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microRNA-10 and -221 modulate differential expression of Hippo signaling pathway in human astroglial tumors

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ABSTRACT

Gliomas represent over 70% of all brain tumors, they are highly invasive and structurally vascular neoplasms. Despite the latest technological advance in neuro-surgery the survival of patients with high-grade glioma remains poor. The lack of robust treatment options has propelled the search for new markers that may ~~able~~ allow the identification of patients who can benefit from molecularly targeted therapies. The Hippo signaling pathway is considered as a key regulator of tissue homeostasis, cell proliferation and apoptosis, and alterations of this pathway seem to contribute to tumorigenesis. Yes-associated protein (YAP1) is a downstream target of the Hippo pathway which acts as a transcription co-activator. In cancer, YAP1 has been reported to function either as an oncogene or tumor suppressor, depending on the cell context. The aim of this study was to examine the expression of YAP1, Survivin and LATS1 kinase activity in human astroglial tumors with different grades of malignancy. Moreover, we also investigated the expression of miR-221 and miR-10b and their relationship with core molecules of the Hippo pathway. Our results showed the overexpression of YAP1 and Survivin as well as a decreased activity of large tumor suppressor 1 (LATS1) in high-grade glioblastoma versus anaplastic astrocytoma and low-grade glioma. Furthermore, we also demonstrated that miR-221 and miR-10b are specifically involved in Hippo signaling via LATS1 regulation and that their knockdown significantly decreased glioma cell proliferation. This preliminary data confirmed the crucial role of the Hippo pathway in cancer and suggested that miR-221 and miR-10b could be potential therapeutic targets for glioma treatment.

Introduction

Glioblastoma (GBM) is an astrocytic malignant tumor representing the most common primary adult brain tumor in Western nations. It is located usually in the cerebral hemispheres, but it could arise throughout the central nervous system and it can occur at any age [1,2]. Despite the available treatment options, including surgery, chemo- and radiotherapy, the prognosis of this cancer remains extremely poor, with a median survival rate of 12–15 months and an average 5-year survival rate of less than 5%. In the last decades, the growing knowledge of cellular and molecular mechanisms underlying glioma development and progression has led to the identification of new therapeutic strategies based on multimodality approaches, improved biomarker tools and use of targeted agents. The Hippo signaling pathway is an evolutionarily-conserved signaling pathway involved in organ size control and tissue regeneration [3], as well as in tumor initiation, progression, and metastasis [4]. YES-associated protein (YAP), a transcriptional co-activator of the Hippo pathway, has been found to be elevated in several human cancers [5]. In mammals, the Hippo pathway is regulated by a precise mechanism that involves the core chinases; the protein

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kinase 1 similar to mammals STE20 (MST1), MST2, the large tumor suppressor 1 (LATS1) and LATS2. When the YAP/TAZ is activated, MST1/2 phosphorylates and then activates LATS1/2 and Mps One Binder Kinase Activator MOB1A/1B by phosphorylation, considered to be one of the core Hippo pathway components. Then, LATS1/2 directly phosphorylates YAP, thus inducing its translocation into the cytoplasm and/or degradation [6–10]. When YAP is active (i.e. unphosphorylated), it translocates to the nucleus and interacts with TEAD family transcription factors to induce the expression of anti-apoptosis and proliferation genes such as Survivin [8,9]. Evidence showed that YAP1 is overexpressed in human glioma tissues and that its levels are positively associated with patient prognosis [11,12]. A crosstalk mechanism between Hippo/YAP and Wnt/β-catenin pathway with a functional role in glioma growth has recently been found [11]. MicroRNAs (miRNAs) are non-coding RNA molecules, resulting from the mRNA splicing process, and are structured by a single strand of about 22 nucleotides, which bind to their compliant filaments in the 3′UTR region of the target gene, blocking their gene expression. Many miRNAs have been found to associate with apoptosis and cancer, thereby functioning as tumor suppressors or oncogenes (oncomirs) [13–15]. MiRNAs have interested us because of the possibility of suppressing their activity through sequence-specific oligonucleotides. Emerging studies revealed that defects in miRNA regulatory network play a key role in glioblastoma pathogenesis [16]. Worth noting is that miR-21 and miR-221 are overexpressed in glioma and high levels of miR-10b are associated with poor survival in GBM patients [17, 18]. In light of this background, we aimed to examine YAP1 and Survivin expression, as well as LATS1 kinase activity in human astroglial tumors with different grades of malignancy. Moreover, to study the relationship between miR-221 and miR-10b with core molecules of Hippo pathway we examined the expression profile of miR-221 and miR10b in human astroglial tumors and in an in vitro model in human glioma cell lines.

Material and methods

Patient population and tissue samples

The present study was submitted to “The Institutional Review Board of the University Hospital of Messina, Italy” to discuss and approve the study (prot. E 09/14 February 12, 2014 (Prof. Alfredo Conti). All patients or their caregivers gave their consent at the time of diagnostic procedures, including research purposes, in accordance with the Helsinki Declaration. A number of 55 flash-frozen tumor specimens, taken from patients who underwent craniotomy and gross total resection (over 95% of the tumor volume) at the Neurosurgical Unit of the University of Messina between 2011 and 2015, were used. All tumors were located in the supratentorial compartment. The tumors were histologically verified, according to the revised World Health Organization (WHO) classification, as: $n = 15$ low grade astrocytoma (LGA); $n = 15$ anaplastic astrocytomas (AA); $n = 25$ glioblastoma multiforme (GBM). Five non-neoplastic brain tissues originating from the temporal lobes of patients undergoing brain surgery for cerebral hemorrhage, including the normal cortex, already confirmed histologically were used as controls (CTRL). Summary of demographic and clinical data are reported in Table 1. Tumor samples containing components that were suspicious of oligodendroglioma, were carefully excluded. No case of recurrent tumors and no patients who underwent radio and/or chemotherapy before surgery was employed in the present study.

Cell culture

Human cell lines normal human astrocytes (NHAs), U87-MG (grade IV glioblastoma) and A-172 MG (grade IV glioblastoma) obtained respectively from the Biosciences Lonza and the American Type Culture Collection (Rockville, Maryland) were used. Cells were kept in DMEM medium (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% of fetal bovine serum containing Penicillin and Streptomycin 100 units/mL of each one (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts). Cells cultures were incubated at 37 °C in 5% CO2 flowed by subculture every 2–3 days in vented 25 cm2 cell culture flasks.

Protein extraction and western blotting

Tissue and cell samples were lysed in a lysis buffer (20 mM 4- (2-hydroxyethyl) −1-piperazineethanesulphonic acid; 2 mM ethylene glycol tetraacetic acid (EGTA); 1 mM ethylenediaminetetraacetic acid (EDTA); 150 mm NaCl; 0.1% sodium dodecyl sulfate (SDS); 1% Nonidet P-40; deoxycholate acid 0.5%, pH 7.5; a mixture of protease inhibitors; 0.5 mm phenylmethylsulfonyl fluoride; 10 μg / ml of aprotinin, 10 μg / ml of lepteptin, and 10 μg / ml of pepstatin, Sigma Aldrich, Italy), followed by the centrifugation at 15,000 g for 15 min at 4 °C. The supernatant containing total cellular protein was collected and stored at −80 °C. The concentration of total proteins was determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). 30 μg of total protein were separated electrophoretically on 8% SDS polyacrylamide gel, then transferred to the methanol-activated polyvinylidene difluoride membrane. After the saturating membranes stage, with 1% bovine serum albumin in a Tris saline solution buffer containing Tween 20 (Sigma-Aldrich), membranes were incubated overnight with monoclonal antibodies of mice against YAP1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), LATS1 (Abcam Cambridge UK) and β-actin (Santa Cruz Biotechnology, Inc). Following the washing step, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase (Dako Italia Srl, Milan, Italy) for 1 hour at room temperature. The visualization of the immunoreactive bands was performed using the ECL chemiluminescence kit (Amersham; GE Healthcare Life Sciences, Chalfont, United Kingdom).

LATS1 kinase activity assay

Tumor specimens were lysed in 1% Nonidet P-40 lysis buffer supplement with 1 mM DTT and 1× phosphatase inhibitor (Sigma). The immunoprecipitation-kinase assay protocol was performed according to published manufacturing procedure [16]. Briefly, we used 100 μg of

Table 1
Demographic and clinical data of patients.

