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The transcriptional modulator HMGA2 promotes stemness and tumorigenicity in glioblastoma

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Abstract

Glioblastoma (GBM) contains a population of stem-like cells that promote tumor invasion and resistance to therapy. Identifying and targeting stem cell factors in GBM may lead to the development of more effective therapies. High Mobility Group AT-hook 2 (HMGA2) is a transcriptional modulator that mediates motility and self-renewal in normal and cancer stem cells. We identified increased expression of HMGA2 in the majority of primary human GBM tumors and cell lines compared to normal brain. Additionally, HMGA2 expression was increased in CD133+ GBM neurosphere cells compared to CD133- cells. Targeting HMGA2 with lentiviral short hairpin RNA (shRNA) led to decreased GBM stemness, invasion, and tumorigenicity. Ectopic expression of HMGA2 in GBM cell lines promoted stemness, invasion, and tumorigenicity. Our data suggests that targeting HMGA2 in GBM may be therapeutically beneficial.

Keywords

glioma; motility; clonogenicity; GSC; HMGI-C

1. Introduction

Glioblastoma (GBM) is the most aggressive and incurable form of astrocytoma with over 10,000 new cases estimated to be diagnosed in 2015 [1]. The average survival time after diagnosis is 12-14 months [2-4]. The standard of care for GBM is maximal surgical

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resection followed by radiation and chemotherapy [5]. GBM tumors are heterogeneous and contain a hierarchy of cell types, a small percentage of which are GBM stem-like cells [6] that are highly invasive and resistant to current therapies [2-4]. Failure of current GBM therapeutics warrants identification of novel targets and pathways regulating stemness, invasion and tumor formation.

HMGA2 (also called HMGI-C) is a DNA binding protein that acts as an architectural transcription regulator that can assemble and maintain enhanceosomes [7]. HMGA2 binds to the AT-rich minor grooves of DNA through three conserved sequences called AT-hooks [8]. HMGA2 can regulate the expression of both oncogenes and tumor suppressors [9]. HMGA2 is important during embryonic morphogenesis [10] and is aberrantly expressed in cancer [7, 10-32]. High levels of HMGA2 in cancer are associated with increased invasiveness, stemness and poor prognosis [12, 14, 18, 22, 24-36]. However, the functional importance of HMGA2 in regulating stemness and tumorigenicity in GBM are poorly understood.

HMGA2 can be regulated by different mechanisms including the LIN28 pathway [7, 17, 37-39]. LIN28A and LIN28B negatively regulate the *let-7* tumor suppressor family of microRNAs [40-43]. HMGA2 has seven conserved *let-7* binding sites in the 3'-UTR region [44], enabling negative regulation by *let-7* in GBM [17]. We previously identified LIN28A and LIN28B as being expressed in GBM, and showed that knockdown of LIN28A or overexpression of *let-7* could decrease GBM invasion [17]. We further found that manipulation of LIN28A and *let-7* levels in GBM led to changes in HMGA2 expression, suggesting that the LIN28/*let-7*/HMGA2 pathway was active in GBM [17]. We here further explore this pathway by directly investigating the function of HMGA2 in GBM.

2. Materials and Methods

2.1. Cell culture

The human adherent GBM cell lines, U-87 MG and LN-229, were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen™, Life technologies, New York) supplemented with fetal bovine serum (FBS; 10% for U-87 MG and 5% for LN-229; GE Healthcare Life Sciences, Utah). The adult human GBM neurosphere cell lines, GBM14, GBM10, JHH520, and HSR-GBM1 were cultured in neurosphere media containing human epidermal growth factor (EGF; PeproTech, New Jersey), fibroblast growth factor (FGF; PeproTech, New Jersey) and heparin sulfate (Sigma-Aldrich, Missouri) as previously described [45, 46]. HEK293T and HEK293GP cells were used for producing viral particles and were cultured in DMEM containing 10% FBS. All these cell lines were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

