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# MicroRNA-224 targets ERG2 and contributes to malignant progressions of meningioma

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ABSTRACT

MicroRNA-224 is overexpressed in various malignant tumors with poor prognosis, which plays a critical role in biological processes including cell proliferation, apoptosis and several developmental and physiological progressions. However, the potential association between miR-224 and clinical outcome in patients with meningiomas remains unknown. Here, we investigate miR-224 expression and biological functions in meningiomas. MiR-224 expression was measured by Northern blot analysis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in meningioma and normal brain tissues. Kaplan–Meier analysis and Cox regression analysis were used to exam its correlation with clinicopathological features and prognostic value. The biological effects of miR-224 on the cell proliferation and apoptosis in meningioma cells were examined by MTT assay and apoptosis assay. We found the expression levels of miR-224 were significantly higher in meningioma tissues than that in normal brain, positively correlated with advanced pathological grade. Kaplan–Meier analysis indicated that meningioma patients with low miR-224 expression exhibited significantly prolonged overall and recurrence-free survival. Furthermore, we demonstrated that ERG2 was an identical candidate target gene of MiR-224 in vitro. Our results indicated that downregulation of miR-224 suppressed cell growth and resulted in the enhancement of cell apoptosis through activation of the ERG2-BAK-induced apoptosis pathway. Our findings imply the miR-224 expression could predict the overall survival and recurrence-free survival of patients with meningioma and it might be a promising therapeutic target for treating malignant meningiomas.



## 1. Introduction

Meningioma represents a common primary neoplasm of the central nervous system in adults, with an annual incidence of approximately 0.0013–0.0078% [1,2]. Although relatively infrequent, high-grade meningiomas are associated with worse prognosis compared to benign meningiomas. Even with the clinical implementation of aggressive therapeutic strategies, patients with advanced or recurrent meningiomas barely achieve cure [3]. Over the last years, there has been an increasing focus on the molecular genetics of meningiomas [1,4]. These efforts aim to find new molecular markers and targeted therapies to achieve earlier diagnosis and better treatment [5].

MicroRNAs (miRNA) are a class of post-transcriptional regulators that play key roles in the regulation of gene expression [6]. They have been reported to participate in cell cycle control, apoptosis and several developmental and physiological progressions [7]. Despite their important functions in healthy individuals, they have also been implicated in various cancers [8]. Consequently, they are intensely studied as candidates for diagnostic and prognostic biomarkers of cancers [9]. Abnormal excessive expression of miR-224 has been demonstrated in several malignant cancers, such as liver, colon, gastric, prostate, and ovarian cancers [10–12]. It has been reported that miR-224 plays a crucial role in cell proliferation, angiogenesis, and apoptosis and cause resistance to chemotherapy [11–15]. Furthermore, downregulation of miR-224 in human tumor cell lines can cause reversion of the malignant phenotype [15]. However, the expression pattern and biological functions of miR-224 in meningioma have not been reported.

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Here, we measured miR-224 expression in patients with meningiomas, and explored its association with histological grading and postoperative recurrence. In addition, we inhibited the expression of miR-224 in IOMM-Lee and CH157 meningioma cells and indicated that miR-224 inhibition enhanced apoptosis and suppressed cell proliferation. Furthermore, we identified ERG2 as a novel miR-224 target and demonstrated that miR-224-ERG2 axis plays a critical role in regulating proliferation and apoptosis of meningioma cells.

## 2. Material and methods

### 2.1. MiRNAs and transfection

MiR-224 inhibitor was designed and performed as previously described [10]. The sequences of antisense oligonucleotides (miR-224 ASO) were: 5′-AACGGAACCACUAGUGAC.

UUG-3′, the sequences of negative control (control ASO) were: 5′-CAGUACUUUUGUGUAGU.

ACAA-3′. ERG2 inhibitor constructs were purchased from Lifetechnologies (California, USA) and transfected using the manufacturer's reagents and protocol. Silencing of target gene expression was determined by Western blot analysis.

### 2.2. Tissue samples

The Research Committee of 411 hospital of PLA approved the use of tissue samples. Tissue specimens were obtained from patients with meningiomas at 411 hospital of PLA and 81 hospital of PLA from July 1999 to July 2011. Patients were under follow-up every 3 months by telephone or e-mail and the last time of the follow-up was January 2013. Simpson grading system was used to define the extent of resection in every patient. The clinical features of the patients are shown in Table 1. The selection criteria were described in detail previously [16]. According to the selection criteria, 103 tissue samples are enrolled in this study. Overall survival (OS) means the time from the surgery to death. Recurrence-free survival (RFS) means the time from surgery to meningioma progression or death.