Tumor types	WHO grade	Number of patients	Female	Male	Age	KPS	OS (weeks)
LGA	II	15	6	9	44.13 ± 6.1	94 ± 6.5	190.3 ± 36.2
AA	III	15	8	7	60.73 ± 3.1	88.5 ± 10.6	110.2 ± 28.4
GBM	IV	25	10	15	60.16 ± 4.2	86.29 ± 11.2	59.9 ± 12.7
CTRL/NBT	–	5	2	3	47.4 ± 3.6	–	–
Total		55	24	31	53.6 ± 9.2	89.6 ± 3.97	120.13 ± 65.8

All Values are expressed as mean ± standard deviation
Abbreviation used: LGA, low grade astrocytoma, AA anaplastic astrocytoma, GBM, glioblastoma multiforme; WHO: World Health Organization; M: male; F: female; KPS: Karnofsky Performance Scale; OS: Overall survival; CTRL/NBT: Control normal brain tissue.

each sample protein, which was mixed with 2 μg of monoclonal antibody together with protein G microspheres and incubated at 4 °C for 3 h. After a series of washings with 1% of lysis buffer containing Nonidet P-40, 1 mM DTT; NaCl 500 mM, and 1 × phosphatase inhibitor, the washed beads were mixed with 2 μg of YAP-GST substrates in a kinase buffer (20 mM Tris–HCl (pH 7.5), 5 mM MgCl2, 5 mM MnCl2, 1 × phosphatase inhibitor, 2 mM DTT, 10 μM ATP, 5 μCi of [γ − 32P] ATP) and incubated at 30 °C for 30 min. The reaction was stopped by adding 7 ul of 5 × SDS dye sample, boiled at 100 °C for 5 min and then separated by electrophoresis to an 8% gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and exposed to autoradiograph film for 0.5–2 h to test phosphorylation of YAP by LATS1 [19].

miRNA isolation

Total RNA extraction was performed using the standard method TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA. USA). Both the integrity and the total concentration of the RNA extract were verified by the Bioanalyzer instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). Subsequently, 300 ng of total extracted RNA of each sample were retrotranscribed in cDNA using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Inc.). In addition, miRNAs were extracted from glioma tissues using the miRVana isolation kit (Ambion; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. The enriched miRNA fraction was retro-transcribed to cDNA using the TaqMan MicroRNA Reverse Transcriptase assay specific to the microRNA type (hsa-miR-10 and hsa-miR-221) (Thermo Fisher Scientific, Inc.).

Real time-quantitative polymerase chain reaction (RT-qPCR) of LATS1, Survivin and miRNAs

RT-qPCR for LATS1 and Survivin was performed using a standard TaqMan PCR kit procedure on an AB-7300 RT-PCR system (Thermo Fisher Scientific, Inc.). For miRNA quantification, 2 μl of cDNA was used for each specific miRNA TaqMan assay (hsa-miR-10 and hsa-miR-221) according to the manufacturer's instructions. All RT reactions, including non-template controls and RT controls, were performed in triplicate. RNU6 small nuclear RNA was used to normalize miRNA expression levels owing to its claimed expression stability and its wide use as a loading control in published miRNA expression studies [17, 19, 20].

The expression levels of miRNAs are indicated as either fold expression (<0.3 downregulation and >3 upregulation) compared to NBT. Results were represented as Log10RQ.

Target prediction tools

MiRNAs that target LATS1 were identified by examining the LATS1, 3′-untranslated region (UTR) with bioinformatics algorithms that predict miRNA target sites. Specifically, three online databases, TargetScan (www.targetscan.org), microRNA.org (www.microrna.org) and PicTar (http://pictar.mdc-berlin.de), were used for the analysis of the alignment between miRNAs and the 3′-UTR of LATS1 [21].

Oligonucleotide transfection

MiR-10b and miR-221 mimics/inhibitors (Qiagen, Milan, Italy) were transfected into U87-MG and A-172 cells using HiPerFect according to the manufacturer's protocols (Qiagen). Cells were transfected twice with 100 pmol of oligonucleotide per well (0.5×10^6 cells) at 24 h intervals. Transfected cells were assayed 48 h after the second transfection.

Plasmid constructs and transient transfections

The plasmid constructs for this study were carried out in accordance with the protocol we have already published in a previous study [22]. In brief, the 3′-UTR of LATS1 and the mutation sequences were amplified with PCR using primers with a BglII restriction site on each 5 'or 3′ strand. PCR products were inserted into the BglII sites of the pGL3 control vector (Promega, Madison, WI, USA) and identified by DNA sequencing. Wild type plasmids were created containing 3′UTR of LATS1 with the complementary sequence of miR-10b (pGL3-LATS1–3′UTR wild 1) and miR-221 (pGL3-LATS1–3′UTR wild 2). The mutant plasmids were generated without the complementary sequence of miR-10b (pGL3-LATS1–3′UTR mut 1) and miR-221 (pGL3-LATS1–3′UTR mut 2). The Luciferase reporter assay was also performed according to previous published protocol [22]. Briefly after Lipofectamine 2000 co-trasfection cells were seeded on 24-well plates) adding 100 ng per well of the resulting UTR-report luciferase vector, 2 ng per well of the pRLCMV vector (internal control, Promega) and 20 ng per well of miR-10b and miR-221 mimics or inhibits following the manufacturer's instructions (Qiagen). After 24 h, the cells were lysed and the relative activity of Luciferase was evaluated with the Dual-Luciferase Assay Reporter System (Promega).

MTT assay

Cell viability following was determined by the MTT [3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide] assay. Cells were cultured at a density of 6×10^5 and incubated for 2 h with MTT reagent at 37 °C. After incubation, cells were treated with DMSO solvent for 15 min at room temperature. Absorbance was measured at OD = 490 nm.

Statistical data analysis

All data is expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by post-hoc analysis was used to test statistical significance. A $p<0.05$ was considered significant. Pearson analysis was performed to analyze the correlation between YAP expression and Survivin in all glioma grades. GraphPad Prism 5 software was used for statistical analysis.

Results

Overexpression of YAP1 and survivin in high-grade gliomas

Western blot analysis showed that YAP1 expression was significantly higher in GBMs when compared to ANAs ($P<0.05$) as well as to LGAs and controls ($P<0.001$) (Fig. 1A). On the contrary, LATS1 expression was significantly higher in CTRs and LGAs in respect to ANAs and GBMs ($P<0.001$) (Fig. 1B). To determine whether elevated YAP1 in diffuse gliomas represents the active form of YAP1, with its inactive phosphorylated form (pYAP1), enzymatic kinase assay of LATS1 was performed using immunoprecipitation assay with [γ − 32P]ATP. The inactive form (pYAP1) was detected in 91.3% of CTRs, which was significantly higher than in LGAs (73.3%, $P<0.05$), ANAs (37.5%, $P<0.01$), and GBMs (18.2%, $P<0.001$) (Fig. 2C). Real-time PCR confirmed the significantly increased expression level of YAP1 mRNA expression in GBMs as compared with ANAs and LGAs (Fig. 2A), as well as a more significantly decrease in LATS1 mRNA levels in GBMs compared with the other grade of tumors (Fig. 2B). Pearson's correlation analysis showed a significant association of tumor grade with either YAP1 ($r = 0.6286$, $p<0.01$) or LATS1 ($r = -0.6111$, $p<0.01$) expression levels. Transcription of Survivin has been shown to be regulated by YAP1. We found a significant overexpression of Survivin in GBMs and ANAs as compared to LGAs and CTRs ($p<0.001$) (Fig. 2C). Correlation analysis revealed significant positive association