2.2. DNA constructs

Two lentiviral shRNA constructs TRCN0000342673 (shHMGA2#1) and TRCN0000342671 (shHMGA2#2) targeting the 3'-UTR region and the coding region, respectively (Sigma-Aldrich, Missouri), of human HMGA2 mRNA were used to knockdown HMGA2. MISSION® pLKO.1-puro Empty Vector Control (does not contain shRNA construct) and

MISSION[®] pLKO.1-puro Non-Target shRNA Control Plasmid DNA (SCRAMBLE shRNA control that does not target any known sequence from any species) were used as controls for the two shRNA constructs. pBABE zeo HMGA2 [47] was a gift from Tyler Jacks (Addgene plasmid # 17411) that was used to retrovirally express HMGA2. pBABE zeo (Addgene plasmid # 1766) was used as a control for the retroviral HMGA2-expressing plasmid.

2.3. Virus preparation, titration and infection

Viral particles were produced and titered as previously described [48]. For infection, GBM cells were treated with trypsin (Sigma-Aldrich, Missouri; for U-87 MG and LN-229) or Accutase (Sigma-Aldrich, Missouri; for neurosphere cell lines) and gently triturated and plated at low density. The concentrated virus (approximately 2.5×10^7 TU/ml) was added to the cells and incubated for 48 hours, after which the transduced cells were selected using puromycin (Sigma-Aldrich, Missouri; 1-2 μ g/ml) or Zeocin (Invitrogen[™], life technologies, New York; 250 μ g/ml). After 7 days of selection, HMGA2 knockdown or expression was confirmed by western blotting.

2.4. Colony formation in soft agar and methyl cellulose

GBM1 cells expressing control or HMGA2 vector were triturated to single cells and plated in soft agar to form colonies for 14-18 days as previously described [17]. For serial clonogenic methylcellulose assays, HSR-GBM1 cells expressing control or HMGA2 vector were triturated into single cells and plated in methylcellulose as previously described [45] with the following modifications. The primary assay was plated into 6-well plates with 4 wells each for control and HMGA2 overexpressing cells. After 19-20 days of the primary assay, three wells were stained with p-Nitro Blue Tetrazolium Chloride (NBT; Affymetrix, Inc., Ohio) and from the fourth well, spheres greater than 100 μ m in diameter were pulled, triturated into single cells and replated into methylcellulose (secondary assay). After 17-19 days of secondary assay, the process described above was repeated again (tertiary assay). Stained colonies from primary, secondary and tertiary assays that were greater than 50 μ m in diameter were imaged and counted using MCID Core software (Cambridge, UK). At least 3 images per well were counted and analyzed.

2.5. Boyden chamber transwell invasion assay

Transwell invasion assays were performed as previously described [17] with the following modifications. Transduced adherent cells were triturated to single cells and plated onto Matrigel-coated transwell inserts in the upper chamber containing serum-deprived medium while the lower chamber contained DMEM supplemented with either 5% (for LN-229) or 10% (for U-87 MG) FBS as a chemoattractant. Transduced neurosphere cells were plated in neurosphere media lacking growth factors while the lower chamber contained media supplemented with EGF and FGF. The cells were allowed to invade through Matrigel for 24-26 hours after which the invading cells collected in the bottom part of the filter were stained with hematoxylin for quantitation. At least 2 transwells per condition and 6 high power fields (HPF) per transwell were analyzed.

2.6. RNA extraction and quantitative PCR

RNA was extracted and purified using RNeasy kit (Qiagen, California). Reverse transcription and quantitative PCR was performed using SYBR Green reagents (Applied Biosystems, California) on an I-Cycler IQ Real-Time detection system (Bio-Rad, California) according to the manufacturer's instructions. The C_T method was used to quantify RNA expression levels and all values were normalized to 18S rRNA. Expression levels were analyzed from at least three experimental and 2 technical replicates. The following primer sets (for all human sequences) were used: HMGA2 forward 5'-AAAGCAGCTCAAAGAAAGCA-3', reverse 5'-TGTTGTGGCCATTCCTAGGT-3', and 18S forward 5'-GTAACCCGTTGAACCCATT-3', reverse 5'-CCATCCAATCGGTAGTAGCG-3'.