**Table 1**
Correlation between miR-224 expression and clinicopathologic characteristics of meningiomas patients.

| Characteristics | | MiR-224 expression | | |
|---|---|---|---|---|
| | N (%) | Low | High | *P*-Value |
| *Age, years* | | | | 0.11 |
| <60 | 59 (57.3) | 33 | 26 | |
| ≥60 | 44 (42.7) | 21 | 23 | |
| *Gender* | | | | 0.15 |
| Male | 42 (40.8) | 21 | 21 | |
| Female | 61 (59.2) | 33 | 28 | |
| *MTD, cm* | | | | 0.11 |
| <4 | 57 (55.9) | 32 | 25 | |
| ≥4 | 46 (44.1) | 22 | 24 | |
| *Tumor location* | | | | 0.324 |
| Convexity | 33 (32.1) | 18 | 15 | |
| Parasagittal sinus | 28 (27.2) | 14 | 14 | |
| Parafalcine | 14 (13.5) | 6 | 8 | |
| Skull base | 28 (27.2) | 16 | 12 | |
| *Extent of resection* | | | | 0.218 |
| Simpson grade I | 48 (46.6) | 25 | 23 | |
| Simpson grade II | 41 (39.8) | 20 | 21 | |
| Simpson grade III | 14 (13.6) | 9 | 5 | |
| *Pathological classification* | | | | 0.004* |
| WHO I | 52 (50.5) | 38 | 14 | |
| WHO II | 26 (25.2) | 9 | 17 | |
| WHO III | 25 (24.3) | 7 | 18 | |
| *Recurrence* | | | | <0.001* |
| Yes | 41 (39.8) | 12 | 29 | |
| No | 62 (60.2) | 42 | 20 | |

Abbreviations: MTD, mean tumor diameter.
*P < 0.05 was considered statistically significant.

### 2.3. Isolation of miRNA and qRT-PCR

NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA) was used to detect the concentration and purity of all RNA samples. NCode™ SYBR Green miRNA qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA) was used to synthesize specific cDNA of miR-224. qRT-PCR was analyzed with the DNA Engine Opticon 2 Real-Time Cycler (MJ Research Inc., Waltham, MA, USA) according to the manufacturer's protocols. MiR-224 expression was normalized to U6B.

### 2.4. Cell proliferation assay

MTT assay was performed to measure cell growth, as described previously [13]. Cell Counting Kit analyzed cell proliferation according to the manufacturer's instructions. The microplate reader (Thermo Scientific) was used to measure the optical density at 570 nm. Experiments were performed in triplicate.

### 2.5. Protein extraction and Western blotting

Western blot analysis was performed as described previously [17]. Western blot data were quantified by normalizing the signal intensity of each sample to that of β-actin. The primary antibodies were ERG2 (Abcam, ab164314, 1:500), BAK (SANTA CRUZ, sc-1035, 1:500), and β-actin (Abcam, ab-8227, 1:1000) used as a gel loading control.

### 2.6. Northern blot analysis

Northern Blot Analysis was performed as described previously [10]. Antisense RNA probes were 3′- end-labeled with $^{32}$P-γATP by T4 polynucleotide kinase; miR-224 antisense probe sequence 5′-TAAACGGAACCACTAGTGACTTG-3 and U6 spliceosomal RNA antisense probe sequence 5′-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3′ were used.

### 2.7. Statistics

All data are expressed as mean ± SD for triplicate determination, and analyzed using Student's test. We evaluated predictors of survival by Cox proportional-hazards regression analysis. Kaplan–Meier analysis was used to investigate OS and RFS in meningioma patients. SPSS 16.0 was used to conduct analyses and $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. MiR-224 expression in meningiomas

We first measured the miR-224 expression in 103 human meningioma specimens and 12 normal brain tissues by qRT-PCR. As illustrated in Fig. 1A, the miR-224 expression was significantly higher in meningiomas than that in normal brain tissues by using qRT-PCR analysis (mean ± SD: 4.23 ± 1.32 vs. 1.78 ± 0.69, $P < 0.001$). In addition, meningioma with advanced stages (WHO grade II, 5.77 ± 1.21; WHO grade III, 7.42 ± 1.87) exhibited greater expression for miR-217 compared to meningioma with low histological grade (WHO grade I, 3.44 ± 0.98). Moreover, Northern blot analysis revealed that there was also an obvious difference of miR-224

