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miR-4516 predicts poor prognosis and functions as a novel oncogene via targeting *PTPN14* in human glioblastoma

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Abstract

Glioblastomas (GBMs) are the most aggressive primary brain tumors, with an average survival of less than 15 months. Therefore, there is a critical need to develop novel therapeutic strategies for GBM. This study aimed to assess the prognostic value of miR-4516 and investigate its oncogenic functions and the underlying cellular and molecular mechanisms in GBM. To determine the correlation between miR-4516 expression and overall survival of patients with GBM, total RNAs were isolated from 268 FFPE tumor samples, miR expression was assayed (simultaneously) using the nCounter human miRNA v3a assay followed by univariable and multivariable survival analyses. Further, *in vitro* and *in vivo* studies were conducted to define the role of miR-4516 in GBM tumorigenesis and the underlying molecular mechanisms. Upon multivariable analysis, miR-4516 was correlated with poor prognosis in GBM patients (HR=1.49, 95%CI: 1.12–1.99, p=0.01). Interestingly, the significance of miR-4516 was retained including *MGMT* methylation status. Overexpression of miR-4516 significantly enhanced cell proliferation and invasion of GBM cells both *in vitro* and *in vivo*. While conducting downstream targeting studies, we found that the

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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tumor-promoting function of miR-4516, in part, was mediated by direct targeting of *PTPN14* (protein tyrosine phosphatase, non-receptor type 14) which, in turn, regulated the Hippo pathway in GBM. Taken together, our data suggest that miR-4516 represents an independent negative prognostic factor in GBM patients and acts as a novel oncogene in GBM, which regulates the *PTPN14*/Hippo pathway. Thus, this newly identified miR-4516 may serve as a new potential therapeutic target for GBM treatment.

Keywords

miR-4516; prognosis; *PTPN14*; Hippo pathway; GBM

INTRODUCTION

Glioblastoma (GBM), is the most common and the most aggressive of all adult primary tumors of the human brain with an average patient survival time of less than 15 months despite standard treatment with maximum safe resection followed by radio-chemotherapy (1). Recently, the World Health Organization has reclassified GBM, based on molecular features, into two broad subgroups: GBM with wild-type *isocitrate dehydrogenase (IDH)1/2* and GBM with mutant *IDH1/2*. However, less than 10% of primary GBMs have *IDH1/2* mutations (2). Significant prognostic biomarkers providing relative high-risk assessments for early clinical treatment selection and potential targets for novel therapies have remained to be identified and characterized in GBM (3, 4). To meet this critical need, we undertook high-throughput molecular profiling approaches to identify micro-RNAs (miRs) that correlate with clinical outcomes of patients with GBM harboring the wild-type *IDH*, who had significantly longer survival times compared with those harboring mutant *IDH* (2). Further, we employed molecular genetics approaches, *in vitro* and *in vivo*, to define the potential roles of candidate biomarker miRs that contribute to GBM pathogenesis and treatment response.

miRNAs are small, endogenous non-coding RNAs that act as negative regulators of mRNA translation at the post-transcriptional level by annealing with 3'-untranslated regions (UTRs) (5). To date, miRNAs have been reported as an important player in regulating tumor cellular functions such as cell motility, apoptosis, proliferation, and stress response (6, 7). Moreover, current literature indicates that miRNAs could be useful in the diagnosis and prognosis of human cancers (8, 9). Although the mechanisms of tumorigenesis are still being elucidated, miRNAs are emerging as novel promising, prognostic biomarkers in GBM. A few miRNAs including miR-326, miR-218, miR-377, and miR-423-5p have been validated as having functional significance in GBM biology (10–13). However, little is known about the novel role of miR-4516 in the development of cancer including GBM. This is the first study, to our knowledge, reporting an involvement of miR-4516 in GBM, one of the most lethal human malignancies, which remains relatively understudied. In the present study, we aimed to investigate the expression and function of miR-4516 in GBM and its potential targets. For the first time, we showed that miR-4516 is a novel prognostic biomarker of GBM patients and that *protein-tyrosine phosphatase non-receptor type 14 (PTPN14)* is a direct functional target of miR-4516. *PTPN14*, also called *Pez*, *PTP36*, *PTPD2* plays a role in the regulation

of cell-cell adhesion, growth, and cell motility (14, 15). *PTPN14* acts as a negative regulator of the oncogenic activity of *YAP*, a downstream signal transducer of the Hippo pathway, and functions as a tumor suppressor in various cancer types (16, 17). Thus, the current study provides insights into the molecular mechanisms underlying miR-4516-mediated tumorigenesis in GBM.

RESULTS

miR-4516 expression is negatively correlated with GBM patient overall survival (OS) and overexpressed in GBM cell lines.

