in the CSF of both controls and patients with chronic hydrocephalus after aSAH were significantly correlated with Alk-1 levels (\blacktriangleright Fig. 2C; \blacktriangleright Table 3) ($p < 0.05$), and LRG levels in the CSF of both controls and patients with chronic hydrocephalus after aSAH were correlated with Smad5 levels (\blacktriangleright Fig. 2D; \blacktriangleright Table 3) ($p < 0.05$).

Discussion

At present, the mechanisms underlying the occurrence of chronic hydrocephalus after aSAH remain obscure. Furthermore, there are few studies relating to biomarkers in the CSF that are associated with this pathogenic process. Previous studies have shown that arachnoid fibrosis after aSAH reduces CSF drainage by arachnoid granules; this is the most important mechanism of hydrocephalus after aSAH.⁵ TGF- β is expressed by endothelial, hematopoietic, and connective tissue cells in response to tissue injury in wound healing or fibrosis.²⁰ After aSAH, it is released into CSF by astrocytes and platelets.²¹ LRG1 binds directly to the TGF- β accessory receptor endoglin, which, in the presence of TGF- β 1, results in the promotion of the proangiogenic Smad1/5/8 signaling pathway.²² Interestingly, it has been documented that the mechanisms of the two types of normal pressure hydrocephalus are similar, especially in terms of fibrosis. Existing evidence indicates that the levels of LRG,

TGF- β 1, and TGF- β 2 CSF are significantly increased in patients with INPH.³ Thus, it is significant to explore the mechanism of LRG-TGF- β after aSAH. However, no previous study has investigated levels of proteins in the LRG, Alk-1, Alk-5, or TGF β RII in the CSF of patients with aSAH. Existing literature only investigated one or two biomarkers of CSF in patients with chronic hydrocephalus after aSAH; these previous studies failed to identify any specific relationships between biomarkers.

In the current study, we used ELISA to detect the levels of 10 biomarkers associated with the TGF- β /Smad pathway. First, we determined the stories of these biomarkers and then compared the hydrocephalus group and the controls to identify significant differences. Then, we attempted to identify specific correlations between pairs of biomarkers. Finally, based on our findings, we tried to identify a potential signaling pathway responsible for developing aSAH.

According to existing research, LRG can regulate the expression of biomarkers through the TGF- β signaling pathway.^{6,7} The TGF- β signaling pathway is very conservative and ubiquitous; members of the TGF- β family are known to regulate cell proliferation, migration, and differentiation.²³ The family of Smad proteins is regarded as the primary effector of TGF- β signaling.^{12,13}

First, we considered the TGF- β signaling pathway and focused on two key receptors: TGF β R1 and TGF β R2. TGF β R1

