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MicroRNA-378 enhances radiation response in ectopic and orthotopic implantation models of glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is the most common and highly malignant primary brain tumor, which is virtually incurable due to its therapeutic resistance to radiation and chemotherapy. To develop novel therapeutic approaches for treatment of GBM, we examined the role of *miR-378* on tumor growth, angiogenesis, and radiation response in ectopic and orthotopic U87 glioblastoma models. Cell and tumor growth rates, in vitro and in vivo radiation sensitivities, and tumor vascular density were evaluated in U87-GFP and U87-*miR-378* tumor lines. Ectopic tumor response to radiation was evaluated under normal blood flow and clamp hypoxic conditions. Results show that in vitro, *miR-378* expression moderately increased cell growth rate and plating efficiency, but did not alter radiation sensitivity. U87-*miR-378* tumors exhibited a higher transplantation take rate than U87-GFP tumors. In vivo, under oxygenated condition, subcutaneous U87-*miR-378* tumors receiving 25 Gy showed a tendency for longer tumor growth delay (TGD) than control U87-GFP tumors. In contrast, under hypoxic condition, U87-*miR-378* xenografts exhibited substantially shorter TGD than U87-GFP tumors, indicating that under normal blood flow conditions, U87-*miR-378* tumors were substantially more oxygenated than U87-GFP tumors. Intracranial multiphoton laser-scanning microscopy demonstrated increased vascular density of U87-*miR-378* versus control U87-GFP tumors. Finally, *miR-378* increased TGD following 12 Gy irradiation in U87 intracranial xenografts, and significantly prolonged survival of U87-*miR-378* tumor-bearing mice ($P = 0.04$). In conclusion, higher *miR-378* expression in U87-*miR-378* cells promotes tumor growth, angiogenesis, radiation-induced TGD, and prolongs survival of orthotopic tumor-bearing

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

hosts. Regulation of VEGFR2 by *miR-378* significantly increased vascular density and oxygenation in U87 xenografts.

Keywords

MicroRNA-378; Glioblastoma model; Orthotopic implantation; Radiation response; Tumor growth delay

Introduction

Glioblastoma multiforme (GBM) is the most common and highly malignant primary tumor of the central nervous system in adults [1–3]. There are approximately 16,000 newly diagnosed cases each year in the United States [2, 4]. The median survival time for GBM patients in most series is consistently around 9–12 months [5–7], with less than 5% of patients surviving 5 years post diagnosis [1–4]. The current standard of care for this disease, which usually involves surgical resection, radiation therapy, and chemotherapy with temozolomide [7–9]. This clinical care has improved the median survival time to 15 months [1, 4, 6–10], but the development of resistance and disease progression is common [8, 11]. Due to its highly aggressive nature and poor therapeutic options, better understanding of the genetic etiology and pathophysiology of GBM is necessary. To develop novel therapeutic approaches in the subsets of patients with high resistance to ionizing radiation and chemotherapy, sophisticated animal models are clearly needed [6, 8, 10–12]. Therefore, GBM has been selected as one of the three initial cancer types to be thoroughly studied in the cancer genome atlas program [13, 14]. U87MG, a long established cell line derived from a human grade IV glioma [15], is one of the most frequently studied cell lines of human brain tumors in our laboratory [16–22], as well as at various other institutions [6, 8, 10–12, 14, 23–26]. Tumor take and the growth of U87MG tumor xenografts is reproducible [5, 16–19], and the histological features of its original human tumor type are retained [16]. Although this cell line has many limitations, U87MG is resistant to radiotherapy—similar to human GBMs [5, 17, 27].

It has been known for decades that hypoxic cells are resistant to ionizing radiation compared to their well-oxygenated counterparts [23, 28]. Hypoxic cancer cells are more likely to survive radiotherapy; and are believed to be one of the factors contributing to local tumor treatment failure [29, 30]. Our studies have demonstrated that anti-angiogenic therapy can normalize tumor vasculature and hence improve tumor oxygenation and radiation response [20, 21].