To investigate the correlation between miRNA expression and GBM patient clinical outcome, we first profiled miRNA expression in GBM tumor samples using Nanostring v3 miRNA assay. miR-4516 was one of the top miRNAs correlated with OS (FDR $p < 0.05$). Upon UVA (HR=1.29, 95%CI: 1.12–1.48, $p < 0.001$, Table 1), miR-4516 was significantly correlated with worse OS. Figure 1A shows the median dichotomized miR association with survival. Multivariable analyses (n=185 patients with non-missing data available across all covariables) showed that miR-4516 was associated with poor prognosis which was independent of clinical variables (HR=1.49, 95%CI: 1.12–1.99, $p = 0.01$, Table 1). Importantly, a multi-marker multivariable analysis was performed with *MGMT* methylation status and miR-4516 retained its statistical significance (Supplementary Table 2). To determine if miR-4516 expression was associated with glioma grade, we evaluated expression of miR-4516 in glioma tumor samples by Taqman microRNA assay-based qRT-PCR. By categorizing all glioma RNA samples into grade II, grade III, and grade IV (8 glioma tissues each group) using the traditional WHO classification, we found that miR-4516 level was significantly upregulated in grade IV compared with grade II and III (Figure 1B). The endogenous expression of miR-4516 was further assessed by qRT-PCR in normal human astrocytes (NHAs) and 7 GBM cell lines (GBM30, U87MG, T98G, LN229, U251, U87MGvIII, and LN18). The results show that all GBM cell lines expressed varying levels of miR-4516, which were significantly higher than miR-4516 level in NHAs (Figure 1C). These results suggest that miR-4516 is significantly upregulated in different GBM cell lines and correlates with poor prognosis in GBM.

miR-4516 promotes proliferation and invasion of GBM cells

Given the fact that high expression of miR-4516 in GBM patients is associated with worse overall survival, we hypothesized that miR-4516 might play a role in the progression of GBM. To investigate the function of miR-4516 in GBM cells, LN229 cells with low endogenous expression of miR-4516 compared with other GBM cells (Figure 1C) were transfected with miRNA negative control (NC) or miR-4516 mimic, and overexpression of miR-4516 was confirmed by qRT-PCR (Figure S1A). In parallel, U87MG and LN18 cells that expressed relatively high levels of endogenous miR-4516 were transfected with NC or miR-4516 inhibitor. Cell proliferation assays showed that overexpression of miR-4516 significantly promoted proliferation and that inhibition of miR-4516 suppressed proliferation of GBM cells (Figures 2A–B). Colony formation assays demonstrated that GBM cells which overexpressed miR-4516 produced significantly more colonies than control (NC) cells (Figures 2C–D). Reversely, there were significantly fewer colonies in miR-4516 inhibited

cells than in NC cells (Figures 2E–F). Subsequently, we determined the role of miR-4516 in invasion using a Transwell Matrigel assay, which demonstrated that overexpression of miR-4516 enhanced invasion of GBM cells (Figures 2G–H), whereas inhibition of miR-4516 reduced the invasion (Figures 2I–J). Similar results were observed in LN18 cells (Figures S1B–D). A recent study shows that miR-4516 inhibition in human keratinocytes partially inhibits PUVA-induced apoptosis, suggesting that miR-4516 is involved in PUVA-induced apoptosis in these cells (18). However, in GBM cells, we found that inhibition of miR-4516 by anti-miR-4516 inhibitor produced a marginal increase in apoptosis compared with the control (Figures S2A–C). Taken together, these *in vitro* data suggest that miR-4516 promotes both proliferation and invasion of GBM cells thereby functioning as an onco-micro-RNA.

***PTPN14* is a putative direct target of miR-4516**

To identify putative mRNA targets of miR-4516, we performed bioinformatics analyses employing three different micro-RNA target prediction tools and found several candidate targets (Figure S3A). Among them, the 3'UTR of *PTPN14* mRNA contains sequences complementary to the miR-4516 seed sequence (Figure 3A). To verify whether *PTPN14* is a putative direct target of miR-4516, LN229, U87MG, LN18, and GBM30 cells were co-transfected with luciferase reporter constructs containing 3 putative miR-4516 targeting sequences in the 3'UTR of *PTPN14*, and NC/miR-4516 mimics. Consistent reduction in luciferase activity by miR-4516 was observed in all GBM cell lines examined (Figure 3B). To further validate target specificity, we generated mutated form of the 3'UTR (Figure 3A), where all the three binding sites of miR-4516 were destroyed using Q5 Site-Directed Mutagenesis Kit (NEB) that altered 3 key nucleotides. Co-transfection of NC or miR-4516 mimics with mutated forms of the 3'UTR (all three binding sites) significantly restored the luciferase activity (Figure 3B). Next, we examined the effects of miR-4516 on both mRNA and protein levels of *PTPN14*. Overexpression of miR-4516 markedly reduced the expression of *PTPN14* in LN229, LN18, U87MG, and GBM30 cells at both mRNA and protein levels (Figures 3C–D). Conversely, the expression of *PTPN14* at both mRNA and protein levels was increased after inhibition of miR-4516 in U87MG and LN18 cells (Figures S3B–D). To confirm that *PTPN14* is a putative target of miR-4516 in GBM tissues, the protein levels of PTPN14 were examined using IHC with a tissue microarray containing 56 GBM tissues. Overall, the majority of the GBM tissues (82.1% (46/56)) showed low cytoplasmic expression of PTPN14 (Figure 3E). Most importantly, we observed a highly significant negative correlation between the expression of miR-4516 and PTPN14 (by Nanostring, $p=0.012$, Figure 3F and by qPCR, $p=0.0007$, Figure S4, respectively). Simultaneously, we validated the miR-4516 expression levels obtained from Nanostring using qPCR in these GBM tissues and as expected, there is a strong positive expression correlation between Nanostring and qPCR ($r=0.602$, $p=2.315e-06$, Figure S5). Collectively, these data suggest that miR-4516 suppresses the expression of *PTPN14* by directly targeting its 3'-UTR in GBM cells both *in vitro* and *in vivo*.

