Serum ROCK1 mRNA is of great diagnostic value for glioma patients

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Abstract
The study aimed to measure the presence of rho-associated protein kinase 1 (ROCK1) mRNA in serum samples collected from glioma patients and investigate its diagnostic significance in glioma.

The presence of ROCK1 mRNA was examined by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between ROCK1 mRNA and clinical characteristics was analyzed via Chi-square test. The criteria of diagnosis evaluation, including sensitivity, specificity, optimal cutoff point, and area under the curve (AUC) were determined through the receiver operating characteristic (ROC) curve analysis.

Besides, high ROCK1 mRNA expression was tightly related with Karnofsky Performance Status (KPS) score (P=0.024) and World Health Organization (WHO) grade (P=0.029). However, there was no association between ROCK1 expression and gender, neurological disorders, family history and cigarette smoking (all, P>0.05). In addition, the optimal cutoff point was 3.025, with the sensitivity and specificity of 88.89% and 79.25%, respectively. The AUC was 0.881, indicating that ROCK1 was a diagnostic biomarker for glioma patients (P<0.0001, 95% CI=0.829–0.933).

Serum ROCK1 mRNA is significantly up-regulated in glioma cases compared to healthy controls. ROCK1 may be a potential diagnostic biomarker in glioma.

Keywords: biomarker, diagnostic, glioma, ROC, ROCK1

1. Introduction
Glioma is the most frequent type of primary malignant brain tumors and 70% cases are at advanced-grade when diagnosed, which is the most aggressive and common brain tumor.[1–3] Currently, gliomas were divided into 4 grades according to the World Health Organization (WHO) grading scale, which is based on the cytologic features and malignant degrees: pilocytic astrocytoma (I), diffuse astrocytoma (II), anaplastic astrocytoma (III), and glioblastoma (IV).[4–6] The precise grading of glioma is important for designing therapy strategies, evaluating prognosis, and monitoring the response to therapies.[7] Glioma mainly derives from neuroepithelial tissues and is of high mortality and morbidity, accounting for about 40% to 50% of intracranial cancers and approximately 1.5% of whole body tumors.[8,9] Because of ineffective therapies and the infiltrative development patterns, the survival rate of glioma patients is relatively low. In addition, the poor prognosis and high mortality are considered to be associated with late diagnosis of glioma.[10,11] Therefore, it is of great importance to find novel biomarkers for the early diagnosis of glioma.

Rho-associated coiled-coil containing protein kinase (ROCK) is the downstream regulator of RhoA and participates in regulating the effects of RhoA on cell adhesion, smooth muscle contraction, cell motility, and apoptosis.[12,13] ROCK is initially identified as a serine/threonine protein kinase that binds to guanosine triphosphate (GTP)-bound RhoA.[14,15] Rho-associated protein kinase 1 (ROCK1) is an isoform of ROCK and consists of a kinase domain, an N-terminal region, a PH domain, a coiled-coil domain, and a C-terminal domain.[16,17] ROCK1 acts as an oncosine and is involved in a wide range of progressions, including cell migration, metastasis, and invasion.[18,19] Moreover, ROCK1 was highly expressed in various tumor tissues and tumor cell lines, including osteosarcoma, gastric cancer, lung cancer, and prostate cancer.[19–22] However, no reports were directly studied the expression and diagnostic value of ROCK1 in glioma.

In the present study, we attempted to determine the presence of ROCK1 mRNA in glioma and assess its relationship with diagnosis of glioma patients.

2. Materials and methods
2.1. Patients and samples
Serum samples were taken from 126 glioma patients and 53 healthy blood donors in Tianjin Medical University General Hospital.
Table 1
Relationship between ROCK1 expression and clinical factors.

| Clinical characteristics | Case NO. | Expression | \( \chi^2 \) | \( P \) value |
|--------------------------|----------|------------|---------------|--------------|
| Gender                   |          |            |               |              |
| Male                     | 67       | High       | 1.530         | .216         |
| Female                   | 59       | Low        | .503          | .478         |
| Neurological disorder    |          |            | 2.682         | .102         |
| Present                  | 55       | 45         |               |              |
| Absent                   | 71       | 49         |               |              |
| Family history           |          |            | 2.352         | .125         |
| Yes                      | 62       | 50         |               |              |
| No                       | 64       | 44         |               |              |
| Preoperative KPS score   |          |            | 5.088         | .024         |
| \( \leq 40 \)             | 65       | 54         |               |              |
| \( >40 \)                | 64       | 40         |               |              |
| Cigarette smoking        |          |            | 0.503         | .478         |
| Ever                     | 54       | 42         |               |              |
| Never                    | 72       | 52         |               |              |
| WHO grade                |          |            | 4.785         | .029         |
| II                       | 78       | 53         |               |              |
| III/IV                   | 48       | 41         |               |              |

KPS = Karnofsky Performance Status, ROCK1 = the-associated protein kinase 1, WHO = World Health Organization.

