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miR-221/222 overexpression in human glioblastoma increases invasiveness by targeting the protein phosphate PTPμ

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Abstract

Glioblastoma is the most frequent brain tumor in adults and is the most lethal form of human cancer. Despite the improvements in treatments, survival of patients remains poor. In order to identify microRNAs (miRs) involved in glioma tumorigenesis, we evaluated, by a miRarray, differential expression of miRs in the tumorigenic glioma LN-18, LN-229 and U87MG cells compared with the non-tumorigenic T98G cells. Among different miRs we focused our attention on miR-221 and -222. We demonstrated the presence of a binding site for these two miRs in the 3′ untranslated region of the protein tyrosine phosphatase μ (PTPμ). Previous studies indicated that PTPμ suppresses cell migration and is downregulated in glioblastoma. Significantly, we found that miR-221 and -222 over-expression induced a downregulation of PTPμ as analyzed by both western blot and real-time PCR. Furthermore, miR-222 and -221 induced an increase in cell migration and growth in soft agar in glioma cells. Interestingly, the re-expression of *PTPμ* gene was able to revert the miR-222 and -221 effects on cell migration. Furthermore, we found an inverse correlation between miR-221 and -222 and PTPμ in human glioma cancer samples. In conclusion, our results suggest that miR-221 and -222 regulate glioma tumorigenesis at least in part through the control of PTPμ protein expression.

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Keywords

Glioma; microRNA; tumorigenesis; apoptosis

Introduction

Gliomas are the most common primary tumors in the brain and are divided into four clinical grades on the basis of their histology and prognosis (Tran and Rosenthal, 2010). Among them, glioblastoma (GB, grade IV) are highly invasive, very aggressive and are one of the most incurable forms of cancer in humans (Purow and Schiff, 2009). In spite of recent improvements of surgical and radiotherapeutic techniques, the prognosis for glioblastoma patients is still very poor with a mean survival time after diagnosis raging from 9 to 12 months. The treatment strategies for this disease have not changed appreciably for many years, and most are based on a limited understanding of the biology of the disease. In fact, although a number of genetic and molecular lesions have been correlated to glioblastoma progression, the deep understanding of the molecular markers is fundamental to develop targets for glioblastoma treatment. Small non-coding RNAs named microRNAs (miRs) are a class of endogenous non-coding, highly conserved RNAs of ~22 nucleotides in length that are encoded in plant and animal genomes. miRs are involved in the pathogenesis of most cancers (Calin and Croce, 2006). They negatively regulate mRNA expression by repressing translation or directly cleaving the targeted mRNA. In the last few years, our understanding of the role of miRNA has expanded from the initially identified functions in the development of round worms to a highly expressed and ubiquitous regulators implicated in a wide array of critical processes, including proliferation, cell death, differentiation, metabolism and, importantly, tumorigenesis (Croce, 2009). miRs profiling achieved by various methods has allowed the identification of signatures associated with diagnosis, staging, progression, prognosis and response to treatment of human tumors. Therefore, miRNA ‘fingerprinting’ represents a new addition to the diagnostic and prognostic tools to be used in medical oncology. In this study we evaluated by a miR array differential expression of miRs in tumorigenic glioma LN18, LN229 and U87MG cells compared with the non-tumorigenic T98G cells. Among different miRs we focused our attention on miRs -221 and -222. Our results show that miRs -221 and -222 target the protein tyrosine phosphatase μ (PTPμ). PTPμ regulates cell invasiveness and adhesion (Burgoyne *et al.*, 2009a), and has been found downregulated in human GB (Burgoyne *et al.*, 2009b). The present study describes for the first time PTPμ as a miR-222 and -221 target and explores the role of those miRs, through this target, in GB tumorigenesis and invasiveness.

Results

miRs expression in glioma cell lines

T98G, U87MG, LN-18 and LN-229 glioma cells have been described as having a different tumorigenic behavior (Cerchia *et al.*, 2009). In fact, while T98G are not able to grow in nude mice and to form colonies, U87MG, LN-18 and LN-229 cells are able to form tumors when injected in nude mice and to form colonies in soft agar, even in the absence of serum. We confirmed these data using soft agar and xenograft growth in nude mice (Supplementary

Figure 1). In order to investigate the involvement of miR in glioma tumorigenesis, we analyzed miR expression profile in the tumorigenic glioma LN-18 and LN-229 and U87MG cells versus non-tumorigenic T98G cells. The analysis was performed with a microarray chip containing 1150 miR probes, including 326 human and 249 mouse miRs, spotted in duplicates (Liu *et al.*, 2004). Pairwise significance analysis (PAM) of the microarray indicated that five *miR* genes were significantly overexpressed in tumorigenic cells with a >2.5-fold change (Table 1). We focus our attention on miR-221 and miR-222, as we and others have already demonstrated that those miRs are frequently overexpressed in a number of human tumors (Pallante *et al.*, 2006; Pineau *et al.*, 2010; Sredni *et al.*, 2010). In order to confirm the array results, we analyzed the levels of miR-222 and -221 with real-time PCR (RT–PCR). Accordingly with microarray data, we found an upregulation of these two miRs in tumorigenic cells (U87MG, LN-18 and LN-229) compared with non-tumorigenic one (T98G) (Figure 1a). We confirmed those data in a larger number of glioma cell lines, with a different tumorigenic phenotype (Supplementary Figure 3A). As shown in Supplementary Figure 3B also the non-tumorigenic cell lines LN-308, A172 and LN-428 express low levels of miR-221 and -222 if compared with the tumorigenic lines LN-319, LN-18 and LN-229. As expected, the predicted miR-221 and -222 targets, $p27^{kip1}$, and PTEN were expressed at decreased levels in the non-tumorigenic U87MG cells (Figure 1b) as compared with T98G cells. Similar results were obtained in the other non-tumorigenic cells (data not shown).

In order to verify the involvement of these miRs in glioma tumorigenesis we transfected the T98G cells with pre-miR-221 and pre-miR-222 or with a scrambled sequence, and U87MG cells with and anti-miR-221 and -222 or with anti-miR negative control. As shown in Figure 1c by soft agar assay, miR-221 and -222 overexpression resulted in a phenotypic change of T98G cells that become able to form colonies. Similar results were obtained in LN-18, LN-229 cells transfected with miR -222 or -221 (data not shown). Furthermore, the tumorigenic U87MG overexpressing anti-miR-221 and -222 exhibited a much weaker capacity to form colonies in soft agar. Similar results were obtained when T98G stably overexpressing miRs with a tween vector were plated in soft agar. Cells expressing the control tween vector did not produce colonies in soft agar (Supplementary Figure 3D), meanwhile T98G-tween-miR-221 formed a large number of colonies indicating a greater tumorigenic behavior of cells overexpressing miR-221/222.

