The Progression Related Gene RAB42 Affects the Prognosis of Glioblastoma Patients

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Abstract: Background: Glioblastoma (GBM) represents the most malignant glioma among astrocytomas and is a lethal form of brain cancer. Many RAB genes are involved in different cancers but RAB42 (Ras-associated binding 42) is seldom studied in GBM. Our study aimed to explore the role of RAB42 expression in the development and prognosis of GBM. Methods: All GBM patient data were obtained from The Cancer Genome Atlas (TCGA) and Chinese Glioma Genome Atlas (CGGA) databases. The relevance of RAB42 expression to the clinicopathologic characteristics of GBM patients was analyzed. The overall survival (OS) significance was determined using log-rank. Significantly enriched KEGG pathways were screened using gene set enrichment analysis (GSEA). Results: High expression of RAB42 was observed in GBM specimens compared with normal samples, which was also verified in cell lines and tissue samples. Elevated RAB42 expression was correlated with higher GBM histological grade. The prognosis of GBM patients with high RAB42 expression was worse than those with lower RAB42. A total of 35 pathways, such as the P53 pathway, were significantly activated in highly RAB42-expressed GBM samples. Conclusions: High RAB42 expression is related to the development of GBM, and RAB42 is a probable prognostic marker for GBM.

Keywords: glioblastoma; RAB42; The Cancer Genome Atlas; prognostic marker

1. Introduction

Glioblastomas (GBM), also named WHO grade IV astrocytomas [1], are intrinsic brain tumors [2]. In addition, GBM is the most malignant glioma among astrocytomas and is a lethal form of brain cancer [3]. The median overall survival (OS) of GBM was just 10–20 months with radio- and chemotherapy in [4–6]. Unlike some other cancers, therapies for and research into GBM are facing more challenges due to limited opportunities for reoperation, limited tumor locations, limited sample amounts, and so on [7–9]. Not only that, there is a significantly heterogeneous inter- and intra-tumor genome in GBMs [8], which is a problem in various kinds of cancers. However, based on the background of a high probability of 5-year recurrence for GBM [10], not only do some estimates suggest that magnetic resonance imaging (MRI) pseudoprogression rates are close to 20% [11] but also significant differences could be observed in the survival outcomes of GBM patients with conventional prognostic factors [12]. Accordingly, it is imperative to explore novel targeted progression and prognostic markers for GBM patients.

Ras-associated binding (RAB) proteins constitute the largest family in the RAS superfamily of small GTPases, including over 60 identified members in humans [13,14]. RAB42 is a member of the RAB family. Aberrant expression of RABs is involved in the dysregulation
of multiple signaling pathways and many diseases such as cancer, Alzheimer’s disease, and so on [15–17]. In recent decades, many studies have documented that several RABs, such as RAB21 [18], RAB34 [19], RAB14 [20], and RAB27a [21] are associated with the progression and prognosis of GBM and other tumors. However, there are few studies about RAB42 in cancers. It has been reported that RAB42 is negatively associated with 5-year overall survival and shows a poorer prognosis in glioma patients [22]. RAB42 is demonstrated to be involved in prenylation in vivo and in the cells [23]. A study has suggested that in keratinocytes, RAB42 participates in protein degradation on melanosomes [24]. In addition, the potential effects of RAB42 in GBM development and prognosis have been seldom investigated as far as we know.

Therefore, we herein aim to study the potential role of RAB42 in GBM through deep mining of publicly obtained GBM data and further experimental validation in our local specimens. Our findings are expected to give more references for the impacts of aberrant RAB42 in the progression of GBM.

2. Materials and Methods

2.1. Data Resources

The mRNA expression data and corresponding clinical information of 161 GBM patients were downloaded from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/, accessed on 21 June 2020) database, among which 160 patients had complete survival information. The clinical information data of 160 patients are listed in Table 1.

| Characteristics          | Patients (n = 160) |
|--------------------------|-------------------|
|                          | NO.   | %      |
| Sex                      |       |        |
| Female                   | 56    | 35.00% |
| Male                     | 104   | 65.00% |
| Age ≤60 (Median)         | 82    | 51.25% |
| Age >60 (Median)         | 78    | 48.75% |
| Race                     |       |        |
| Black or African American| 11    | 6.88%  |
| Asian                    | 5     | 3.13%  |
| Unknown                  | 1     | 0.63%  |
| Survival Time            |       |        |
| Long (>5 years)          | 2     | 1.25%  |
| Short (<5 years)         | 158   | 98.75% |
| OS status                |       |        |
| Dead                     | 131   | 81.88% |
| Alive                    | 29    | 18.13% |

Additionally, independent mRNA expression data and corresponding clinical information of 301 GBM patients were downloaded from the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/, accessed on 21 June 2020) database, among which 285 patients had complete survival information (Table 2). Whole genome expressions of these samples were detected by the Agilent-014850 Whole Human Genome Microarray 4 × 44 K G4112F platform.