2.7. Western Blotting

Cells and primary tumor tissues were lysed, separated on SDS-polyacrylamide gels and subjected to Western blotting as described before [17] using the following antibodies: HMGA2 (1:1000 dilution, Cell Signaling Technology, Inc., Massachusetts), glyceraldehyde-3-dehydrogenase (GAPDH) (1:3000 dilution, RDI, New York), and β -actin (1:5000 dilution, Santa Cruz Biotechnology, Inc., Texas). Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to horse-radish peroxidase (1:3000 to 1:5000 dilution, KPL, Maryland) were used to detect the primary antibodies. Western Lightning *Plus* ECL (PerkinElmer, Massachusetts) was used to detect the secondary antibodies. Densitometry analysis was performed using Image J software [49].

2.8. Intracranial xenograft tumors and bioluminescence imaging

Transduced cells were xenografted intracranially into immunocompromised mice as described previously [17] with the following modifications. Stereotactic technique [50] was used for orthotopic xenografting into the striatum with the coordinates -3 from bregma, +2 from the sagittal suture and 3 mm deep. We implanted 1×10^4 transduced tumor cells overexpressing HMGA2 and 1×10^5 transduced cells wherein HMGA2 expression was reduced using shRNA. Tumor growth was monitored periodically using bioluminescence imaging by the Xenogen IVIS $\text{\textcircled{R}}$ Spectrum *in vivo* imaging system (PerkinElmer, Inc., Massachusetts) as described previously [46]. Animals were monitored regularly for signs of distress (cachexia, neurologic deficits, weight loss, hunching) suggestive of increasing tumor mass and were sacrificed at that time. Resected brains with intracranial tumors were embedded in paraffin and processed for immunohistochemistry. 3/10 animals from the shHMGA2 group were censored and not included in the survival analysis due to the following reasons: one died post-operation, one died from lymphoma, and the other was lost during cage maintenance. All procedures performed on animals were in accordance with approved protocols from the Institutional Animal Care and Use Committee.

2.9. Immunohistochemistry

Human glioma tissue microarrays (TMAs) containing tumor cores from 45 adult and 35 pediatric primary GBM tumors were constructed at Johns Hopkins as described in [17]. The TMAs and deparaffinized intracranial xenografted tissue sections were subjected to

immunohistochemistry as described before [17] at the Johns Hopkins Histopathology Core. The tissue sections were stained with hematoxylin and eosin (H&E) to visualize tumor cells. For HMGA2 immunohistochemistry on the TMAs, human specific HMGA2 primary antibody (1:7 dilution, Abcam, Massachusetts) was used and nuclear staining was scored by a neuropathologist (F.R.) as high (3), moderate (2), low (1) or negative (0, if no immunoreactivity or only a blush/trace was observed).

2.10. Human tumor tissue processing

Primary human brain tumor and normal samples were obtained by surgical resections performed at the Johns Hopkins Hospital following approved Institutional Review Board protocols and after informed consent from patients. The tissue was cryopreserved immediately post resection. For preparation of tumor lysates, the cryopreserved tumor and normal samples were lysed in RIPA buffer and processed for Western blotting as described previously [17].

2.11. Cell proliferation and apoptosis assays

Cell proliferation was measured by treating the transduced cells with bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, Missouri, USA) for 3 hours and the samples were then processed for immunofluorescence as described previously [51]. For assessing apoptosis, transduced cells were cytopun and processed for cleaved caspase-3 staining as described in [51] using cleaved caspase-3 antibody (Cell Signaling Technology, Massachusetts).

2.12. Flow cytometric analysis

Transduced cells overexpressing HMGA2 were triturated to single cells and processed for CD133 flow sorting as described previously [45].

2.13. Statistical methods and survival analysis

Data was analyzed from each experiment performed at least twice with concordant results and representative results are shown. Statistical tests were performed using Excel (Microsoft, Washington). Error bars denote standard error of the mean (SEM). Statistical significance was determined using an unpaired, two-sided Student's *t*-test, where $P < 0.05$ was considered to be statistically significant. For *in vivo* experiments, data was collected from at least 5 animals injected per condition and statistical significance was determined by log-rank test using GraphPad Prism (GraphPad software, Inc., California).