Identification of PTPμ as a new target of miR-221 and -222

To find new miR-221 and -222 targets, we used bionformatics analysis. Comparing the results obtained from the different searches, we found that the protein phosphatase PTPμ was predicted as a target of miR-222 by the miRanda algorithm (www.microrna.org/microrna/home.do). RNAhybrid also predicted a possible binding region of miR-221 and -222 in the 3′ untranslated region (UTR) of PTPμ (Figure 2a). The most widely used approach for experimentally validating miRNA targets is to clone the predicted miRNA-binding sequence down-stream of a luciferase reporter construct, and to co-transfect it with the miRNA of interest for luciferase assays. To this end, we cloned the 3′UTR sequence of human PTPμ into the luciferase-expressing vector pGL3-control downstream of the luciferase stop codon; Meg01 cells were transiently transfected with this construct in the presence of pre-miR-221 and pre-miR222 or in the presence of a scrambled oligonucleotide

acting as a negative control. As reported in Figure 2c, miR-221 and miR-222 significantly reduced luciferase activity compared with the scrambled oligonucleotide. This indicates that miR-221 and -222 bind to the 3′UTR of *ptpm* and impair its mRNA translation. We observed a similar effect in the presence of both miRs added at the same time, indicating that the target site of the miRs in the 3′UTR of PTPμ is the same. In order to further confirm that the region was specific for the binding with miR-222 and -221, we generated a deletion mutant (Figure 2b) lacking the binding site, ATGTAGC. The mutant was cloned into the 3′UTR of the luciferase gene and co-transfected with pre-miR-221 and -222 in Meg01 cells. As shown in Figure 2d, miR-221 and -222 added singularly or at the same time, did not significantly reduce luciferase activity in the presence of the 3′UTR PTPμ mut sequence. This result indicates that miR-221 and -222 target PTPμ mRNA at the ATGTAGC sequence.

Expression of PTPμ and miR-222 and -221 in glioma

To assess whether the expression of PTPμ was inversely correlated with miR-221 and -222 in glioma cells, we analyzed the levels of the protein phosphatase in the different glioma cells. We found reduced PTPμ protein (Figure 3a) and mRNA levels (Figure 3b) in U87MG, LN-18 and LN-229 cells overexpressing miR-221 and -222 compared with T98G.

To confirm these data, we extended our analysis to different tumorigenic and non-tumorigenic cell lines. As shown in Supplementary Figure 3B, LN-308 and LN- 428 cells that express high levels of PTPμ protein express low level of miR-221 if compared with LN-319, LN-18 and LN-229 which overexpress miR-221 and have low levels of PTPμ. The only exception was represented by A172. These cells have been already reported to be non-tumorigenic (Ridder *et al.*, 1987; Liang *et al.*, 2002; Cerchia *et al.*, 2009). Our data show that in A172 cells PTPμ expression is low even in the presence of low miR-221 levels. However, A172 cells have been reported to be strongly invasive even if they are not tumorigenic (Ridder *et al.*, 1987). It is then possible that in this cell line other mechanisms are involved in the regulation of PTPμ expression and tumorigenic phenotype.

In order to establish a causative link with miRs-222 and -221 and PTPμ, we stably infected T98G cells with a Tween lentiviral construct expressing miR-221 and -222, and then analyzed PTPμ protein and mRNA levels. miR expression in infected cells was evaluated by RT–PCR (Figure 3c). We observed decreased PTPμ protein (Figure 3c) and mRNA expression levels (Figure 3d) in miR-222 and -221 stably expressing cells. The same effect was observed in T98G cells transiently transfected with a synthetic pre-miR-222 and miR-221. We also observed a decrease of known miR-221/222 targets, the proteins PTEN and $p27^{Kip1}$ (Figure 3e and f). The efficiency of miR expression upon transfection was monitored by RT–PCR (Figure 3e). Consistently with these data, U87-MG cells transfected with the anti-miR-222 and -221 showed an increase of PTPμ protein (Figure 4a) and RNA levels (Figure 4b). The efficiency of miR downregulation upon anti-miR transfection was monitored by RT–PCR (Figure 4c). Similar results were obtained by transfecting anti-miR-222 in LN-229 and LN-18 (data not shown).

miR-221 and -222 regulates cell motility in glioma cells

We hypothesized that miR-221 and -222 promote cell migration by regulating PTPμ expression. To this end, we analyzed cell motility through a transwell assay in U87MG, LN-229, LN-18 and T98G parental cells, and T98G transduced with control vector (T98G-tween), or lenti-miR221 and -222 vector (T98G miR-221 and T98G miR-222). As shown in Figure 5a, U87MG, LN-18 and LN-229 cells have a higher migration rate than T98G cells. The upregulation of miR-221 and -222 in T98G cells induced an increase of cell motility. The same result was obtained in T98G cells transiently transfected with miR-221 and -222. Conversely, expression of anti-miR- 222 was able to reduce cell migration of U87MG (Figure 5b). We also tested the effects of miRs or antimiR expression on cell adhesion, obtaining the same results (data not shown). In agreement with the existing data (Burgoyne *et al.*, 2009a), we demonstrate that transfection of T98G cells with two different PTP short hairpin RNA interference (shRNAi) (#1, #2) induced a strong reduction of PTP expression levels (Supplementary Figure 2B) and at the same time an increase of cell motility (Figure 5c). The same effect was not observed in T98G transfected with $p27^{kip1}$ and PTEN siRNAs (Supplementary Figure 4B). In addition, we also show that the overexpression of PTPμ complementary DNA in U87MG cells (Supplementary Figure 2A) induced a decrease of cell motility (Figure 5c). This result suggests that PTPμ protein is able to control cell motility in glioma cells. miRs may target different proteins. In order to demonstrate that migration/adhesion effects observed were carried out by PTPμ, we transfected T98G tween, tween-221 and tween-222 with ectopic PTPμ complementary DNA lacking the miRNA-binding site in its 3′UTR, before assaying migration and adhesion. Levels of transfected PTPμ were analyzed by western blot (Supplementary Figure 2A). Interestingly, transfection of PTPμ in miR-221- and -222-overexpressing U87MG cells, was able to overcome the effects of both miRs (Figure 5c). These rescue experiments proved the causative connection between miR-222, PTPμ and glioma cell motility.

Effect of PTPμ expression on other miR-221/222 targets

As it has been recently described that miRs targeting multiple proteins may differently affect cellular behavior (Poliseno *et al.*, 2010), we investigated whether inhibiting the expression of one target may affect the expression of the others. Therefore, to exclude that the effects of PTPμ knockdown were mediated in a large part by changes in PTEN and $p27^{Kip1}$ protein expression, we specifically silenced PTPμ and then evaluated expression levels of PTEN and $p27^{Kip1}$. As shown in supplementary Figure 2B, the knock down of PTPμ did not produce changes in expression of either PTEN or $p27^{Kip1}$. Moreover, silencing of PTEN or $p27^{Kip1}$ in T98G did not produce any changes in PTPμ protein expression (Supplementary Figure 4A) or in tumorigenicity, as the number of colonies grown in soft agar were comparable to that observed in cells transfected with a control siRNA (Supplementary Figure 4C).

miR-222 and PTPμ mRNA levels in glioma

To evaluate whether PTPμ downregulation in GB was related to increased miR-222 and -221 levels also *in vivo*, we analyzed PTPμ protein and miR-222 and -221 expression levels in tumor tissue specimens collected from 18 glioblastoma grade IV patients. miR-221 and -222 increased expression was not observed in all patient analyzed. We divided samples in

two groups: high-expressing miR-221 and -222, low-expressing PTPμ (A) (12/18, 67%) and low-expressing miR-221 and -222 and high-expressing PTPμ (B) (6/18, 33%). In both group we observed an inverse correlation between miR-221 and -222 with PTPμ (Figure 6).