In the present study, we use the U87 tumor model to study the effect of genetic modification on angiogenesis, tumor oxygenation, and radiation response. MicroRNAs (miR-NAs) are non-coding single-stranded RNA molecules with 18–24 nucleotides, which are produced from endogenous transcripts of short stem-loop structures. Through binding to the 3′-untranslated region (3′ UTR) of different target messenger RNAs (mRNAs), miRNAs can repress the translation of target mRNAs. As a new class of regulatory molecules, *miR-378* has diverse functions in cell proliferation and survival; tumor development, angiogenesis, invasion, and metastasis; as well as a correlation with the prognosis of glioma patients [31–

34]. However, whether *miR-378* regulates tumor response to radiotherapy remains unclear. Thus, in this study we investigated the impact of *miR-378* expression on U87 tumor cell proliferation and angiogenesis, genetically modifying these cells to over-express microRNA-378 in GFP-labeled U87 cells [U87-*miR-378*-GFP (referred to as 'U87-*miR-378*') [31]. We also examined whether *miR-378* enhances tumor response to radiation treatment in both U87 ectopic subcutaneous and orthotopic brain xenografts. We further studied its impact in the U87-*miR-378* orthotopic brain implantation model where the tumor microenvironment/tumor-stromal interactions may be important to treatment response [19, 35]. Our results show that higher level *miR-378* expression in U87-*miR-378* cells (1) improved cell proliferation, but did not change cell radiosensitivity in vitro; (2) promoted in vivo tumor growth, angiogenesis, and significantly enhanced tumor response to radiation treatment in ectopic or orthotopic implantation xenografts; and (3) prolonged survival with radiotherapy in U87 glioblastoma orthotopic brain implantation model, possibly by increasing tumor blood perfusion and oxygenation.

Materials and methods

Tumor cell line and doubling time assay

U87 glioblastoma cells transfected with either microRNA-378-GFP vector/pSV2 neo plasmid (U87-*miR-378*) or a control GFP vector (U87-GFP) used in the present study have been previously described [31]. The *miR-378* expression was confirmed by RT-PCR. A single cell suspension was prepared and 5×10^4 cells were plated in 25 cm² flasks in duplicate. 72 h later, the cells were trypsinized and counted. The cell doubling time (CDT) of each cell line was calculated by the equation: $CDT = (0.693 \times T) / \ln(N - N_0)$; where T is 72 h, N_0 is the number of cells plated at day 0, and N is the number of cells obtained 72 h later.

In vitro colony formation assay

Cells were plated into 25 cm² culture flasks; and irradiated 20–24 h later with 320-kV 12.5 mA, X-ray at a nominal dose rate of 1.66 Gray (Gy) per minute. Cells were then incubated for 14–18 days for colony formation depending on the dose administered. The surviving fraction data were corrected for initial and final multiplicity determined 4–6 h after plating and at the time of irradiation [36]. At each dose point, five flasks containing a two to four folds range of test cells were plated with the intent of obtaining 20–100 colonies per flask. U87-*miR-378* and U87-GFP cells were routinely plated in selection medium with or without G418 (400 µg/ml, Fisher BioReagents, Fair Lawn, N. J. Switzerland). For determination of initial and final multiplicity, 2000 test cells were plated in filtered conditioned medium without feeder cells, i.e., medium from cultures containing approximately two million cells in 5 ml medium for 24 h. Initial multiplicities were determined following attachment of the cells to the flask, and final multiplicities were determined at the time of irradiation. Following irradiation, the cells were cultured from 14 (for control cells) to 18 days (for cells exposed to high doses of radiation), i.e., when the number of colonies (formed with 50 or greater cells) per flask did not change. The colonies were then fixed, stained and quantified.

Animals and in vivo tumorigenesis assay

Six to eight week-old male NCr/Sed Nude (nu/nu) mice were bred and maintained in the Cox-7 defined flora animal facility [37]. Experimental mice were housed in micro-isolator cages, fed with sterile laboratory pellets, and given acidified sterile water *ad libitum*. All animal care and experimental procedures were carried out following the *Public Health Service Policy on Humane Care of Laboratory Animals* and approved by the Institution Animal Care and Use Committee (IACUC) at Massachusetts General Hospital.