3. Results

3.1. HMGA2 is expressed in human GBM tumors and cell lines

We analyzed HMGA2 protein expression in primary GBM tumors resected from human patients by western blotting and immunohistochemistry. High levels of HMGA2 protein compared to normal brain were detected in the majority (5 out of 7, 71%) of primary GBM tumors (Fig. 1A) by western blotting. We performed immunohistochemistry to determine the expression levels of HMGA2 protein in 45 adult GBM and 35 pediatric GBM tumors on a tissue microarray (TMA) (Fig. 1B). We found moderate to high nuclear expression of

HMGA2 in 29 out of 45 (67%) adult GBM tumors and 24 out of 35 (70%) pediatric GBM tumors (Fig. 1B).

We next tested the expression of HMGA2 protein in multiple patient-derived adherent (U-87 MG and LN-229) and neurosphere (GBM14, GBM10, JHH520, and HSR-GBM1 [52, 53]) cell lines by western blotting. All GBM cell lines analyzed showed some degree of HMGA2 protein expression (Fig. 1C).

HMGA2 promotes self-renewal capacity in embryonic and cancer stem cells [10]. CD133 is used as a marker to differentiate GBM stem-like cells from the other cell types in the heterogeneous tumor cell population [53, 54]. We used CD133 as a surrogate marker for GBM stem-like cells and flow-sorted HSR-GBM1 neurospheres for cells expressing CD133. Analysis of *HMGA2* expression by quantitative PCR revealed a 9.3-fold higher expression of *HMGA2* in HSR-GBM1 neurosphere cells expressing CD133 compared to CD133- cells (Fig. 1D, $P < 0.0001$, $N = 2$). This suggests that HMGA2 may play a role in promoting stemness or clonogenicity of GBM cells.

3.2. Reduction of HMGA2 expression inhibits GBM cell clonogenicity and invasion

To functionally dissect the role of HMGA2 in GBM, we used two GBM adherent (U-87 MG and LN-229) cell lines and a neurosphere (HSR-GBM1) cell line [46]. U-87 MG and LN-229 express levels of HMGA2 (Fig. 1C) comparable to the high levels of HMGA2 we detected in a subset of primary tumors, making them ideal for loss of function experiments. HSR-GBM1 expresses lower levels of HMGA2 (Fig. 1C), making it appropriate for gain and loss of function experiments. We used two separate lentiviral shRNA constructs as controls (pLKO.1 and SCRAMBLE) and two lentiviral HMGA2 shRNA constructs (shHMGA2#1 and shHMGA2#2) to decrease HMGA2 expression in GBM cell lines (Fig. 2A).

Densitometric analysis of the bands in the western blots showed a reduction of HMGA2 protein by respectively 53% and 57% with shHMGA2#1 and shHMGA2#2 for HSR-GBM1, 28% and 42% with shHMGA2#1 and shHMGA2#2 for LN-229 compared to control (pLKO.1) infected cells (Fig. 2A). In U-87 MG cells, we found that shHMGA2#1 decreased HMGA2 protein expression by 14% compared to the cells expressing control shRNA (pLKO.1) (Supplementary Fig. S1-A). Although we have used shHMGA2#1 and shHMGA2#2 for suppressing HMGA2 expression up to 70% compared to the empty vector control shRNA in pediatric tumor cell lines [22], we were unable to achieve more than 57% knockdown of HMGA2 in GBM cells even after multiple attempts. This suggests that there is a greater requirement for HMGA2 in GBM tumor cells. To test the hypothesis that HMGA2 promotes clonogenicity in GBM, we performed clonogenic soft agar assays with HSR-GBM1 cells expressing either pLKO.1 or HMGA2 shRNA. HSR-GBM1 cells infected with pLKO.1 formed 79 ± 11 colonies, whereas the number of colonies formed by HSR-GBM1 cells expressing HMGA2 shRNA#2 were reduced to an average of 16 ± 2 (Fig. 2B, $P < 0.0001$, $N = 2$).