To corroborate the inverse relation between miR-221/222 and PTPμ *in vivo*, *in situ* hybridization analysis was performed using 5′-digoxigenin-labeled locked nucleic acid probes on 66 glioma cancers (Table 2), followed by immunohistochemical detection of co-expression of PTPμ protein. As shown in Figure 7A, miR-222 was abundantly expressed in high-grade glioma and rarely found in normal cells. No co-expression was found with PTPμ. Same result was obtained for miR-221 (data not shown). Importantly, it was evident that miR-221/222 was abundantly expressed in grade III and IV aggressive cases glioblastoma as shown by comparing grade IV GB with oligodendroglioma, a slowly growing glioma (Figure 7). Conversely, PTPμ was highly expressed in oligodendroglioma and normal brain compared with grade IV glioblastoma.

Discussion

GB are among the most deadly types of cancer (Tran and Rosenthal, 2010). Advances in standard treatments for this tumor, such as surgery, radiotherapy and chemotherapy, have not significantly increased patient survival (Huse and Holland, 2010). The lethality of GB can be attributed to the capacity of the cells to migrate and develop foci throughout the brain (Demuth and Berens, 2004). It is thought that the invasive behavior of glioblastoma cells is one of the most important causes of poor clinical outcome, enabling tumor cells to actively egress from the main mass and invade the surrounding normal brain where they are out of reach of surgical resection, radiation and chemotherapy (Giese *et al.*, 2003).

The mechanisms of the spreading phenotype are not well understood so far. It was recently demonstrated that the receptor PTPμ negatively regulates GB cell migration (Burgoyne *et al.*, 2009a). PTPμ is the prototype of the type IIb subfamily of receptor PTPs (RPTP). This phosphatase is able to sense an extra-cellular signal via its extracellular segment and to transduce this signal intracellularly via its phosphatase activity (Brady-Kalnay *et al.*, 1995). In a xenograft mouse model of intracranially injected U87MG cells, PTPμ small hairpin RNA was able to induce cell migration and dispersal (Burgoyne *et al.*, 2009a). PTPμ may be considered as a ‘migration suppressor’ with regard to the diffuse infiltrative growth pattern observed in human gliomas. It was previously shown that PTPμ protein is downregulated in glioblastoma and that its levels correlated to tumor stage (Burgoyne *et al.*, 2009b). In particular, a striking loss of PTPμ protein was observed in highly dispersive GB compared with less dispersive low-grade astrocytomas and normal brain (Burgoyne *et al.*, 2009a). It was recently demonstrated that one mechanism of PTPμ downregulation in GB is proteolytic breakdown (Burgoyne *et al.*, 2009b).

In this work, we identified a new molecular mechanism of PTPμ downregulation in human glioma, by identifying two related miRs that target this phosphatase. In order to identify new signatures of GB invasiveness, we investigated the microRNA expression profile of tumorigenic glioma cells compared with non-tumorigenic cells. We identified five *miR* genes significantly overexpressed in tumorigenic cells with a >2.5-fold change. Among the

different miRs we focused our attention on two highly related miRs, miR-221 and -222. MiR-222 and -221 expression levels in human cancer have been extensively investigated (Garofalo *et al.*, 2008, 2009) and have been frequently found overexpressed in a number of human tumors (Pallante *et al.*, 2006; Garofalo *et al.*, 2008, 2009, 2010; Conti *et al.*, 2009; Pineau *et al.*, 2010). In previous works we demonstrated that miR-221 and miR-222 regulate death receptor signaling and TRAIL apoptosis sensitivity in non-small cell lung cancer and in hepatocarcinoma by modulating p27kip1, PTEN and TIMP3 expression (Garofalo *et al.*, 2008, 2009). In GB tissues and cell lines miR-222 and -221 were found overexpressed (Ciafrè *et al.*, 2005; Conti *et al.*, 2009) and correlated to the stage of the disease. However, the molecular targets of those miRs potentially involved in GB's invasive behavior had not been clarified. In this manuscript we provide evidence for the first time that miR-222 and -221 bind to the 3′UTR region of PTPμ and are able to downregulate PTPμ at RNA and protein levels. By luciferase assay, we also identified the 3′UTR region of the *PTP* gene that represents the miR-binding site.

As PTPμ has been described to be able to suppress glioma cell migration (Burgoyne *et al.*, 2009a), we hypothesized that miR-221 and -222 promote cell migration by downregulating PTPμ expression. In fact, the analysis of cell motility in T98G cells transduced with miR-221 or -222 demonstrated that upregulation of those miRs induced an increase of cell motility. The same result was obtained in T98G cells transiently transfected with either miR-221 or -222. Conversely the expression of anti-miR -222 was able to reduce cell migration. Comparable results were obtained when we analyzed cell adhesion phenotype of glioma cells. As miRNAs can affect many different proteins, we validated the migration/adhesion effects by co-transfection of miR-222 and ectopic PTPμ lacking the miRNA binding site in its 3′UTR. These rescue experiments proved the causative connection between miR-222/221 PTPμ and migration/invasion.

Moreover, our data show that in specimens of glioma, miR-222 and -221 expression inversely correlates with that of PTPμ. Therefore, miR-222 and -221 expression levels could predict the aggressive behavior of glioma. Over the past few years, several miRs have been implicated in various human cancers. Both losses and gains of miR function have been shown to contribute to cancer development through a range of mechanisms (Croce, 2009). Several miRs have been implicated in glioma formation, including miR-21, -7, -124a, -137, -221, -222, -34a, -125b, 146b and -181 family (Huse and Holland, 2010). Interestingly, in our model we found almost the same set of de-regulated miRs, indicating that they may be a molecular signature of glioblastoma cells.

Some of those miRs have been specifically described to be involved in the invasive glioma phenotype. In particular, inhibition of miR-21 reduces motility and invasiveness of glioma cells through the regulation of RECK, a membrane-anchored matrix metalloproteinase inhibitor important for extracellular matrix (ECM) remodeling (Gabriely *et al.*, 2008). Similarly, Xia *et al.*, 2009 described that miR-146b significantly reduced the migration and invasion of glioma cells by targeting one matrix metallo-proteinase family member, matrix metalloproteinase16. miR-7, through targeting of EGFR and AKT activity, has also been implicated in GBM invasiveness (Kefas *et al.*, 2008). Our data provide evidence for the role of miR- 222 and -221 in glioma cell migration/invasion through PTP regulation.

In conclusion, this study has identified a new mechanism of oncogenic action by miRs-222/-221 overexpression, leading to dissemination and invasion of GB cells, causing a very aggressive behavior. Our results suggest that miR-221 and -222 regulate glioma tumorigenesis at least in part through the control of PTPμ protein expression.

Materials and methods

Cell culture and transfection

U87MG, T98G, LN-308, LN-319, A172 and LN-428 cells were grown in Dulbecco's modified Eagle's medium. LN-18 and LN-229 were grown in Advanced Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Milan, Italy), Meg01 cells (human, chronic myelogenous leukemia cells) were grown in RPMI 1640+2mM glutamine+10% fetal bovine serum. Media were supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine and 100 U/ml penicillin/streptomycin. For miRs transient transfection, cells at 50% confluency were transfected using Oligofectamine (Invitrogen) with 100 nM (final) of pre-miR-221 and 222, scrambled or Anti miR-222 and Anti miR-221 (Applied Biosystems, Milan, Italy). For PTPμ transient transfection, cells were transfected using Lipofectamine and Plus Reagent with 5 μg of PTPμ complementary DNA (Origene, Rockville, MD, USA) for 24 h. To knock-down *PTPμ* gene, specific small hairpin RNA were obtained by Open Biosystems (Huntsville, AL, USA) and were transfected using lipofectamine 2000. After 24 h the cells were treated for 24 h with 500 ng/ml of puromicine for selection of transfected cells. Two clones stably expressing small hairpin RNAi-PTPμ were obtained. To knock-down $p27^{kip1}$ and PTEN, cells were transfected with $p27^{kip1}$- and PTEN-specific siRNAs from SantaCruz Biotechnology (Heidelberg, Germany) using lipofectamine 2000. TWEEN empty vector, TWEEN miR-221 or TWEEN miR-222 vectors, were obtained from Dr Ruggero De Maria (Rome).

