Splicing factor hnRNP A2/B1 regulates tumor suppressor gene splicing and is an oncogenic driver in glioblastoma.

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Running title: hnRNP A2/B1 is a driving oncogene in glioblastoma

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ABSTRACT

The process of alternative splicing is widely misregulated in cancer, but the contribution of splicing regulators to cancer development is largely unknown. In this study, we found that the splicing factor hnRNP A2/B1 is overexpressed in glioblastomas and is correlated with poor prognosis. Conversely, patients who harbor deletions of the HNRPA2B1 gene show better prognosis than average. Knockdown of hnRNP A2/B1 in glioblastoma cells inhibited tumor formation in mice. In contrast, overexpression of hnRNP A2/B1 in immortal cells led to malignant transformation, suggesting that hnRNP A2/B1 is a putative proto-oncogene. We then identified several tumor suppressors and oncogenes which are regulated by hnRNP A2/B1, among them c-FLIP, BIN1, Wwox and the proto-oncogene RON. Knockdown of RON inhibited hnRNP A2-mediated transformation, which implied that RON is one of the mediators of hnRNP A2/B1 oncogenic activity. Together, our results indicate that hnRNP A2/B1 is a novel oncogene in glioblastoma and a potential new target for glioblastoma therapy.

INTRODUCTION

The process of alternative splicing is widely misregulated in cancer and many tumors express new splicing isoforms which are absent in the corresponding normal tissue (1-3). Many oncogenes and tumor suppressors are differentially spliced in cancer cells and it has been shown that many of these cancer-specific isoforms contribute to the transformed phenotype of cancer cells (4-6). However, the contribution of alternative splicing regulators to cancer development has been largely unknown. Only recently the first functional evidence showed that some splicing factors can act as potent oncogenes and are up-regulated in human cancers (7, 8). hnRNP proteins are abundant RNA-binding proteins expressed in most human tissues (9, 10). The hnRNP A/B family is a subset of hnRNP proteins with closely related sequences and a conserved modular structure (10). They can affect alternative splicing, frequently by antagonizing SR proteins, in part through the recognition of exonic splicing silencers (ESS) elements (11-13). Additional functions of these proteins in post-splicing events, such as mRNA trafficking, and replication and transcription of cytoplasmic RNA viruses, have also been reported (10). Recent
studies have shown that hnRNP A1 also affects the maturation of specific miRNAs among them pre-miR-18a which is part of a cluster of miRNAs with oncogenic activity (oncomirs) (14-16). Previous studies showed overexpression of hnRNP A1 and hnRNP A2/B1 in lung and breast cancer (17, 18). Moreover, knockdown of hnRNP A1 and A2/B1 in breast cancer cells induced apoptosis which was specific for cancer cells (19). However a direct role for hnRNP A2/B1 as an oncogene in human cancer has not yet been shown. Recent studies found that hnRNP A1 and A2/B1 modulate alternative splicing of the glycolytic PKM2 enzyme in cancer cells suggesting a possible role for hnRNP A1 and A2/B1 in the regulation of tumor metabolism (20, 21).

Another unsolved question is the biochemical and biological difference between members of the hnRNP A/B protein family. To date their splicing activities, both in vitro and in knockdown or transfection assays, showed similar splicing effects on several substrates (11, 20-22). Thus, it is not clear to what extent there is redundancy in their splicing targets and biological activities. We found that hnRNP A2/B1 but not any other hnRNP A/B or SR protein is a prognostic marker for glioblastoma patient survival and a potent oncogene in glioblastoma development and tumor maintenance (Figs. 1-3 and Figs. S1-S3). In a search for its alternative splicing targets we found that hnRNP A2/B1 modulates alternative splicing of the tumor suppressors BIN1, Wwox, the anti-apoptotic proteins c-FLIP and Caspase-9B, the Insulin receptor and the RON proto-oncogene among others (Fig. 4, Table-S1). In all of these splicing events hnRNP A2/B1 enhanced the expression of the oncogenic isoforms of these genes (Fig. 4). We further show that RON knockdown inhibits transformation of glioma cells overexpressing exogenous hnRNP A2/B2 suggesting that RON is an important target in hnRNP A2/B1-mediated transformation (Fig. 5).