We further hypothesized that reduced expression of HMGA2 in GBM will decrease the invasive capacity of GBM cells. We performed transwell invasion assays in HSR-GBM1, LN-229 and U-87 MG cells expressing either pLKO.1 control or HMGA2 shRNA. HSR-GBM1 cells expressing HMGA2 shRNA showed a significant decrease in the ability of cells

to invade Matrigel (57% and 50% reduction with shHMGA2#1 and shHMGA2#2, respectively), compared to pLKO.1 infected cells (Fig. 2C, $P<0.001$ for shHMGA2#1 and $P=0.001$ for shHMGA2#2, $N=2$). LN-229 cells expressing HMGA2 shRNA had a reduction in invasion of 73% and 44% with shHMGA2#1 and #2, respectively, compared to pLKO.1 infected cells (Fig 2C, $P<0.001$ for both HMGA2 shRNAs, $N=2$). U-87 MG cells infected with shHMGA2#1 showed a significant decrease in invasion by 30% compared to pLKO.1 infected cells (Supplementary Fig. S1-B, $P=0.006$ $N=3$), suggesting that HMGA2 is required for GBM cell invasion.

3.3. Reduction of HMGA2 suppresses GBM tumorigenicity *in vivo* in orthotopic xenografts

To determine whether HMGA2 is required for tumor formation and survival of tumor cells in an *in vivo* environment, we injected HSR-GBM1 cells expressing either pLKO.1 or HMGA2 shRNA into the striatum of the brain of immunocompromised mice (100,000 cells/mouse). We have engineered HSR-GBM1 cells to express luciferase constitutively allowing bioluminescence imaging [46]. After five weeks, animals injected with HSR-GBM1 cells transduced with pLKO.1 showed more luciferase intensity (Fig. 3A and B, mean bioluminescence intensity = 7273) compared to animals harboring HSR-GBM1 orthotopic xenografts transduced with HMGA2 shRNA (mean = 1666) (Fig. 3A and B, $P=0.035$, $N=5$ for each condition). The experiment was continued, and animals were sacrificed when they showed clinical signs of harboring a significant brain tumor. Log rank analysis of Kaplan-Meier survival curves showed significant increase in survival after HMGA2 knockdown with median survival for pLKO.1 control being 67.5 days and median survival for shHMGA2#2 being 118 days, $P<0.0001$ by log-rank test (Fig. 3C, $N=10$ for pLKO.1 and $N=7$ for shHMGA2#2). Animals were monitored for symptoms for six months, at which time the remaining mice harboring shHMGA2 xenografts were sacrificed. None of these long-surviving animals had a brain tumor when examined histologically (Supplementary Fig. S2).

3.4. Overexpression of HMGA2 increases GBM clonogenicity and invasion

To test the hypothesis that increased expression of HMGA2 would promote GBM tumorigenicity, we expressed HMGA2 in our HSR-GBM1 neurosphere cell line (GBM1-HMGA2). Viral mediated expression of HMGA2 in HSR-GBM1 increased HMGA2 expression by 6.5 fold compared to the control virus infected cells (Fig. 4A).

To confirm HMGA2's role in promoting GBM clonogenicity, we performed clonogenic serial methylcellulose assays. We plated single cells of either control plasmid or HMGA2 transduced GBM1 cells in methylcellulose to form colonies (primary assay). We then extracted colonies that were greater than 100 micrometers (μm) in diameter and replated the assay twice (secondary and tertiary assays) as described in the Materials and Methods section. We have shown previously by clonogenic assays that HSR-GBM1 cells that form colonies greater than 100 μm in diameter are derived from a tumor stem-like cell and retain the ability to self-renew over multiple subsequent generations [45]. High expression of HMGA2 caused a significant increase in the number of larger colonies (diameter $> 300\mu\text{m}$) formed in primary ($P=0.001$, 2.9-fold increase), secondary ($P=0.001$, 7.4-fold increase) and tertiary assays ($P=0.003$, 40.4-fold increase) compared to control (Fig. 4B, $N=3$). Since increased HMGA2 expression in HSR-GBM1 does not lead to enhanced proliferation

(Supplementary Figure S3-A), the increased colony size suggests that HMGA2 promotes the self-renewal capacity of GBM cells, a property that is important for tumor initiation.

