EGR1 modulated LncRNA HNF1A-AS1 drives glioblastoma progression via miR-22-3p/ENO1 axis

Chunchun Ma1,2,3, Hongliang Wang1,2,3, Gang Zong1,2, Jie He1,2, Yuyang Wang1,2, Fan Yang1,2, Zhihao Yang1,2, Erbao Bian1,2 and Bing Zhao1,2

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INTRODUCTION

GBM, the most aggressive subtype of glioma in adults, highly malignant and high risk of recurrence, accounting for 47.1% of all malignant tumors of the nervous system [1]. In the light of the World Health Organization (WHO) classification of tumor in the central nervous system (CNS), GBM is classified as a grade IV glioma, with an unfavorable prognosis and a five-year overall survival rate less than 10% [2, 3]. Even after multimodal therapies, including surgical resection, adjuvant radiotherapy, temozolomide (TMZ)-based chemotherapy or targeted therapy such as rituximab, which have been commonly used in GBM patients, the overall survival rate is still unsatisfactory with a median survival time of 12–15 months from first diagnosis [4]. Therefore, it is extremely vital for us to establish new targeted therapies and to gain a clear understanding of the definite mechanisms of GBM malignant progression for the identification of new diagnostic and prognosis markers.

LncRNAs comprise a class of transcripts that are over 200 nt in length, which have many ways of regulating ability except for none protein-coding potential [5, 6]. The functions and mechanisms of lncRNAs exert their biological role through diverse modes, including chromatin modification, alternative splicing, mRNA stability, encode functional micropeptides, ceRNA molecular sponge to miRNAs, and so on [7–14]. An increasing number of reports have demonstrated that lncRNAs play a vital role in cancer progression, and are dysregulated in various human cancers, including GBM [15–17]. For example, LncRNA miR155HG is overexpressed in GBM, and promotes GBM progression by acting as a ceRNA for the tumor suppressor miR-185 to upregulate ANXA2 [18]. LncRNA AC0164053 endip cell proliferation and metastasis by regulating TET2, via sponging of miR-19a-5p in GBM cells [19]. LncRNA HOTAIR1M1 is highly upregulated in GBM, which is positively correlated with tumor grade in patients with glioma, and aggravates the progression of GBM by regulating HOXA1 gene [20]. Despite several lncRNAs have been well studied, the functional mechanism of most lncRNAs in GBM remain largely unknow.

Hepatocyte nuclear factor 1 homeobox A antisense RNA 1 (HNF1A-AS1), was first identified as an lncRNA that upregulated in esophageal adenocarcinoma [21], and its overexpression was drastically associated with tumor advanced-stage and unfavorable outcomes in various cancers, including oral squamous carcinoma,
urothelial carcinoma of the bladder and lung carcinoma, indicating an oncogenic function of HNF1A-AS1 in tumor progression [22–24]. However, the functional mechanism of HNF1A-AS1 in GBM has not been fully revealed yet. Herein, we demonstrate that HNF1A-AS1 is highly overexpressed in GBM tissues and cells, associates with poor patient survival, by acting as ceRNA for miR-22 and facilitating ENO1, to promote GBM malignant phenotypes. Therefore, these results imply that HNF1A-AS1 may serve as a druggable target for GBM.

RESULTS
HNF1A-AS1 is upregulated in GBM and negatively related with patient prognosis
To identify the expression of HNF1A-AS1 in GBM, using RT–QPCR analysis, we firstly measured HNF1A-AS1 expression in 15 normal brain tissues, 41 low-grade glioma tissues, 72 GBM tissues. We found that HNF1A-AS1 was obviously upregulated in GBM, as compared to low-grade glioma tissues and normal brain tissues, but there was no significant difference between low-grade glioma tissues and normal brain tissues (Fig. 1A). Furthermore, we also tested HNF1A-AS1 expression in normal human astrocyte (HA) and four GBM cell lines (U251, LN18, U87 and A172). The results indicated that HNF1A-AS1 was highly upregulated in four GBM cell lines in comparison with HA (Fig. 1B).

