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ORIGINAL ARTICLE

Retinol binding protein 1-dependent activation of NF- κB signaling enhances the malignancy of non-glioblastomatous diffuse gliomas

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Abstract

Nonglioblastomatous diffuse glioma (non-GDG) is a heterogeneous neuroepithelial tumor that exhibits a varied survival range from 4 to 13 years based on the diverse subtypes. Recent studies demonstrated novel molecular markers can predict prognosis for non-GDG patients; however, these findings as well as pathological classification strategies show obvious limitations on malignant transition due to the heterogeneity among non-GDGs. Therefore, developing reliable prognostic biomarkers and therapeutic targets have become an urgent need for precisely distinguishing non-GDG subtypes, illuminating the underlying mechanism. Nuclear factor κβ (NF-κB) has been proved to be a significant nuclear transcriptional regulator with specific DNA-binding sequences to participate in multiple pathophysiological processes. However, the underlying mechanism of NF-κB activation still needs to be further investigated. Herein, our results indicated retinol-binding protein 1 (RBP1) was significantly upregulated in the IDH^{WT} and $1p19q^{Non\ co\text{-}del}$ non-GDG subtypes and enriched RBP1 expression was markedly correlated with more severe outcomes. Additionally, malignant signatures of the non-GDG cells including proliferation, migration, invasion, and self-renewal were significantly suppressed by lentiviral knockdown of RBP1. To further explore the underlying molecular mechanism, bioinformatics analysis was performed using databases, and the results demonstrated RBP1 was strongly correlated with tumor necrosis factor α (TNFα)–NF-κB signaling. Moreover, exogenous silencing of RBP1 reduced phosphorylation of IkB-kinase α (IKKα) and thus decreased NF-κB expression via decreasing the degradation of the IκBα protein. Altogether, these data suggested RBP1-dependent activation of NF-κB signaling promoted malignancy of non-GDG, indicating that RBP1 could be a reliable prognostic biomarker and potential therapeutic target for non-GDG.

Wei Wu and Yichang Wang contributed equally to this work.

KEYWORDS
EMT-like phenotypes, NF-κB signaling, prognostic biomarker, RBP1, non-GDG

1 | INTRODUCTION

Nonglioblastomatous diffuse gliomas (non-GDGs) are a heterogeneous group of neuroepithelial tumors with unique biological and clinical properties, which accounted for approximately 20% of all gliomas and presented a peak incidence in patients aged between 35 and 45 years.[1-3] Non-GDG is traditionally defined as grade I to grade III glioma based on the World Health Organization (WHO) classification according to histopathologic features and morphologic signatures.[1,4] However, these classification strategies have barely reflected the heterogeneity of non-GDG, in which grade I and partial grade II non-GDGs are benign neoplasms and could be effectively cured with craniotomy, thereby conferring a comparatively favorable prognosis, whereas diffuse and infiltrative intracerebral gliomas are hardly completely resected by surgical operation and might undergo potential malignant transition, thus inducing rapid recurrence.[5] Recently, multiple research studies have corroborated that molecular markers with genomic analysis are becoming crucial in the definition of discrete glioma subtypes, prognosis prediction, and making individualized therapy strategies.[1,6,7] Accumulating evidence shows that the mutation of isocitrate dehydrogenase (IDH) and the integrity status of chromosomal arms 1p and 19q (1p/19q) provide significant clinical implication in non-GDG.[8] IDH mutation is considered as an early driver of tumorigenesis and elevation susceptibilities for chemotherapy thus is correlated with prolonged survival in glioma.[9,10] Similarly, combined deletion of 1p/19q is widely used as a pathological signature for glioma malignancy.[11] A recent comprehensive genomic analysis based on The Cancer Genome Atlas (TCGA) Research Network presented that distinct prognoses of glioma molecular subtypes depended on IDH and 1p/19q status.[8] Glioma patients with pathological signatures including IDH mutation and 1p/19q combined deletion showed more favorable outcomes, with a median survival time of approximately 10 years. Conversely, patients with wild-type IDH and intact 1p/19q presented a more severe median survival time of 1.7 years, which is similar to that of glioblastoma (GBM) patients.[12,13] Although these findings showed that combination of IDH and 1p/19q status of non-GDG exactly predicted prognosis, the underlying molecular mechanism of the malignant subtype transition, radio resistance, or chemoresistance of non-GDG still remains unclear. Therefore, developing novel molecular markers for illuminating malignant biological behavior and making rational therapeutic strategy are becoming an urgent need.

Epithelial-mesenchymal transition (EMT) has been proved to be indispensable for multiple malignant signatures in tumor, including stemness maintenance, embryonic development, therapy resistance, and tumor invasion.[14] Accumulating evidence has shown that the subtype switch from epithelial to mesenchymal is functionally mediated by a wide range of crucial transcription factors including Twist1, Snail, ZEB1, ZEB2, MMPs, and SLUG.[15] Recent studies have revealed that EMT-specific biomarkers including N-cadherin, fibronectin, and vimentin as well as other EMT-inducing transcription factors were significantly upregulated in various tumors, including gastric cancer, glioma, lung cancer, and melanoma; moreover, they were strongly correlated with poor prognosis.[16-19] More recently, emerging evidence has shown that the EMT process could be observed in GBM, promoting tumor invasion, enhancing therapy resistance, and thus leading to rapid recurrence of GBM.[20,21] However, the functional role and underlying molecular mechanism of EMT in non-GDG has not been deeply investigated yet.

Retinol-binding protein 1 gene (RBP1), located on 3q21-q22, encodes the cellular retinol-binding protein 1 (CRBP1), which is a 15KDa cytosolic protein belonging to the family of fatty acid–binding proteins and is essential for vitamin A stability and metabolism.[22-24] Retinol (known as vitamin A) and its metabolic products are necessary for many biological processes including epithelial cell proliferation, differentiation, and apoptosis.[25] Emerging evidence recognizes that aberrant RBP1-expressive alterations are associated with various tumor processes.[26] Recent studies have demonstrated that RBP1 exhibits changeable expressive patterns in multiple cancer subtypes: It is significantly downregulated in hepatocellular carcinoma, breast cancer, endometrial cancer, and ovarian cancer[27-30] and, intriguingly, is markedly elevated in laryngeal cancer and glioma accompanied by increased intratumoral retinoid levels and an unfavorable outcome.[31,32] Moreover, it is reported that RBP1 promoter hypermethylation is accompanied by mutant IDH in gliomas and is associated with improved patient survival, suggesting that dysregulation of retinoic acid metabolism contributes to tumorigenesis of glioma.[33] However, the function and molecular mechanism of RBP1 in non-GDG are still unclear.