To confirm that HMGA2 promotes GBM invasiveness, we performed Matrigel-transwell assays with GBM1-HMGA2 and GBM1-control cells. GBM1-HMGA2 cells showed 1.4 fold increase in the number of invading cells in 24 hours compared to those infected with the empty vector control plasmid (Fig. 4C, $P<0.001$, $N=3$).

3.5. Overexpression of HMGA2 promotes GBM tumor formation in orthotopic xenografts

We hypothesized that increased HMGA2 would enhance GBM tumor formation. We injected 10,000 GBM1-HMGA2 or GBM-vector control cells into the striatum of immunocompromised mice. Luciferase imaging showed that all the animals xenografted with HMGA2-transduced GBM1 cells (5/5) showed large tumors (mean bioluminescence intensity = 24,834) by 4 weeks post injection (Fig. 5A and B). In comparison, at 4 weeks post injection, 4/5 control animals had tumors that were significantly smaller in bioluminescence intensity (mean = 3556) compared to the tumors formed by HMGA2 xenografted mice, and 1 animal did not have evidence of any tumor (Fig. 5A and B, $P=0.0209$ by Student's t-test, $N=5$). The experiment was continued, and animals were sacrificed when they showed clinical signs of harboring a significant brain tumor. Log rank analysis of Kaplan-Meier curves showed a trend towards a decrease in median survival of high HMGA2-expressing GBM1 xenografted mice (86 days) compared to control vector mice (median survival = 94.5 days, $P=0.08$ by log-rank test, $N=5$, Fig. 5C).

4. Discussion

The High Mobility Group AT-hook 2 (HMGA2) protein upregulates a pro-invasive program in diverse cancer types [7, 10, 12, 18-23, 34, 36]. We found increased expression of HMGA2 protein in the majority of GBM tumors by western blot analysis and immunohistochemistry using a cohort of 80 pediatric and adult GBM samples. Prior studies have investigated HMGA2's role in GBM invasion and tumor formation in heterotopic flank tumors using adherent GBM cell lines [17, 55-57]. We have confirmed these findings using adherent cell lines and extended our interrogation of HMGA2 in stemness and tumorigenicity using GBM neurosphere lines and orthotopic intracranial mouse models. Neurospheres grow in a serum-free medium and are known to simulate GBM in culture and *in vivo* better than adherent cells [58, 59]. Since brain microenvironment plays profound roles in regulating tumor progression [60], we have confirmed the role of HMGA2 in promoting GBM progression using intracranial xenograft models.

We hypothesized that HMGA2 would promote tumorigenicity in GBM, and we found that HMGA2 enhances GBM clonogenicity and invasion. Supporting a role for HMGA2 in tumor initiation, the majority of GBM cells bearing shRNA against HMGA2 do not form colonies when plated as single cells in soft agar. The reduction in colony formation from single cells suggests that HMGA2 is required for clonogenicity and could affect survival of GBM cells. These cells also have reduced tumorigenicity in intracranial xenografts. However, it is difficult to assess invasiveness of these cells *in vivo* since the animals were sacrificed when they become symptomatic and the tumor growth is at an advanced stage.

Since HSR-GBM1 cells are invasive at baseline, the tumors in both the animal groups (control and HMGA2-expressing) occupy a significant portion of the brain. We also did not observe significant changes in GBM cell proliferation *in vitro* (measured by BrdU cell growth assays) with perturbation of HMGA2 (Supplementary Fig. S3-A) suggesting that the reduced tumor formation *in vivo* upon knockdown of HMGA2 may be due to decreased tumor initiation and/or increased apoptosis. Consistent with prior studies [25, 56, 61, 62], we found increased apoptosis upon knockdown of HMGA2 in HSR-GBM1 cells (Supplementary Fig. S3-B). Our *in vitro* studies indicate HMGA2 promotes both increased invasion and clonogenicity, supporting the *in vivo* findings that modulation of HMGA2 levels affect tumorigenicity. Even though we could knockdown HMGA2 by 53-57% in our GBM cell lines, we observed prominent phenotypic effects. The inability of GBM cells to tolerate more than 57% reduction in HMGA2 supports the importance and greater dependence of GBM cells on HMGA2 for their survival.