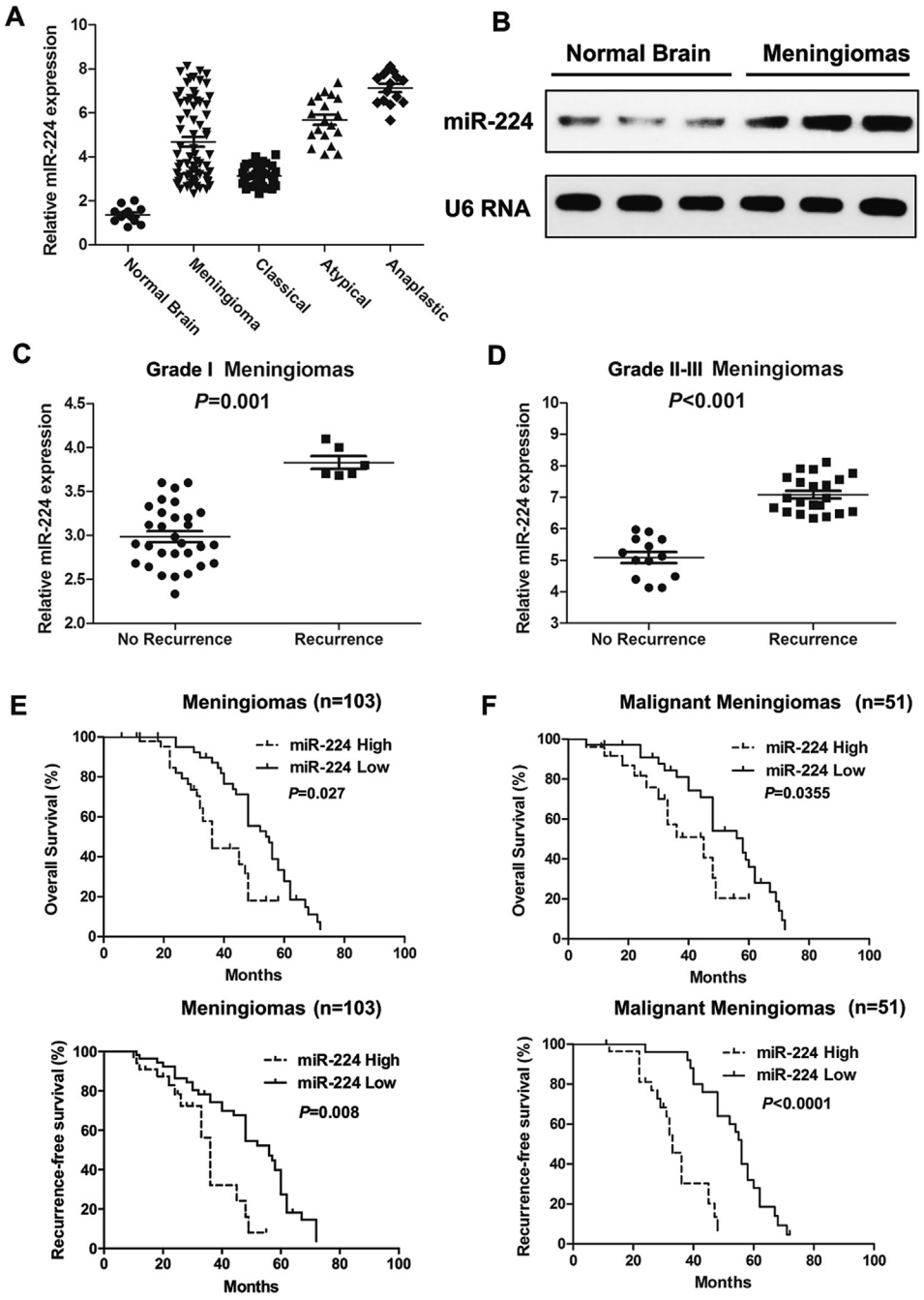


**Fig. 1.** (A) qRT-PCR results of 103 meningiomas and 12 normal brain samples. All data were normalized to U6B. (B) Northern blot revealed that miR-224 was highly expressed in meningiomas. (C) qRT-PCR showed that grade I meningiomas with recurrence had higher miR-224 expression compared to benign tumors with no recurrence. $^{*}P = 0.001$ (D) miR-224 overexpression was also observed in advanced stage meningioma with recurrence. $^{*}P < 0.001$ (E) Kaplan–Meier curves of meningiomas with stratified miR-224 expression. (F) Subgroup analyses of miR-224 expression in meningioma patients with advanced pathological grade.

expression between normal brain tissues and meningiomas (Fig. 1B). The mean miR-224 expression level of all meningiomas was 4.23, which is used to divide meningioma patients into high expression group ($\geq$4.23, n = 49) and low expression group (<4.23, n = 54). High expression miR-224 was observed in grade I meningioma with recurrence, although these benign tumors had low miR-224 expression, indicating that miR-224 may induce more malignant tumor progression (Fig. 1C). Furthermore, miR-224 overexpression was also observed in advanced stage meningioma with recurrence (Fig. 1D). We next investigated the association between miR-224 expression and clinicopathologic features (Table 1). The expression level of MiR-224 was significantly related with cancer histological grade ($p = 0.004$) and recurrence ($p < 0.001$). No significant correlation was found between miR-244 expression and other clinical parameters, such as age, gender, size, tumor resection, or tumor location. These results suggested that miR-224 expression was significantly increased in meningioma tissues than that in normal brain, positively correlated with advanced pathological grade and high recurrent rate.