In this study, differential gene analysis was performed by using open-access databases, and RBP1 was identified as one of the most upregulated genes in severe subtypes of non-GDG, which were defined by wild-type IDH and non-codeletion 1p19q. Additionally, increased RBP1 revealed poor outcomes in non-GDG patients. Moreover, overexpression of RBP1 enhanced multiple malignant biological behaviors of non-GDG cells, while lentivirus-dependent suppression of RBP1 markedly attenuated the proliferation, migration, and invasion of non-GDG cells. Bioinformatics analysis revealed that enriched RBP1 was correlated with the EMT process and tumor necrosis factor α (TNFα)-dependent activation of nuclear factor κβ (NF-κB) signaling was involved. Results from mechanism studies showed that RBP1 induced the phosphorylation of inhibitor of nuclear factor kappa-B kinase subunit α (IKKα) and then enhanced the expression of NF-κB signaling, thus promoting the invasion and migration properties of EMT-like phenotypes and cell proliferation in non-GDG cells. Taken together, our study indicated that RBP1 was

a reliable prognostic biomarker and might be a potential therapeutic target for non-GDG.

2 | MATERIALS AND METHODS

The materials and methods are described in Appendix S1.

3 | RESULTS

3.1 | Retinol-binding protein 1 was markedly upregulated in IDH^{WT} and 1p19q$^{Non\ co\text{-}del}$ non-GDG

To investigate the clinical significance of IDH status and 1p19q integrity, the complete survival data of 546 the non-GDG derived from the Chinese Glioma Genome Atlas (CGGA) database were used to construct Kaplan-Meier plot according to subgroup stratification. As shown in Figure 1A, in the IDH^{WT}/1p19q$^{Non\ co\text{-}del}$ subgroup, by contrast, it was verified that the prognosis was significantly declined, which was consistent with a previous study in which patients with wild-type IDH and non-codeletion 1p/19q non-GDG presented a worse prognosis.[12] Furthermore, to investigate the differentially expressed genes in different subgroups, the CGGA samples of IDH^{WT}/1p19q$^{Non\ co\text{-}del}$ and IDH^{Mut}/1p19q$^{Co\text{-}del}$ were analyzed, and the results indicated that RBP1 was one of the most markedly augmented genes in the IDH^{WT}/1p19q$^{Non\ co\text{-}del}$ subgroup (Figure 1B). To explore the consistency of RBP1 expression, qRT-PCR analysis was performed by using non-GDG samples (0864, 3247, 0708, 1789, 7419, 6567, and 0721) compared with nontumor samples deriving from epilepsy surgery. The results showed that RBP1 was significantly elevated in non-GDGs compared with nontumor tissue (Figure 1C). Similar results could be observed by using Western blot analysis (Figure 1D). Moreover, RBP1 expression was investigated in three primary cultured non-GDG cell lines (0708, 7491, and 0721) and normal human astrocytes (NHAs). Consistent with the previously mentioned data, both qRT-PCR and Western blot results indicated a significantly enriched expression of RBP1 in the non-GDG cell lines (Figure 1E,F). Taken together, RBP1 was highly expressed in non-GDGs compared with nontumor tissues and cell lines.

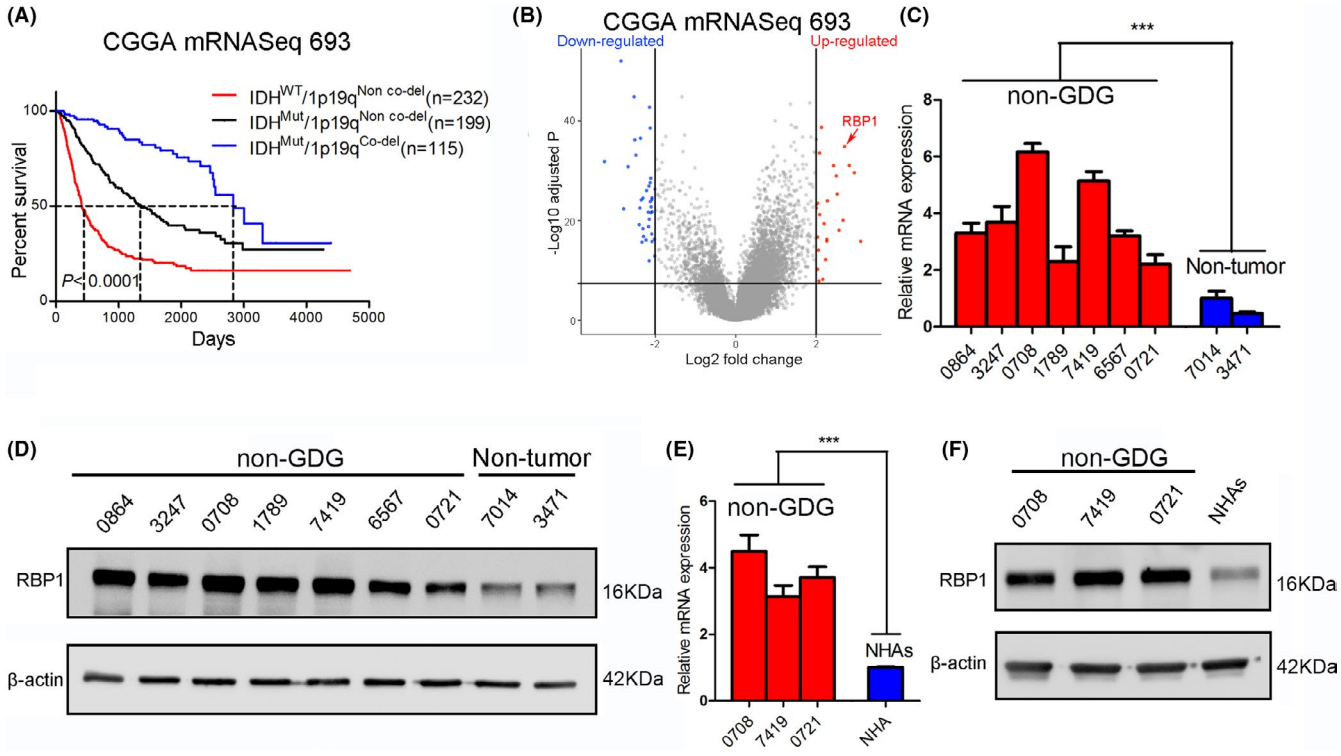


FIGURE 1 Retinol-binding protein 1 (RBP1) was markedly upregulated in IDH^{WT} and 1p19q$^{Non\ co\text{-}del}$ nonglioblastomatous diffuse glioma (non-GDG) samples. A, Kaplan-Meier survival analysis for the nonGDG subgroup using the CGGA mRNASeq 693 non-GDG database ($P < .0001$, with Log-rank test; n = 232 in the IDH^{WT}/1p19q$^{Non\ co\text{-}del}$ group; n = 199 in the IDH^{Mut}/1p19q$^{Non\ co\text{-}del}$ group; n = 115 in the IDH^{Mut}/1p19q$^{Co\text{-}del}$ group; n = 16 in the IDH^{WT}/1p19q$^{Co\text{-}del}$ group, which was excluded because of too few samples). B, Differentially expressed genes (DEGs) in IDH^{WT}/1p19q$^{Non\ co\text{-}del}$ non-GDG versus IDH^{Mut}/1p19q$^{Co\text{-}del}$ non-GDG. The expression profile was extracted from the CGGA database. DEGs were defined as $Log_2FC>2.0$ and $P < .05$. C, qRT-PCR analysis for measuring the mRNA expression of RBP1 in seven non-GDG tumor tissues versus two nontumor tissues (***$P < .001$, with Student's *t* test, n = 3). D, Western blot analysis for detecting the RBP1 protein expression level in seven non-GDG tumor tissues versus two nontumor tissues. β-actin served as internal control. E, qRT-PCR analysis for measuring the mRNA expression of RBP1 in 0708, 7419, and 0721 non-GDG primary cell lines versus normal human astrocytes (NHA) (***$P < .001$, with Student's *t* test, n = 3). F, Western blot analysis for detecting the RBP1 protein expression level in 0708, 7419, and 0721 non-GDG primary cell lines versus NHA. β-actin served as internal control