To evaluate the tumorigenesis (tumor “take rate”) of U87-*miR-378* and U87-GFP cell lines in vivo, 10^6 cells (in 0.5 ml PBS) were injected subcutaneously (s.c.) into nude mice. Mice were observed weekly for tumor development and growth for eight weeks post-injection. Tumors that grew beyond 3 mm in diameter and showed progressive growth were counted as tumor “takes”. For tumorigenesis in the brain microenvironment evaluation, approximately 0.5 million cells in 3 μ l PBS were injected in the left cerebral cortex under cranial windows (CWs) which were created in nude mice 1 week prior to implantation [19]. Tumor development was observed twice per week by microscopy for 8 weeks. Any detectable tumor with progressive growth was counted as a tumor “take”.

In vivo imaging of orthotopic brain implanted tumors

To observe U87-*miR-378* and U87-GFP tumor growth and angiogenesis in the brain of mice under CWs, multi-photon laser scanning microscopy (MPLSM) imaging was used. It was conducted on a custom-built MPLSM using a confocal laser-scanning microscope body (BX-51, Optical Analysis) and a femtosecond laser source (High Performance Mai Tai, Spectra-Physics) [21, 22, 38]. Imaging studies were performed at 20 magnification, 0.95 NA water immersion objective (Olympus XLUMPlanFI, 1-UB965, Optical Analysis). Prior to each imaging session, mice were anesthetized by intraperitoneal injection of Xylazine/Ketamine (10 and 1.0 mg/kg, respectively). The tail vein was injected with 100 μ l rhodamine-dextran (Sigma-Aldrich) and positioned on a stereotactic frame. For each tumor, four adjacent images were obtained through the CW at day 3, and the same regions were revisited and recorded at day 7 post-implantation. The percentage of tumor vessels was analyzed by Image J software (<http://rsbweb.nih.gov/ij/download.html>).

Growth delay assay in ectopic subcutaneous xenografts

Small (1–2 mm diameter) fragments from U87-*miR-378* and U87-GFP source tumors were implanted subcutaneously into right rear legs of mice. A single source tumor from each cell line was used to initiate all tumors for each experiment. When the xenografted tumors reached 6 $\times$ 6 mm in diameter (or approximately 126–154 mm³), they were randomly assigned to receive a single dose (25 Gy) irradiation under normal blood flow (oxygen) or clamp hypoxia condition. Then tumor growth delay (TGD) was determined.

Growth delay and survival assays in intracranial orthotopic xenografts

To prepare source tumors for orthotopic brain implantation, 5×10^5 cells were injected into the cerebral cortex under the CWs of nude mice [18]. Three to four weeks later, ~ 0.2 mm diameter tumor fragments from both U87-*miR-378* and control U87-GFP source tumors were implanted into the cerebral cortex under the CWs at a depth of 0.4 mm. Tumor growth

and vasculature were evaluated by MPLSM. Tumor size was measured on alternating days, beginning at day 5 post-implantation. Measurements were obtained through CWs using a high-frequency ultrasonography (Vevo 2100, FujiFilm VisualSonics Inc., Toronto, Canada) [21]. When the tumor xenografts reached an average volume of 2 mm³, they were treated with a single dose of 12 Gy local irradiation (3.76 Gy per min; 10 × 10 mm field size). Survival experiments were terminated when mice became moribund, showed signs of paralysis or lost more than 15% of body weight. Growth delay was determined when control and irradiated tumors achieved a volume of over 40 mm³.