Virus production

We produced vector stocks by calcium phosphate transient transfection, co-transfecting three plasmids in 293T human embryonic kidney cells, as these cells are good DNA recipients. The three plasmid are: the packaging plasmid, pCMVDR8.74 designed to provide the HIV proteins needed to produce the virus particle; the envelope-coding 57 plasmid, pMD.G, for pseudotyping the virion with VSV-G and TWEEN miR-221 or miR-222 vector, the transgene coding plasmids. The calcium phosphate-DNA precipitate was allowed to stay on the cells for 14–16 h, after which the medium was replaced, collected 48 h later, centrifuged at 1000 r.p.m. for 5 min at room temperature and filtered through 0.22mm pore nitrocellulose filters. On the day of infection, the medium was removed and replaced with viral supernatant to which 4 mg/ml of polybrene had been added. Cells were then centrifuged in their plate for 45 min in a Beckman GS-6KR centrifuge (Beckman, Milan, Italy), at 1800 r.p.m. and 32 °C. After centrifugation, cells were kept for either 1 h 15 min or ON in a 5% CO2 incubator at 32 or 37 °C, respectively. After exposure, cells were washed twice with cold phosphate-buffered saline and fresh medium added. At either 12 or 48 h after the infection, cells were washed with phosphate-buffered saline, harvested with trypsin/EDTA and analyzed by FACS for GFP expression. The GFP positive were sorted by a FACSscan.

Soft agar assay

In all, 10^3 cells were plated in 60mm dishes in a solution containing Dulbecco's modified Eagle's medium 2× (Sigma, St Louis, MO, USA), TPB Buffer (Difco, BD, Franklin Lakes, NJ, USA), and 1.25% of Noble Agar (Difco). Briefly, cells were harvested and counted then a layer of 7ml with the solution containing Noble Agar were left to polymerize on the bottom of the dishes. Then cells were resuspended in 2ml of same solution and plated. Cells were left grown for 2 weeks in the incubator.

Injection of glioma cells in nude mice

Nude mice were provided by Charles River. A total of10^5 cells of T98G, LN-18, LN-229 and U87MG were subcutaneously injected in one flank of the mice. Five mice for each cell type were injected. Mice were followed for 4 weeks and the tumors were measured and photographed.

Protein isolation and western blotting

Cells were washed twice in ice-cold phosphate-buffered saline, and lysed in JS buffer (50mM HEPES pH 7.5 containing 150mM NaCl, 1% glycerol, 1% Triton × 100, 1.5mM $MgCl_2$, 5mM EGTA, 1mM Na_3VO_4, and 1 × protease inhibitor cocktail). Protein concentration was determined by the Bradford assay (BioRad, Milan, Italy) using bovine serum albumin as the standard, and equal amounts of proteins were analyzed by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide). Gels were electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). For immunoblot experiments, membranes were blocked for 1 h with 5% non-fat dry milk in tris buffered saline containing 0.1% Tween-20, and incubated at 4 °C over night with primary antibody. Detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Amersham-Pharmacia Biosciences, GE Healthcare, Milan, Italy). Primary antibodies used were: anti-PTPμ (SantaCruz), anti-βActin (Sigma), P-Ser-AKT (Promega, Milan, Italy) and anti-p27, -AKT, -PTEN (Cell Signaling, Danvers, MA, USA).

miRNA microarray experiments

In all, 5 μg of total RNA from each sample was reverse transcribed using biotin-end-labeled random-octamer oligo-nucleotide primer. Hybridization of biotin-labeled complementary DNA was performed on a new Ohio State University custom miRNA microarray chip (OSU_CCC version 3.0, NCBI accession GPL5106), which contains 1150 miRNA probes, including 326 human and 249 mouse *miRNA* genes, spotted in duplicates. The hybridized chips were washed and processed to detect biotin-containing transcripts by streptavidin-Alexa647 conjugate and scanned on an Axon 4000B microarray scanner (Axon Instruments, Sunnyvale, CA, USA).

Raw data were normalized and analyzed with GENE-SPRING 7.2 software (zcomSilicon Genetics, Redwood City, CA, USA). Expression data were median-centered by using both the GENESPRING normalization option and the global median normalization of the BIOCONDUCTOR package (http://www.bioconductor.org) with similar results. Statistical

comparisons were done by using the GENESPRING analysis of variance tool, predictive analysis of microarray (PAM) and the significance analysis of microarray (SAM) software (http://www-stat.stanford.edu/~tibs/SAM/index.html).

Glioma cancer samples

A total of 18 paraffined high-grade glioma samples were collected at the Federico II University of Naples, Italy and tumor bank Unit of the Spanish National Cancer Research (CNIO) Madrid, Spain. RNA was isolated with RecoverALL Total Nucleic Acid Isolation kit from Ambion (Ambion Inc., Austin TX, USA). The samples were stored at −80 °C.

RNA extraction and RT–PCR

Total RNAs (miRNA and mRNA) were extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription of total miRNA was performed starting from equal amounts of total RNA/sample (1 μg) using miScript reverse Transcription Kit (Qiagen, Milan, Italy), for mRNA was used SuperScript III Reverse Transcriptase (Invitrogen). For cultured cells, quantitative analysis of PTPμ, β-Actin (as an internal reference), miR-221/222 and RNU5A (as an internal reference) were performed by RT–PCR using specific primers (Qiagen), miScript SYBR Green PCR Kit (Qiagen) and iQ SYBR Green Supermix (BioRad), respectively. The reaction for detection of mRNAs was performed as follow: 95 °C for 15′, 40 cycles of 94 °C for 15″, 60°C for 30″ and 72 °C for 30″. The reaction for detection of miRNAs was performed as follow: 95 °C for 15′, 40 cycles of 94 °C for 15″, 55°C for 30″ and 70 °C for 30″. All reactions were run in triplicate. The threshold cycle is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. For relative quantization the $2^{(-\Delta CT)}$ method was used as previously described (Livak and Schmittgen, 2001). Experiments were carried out in triplicate for each data point, and data analysis was performed by using software (BioRad).

Luciferase assay

The 3′ UTR of the human *PTPμ* gene was PCR amplified using the following primers: PTPμ Fw: 5′-TCTAGACGAGGTGGCCCTGGAATACTTGAATTCT-3′ and PTPμ Rw 5′-TCTAGAGCATTTTGTGAATGAGTCCTCCCCCAA-3′, and cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega). This construct was used to generate, by inverse PCR, the UTRmut-PTPμ plasmid (primers: PTPμ-mut: Fw 5′-GCATAATATATGCTTGCTTTCCAGGACTAACAGATAAATGTG-3′; Rw 5-′CACATTTATCTGTTAGTCCTGGAAAGCAAGCATATATTATGC-3′. MeG01 cells were co-transfected with 1 μg of UTR- PTPμ plasmid and with UTRmut- PTPμ plasmid and 1 μg of a Renilla luciferase expression construct pRL-TK (Promega) with Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h post-transfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions. We used MEG01 cell lines as they express low levels of miR-221 and -222 and also because they are easily transfectable (Incoronato *et al.*, 2010). Three independent experiments were performed in triplicate.