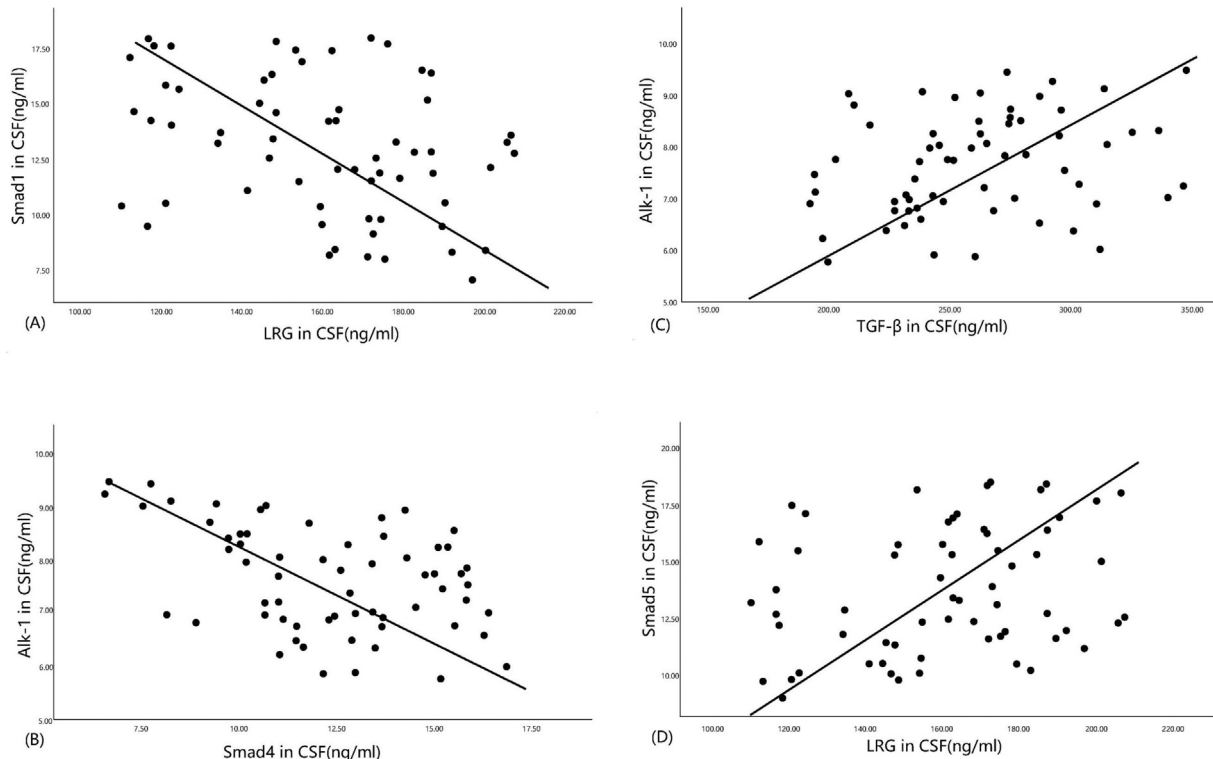


Fig. 2. Relationships between levels of leucine-rich alpha-2-glycoprotein (LRG), Smad1, Smad4, Smad5, activin receptor-like kinase 1 (Alk-1), and transforming growth factor-β (TGF-β) levels in cerebrospinal fluid (CSF). (A) LRG levels in the CSF were correlated to Smad1 levels, (B) Alk-1 levels in the CSF were correlated to Smad4 levels, (C) TGF-β levels in the CSF were correlated to Alk-1 levels, and (D) LRG levels in the CSF were correlated to Smad5 levels.

(Alk-1 and Alk-5) plays a vital role in two very different signaling pathways: Alk-1 induces the phosphorylation of Smad1/5/8 and Smad4,^{14,16,24} while Alk-5 promotes the activation of Smad2/3 and the inhibition of Smad1/5/8.^{14,16,25} Alk-1 and Alk-5 signal transduction plays an inhibitory role on each other.¹⁴ TGFβRII can synergize with Alk-1 to promote the phosphorylation of Smad proteins.²⁶ According to our experimental data, Alk-1 and TGFβRII were statistically significant ($p < 0.05$), while Alk-5 was not ($p > 0.05$); therefore, this signaling pathway is mainly guided by Alk-1 and TGFβRII, thus promoting the phosphorylation of Smad1/5/8 and Smad4 and a reduction in their levels. Once activated, these Smad1/5/8 proteins dissociate from Alk-1, bind to Smad4 to form a

dimer, and then enter the nucleus to perform a TGF-β signaling role, thus directly regulating the transcription of specific genes.^{15,27} Based on these previous findings, we hypothesized that the LRG-TGF-β-Alk-1/TGFβRII-Smad1/5/8-Smad4 signaling pathway might play a role in the pathogenic development of chronic hydrocephalus after aSAH.

This experiment is a retrospective study of aSAH patients with Fisher grades III–IV. The initial amount of blood in these patients was relatively high, and their H&H grades were II–V. Patients with less severe aSAH had a lower chance of developing hydrocephalus; moreover, not all patients had CSF collection. Therefore, we used Fisher grades as an essential selection and exclusion criterion and excluded aSAH patients with Fisher grades I–II. We also excluded patients with obvious signs of death and critically ill patients without further treatment because these patients have poor prognoses.