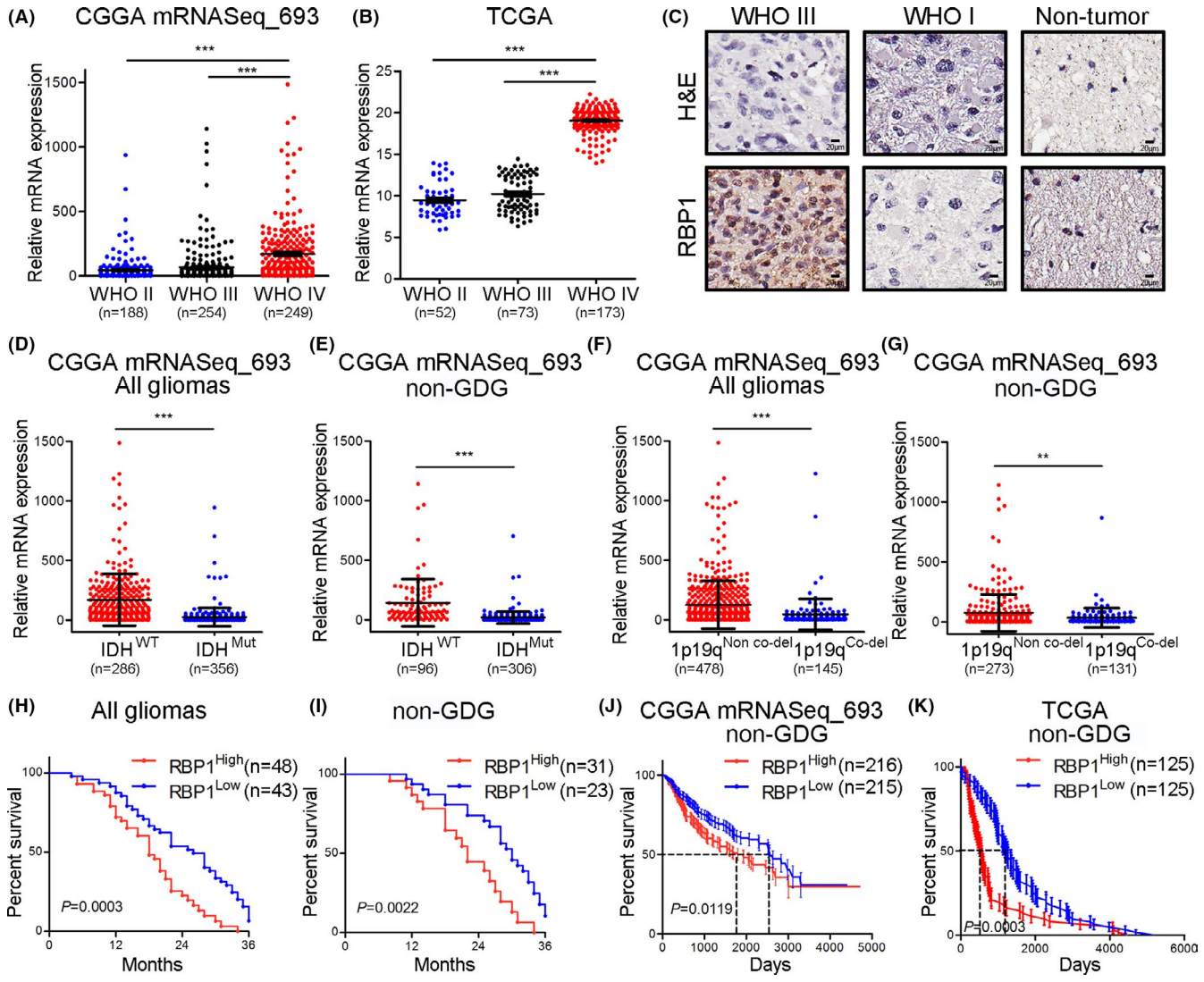


FIGURE 2 Increased retinol-binding protein 1 (RBP1) expression was closely correlated with poor outcomes in nonglioblastomatous diffuse glioma (non-GDG) patients. A and B, The expression of RBP1 mRNA in gliomas grouped by WHO grade using the CGGA (A) and The Cancer Genome Atlas (TCGA) database (B) (***$P < .001$, with one-way ANOVA followed by Dunnett's post-test). C, Representative IHC images of RBP1 in glioma tissues and nontumor samples. Upper panel: H & E staining. Lower panel: RBP1 staining. D and E, The mRNA expression of RBP1 in all glioma (D) or non-GDG (E) samples grouped by isocitrate dehydrogenase (IDH) status in the CGGA database (***$P < .001$, with Student's *t* test). F and G, The mRNA expression of RBP1 in all glioma (F) or non-GDG (G) samples grouped by 1p19q status in the CCGA database (***$P < .001$, **$P < .01$, with Student's *t* test). H and I, Kaplan-Meier analysis for RBP1 expression in all glioma (H) or non-GDG patient samples (I) ($P = .0003$ for all glioma and $P = .0022$ for non-GDG, respectively, with log-rank test). J and K, Kaplan-Meier analysis for RBP1 expression using non-GDG samples extracted from the CGGA (J) and TCGA database (K) ($P = .0119$ for the CGGA and $P = .0003$ for TCGA, respectively, with log-rank test)

3.2 | Increased RBP1 expression was closely related to poor outcomes in non-GDG patients

To gain more insight into the clinical relevance of RBP1 expression in non-GDGs, CGGA and TCGA databases were utilized to investigate the expression pattern of RBP1 in different pathological subtypes. The results indicated that RBP1 was gradually upregulated along with the pathological characteristics of glioma in both the CGGA and TCGA databases (Figure 2A,B). Immunohistochemistry (IHC) staining was used to address the RBP1 expression level of glioma samples. The results demonstrated that RBP1 was expressed predominantly in the cytoplasm cytomembrane of non-GDGs and was highly enriched in WHO III grade gliomas compared with their corresponding counterparts (Figure 2C). Given the results from previous data, we hypothesized that RBP1 was a molecular candidate for promoting the malignant transition of $IDH^{WT}/1p19q^{Non\ co\text{-}del}$ non-GDG. To this end, we investigated RBP1 expression level in non-GDG and all gliomas depending on IDH and 1p19q status by using the CGGA database. The results showed that RBP1 was significantly enriched in IDH^{WT} and $1p19q^{Non\ co\text{-}del}$ samples among gliomas (Figure 2D,G). Moreover, a prolonged survival could be observed in glioma/ non-GDG patients with lower RBP1 expression compared