To further clarify the clinical significance of HNF1A-AS1 in GBM patients. The Kaplan–Meier method and log-rank test were performed to assess the expression of HNF1A-AS1 in GBM TCGA date cohort and our clinic study. TCGA date cohort demonstrated that GBM patients with high expression of HNF1A-AS1 was negatively associated with overall survival time (Fig. 1C). In our study, we also observed the similar results, indicating that high HNF1A-AS1 expression was significantly correlated with poor survival patients (Fig. 1D). In addition, subcellular fractionation and RT–QPCR analyses showed that HNF1A-AS1 is localized both in the cytoplasm and nucleus of GBM cells (Fig. 1E), which indicated its complicated functions.

HNF1A-AS1 underpins GBM cells proliferation, migration, invasion
To evaluate the biofunctional role of HNF1A-AS1 in GBM cells, GBM cells were transfected with si-HNF1A-AS1 or si-NC. RT–QPCR was performed to examine the knockdown efficiency of HNF1A-AS1 after transfection 48 h (Fig. 2A). Compared with si-NC groups, CCK-8 assays revealed that HNF1A-AS1 knockdown significantly decreased cell proliferation (Fig. 2B). Colony formation assays indicated that the clone numbers and colony size was attenuated in the HNF1A-AS1 knockdown group, suggesting that depletion of HNF1A-AS1 slow down the growth of GBM cells (Fig. 2C). Wound healing assays indicated that the wound-healing capacity was worse and slower in si-HNF1A-AS1 groups than in si-NC groups (Fig. 2D). In addition, transwell assay showed that knockdown of HNF1A-AS1 significantly reduced migratory and invasive capacity compared with si-NC groups (Fig. 2E). On the contrary, over-expression of HNF1A-AS1 obviously promote the malignancy of GBM cells (Fig. S1A–C). To sum up, these results proved that HNF1A-AS1 plays an important role in promoting GBM cells malignant behaviors.

Fig. 1  HNF1A-AS1 was highly expressed in GBM. A RT-QPCR analysis of HNF1A-AS1 expression in normal brain tissues (NBT) (n = 15), low-grade glioma tissues (n = 41) and GBM tissues (n = 72). **P < 0.01 vs. NBT group. B RT-QPCR analysis the expression levels of HNF1A-AS1 in normal human astrocyte (HA) and four GBM cell lines (U251, LN18, U87 and A172). *P < 0.05, **P < 0.01 vs. HA group. C In TCGA date, the overall survival rate of GBM patients with high (HNF1A-AS1-high, n = 55) and low (HNF1A-AS1-low, n = 112) expression of HNF1A-AS1 in tumor. D Kaplan–Meier analyses of the associations between HNF1A-AS1 expression level and overall survival of patients with human glioma in our department (The log-rank test was used to calculate P-values). **P < 0.01 vs. low HNF1A-AS1 expression group. E Nuclear and cytoplasmic fractions of HNF1A-AS1 in GBM cell lysates were analyzed by RT-QPCR.
EGR1 strengthen HNF1A-AS1 expression in GBM cells

We next discover the potential regulatory mechanisms which cause the upregulation of HNF1A-AS1 in GBM. By using UCSC Genome Browser, we found that Early Growth Response gene 1 (EGR1) is a latent transcription factor of HNF1A-AS1. Furthermore, previous study has demonstrated that EGR1 transcriptionally activated HNF1A-AS1 in human gastric cancer [25]. According to JASPAR database, the predicted transcription factor-binding site of EGR1 in the HNF1A-AS1 promoter is indicated in (Fig. 3A). Importantly, TCGA database confirmed that EGR1 is highly expressed in GBM tissues, and patients with higher HNF1A-AS1 expression indicated a shorter survival time (Fig. S2A, B). To detect the effect of EGR1 on HNF1A-AS1 expression, we successfully establish EGR1 overexpression or knockdown model and was tested by RT-QPCR and western blot (Fig. S2C–E). As a result, depletion of EGR1 significantly decrease the levels of HNF1A-AS1, while EGR1 overexpressed dramatically increase the levels of endogenous HNF1A-AS1 (Fig. 3B, C). Luciferase reporter assay further demonstrated that the region of HNF1A-AS1 promoter is responsible for HNF1A-AS1 transcription (Fig. 3D–F). Moreover, ChIP assay showed that the EGR1 specifically associated with the promoter of HNF1A-AS1 (Fig. 3G). These results demonstrated that EGR1 enhance HNF1A-AS1 expression at transcriptional level by directly binding to its promoter.