***PTPN14* plays an essential role in the miR-4516-induced phenotype of GBM cells**

Downregulation of *PTPN14* is regarded as a trigger of cancer metastasis (14). However, the role of *PTPN14* in GBM is not fully understood. In this study, we hypothesized that the

oncogenic function of miR-4516 is mediated via direct targeting of the tumor suppressor *PTPN14*. To explore whether *PTPN14* has tumor suppressive function in GBM cells, we first overexpressed *PTPN14* in three different GBM cell lines (LN18, U87MG, and LN229), and confirmed that overexpression of *PTPN14* inhibited GBM cell proliferation (Figures S6A–D). Then, we examined whether downregulation of *PTPN14* would mimic the effect of overexpression of miR-4516; *PTPN14* was knocked down in both U87MG and LN229 cells by using RNAi (siRNA) (Figure 4A). As expected, blocking *PTPN14* expression significantly increased proliferative and invasive ability of both U87MG and LN229 cells (Figures 4B–C). Additionally, U87MG and LN229 cells were transfected with NC or miR-4516-inhibitor alone, or co-transfected with miR-4516-inhibitor and *PTPN14* siRNA. Western blot analyses showed that miR-4516 inhibition enhanced expression of *PTPN14* at protein level (Figure 4D). Further, functional assays demonstrated that miR-4516 inhibition significantly suppressed cell proliferation and invasion which were significantly reversed by knockdown of *PTPN14* expression (Figures 4E–F). Collectively, these data confirmed that the oncogenic potential of miR-4516 is largely mediated through the suppression of *PTPN14* expression in GBM cells.

miR-4516 regulates the Hippo pathway through targeting of *PTPN14*

PTPN14 was found to be involved in the Hippo signaling pathway through regulation of the Hippo effector YAP in 2012 (19, 20). Recently, studies have identified the Hippo/YAP signaling pathway as a key mechanism that controls cell growth and proliferation (21, 22). YAP, a pivotal effector of the Hippo pathway, is a transcriptional co-activator that is amplified in human cancers, and promotes epithelial-to-mesenchymal transition (EMT) and malignant transformation (23). Therefore, we sought to determine whether miR-4516 contributes to cell proliferation and invasion by regulating the Hippo pathway in GBM. It is known that Ser127-phosphorylation in YAP promotes its binding to 14–3–3 proteins, which causes cytoplasmic retention YAP, and upon de-phosphorylation, YAP moves to the nucleus and activates genes that promote cell proliferation and inhibits apoptosis (24). Accordingly, to investigate whether the Hippo pathway is affected by *PTPN14* in GBM, we determined the phosphorylation of YAP in cytoplasm of U87MG, LN18, and GBM30 cell lines transiently overexpressing *PTPN14*. As expected, overexpression of *PTPN14* resulted in an increase in cytoplasmic YAP levels of LN229, U87MG, LN18, and GBM30 cells (Figures 5A–C). Furthermore, we confirmed the translocation of phospho-YAP (Ser127) into the cytoplasm in *PTPN14*-overexpressing GBM30 cells (Figures S7A–B) as previously reported (16). To further determine whether miR-4516 regulates the Hippo pathway by affecting the expression of *PTPN14*, we overexpressed miR-4516 in U87MG, LN18, and GBM30 cell lines and determined the change of phosphorylation of YAP. As shown in Figures 5D–F, overexpression of miR-4516 considerably decreased S127-phospho-YAP in the cytoplasm, concomitant with downregulation of *PTPN14*. Taken together, these data indicate that miR-4516 regulates the Hippo pathway by targeting the expression of *PTPN14*.