We and others have demonstrated the importance of LIN28A or LIN28B in multiple brain tumor types [17, 51, 63-65]. We found that HMGA2 was altered coordinately after perturbation of LIN28A levels in GBM [17]. However, we identify LIN28A or LIN28B expression in approximately 40 percent of GBM [17]. In contrast, we find increased HMGA2 by immunohistochemistry and western blot in 70 percent of GBM, suggesting that multiple factors may regulate HMGA2. In other tumor types, HMGA2 levels are controlled by Transforming Growth Factor- β (TGF- β) [34], Raf-1 kinase inhibitory protein [66-68], RUNX1 [37], and several microRNAs [35, 39, 69-73].

HMGA2 is a stem cell factor primarily expressed during fetal development with almost no expression in adult human tissues [7, 10]. HMGA2 is aberrantly expressed at high levels in cancer [7] including GBM, which we show in this manuscript corroborating earlier reports [55, 56]. We demonstrate that CD133-expressing GBM cells (which are thought to represent GBM stem-like cells) show 9-fold higher expression of *HMGA2* compared to CD133- cells. Since GBM stem-like cells are believed to be the root cause of self-renewal, invasion, tumor recurrence and therapy resistance, identifying targets in these stem-like cells is needed to improve outcomes. Our experiments show that depletion of HMGA2 in GBM neurospheres (which contain GBM stem-like cells) reduced invasion, increased apoptosis, decreased clonogenicity, and suppressed *in vivo* tumor-forming ability of GBM cells, suggesting that GBM-stem like cells need HMGA2 to maintain malignant properties. Therefore targeting HMGA2 in GBM stem-like cells may be a therapeutically beneficial strategy.

In summary, our studies demonstrate that HMGA2 plays an oncogenic role in GBM and can promote GBM cell clonogenicity, invasion, and tumorigenicity. This work validates HMGA2 as a potential therapeutic target in GBM. Developing anti-HMGA2 therapeutics may lead to improved outcomes for patients suffering from this deadly brain tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HMGA2	High Mobility Group AT-hook 2
GBM	Glioblastoma
CD133	Cluster-of-Differentiation-133

Highlights

- HMGA2 is validated as a potential therapeutic target in GBM.
- High levels of HMGA2 are present in GBM tumors, cell lines and stem-like cells.
- HMGA2 promotes GBM stemness, invasion and *in vivo* tumor formation.

Figure 1.

The majority of GBM primary tumors and cell lines show increased expression of HMGA2. (A) Western blot showing increased expression of HMGA2 protein in 71% (5/7) primary tumor samples from GBM patients compared to normal (N) brain tissue (cortex). GAPDH was used as a loading control. HMGA2 and GAPDH protein levels were quantitated using densitometry. Values normalized to sample#1 of normal brain tissue (N) are depicted below the blots. (B) Immunohistochemical analysis showing varying expression of HMGA2 in primary GBM samples on a tissue microarray (Scores: 0 or negative =no staining, 1=low staining, 2=moderate staining, 3=high staining). Quantitation of the percentage of tumor samples showing the staining is depicted at right. (C) Western blot showing HMGA2 expression in GBM cell lines (GBM14, GBM10, JHH520, HSR-GBM1, LN-229 and U-87 MG). A primary tumor sample is also shown for comparison of HMGA2 expression. β-actin was used as a loading control. HMGA2 and β-actin protein levels were quantitated on the western blot using densitometry and values normalized to the primary tumor sample for each cell line are depicted below the blots. (D) Increased expression of HMGA2 mRNA by

quantitative PCR in CD133+ HSR-GBM1 neurospheres compared to CD133- cells. Data were normalized to 18S ribosomal RNA and are shown as relative expression (relative to CD133- cells) (*P=0.0001 by Student's *t*-test, N=2).