Materials and Methods

Cells.
U87MG and T98G cells were grown in DMEM, supplemented with 10% FCS, penicillin and streptomycin. NIH 3T3 cells were grown in DMEM supplemented with 10% calf serum (CS), penicillin, and streptomycin. To generate stable transductant pools, NIH 3T3 and U87MG cells were infected with pBABE-puro retroviral vectors expressing T7-tagged human hnRNP A2 cDNA. At 24 h after infection, the medium was replaced, and 24 h later, infected cells were
selected with puromycin (2 μg/ml) for 72-96 h. In the case of infection with MLP-puro-shRNAs vectors, U87MG, T98G cells transductants were selected with puromycin (2 μg/ml) for 96 h.

**Anchorage-Independent Growth.**

Colony formation in soft agar was assayed as described (23). Plates were incubated at 37°C and 5% CO₂. After 14–21 days, colonies from 10 different fields in each of two wells were counted for each treatment, and the average number of colonies per well was calculated. The colonies were stained as described (23) and photographed under a light microscope at magnification ×100.

**Tumorigenesis Assays in Nude Mice.**

U87MG cells expressing MLP-puro or MLP-puro containing hnRNP A2 shRNAs or NIH 3T3 cells overexpressing hnRNP A2 or an empty vector (pBABE) were injected (2 × 10⁶ cells per site in 200 μl of PBS) s.c. into each rear flank of (Atimic-Nu/Nu) nude mice by using a 26-gauge needle. Tumor growth was monitored twice a week as described (7).

**Immunoblotting.**

Cells were lysed in SDS and analyzed for total protein concentration as described (7). Thirty or 20 μg of total protein from each cell lysate was separated by SDS/PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked and probed with antibodies by using enhanced chemiluminescence detection. Primary antibodies: anti β-catenin (1:2,000; Sigma); anti β-actin (1:2000 Santa Cruz); anti RON (1:1000 Santa Cruz); anti hnRNAP A2/B1 (1:1000; Sigma); Pan-hnRNP A/B (1:400 Mab clone 62); T7 tag (1:5,000; Novagen). Secondary antibodies: HRP-conjugated goat anti-mouse (1:10,000; Jackson Laboratories).

**Growth curves.**

U87MG or NIH 3T3 cells were infected with the indicated retroviruses. After selection, 5,000 cells per well were seeded in 96-well plates. Cells were fixed and stained with methylene blue as described (7), and the A650 of the acid-extracted stain was measured on a plate reader (BioRad).
RT-PCR.
Total RNA was extracted with Trizol reagent (Sigma) and 2 µg of total RNA was reverse transcribed with AffinityScript II (Stratagene) RT. PCR was performed on 1/10 (1-2 µl) of the cDNA, in 25-50-µl reactions containing 0.2 mM dNTP mix, 10× PCR buffer with 15 mM MgCl₂ (Invitrogen), 2.5 units of TaqGold (Invitrogen) and 0.2 µM of each primer; 5% (v/v) DMSO was included in some reactions. PCR conditions were 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s, followed by 10 min at 72 °C. PCR products were separated on 1.5% or 2% agarose gels or on 6% nondenaturing polyacrylamide gels. Primers are described in Supplementary Table-2.

RESULTS
The splicing factor hnRNP A2/B1 is up-regulated in brain cancer.
We analyzed the expression of hnRNP A2/B1 in several types of brain cancers using the Oncomine database (http://www.oncomine.org) and found hnRNP A2/B1 overexpression in tumor samples from glioblastoma, oligodendroglioma and astrocytoma patients compared to normal brain tissues (Fig. 1A). These results support a recent finding that hnRNP A2/B1, hnRNP A1 and PTB are overexpressed in glioblastoma (20) We further analyzed 3 normal brain (blue bars) and 22 glioblastoma grade IV tumor samples by Q-RT-PCR and found that in 19 out of the 22 tumors (red bars) hnRNP A2/B1 was overexpressed over two fold than the average expression in the normal brain samples (Fig. 1B).