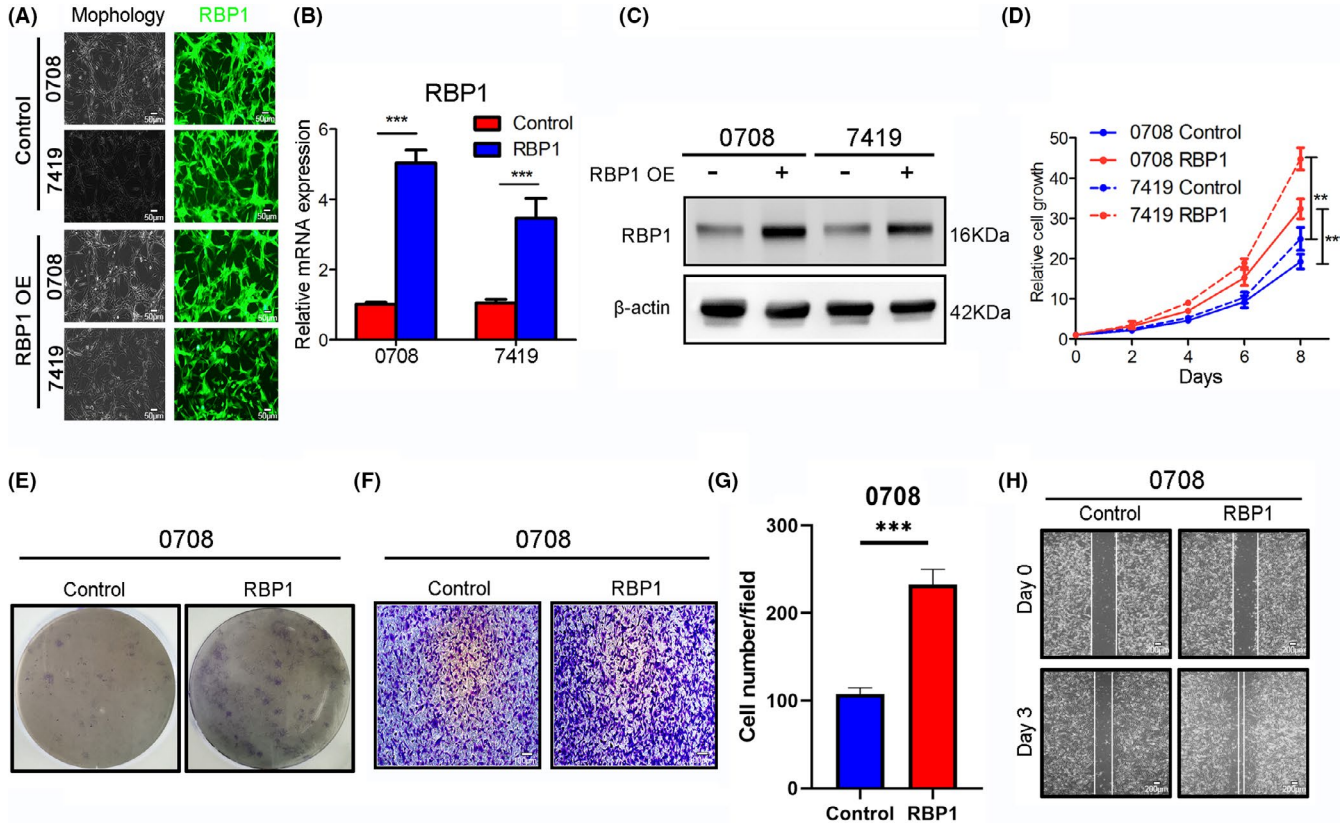


FIGURE 3 Retinol-binding protein 1 (RBP1) overexpression (OE) promoted the malignant characteristics of nonglioblastomatous diffuse glioma (non-GDG) cell lines. A, Representative images of immunofluorescence showing the transduction efficiency of RBP1 overexpression lentivirus in the control (upper panel) and RBP1 OE (lower panel) group. B, qRT-PCR analysis for measuring the mRNA expression of RBP1 in 0708 and 7419 primary cell lines after lentiviral RBP1 transduction (***P <. 001, with Student's t test). C, Western blot analysis for detecting the RBP1 protein expression in 0708 and 7419 primary cell lines pretransduced with or without RBP1 overexpression lentivirus. β-actin served as internal control. D, In vitro proliferation assay of 0708 and 7419 cells transduced with lentiviral RBP1 and negative control (**P < .01, with one-way ANOVA followed by Dunnett's post-test, n = 6). E, Colony formation assays with 0708 cells pretransfected with or without RBP1 overexpression lentivirus. F and G, Invasion assays of 0708 cells pretransfected with or without RBP1 overexpression lentivirus (F). The invasion ability was represented by microscopic counts of cell clones (G) (***P < .001, with Student's t test, n = 3). H, Wound healing analysis of 0708 cells pretransfected with or without RBP1 overexpression lentivirus at day 0 (upper panel) and day 3 (lower panel), respectively

with those with higher RBP1 expression (Figure 2H,I). Consistently, Kaplan-Meier survival analysis using the CGGA and TCGA databases revealed that elevated RBP1 expression indicated poor prognosis in non-GDG patients (Figure 2J,K). Altogether, our findings showed that RBP1 was correlated with $IDH^{WT}/1p19q^{Non\ co\text{-}del}$ subtype malignancy evolution in non-GDG and could be a prognostic biomarker.

3.3 | Retinol-binding protein 1 overexpression promoted the malignancy of non-GDG cells

To investigate the function of RBP1, exogenous RBP1 was introduced into 0708 and 7419 cells using lentivirus transduction. The transduction efficiency was evaluated by immunofluorescence (Figure 3A). qRT-PCR (Figure 3B) and Western blot assays (Figure 3C) showed that RBP1 expression was markedly upregulated in both 0708 and 7419 cells transduced with RBP1 overexpression lentivirus compared with the control cells. In vitro cell growth assays indicated that the exogenous overexpression of RBP1 significantly improved the proliferation of non-GDG cells compared with negative controls (Figure 3D). In addition, colony formation assays, Matrigel invasion assays, and wound healing assays were performed to explore the effects of RBP1 on tumor malignant biological behaviors in non-GDG cells. The results showed that cell growth and self-renewal ability were significantly enhanced by RBP1 overexpression (Figure 3E). Additionally, the invasion of RBP1 lentivirally transduced 0708 cell lines was significantly elevated compared with the control cells after incubation for 24 hours. (Figure 3F,G). Furthermore, the number of migration cells was obviously increased in RBP1 lentivirally transduced 0708 cell lines compared with the control cells (Figure 3H). Altogether, these data demonstrated that RBP1 overexpression promoted the malignant biological processes including proliferation, self-renewal, invasion, and migration of non-GDG cells.