Hospital. Among the 126 glioma patients, there were 56 males and 70 females, who were grouped using the tumor-node-metastasis (TNM) stage. Their clinical parameters were recorded at diagnosis time and summarized in Table 1. None of the patients received any chemotherapy or radiotherapy before blood collection. The healthy controls were prospectively recruited from the medical examination center of the same hospital, and they were matched with cases in age and gender. Our present study was authorized by the Ethic Committee of Tianjin Medical University General Hospital. Informed consents were provided by all participants.

2.2. Serum specimens

After 12 hours overnight fasting, 5 mL peripheral blood samples were collected from all the participants using VP-AS109K Vacutainer tubes (Terumo Corporation, Tokyo, Japan). The blood samples were incubated for 30 minutes at room temperature then centrifuged at 1500 x g for 10 minutes to isolate serum samples. Subsequently, the serum specimens were centrifuged at 20,000 x g for 10 minutes under 4°C condition to remove cell debris, and then maintained at −80°C until use.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 200 μL serum using Trizol reagent (Invitrogen, Carlsbad, CA). 2% agarose gel electrophoresis (AGE) was used to detect the integrity of the obtained RNA samples, and the purity of RNA sample was estimated using Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE). In AGE images, the complete RNA exhibited clear 28 s and clear 18 s bands, moreover, the brightness ratio of 28 s band to 18 s band was nearly 2:1. OD A260/A280 ratio of 1.8 to 2.0 suggested the high purity of the RNA sample, without the contamination of DNA or protein. The first stand of cDNA was synthesized by PrimerScript™ RT reagent kit (Takara, Kyoto, Japan). Then real-time PCR was conducted using Power SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA) in the ABI Prism 7500 Sequence Detector System Applied Biosystems (Foster City, CA). The used specific primer sequences were as follows: ROCK1: Forward 5′-AACATGCTGCTGATATAAA-TCTGG-3′; Reverse 5′-TGTTATACATGTTACACATTGCT-3′; GAPDH: Forward 5′-AGACCTTGGGCTGGGACAT-3′; Reverse 5′-ACCAATTCGTTGACTCCGA-3′. GAPDH was employed as an internal control. The amplification was carried out in a 20 μL volume containing 1 μL cDNA, 10 μL 2 × SYBR Green Supermix, 2 μL qRT-PCR primers, and 7 μL ddH2O. The reaction sets were as followed: 50°C 2 minutes and 95°C 10 minutes, followed by 40 cycles of 95°C for 30 s and finally 60°C for 1 minutes. The data were shown as cycle threshold (Ct). The levels of ROCK1 mRNA were normalized to GAPDH, and calculated using the formula of 2^−ΔΔCt. Each experiment was performed 3 times.

2.4. Statistical analysis

All data analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL), and the figures were plotted by GraphPad Prism version 5.0 (GraphPad, San Diego, CA). It was considered as statistical significance if \( P \) was less than 0.05. The expression levels of ROCK1 were shown as mean ± standard deviation (SD), and their comparison between glioma patients and healthy controls was carried out using student t test. Chi-square test was adopted to compare the relationship of ROCK1 expression and gender. Receiver operating characteristics (ROC) analysis was performed to evaluate the diagnostic value of ROCK1 through calculating the sensitivity, specificity and area under the curve (AUC) in glioma.

3. Results

3.1. Up-regulation of ROCK1 mRNA in serum samples collected from glioma

Quantitative Real-time PCR was performed to measure the presence of ROCK1 mRNA in serum collected from glioma and healthy controls. The serum level of ROCK1 mRNA in glioma was 4.11 ± 0.96, while that in the controls was only 2.41 ± 1.00. It could be concluded that ROCK1 mRNA was obviously increased in serum collected from glioma compared to the controls (Fig. 1, \( P < .05 \)).