Effect of miR-4516 on *in vivo* tumorigenicity

To determine the role of miR-4516 on tumor growth in mouse model, we generated stable U87MG/*EGFR*^{III}-luc/miR-4516 and GBM30-luc/miR-4516 cell lines by lentiviral transduction. In response to overexpression of miR-4516, the ability of proliferation was

increased in both U87MG/*EGFR*vIII-luc/miR-4516 and GBM30-luc/miR-4516 cells (Figures 6A–B). In addition, the ability of colony formation in U87MG/*EGFR*vIII-luc/miR-4516 and sphere formation rate in GBM30-luc/miR-4516 cells were increased (Figures 6C–D and Figures S8A–B). Then, an athymic nude xenograft mouse model was established, in which stable U87MG/*EGFR*vIII-luc/miR-4516 or GBM30-luc/miR-4516 cells were stereotactically implanted into the brain. Mice were sacrificed when they became moribund. As shown in Figure 6E, increased brain tumor formation and growth were observed with GBM cells after overexpression of miR-4516 as assessed by luminescent imaging, as well as reduced overall survival of mice (Figure 6F). Representative H&E staining is shown in Figure 6H. Compared with NC tumors, more aggressive tumors with infiltrative borders and higher mitotic index were found in tumors derived from miR-4516-overexpressing cells. Immunohistochemical staining for Ki-67 and *PTPN14* showed increased expression of Ki-67 and decreased expression of *PTPN14* in miR-4516 overexpressing tumors compared to NC tumors (Figures 6G–I). Taken together, these data clearly demonstrated that miR-4516 promotes GBM tumor growth and negatively correlated with expression of *PTPN14* in mouse xenograft models of human GBM patient-derived primary and established cell lines

DISCUSSION

Numerous therapeutic challenges are contributing to poor survival times in GBM patients, including aggressive tumor growth rates, heterogeneity, and therapeutic resistance (25). In order to find novel approaches for GBM treatment, it is imperative that current studies identify new biomarkers and therapeutic strategies (26). In this article, we report, for the first time to our knowledge, that 1) miR-4516 is associated with worse OS independent of *MGMT* methylation status in GBM patients; 2) miR-4516 promotes proliferation and invasion via targeting *PTPN14*-mediated regulation of the Hippo pathway in GBM.

Accumulated evidence indicates that miRNA abnormalities are involved in cancer development by negative regulation of tumor suppressor genes (27). Interestingly, our previous study, for the first time, identified a predictive model for the recurrence after salvage RT in prostate cancer and demonstrated that miR-4516 was overexpressed in patients that experienced treatment failure after salvage RT (28). Moreover, another study shows that high level of miR-4516 is associated with infiltrative growth of follicular variant of papillary thyroid carcinomas (29). However, little is known about the function of miR-4516 in GBM.

In this study, we are the first to investigate the association of miR-4516 expression and clinical outcome in GBM patients. In a comprehensive approach, miR-4516 was identified as one of the top miRNAs that associated with GBM patient OS among several miRNA candidates as assessed by both univariable and multivariable analyses. To our knowledge, this is the first study that indicates overexpression of miR-4516 is related with poor prognosis in GBM patients. Importantly, miR-4516 holds an independent prognostic value even in the context of *MGMT* methylation status in our cohort of patients and warrants further validation to assess its clinical utility as a prognostic biomarker.

To further substantiate the clinical significance of miR-4516 and investigate its biological mechanism for this observation, we performed *in vitro* and *in vivo* studies after overexpression and inhibition of miR-4516 in GBM cells. Our study characterized a possible biological role of miR-4516 in GBM, in which our results suggest that miR-4516 promotes GBM cell proliferation and invasion. Overexpressing miR-4516 in GBM cells induced tumor growth and invasion both *in vitro* and *in vivo*. As we know, the infiltrative nature of GBM is an important factor that limits treatment efficacy. Our study might open a new therapeutic strategy; however, the value of miR-4516 inhibitors as potential agents to inhibit tumor growth and invasion should be further tested in preclinical models.