Western blotting

Western blot analysis was performed with 8% SDS/PAGE using standard methods. In brief, fresh nitrogen-frozen tumor tissues were placed directly into RIPA lysis buffer (Boston BioProducts Inc, MA Cat# BP-115) contained Complete ULTRA tablets, Mini EDTA-free (Roche Diagnostics, IN Cat# 05892791001), and PhosStop (Roche Diagnostics, IN Cat# 04906837001), incubated at 4 °C for 15 min, and then centrifuged at 14,000 rpm (~18,000×g), 4 °C, for 10 min for protein extraction. Approximately 20 µg of denatured protein per sample lysates were loaded on SDS/PAGE, separated by electrophoresis and transferred onto a polyvinylidene difluoride membrane (PVDF) using wet electroblotting transfer at 200 mA for 2.5 h. Non-specific binding was blocked with 5% non-fat milk for 1 h. Membranes were incubated overnight at 4 °C with the specific primary antibodies, followed by peroxidase-conjugated anti-rabbit HRP secondary antibodies. The following antibodies were used: Phospho-VEGF Receptor 2 Tyr1175 (1:200, Cell Signaling Technology, MA Cat# 2478), VEGF Receptor 2 (1:1000, Cell Signaling Technology, MA Cat# 2479), and β-Actin (1:5000, Sigma, MO Cat# A5441). Image J software was used for densitometric analysis. The ratio area pixels of the phosphor-VEGFR2 to total VEGFR2 was calculated.

Statistical analysis

Data are shown as mean ± SD. Difference in tumor take rates was analyzed by χ^2 tests (StatView, Abacus). Statistical differences between two groups of independent samples were compared by unpaired *t* tests (Two-tailed, StatView, Abacus). Survival curves of mice carrying orthotopic brain tumors were compared with the Gehan–Breslow–Wilcoxon test using GraphPad Prism 6 software. Differences were considered to be statistically significant when the *P* value was less than 0.05.

Results

***miR-378* increases proliferative capacity and plating efficiency of U87 cells but does not change cellular radiosensitivity**

RT-PCR analysis of total RNA extracted from cell lysates demonstrated that the U87-*miR-378* cells consistently expressed higher levels of mature *miR-378* as compared to control U87-GFP cells (*P* = 0.0047; Fig. 1a). As shown in Fig. 1b, U87-*miR-378* cells had a significantly shorter CDT (23.01 ± 2.92 vs. 26.23 ± 4.48 h) than U87-GFP cells (*P* = 0.0017). Colony formation assay (Fig. 2a) showed that U87-*miR-378* cells had significantly higher plating efficiency compared to control U87-GFP cells (0.48 ± 0.15 vs. 0.28 ± 0.09; *P*

< 0.0001). Cell survival curves of irradiated U87-*miR-378* and U87-GFP cells are shown in Fig. 2b. The responses of U87-*miR-378* and U87-GFP cells were not significantly different ($P = 0.177$) after correcting for both initial and final multiplicity. Thus, *miR-378* expression improved the plating efficiency of U87 cells, but did not change their intrinsic radiosensitivity.

Enhanced in vivo tumorigenicity of U87-*miR-378* cells

U87-*miR-378* cells had a greater in vivo tumorigenicity compared to control U87-GFP cells. Their tumor “take rates” were 16/16 (100%) versus 8/24 (33.3%) after s.c. injections in nude mice. To further characterize and assess tumorigenesis in the brain environment, 4/4 nude mice took the U87-*miR-378* tumors (100%) after CW injection whereas only 4/6 nude mice took the U87-GFP tumors (66.6%). Thus, the total take rate of the U87-*miR-378* cells (20/20, 100%) versus the U87-GFP cells (12/30, 40%) when merged with both of s.c. and CW injection results, was significantly different ($X^2 = 20 > 10.62$; $P < 0.005$) (see Supplemental Table).

U87-*miR-378* ectopic xenograft response to radiation

To determine if *miR-378* expression altered U87 tumor response to irradiation in s.c. xenografts, tumors were treated with a single dose of 25 Gy local irradiation under normal blood flow (oxygenated) conditions, when they reached approximately 6×7 mm (~ 132 mm³). Results are shown in untreated mice, U87-*miR-378* tumors grew faster than U87-GFP tumors (Fig. 3a). But in the treatment groups, the U87-*miR-378* tumors took a longer time to reach the tumor volume endpoint (1200 mm³) compared to the U87-GFP tumors (46.7 ± 5.0 vs. 41.9 ± 3.6 days; $P > 0.05$). However, when the same treatment (25 Gy single-dose irradiation) was performed under clamp hypoxic conditions, U87-*miR-378* tumors were more resistant to radiation, as evaluated by TGD (Fig. 3b). Irradiated U87-*miR-378* tumors reached 1200 mm³ in a shorter time (20.6 ± 2.7 vs. 46.7 ± 5.0 days) under oxygenated (normal blood flow) conditions ($P < 0.0001$). The TGD of U87-GFP tumors exposed to 25 Gy local irradiation, under hypoxic or oxygenated condition, was similar (41.6 ± 7.9 vs. 41.9 ± 3.6). Thus, only the U87-*miR-378* tumors' hypoxic fraction was changed under normal blood flow conditions. These results provide evidence that *miR-378* expression in U87 tumors significantly enhances tumor oxygenation and response to radiation treatment.