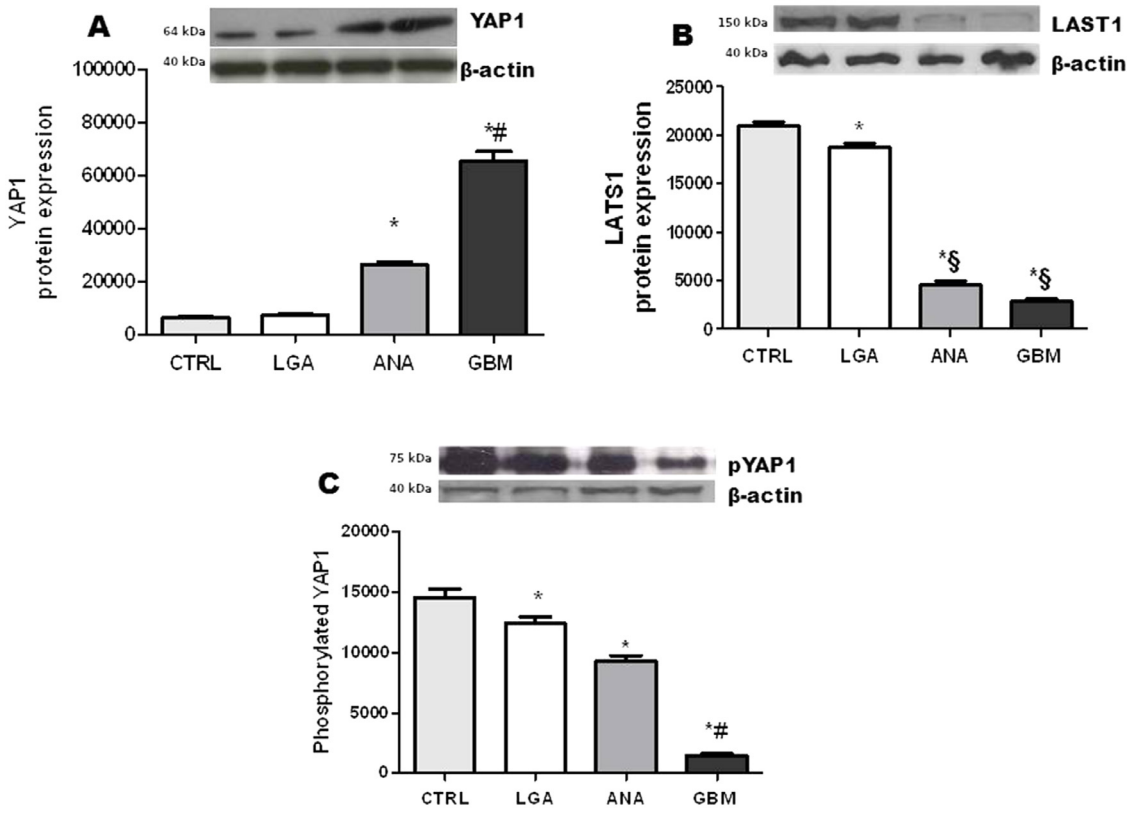

Fig. 1. Western blot analysis of in YAP1 (A), LATS1 (B) Phosphorylated -YAP1 generated by LATS 1 kinase activity (C) in CTRL, LGA, AA and GBM tissues. Upper panel: Representative autoradiography highlights YAP1 (A), LATS1 (B) Phosphorylated- YAP1 (pYAP1) (C) and β-actin expression. Lower panel: Quantitative data represent represents the mean ± SD of relative expression (error bars). *p < 0.01 vs CTRL and LGA, # p < 0.01 GBM vs ANA, § p < 0.01 vs CTRL and LGA. Abbreviations: CTRL, non-neoplastic brain tissue; LGA, low-gradeAstrocytoma; ANA, anaplastic astrocytoma; GBM, glioblastoma multiforme.

between YAP1 and Survivin expression (Fig. 2D, $r = 0.7544$, $p < 0.001$), thus suggesting that Survivin may be modulated by YAP1 in glioma tumors.

miR-10 and miR-221 are highly expressed in glioma tissues and associated with poor outcome

Recent studies have highlighted the involvement of miR-221 and miR-10 in the regulation of cell differentiation, proliferation and apoptosis, as well as in the promotion of cancer transformation [20–22]. Using qRT-PCR, in the present study, we verified the gene expression of miR-221 and miR-10 in our cases with different degrees of glioma progression. As shown in Fig. 3A and B, miR-10 and miR-221 were upregulated in GBMs and ANAs compared to LGAs and CTRs, as well as this we observed an upregulation of miR-10 and miR-221 in U-87 MG and A-172 MG cell lines with respect to normal human astrocytes (NHAs) (Fig. 3C and D). The correlation analysis showed that miR-10 and miR-221 expression was positively associated with tumor stage (for miR-10; $r = 0.811$ $P < 0.05$; miR-221, $r = 0856$, $P < 0.05$).

Correlation analysis between miR-10 and miR-221 expression and key molecules of Hippo pathway in glioma tissues

To our knowledge, there is still lack of evidence whether key molecules of Hippo pathway are the predicted targets of miR-10b and miR-221. To verify the correlation between miR-10b and miR-221 and key molecules of Hippo pathway by statistical evidence, Pearson correction analysis was used. As expected, expression levels of miR-10b and miR-221 exhibited a significant negative correlation with LATS1 mRNA ($r = -0.8775$, $P < 0.01$). In addition, miR-10b and miR-221 expression was positively associated with YAP1 mRNA expression ($r = 0.8963$, $P < 0.05$). Overall, this data suggests that miR-10b and miR-221 have potential to be regulators of LATS1 and YAP1 in glioma.

miR-10 and miR-221 inhibit Hippo pathway by targeting LATS1 in glioma

To evaluate the direct binding of miR-10 and miR-221 to the 3′UTR of LATS1 mRNA, bioinformatics analysis was carried out. TargetScan (www.targetscan.org), microRNA.org (www.microrna.org) and PicTar (http://pictar.mdc-berlin.de) showed that 3′UTR of LATS1 mRNA had binding sequence of miR-10b and miR-221 (Fig. 4A). To confirm that miR-10b and miR-221 target LATS1, U87-MG and A-172 cells were transfected with Luciferase reporter vectors containing the 3′UTR of LATS1 with the complementary sequence of miR-10b (pGL3-LATS1–3′UTR wild 1) or miR-221 (pGL3-LATS1–3′UTR wild 2). The mutant plasmids were generated without the complementary sequence of miR-10b (pGL3-LATS1–3′UTR mut 1) and miR-221 (pGL3-LATS1–3′UTR mut 2). Our results showed that in mimic (miR-10b or miR-221) cells transfected, the luciferase activity of LATS1–3′-UTR-wt was drastically reduced ($p < 0.001$), whereas the luciferase of LATS1–3′- UTR mutant cells was not affected from transfection with the same imitators, compared to negative controls (Fig. 4B). When the expression of miR-10b and miR-221 is blocked with specific inhibitors, the intensity of Luciferase is significantly increased in cells transfected with the LATS1–3′-UTR-wt vector ($p < 0.001$) and is not classified in the cells transfected with LATS1–3′ -UTR-mut vector (Fig. 4C). As the upstream molecules of YAP1 in Hippo pathway, low expression of LATS1 can decrease the level of phosphorylation of YAP1 and lead to sustained tissue growth. We found that silencing of either miR-10b or miR-221 inhibited YAP1 expression in U87-MG and A-172 cells, whereas up-regulated miR-10 and miR-221 promoted YAP1 expression, as compared with control group (Fig. 4D), thus suggesting that miR-10 and miR-221 could affect