2.2. Clinical Samples Collection

The clinical samples were all collected from the Tianjin Huanhu Hospital (Tianjin, China), including 15 GBM tissue samples and 5 brain tissue samples. All experiments have been approved by the Ethics Committee of Tianjin Huanhu Hospital (Tianjin, China), in line with the “Declaration of Helsinki”. Informed consent was obtained from all participants. The detailed patient information of the subjects is listed in Table S1.
Table 2. Clinicopathological characteristics of GBM patients from CGGA database.

| Characteristics | RAB42    | X-Squared | p-Value |
|-----------------|----------|-----------|---------|
|                 | High     | Low       |         |
| Age             | 45.7 ± 12.5 | 39.6 ± 10.2 | 0.43623 | 0.509 |
| Sex             |          |           |         |
| Female          | 50       | 67        | 3.1994  | 0.07367 |
| Male            | 88       | 74        |         |         |
| Grade           |          |           |         |
| II              | 23       | 82        | 56.14   | 6.45 × 10^{-13} |
| III             | 27       | 24        |         |         |
| IV              | 88       | 35        |         |         |
| IDH             |          |           |         |
| Wild            | 99       | 53        | 31.435  | 2.06 × 10^{-8} |
| Mutation        | 39       | 88        |         |         |

2.3. Survival Analysis

The OS of GBM patients was evaluated in the survival and survminer package of R (https://CRAN.R-project.org/package=survminer, accessed on 5 August 2020) (the difference significance was tested by log-rank).

2.4. Differentially Expressed Genes

Differentially expressed genes (DEGs) were analyzed in limma of R [25]. Only those DEGs with |Log₂FC| > 1 and FDR ≤ 0.05 were considered significant.

2.5. GO and KEGG Enrichment Analysis

Regarding the DEGs, the GO (including Biological Process, Molecular Function, and Cellular Component) and KEGG pathway enrichment analysis was conducted in ClusterProfiler of R [26]. The terms with p-value.adjust < 0.05 were considered significant terms.

2.6. Gene Set Enrichment Analysis (GSEA)

GSEA analysis was conducted based on the gene set c2.cp.kegg.v7.0.symbols in the MSigDB database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp, accessed on 25 November 2020) (software version: 4.0, screening criteria: p < 0.05).

2.7. Statistical Analysis

The Wilcoxon rank-sum test or Kruskal–Wallis test were employed to determine the difference significance. Multivariate Cox regression analysis was performed to find the independent OS-related factors. The statistically significant criterion was p < 0.05. All statistical analyses were performed with R package v3.5.2. (R Core Team, Vienna, Austria).

2.8. Cell Culture

In our present research, a total of four cell lines were included, comprising 3 GBM cell lines (U87, TJ905, H4) and 1 human normal astrocyte cell line, HA1800. Additionally, HA1800, TJ905, and H4 were purchased from the National Laboratory Cell Resource Platform (Beijing, China), and U87 cells were purchased from ATCC. The cell line authentication was conducted using STR profiling. All cell lines were cultured in DMEM (C11995500BT, Gibco), supplementing 100 µL/mL penicillin, 100 µL/mL streptomycin, 10% fetal bovine serum (P5, 15,140,122, Gibco), in an incubator with 5% CO₂ at 37 °C. All cells were cultured in T25 plates at a density of 1 × 10^5 cells per well.

2.9. qRT-PCR and Reagents

The total RNA extraction was undertaken with TriQuick Reagent (Solarbio, R1100, Beijing, China), and the total RNA was subjected to concentration and purity detection. PrimeScript RT Master Mix (TaKaRa, RR037A, Beijing, China) was used for reverse tran-
scription. Then, the amplification was conducted in the Roche 480 real-time PCR system, using SYBR Green Master Mix (ROCHE, Basel, Switzerland). The following program of PCR was used (3 repeats per sample): pre-denaturation 95 °C for 5 min, 45 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 30 s. The β-actin was used for internal reference, and for all primer sequences, see Table 3. The mRNA expression was calculated by the formula $2^{-\Delta\Delta CT}$ [27].