MiRNA locked nucleic acid *in situ* hybridization of formalin-fixed, paraffin-embedded tissue section

In situ hybridization was carried out on deparaffinized human glioma tissues sections using previously published protocol (Nuovo *et al.*, 2009), which includes a digestion in pepsin (1.3 mg/ml) for 30 min. The sequences of the probes containing the six dispersed locked nucleic acid locked nucleic acid-modified bases with digoxigenin conjugated to the 5′ end were: miR-221-(5′) GAAACCCAGCAGACAATGTAGCT; miR-222(5′) ACCCAGTAGCCAGATGTAGCT. The probe cocktail and tissue miRNA were co-denatured at 60 °C for 5 min, followed by hybridization at 37 °C overnight and a low-stringency wash in 0.2× SSC and 2% bovine serum albumin at 4 °C for 10 min. The probe-target complex was seen due to the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromo-chloroindolyl phosphate (NBT/BCIP). Negative controls included the use of a probe, which should yield a negative result in such tissues. No counterstain was used, to facilitate co-labelling for PTPμ protein. After *in situ* hybridization for the miRNAs, as previously described (Nuovo *et al.*, 2009; Incoronato *et al.*, 2010), the slides were immunostained to identify PTPμ protein expression. The anti-PTPm antibody (Santacruz) was incubated at 1:200 for 30 min. For the immunodetection, we used the Ultrasensitive Universal Fast Red system from Ventana Medical Systems. We used low-grade glioma tissues know to express PTPμ (Burgoyne *et al.*, 2009a) as positive control of PTP protein expression. The percentage of tumor cells expressing PTPμ and miR-221&222 was then analyzed with emphasis on co-localization of the respective targets.

Migration assay

Transwells Permeable Supports, 6.5mm diameter inserts, 8.0 μM pore size, polycarbonate membrane (Corning Incorporate, Corning, NY, USA) were used to perform migration assay. T98G and U87MG cells were grown as indicated above, then harvested by TrypLE Express (Invitrogen, Carlsbad, CA, USA), and 10^5 cells were washed three times and then resuspended in 1% fetal bovine serum containing Dulbecco's modified Eagle's medium medium and seeded in the upper chamber. The lower chamber of the transwell was filled with 600 μl of culture medium containing 10% fetal bovine serum, 5 μg/ml fibronectin, as an adhesive substrate. Cells were incubated at 37 °C for 24 h. The transwells were then removed from the 24-well plates and stained with 0.1% Crystal Violet in 25% methanol. Non-migrated cells were scraped off the top of the transwell with a cotton swab. Percentage of migrated cells was evaluated by eluting crystal violet with 1% SDS and reading the absorbance at λ 570 nm.

Statistical analysis

Continuous variables are expressed as mean values±s.d. One-tailed Student's *t*-test was used to compare values of test and control samples. $P<0.05$ was considered significant. Fold increase of the bands was calculated using ImageJ program available on web.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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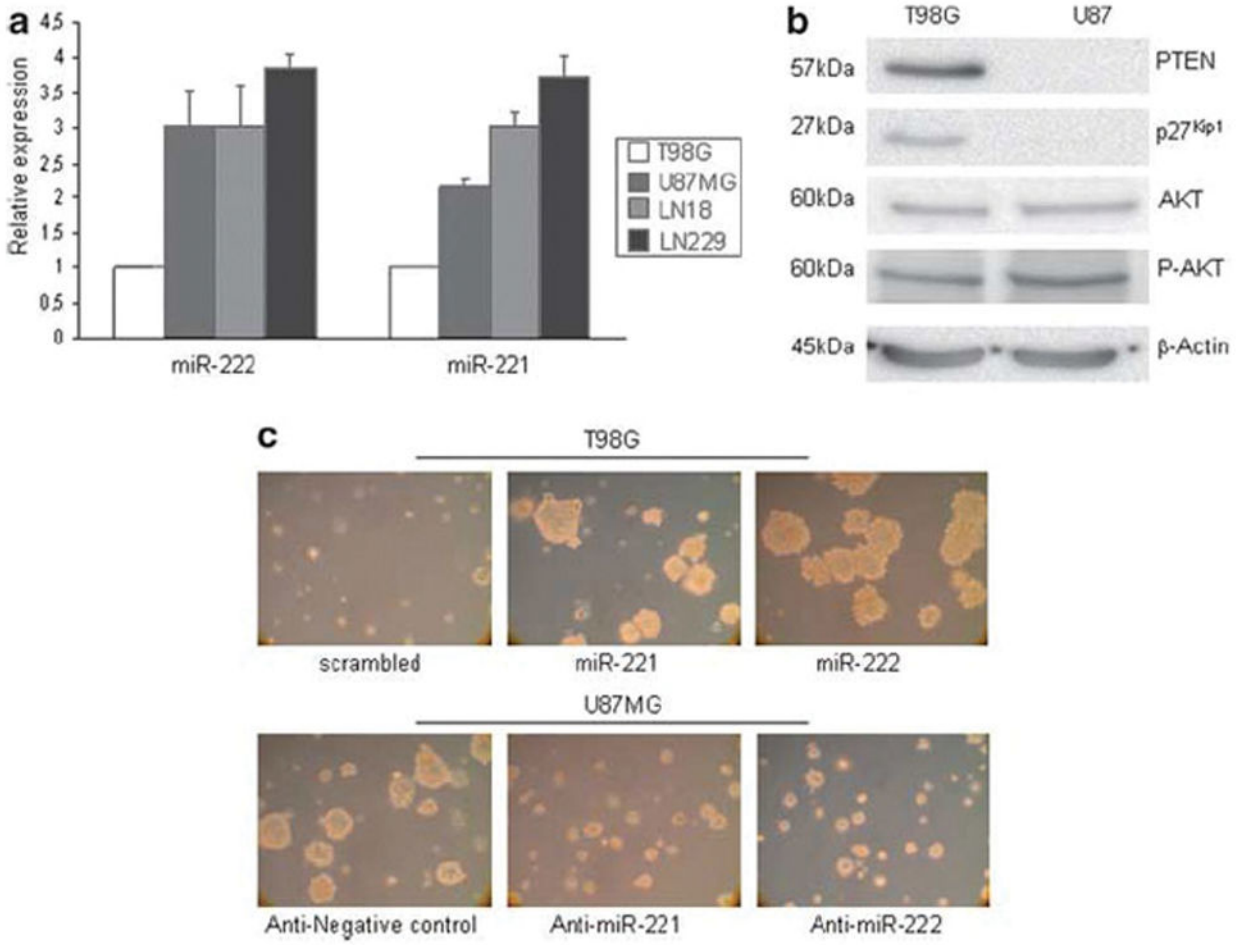

Figure 1.

Expression of miR-222 and -221 and their targets in T98G and U87MG glioma cells. (**a**) Real-time PCR of miR-221 and -222 in glioma cells. Representative of at least three independent experiments. (**b**)Western blot analysis of the known miR-221 and -222 targets, PTEN and $p27^{kip1}$. As a consequence of decreased PTEN protein levels, p-AKT levels were increased, although total AKT levels were comparable. β-Actin was used as the loading control. (**c**) Soft agar growth of T98G cells transiently transfected with miR-221, -222 or a scrambled sequence and of U87MG cells transfected with anti-miR-221, anti-miR-222 or a scrambled sequence.