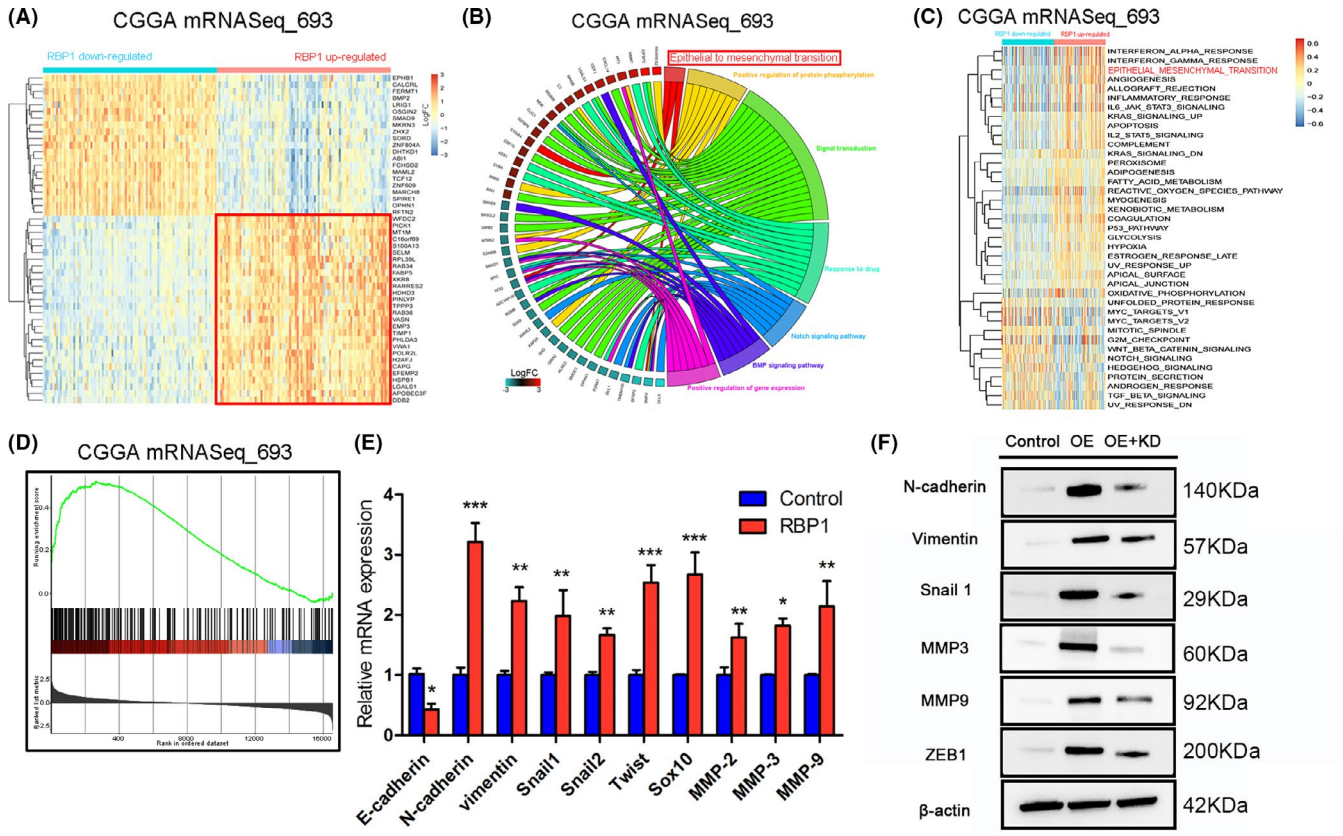


FIGURE 4 Retinol-binding protein 1 (RBP1) was positively associated with the invasion and migration properties of EMT-like phenotypes. A, Hierarchical biclustering heatmap analysis was performed using the CGGA database, illuminating the significant gene signature in RBP1^{High} nonglioblastomatous diffuse glioma (non-GDG) compared with RBP1^{Low} non-GDG. B, Chord plot was performed to illustrate the results of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) analysis using the transcriptome profiles of the CGGA database. C, GSVA analysis was performed using the CGGA database. D, GSEA analysis showed that the epithelial-mesenchymal transition (EMT) was significantly correlated with RBP1 expression in the CGGA database. E, qRT-PCR analysis was performed to investigate the mRNA expression levels of the downstream targets of EMT in 0708 cells pretransduced with lentiviral RBP1 and its negative control (*$P < .05$, **$P < .01$, ***$P < .001$, with Student's t test, n = 3). F, Western blot analysis was performed to investigate the protein expression levels of the downstream targets of EMT in control, RBP1 overexpression, and reversed RBP1 overexpression subgroups. β-actin served as internal control

3.4 | Retinol-binding protein 1 induced malignant transformation in non-GDG via regulation of the invasion and migration properties of EMT-like phenotypes

As previously described, RBP1 could be an oncogene that is upregulated along with glioma grade and induces non-GDG malignancies; therefore, bioinformatics analysis was performed to gain more insight into the potential mechanism. The expression profiles of the non-GDG samples were extracted from the CGGA and TCGA databases and then grouped respectively into two groups depending on RBP1 expression. Hierarchical biclustering analysis indicated that genomic signatures in RBP1^{High} compared with RBP1^{Low} non-GDG samples (Figure 4A). To investigate the underlying pathways, the Database for Annotation, Visualization, and Integrated Discovery online tool (DAVID, https://david.ncifcrf.gov/) was used to conduct the Gene ontology (GO) annotation, and the results were visualized by using the clusterProfiler package, while the Gene set variation analysis (GSVA) package and Gene set enrichment analysis (GSEA) analysis were used to validate the results. GO analysis showed that the EMT of GO terms was markedly enriched in RBP1^{High} samples (Figure 4B). GSVA (Figure 4C) and GSEA (Figure 4D) were performed to confirm the GO analysis, and the results indicated that the EMT process was strongly correlated with RBP1 expression in the CGGA database. To further verify the results of the bioinformatics analysis, qRT-PCR and Western blot assays were performed. The results demonstrated that overexpressed RBP1 markedly elevated the expression of mesenchymal relevant genes, while epithelial biomarkers were significantly reduced. Typically, EMT is described as a process in which epithelial cells lose their apical-basal polarity, modulate their cytoskeleton, and exhibit reduced cell-cell adhesive properties; furthermore, and cells may individually or collectively acquire mesenchymal features and increase motility and invasive ability. However, according to another important definition, some EMT-associated transcription factors are also involved in other cellular processes (eg, proliferation, apoptosis, or stemness) and play important roles during tumor progression,