HNF1A-AS1 physically bound to miR-22 and induced its degradation

To further clarify the underlying molecular mechanism by which HNF1A-AS1 regulates GBM cells proliferation, migration and invasion. As we all know, lncRNAs could restrain miRNAs expression and activity on their target mRNAs, by function as a ceRNA [26]. Bioinformatics data such as RegRNA 2.0, Diana-lncBase and miRANDA, were performed to predict the potential miRNA targets of HNF1A-AS1. According to the mirSVR and PhastCons scores, we found that miR-22 contain the potential target sites on HNF1A-AS1. Our studies indicated that the expression of miR-22 was significantly inverse in correlation with HNF1A-AS1 in GBM tissues (Fig. 3H). Moreover, HNF1A-AS1 knockdown dramatically increased miR-22 expression, while GBM cells transfected with PCDNA3.1-HNF1A-AS1 significantly inhibited the expression of miR-22 (Fig. 4A, B). However, no expression changed on HNF1A-AS1 when GBM cells transfected with miR-22 mimics or inhibitors (Fig.4C, D). These results indicated that miR-22 was negatively regulated by HNF1A-AS1 in GBM. Bioinformatics analysis predicted the potential binding sites of miR-22 on HNF1A-AS1 and the conservation of HNF1A-AS1 in the binding site of miR-22 was snapshotted from human genome in UCSC Genome Browser (Fig. 4E, F). Then, luciferase reporter assay indicated that there was no significant difference in the relative
luciferase activity between HNF1A-AS1-Mut+ miR-22 mimics and HNF1A-AS1-Mut+ miR-22 NC groups, but co-transfection of pmirGLO-HNF1A-AS1-WT and miR-22 mimics dramatically reduced the luciferase activity compared with HNF1A-AS1-WT+ miR-22 NC groups (Fig. 4G, H). To confirm whether HNF1A-AS1 and miR-22 are in the same RNA-induced silencing complex (RISC), we conducted anti-Ago2 RNA-binding protein immunoprecipitation (RIP) assay, and the results showed that Ago2 antibody enriched HNF1A-AS1 (Fig. 4I, J). These findings suggested that HNF1A-AS1 directly targeted miR-22 in GBM cells.

Downregulation of miR-22 promotes GBM cells malignant behaviors

According to the TCGA data, the expression of miR-22 was obviously decreased in GBM samples, and was negatively correlated the pathological grades of glioma (Fig. 5A), which was coincided with our study (Fig. 5B). RT-QPCR analysis confirmed that miR-22 expression was significantly downregulated in four GBM cell lines (Fig. 5C). Compared with the miR-22 NC group, the proliferation, migration and invasion ability of GBM cells were distinctly reduced when cells transfected with miR-22 mimics, and cells’ malignant behavior ability were enhanced when cells transfected with miR-22 inhibitors (Fig. 5D–E). These results indicated that miR-22 acted as an anti-oncogene in GBM cells.