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Figure 2. shRNA mediated knockdown of HMGA2 reduces GBM cell invasion and colony formation *in vitro*. (A) Western blots showing reduction of HMGA2 expression with two different shRNA (shHMGA2#1 and shHMGA2#2) compared to control vector (pLKO.1 and SCRAMBLE) infected cells in HSR-GBM1 and LN-229 cell lines. GAPDH was used as a loading control. HMGA2 and GAPDH protein levels were quantitated on the western blot using densitometry and values normalized to pLKO.1 for each cell line are depicted below the blots. (B) *Left*, HSR-GBM1 cells showing 79% reduction in colony formation with shHMGA2#2 in soft agar compared to control infected cells (*P<0.0001 by Student's *t*-test, N=2). *Right*, Quantitation of the number of colonies is shown on the right. (C) *Left*, Photomicrographs (20X) showing reduced invasion of HSR-GBM1 and LN-229 cells infected with shHMGA2 compared to pLKO.1 infected cells in Matrigel transwell assay. *Right*, Quantitation of photomicrographs shown in (C). For HSR-GBM1: 57% and 50% reduction with shHMGA2#1 and #2, respectively, *P<0.001 for shHMGA2#1 and *P=0.001

for shHMGA2#2 by Student's *t*-test, N=2. For LN-229: 73% and 44% reduction with shHMGA2#1 and shHMGA2#2, respectively, compared to pLKO.1 infected cells, *P<0.001 by Student's *t*-test, N=2.

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Figure 3.

shRNA mediated knockdown of HMGA2 in GBM neurosphere cells inhibits intracranial tumor growth in immunocompromised mice. **(A)** Photographs of xenografted mice showing decreased bioluminescence intensity depicting reduced intracranial tumor growth with knockdown of HMGA2 (shHMGA2#2) in HSR-GBM1 cells at 5 weeks post injection compared to control mice (pLKO.1). **(B)** Quantitation of the intensity of bioluminescence showing 77% reduction with shHMGA2#2 xenografted mice at 5 weeks, respectively, compared to pLKO.1 mice, * $P=0.035$ by Student's *t*-test, $N=5$. **(C)** Kaplan-Meier survival analysis depicting decreased survival of mice injected with HSR-GBM1 neurospheres expressing shHMGA2#2 compared to control infected cells ($P<0.0001$, $N=10$ for pLKO.1 and $N=7$ for shHMGA2#2).

Figure 4. Overexpression of HMGA2 increases GBM cell invasion and clonogenicity *in vitro*. **(A)** Western blot showing increased expression of HMGA2 in HSR-GBM1 neurospheres infected with retroviral HMGA2 (HMGA2) compared to control vector (Vector). β-actin was used as a loading control. Densitometry values normalized to control vector are depicted below the blots. **(B)** Photos and quantification depicting increase in the number of larger colonies (diameter>300μm) formed by HSR-GBM1 neurospheres expressing retroviral HMGA2 compared to control vector cells in methylcellulose assays *in vitro* (*P=0.001 and 0.003 by Student's *t*-test, N=3). Scale bar = 2500 micrometers on high power macrophotographs. **(C)** Photomicrographs (20X) depicting increased invasion of HSR-GBM1 neurospheres expressing HMGA2 compared to control vector infected cells in

Matrigel-transwell invasion assays *in vitro*. Quantification depicting increase in invasion is shown on the right (*P<0.001 by Student's *t*-test, N=3).

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Figure 5.

Overexpression of HMGA2 increases intracranial tumor growth *in vivo* (A) Increased bioluminescence intensity depicting increased intracranial tumor growth at 4 weeks post injection with overexpression of HMGA2 compared to control vector mice. (B) Quantitation of the increased bioluminescence intensity observed in (A) depicting 7.0 fold increase in HMGA2 overexpressing xenografted mice compared to controls (*P=0.0209 by Student's *t*-test, N=5). (C) Kaplan Meier survival curves show a decrease in median survival with overexpression of HMGA2 in GBM1 neurospheres (P=0.08, N=5).