Table 3. Primer sequences for RT-PCR.

| Genes  | Forward Primer (5′–3′)       | Reverse Primer (5′–3′)       | Product Length (bp) | Tm (°C) |
|--------|------------------------------|------------------------------|---------------------|---------|
| β-actin| CCTGGCACCCAGCACAAAT          | GGGCGGACTCGTCATA              | 144                 | 60      |
| RAB42  | GGGTCATCATTAGCCCTT           | GACCGAGTGAAACTCCTGG           | 82                  | 60      |

2.10. Western Blot

All Western blot steps were in line with previous methods [28], and all proteins were assessed sequentially on the same membranes. The reagents used in our study included primary antibody RAB42 AnTibody (H00115273-M03, 1:500, Novus, Beijing, China), secondary antibody IgG-HRP (1:10,000, Santa Cruz, CA, USA), and internal reference β-actin. The chemiluminescent HRP Substrate (BeyoECL Moon, Beyotime, Shanghai, China) was used for signal detection, and the signal was exposed with Alliance Mini HD6 (UVitec Limited, Cambridge, UK). All antibodies underwent genetic validation to verify their specificity [29]. The gray value was analyzed in software Image J and then standardized.

2.11. Immunohistochemistry (IHC)

The immunohistochemistry (IHC) was conducted using methods consistent with a previous report [30]. The primary antibody RAB42 AnTibody (H00115273-M03, 1:500) and secondary antibody anti-rabbit poly-HRP-IgG (Santa Cruz, USA) were used in our study. A fully automatic immunohistochemical staining experiment was performed on the BOND MAX (Leica, DS9800, Wetzlar, Germany) instrument. The PRECICE 500B (UNIC, Beijing, China) was used for exposure.

3. Results

3.1. High Expression of RAB42 was Associated with the Development of GBM

Using the data in TCGA, the RAB42 expression in GBM tumor tissues and normal tissues was compared. Compared with corresponding normal samples, there was significantly higher RAB42 expression in GBM specimens ($p = 0.00014$) (Figure 1A). In addition, 12 RAB gene family members including RAB42 were subject to single factor Cox analysis. Our results suggested that RAB42 was significantly correlated with the prognosis of GBM ($p = 0.042$). Moreover, the HR (hazard ratio) value of RAB42 was greater than 1, which indicated that enhanced RAB42 expression would lead to a poor prognosis of GBM (Figure 1B). All results above indicated that high RAB42 expression was probably closely related to the occurrence of GBM.

3.2. Correlation of RAB42 Expression with the Grade, Gender, Age, and IDH Mutant Status of GBM Patients

Based on data from CGGA, the correlation of RAB42 expression with the GBM patients’ clinicopathologic characteristics was analyzed. We found that there was a significant correlation between higher RAB42 expression and grade ($p < 0.05$) (Figure 2A), and it was significantly enhanced along with the increase in grade (Figure 2A). In addition, no significant correlation between higher RAB42 expression and gender was observed (Figure 2B). However, compared with younger and mutant IDH (isocitrate dehydrogenase) patients, the RAB42 expression was significantly elevated in older and IDH wild-type GBM patients (Figure 2 C, D).
Figure 1. The RAB42 expression in glioblastoma (GBM) samples and normal samples. (A) The expression of RAB42 in GBM samples and corresponding normal samples based on the TCGA dataset, showed in a box plot. (B) Single-factor Cox analysis results of the RAB family genes based on the TCGA dataset displayed in a forest plot. HR: Hazard ratio; 95% CI: 95% confidence interval.

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3.3. Highly RAB42 Expressed GBM Patients Had Worse Prognosis

All GBM samples were divided into high and low RAB42 expression groups based on the median RAB42 expression. Then, a survival analysis was conducted to evaluate the prognostic value of RAB42 in GBM data (CGGA database). Our results suggested that highly RAB42 expressed GBM patients had poorer OS than lower RAB42 expressed patients, which was consistent with the results obtained from the TCGA database (p = 2.2 × 10^{-12}, HR = 0.36, 95%CI: 0.27–0.49) (Figure 3A).