Enhanced response of U87-*miR-378* orthotopic xenografts to radiation

The U87-*miR-378* and U87-GFP tumors were implanted into the brains of nude mice in CWs. Tumor size was measured by high-frequency ultrasonography. As shown in the Supplemental figure, these U87-*miR-378* xenografts reached a tumor volume of 1.94 ± 1.49 mm³ on day 7 post-implantation, whereas it took an average of 14 days for U87-GFP brain xenografts to reach a volume of 1.88 ± 1.56 mm³. As was observed in s.c. tumor xenografts, orthotopic U87-*miR-378* tumor grew more rapidly than the U87-GFP tumors (reaching a size of 30 times its original at day 21 and 29 for the U87-*miR-378* and U87-GFP tumors, respectively). Also similar to the results observed in the s.c. transplanted tumors, radiation growth delay was substantially more pronounced in the U87-*miR-378* orthotopic brain xenografts vs. the control U87-GFP tumors. U87-GFP tumors achieved 30 times their original volume at 22 days following irradiation, whereas U87-*miR-378* orthotopic brain

tumors required 30 days. The resulting growth delay (irradiated vs. control) was 7 days for U87-GFP and 16 days for U87-*miR-378* tumors (Fig. 4a). Additionally, the survival time of the U87-*miR-378* brain tumor-bearing hosts after 12 Gy irradiation treatment, significantly exceeded the survival time of irradiated U87-GFP tumor-bearing mice in our study ($P=0.0408$) (Fig. 4b).

***miR-378* increases tumor vascular density in U87 orthotopic xenografts**

The vascular density of U87-*miR-378* and U87-GFP brain xenografts was evaluated by MPLSM as seen in Fig. 5a–d. The percentage of tumor blood vessels in U87-*miR-378* orthotopic tumors (Fig. 5e) was significantly increased on day 3 (0.036 ± 0.0036 vs. 0.00768 ± 0.00097 , $P < 0.0001$); and day 7 (0.18 ± 0.026 vs. 0.0665 ± 0.0108 , $P = 0.0002$) post-implantation. Thus, over-expression of U87-*miR-378* promoted tumor angiogenesis, and resulted in a higher density of tumor vasculature.

VEGFR2 is positively correlated with *miR-378* expression in U87 orthotopic xenografts

By western blotting of lysates extracted from U87 orthotopic xenografts, we found that vascular endothelial growth factor receptor 2 (VEGFR2) expression was significantly increased ($P = 0.0001$) in the *miR-378* expressing tumors (1.183 ± 0.309 , normalized to β -actin) compared to control U87-GFP tumors (0.4197 ± 0.239 , normalized to β -actin) (Fig. 6).

Discussion

Human GBM is the most common and aggressive malignant primary brain tumor in adults. GBMs generally display rapid cell proliferation and inadequately organized vascularization which frequently results in tumor regions of poor blood and local hypoxia [3, 38–40]. Hypoxia is a well-recognized component of the tumor microenvironment and has been linked to poor patient outcomes and resistance to therapies in different cancer types [29, 30, 39, 40]. In GBM, mechanisms of resistance to radiotherapy are not fully understood, given the genetic heterogeneity within the tumors, but there are several factors leading to therapy failure. Recent research has revealed that local hypoxia during radiation and miR-NAs may adversely impact the therapeutic response to radiotherapy [39, 40].