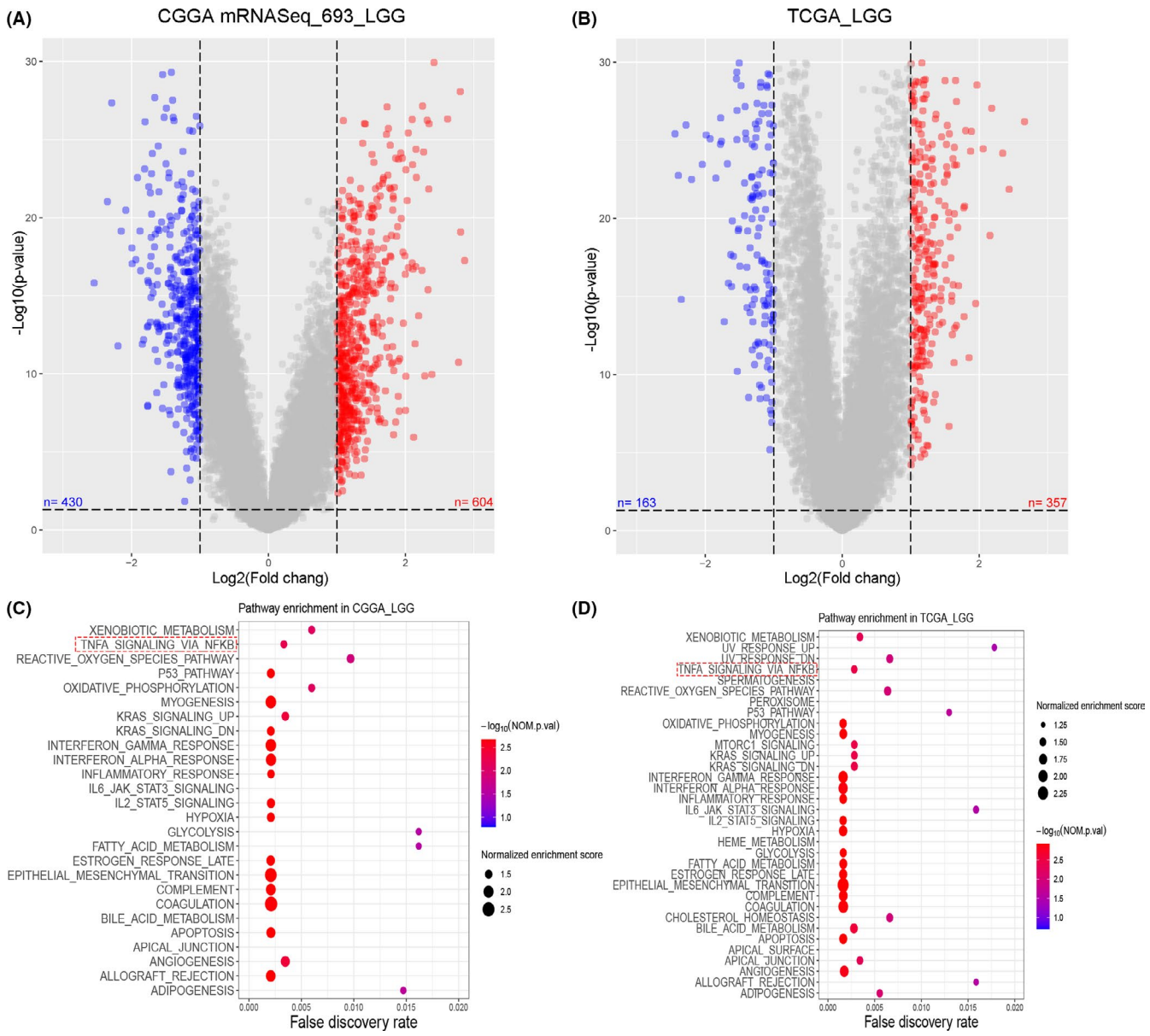


FIGURE 5 Retinol-binding protein 1 (RBP1) was positively associated with tumor necrosis factor α (TNFα)/nuclear factor κβ (NF-κB) signaling. A and B, The volcano plots present the differentially expressed genes (DEGs) using the CGGA (A) and The Cancer Genome Atlas (TCGA) (B) databases in $IDH^{WT}/1p19q^{Non\ co\text{-}del}$ nonglioblastomatous diffuse glioma (non-GDG) versus $IDH^{Mut}/1p19q^{Co\text{-}del}$ non-GDG. C and D, Bubble plots were carried out to illustrate the results of the GSEA analysis using the transcriptome profiles of the CGGA (C) and TCGA (D) databases, indicating that RBP1 was positively correlated with TNFα/NF-κB signaling

often beyond classic EMT phases.[34-37] The results indicated that RBP1 enhanced the invasion and migration characteristics of non-GDG via inducing EMT-like phenotypes (Figure 4E,F).

3.5 | Retinol-binding protein 1 promoted the malignant characteristics of non-GDG via activation of NF-κB signaling

Activation of NF-κB signaling is proved to be an essential mediator of EMT phenotype, which functions through regulation of a range of transcription factors related to the mesenchymal transition program.[38] To confirm this, GSEA with the CGGA and TCGA databases was performed to explore whether the NF-κB pathway participates in the EMT process. As a result, 604 upregulated genes in the $RBP1^{High}$ sample and 430 downregulated genes in the $RBP1^{Low}$ sample were extracted from the CGGA database, while 357 upregulated genes and 163 downregulated genes were extracted from the TCGA database (Figure 5A,B). Subsequently, all genes were sorted by logFC value to perform GSEA. As expected, GSEA results indicated that the TNFα/NF-κB pathway was simultaneously enriched in the $RBP1^{High}$ group in both the CGGA and TCGA database (Figure 5C,D). Taken together, we speculated that the TNFα/NF-κB pathway might be a downstream pathway of RBP1 in the EMT process.