To elucidate possible molecular mechanisms regulated by miR-4516, we performed a comprehensive *in silico* analysis to predict its putative targets. One of them, *PTPN14* (also called Pez, PTP36, PTPD2), a non-receptor tyrosine phosphatase, which is frequently mutated in a variety of human cancers, has been previously reported to be involved in inhibiting metastasis through reducing intracellular protein trafficking (14). It is reported to be a tumor suppressor in colorectal cancer (15), and acts as a regulator of oncogene *YAP*, which is a critical component of the mammalian Hippo pathway (30). The Hippo pathway controls cell number by inhibiting cellular proliferation and promoting apoptosis through phosphorylation of the transcriptional co-activator YAP (23). However, the cell signaling pathways regulated by *PTPN14* in GBM largely remain to be elucidated. The lack of information prompted us to focus on *PTPN14*. Importantly, for the first time, we found that miR-4516 exerts its oncogenic function by targeting *PTPN14*-mediated regulation of Hippo pathway in GBM. Additionally, we provided evidence from the luciferase activity assay that miR-4516 regulates *PTPN14* through directly binding to the predicted miRNA-mRNA binding site in the 3'UTR region of the *PTPN14* mRNA. These data confirmed the tumor suppressive role of *PTPN14* in GBM cells and provide evidence that *PTPN14* is an important mediator of miR-4516 induced regulation of the Hippo pathway. Importantly, we also confirmed that miR-4516 is negatively correlated with PTPN14 protein expression in our GBM patient samples. Furthermore, Chowdhari et al. have suggested that miR-4516 is important in PUVA-mediated apoptosis (18). However, in GBM cells, the underlying mechanisms through which miR-4516 functions as an onco-miRNA need to be further investigated. Notably, this is the first study indicating that miR-4516 can promote proliferation and invasion of GBM cells in part via directly targeting of *PTPN14*. In addition, a better understanding of the role of *PTPN14* in GBM, which has been proposed for the first time in this study, might also help to develop new therapeutic strategies for GBM patients. However, to restore tumor suppressive function of protein-tyrosine phosphatase function is a challenging task in translational research. It is important to highlight the limitations of the study. First, the prognostic value of miR-4516 should be further tested in additional cohorts as well as prospective biomarker studies; however, there are no data available from the TCGA database for miR-4516. Second, Chowdhari et al. (18) have shown that miR-4516 directly targets *STAT3* and *TP53* by binding to their cognate 3'UTR in HaCaT cells, and downregulates *STAT3*, *CDK6*, and *UBE2N* proteins. However, we did not see the effect of miR-4516 on *STAT3* expression (Figures S9A–B). Third, in this study, only one of the direct targets of miR-4516 was thoroughly investigated; therefore, more putative targets, such as *PIM2*, *MECP2*, *COL5A1*, and *CDKN1A* (Figures S9C–F) and their

downstream signaling pathways need to be investigated in future studies. In conclusion, this study indicates that miR-4516 represents an independent negative prognostic factor in GBM patients and plays a novel oncogenic role in GBM; moreover, miR-4516 promotes proliferation and invasion via targeting *PTPN14*-mediated regulation of the Hippo pathway in glioblastoma.

MATERIALS AND METHODS

Patient Cohort, Ethics Statement, and RNA Isolation

FFPE tumor specimens from 268 newly diagnosed GBM patients who do not have *IDH1*R132H mutation were used in the study. These patients underwent tumor biopsy or resection at the University of Utrecht (The Netherlands) during 2005 to 2014. The study was approved by the institutional review boards of The Ohio State University and Utrecht University Medical Center. Total RNA was isolated from the tumor specimens as described previously (28).

miRNA Expression Analysis

miRNA data were generated from 268 tumors as previously described (28) and R statistical computing software was used for data analyses (31). Raw NanoString data were log2 transformed for the filtering step. Per sample thresholds for missing values were calculated as 1.5 standard deviations above the mean of the negative controls. miRNAs were removed if > 90% of the values were below the threshold, and samples were removed if, after miR filtering, samples had > 60% missing values. The miRNA data were normalized by the geometric mean. This was accomplished using the filtered raw counts data set, by multiplying the expression of each miRNAs for each sample by the ratio of the mean of the geometric means across samples to the geometric mean of the sample. The filtered geometric mean normalized data were then log2 transformed for further analysis.

MGMT Promoter Methylation Analyses

DNA (250 ng) input was used per sample and the *MGMT*-STP27 model was used to calculate *MGMT* promoter methylation status from Illumina EPIC data (32). EPIC data were SWAN (33) normalized and M-value transformed using the minfi (34) package in R.

Cell Lines and Cell Culture

Normal human Astrocytes (NHAs) were purchased from Lonza (Walkersville, MD). Human GBM cell lines U87MG, LN18, LN229, U251, and T98G were from ATCC. U87MG/*EGFR*vIII cells were provided by Dr. Deliang Guo (The Ohio State University). U87MG/*EGFR*vIII-luc and primary GBM patient-derived GBM30-luc cells were provided by Dr. Balveen Kaur (The Ohio State University). U87MG/*EGFR*vIII-luc cells were grown in DMEM (Gibco) with 10% (v/v) FBS (Invitrogen), 100U/ml penicillin/streptomycin (Sigma), in the presence of 5% CO₂ at 37°C. GBM30-luc cells were cultured in neurobasal medium with EGF (20ng/ml), FGF (20ng/ml), B27 (1×), and Heparin (2μg/ml).

qRT-PCR Analysis of mRNA Expression and miRNA expression

Total RNA was isolated from GBM cells with TRIzol reagent as previously described (35). RNA (1000 ng) was subjected to reverse transcription to cDNA using high capacity RNA to cDNA kit (Applied Biosystems) and amplified by qPCR using SYBR Green (Qiagen). Resultant values were normalized against the internal control gene GAPDH for each replicate. The primers used for qPCR were listed in Supplementary Table 1. For miRNA expression analysis, RNA (100 ng) was reverse transcribed using the TaqMan Advanced MicroRNA Assay kit (Applied Biosystems) with miRNA-specific primers. Primers of Taqman MicroRNA Assays for hsa-miR-4516 (Cat. #A25576, 478303_mir) and hsa-miR-361-5p (Cat. #A25576, 478056_mir) were purchased from Applied Biosystems. Relative expression levels of miR-4516 were calculated by normalization with miR-361-5p expression in GBM cells, using 24 RNAs isolated from glioma tissues (Utrecht patient cohort, WHO grades II-IV). All the experiments were performed in triplicate.