In this study, we found that human glioblastoma U87 cells overexpressing *miR-378* had an increased proliferative capacity and cell plating efficiency in vitro; as well as increased tumorigenicity in vivo. These findings are consistent with previously reported studies [31, 32]. Importantly, we have demonstrated that *miR-378* expressing U87 tumors are more responsive to local radiation treatment in vivo. In subcutaneous xenografts, U87-*miR-378* tumors exposed to a 25 Gy single dose of local irradiation under oxygenated condition exhibited a significantly greater tumor growth delay compared to the same treatment under clamp hypoxia (Fig. 3a, b; 46.7 vs. 20.6 days, $P < 0.0001$). In contrast, control tumor response to radiotherapy was minimally changed in oxygenated and clamp hypoxic conditions (Fig. 3a, b; 41.6 vs. 41.9 days, $P > 0.05$). These findings suggest that control U87 tumors were poorly perfused while *miR-378* expressing tumors were well perfused and thus more responsive to irradiation treatment. Our results also suggest that the U87 tumor used in

this study possesses a high hypoxic fraction—a characteristic when reportedly pertains to human GBMs in situ.

We next used an intracranial orthotopic U87 tumor model to evaluate the effect of *miR-378* expression on radiation response. In conjunction with in-house MPLSM, this model better replicated the brain microenvironment under normal blood flow conditions. The results were consistent with those observed in subcutaneous xenografts (Fig. 4a, b). That is, *miR-378* enhanced U87 tumor response to radiation by delaying tumor growth more than in control U87-GFP tumors (Fig. 4a, 16 vs. 7 days), and significantly prolonged the survival times of U87-*miR-378* orthotopic brain tumor-bearing hosts ($P = 0.0408$). This *miR-378* mediated effect was most likely due to the increased vascular density and tumor perfusion (Fig. 5). Previous studies showed that *miR-378* promotes U87 tumor angiogenesis by targeting SuFu and Fu-1 expression [31]. Our present study found additionally that *miR-378* expression was positively correlated with VEGFR2 (Fig. 6). The mechanism of this remains unclear and warrants further investigation. These results also should encourage deeper investigation with other tumor models, especially GBM models.

Increasing tumor perfusion and improving tumor tissue oxygenation are potential strategies to enhance GBM response to radiotherapy. Our study provides strong evidence that over-expression of *miR-378* increased the vascular density and perfusion in glioblastoma xenografts, thus enhancing local tumor response to radiotherapy and prolonging the survival of tumor-bearing hosts. Our results indicate that *miR-378* could be a potential target for GBM's radiotherapy. However, previous studies have shown that *miR-378* functions as an oncogene [31, 32], and may be implicated in tumor initiation and progression. Adjunct therapeutic dosing of microRNAs could be examined as a strategy to transiently increase tumor perfusion and improve radiation response. Furthermore, the *miR-378* expression could also be utilized as a biomarker for tumor sensitivity in glioblastoma.

A recent study also reported that *miR-124* expression negatively correlates with a hypoxic gene signature in glioblastoma patient samples, suggesting that low *miR-124* levels contribute to pro-survival stress responses in the disease [38]. These studies illustrate that *microRNA* role in human brain tumor initiation, development, and response to radiotherapy is complex. Further studies to analyze *miR-378* expression in clinical GBM samples and its correlations with their response to radiotherapy and clinical outcomes may lead to a better appreciation of *miR-378* in GBM pathophysiology.

Conclusion

U87-*miR-378* cells over-expressing microRNA-378 exhibit enhanced cell proliferation and colony formation in vitro, but did not affect cellular radiosensitivity. In vivo, U87-*miR-378* cells were more tumorigenic and exhibited more robust angiogenesis, likely mediated by increased VEGFR2 expression. Thus *miR-378* expression improved tumor oxygenation, thereby increasing the tumor local radiation response and the survival time of irradiated brain tumor-bearing hosts. Further preclinical exploration of *miR-378* as a novel therapeutic intervention is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

miR-378 over-expression promotes U87 cell proliferation. **a** RT-PCR analysis of mature *miR-378* using RNA prepared from U87 cells stably transfected with *miR-378* or a control vector, confirmed proper processing of the *miR-378* expression. Real-time PCR analysis confirmed the expression of mature *miR-378* ($P = 0.0047$, $n = 4$). **b** The doubling time of each cell line was calculated by the equation: $T_d = (0.693 \times T) / \ln (N/N_0)$. Compared with the control U87-GFP cells, U87-*miR-378* cells showed a significantly shorter cell doubling time ($P = 0.017$)