The expression and gene copy number of the splicing factor hnRNP A2/B1 are inversely correlated with glioma patient survival.
Using the REMBRANDT database of the National Cancer Institute (https://caintegrator.nci.nih.gov) we analyzed the correlation of hnRNP A2/B1 expression and gene copy number and patient survival. We found very significant inverse correlation P=2.62E-5) between patient survival and elevated expression or gene copy number of hnRNP A2/B1 (Fig. 1C-D).These results indicate that patients with tumors harboring elevated copy number of hnRNPA2/B1 have poor prognosis of survival. Analysis of the same samples for overexpression of known oncogenes (MYC and EGFR) or down-regulation of know tumor suppressors (TP53...
and TP73) did not show significant correlation with patients survival (Figs S1,S2). Gene copy number of the tumor suppressor PTEN however, showed significant correlation with patient survival and AKT2 expression and copy numbers were also inversely-correlated with patient survival (Fig. S3) corroborating previous findings (24). It is important to mention that hnRNP A2/B1 prognostic value was statistically better than AKT2 (Fig. 1C-D) and thus it might be a better bio-marker for glioma prognosis. Interestingly, none of the other splicing factors from the hnRNP A/B protein family (hnRNP A1, hnRNP A0, hnRNP A3) or SR protein family showed significant inverse correlation with patient survival (Fig. S4).

**hnRNP A2/B1 is required for glioblastoma transformation and tumorigenicity.**

To examine the importance of hnRNP A2/B1 to glioma tumor maintenance, hnRNP A2/B1 was knocked-down in U87MG and T98G or overexpressed in the U87MG glioblastoma cell lines, and anchorage-independent growth was analyzed by colony formation in soft agar. Colony formation of U87MG and T98G cells with hnRNP A2/B1 knockdown was reduced in contrast to increased colony number and size in glioblastoma cells overexpressing hnRNP A2/B1 (Figs. 2A-C, E-G; Fig. S5). One of the features that can contribute to colony formation is the proliferation rate of the cells. Notably, for tumor development and progression other aspects such as motility, invasiveness, the ability to grow in an anchorage-independent manner and to resist apoptotic cues can be more prominent. To examine if hnRNP A2/B1 plays a role in proliferation, we measured proliferation rates of U87MG glioblastoma cells with up and down -regulation of hnRNP A2/B1. hnRNP A2/B1 knockdown did not reduce the viability of the glioblastoma cells and slightly inhibited their ability to proliferate on plastic in 10% serum but inhibited their proliferation in lower serum concentrations (Fig. 2D, Fig. S6) suggesting that hnRNP A2/B1 contributes to cell proliferation in low growth factor conditions. Importantly, in hnRNP A2/B1 knockdown and overexpression cell pools hnRNP A2/B1 silencing and overexpression was sustained over the growth curve period eliminating the possibility that cell density or an aberrant clone could overcome hnRNP A2/B1 silencing (Fig. S6G-H) However, overexpression of hnRNP A2/B1 increased the proliferation rate of U87MG and NIH 3T3 cells in all growth factor concentrations (Figs. 2H, 3D, S6A-C) indicating that it can contribute to enhance proliferation at both low and high growth factor conditions when overexpressed. In order to determine the
importance of hnRNP A2/B1 for glioma tumor formation in vivo, nude mice were injected with U87MG cells with and without knockdown of hnRNP A2/B1. Cells with empty vector gave rise to fast growing large tumors while mice injected with U87MG cells where hnRNP A2/B1 was knocked-down developed either small tumors in some of the injection sites or no tumors at all (Fig. 2I, J). The importance of hnRNP A2/B1 to brain cancer maintenance was also confirmed in the T98G glioma cell line (Fig. S5). T98G cells with hnRNP A2/B1 knockdown showed reduced colony formation in soft agar, similar to results in U87MG cells (Figure S5).