Western Blot Analysis

Protein extraction and Western blot analysis were performed as previously described (35). The antibodies: PTPN14 (1:1000 dilution), pYAP (s127, 1:1000 dilution), total YAP (1:1000 dilution), c-parp/parp (1:1000) and GAPDH (1:1000 dilution) were purchased from Cell Signaling Technology (Denver, MA). Polyclonal goat anti-rabbit antibody (Cell Signaling Technology) and Western Blotting Detection System (Millipore) were used for exposure.

Immunohistochemistry (IHC)

A human GBM tissue array containing 56 cases was constructed at the University of Utrecht. IHC was carried out as previously described (36). Primary mouse antibody against PTPN14 (1:500, R&D Systems) was used. The percentage of positive cytoplasm-stained cells was scored as score 0 (0%), score 1 (1–10%), score 2 (11–50%), or score 3 (>50%). Score 0 and 1 was considered as low expression of PTPN14, whereas score 2 and 3 together as high expression of PTPN14 for statistical evaluation.

Immunofluorescence

Twenty-four hours post-transfection, cells were re-seeded onto 4-well cell culture slides, and then fixed and washed with PBS as previously described (35, 37). Primary p-YAP (s127, 1:100) antibody and fluorescence-labeled secondary antibody goat anti-rabbit Alexa Fluor 594 (1:1000, Invitrogen) were used. Samples were analyzed under a confocal microscopy (Carl Zeiss MicroImaging).

Transfection of miR-4516 Mimics and Inhibitors, siRNA Treatment, Plasmids, and Lentiviral Transduction

For functional studies, a specific miR-4516 mimic/inhibitor (Ambion) and a respective negative control (Ambion) were transfected into the GBM cells. For siRNA transfection, a specific *PTPN14* siRNA (50 nmol/L) (OriGene) and a negative control were used. pcDNA3/*PTPN14* plasmid containing *PTPN14* open reading frame (plasmid #61003) was purchased from Addgene (Cambridge, MA). For studying *in vivo* effects, stable miR-4516-overexpressing U87MG/*EGFR*vIII-luc and GBM30-luc cells were generated by lentiviral

transduction (Sigma). Cells were selected in the presence of 2 μ g/ml Puromycin (Sigma) for 7 days.

Luciferase Reporter Assay

The 3'UTR of *PTPN14* was synthesized, annealed, and then inserted into the Xho I and Not I sites of the pCheck2-reporter luciferase vector downstream of the luciferase gene. To introduce mutations, the sequences complementary to the binding sites of miR-4516 in the 3'UTR (site 1: 5'-UGUUUCUAGCUUCUCCC-3', site 2: 5'-UCCCCAACCUUCUCCU-3', site 3: 5'-UUCAUAGCCUUCUCCU-3') were replaced by site 1: 5'-UGUUUCUAGUUACACCC-3', site 2: 5'-UGUUUCUAGUUACACCC-3', site 3: 5'-UGUUUCUAGUUACACCC-3'. The constructs were confirmed by nucleotide sequencing. Cells were co-transfected with the wild-type or mutated constructs, pCheck2 plasmid, and equal amount of negative control or miR-4516 mimic. Luciferase assay was performed using Dual Luciferase Reporter Assay System (Promega).

Cell Proliferation and Invasion Assays

Twenty-four hours post-transfection, cells (1000 cells/well) were seeded in 96-well plates, washed, fixed, and stained after indicated time points as previously described (37). For GBM30 cells, the MTS assay (Promega) was used to measure cell proliferation. Cell invasion assays were performed as previously described (35). All the experiments were performed in triplicate.

Colony Formation Assay

GBM cells (100 cells/well) were used for colony formation assay as described previously (37). Briefly, 100 cells were plated onto 6-well tissue culture plates. Visible colonies were fixed and stained with 0.1% crystal violet in 20% methanol after 10–14 days. All the experiments were performed in triplicate.

Sphere formation assay

A total of 1,000 cells were incubated in neurobasal medium with B27 (1 $\times$), Heparin (2 μ g/ml), EGF (20ng/ml), and FGF (20ng/ml) in a humidified atmosphere with 5% CO₂ at 37°C. The number of spheres was counted two weeks after cell seeding. All the experiments were performed in triplicate.

Apoptosis assay

Apoptosis was detected using a FITC Apoptosis Detection Kit (Invitrogen). Cells were processed using flow cytometry according to the manufacturer's protocol. All the experiments were performed in triplicate.