ENO1, a direct target of miR-22 in GBM cells

Bioinformatic tools were adopted to predict potential targets of miR-22 in GBM cells. We found that 3’UTR regions of the overlapped potential candidates both have the predicted binding sites of miR-22. Ultimately, a key glycolytic enzyme, ENO1, was identified as target gene, in view of its upregulation is associated with glioma progression and prognosis [27, 28]. A previous study showed that miR-22 suppresses the proliferation of retinoblastoma cells by inhibiting ENO1, and ENO1 was a target of miR-22 [29]. As showed in Fig. 6A, the presumptive binding sites of miR-22 within the 3’UTR of ENO1 were predicted by TargetScan and MirDB. To confirm whether miR-22 regulated ENO1, GBM cells were transfected with miR-22 mimic or miR-22 inhibitors. The results indicated that increasing miR-22 markedly suppressed ENO1 mRNA and protein levels compared to miR-22 NC and conversely ENO1 expression significantly increased after inhibited miR-22 (Fig. 6B, C). Then, luciferase reporter assay demonstrated ed that co-transfection of ENO1 WT and miR-22 mimics drastically reduced luciferase activity compared with the ENO1 WT+ miR-22 NC group, whereas miR-22 Mut binding site within ENO1 abrogated
the inhibitory effect of miR-22 mimics on the reporter gene expression (Fig. 6D, E). In addition, we found that ENO1 was significantly overexpressed in GBM tissues and its high expression was inverse correlation with overall survival time in GBM TCGA data, which was consistent with CGGA data (Fig. S3A–D). These findings indicated that miR-22 inhibited ENO1 expression in GBM cells by targeting the 3′ UTR of oncogene ENO1.

Knockdown of HNF1A-AS1 induced suppression of malignant phenotype was smothered by knockdown of miR-22 in GBM cells

Previous study has confirmed that ENO1 attenuated led to underpin cancer progression in glioma cells [27]. However, whether HNF1A-AS1 promote the malignant behaviors of GBM cells by inhibiting miR-22 remained largely unknown. miR-22 mimics and miR-22 inhibitors were transfected into si-HNF1A-AS1 GBM cells. The results indicated that cell proliferation, migration and invasion ability were attenuated in si-HNF1A-AS1 and miR-22 mimics groups. si-HNF1A-AS1 combined with miR-22 mimics group was strongly reduced the malignant phenotype of GBM cells, while miR-22 inhibitors reversed the suppression of HNF1A-AS1 attenuated in GBM cells (Fig. 7A–D). Therefore, these results suggested that HNF1A-AS1 exerts its biofunctional roles though miR-22 in GBM cells.

HNF1A-AS1 functions as a ceRNA for ENO1 via modulating miR-22 and advances tumorigenesis in vivo in GBM

To explore whether HNF1A-AS1 regulates the expression of ENO1 by inhibiting miR-22 in GBM. ENO1 mRNA and protein levels were detected by RT-QPCR and Western blot assays after U251 and LN18 cells co-transfected with miR-22 NC, miR-22 mimics and miR-22 inhibitors. The results showed that HNF1A-AS1 knockdown decreased the mRNA and protein levels of ENO1 compare with si-NC, and si-HNF1A-AS1 combined with miR-22 mimics drastically decreased the expression levels of ENO1 but were reversed by co-transfection with si-HNF1A-AS1 and miR-22 inhibitors (Fig. 8A–C). Furthermore, our study first found that ENO1 expression was negatively correlated to miR-22 and positively correlated to HNF1A-AS1 in 72 GBM patients (Fig. 8D, E). Hence, these results indicated that HNF1A-AS1 regulates ENO1 expression by sponging miR-22 in GBM.

Tumor xenograft models were performed to evaluate the functional roles of HNF1A-AS1 in vivo. We inoculated subcutaneously the treated U251 cell, as showed in (Fig. 8A–C), Tumor...
volumes and tumor weights in the si-HNF1A-AS1 group were obviously smaller and lower compared with the si-NC group. Also, western blot indicated that ENO1 protein expression levels in nude mice tumor tissues was significantly decreased in the si-HNF1A-AS1 group than in the si-NC group (Fig. S4).

**DISCUSSION**

GBM is the most common and lethal primary malignant brain cancer in adults [30]. Although Maximal safe resection and chemoradiotherapy are known as comprehensive regimens which are the most popular mean for the treatment of patients with GBM, the overall survival time of GBM patients is still poor [31, 32]. Therefore, the diagnosis, treatment and prognosis estimation of such brain tumors remain a challenge in our clinical work. What is encouraging is that the research of molecular diagnosis participated in the progression of glioma such as IDH, TP53, EGFR, H3K27M and WNT, has gain much progress [33–37].