**hnRNP A2/B1 overexpression transformed NIH 3T3 and converted them to be tumorigenic.**

Based on our results that hnRNP A2/B1 is required for the glioblastoma transformed phenotype, we examined the ability of this factor to transform normal immortal cells. Up regulation of hnRNP A2/B1 in NIH 3T3 cells induced colony formation in soft agar in contrast to control cells that did not form colonies (Fig. 3 A-C). Moreover, these cells formed fast-growing tumors when injected subcutaneously into nude mice (Fig. 3E, F). Pathological analysis of the tumors formed by cells overexpressing hnRNPA2/B1 showed that these tumors look like high-grade invasive sarcoma with high mitotic index (Fig. 3G). These results suggest that hnRNP A2/B1 is a driving oncogene on its own and probably directly contributes to glioblastoma development.

**hnRNP A2/B1 levels affects the alternative splicing of several tumor suppressors and oncogenes.**

To examine the effect of hnRNP A2/B1 on alternative splicing in glioma tumor cells, we analyzed the alternative splicing pattern of several genes for which alternatively spliced isoforms have been characterized and for some of them shown to contribute to transformation invasion and apoptosis or to be affected by hnRNP A2/B1 (Fig. 4, Fig. S7, Table-S1). Knock-down of hnRNP A2/B1 in U87MG cells increased the inclusion while up-regulation of hnRNP A2/B1 increased the skipping of exon 11 of RON, a tyrosine kinase receptor involved in the invasiveness and motility of tumor cells (25, 26)(Fig. 4A). These results suggest that similar to the splicing factor oncprotein SRSF1 (SF2/ASF), hnRNP A2/B1 up-regulation contributes to cellular transformation by increased skipping and up-regulation of the ΔRON oncogenic splicing
isoform (25). Importantly, the levels of SRSF1 (SF2/ASF) or SRSF6 (SRp55), another SR protein and the levels of hnRNP A1, another hnRNP A/B family member did not change upon hnRNP A2/B1 down- or up-regulation (Fig. S8). In NIH 3T3 cells overexpressing hnRNP A2 we could only detect one isoform of RON suggesting that either this splicing event occurs only in humans or that it is hardly occurs in fibroblasts. The insulin receptor (IR) is the main mediator of insulin metabolism and glucose levels in the body and is expressed in most tissues. Skipping of exon 11 from the INSR transcript generates the splicing variant IR-A which binds the growth factor IGF-II in addition to insulin, is overproduced in many cancers and has been implicated in an autocrine loop in cancer cells (27, 28) hnRNP A2/B1 knockdown increased the inclusion of exon 11 while its overexpression increased exon 11 skipping generating the mitogenic isoform (Fig. 4B). The ENAH gene has been shown to play a role the in epithelial to mesenchymal transition (EMT) process and to affect cellular motility and invasion. Overexpression did not affect the inclusion of exon 11a suggesting that it is not a splicing target of hnRNP A2/B1 in U87MG cells (Fig. 4C). CFLAR (c-FLIP) is an anti-apoptotic