Intracranial Mouse Model, Luminescence Imaging, and Mouse Survival Analysis

Six to eight week old athymic nude mice, obtained from the Target Validation Shared Resource at The Ohio State University, were used for the intracranial xenograft models. GBM cells (1×10^5 U87MG/*EGFR* Δ III-luc and GBM30-luc in 2 μ l PBS), transduced with miR-4516 or control were implanted into the mouse brain. For live tumor imaging, luciferin

(Perkin Elmer) solution (100mg/kg) was injected and after 10 min, a group of 5 mice were placed into a clear Plexiglas anesthesia box (2.5% isoflurane). The IVIS Lumina II imaging platform was used. The experiments were performed at the Ohio State University (OSU) Small Animal Imaging Core. Mouse survival time and tumor size were recorded and analyzed. All procedures were approved by the Subcommittee on Research Animal Care at The Ohio State University.

Target Analysis

Bioinformatic analysis was performed by using these specific programs: miRDB (38), Targetscan (39), miRTarBase (40).

Statistical Analysis

Analysis of Nanostring data was carried out in R. Cox regression was used to identify the association between the NanoString miRs (continuous) and overall survival with age as a covariable. P-values were adjusted by the false discovery rate method (41). miR-4516 was then median dichotomized and the log rank test was employed to visualize the association between the expression and overall survival. Cox regression was used for a comprehensive multivariable analysis of the continuous expression. Covariables included in the multivariable model were gender (categorical), age (continuous), KPS (categorical; < 70 vs 70 and above), treatment (categorical; “none”, “monotherapy TMZ or radiation only”, or “TMZ + radiation (Stupp protocol)”), and *MGMT* methylation status (methylated vs unmethylated). The SPSS 19.0 (SPSS, Chicago, USA) was used for other statistical analyses. Spearman’s rank correlation was performed to detect the correlation between qPCR and Nanostring data. The Wilcoxon rank sums test was employed to test the association between Nanostring/qPCR and PTPN14 protein expression level in tissues. Student’s t-test was carried out to detect the different miRNA and protein expression *in vitro*. P values were calculated two-sided and less than 0.05 were defined as statistically significant.

Supplementary Material

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Figure 1. Expression of miR-4516 in glioma patient samples and cell lines

(A) Correlation between miR-4516 expression and OS by Kaplan-Meier analysis of GBM patients with high (n=134) and low (n=134) expression of miR-4516. (B) qRT-PCR analysis of miR-4516 expression in grade II (n=8), grade III (n=8), and grade IV (n=8) glioma patient samples by qRT-PCR. Normalized by miR-361-5p. Alterations in miR-4516 expression are shown as box plot. **P<0.01. (C) Expression of miR-4516 in 7 glioblastoma cell lines (GBM30, U87MG, T98G, LN229, U251, U87MGvIII, and LN18) and NHA. Normalized by miR-361-5p. n=3, **P<0.01, ***P<0.001.

Figure 2. miR-4516 promotes proliferation and invasion of GBM cells.

LN229 cells were transiently transfected with miRNA negative control (NC) or miR-4516 mimics, respectively. U87MG cells were transiently transfected with NC or miR-4516 inhibitor. Cell proliferation was measured at the different time points. $n=5$, $**P<0.01$, compared with controls (A-B). Colony formation assay was performed after NC, miR-4516 mimics, or miR-4516 inhibitor transfection (C-F). $n=3$, $**P<0.01$. The transwell invasion assay was conducted and numbers of invaded cells were quantified (G-J). $n=3$, $**P<0.01$.

Figure 3. *PTPN14* is the putative direct target of miR-4516

(A) Luciferase reporter containing 3 putative miR-4516 targeting sequences in the 3'UTR of *PTPN14* (*PTPN14* 3'UTR wild-type) and mutated versions, containing 3 altered nucleotides (red). (B) LN229, U87MG, LN18, and GBM30 cells were co-transfected with the luciferase reporter constructs containing wild type (wt) or mutated *PTPN14* 3'UTR (mut) and NC or miR4516 mimic into. $n=3$, *** $P<0.001$. 72 hours post-transfection luciferase activity was measured and normalized. (C-D) qPCR and Western blotting to measure *PTPN14* mRNA and protein levels in LN229, U87MG, LN18, and GBM30 cells transfected with NC or

miR-4516 mimic. 72 hours post-transfection, cells were harvested for RNA and protein extractions. n=3, **P<0.01, ***P<0.001. (E) Immunohistochemical staining of PTPN14 on TMA showed the differential expression of PTPN14 in GBM tissues with low or high miR-4516 expression. Magnification: $\times 100$ and $\times 200$. (F) An inverse relationship between miR-4516 expression level and the PTPN14 protein level in 56 GBM tissues. P=0.012. Score 0/1 was considered as low expression of PTPN14, whereas score 2/3 together as high expression of PTPN14.