Considering selection bias, in addition to the above selection and exclusion criteria, we selected 63 patients with aSAH who came to our hospital for treatment based on the principle of randomness. According to the diagnostic criteria, there were 19 cases in the experimental group and 44 cases in the control group. Among them, the ratio of the H&H and Fisher grades at each level of the experimental and control groups was about 1:2, and the average age was similar. There were more male patients in the experimental group and more female patients in the control group, indicating that the male patients were more likely to develop hydrocephalus

Table 3 Pearson’s correlation coefficients between LRG and Smad1, LRG and Smad5, Alk-1 and Smad4, and TGF-β and Alk-1

	LRG	Alk-1	p-Value
Smad1	-0.341		0.006*
Smad5	0.257		0.042*
Smad4		-0.424	0.001*
TGF-β		0.256	0.043*

Abbreviations: Alk-1, activin receptor-like kinase 1; LRG, leucine-rich alpha-2-glycoprotein; TGF-β, transforming growth factor-β.
*Significant correlation at the 0.05 level (two-tailed).

after aSAH. Although there is literature supporting this phenomenon,²⁸ most of the literature does not show an effect of gender on hydrocephalus after aSAH.^{29,30}

Here, we measured the levels of 10 biomarkers to test the hypothesized signaling pathways. We found that the levels of LRG, TGF- β , Alk-1, TGF β RII, and Smad5 in CSF samples from the hydrocephalus group were significantly higher than in the controls. These findings were similar to our previous results in the CSF of patients with INPH. In addition, we found that the levels of Smad1, Smad4, and Smad8 were significantly lower in CSF samples from patients in the hydrocephalus group. These findings support the fact that the CSF of patients with chronic hydrocephalus contains higher levels of LRG, TGF- β , Alk-1, and TGF β RII; the LRG acts on TGF- β via the Alk-1 receptor and TGF β RII, thus resulting in increased levels of Smad1/5/8 and Smad4 phosphorylation; this causes their grades to fall along with the dimerization of Smad1/5/8 and Smad4.

The involvement of this signaling pathway in the development of aSAH was further verified by applying *t*-tests for independent samples; these analyses found that the levels of LRG, TGF- β , Alk-1, TGF β RII Smad1/5/8, and Smad4 were all statistically significant biomarkers. Further investigation showed that Pearson's correlation coefficient between LRG and Smad1 was -0.341 ($p = 0.006$) and that these factors were negatively correlated. Pearson's correlation coefficient between TGF- β and Alk-1 was 0.256 ($p = 0.043$), thus indicating a positive correlation. Pearson's correlation coefficient between Alk-1 and Smad4 was -0.424 ($p = 0.001$), thus showing a negative correlation. The results suggest that these biomarkers may play an essential role in the pathogenesis of chronic hydrocephalus after aSAH and, therefore, that the LRG-TGF- β -Alk-1/TGF β RII-Smad1/5/8-Smad4 signaling pathway plays a critical role in the occurrence and development of chronic hydrocephalus after aSAH.

However, some limitations of our study need to be considered. For example, in the CSF of patients with chronic hydrocephalus after aSAH, we found that the levels of Smad5 were higher than in the controls; this is not consistent with the reduction of Smad5 in response to the action of a signaling pathway. In addition, according to Pearson correlation analysis, LRG and Smad5 were positively correlated ($p = 0.042$; Pearson correlation coefficient = 0.257); this does not correlate with the changes of these factors in the signaling pathway, which showed a negative correlation. These discrepancies could be related to our small sample size. Future research should involve a larger sample size. It is also essential to consider that other proteins may also be of interest in this pathway but have yet to be identified. We plan to conduct a prospective study, adding aSAH patients with the H&H and Fisher grade I-II as controls. We will collect blood and CSF samples from all patients at different time points to investigate whether blood biomarkers levels were associated with hydrocephalus.

Conclusion

The analysis demonstrated that the levels of LRG, TGF- β , Alk-1, TGF β RII, Smad1/5/8, and Smad4 in the CSF are potential

predictive biomarkers of chronic hydrocephalus after aSAH. Our data also indicate that the LRG-TGF- β -Alk-1/TGF β RII-Smad1/5/8-Smad4 signaling pathway is probably involved in the pathogenic process underlying the development of chronic hydrocephalus after aSAH. These findings may provide ideas for further research on preventing and treating chronic hydrocephalus after aSAH. Further research needs to focus on the LRG-TGF- β -Alk-1/TGF β RII-Smad1/5/8-Smad4 signaling pathway as a potential target for intervention.

Data Availability

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

None declared.

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