protein that is alternatively spliced, and have been shown to inhibit TNF and TRAIL-induced apoptosis (29, 30) but also to enhance motility and invasion through activation of the MAPK-ERK pathway (31, 32). We found that hnRNP A2/B1 down-regulation decreased the levels of the long isoform of c-FLIP while overexpression of hnRNP A2/B1 increased the level of the long isoform raising the possibility that upon hnRNP A2/B1 knockdown glioma cells might become more sensitive to apoptotic stimuli and in the same time be less motile and less invasive (31) (Fig. 4D). The tumor suppressor BIN1 has been shown to be regulated by alternative splicing and inclusion of exon 12a of BIN1 inactivates its tumor suppressor activity (33, 34). BIN1 exon 12a was also identified as a target of the SR protein SRSF1 (SF2/ASF) (7). We found that similar to the effect of SRSF1 overexpression, hnRNP A2/B1 overexpression in both U87MG and in NIH 3T3 enhanced exon 12a inclusion generating the anti-apoptotic isoform of BIN1 while its knockdown enhanced exon 12a skipping (Fig. 4E, S7). CASP9, the gene coding for Caspase-9 has been previously shown to be alternatively spliced by skipping of exons 3-6 and to generate a truncated dominant negative isoform that inhibits apoptosis and it overexpressed in several cancers (35, 36). We found that hnRNP A2/B1 knockdown enhanced the production of full-length caspase-9 while its overexpression enhanced skipping of exons 3-6 generating the anti-apoptotic isoform Caspase
9B (Fig. 4F). *WWOX* is a known tumor suppressor which resides in common fragile site (37) and is frequently inactivated in several types of cancer including glioblastoma (38). Skipping of exons 6-8 of *WWOX* have been reported in breast cancer (39). We found that hnRNP A2/B1 knockdown enhanced inclusion of alternatively spliced exons 6-8 while its overexpression induced skipping of these exons (Fig. 4G). Skipping of these exons causes deletion of 180 amino acids including its substrate binding domain and its alcohol dehydrogenase (ADH) domain probably inactivating its catalytic activity (39). However, the functional role of these skipped isoforms requires further examination. Wwox tumor suppressive activity is related to its anti-invasive and anti-apoptotic functions (37, 39) which are a common theme shared with the other targets of hnRNP A2/B1 we identified. hnRNP A2/B1 also affected the alternative splicing of exon 7B of hnRNP A1, another member of the hnRNP splicing factor family (Figs. S7, S8). Skipping of this exon has been shown to be affected by hnRNP A1 and hnRNP A2/B1 levels in an autoregulatory fashion and thus our results corroborated these previous findings (40). The levels of hnRNP A2/B1 also affected the alternative splicing of other genes, including known hnRNP A2/B1 targets such as exon 7 of the *SMN* gene and other splicing events (Fig. S7, Table-S1). Importantly, many splicing events we examined were not affected by hnRNP A2/B1 depletion or overexpression (Table-S1).