Recently, accumulating studies have confirmed that dysregulation of IncRNAs play a considerable functional role in carcinogenesis and progression of multiple cancers. Moreover, a number of studies have demonstrated that IncRNAs could serve as a diagnostic biomarker and therapeutic target in GBM [38]. Previous studies have confirmed that IncRNAs are dysregulated in GBM. For instance, NEAT1, a glioblastoma-associated IncRNA, was an oncogenic factor that was regulated by EGFR pathway, by activating WNT/β-Catenin pathway to promote GBM cells growth and invasion [39]. Our group’s previous research found that ATB was associated with a poor clinical outcome in glioma patients, and its depletion attenuated glioma biological characteristics by directly repression miR-200a, and positively regulating TGF-β expression in glioma cells [40].

In this study, we found HNF1A-AS1 was significantly upregulated in GBM tissues and cell lines compared with normal brain tissues and HA cell. High expression of HNF1A-AS1 was negatively associated with the clinical outcomes of GBM patients. In addition, knockdown of HNF1A-AS1 inhibited cell proliferation, colony formation, migration and invasion in vitro and in vivo, while overexpression HNF1A-AS1 strengthen the malignant behaviors of GBM cells. Taken together, our results indicated that HNF1A-AS1 functions as an oncogene and may serve as a potential prognostic biomarker in GBM.

Mounting studies have demonstrated that IncRNAs expression could be activated by their upstream transcription factors. For instance, MKL1 induce the transactivation of SNHG18, which promotes NSCLC growth, invasion, and metastasis [41]. The transcription factor TEAD4 mediates MNX1-AS1 expression to drive gastric cancer progression [42]. EGR1 as a transcription activator several IncRNAs [43, 44]. In the present study, we found that EGR1 could bind with HNF1A-AS1 promoter region and transcriptionally induced HNF1A-AS1 overexpression in GBM cells, and TCGA datasets confirmed upregulated EGR1 is correlated with poor prognosis of GBM patients.
Recently, increasing evidences demonstrated that lncRNAs can competitively binding to miRNAs and then regulate the expression of miRNA downstream target genes [8]. Liu et al. find that high HNF1A-AS1 expression function as a ceRNA that sponging miR-661, thereby increasing CDC34 and in turn accelerating HNF1A-AS1 expression in gastric cancer [25]. Cai et al. show that HNF1A-AS1 is overexpressed in colon tissues and cell lines, and served as a ceRNA to modulate miRNA-34a expression, subsequently with repression of miR-34a/SIRT1/p53 feedback loop and activation of canonical Wnt signaling pathway in metastasis of colon cancer [45]. Here, we found that HNF1A-AS1 located in both the nucleus and cytoplasm, indicating its complicated functions. To clarify the underlying mechanism by which HNF1A-AS1 functions as an oncogene in GBM. According to our bioinformatics analysis, we found that miR-22 might has putative binding sites with HNF1A-AS1 in GBM. Moreover, a negative association between HNF1A-AS1 and miR-22 expression in GBM tissues was confirmed from our study. HNF1A-AS1 knockdown or overexpression significantly increased or decreased the expression of miR-22 in GBM cells. In addition, dual-luciferase reporter and RIP assay demonstrated that HNF1A-AS1 acted as miRNA sponge and negatively regulates miR-22 expression in GBM cells.

MiR-22, which was an exon of the C17orf91 gene, was located at chromosome 17p13.3. Previous studies have confirmed that miR-22 is markedly downregulated and functions as a tumor suppressor miRNA in various cancers. Sun et al. find that miR-22 is downregulated in colon cancer, overexpression of miR-22 significantly inhibits cell proliferation, migration, metastasis, and epithelial-mesenchymal (EMT) transition by directly targeting BCL9L [46]. Jiang et al. show that miR-22 is significantly downregulated in AML and forced expression of miR-22 significantly suppresses leukemic cell viability and growth, and restoration of miR-22 expression holds great therapeutic potential to treat AML [47]. Chen et al. find that miR-22 mimics suppresses cell proliferation, migration, and invasion via targeting the 3′-UTR of SIRT1 in the progression of GBM [48]. However, many other important downstream target genes of miR-22 in GBM are not clear. In our study, we found miR-22 expression was downregulated in GBM tissues and cells in comparison with normal tumor tissues and HA cell. Furthermore, overexpression of miR-22 remarkably suppressed cell proliferation, migration, and invasion, and miR-22 inhibition exhibited the opposite effects. In addition, miR-22 suppression reversed the inhibitory effects caused by HNF1A-AS1 knockdown. In a word, these findings suggested that HNF1A-AS1 aggravated the biological characteristic of GBM cells by directly targets miR-22.