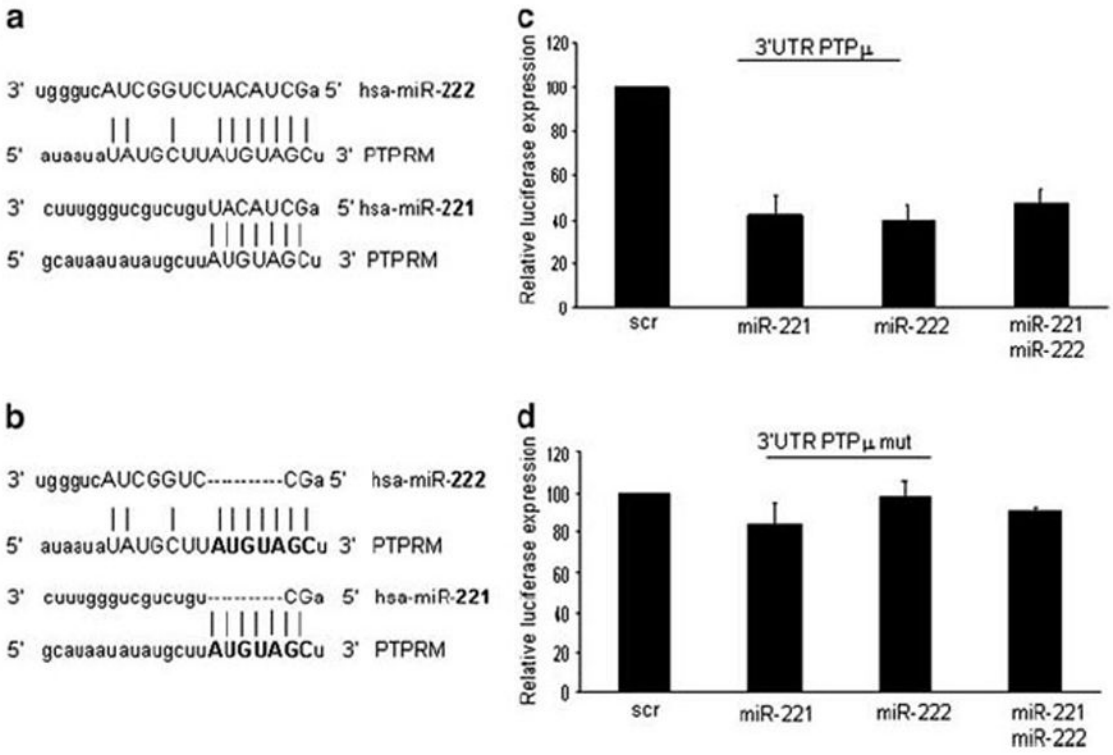

Figure 2.

Identification of target sites in the 3′-UTR of PTPμ. Complementary sites for miR-222 and -221 on wild-type (**a**) or mutated (**b**) PTPμ 3′UTR. The capital letters identify perfect base matches according to miRanda software (www.microrna.org/microrna/home.do). The bold letters identify the deleted regions. For luciferase activity, Meg01 cells were transiently co-transfected with the luciferase reporter containing wild-type PTPμ-3′UTR (**c**) or PTPμ-3′UTR mutant (**d**) in the presence of pre-miR-222, miR-221, pre-miR- 221 and miR-222 together or scrambled oligonucleotide. Luciferase activity was evaluated 24 h after transfection as described in Materials and methods. Representative of at least three independent experiments.

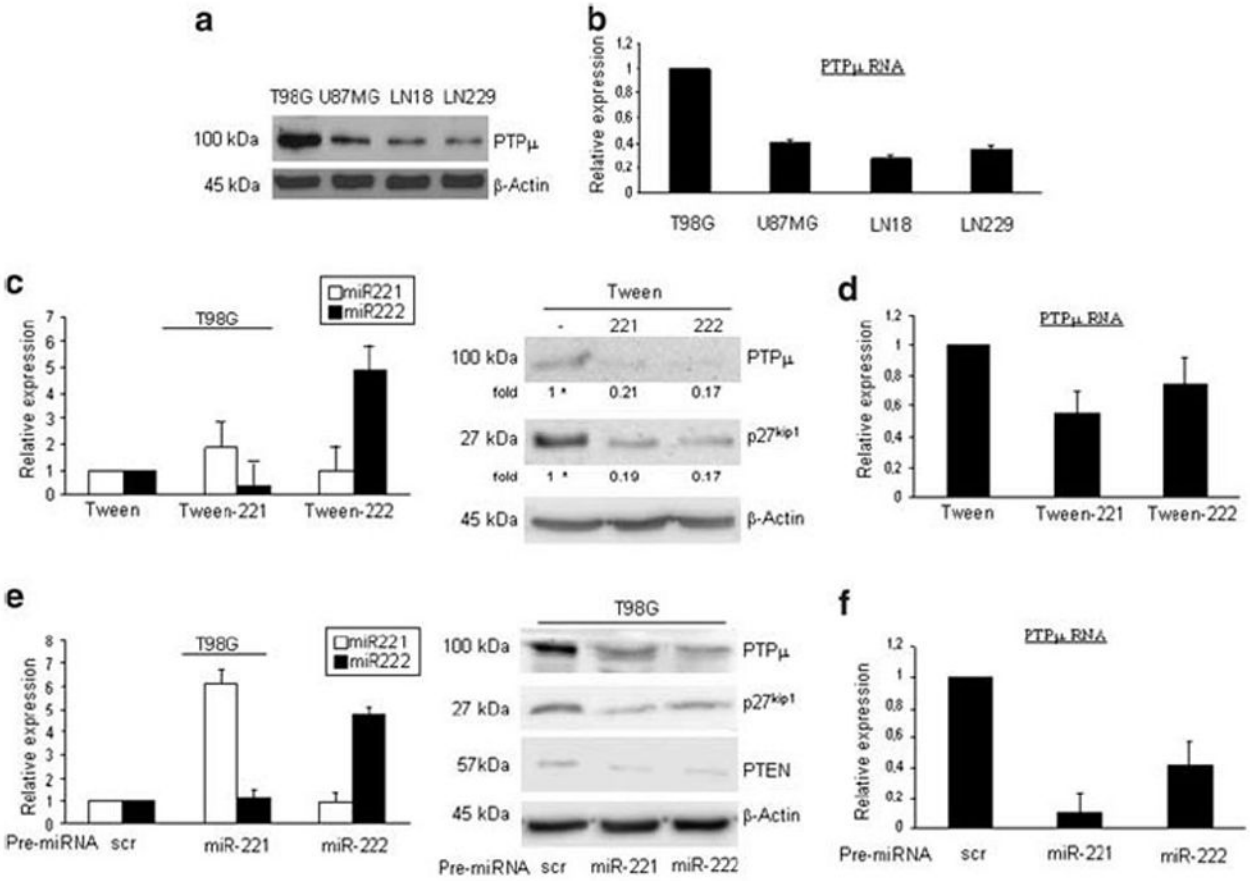

Figure 3.