**knockdown of RON reverses transformation of glioblastoma cells overexpressing hnRNP A2/B1.**

In order to examine if RON contributes to hnRNP A2/B1-mediated transformation we knocked down the expression of the RON proto-oncogene in U87MG cells overexpressing ectopic hnRNP A2. (Fig. 5A). We found that stable knockdown by two different shRNA reduced RON levels and inhibited colony formation in soft agar of U87MG cells (Fig. 5B-C). These results indicated that RON is one of the important mediators of hnRNP A2/B1 oncogenic activity in glioblastoma cells even tough other targets can probably contribute as well.

**DISCUSSION**

An emerging body of data shows that alternative splicing misregulation plays an important role in cancer development and tumor maintenance (5-8). Alternative splicing regulators from the SR
and hnRNP A/B protein families are overexpressed or down-regulated in various cancers and can probably account for many of the cancer-specific alternative splicing changes. Some alternative splicing regulators such as the SR protein SRSF1 (SF2/ASF) have been shown to be up-regulated in many cancers and act as potent oncogenes when slightly overexpressed (7).

hnRNP A/B proteins are important regulators of alternative splicing and in several examples of in vitro and in vivo splicing assays seemed to act antagonistically to SR proteins (11-13). Even though hnRNP A/B proteins can alter alternative splicing in an opposite manner to oncogenic splicing factors like SRSF1 (SF2/ASF), there are no evidence that they act as tumor suppressors, and on the contrary, members of the hnRNP A/B protein family are overexpressed in some cancers (7, 17-20) and change the splicing pattern of genes that contribute to the transformed phenotype (20, 21, 42). Moreover, recently it has been shown that the splicing factors hnRNP A1 and A2/B1 as well as PTB are under the transcriptional control of the myc proto-oncogene and can modulate the splicing of PKM2, activating a metabolic switch to aerobic glycolysis which is a hallmark of cancer cells (the "Warburg effect") (20, 21). However, to date there is no direct evidence that any member of the hnRNP A/B families is an oncogene that can transform cells and is genetically up-regulated (amplified, translocated or mutated) in cancer. We show here that the splicing factor hnRNP A2/B1 is overexpressed in gliomas compare to normal brain samples in many cases due to amplification of the HNRPA2B1 gene (Fig. 1A-D). Moreover, overexpression and amplification of hnRNP A2/B1 correlates with poor prognosis of glioma patients while deletion of the HNRPA2B1 gene correlates with better prognosis then average (Fig. 1C-D). Importantly, none of the other hnRNP A/B proteins, as well as the SR proteins or some other known oncogenes, showed significant inverse correlation with survival of glioma patients (Figs. S1-S2). In accordance with previous findings, deletion of the tumor suppressor PTEN and overexpression of the proto-oncogene Akt2 showed significant inverse-correlation with glioma patient survival (24) (Fig. S3). Taken together these findings suggest that hnRNP A2/B1 is a valuable prognostic marker for glioblastoma development and patient survival. In order to examine if hnRNP A2/B1 is causatively involved in glioblastoma tumor development, we down-regulated the expression of hnRNP A2/B1. We found that knockdown of hnRNP A2/B1 in U87MG glioblastoma cells partially inhibited their proliferation, especially in low-serum condition (Figs. 2D, S6). This result suggests that the cell cycle and viability may not
be the major anti-oncogenic effectors in cells with hnRNP A2/B1 knockdown. However, knockdown of hnRNP A2/B1 inhibited colony formation in soft agar as well as tumor formation in nude mice of glioblastoma cells (Fig. 2A-C, I-J, S5) suggesting that hnRNP A2/B1 is important for glioblastoma tumor development and maintenance. Moreover, overexpression of hnRNP A2/B1 in the U87MG glioblastoma cells enhanced their proliferation in all serum concentrations, and increased colony size and number in soft agar (Figs. 2E-H, S6) indicating that hnRNP A2/B1 may not be a limiting factor when growth factors are present but plays a positive role in glioblastoma transformation when up-regulated. To examine if hnRNP A2/B1 acts as an oncogene, we slightly overexpressed (two fold) hnRNP A2/B1 in immortal mouse fibroblasts (NIH 3T3 cells) and examined their oncogenic properties. NIH 3T3 overexpressing hnRNP A2/B1 became transformed, formed colonies in soft agar and were tumorigenic in nude mice forming tumors with hallmarks of high grade sarcomas (Fig. 3). These data suggest that hnRNP A2/B1 acts as a proto-oncogene when slightly up-regulated and thus it is not only a marker but also a driving oncogene in glioblastoma development. In order to identify possible alternative splicing targets of hnRNP A2/B1 that might mediate its oncogenic activity we examined the alternative splicing pattern of several genes known to undergo alternative splicing in cancer and to contribute to the transformed phenotype. We identified several alternative splicing events modulated by hnRNP A2/B1, among them the tumor suppressor BIN1, the anti-apoptotic gene CFLAR (c-Flip) and CASP9 all regulators of the apoptotic process (29-36). The tumor suppressor gene WWOX, the INSR gene, the long isoform of CFLAR and the RON tyrosine kinase receptor all involved in motility and invasion (25-28, 31-32, 37-39). Our results suggest that hnRNP A2/B1 activates an alternative splicing program that enhances the production of anti-apoptotic isoforms of genes such as BIN1, CASP9 and CFLAR as well as invasion-promoting isoforms such as ΔRON, CFLAR-long and IR-A which contribute to transformation and invasion.