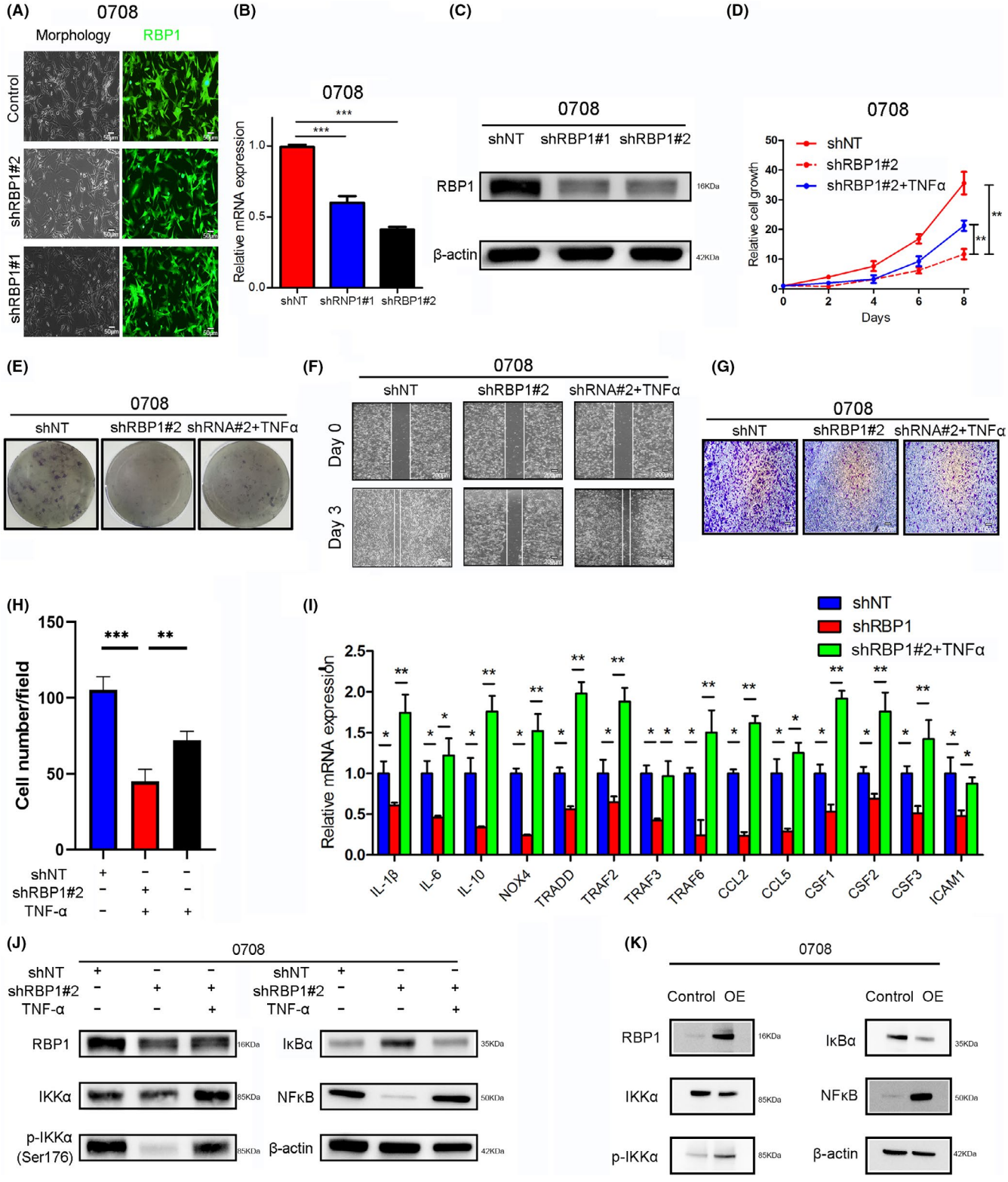
To further validate the mechanism of RBP1-dependent regulation of NF-κB signaling, shRBP1 and shNT lentivirus were introduced into 0708 cells, and immunofluorescence imaging was used to evaluate the lentivirus transfection efficiency (Figure 6A). qRT-PCR (Figure 6B) and Western blot assays (Figure 6C) verified that RBP1 expression was markedly suppressed in both shRBP1#1- and shRBP1#2-transfected 0708 cells compared with the shNT cells, and shRBP1#2 was selected according to the efficiency of knockdown. Additionally, in vitro cell viability assays were performed to investigate the effects of RBP1 knockdown on tumor proliferation. The results indicated that RBP1 silencing markedly reduced the in vitro cell growth of 0708 cell, while cell growth kinetics was partially rescued by TNFα treatment (Figure 6D). Additionally, colony formation assays, Matrigel invasion assays, and wound healing assays were performed to explore the functional role of RBP1 knockdown in tumor malignance. The results showed that the properties of self-renewal, invasion, and migration were significantly suppressed in RBP1-knockdown 0708 cells but could be partially elevated by TNFα treatment (Figure 6E-H). Moreover, silencing RBP1 notably presented a coordinated tendency which reduced the downstream targets (IL-1β, IL-6, IL-10, NOX4, TRADD, TRAF2, TARF3, TRAF6, CCL2, CCL5, CSF1, CSF2, CSF3, and ICAM1) of the NF-κB pathway, and by contrast, these landmark genes were partially improved on transcriptome and translation levels when TNFα was added (Figure 6I). Similarly, Western blot analysis showed that RBP1 knock-down significantly reduced the phosphorylation level of IKKα thus decreased NF-κB expression via inducing increasement of IκBα, while the NF-κB expression could be partially elevated by TNFα treatment (Figure 6J). Moreover, the phosphorylation level of IKKα was also promoted coupled with the degradation of IκBα and increase in NF-κB in RBP1 overexpression (Figure 6K). These results indicated that RBP1-dependent activation of NF-κB signaling might proceed through a TNFα-based stimulation mechanism. Taken together, enriched RBP1 induced tumor malignance of non-GDG cells via activation of TNFα-dependent NF-κB signaling.

4 | DISCUSSION

It is well known that non-GDG is a group of heterogeneous neuroepithelial tumors characterized by slow continuous growth and an evolution toward a more malignant GBM; thus, non-GDG patients exhibit a vast difference in overall survival, where median survival time ranges from 4 to 13 years due to the variety of subtypes.[39-41] Consequently, traditional histopathological classification strategies show powerless, reflecting the heterogeneity of non-GDG. Therefore, the dilemma urges to develop reliable molecular markers to precisely distinguish non-GDG subtypes and improve the prognosis of patients.

The status of IDH and the integrity status of 1p/19q play an important role in classifying subtypes and predicting prognosis for glioma; nevertheless, they are ambiguous for specific mechanisms of malignant transition for non-GDG.[12,13,42] Given this intractable question, this study originated from bioinformatic data mining utilizing the CGGA and TCGA databases and depending on IDH status and 1p19q integrity. As a result, we identified RBP1 as a significant upregulated gene in the $IDH^{WT}/1p19q^{Non\ co\text{-}del}$ group. The synchronization of enriched RBP1 expression was confirmed by qRT-PCR and Western blot assays in tumor tissues and primary cell lines. According to previous research, RBP1 locates on the 3q21-q22 chromosome and encodes the CRBP1 cytosolic protein to participate in the stability and metabolism of retinol.[24,25] Intriguingly, RBP1 expression shows a dramatic distribution: it is downregulated in hepatocellular carcinoma, breast cancer, endometrial cancer, and ovarian cancer, while it is highly expressed in glioma, laryngeal cancer, and lung adenocarcinoma and was markedly enriched along with the pathological signatures.[27-32,43] However, whether RBP1 expression level is associated with the outcome of non-GDG patients is still unclear. To this end, expression profiles were extracted from the CGGA and TCGA databases, and they demonstrated that RBP1 expression was continuously upregulated along with the pathological grade of glioma. Immunohistochemistry also confirmed this augmentation in tumor tissues. Moreover, RBP1