### 3.2. Low miR-244 expression associated with improved prognosis

The Kaplan–Meier survival method was used to investigate the differences of OS and RFS between low and high expression group.

**Table 2**
Univariate analysis of factors associated with survival and recurrence of meningioma patients (n = 103).

| Variable | Overall survival | | | Recurrence-free survival | | |
|---|---|---|---|---|---|---|
| | HR | Univariate analysis,P | Multivariate analysis,P | HR | Univariate analysis,P | Multivariate analysis,P |
| Gender | 0.754 | 0.435 | 0.512 | 0.782 | 0.432 | 0.211 |
| Age | 0.874 | 0. 648 | 0.886 | 0.912 | 0.456 | 0.479 |
| MiR-224 | 2.179 | 0.017* | 0.012* | 1.998 | 0.034* | 0.021* |
| Tumor location | 0.774 | 0.358– | 0.658 | 1.112 | 0.487 | 0.879 |
| Pathological classification | 1.879 | 0.028* | 0.031* | 1.857 | 0.042* | 0.026* |
| MTD | 0.997 | 0.421 | 0.118 | 0.876 | 0.318 | 0.857 |
| Extent of resection | 0.674 | 0.338 | 0.438 | 0.878 | 0.586 | 0.674 |

Note: Univariate analysis, Cox proportional hazards regression model.
Abbreviations: MTD, mean tumor diameter; HR, Hazard ratio.
*P < 0.05 was considered statistically significant.

The follow-up was conducted from 1 to 90 months. Patients with low levels of miR-224 had significantly prolonged OS ($P = 0.027$) and RFS ($P = 0.0018$) relative to those with high levels of miR-224 (Fig 1E). Univariate analysis showed that high miR-224 expression and advanced histologic grade were independent prognostic parameters indicating poor prognosis for meningioma patients (OS: miR-224, $P = 0.017$, histologic grade, $P = 0.028$; RFS: miR-224, $P = 0.034$, histologic grade, $P = 0.042$, Table 2). Moreover, multivariate analysis revealed that miR-224 expression is an independent parameter for OS and RFS (OS, $P = 0.012$; RFS, $P = 0.021$, Table 2). However, no similar correlation was found among other clinical factors (gender, age, tumor size, location, and resection).

As classical meningioma (WHO grade I) are considered benign with long survival and low recurrence, we did a Kaplan Meier analysis of miR-224 expression and prognosis in 51 high-grade meningiomas (WHO grade II, n = 26 and WHO grade III, n = 25) by taking out all 52 benign tumors (WHO grade I) to make OS and RFS analysis more convincing. As predicted, OS and RFS rate was higher in malignant meningioma patients with lower miR-224 expression (Fig. 1F). Univariate and multivariate analysis further indicated that high expression level of miR-224 was independent prognostic parameters for OS and RFS in high-grade meningioma (OS: univariate analysis $P = 0.018$, multivariate analysis $P = 0.009$; RFS: univariate analysis $P = 0.024$, multivariate analysis $P = 0.016$, Table 3).

### 3.3. Mir-224 regulates cell growth and apoptosis in meiningioma cancers

We first measured miR-224 expression in five meningioma cell lines (Ben-Men-1, IOMM-Lee, HBL-52, F5, and CH157) by using Northern blot (Fig. 2A). IOMM-Lee and CH157 cells showed significantly higher miR-224 expression than HBL-52, F5, and Ben-Men-1 cells. So IOMM-Lee and CH157 cells were chosen to perform the next experiments in vitro. IOMM-Lee and CH157 cells transfected with antisense oligonucleotides (ASO) exhibited a significant reduction in miR-224 expression by qRT-PCR (Fig. 2B). We then measured the contribution of miR-224 expression to the IOMM-Lee and CH157 cell growth by using MTT assay. Downregulation of miR-224 significantly suppressed cell growth in meningioam cells transfected with miR-224 ASO compared to cells transfected with control ASO (Fig. 2C). Furthermore, miR-224 inhibition was correlated with enhanced apoptosis of IOMM-Lee and CH157 cells (Fig. 2D). These results indicated that miR-224 could promote cell growth and suppress apoptosis in meningioma cells.

### 3.4. ERG2 is a potential target of miR-224 and inversely associated with miR-224 expression

We speculate that early growth response 2 (ERG2) is a potential target of miR-224 because it contains a putative miR-224 target sites in its 3′UTR by using target scan. We cloned the target site, or its mutant into an identical luciferase reporter vector (Fig. 3A). We found that the reporter vectors with the putative target sequence resulted in an approximately 34.9% ± 2.5% decrease in relative luciferase activity compared to the mutant introduced with miR-224 in IOMM-Lee cells (Fig. 3B). In addition, we measured the expression of ERG2 and miR-224 in 48 meningioma specimens to investigate its clinical relevance in vivo. We found the mRNA and protein expressions of ERG2 were significantly decreased in high grade meningiomas compared to low-grade meningiomas by using qRT-PCR and Western-blot (Fig. 3C and D). Furthermore, statistical analyses of mRNA expression indicated that ERG2 expression associated inversely with miR-224 expression ($r = -0.354$, $p = 0.047$) (Fig. 4E). These data indicated that ERG2 is a crucial downstream effecter of miR-224 in vitro and inversely associated with miR-224 expression in vivo.