Surprisingly, in several cases of alternative spliced exons showed in this paper (BIN1 exon 12a, RON exon 11), hnRNP A2/B1 showed similar splicing effects to the oncogenic SR protein SRSF1 (SF2/ASF) raising the possibility that in many cases in vivo, these splicing factor are not antagonistic as previously thought from in vitro and transient-transfection splicing assays (11-13). hnRNP A2/B1 is a general splicing factor and it expected to change the splicing of
many (currently unknown) transcripts. We know that the alternative splicing of several transcripts did not change upon up- or down-regulation of hnRNP A2/B1 (Fig. 4, Table S1). We are limited to the small number of transcript we tested and only a genome-wide analysis will reveal how many transcripts are regulated directly and indirectly by hnRNP A2/B1.

In order to examine if RON is an important target that mediated hnRNP A2-induced transformation, we stably knocked down RON in U87MG glioblastoma cells overexpressing exogenous hnRNP A2 cDNA and found that RON knockdown significantly inhibited transformation of these cells similarly to the effect of hnRNP A2/B1 knockdown (Fig. 5). We conclude that RON is one of the important mediators of hnRNP A2-induced transformation and contributes to the transformed phenotype of glioblastoma cells.

Because hnRNP A2/B1 is a general alternative splicing factor and participates in RNA processing steps other then splicing (10, 12), we assume that it has many splicing targets other then RON and that it targets other RNA processing steps (such as miRNA maturation) (14, 15) which contribute to its transforming activity. For example, hnRNP A1 has been shown to promote the maturation of onco-miRs such as miR 18a and to down-regulate tumor suppressive miRs such as Let-7a (14, 42). hnRNP A2/B1 has been also linked to tumor metabolism (20, 21, 43). It has been shown that hnRNP A2/B1 is down-regulated post-transcriptionaly by the tumor suppressor VHL which is lost in many tumors and induces aerobic glycolysis (the "Warburg effect") through HIF-1α stabilization (43). It will be intriguing to examine if hnRNP A2/B1 can affect HIF-1α stabilization by competitive binding to VHL when overexpressed. In recent studies hnRNP A2/B1 has been shown to be transcriptionaly controlled by the myc proto-oncogene and to regulate the splicing of the glycolitic enzyme PKM2 contributing to the induction of aerobic glycolysis by another mechanism (20, 21).

Taken together our data suggest that hnRNP A2/B1 is a new bio-marker for glioblastoma patient survival and a new proto-oncogene that regulate the splicing and other RNA processing steps of several tumor suppressors and oncogenes. Furthermore, down-regulating hnRNP A2/B1 levels in glioblastoma cells should be considered as a new strategy for glioblastoma therapy.
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**FIGURE LEGENDS.**

**Figure 1.** hnRNP A2/B1 is up-regulated in brain tumors and the expression and gene copy number of *HNRPA2/B1* are inversely correlated with glioma patient survival.

(A). hnRNP A2/B1 expression in samples of glioblastoma, oligodendrogioma and astrocytoma patient (red) compared to normal brain samples (blue). Data taken from Oncomine (http://www.oncomine.org).

(B). Q-RT-PCR quantitation of hnRNP A2 expression was performed on total RNA extracted from normal brain (Blue) and glioblastoma WHO grade IV tumors (Gray and Red) columns. Red columns represent tumor samples in which hnRNP A2 expression is more than two-fold over the average expression of the normal brain samples (normalized as 1 and represented as black line).

(D). Kaplan-Meier plots show the correlation between HNRPA2B1 gene copy number and glioma patient survival. Samples with gene amplification (red line) n=172. Samples with gene deletion (green line) n=179. Blue line represents the survival of all glioma patients. Log-rank p-value (Up-regulated vs Intermediate): 1.54771E-5. Data based on the National Cancer Institute REMBRANDT data set (http://rembrandt.mci.nih.gov).

Figure 2. hnRNP A2/B1 is required for of glioblastoma transformation.

(A). U87MG cells were transduced with retroviruses encoding hnRNP A2/B1-specific shRNA or empty vector (MLP(-)) and after selection cells were analyzed by Western blotting for hnRNP A2/B1 protein expression. β-catenin levels were used as loading control.