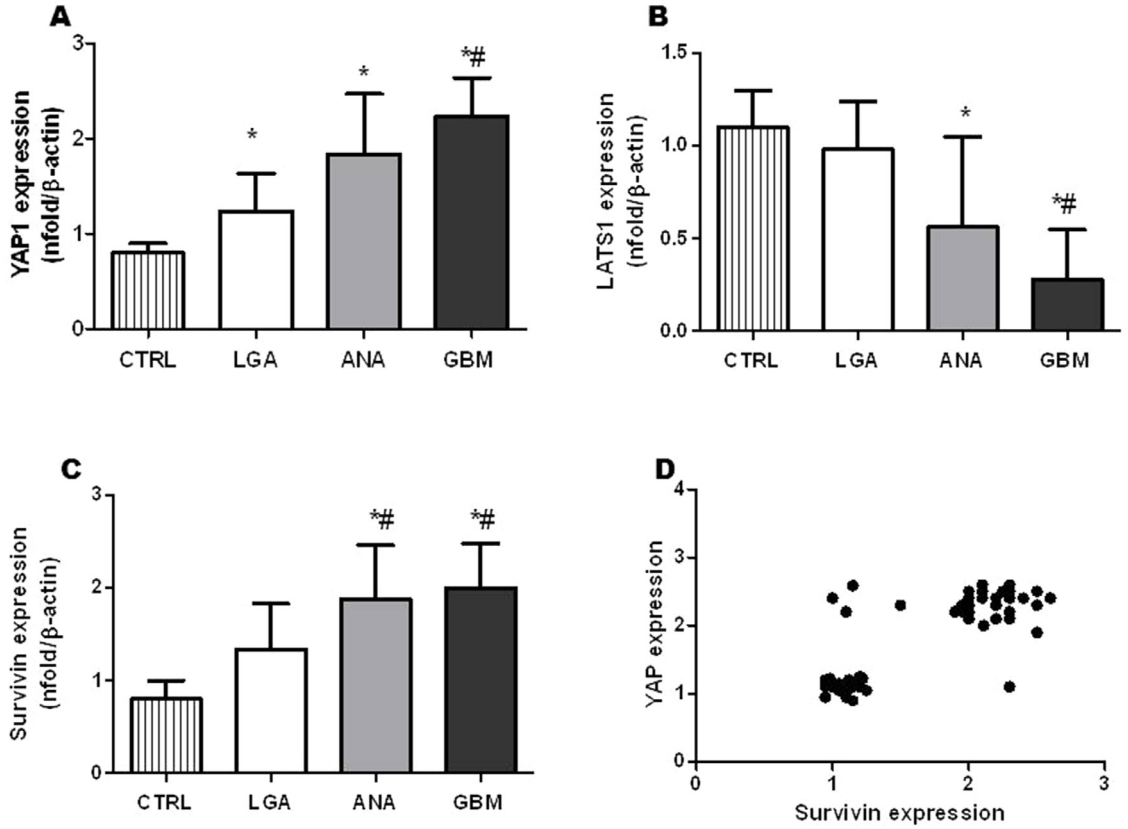

Fig. 2. mRNA expression levels for YAP1 (A), LATS1 (B), survivin (C) in astroglial tumors of different grade (LGA, ANA and GBM). mRNA expression were quantified by qRT-PCR analysis on tissues samples and normalized versus CTRL. Data are represented as log10RQ. Error bars represent the standard deviation. A p value < 0.05 was considered statistically significant. Correlational analysis between YAP expression and Survivin expression (D) was measured by Pearson's correlation coefficient ($r = 0.7544$, $p < 0.001$). Abbreviations: CTRL, non-neoplastic brain tissue; LGA, low-gradeAstrocytoma; ANA, anaplastic astrocytoma; GBM, glioblastoma multiforme.

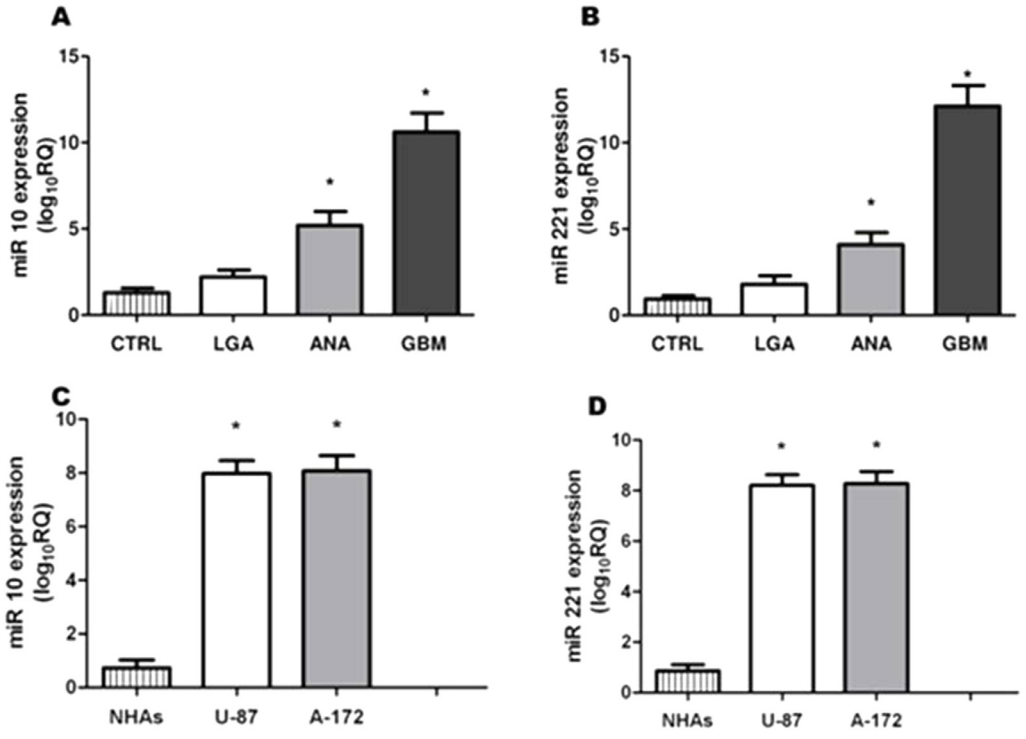

Fig. 3. miR-10b and miR-221 expression performed three independent experiment with different samples of astroglial tumors of different grade (LGA, ANA and GBM) and in cell lines. miRNA expression of miR-10b (A and C) and miR-221 (B and D) were quantified by qRT-PCR analysis on tissues samples and normalized versus tissue CTRL (A- B) and on Cell lines U87 MG and A-172 MG versus NHAs. Data are represented as log10RQ. Error bars represent the standard deviation. A p value < 0.05 was considered statistically significant. *$p < 0.001$ vs CTRL, LGA or NHAs. Abbreviations: CTRL, non-neoplastic brain tissue; LGA, low-grade Astrocytoma; ANA, anaplastic astrocytoma; GBM, glioblastoma multiforme; NHAs, Normal Human Astrocytes.