Fig. 2.

miR-378 increases the plating efficiency in U87-*miR-378* cells but does not change their radiosensitivity in vitro. **a** Plating efficiency (PE) of the U87-*miR-378* cells was significantly higher than the control U87-GFP cells ($P < 0.0001$). **b** Colonogenic survival following in vitro culture and irradiation: survival fractions from U87-*miR-378* cells and the control U87-GFP cells are not significantly different ($P = 0.177$, t test, unpaired confidence intervals are 1 SD)

Fig. 3.

miR-378 enhances U87 ectopic xenografts response to irradiation by improving tumor oxygenation. Source tumors were initiated from in vitro cultured cells by s.c. injected 2 million cells into each NCr/Sed-*nu/nu* (nude) mouse. Experimental tumors were initiated by s.c. implanted chunk of source tumors to the hind leg of 6–8 week-old male nude mice provided by our Cox-7 defined flora animal facility in MGH. When tumor reaches to 6 × 7 mm diameter post implantation, were randomly grouped into control and exposed to 25 Gy single dose irradiation at oxygenated (a) or hypoxic conditions (b). Following irradiation, the mice were examined twice weekly for the presence of tumor and progressive tumor growth. Growth curves of control (non-irradiated) and irradiated tumors exposed to single dose 25 Gy under oxygenated (a) or hypoxic (b) conditions are shown

Fig. 4.

miR-378 enhances U87 orthotopic xenografts response to radiation therapy and prolongs survival of tumor-bearing hosts. When orthotopic brain xenografts reached the average volumes at approximately 2 mm³, they received a single dose 12 Gy local irradiation. An assessment of their tumor growth delay and survival time was conducted. Survival studies were terminated when the mice became moribund or showed sign of paralysis, or lost more than 15% body weight. **a** Growth curves of control (non-irradiated) and irradiated U87-GFP and U87-*miR-378* orthotopic tumors exposed to single dose 12 Gy in CWs under oxygenated condition. Horizontal bars (1 SD) and data points are the days to achieve the specified volumes of the control growing and re-growing tumors. **b** Survival curves of tumor-bearing hosts in the control (non-irradiated) or irradiated groups after received single dose 12 Gy radiation therapy under oxygenated condition. Results showed the significant longer survival times of the U87-*miR-378* orthotopic brain tumor-bearing hosts after 12 Gy local irradiation treatments versus the control irradiated U87-GFP tumor-bearing mice ($P=0.0408$)

Fig. 5.

miR-378 promotes U87 brain tumor angiogenesis. MPLSM imaging was used to evaluate the angiogenesis brain tumors xenografts in the cranial window. Tumor vascularity in both U87-*miR-378* and U87-GFP brain tumors were evaluated on day 3 and 7 post-implantation. Red color shows the perfused vessels with rhodamine-conjugated dextran, and the green color shows the GFP-positive tumor cells. **a, b** Day 3 MPLSM imaging of U87-GFP (**a**) and U87-*miR-378* (**b**) brain tumors. **c, d** Day 7 MPLSM imaging of U87-GFP (**c**) and U87-*miR-378* (**d**) brain tumors. **e** Vessel density of the orthotopic tumors with or without *miR-378* transfected at day 3 and day 7 was conducted using Image J

Fig. 6. *miR-378* significantly increased VEGFR2 expression in U87 tumor cells. **a** Western blotting showed that vascular endothelial growth factor receptor 2 (VEGFR2) expression was significantly increased ($P = 0.0001$, see **b**) in the U87-miR-378 orthotopic tumors (1.183 ± 0.309 , normalized to β -Actin) when compared to that in control U87-GFP brain tumors (0.4187 ± 0.239 , normalized to β -Actin) (**b**). Densitometric analysis of VEGFR2 expression