Moreover, a multivariate Cox regression analysis was undertaken to find the independent prognostic indicators for GBM, comprising age, sex, grade, IDH mutation status, and RAB42. RAB42 expression was still significantly associated with overall survival. High RAB42 expression was indicated to correlate with a higher death risk (HR = 1.2, 95%CI: 1.09–1.4, p < 0.001) (Figure 3B).

3.4. Functional Enrichment Results between High and Low RAB42 Expression GBM Samples

To study the functional pathways between high and low RAB42 expression GBM samples, we performed a differential expression analysis. Compared with highly RAB42 expressed GBM samples, there were 675 upregulated genes and 740 downregulated genes in low RAB42 expressed samples (Figure S1A). These 1415 DEGs then underwent a functional enrichment analysis, and they were significantly enriched in 1076 GO terms and 61 KEGG pathways. The top 20 GO terms and KEGG pathways are shown in Figure S1B,C.

3.5. RAB42 Expression in GBM Cell Lines and Clinical Samples

Based on our findings mined from the public databases, we also explored RAB42 expression in GBM cell lines and clinical specimens. Compared with normal cell line HA1800, RAB42 showed higher mRNA (Figure 4A) and protein expression (Figure 4B, original Western blots are shown in Figure S2) in GBM cell lines, including U87, TJ905, and H4, which were in line with our bioinformatic analysis results. Besides, our IHC results indicated that higher RAB42 protein expression was detected in clinical GBM specimens (Figure 5), which was consistent with bioinformatic analysis.
Figure 2. Association of RAB42 with clinicopathological characteristics. (A–D) Based on the CGGA dataset, the correlations of RAB42 expression with different grades, genders, ages, and IDH mutation status, separately.

3.6. GSEA Results Based on RAB42 Expression

Based on the data from CGGA, GSEA was performed to identify the significantly activated pathways in enhanced RAB42 expression GBM patients compared with low RAB42 expression GBM patients. The results revealed that 35 pathways including Systemic Lupus Erythematosus, Autoimmune Thyroid Disease, Allograft Rejection, Antigen Processing and Presentation, P53 Signaling Pathway, and Glycosaminoglycan Degradation were significantly activated \( p < 0.05 \) in highly RAB42 expressed GBM patients, compared with low RAB42 expression samples (detailed results were listed in Table S2). According to the \( p \)-value, the top six significant pathways are exhibited in Figure 4.
Figure 3. The highly RAB42 expressed GBM patients had a worse prognosis. (A) Kaplan–Meier (KM) survival curve of GBM patients with high and low RAB42 expression. \( p \) determined by log-rank. (B) Results of the multivariate Cox regression analysis. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).

Figure 4. The RAB42 expression levels in local GBM cells and specimens. (A,B) RAB42 mRNA expression and protein expression were both upregulated in GBM cell lines, respectively (vs. HA1800; * \( p <0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \)).
Figure 4. The RAB42 expression levels in local GBM cells and specimens. (A–B) RAB42 mRNA expression and protein expression were both upregulated in GBM cell lines, respectively (vs. HA1800; * p <0.05, ** p < 0.01, *** p < 0.001).

Figure 5. The representative results of IHC.

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Figure 6. The results of GSEA enrichment of RAB42.

4. Discussion
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4. Discussion

The malignancy and heterogeneity of GBM make it a great health burden for patients [3]. Consequently, we herein explored the correlation between RAB42 expression and GBM based on public data in TCGA and CGGA databases. Our data implied that the high expression of RAB42 was probably related to the development of GBM. Moreover, highly RAB42 expressed GBM patients showed worse prognosis, indicating its prognostic biomarker probability. Additionally, enhanced RAB42 expression was found to be significantly associated with grade, and several pathways were significantly activated in highly RAB42 expressed GBM samples.

We demonstrated that RAB42 was probably a promising prognostic biomarker in GBM patients. Mutant or aberrant RAB expressions were demonstrated to cause various disorders [31]. Furthermore, RABs have been reported to be up-regulated in several types of cancers [32]. RAB42 is a member of the RAB family, while it has been hardly studied in GBM. In this study, up-regulated RAB42 expression was observed in GBM specimens compared with normal specimens, which was successfully validated in cell lines and clinical

Figure 6. The results of GSEA enrichment of RAB42. (A–F) Systemic Lupus Erythematosus, Autoimmune Thyroid Disease, Allograft Rejection, Antigen Processing and Presentation, P53 Signaling Pathway, and Glycosaminoglycan Degradation.