Alpha-enolase (ENO1), a famous glycolytic enzyme functioning during aerobic glycolysis, was found in nearly all parts of adult human, and contributed to the Warburg effect in cancer cells [49]. Previous studies showed that ENO1 expression was upregulated in various cancers and functioned as an oncogene in various cancer types [50, 51]. Song et al. find that elevated ENO1 expression was associated with poor prognosis in patients with GBM [27]. Principe et al. show that ENO1 silencing which increased integrins and uPAR (an ECM receptor), could impeded cell

Fig. 6 MiR-22 targets the 3′UTR of ENO1 and inhibits its expression in GBM. A Schematic diagram showing the predicted miR-22 binding sites within the 3′UTR of oncogene ENO1. B, C Relative expression of ENO1 mRNA and protein levels in U251 and LN18 cells after transfection with miR-22 mimics, miR-22 inhibitors, and miR-22 NC. *P < 0.05, **P < 0.01 vs. miR-22 NC group. D, E Luciferase activity in U251 and LN18 cells co-transfected with miR-22 mimics and luciferase reporters containing ENO1 wild type (WT) or mutant type (MUT) 3′-UTR. **P < 0.01 vs. miR-22 NC group. Data are presented as mean ± SD from three independent experiments.
adhesion, invasion, and metastasis, by acting as a plasminogen receptor on the tumor cell surface in pancreatic cancer [52]. Fu et al also demonstrate that upregulated ENO1 drastically enhanced NSCLC cell glycolysis and malignant biological behaviors by activating FAK-mediated PI3K/AKT pathway and its downstream signals to regulate the glycolysis, cell cycle, and EMT-associated genes [53]. In our study, we found both TCGA data and CGGA data validated that ENO1 was upregulated in GBM tissues and high ENO1 expression indicated a poor outcome of GBM patients. We confirmed that miR-22 directly target the 3′ UTR of ENO1 and negatively regulate its expression. More importantly, we further validated that HNF1A-AS1 knockdown induced the reduction of ENO1 expression was regain via miR-22 inhibition, indicating HNF1A-AS1 could positively regulate ENO1 expression by inhibiting miR-22 expression in GBM. These results suggested that HNF1A-AS1 functioned as a ceRNA by competitively binding miR-22 and releasing ENO1 in GBM.

In conclusion, our data demonstrate that a crucial oncogenic transcription factor EGR1 mediates HNF1A-AS1 expression via binding to the promoter region of HNF1A-AS1, which is highly expressed in GBM tissues and cell lines, and was negatively correlated with GBM progression and prognosis. HNF1A-AS1 suppressed GBM malignancy by functioning as a ceRNA to sponge miR-22 and facilitates the expression of ENO1, which is a direct target of miR-22 in GBM. Therefore, our findings support that HNF1A-AS1 may become a new therapeutic target for GBM treatment.

MATERIALS AND METHODS

Patient samples
72 GBM samples and 41 low-grade glioma (LGG) tissues were derived from surgical excision, and 15 normal brain tissues (NBT) come from acute brain injury patients during surgical. All patient samples were obtained from the Department of Neurosurgery, the Second Affiliated Hospital of AnHui Medical University from January 2013 to October 2018. All patients signed the written informed consent, and simultaneously approved by the Clinical Research Ethics Committee at the Second Affiliated Hospital of AnHui Medical University.

Bioinformatics data
UCSC Genome Browser (http://genome.ucsc.edu/), JASPAR (http://jaspar.genereg.net/), The Cancer Genome Atlas (TCGA, http://cancergenome), RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw/), Diana-lncBase (http://carolina.imis.athena-innovation.gr), miRanda (http://www.microrna.org), Chinese Glioma Genome Atlas (CGGA, www.cgga.org), TargetScan (http://www.targetscan.org), MirDB (http://www.mirdb.org/).