### 3.5. MiR-224 regulates EGR2 signaling pathway

Recent studies reported that overexpression of EGR2 could enhance cell apoptosis by directly transactivating BAK in various

**Table 3**
Univariate analysis of factors associated with survival and recurrence of high-grade meningioma patients (n = 51).

| Variable | Overall survival | | | Recurrence-free survival | | |
|---|---|---|---|---|---|---|
| | HR | Univariate analysis,P | Multivariate analysis,P | HR | Univariate analysis,P | Multivariate analysis,P |
| Gender | 0.528 | 0.238 | 0.328 | 0.648 | 0.412 | 0.299 |
| Age | 0.678 | 0.332 | 0.763 | 0.879 | 0.352 | 0.428 |
| MiR-224 | 2.672 | 0.018* | 0.009* | 2.189 | 0.024* | 0.016* |
| Tumor location | 0.689 | 0.491 | 0.549 | 1.231 | 0.628 | 0.672 |
| MTD | 1.121 | 0.338 | 0.216 | 0.989 | 0.296 | 0.857 |
| Extent of resection | 0.559 | 0.214 | 0.518 | 0.993 | 0.397 | 0.592 |

Note: Univariate analysis, Cox proportional hazards regression model.
Abbreviations: MTD, mean tumor diameter; HR, Hazard ratio.
*P < 0.05 was considered statistically significant.