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samples. Our data indicated that high RAB42 expression might be associated with the development of GBM. Our results partly enriched the previous similar research outcome. The research in glioma first demonstrated that RAB42 was negatively correlated with 5-year OS and displayed a poorer prognosis [22]. In addition, it has been reported that RAB42, as a protein-coding gene, is related to prenylation in vivo and in cells [23], which may be indirectly involved in tumorigenesis. Not only that, another research study reported that RAB25, a member of the RAB family, exerted a promoting effect on the growth and proliferation of GBM cells [33]. A study related to RAB43, another member of the RAB family, suggested that glioma patients with high RAB43 expression showed worse clinical outcomes when compared with low RAB43 expression glioma patients [34].

Additionally, the correlation of RAB42 with various clinicopathological characteristics and the prognosis of GBM patients was analyzed. Elevated RAB42 expression was significantly correlated with grade. Additionally, our data indicated that the RAB42 expression was significantly enhanced in wild-type IDH patients compared to mutant IDH GBM patients. IDH status is one of the most important genetic molecular markers of GBM [35,36], and the wild-type IDH GBMs often show poorer survival [37]. Accordingly, highly expressed RAB42 associated with worse GBM prognosis was in line with higher RAB42 expression in wild-type IDH GBMs. Compared with younger GBM patients, the RAB42 expression was significantly enhanced in older patients, which was in line with a previous study showing that GBM was most commonly diagnosed in elderly patients [38]. High RAB42 expression correlated with higher death risk, serving as a poor prognostic marker for GBM. It has been documented that various cancers are associated with upregulated RAB family members [19,33,39]. Collectively, RAB42 is probably an independent prognostic indicator for GBM.

Moreover, RAB42-related pathways were identified using CGGA datasets between high and low RAB42 expression GBM patients. Then, 35 signaling pathways were observed to be activated in high RAB42 expression GBM patients and the six most significantly activated pathways were SYSTEMIC LUPUS ERYTHEMATOSUS, AUTOIMMUNE THYROID DISEASE, ALLOGRAFT REJECTION, ANTIGEN PROCESSING AND PRESENTATION, P53 SIGNALING PATHWAY, and GLYCOSAMINOGLYCAN DEGRADATION. We noticed that the P53 signaling pathway was significantly activated in highly RAB42 expressed GBM patients. It has been documented that one or more genetic aberrations in the p53 pathway were contained in most GBMs [40–42], indicating our data were consistent with previous reports. Whether high RAB42 expression participates in the tumorigenesis of GBM through the P53 signaling pathway and thereby negatively affects the prognosis of patients needs to be further verified. Other significantly activated pathways implied that RAB42 exerted roles probably involving immune response (Antigen Processing and Presentation, Natural Killer Cell Mediated Cytotoxicity) and cell adhesion (Cell Adhesion Molecules CAMs, Focal Adhesion). In our research, aberrant RAB42 expression was evidenced to activate the P53 and other signaling pathways and was related to the occurrence and prognosis of GBM. Nevertheless, there are also several limitations in this work. Although we first revealed the potential role of RAB42 in GBM, the detailed RAB42-related underlying mechanisms remain unclear and deserve further investigation via wet experiments in the near future.

5. Conclusions

In summary, we have revealed for the first time the potential role of RAB42 in the development of GBM. Moreover, high RAB42 expression might affect the prognosis of GBM by involving its progression. Highly RAB42 expressed GBM patients’ poor prognosis indicates that RAB42 might be a possible biomarker for GBM. All of our results may contribute to further study of the potential mechanisms in GBM and more research should be performed in the future.
Supplementary Materials: The following are available online at http://www.mdpi.com/1565-3994/12/6/767/s1, Figure S1: Functional enrichment results. (A) Totally 1415 DEGs were identified. (B,C) Top 20 GO and KEGG terms, respectively; Figure S2: The original Western blots; Table S1: The clinical information of patients; Table S2: Detailed results of GSEA.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and analysed during the current study are available in The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/, accessed on 21 June 2020) and the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/, accessed on 21 June 2020).

Conflicts of Interest: The authors declare no conflict of interest.

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