PTPμ and miR-222/221 expression levels are inversely correlated in glioma. PTPμ protein (**a**) and RNA (**b**) expression levels in T98G, U87MG LN18 and LN229 cells. Cell lysates were immunoblotted with anti-PTPμ antibody. To confirm equal loading, the membrane was immunoblotted with anti–β-Actin antibody. Effect of miR transfection on PTPμ expression: miR-222 and -221 constitutive (**c**) or transient (**e**) expression in glioma cells induced a decrease of PTPμ protein (**c**, **e**) and RNA (**d**, **f**) expression levels. Relative expressions of mRNA and miR-221 were calculated using the comparative threshold cycle methods. Columns, mean of four different experiments; bars, s.d.. Fold values are expressed relative to the reference points indicated by * symbol.

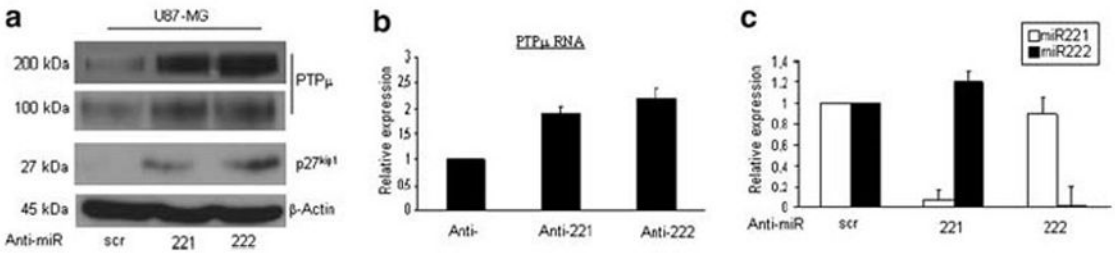

Figure 4.

Effects of anti miR-222 and -221 on PTPμ expression levels in glioma. PTPμ protein (**a**) and RNA (**b**) expression levels in U87MG cells transfected with anti miR-222, -221 or control scrambled (scr). The anti-miRs were able to increase PTPμ expression levels. (**c**) Anti-miR transfection reduced miR levels as analyzed by real-time PCR. Columns, mean of four different experiments; bars, s.d.

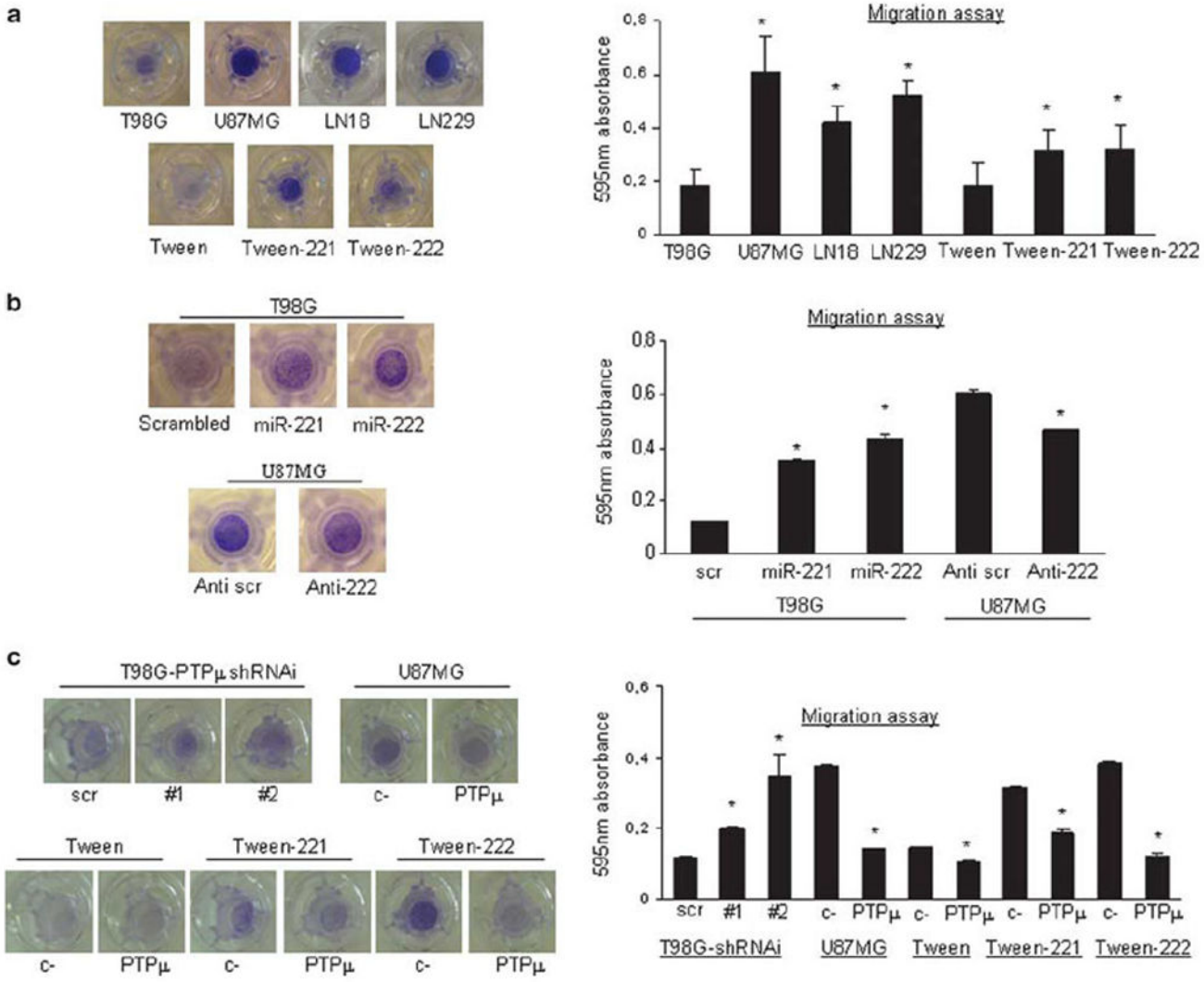

Figure 5.

Effects of miR-222 and-221 on cell migration and adhesion. T98G glioma cells showed lower migration compared with U87MG, LN18 and LN229 cells (**a**), whereas T98G cells stably (**a**) or transiently (**b**) transduced with miR-222 and -221 exhibited increased migration levels. Transfection of PTPμ complementary DNA in miR-222 and -221 overexpressing cells was able to rescue the effect of both miRs on invasion (**c**), whereas T98G transfected with two different PTPshRNAi (#1, #2) exhibited higher migration compared with control scrambled small hairpin RNA (scr) (**c**). Each assay was performed three times in independent experiments (*n*=3). Error bars indicate standard deviation. **P*<0.05

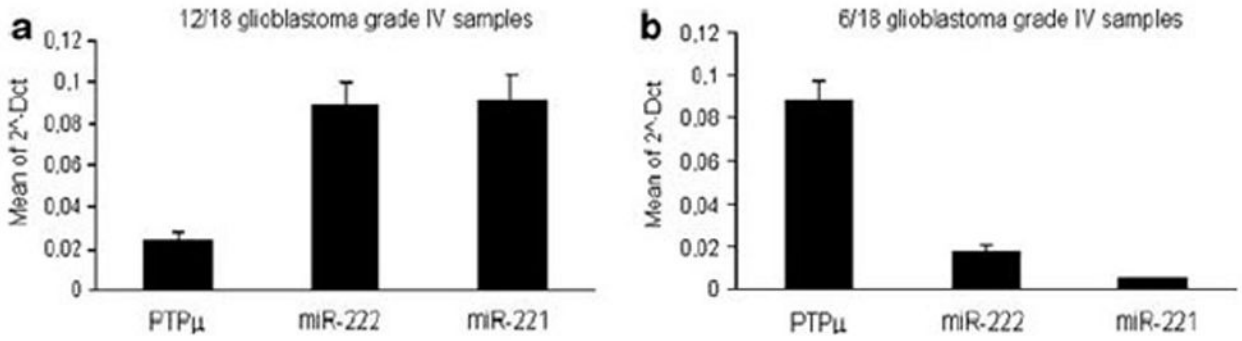

Figure 6.