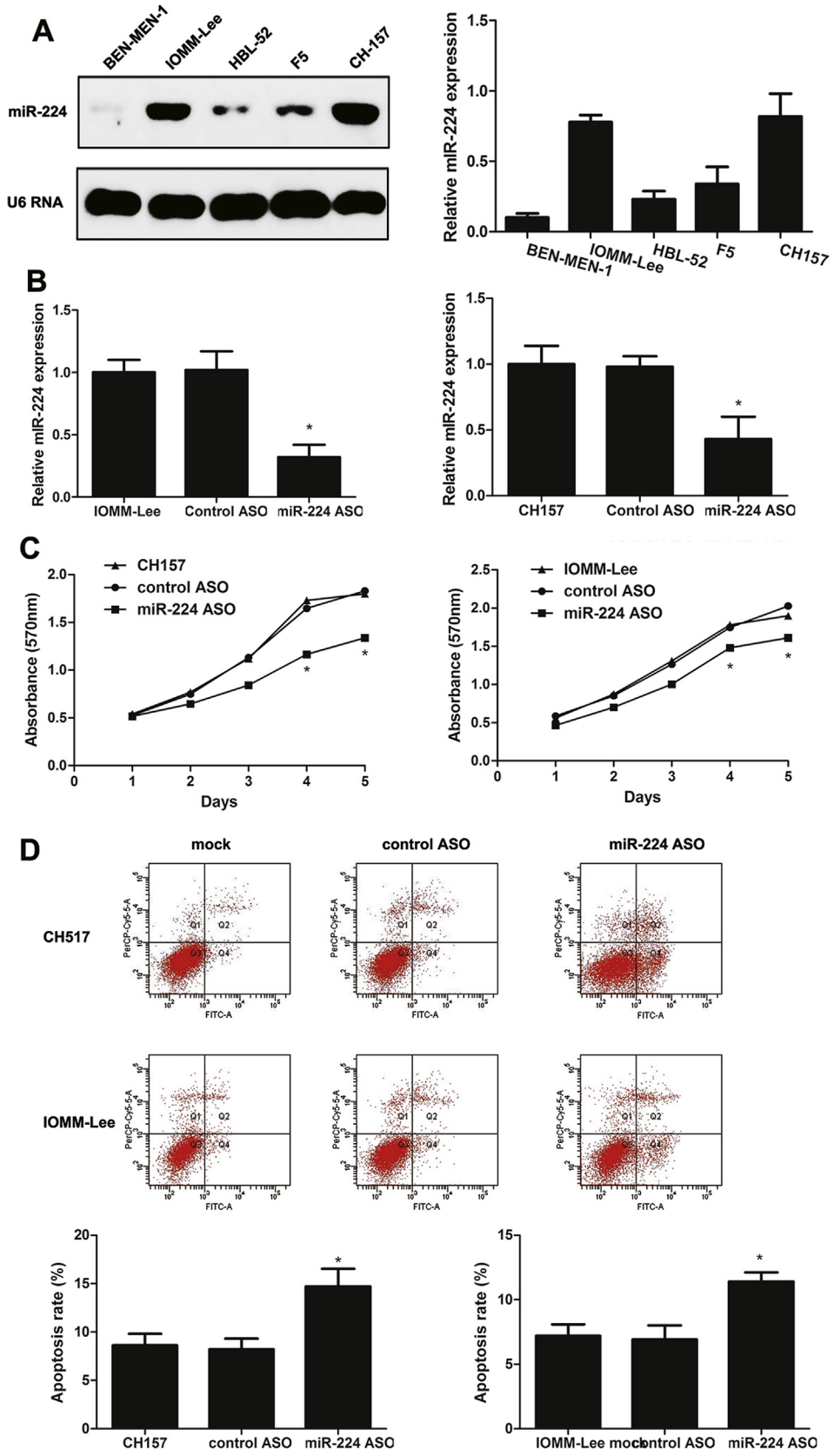


**Fig. 2.** (A) Northern blot analysis shows the results of miR-224 expression in Ben-Men-1, IOMM-Lee, HBL-52, F5, and CH157 cells. (B) miR-224 expression was significantly reduced in IOMM-Lee and CH157 cells transfected with miR-224 ASO compared to cells transfected with control ASO. $^{*}P < 0.01$ (C) MTT assay shows that the growth inhibition ratio decreased in IOMM-Lee and CH157 cells underexpressing miR-224. (D) miR-224 inhibition significantly enhanced cell apoptosis in cells underexpressing miR-224 compared to controls. $^{*}P < 0.05$.

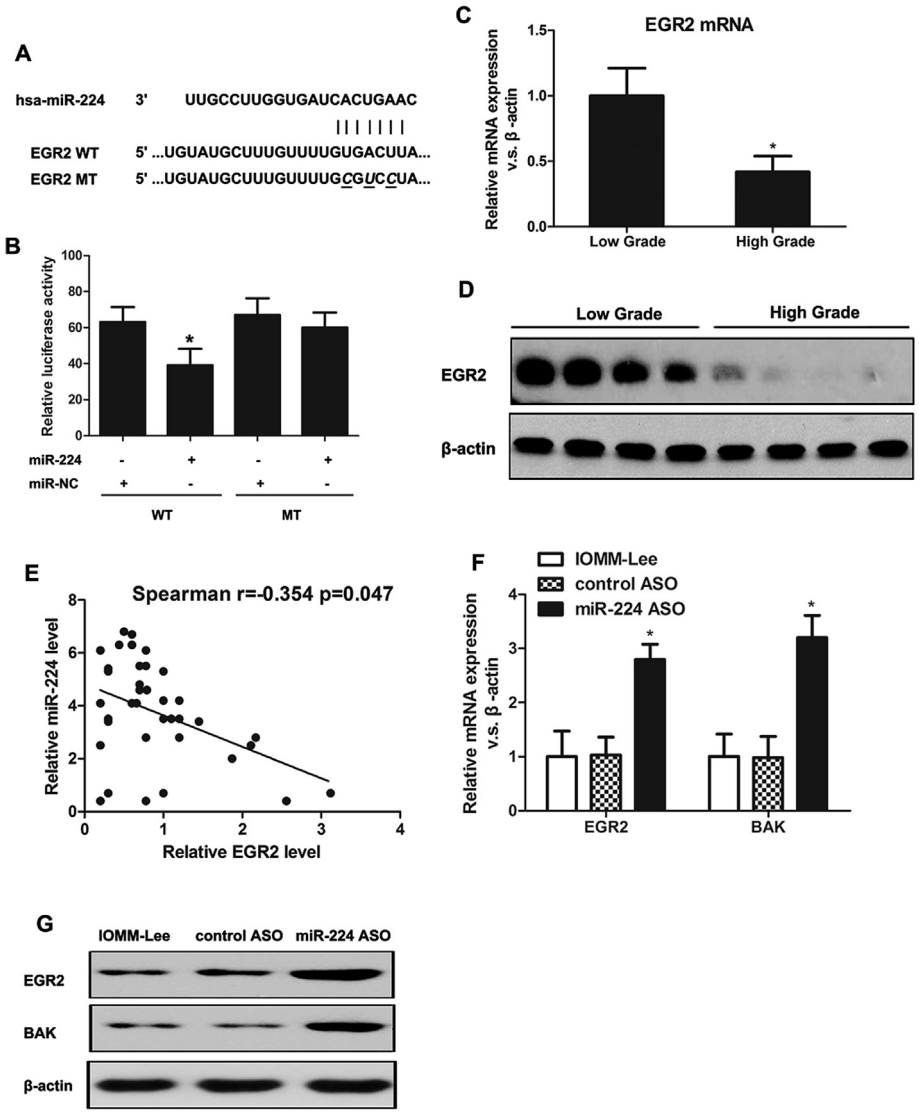


**Fig. 3.** (A) Sequence of wild type and mutant miR-224 target sites in the ERG2. (B) Luciferase reporter assay in IOMM-Lee cells. $P < 0.05$. (C) qRT-PCR analysis of ERG2 mRNA expression in meningiomas. Results showed that ERG2 mRNA expression was significantly reduced in high-grade meningiomas compared to that in low-grade meningiomas. *$P < 0.05$. (D) Western blot analysis showed similar results. (E) Correlation between ERG2 mRNA expression and miR-224 expression. qRT-PCR (F) and Western-blot analysis (G) demonstrates that ERG2 and BAK expression were significantly increased in IOMM-Lee cells underexpressing miR-224 on mRNA and protein levels.