3.2. Relationship between ROCK1 expression and clinical factors

The included glioma patients were divided into high expression group (n = 84) and low expression group (n = 32) according to their median serum ROCK1 mRNA levels. Chi-square test was used to illustrate the association between ROCK1 and clinical parameters of glioma patients. The result showed that high ROCK1 mRNA level was significantly correlated with preoperative Karnofsky Performance Status (KPS) score (\( P = .024 \)) and WHO grade (\( P = .029 \)). However, no obvious relationship was found between ROCK1 expression and gender (\( P = .216 \)), neurological disorders (\( P = .102 \)), family history (\( P = .125 \)) or cigarette smoking (\( P = .478 \)) (Table 1).
3.3. Diagnostic accuracy of serum ROCK1 in glioma

The ROC curve was profiled to assess the potential significance of serum ROCK1 mRNA levels in diagnosis of glioma. As shown in Figure 2, the sensitivity and specificity were 88.89% and 79.25%, respectively, with an optimal cutoff point of 3.025. Besides, the AUC was 0.881, indicating that ROCK1 was a diagnostic marker for glioma ($P<.0001$, 95% CI = 0.829–0.933).

4. Discussion

Glioma is a tumor that occurs in neural ectoderm. It is difficult to completely remove by surgical resection because of high invasion and metastasis, and glioma is not sensitive to radiotherapy or chemotherapy. The cause of glioma is complex, which may be related to the chemical carcinogens, ionizing radiation, and heredity.$^{[23]}$ Besides, changes of these factors drive abnormal expression of cancer-related genes, including activation of oncogenes, and inactivation of anti-oncogenes. So far, various biomarkers and signaling pathways involved in cell progression and tumorigenesis have been studied in glioma. Wang et al showed that microRNA (miR)-132 enhanced the activation of transforming growth factor-$\beta$ (TGF-$\beta$) signaling via suppressing SMAD7 in glioma cells.$^{[24]}$ Cheng et al. revealed that miR-218 was an efficient and novel marker for prognosis of glioma patients.$^{[25]}$ Wang et al$^{[26]}$ reported that RAB34 was related with progression and prognosis of glioma. What is more, Zhang et al$^{[27]}$ investigated the function of ROCK1 on the proliferation and metastasis grade of glioma, and the results revealed that up-regulation of ROCK1 played important roles in the carcinogenesis, progression, and invasion of glioma. Thus it attracted great interest to value the clinical role of ROCK1 in glioma patients.

ROCK1, a key downstream effector of the small GTPase RhoA, is a serine/threonine kinase and mediates various cellular responses, including cell proliferation, growth, and apoptosis via microtubule network organization and effects on the cytoskeleton.$^{[28,29]}$ Aberrant expression of ROCK1 has been observed in several cancers and proved to be related to the tumor development and progression. Zhang et al demonstrated that over-expression of ROCK1 was found in laryngeal squamous cell carcinoma.$^{[30]}$ Chen et al explained that the expression of ROCK1 in the myolytic left atrial myocytes of mitral regurgitation (MR) patients was significantly higher than the controls.$^{[31]}$ In addition, ROCK1 has been studied as a biological marker in various diseases. Smit et al claimed that ROCK1 was a potential drug target for BRAF mutant melanoma.$^{[32]}$ Akagi et al showed that ROCK1 was a novel prognostic biomarker for vulvar cancer.$^{[33]}$ In the present study, the attention was paid on the expression of ROCK1 in glioma and its relationship with diagnosis of glioma.

In our study, the underlying role of ROCK1 in glioma diagnosis was assessed through a series of determinations. First, we determined the presence of ROCK1 mRNA in serum samples collected from glioma and healthy controls using the quantitative real-time PCR and the results revealed that serum level of ROCK1 mRNA was increased notably in glioma compared to normal controls, which was in accordance with the previous studies. Besides, high ROCK1 mRNA level was significantly related with preoperative KPS score and WHO grade, indicating ROCK1 might be involved in the progression of glioma. Furthermore, we established the ROC curve to detect the diagnostic value of serum ROCK1 for glioma patients. The results showed that AUC exhibiting a global summary of the diagnostic performance of ROCK1, and the AUC value showed that ROCK1 could discriminate glioma patients from the healthy individuals.

Though the diagnostic performance of ROCK1 in glioma has been investigated in the study, its mechanism on glioma is still unclear. There was a report suggesting that the RhoA/ROCK pathway might be related to abnormal myometrial contractility in obese pregnant women.$^{[34]}$ Hallgren et al demonstrated that the rho/ROCK signaling pathway was regarded as a sensor of tissue compliance.$^{[35]}$ These may provide us with research interests.

In summary, we explored the diagnostic value of ROCK1 in glioma. Serum ROCK1 mRNA level was significantly higher in glioma than that in the controls and ROCK1 was of great
diagnostic significance in glioma. What is more, the precise mechanism of ROCK1 in glioma is still dismal and needs more investigations and efforts in the future work.

Author contributions

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