Cell culture
Human GBM cell lines (U251, LN18, U87 and A172), normal human astrocytes (HA) were purchased from the American Type Culture Collection (ATCC). All cells were cultured in DMEM with 10% FBS and streptomycin (100 μg/ml), penicillin (100 U/ml). All cell lines were cultured at 37°C in a humidified incubator with 5% CO2.

Subcellular fractionation
Cytoplasmic and nuclear fractions of the LN18 cells were prepared and collected according to the manufacturer’s instructions of PARIS™ Kit (Life Technologies, CA, USA). GAPDH was used as the cytoplasmic internal reference. U6 small nuclear RNA was used as the nuclear internal reference.

Cell transfection
HNF1A-AS1 siRNA (si-HNF1A-AS1), EGR1 siRNA (si-EGR1) and corresponding negative control (si-NC) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequence of si-HNF1A-AS1-1 was as follows:
CCCTCCATCTAACATTCAA, si-EGR1, CAACGAGAAGGTGCTGGTG. U251 and LN18 cells transfected with these fragments respectively by using Lipofectamine3000 (Invitrogen, USA). Full‐length of HNF1A‐AS1 and EGR1 was amplified by PCR and sub-cloned into pcDNA3.1-Vector (pcDNA3.1-HNF1A-AS1 and Vector), pcDNA3.1-Vector (pcDNA3.1-EGR1 and Vector) (Sangon Biotech, Shanghai, China). MiR-22-3p negative control (NC), miR-22-3p mimics and miR-22-3p inhibitors were purchased from Guangzhou RiboBio (Guangzhou, China).

RT‐QPCR
Cells and tissues were lysed in TRizol (Invitrogen, USA), and Total RNA isolation was reverse transcribed to complementary DNA (cDNA) using the PrimeScript RT (Takara, Nanjing, China). The primers for genes were determined as follows: HNF1A-AS1 forward 5′- CAAGAAATGGTGGCTATGA-3′, reverse 5′-TGGACTGGGACAAGGGT-3′; GAPDH forward 5′-AGCAAGAGCACAAGAGGAAG-3′, reverse 5′-GGTTGAGCACAGGGTACTTT-3′.

EGR1, forward: 5′-CACGACCTCTCAACCTCAG-3′, reverse: 5′-CACAAGGTGTTGCCAGCTCT-3′; ENO1 forward 5′-GCCCTGCTGCACAGTCA-3′, reverse 5′-AAGCAGACAGACACATGACG-3′; GAPDH and L6 were used as loading control for HNF1A-AS1, ENO1 and miR-22. All RT‐QPCR reactions were performed in triplicate. The data were determined using the 2^(-△△Ct) method.

Cell proliferation assay
U251 and LN18 cells after transfection were plated into 96-well plate (1000 cells/well), and cultured them at 37 °C with five percent CO2. Approximately 10 μl of CCK (Dojindo, Shanghai, China). solution was added into per well. Finally, the absorbance at 450 nm was measured using a ST-360 micro-plate reader (KHB, Shanghai, China) after incubated at 37 °C for 2 h.

Colony formation assay
For the clone formation assay, 48 h after transfection, GBM cells (200 viable cells per well) were seeded in a 6-well plate and cultured with complete medium for 12 days. cells were fixed with 4 % polyoxymethylene and stained with 1.5 % methylene blue for 30 min at room temperature.

Wound healing
U251 and LN18 cells after transfection 48 h were seeded in a 6-well plate and cultured with complete medium (200 viable cells per well). a 10-μl pipette tip was used to create wound gaps, gently washed, and cultured with serum-free medium for 24 h. The wound gaps were observed at 0 and 24 h after wounding and photographed with a light microscope (Olympus, Japan).

Transwell assay
The 24-well chambers with 8 μm polycarbonate membrane inserts (Corning, New York, USA) was used to detect the migration and invasion ability. A total of 2×10^4 cells were resuspended in 150 μl serum-free medium and 500 μl of 10 % FBS medium, respectively place in the upper chamber with or without pre-coated with 400 ng/ml Matrigel solution (BD Biosciences, New jersey, USA), and placed in the lower chamber of Transwell plates. After 48 h, the migrated and invaded cells on the lower...
chamber membrane were fixed with 4% polyoxymethylene and stained with crystal violet (Sigma). Five predetermined fields were counted under a microscope (Olympus, Japan). All assays were performed in triplicate.