cancers [18–20]. ERG2-BAK-dependent apoptotic pathway has been proved to play a critical role in carcinogenesis [20]. Moreover, Koschny's work has demonstrated that BAK expression is positively associated with tumor progression and the proliferative activity of meningiomas [21]. In light of above findings, the alternations in the EGR2 and BAK expression were examined to investigate the mechanism of miR-224-induced apoptosis in meningiomas. We found miR-224 inhibition significantly increased EGR2 and BAK expression in IOMM-Lee cells on mRNA and protein levels (Fig. 3F and G), indicating the anti-tumor effects of miR-224 inhibition on meningiomas are associated with enhanced apoptosis and increased ERG2-dependent apoptotic pathway.

To further investigate the role of miR-224-EGR2 signals in cell growth and apoptosis, we stably transfected IOMM-Lee cells expressing miR-224 ASO with EGR2 siRNA (Fig. 4A and B). MTT assay and apoptosis assay showed that downregulation of EGR2 in IOMM-Lee cells expressing miR-224 ASO reversed the enhanced apoptosis (Fig. 4C) and lower growth (Fig. 4D) observed in cells expressing only the miR-224 ASO. Furthermore, downregulation of ERG2 by siRNA substantially decreased BAK expressions in IOMM-Lee cells transfected with miR-224 ASO compared to cells transfected with only the miR-224, suggesting that EGR2 is a critical downstream of miR-224. These data indicated that miR-224 is an important regulator of the ERG2-BAK-induced intrinsic apoptotic pathway in IOMM-Lee cells.

## 4. Discussion

MicroRNAs as regulators play critical roles in malignant biological behavior, including cell proliferation, apoptosis and invasion [9]. Mutations affecting miRNAs or their functional interactions with oncogenes and tumor suppressor genes may be involved in tumorigenesis [22]. Therefore, miRNAs may serve as new biomarkers to predict clinical outcomes in the future. Recent studies have indicated that miR-224 is overexpressed in colon, ovarian, prostate, and liver cancers [11,14,15,23], but the functions of miR-224 in meningiomas remain largely unclear. In the present study, we demonstrated that miR-224 expression was higher in