Figure 4. *PTPN14* plays a crucial role in miR-4516-induced cell growth and invasion.

(A) Western blot analysis for *PTPN14* in U87MG/LN229 cells transfected with siRNA control or *PTPN14* siRNA for 48hrs. (B) Cell proliferation assay for U87MG/LN229 cells transiently transfected with *PTPN14* siRNA. The cell proliferation rates were measured by methylene blue. $n=5$, $**P<0.01$. (C) Cell invasion assay for U87MG/LN229 cells transiently transfected with *PTPN14* RNAi using matrigel transwell membranes. $n=3$, $***P<0.001$. (D-F) U87MG/LN229 cells were transiently transfected with NC or miR-4516 inhibitor alone, or simultaneously with *PTPN14* RNAi. The protein expression of *PTPN14* was determined

using western blotting (**D**). Cell proliferation at 72 hours was determined using methylene blue (**E**). $n=5$, **, $P < 0.01$. The matrigel transwell invasion assay was carried out to quantify the invaded cells (**F**). $n=3$, ***, $P < 0.001$.

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Figure 5. miR-4516 regulates the Hippo pathway by targeting *PTPN14*

(A-C) U87MG, LN18, and GBM30 cells were transfected with either control (pcDNA3) or pcDNA3/*PTPN14* plasmid. Forty-eight hours post-transfection, western blotting was performed to determine the protein expression of PTPN14, pYAP, YAP, and GAPDH using respective antibodies. The intensity of each band was quantified using ImageJ and normalized to GAPDH and then to pcDNA3-transfected cells. (D-F) U87MG, LN18, and GBM30 cells were transiently transfected with either miRNA negative control (NC) or miR-4516 mimic. After 48 hours, western blotting was performed to determine the protein level of *PTPN14*, pYAP, YAP, and GAPDH using respective antibodies. The intensity of each band was quantified using ImageJ and normalized to GAPDH and then to NC-transfected cells.

Figure 6. miR-4516 promotes tumorigenicity *in vivo*.

(A-B) Cell proliferation assay for U87MGvIII-luc/GBM30-luc cells stably transfected with lentivirus control or lentivirus miR-4516. The cell proliferation rates at 72 hours were measured by methylene blue. $n=5$, ** $P<0.01$ (C) U87MG/EGFRvIII-luc/NC cells and U87MG/EGFRvIII-luc/miR-4516 cells (100 cells/well) were seeded into 6-well plates for colony formation assay and cultured for ~2 weeks to allow colony formation. The number of detectable colonies was counted. $n=3$, ** $P<0.01$. (D) GBM30-luc/NC cells and GBM30-luc/miR-4516 cells (1000 cells/well) were seeded into 6-well plates for sphere formation assay

and cultured for ~2 weeks. The number of detectable colonies was counted. $n=3$, $**P<0.01$. (E) Luminescent imaging for mice with intracranial tumor formed from GBM30-luc/NC and GBM30-luc/miR-4516 cells on day 10 after implanting (left). Quantification of luminescence signal intensity (right). Student t test was carried out for statistical analysis (mean $\pm$ SEM, $n=9$). (F) Kaplan-Meier survival analysis shows that overall survival of U87MGvIII-luc or GBM30-luc cells with stable overexpression of miR-4516 in comparison with miRNA-NC ($n=9$) (G) Representative images of strongly positive or weakly positive PTPN14 in GBM30-luc/NC and GBM30-luc/miR-4516 tissue are presented. Arrow head identifies a blood vessel with positive PTPN14 expression in endothelial cells as opposed to lack of expression on neoplastic cells. Magnification: $\times 400$. (H) Tumors were formalin-fixed, paraffin-embedded, sliced and stained with H&E. Representative images of each group are presented. Magnification: left: $\times 200$, right: $\times 400$. Cell proliferation was determined by Ki-67 using immunohistochemistry. Magnification: $\times 400$. (I) The *PTPN14* protein in GBM30-luc/NC and GBM30-luc/miR-4516 tissues displayed an inverse correlation with the expression of miR-4516.

Table 1

Univariable and Multivariable analyses of clinic-pathologic parameters of overall survival in patient with GBM

Parameter		UVA			MVA		
		HR	95% CI	P value	HR	95% CI	P value
Gender	Categorical	1.13	0.85–1.50	0.39	1.23	0.92–1.64	0.16
Age	Continuous	1.03	1.02–1.04	<0.001	1.00	0.99–1.02	0.23
KPS-70	< 70 vs 70 and above	0.55	0.40–0.76	<0.001	0.91	0.64–1.29	0.60
Treatment	None vs TMZ or IR alone	0.12	0.07–0.20	<0.001	0.12	0.070–0.21	<0.001
Treatment	None vs TMZ+IR (Stupp protocol)	0.03	0.02–0.05	<0.001	0.04	0.02–0.06	<0.001
miR-4516	Continuous	1.29	1.12–1.48	<0.001	1.49	1.12–1.99	0.01