FIGURE 6 Retinol-binding protein 1 (RBP1) activated the nuclear factor κβ (NF-κB) signaling pathway via regulation of IkB-kinase α (IKKα) phosphorylation. A, Representative images of immunofluorescence showing the transduction efficiency of 0708 cells after lentiviral shRBP1#1 and shRBP1#2 transduction and control. B, qRT-PCR analysis for RBP1 in 0708 cells transfected with shNT or shRBP1 lentivirus (***$P < .001$, with Student's t test). C, Western blot analysis for RBP1 expression in 0708 cells transfected with shNT or shRBP1 lentivirus. β-actin served as internal control. D, In vitro proliferation assay for 0708 cells pretransfected with shNT or shRBP1#2 lentivirus and then treated with or without tumor necrosis factor α (TNFα) (**$P < .01$, with one-way ANOVA followed by Dunnett's post-test). E, Colony formation assays of 0708 cells pretransfected with shNT or shRBP1#2 lentivirus and then treated with or without tumor necrosis factor α (TNFα). F, Wound healing assays of 0708 cells pretransfected with shNT or shRBP1#2 lentivirus and then treated with or without TNFα at day 0 (upper panel) and day 3 (lower panel), respectively. G and H, cell invasion assay of 0708 cells pretransfected with shNT or shRBP1#2 lentivirus and then treated with or without TNFα. G, The invasion ability was represented by microscopic counts of cell clones (H) (***$P < .001$, **$P < .01$, with Student's t test, n = 3). I, qRT-PCR assays were performed to measure the mRNA expression levels of the downstream targets of nuclear factor κβ (NF-κB) in 0708 cells pretransfected with shNT or shRBP1#2 lentivirus and then treated with or without TNFα (*$P < .05$, **$P < .01$, with Student's t test, n = 3). J, Western blot analysis were conducted to detect the protein levels of downstream targets of NF-κB in 0708 cells pretransfected with shNT or shRBP1#2 lentivirus and then treated with or without TNFα. β-actin served as internal control. K, Western blot analyses were conducted to detect the protein levels of the downstream targets of NF-κB in 0708 cells pretransfected with RBP1 overexpression lentivirus



expression was also associated with IDH status and 1p19q integrity, and it was firstly demonstrated that enriched expression of RBP1 might cause a worse prognosis. Therefore, our findings indicated that RBP1 could be a candidate molecular marker to predict patient outcome, which was consistent with the classification based on IDH and 1p/19q status.

Accumulating evidence indicates that RBP1-encoded protein is crucial for the uptake and esterification of retinol that is necessary for many biological processes including epithelial cell proliferation, differentiation, and apoptosis, and the biological effects of retinol are primarily mediated by all transretinoic acid receptors and 9-cis retinoic acid receptors.[44-46] Moreover, a potential effect of

RBP-1–driven aberrant intracellular retinoid signaling in nonglioma carcinogenesis has also been highlighted.[28,32,47] Doldo et al43 found that high RBP1 expression in lung adenocarcinoma was correlated with increased tumor grade, likely by upregulating Akt/Erk/EGFR-mediated cell proliferation and differentiation. Nevertheless, few studies revealed the relationship with non-GDG malignant behaviors and the potential signaling pathway of RBP1 for inducing malignant transition of non-GDG. In this study, RBP1 overexpression was proved to be crucial in promoting malignant biological processes of non-GDG, such as in vitro proliferation, self-renewal, invasion, and migration. Thus, we speculated that RBP1 participates in the malignant transition of non-GDG and thus leads to therapy resistance and rapid recurrence. Using the TCGA and CGGA databases combined with qRT-PCR and Western bolt, for the first time, we confirmed that the underlying mechanism of RBP1-induced malignancy evolution of non-GDG was the EMT to change adhesion molecules and elevate the mesenchymal phenotype of migration and invasion.

As well known, NF-κB is a significant nuclear transcriptional regulator with specific DNA-binding sequences, which is constitutively activated with the canonical signaling pathway mediated by the RELA proto-oncogene NF-κβ protein subunit and the noncanonical signaling pathway mediated by the RELB proto-oncogene NF-κβ subunit to participate in the pathophysiological processes of GBM cells, such as EMT and preventing apoptosis.[38,48-50] In inactive status, NF-κB complexes are sequestered in the cytoplasm and held inactive with inhibitor of IκB proteins.[51] Similar to malignancies, activation of the NF-κB pathway depend on whether classic and alternative signaling pathways promote GBM tumor growth and progression through the transcriptional activation of genes associated with suppression of apoptosis, metastasis, and resistance to cytotoxic agents.[52] For the classic pathway, activation of upstream receptors, for example, induces IκB kinase β (IKKβ) to phosphorylate the inhibitory IκB proteins, resulting in their rapid ubiquitination and proteasome-mediated degradation, which culminates in the release of NF-κB complexes from their inhibitory interaction. Ultimately, NF-κB heterodimers p50/p65 (known as NF-κB1 heterodimeric complexes) translocate to the nucleus, where they activate the transcription of a plethora of genes involved in immune response, cell growth, and cell survival.[53,54] Consistently, we observed that exogenous silencing of RBP1 reduced the phosphorylation level of IKKα and decreased the degradation of IκB proteins, leading to a reduction of release and activation of the NF-κB protein, while these effects could be partially rescued by TNFα treatment. Meanwhile, literature has been reported that the TNFα/NFκB pathway could regulate the EMT process and cell proliferation.[55,56] Therefore, we hypothesized that enriched RBP1 promoted the malignant characteristics via activation of the NF-κB signaling pathway and further promoted the invasion and migration properties of EMT-like phenotypes and cell proliferation in non-GDG.

Although the underlying roles of RBP1 in the malignant transition of non-GDG were well discussed in this study, additional molecular biological investigations are still required for a more distinct evaluation of the mechanism. One deficiency of our study is the indeterminacy as to how RBP1 interacts with the NF-κB pathway and thus further regulates downstream molecular markers. Although we demonstrated that RBP1 participates in phosphorylating IKKα, RBP1-encoded protein is a cytosolic protein and belongs to the family of fatty acid–binding proteins that cannot directly phosphorylate IKKα, implying that some unknown intermediate regulation mechanism may exist in the RBP1-NF-κB axis. In addition, whether RBP1 induces retinol and its metabolic products to activate the phosphorylation process of IKKα to degrade inhibitory IκB proteins in the NF-κB pathway remains unclear. Moreover, alteration of a single candidate biomarker might bring unpredictable effects due the heterogeneity of non-GDG. Therefore, it is crucial to evaluate non-GDG patients by more integrated strategies before clinical management.

In summary, our study suggested RBP1 was significantly upregulated in the IDH^{WT} and $1p19q^{Non\ co\text{-}del}$ non-GDG subtypes, and enriched RBP1 expression was markedly correlated with more severe outcomes. Moreover, RBP1-dependent activation of NF-κB signaling promoted the malignancy of non-GDG, indicating that RBP1 could be a reliable prognostic biomarker and potential therapeutic target for non-GDG.

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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