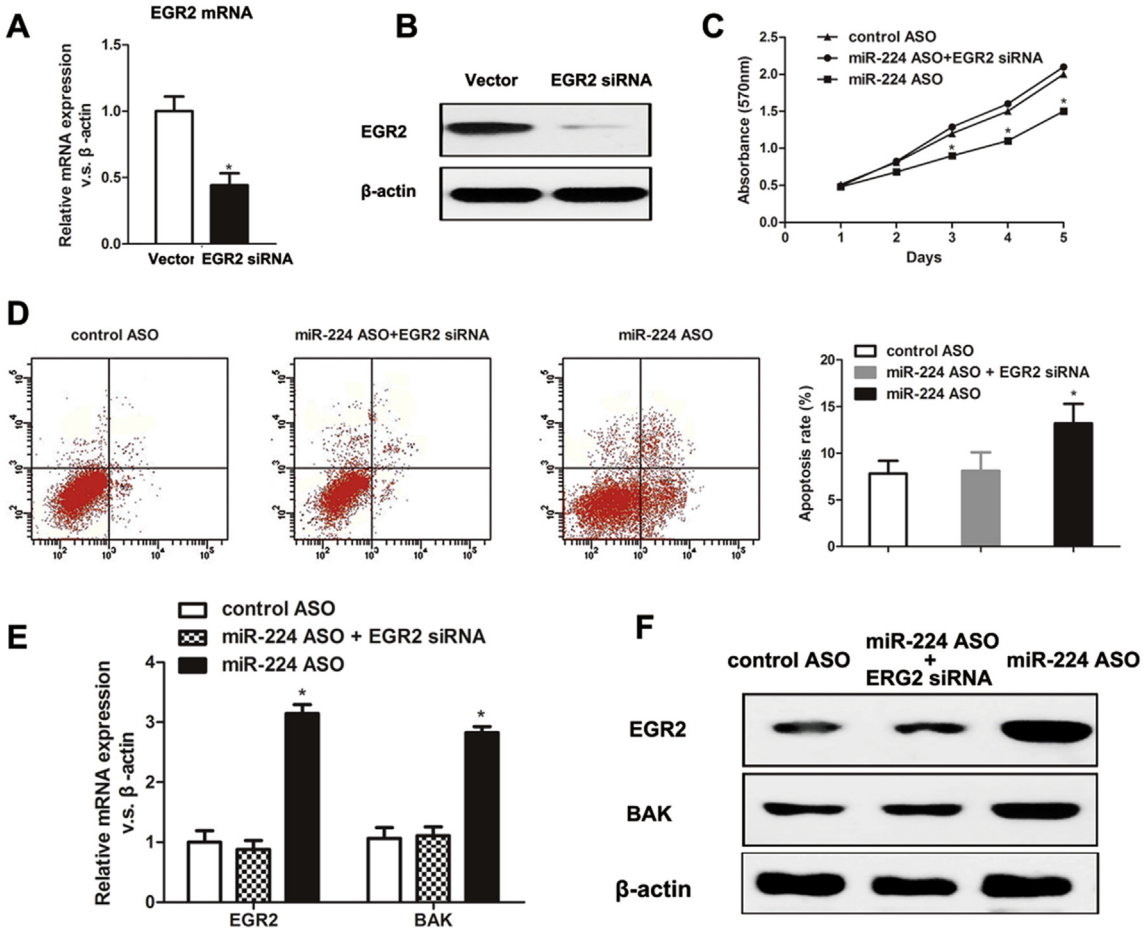


**Fig. 4.** (A) qRT-PCR demonstrates that ERG2 mRNA was significantly suppressed in IOMM-Lee cells treated with ERG2-siRNA compared to cells treated with vector. $^{*}P < 0.05$. (B) Western-blot indicates the similar results. (C) MTT assay of IOMM-Lee cells co-transfected with miR-224 ASO and EGR2-siRNA or the control. $P < 0.05$ (D) Apoptosis assay of IOMM-Lee cells co-transfected with miR-224 ASO and EGR2 siRNA or the control. $^{*}P < 0.05$.

meningiomas than that in normal brain tissues, associated with advanced pathological grade. This result is consistent with previous studies suggesting that miR-224 may play a critical role in enhancing survival and proliferation of malignant cancers. In addition, K-M analysis suggested that meningioma patients with high expression levels of miR-224 exhibited distinctly shorter OS and RFS, especially for meningioma with advanced stages. Excessive expression of miR-224 was also observed in low-grade meningioma with recurrence, suggesting that miR-224 expression may induce more aggressive tumor behavior. Thus, this is the first study to show that miR-224 expression is positively associated with the pathological stages and recurrence of meningiomas.

MiR-224 has been proved to play a role in several biological progressions, and participate in the pathogenesis of different types of cancer [12,13,15]. Since miR-224 regulates cell proliferation, apoptosis, and invasion, this miRNA has been regarded as an oncogenic miRNA capable of affecting various pivotal signal pathways [14,15,24,25]. Recent studies indicated that overepression of miR-224 was correlated with poor prognosis in liver, prostate, gastric, colon or lung cancers [11,13,26,27]. Furthermore, antisense miR-224 inhibitors have been shown to effectively reverse phenotypes associated with miR-224 overexpression in vitro models [14]. As increased miR-224 expression is proved to be positively correlated with aggressive behavior in tumors, there is good reason to believe this might be of similar value in meningiomas.

EGR2 has been regarded as a major contributor to apoptosis in various cancers. Here we demonstrated that EGR2 is an identical target of miR-224. In addition, we found that underexpression of miR-224 significantly increases EGR2 expression, which can directly transactivates proapoptotic protein BAK, and results in an enhancement of BAK-induced apoptosis. Furthermore, we showed that downregulation of ERG2 could reverse the anti-tumor effects of miR-224 inhibition in IOMM-Lee cells by using MTT assay and apoptosis assay. In light of above results, we strongly suggest that significantly suppressed meningioma cell proliferation by miR-224 inhibition could be attributed to enhanced EGR2-BAK-induced apoptosis in meningomas. Considering the promising roles that miR-224, ERG2 and BAK signaling network play in meningioma cell apoptosis, we believe miR-224 might be an ideal target for clinical therapy to treat this aggressive cancer.

Taken together, we demonstrate that the expression levels of miR-224 were significantly higher in meningioma tissues than that in normal brain, positively correlated with advanced pathological grade. Our findings also imply the miR-224 expression could predict the overall survival and recurrence-free survival of patients with meningiomas. Furthermore, our results indicate that miR-224 likely results in the enhancement of cell apoptosis through activation of the ERG2-BAK-induced apoptosis pathway. We believe that miR-224 may be a promising therapeutic target for treating malignant meningiomas.

## Conflict of interest

There are no potential conflicts of interest.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.038.

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