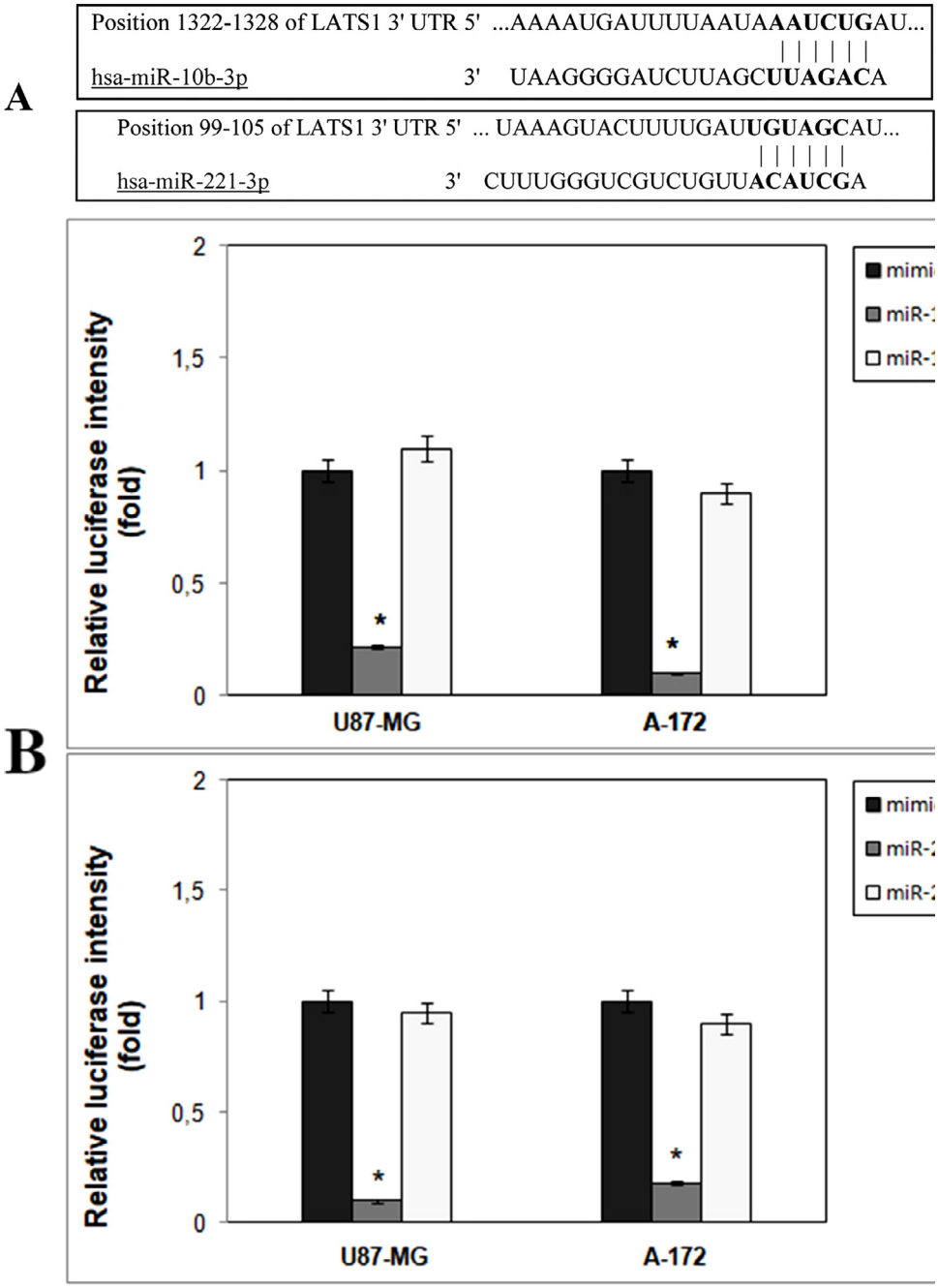

Fig. 4. LATS1 is the direct target of miR-10b and miR-221. (A) Alignment details with 3′-UTR region of LATS1. Schematic representation of the predicted interaction of miR-10ba and miR-221 with LATS1 3′UTR site. (B) Luciferase reporter assay was performed to detect the effect of miRNA mimics on the luciferase intensity controlled by 3′UTR of LATS1. A p value < 0.05 was considered statistically significant. *p < 0.001, as compared to cells transfected with mimic control. (C) Luciferase reporter assay was performed to detect the effect of miRNA inhibitors on the luciferase intensity controlled by 3′UTR of LATS1. (D) Relative expression of YAP after transfection with mimic an siRNA's . Significant *$p < 0.001$, as compared to cells transfected with inhibitor control.

YAP1 activity through targeting LATS1.

Inhibition of miR-10 and miR-221 reduces proliferation of glioma cancer cells

In light of the above findings, we hypothesized that miR-10 and miR-221 might play a role in glioma cell growth. U87-MG and A-172 cells were transfected with miR-10 and miR-221 inhibitors or controls for 24, 48, 72, 96 and 120 h. We found that both si-miR-10 and si-miR-221 showed lower proliferation as from 72 h (Fig. 5A and 5 B). Thus, si-miR-10 and si-miR-221 intervention inhibited the proliferation capacity of U87-MG and A-172 glioma cells. However, the expression of YAP1 is decreased in the two cell lines after transfection with both the anti-miR10 and 221 Panel C and D of Fig. 6, while the expression of LAST1 is slightly increased with both the two anti-miRs. Furthermore, the decrease in miR-10 and Mir-221 levels after transfection panel A and B of Fig. 6, suggest the direct involvement of these two microRNAs in the regulation of YAP phosphorylation via LATS1.

Discussion

The Hippo signaling pathway is an important conserved pathway that controls organ size and cell differentiation across diverse organisms through the regulation of cell proliferation and apoptosis [23]. The

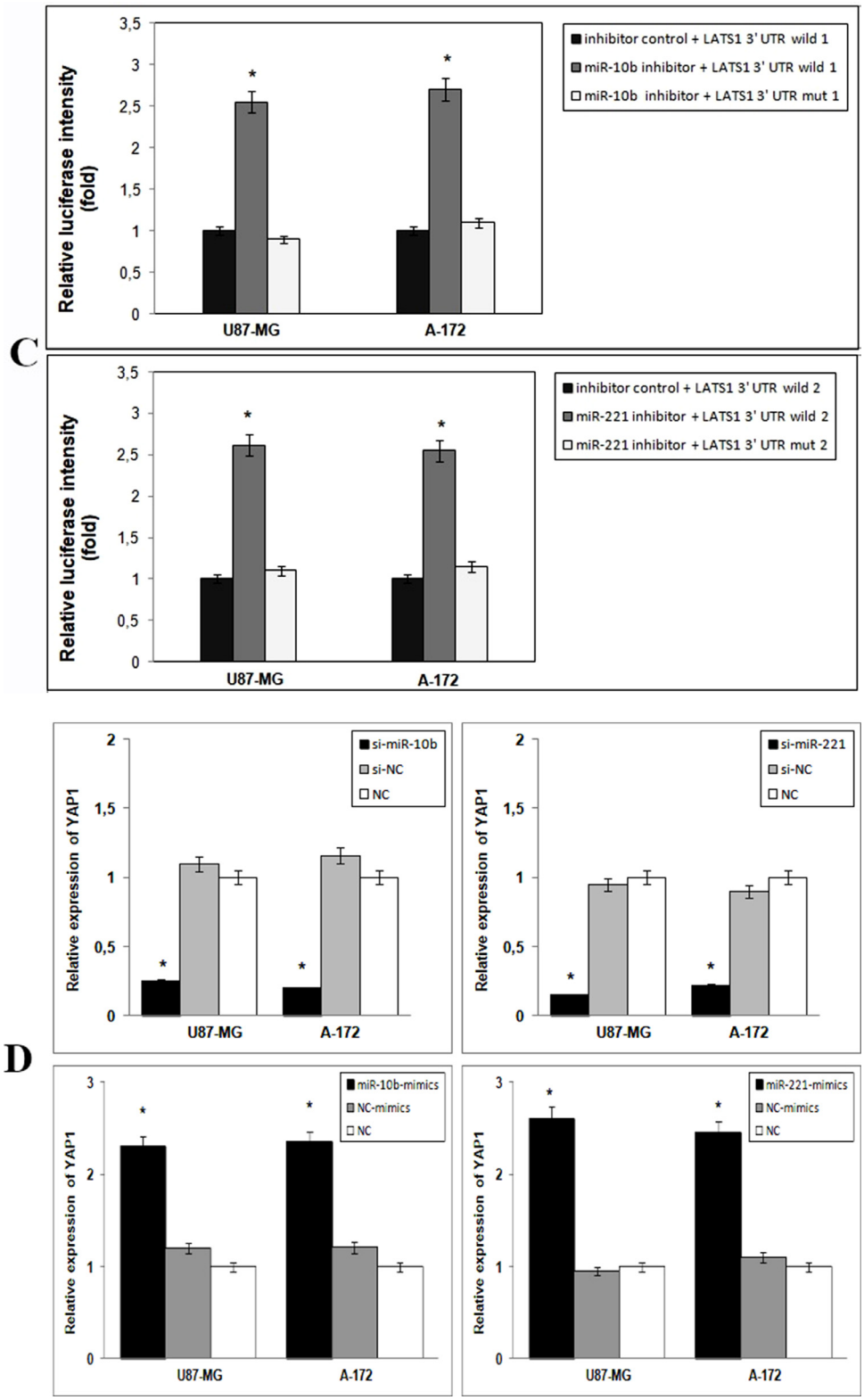

Fig. 4. (*continued*)

core of Hippo pathway is a kinase cascade consisting of protein kinase MST1/2, the large tumor suppressors (LATS1/2), and adaptor proteins Salvador homolog 1 (SAV1) and MOB kinase activators (MOB1A/MOB1B) [8]. These kinases directly phosphorylate the major Hippo pathway downstream effectors, the yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ), and thus inhibit or activated the transcription of their downstream target genes such as Survivin (BIRC5) [5].

Recently, the critical role of the Hippo pathway in cancer development has been increasingly recognized [4]. Dysregulated signaling by the Hippo pathway has been reported in several cancer types such as breast, liver, lung, prostate, gastric, colorectal and astroglial tumors [24]. Extensive studies have experimentally established LATS1/2 has a tumor suppressor function. For example, loss of YAP via STK3/STK4 in