Correlation of endogenous miR-222 and PTPμ mRNA expression levels in human glioma. Total RNA extracted from tissue specimens collected from 18 GB individuals was used to analyze miR-222/221 and PTPμ mRNA expression by RT–PCR. We divided samples in two groups: high-expressing miR-221 and -222 and low-expressing PTPμ (**a**) (12/18, 67%) and low-expressing miR-221 and -222 and high-expressing PTPμ (**b**) (6/18, 33%). In each group we observed an inverse correlation between miR-221 and -222 with PTPμ was observed.

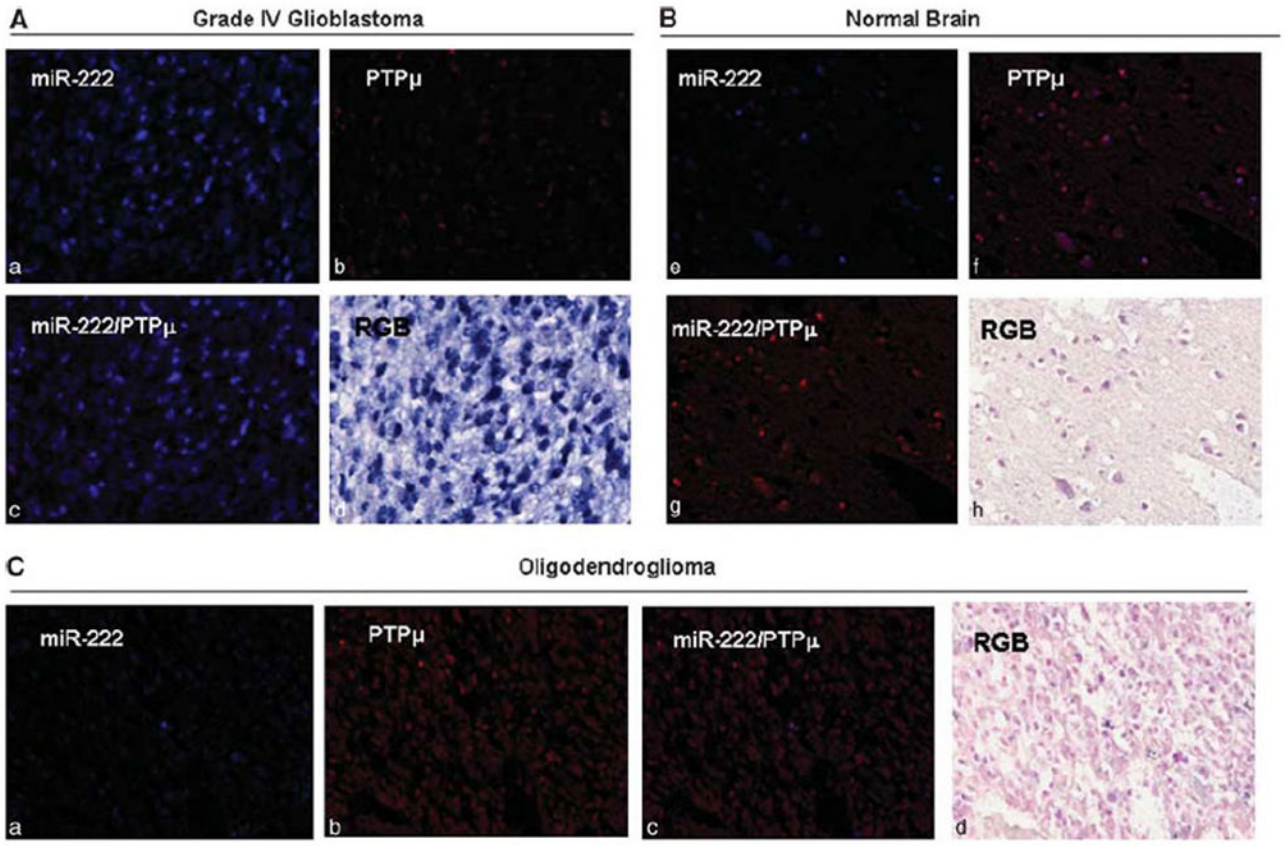

Figure 7.

Correlation of the *in situ* co-detection miR-221 and PTP expression in benign and malignant brain tissue. (**A**) A total of 66 low- and high- grade glioma on a TMA were analyzed for miR-222 expression by *in situ* hybridization and then for PTPμ, respectively, by immunohistochemistry. Panel **A** shows miR-222 signal (fluorescent blue) and panel B shows the PTPμ signal (fluorescent red) in a grade IV glioblastoma. Panel **C** shows the mixed signal in which fluorescent yellow is indicative of miR-222 and protein co-expression; note the lack of miR-222 and PTPμ co-expression. Panel **D** shows the RGB image of the *in situ* hybridization/immunohistochemical reaction shown in panels b–d. (**B**) In the normal brain one sees the miR-222 signal (fluorescent blue) in panel e and the PTPμ image as fluorescent red (panel f). As described in the text, the majority of normal brain was negative for miR-222 (panel e) and positive for PTPμ (panel f). Panel g shows the mixed signal in which fluorescent yellow is indicative of miR and protein co-expression. Panel h shows the RGB image of the *in situ* hybridization/immunohistochemical reaction shown in panels e–g. (**C**) Finally, on four oligodendrogliomas, only 1/4 was miR-222+ and 4/4 were PTPμ positive. Panel a shows miR-222 signal (fluorescent blue) and panel b is the PTPμ signal (fluorescent red). Panel c shows the mixed signal in which fluorescent yellow is indicative of miR and protein coexpression; note the lack of miR-222 and PTPμ co-expression. Panel d shows the RGB image of the *in situ* hybridization/immunohistochemical reaction shown in panels b–d.

Table 1

Upregulated miRs in tumorigenic glioma cells

MiR	Intensities of U87MG	Intensities of T98G	Fold change
hsa-miR-221	11150.1	1763.5	6.323
hsa-miR-125b	4697.4	1020.4	4.603
hsa-miR-21	6236.3	1662.4	3.751
hsa-miR-222	16685.8	6014	2.774
hsa-miR-34a	319.1	26.7	11.951

All differentially expressed miRs have $Q<0.01$ (false positive rate). *t*-test $P<0.05$.

These miRs were identified by PAM as predictor of glioma cells with the lowest misclassification error.

Table 2

PTPμ and miR-222 staining on glioma tumors

	Grade I and II astrocytomas (*N*=26)	Grade III and IV glioblastomas (*N*=40)	Total gliomas (*N*=66)
miR-222+/PTPμ−	7 (27%)	13 (32%)	20 (31%)
miR-222−/PTPμ+	3 (11%)	2 (6%)	5 (7%)

Abbreviations: PTPμ, protein tyrosine phosphatase μ.

Results of miR-222 *in situ* hybridization and PTPμ immunohistochemistry on 66 gliomas (26 Grade I and II, 40 Grade III and IV). N, indicates the number of sample analyzed.