(B). Quantification of colony formation in soft agar of cells described in (A). Error bars represents standard deviations (n=2).

(C). Representative fields of colonies in soft agar described in (B).

(D). Cell proliferation of cells described in (A) was determinate by methylene blue staining. Error bars represents standard deviations (n=6).

(E). U87MG cells transduced with empty vector (pBABE) or hnRNP A2/B1 (A2) were analyzed by Western blotting for hnRNP A2/B1 protein expression and β-catenin was used as loading control.

(F). Quantification of colony formation in soft agar of the cells described in (E). Colonies were considered "Big colonies" if the contained approximately over 100 cells or were over 1mm in diameter. Error bars indicate standard deviations (n=2).

(G). Representative fields of soft agar colonies described in (F).

(H). Cell proliferation of cells described in (E) was determinate by methylene blue staining. Error bars represents standard deviations (n=6).
(I). Cells described in (A) were injected (10^6 cells/site) subcutaneously near both rare flanks of nude/nude mice, and tumor volume was measured bi-weekly. Error bars indicate standard deviations (n=8).

(J). Representative mice described in (I) are shown.

Figure 3. hnRNP A2/B1 can transform NIH 3T3 cells in vitro and in vivo.

(A). NIH 3T3 cells transduced with empty vector (pBABE) or hnRNP A2/B1 (A2) were analyzed by Western blotting for hnRNP A2/B1 protein expression and β-catenin was used as loading control.

(B) Quantification of colony formation in soft agar of cells described in (A). Error bars represents standard deviations (n=2).

(C). Representative fields of colonies in soft agar described in (B).

(D). Proliferation of cells described in (A) was determinate by methylene blue staining. Error bars indicate the standard deviation (n=6).

(E). Cells described in (A) were injected (10^6 cells/site) subcutaneously near both rare flanks of nude/nude mice, and tumor volume was measured bi-weekly. Error bars indicate standard deviations (n=8).

(F). Representative mice described in (E) are shown.

(G). Light micrographs of formalin-fixed, paraffin-embedded tissue sections from tumors derived from NIH 3T3 cells overexpressing hnRNP A2/B1, stained with hematoxylin and eosin.

Figure 4. hnRNP A2/B1 regulates the alternative splicing of tumor suppressors and oncogenes.

U87MG glioblastoma cancer cells were transduced with retroviruses encoding shRNA empty vector (MLP), or hnRNP A2/B1 specific shRNA, the empty pBABE vector or pBABE containing hnRNP A2/B. After selection cells were lysed, RNA isolated and the alternative splicing pattern of RON, INSR, ENAH, CFLAR, BIN, CASP9 and WWOX was examined by RT-PCR using the indicated isoform-specific primers (arrowheads). The splice variants are indicated...
by boxes at the right side of each gel. (A) RON exon 11 skipping/inclusion. (B) INSR exon 11 skipping/inclusion. (C) ENAH exon 11a skipping/inclusion. (D) CFLAR exon 7 skipping/inclusion. (E) BIN1 exons 12a and 13 skipping/inclusion. (F) CASP9 exons 3-6 skipping/inclusion. (G) WWOX exons 6-8 skipping/inclusion. (H) GAPDH control. Numbers under each panel represents the average and standard deviations of fold change in the inclusion of the indicated exon compared to the empty vectors (normalized as 1) in 2-4 independent experiments.

Figure 5. knockdown of RON reverses transformation of U87MG cells overexpressing hnRNP A2.

U87MG cells transduced with hnRNP A2 cDNA were co-transduced with the indicated lentiviruses containing shRNAs against RON or an empty vector. After selection cells were plated into soft agar and 14 days later colonies were counted. (A) Western blot showing RON levels in the different cell populations. (B) Quantitation of colonies formed in soft agar from cells described in (A). (C) Representative fields of cells described in (A) (magnification ×10).
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Splicing factor hnRNP A2/B1 regulates tumor suppressor gene splicing and is an oncogenic driver in glioblastoma

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