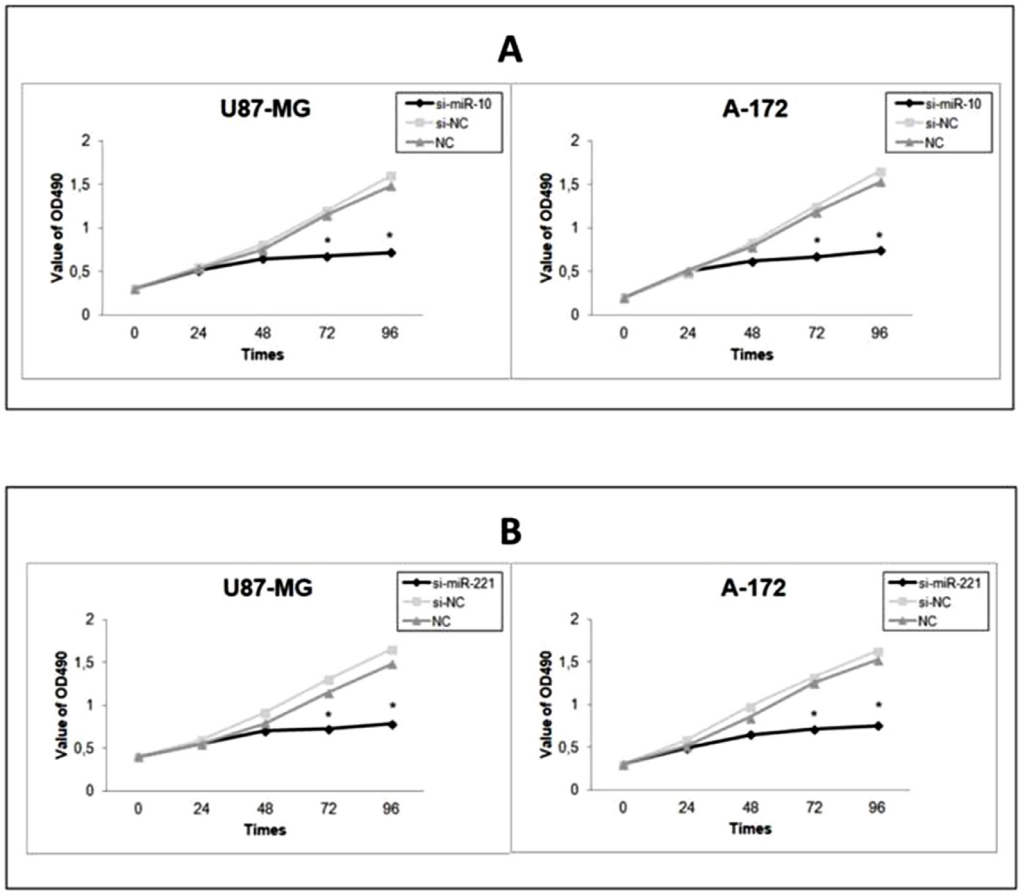

Fig. 5. Cell proliferation rate of U87-MG and A-172 after transfection inhibitors of miR-10 panel (A) and miR-221 panel (B) detected by MTT assay . *P < 0.01. Abbreviations: NC, normal cells (U87-MG or A-187); Si-NC, only the plasmid transfected Normal Cells; si-miR10, or si-miR221, plasmid inhibitors miR10, or miR221.

a mouse liver leads to uncontrolled cell proliferation and differentiation [25], and YAP/TAZ overexpression leads to tissue overgrowth through action of several growth promoting factors, including Survivin, which is part of a series of genes involved in cell proliferation [3–6].

Indeed, tissue growth, where the Hippo metabolic pathway plays a crucial role, derives from an imbalance between survival, cell proliferation and apoptosis. An imbalance of homeostasis due to intrinsic and/or extrinsic factors of the tissues can be fatal to carcinogenesis and tumor progression.

Based on the public database, the levels of YAP1 and Survivin were positively correlated with the progression and prognosis of the glioma patient, while that of LATS1 showed a negative association. In accordance with TCGA Cancer Molecular Analysis Portal [26], our data (Figs. 1 and 2) suggests a cause of LATS1 downregulation, YAP1 / Survivin could be used abnormally, which in turn promotes the onset and development of gliomas. In the present study, we have shown that in the progression of glioma cells (from Low Grade to the glioblastoma multiform IV) LATS1 progressively was inhibited probably through epigenetic control. Several studies have demonstrated the involvement of micro RNAs in the setting of different tumors [15,27–28], interfering on potential tumor suppressor factors favoring cell proliferation and tumorogenesis. In this study, we focused the molecular mechanisms of two MirNAs on LATS1 expression.

Mir-10b and miR-221 are considered important onco-miRs that promote carcinogenesis and maintain malignancy in GBM cells [17–18,29–30]. Our results show this trend in both the tumor samples and the cell cultures analyzed. In particular, the high expression of miR-10b and miR-221 were both associated with glioma staging (Fig. 3). Mir-10 has already been shown to be involved in the modulation of the metabolic pathway PTEN / PI3K / AKT, and in stem cell cancer [31]. While Mir-221 targets the O6-methylguanine – DNA methyltransferase (MGMT), a key enzyme in conventional glioma patients therapy based on administration of temozolomide (TMZ) in combination with radiotherapy [32]. The intracellular levels of the alkylating enzyme MGMT influence the response to TMZ in patients with GBM [33]. Low MGMT levels are associated with a better TMZ response, because in the absence of MGMT cells are unable to repair the TMZ-induced base mismatch. Therefore, double stranded DNA breaks, DNA mismatch repair and apoptotic pathway are activated. MGMT expression is regulated by methylation of its promoter. Methylation of the MGMT promoter lowers MGMT levels and explains a greater TMZ response when associated with radiation therapy. However, a fraction of patients with unmethylated MGMT show a TMZ response, suggesting that promoter methylation is not necessary to regulate the expression of MGMT, but well an epigenetic control conducted by mir-221.

Recently it has been shown that miR-221/222 also target tissue inhibitors of metalloproteinases2 (TIMP2), a member of the TIMP family that is widely distributed in the brain. TIMP protects the extracellular matrix (ECM) from proteolytic degradation thus preventing the invasion and metastasis of tumor cells, in addition to this function of the TIMP2 regulation, miR-221/222, they safeguard the angiogenesis of glioma cells. Angiogenesis is the formation of new blood vessels, which require that endothelial cells invade through an interstitial matrix and proliferate [34]. As a result, TIMPs have antiangiogenic activity by inhibiting the activity of MMP metalloproteinases or by directly inhibiting the proliferation of endothelial cells [35].

The bioinformatic analysis through online database, TargetScan (www.targetscan.org), microRNA.org (www.microrna.org) and PicTar (http://pictar.mdc-berlin.de), we studied the sequence alignments nucleotides between miR-10 and MiR-221 and 3′-UTR of LATS1. With

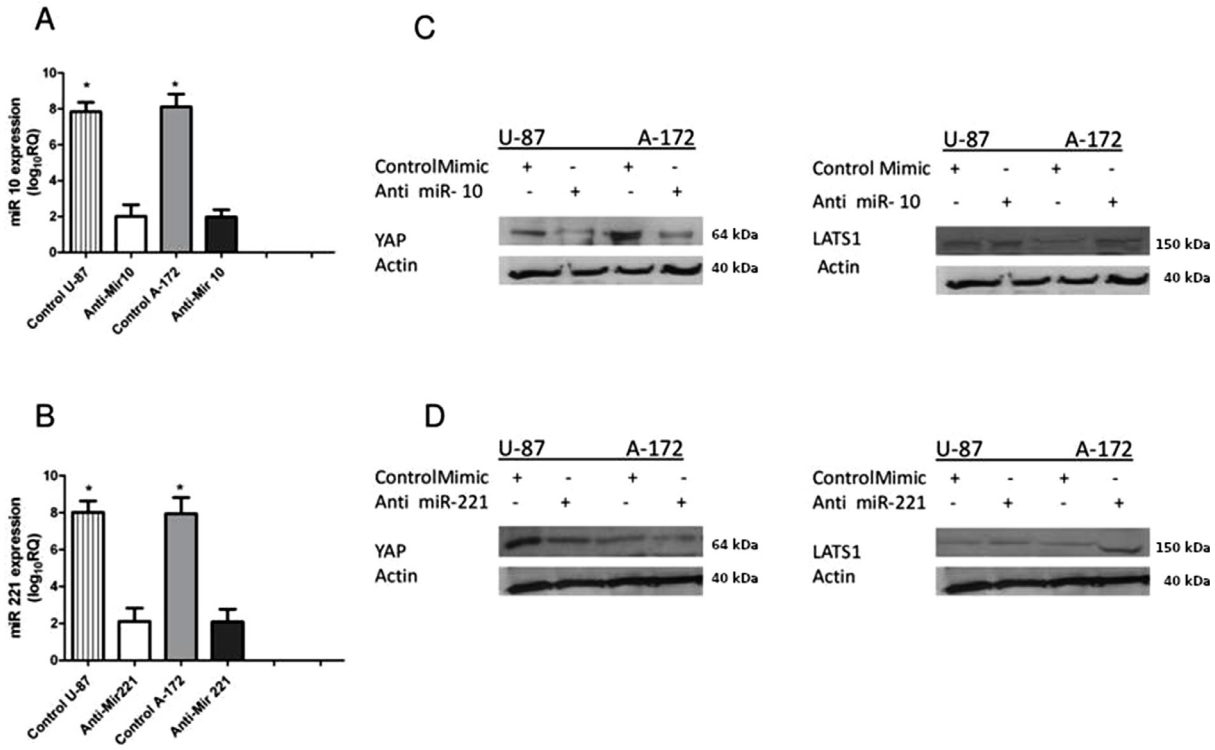

Fig. 6. *Repressed YAP1 and increased LATS1 protein expression by anti miR-10 and anti miR-221 transfection in human glioma cells.* (A and B) The Real time PCR of miR-10 and miR-221 analysis in U87and A-172 glioma cell lines after transfection with anti-Mirs ($p < 0.05$). Data was from three independent assays. (C and D) YAP and LATS 1 expression in U-87 and A-172 glioma cell lines were detected by immunoblotting, b-Actin was used as a loading control. The control mimics or Anti miR-10 were transfected into U87 MG or A-172 MG cells, and TAZ expression was examined by immunoblotting. .

functional studies using vectors of the two mutated microRNA constructs we showed an important significant cell death rate after 72 h on the transfected cells. (Fig. 4). The unleashed apoptosis is consequent to the drop of YAP in the tumor cells as shown in the Fig. 6.