**Luciferase reporter assays**

The fragments of HNF1A-AS1 and 3′ UTR of ENO1, both containing the predicted miR-22 binding site, then the predicted wild-type (WT) binding sites of miR-22 and mutant binding sites (Mut) were cloned into a pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA), termed as pmirGLO-HNF1A-AS1-wild-type (HNF1A-AS1-WT), pmirGLO-HNF1A-AS1-mutated-type (HNF1A-AS1-Mut), pmirGLO-ENO1-wild-type (ENO1-WT) and pmirGLO-ENO1-mutated-type (ENO1-Mut). Then HNF1A-AS1-WT or HNF1A-AS1-Mut was co-transfected with the miR-22 negative control or mimics into GBM cells by using Lipofectamine3000 (Invitrogen, USA). Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to detect the relative luciferase activity. ENO1-WT and ENO1-Mut were handled similarly as described above.

To confirm the bind relation between EGR1 and HNF1A-AS1 promoter, pGL3-HNF1A-AS1 promoter was co-transfected into cells along with si-EGR1 or si-NC using Lipofectamine3000. The luciferase activity was measured by a Dual-Luciferase reporter assay system (Promega, USA). All assays were independently performed in triplicate.

**Chromatin immunoprecipitation assay**

The EZ-Magna ChiP™ Chromatin Immunoprecipitation Kit (Millipore, USA) was used for Chromatin immunoprecipitation (ChIP) assay. U251 and LN18 cells were fixed with 1% formaldehyde for 10 min at room temperature and lysed in ChiP lysis buffer, and then the DNA was sonicated for shearing DNA into 500-bp fragments. Subsequently, DNA samples were precipitated with anti-igg or anti-EGR1 antibody and Protein A/G magnetic beads for overnight. Finally, the co-precipitated chromatin DNA was collected, and was tested by RT-QPCR.

**RNA immunoprecipitation**

RNA immunoprecipitation (RIP) experiments were performed by the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA), and was conducted as previously described [40].

**Western blotting**

Protein extract from U251, LN18 cells and tissue samples by using RIPA protein extraction reagent (Beyotime,Shanghai, China). The concentration of protein was tested by the BCA Protein Assay Kit (Beyotime, Shanghai, China). Magna RIP RNA immunoprecipitation (RIP) experiments were performed by the EZ-Magna ChIP Kit (Millipore, USA) and was conducted as previously described [40].

**In vivo xenograft model**

Female nude mice at 4–6 weeks of age were used in this study, and were divided into two groups (five/group). U251 cells stably transfected with si-NC or si-HNF1A-AS1 were collected, and injected into the subcutaneous tissues of the axillary skin. The growth of tumor was measured every five days. 40 days after injection, the mice were sacrificed, and the tumor nodules were harvested for further study. All experiments was approved by the Animal Care and Use Committee of AnHui Medical University.

**Statistical analysis**

Unless stated otherwise, all experiments were performed in triplicate and all data were presented as the mean ± standard deviation (SD). GraphPad Prism V6.01 (GraphPad Software, Inc., La Jolla, CA, USA) software was used for statistical analysis and generate figures. Differences were analyzed by SPSS statistical software (version 19.0, Armonk, NY, USA) with the Student's t-test or one-way ANOVA. Pearson's correlation was performed to analyze the relationship between the expression of HNF1A-AS1, miR-22 and ENO1 in tissues. Survival analysis was performed using the Kaplan-Meier method and log-rank tests in GraphPad Prism 6.01. Differences were considered significant if P < 0.05.

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AUTHOR CONTRIBUTIONS

CCM and HLW conceived and designed the experiments. GZ performed the experiments. FY played an important role in interpreting the results. EBB, and JH analyzed the data. YW drafted the manuscript. ZHY performed the bioinformatics analysis. BZ supervised the whole work and revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Bing Zhao.

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