The expression of LATS1 has significantly decreased in some tumors such as breast cancer and astrocytomas [36–37]; this downregulation has been attributed to its promoter hypermethylation. The negative regulation of the YAP1 oncoprotein, in the Hippo signaling pathway, plays a fundamental role in the control of the size of the organs and in the suppression of tumors, limiting the proliferation and promoting apoptosis. LATS1 phosphorylates the YAP1 protein and inhibits its translocation in the nucleus, favoring its ubiquitation and degradation [38]. Furthermore, in previous studies, the over-expression of LATS1 induced cell apoptosis by increasing the pro-apoptotic proteins p53 and Bax and by suppressing cell proliferation through the upregulation of p53 to ensure genomic integration. In contrast, the knockdown of LATS1 induced cell migration in HeLa cells [39]. To evaluate the role of LATS1 in glioma, we first performed real-time PCR to measure the expression of LATS1 mRNA transcripts in 45 samples of different grade of glioma coupled with 5 normal brain tissues. Similar to reports of other tumor types [40], we observed the expression of LATS1 was significantly reduced in all 25 high grade glioblastoma tissues and gradually decreased in 15 anaplastic astrocytomas compared to normal tissues. This suggests that LATS1 works as a tumor suppressor in glioma. We validated this downregulation of the LATS1 protein by western blot. These results were analogous to the report by Takahashi et al. strongly suggested a suppressive role of LATS1 in glioma tumorigenesis, where LATS1 was positively correlated to the overall survival of the patient considering that LATS1 was a significant predictive factor of unfavorable prognosis for patients with glioma, as also recently it has been proposed as a potential therapeutic target in tumors using anti tubulin drugs [41].

Conclusion

Overall, the present study provides a glimpse into the understanding of the molecular mechanisms underlying malignant gliomas, suggesting that the LATS/YAP path is heavily involved and that targeted therapeutic interventions are applicable in these tumoral pathologies. Furthermore, our data adds a small piece to the complex puzzle of the astrocytomas cancer biology, indicating another mechanism like the one we find to regulate the Hippo pathway (Fig. 7). Thereby they may be seen as markers for diagnosis and prognostic analysis as well as potential therapeutic targets for glioma treatment.

Compliance with ethical standards

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Local Bioethical Committee at the University Hospital “G. Martino” of Messina and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The present study was submitted and approved by “The Institutional Review Board of the University Hospital of Messina, Italy” (prot. E 09/14 February 12, 2014 (Prof. Alfredo Conti).

Informed consent

Informed consent was obtained from all participants included in the study.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

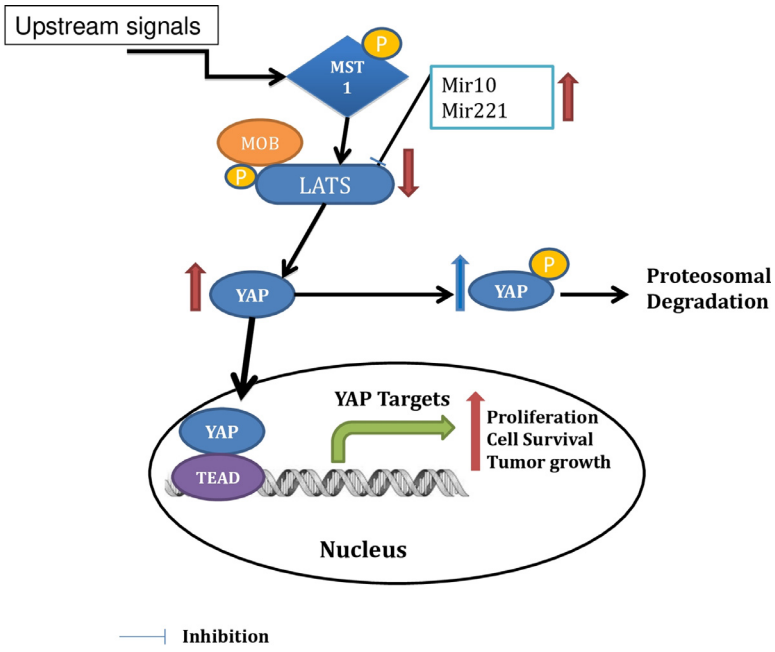

Fig. 7. *Proposed schematic model of Hippo pathway miR 10 and miR221 regulation in astroglial tumors.* Phosphorylated MST1 resulting from increased phosphorylation via MOB of LATS1 and increased kinase activity of YAP phosphorylation. The inhibition through high concentration of miR-10 and mir-221 present in tumor tissues on LATS1 allows YAP to reach the genes target in the nucleus.

influence the work reported in this paper.

The authors declare that they have no conflict of interest

References

[1] D.N. Louis, H. Ohgaki, O.D. Wiestler, W.K. Cavenee, P.C. Burger, A. Jouvet, B.W. Scheithauer, P Kleihues, The 2007 WHO classification of tumours of the central nervous system, Acta Neuropathol. 114 (2) (2007) 97–109.

[2] Global Burden of Disease Cancer Collaboration, C. Fitzmaurice, D. Abate, N. Abbasi, H. Abbastabar, F. Abd-Allah, O. Abdel-Rahman, et al., Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: a systematic analysis for the global burden of disease study, JAMA Oncol. 5 (12) (2019 Sep 27) 1749–1768.

[3] V. Fu, S.W. Plouffe, K.L Guan, The Hippo pathway in organ development, homeostasis, and regeneration, Curr. Opin. Cell Biol. 49 (2018 Jan 6) 99–107.

[4] F. Zanconato, M. Cordenonsi, S Piccolo, YAP/TAZ at the Roots of Cancer, Cancer Cell 29 (6) (2016 Jun 13) 783–803.

[5] S.M. White, S. Murakami, C Yi, The complex entanglement of Hippo-Yap/Taz signaling in tumor immunity, OncogeneOncogene 38 (16) (2019) 2899–2909.

[6] W.C. Juan, W. Hong, Targeting the Hippo signaling pathway for tissue regeneration and cancer therapy, Genes (Basel) 7 (2016) 55.

[7] A.V. Pobbati, W. Hong, Emerging roles of TEAD transcription factors and its coactivators in cancers, Cancer Biol. Ther. 14 (5) (2013) 390–398.

[8] B. Zhao, X. Wei, W. Li, R.S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, P. Zheng, K. Ye, A. Chinnaiyan, G. Halder, Z.C. Lai, K.L Guan, Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control, Genes Dev. 21 (21) (2007 1) 2747–2761.

[9] Z. Meng, T. Moroishi, K.L Guan, Mechanisms of Hippo pathway regulation, Genes Dev. (2016) 1–17.

[10] Y. Hao, A. Chun, K. Cheung, B. Rashidi, X Yang, Tumor suppressor LATS1 is a negative regulator of oncogene YAP, J. Biol. Chem. 283 (2008) 5496–5509.

[11] Y. Wang, P. Pan, Z. Wang, Y. Zhang, P. Xie, D. Geng, Y. Jiang, R. Yu, X Zhou, β-catenin-mediated YAP signaling promotes human glioma growth, J. Exp. Clin. Cancer Res. 36 (1) (2017 Sep 29) 136.

[12] Brent a, Bai H, Y. Odia, D. Jain, R.A. Anders, C.G Eberhart, Yes-associated protein 1 (YAP1) is widely expressed in human brain tumors and promotes glioblastoma growth, J. Neuropahol. Exp Neurol. 7 (2011) 568–577.

[13] S. Wang, Y. Yin, S Liu, Roles of microRNAs during glioma tumorigenesis and progression, Histol. Histopathol. 14 (2018) 18040.

[14] J.S. Nahand, S. Taghizadeh-Boroujeni, M. Karimzadeh, et al., microRNAs: new prognostic, diagnostic, and therapeutic biomarkers in cervical cancer, J. Cell. Physiol. 234 (10) (2019) 17064–17099.

[15] A.M. Aghdam, A. Amiri, R. Salarinia, A. Masoudifar, F. Ghasemi, H Mirzaei, MicroRNAs as diagnostic, prognostic, and therapeutic biomarkers in prostate cancer, Crit. Rev. Eukaryot. Gene Expr. 29 (2) (2019) 127–139.

[16] C. Xie, M. Xu, D. Lu, W. Zhang, L. Wang, H. Wang, J. Li, F. Ren, C Wang, Candidate genes and microRNAs for glioma pathogenesis and prognosis based on gene expression profiles, Mol. Med. Rep. 18 (3) (2018) 2715–2723.

[17] A. Conti, M. Aguennouz, D. La Torre, C. Tomasello, S. Cardali, F.F. Angileri, F. Maio, A. Cama, A. Germanò, G. Vita, F Tomasello, miR-21 and 221 upregulation and miR-181b downregulation in human grade II–IV astrocytic tumors, J. Neurooncol. 93 (2009) 325–332.

[18] F. Guessous, M. Alvarado-Velez, L. Marcinkiewicz, Y. Zhang, et al., Oncogenic effects of miR-10b in glioblastoma stem cells, J. Neurooncol. 112 (2) (2013) 153–163.

[19] G.L. Vita, F. Polito, R. Oteri, R. Arrigo, A.M. Ciranni, O. Musumeci, S. Messina, C. Rodolico, R.M. Di Giorgio, G. Vita, M Aguennouz, Hippo signaling pathway is altered in Duchenne muscular dystrophy, PLoS ONE 10 (10) (2018) 13.

[20] Z. Shabaninejad, F. Yousefi, A. Movahedpour, et al., Electrochemical-based biosensors for microRNA detection: nanotechnology comes into view, Anal. Biochem. 581 (2019) 113349.

[21] G.L. Vita, M. Aguennouz, F. Polito, R. Oteri, M. Russo, L. Gentile, C. Barbagallo, M. Ragusa, C. Rodolico, R.M. Di Giorgio, A. Toscano, G. Vita, A Mazzeo, Circulating microRNAs profile in patients with Transthyretin variant amyloidosis, Front. Mol. Neurosci. 23 (2020).

[22] M. Visalli, M. Bartolotta, F. Polito, R. Oteri, A. Barbera, R. Arrigo, R.M. Di Giorgio, G. Navarra, M Aguennouz, miRNA expression profiling regulates necroptotic cell death in hepatocellular carcinoma, Int. J. Oncol. 53 (2) (2018 Aug) 771–780.

[23] Cancer Genome Atlas Research Network, D.J. Brat, Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas, J.N. Engl J Med. 372 (26) (2015 Jun 25) 2481–2498.

[24] N.J. Ganem, H. Cornils, S.Y. Chiu, K.P. O'Rourke, J. Arnaud, D. Yimlamai, M. Théry, F.D. Camargo, D Pellman, Cytokinesis failure triggers hippo tumor suppressor pathway activation, Cell 158 (4) (2014) 833–848 14.

[25] L. Hong, Y. Li, Q. Liu, Q. Chen, L. Chen, D Zhou, The Hippo signaling pathway in regenerative medicine, Methods Mol Biol. 1893 (2019) 353–370.

[26] O. Celiku, S. Johnson, S. Zhao, K. Camphausen, U Shankavaram, Visualizing molecular profiles of glioblastoma with GBM-BioDP, PLoS ONE 10 (7) (2014) 9.

[27] A. Savardashtaki, Z. Shabaninejad, A. Movahedpour, R. Sahebnasagh, H. Mirzaei, M.R Hamblin, miRNAs derived from cancer-associated fibroblasts in colorectal cancer, Epigenomics. 11 (14) (2019) 1627–1645, https://doi.org/10.2217/epi-2019-0110.

[28] A. Conti, S.G. Romeo, A. Cama, D. La Torre, V. Barresi, G. Pezzino, C. Tomasello, S. Cardali, F.F. Angileri, F. Polito, G. Ferlazzo, R. Di Giorgio, A. Germanò, M Aguennouz, MiRNA expression profiling in human gliomas: upregulated miR-363 increases cell survival and proliferation, Tumour Biol. 37 (10) (2016) 14035–14048.

[29] B. Sun, X. Zhao, J. Ming, X. Liu, D. Liu, C Jiang, Stepwise detection and evaluation reveal miR-10b and miR-222 as a remarkable prognostic pair for glioblastoma, Oncogene 38 (33) (2019) 6142–6157.

[30] C. Quintavalle, M. Garofalo, C. Zanca, G. Romano, M. Iaboni, M. del Basso De Caro, J.C. Martinez-Montero, M. Incoronato, G. Nuovo, C.M. Croce, G Condorelli, miR-221/222 overexpression in human glioblastoma increases invasiveness by targeting the protein phosphate PTPμ, Oncogene 31 (7) (2012) 858–868 16.

[31] P.A. Koshkin, D.A. Chistiakov, A.G. Nikitin, A.N. Konovalov, A.A. Potapov, D.Y. Usachev, D.I. Pitskhelauri, G.L. Kobyakov, L.V. Shishkina, V.P Chekhonin, Analysis of expression of microRNAs and genes involved in the control of key signaling mechanisms that support or inhibit development of brain tumors of different grades, Clin. Chim. Acta 430 (2014) 55–62 20.

[32] Y.Y. Chen, H.L. Ho, S.C. Lin, T.D. Ho, C.Y Hsu, Upregulation of miR-125b, miR-181d, and miR-221 predicts poor prognosis inMGMT promoter-unmethylated glioblastoma patients, Am. J. Clin. Pathol. 149 (5) (2018, 29) 412–417.

[33] S.L Gerson, MGMT: its role in cancer aetiology and cancer therapeutics, Nat. Rev. Cancer 4 (2004) 296–307.

[34] T.H. Chun, F. Sabeh, I. Ota, H. Murphy, K.T. McDonagh, K. Holmbeck, et al., MT1-

MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix, J. Cell Biol. 167 (4) (2004) 757–767.
[35] D.W. Seo, H. Li, C.K. Qu, J. Oh, Y.S. Kim, T. Diaz, et al., Shp-1 mediates the anti-proliferative activity of tissue inhibitor of metalloproteinase-2 in human micro-vascular endothelial cells, J. Biol. Chem. 281 (6) (2006) 3711–3721.
[36] Y. Takahashi, Y. Miyoshi, C. Takahata, N. Irahara, T. Taguchi, Y. Tamaki, S Noguchi, Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers, Clin. Cancer Res. 11 (2005) 1380–1385.
[37] S. Visser, X Yang, LATS tumor suppressor: a new governor of cellular homeostasis, Cell. Cycle 9 (2010) 3892–3903.
[38] Y. Liu, Deng Ubiquitination-deubiquitination in the Hippo signaling pathway (Review), J. Oncol. Rep. 41 (3) (2019) 1455–1475.
[39] N. Furth, N. Bossel Ben-Moshe, Y. Pozniak, Z. Porat, T. Geiger, E. Domany, Y. Aylon, M Oren, Down-regulation of LATS kinases alters p53 to promote cell migration, Genes Dev. 15 (22) (2015) 2325–2330 29.
[40] T. Ji, D. Liu, W. Shao, W. Yang, H. Wu, X Bian, Decreased expression of LATS1 is correlated with the progression and prognosis of glioma, J. Exp. Clin. Cancer Res. 31 (1) (2012) 67 21.
[41] B. Yeung, P. Khanal, V. Mehta, L. Trinkle-Mulcahy, X Yang, Identification of Cdk1-LATS-Pin1 as a novel signaling axis in anti-tubulin drug response of cancer cells, Mol. Cancer Res. 16 (6) (2018) 1035–1045.