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MiR-224 expression increases radiation sensitivity of glioblastoma cells

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ABSTRACT

Glioblastoma (GBM) is the most common and highly aggressive primary malignant brain tumor. The intrinsic resistance of this brain tumor limits the efficacy of administered treatment like radiation therapy. In the present study, effect of miR-224 expression on growth characteristics of established GBM cell lines was analyzed. MiR-224 expression in the cell lines as well as in primary GBM tumor tissues was found to be low. Exogenous transient expression of miR-224 using either synthetic mimics or stable inducible expression using doxycycline inducible lentiviral vector carrying miR-224 gene, was found to bring about 30–55% reduction in clonogenic potential of U87 MG cells. MiR-224 expression reduced clonogenic potential of U87 MG cells by 85–90% on irradiation at a dose of 6 Gy, a dose that brought about 50% reduction in clonogenic potential in the absence of miR-224 expression. MiR-224 expression in glioblastoma cells resulted in 55–65% reduction in the expression levels of *API5* gene, a known target of miR-224. Further, siRNA mediated down-regulation of *API5* was also found to have radiation sensitizing effect on glioblastoma cell lines. Analysis of the Cancer Genome Atlas data showed lower miR-224 expression levels in male GBM patients to correlate with poorer survival. Higher expression levels of miR-224 target *API5* also showed significant correlation with poorer survival of GBM patients. Up-regulation of miR-224 or down-regulation of its target *API5* in combination with radiation therapy, therefore appear as promising options for the treatment of glioblastoma, which is refractory to the existing treatment strategies.

1. Introduction

Glioblastoma (GBM) is the most aggressive and common primary malignant brain tumor. Deregulation of at least three signaling pathways including inactivation of tumor suppressive TP53 and pRB pathways and activation of oncogenic RTK/RAS/PI3K signaling pathway drive pathogenesis of GBMs [1]. Despite the advances in surgery, radiation therapy, and inclusion of temozolomide for the treatment of glioblastomas, the median survival of GBM patients remains dismal at 12–14 months [2]. Glioblastomas are refractory to the current mode of treatment primarily due to their inherent resistance to radiation and chemotherapy [3]. For effective treatment, it is necessary to bring about specific and complete cell death of glioblastoma cells overcoming their inherent resistance [4]. The multiplicity of the genetic alterations in GBMs, makes it difficult to design molecularly targeted therapy that would effectively bring about complete cell death of malignant glioma cells [3].

MicroRNAs are small non-coding RNAs, which regulate gene expression post-transcriptionally through mRNA degradation or translation inhibition [5]. Each miRNA is believed to target several hundred protein-coding genes. We have earlier reported distinctive miRNA expression profile of WNT subgroup medulloblastomas [6,7]. Like GBM, medulloblastoma is a grade IV malignant brain tumor. However, unlike GBMs most medulloblastomas respond to radiation and chemotherapy and as a result five year survival rate of these tumors is more than 70%. WNT subgroup in particular, have excellent prognosis with five-year survival rates of more than 95% [8]. We have earlier shown that miR-224, a miRNA over-expressed in WNT subgroup medulloblastomas increases radiation sensitivity of medulloblastoma cells [6]. MiR-224 expression has also been reported to bring about apoptosis of hepatocarcinoma cells [9]. In the present study we have therefore investigated effect of miR-224 expression on growth, and radiation sensitivity of glioblastoma cells.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Life Technologies (Carlsbad, CA, USA).

Abbreviations: GBM, glioblastoma; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; RT-PCR, Reverse Transcription-Polymerase Chain Reaction.

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Synthetic miR-224 mimic, control siGLO and DharmaFect transfection reagents were obtained from Dharmacon, Thermo Fisher Scientific, Lafayette, CO, USA. All other chemicals used were of the highest quality available commercially.

2.2. Cell culture

U87 MG cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). LN229 cells were obtained from National Centre for Cell Science, Pune, India (originally from ATCC, Manassas, VA, USA). HEK293FT cells were obtained from Invitrogen, Life Technologies, Carlsbad, CA, USA. The cells were maintained in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO_2.

2.3. Transient transfection

The cells were transiently transfected with miR-224 mimic or siRNAs targeting *API5* using DharmaFECT 1 or DharmaFECT 2 transfection reagents as per the manufacturer's protocol (Dharmacon, Thermo Fisher Scientific, Lafayette, CO, USA) for a period 48 h. The medium was replaced and the cells were allowed to recover from the transfection for another 24 h before assessing the effect of miRNA expression on the cell behavior in various assays as described below. The nucleotide sequences of the two API5 siRNAs used are given in Supplementary Table 1.

2.4. Stable inducible miR-224 expression using lentiviral vector

The genomic region encoding miR-224 was cloned in doxycycline-inducible pTRIPZ lentiviral vector (Open Biosystems, Thermo Fisher Scientific, Huntsville, AL, USA) by replacing the shRNA-miR cassette but retaining the Drosha cleavage sites in the vector. To generate lentivirus particles, HEK 293FT cells were transfected with pTRIPZ-*miR-224* construct and packaging constructs psPAX2 and pMD2.G (Addgene, Cambridge, MA, USA). The virus containing supernatants were harvested, filtered, and supplemented with polybrene (4 μg/ml) during infection of U87 MG cells. For stable transfection, the infected cells were selected in the presence of puromycin (400 ng/ml). Stable polyclonal populations of U87 MG cells expressing Non-Targeting (NT) control pTRIPZ vector and pTRIPZ-miR-224 were generated and analyzed for inducible expression of miR-224 by real time RT-PCR.

2.5. MTT viability assay

1000 cells of either U87 MG or LN229 cells, control/stably/transiently transfected cells, were seeded per well of a 96-well plate. The medium was replenished at 2–3 days intervals. For assessing cell viability, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) solution (20 μl of 5 mg/ml in PBS) was added per well, and the formazan crystals were allowed to form over a period of 4 h. The crystals formed were dissolved in 100 μl of acidified 10% SDS. Optical density was read on an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm with a reference wavelength of 690 nm.

2.6. Soft agar assay

Anchorage-independent growth of miR-224 expressing U87 MG cells was studied by their potential to form colonies in soft agar. The cells were seeded in triplicate at a density of 2000 cells/35 mm plate in medium containing 0.4% agarose as a top layer over a bottom layer of 1% agarose. The cells were incubated for 10–15 days and colonies formed were counted.

2.7. Clonogenic assay

Clonogenic assay was performed to study the radiation sensitivity of glioma cells over-expressing miR-224 in transient or stable manner. 1000 cells were plated per 55 mm plate and then irradiated at a dose of 6 Gy (Cobalt-60 gamma irradiator, Tata Memorial Centre). The medium was changed 24 h later and the cells were allowed to grow until microscopically visible colonies formed. The cells were fixed in chilled methanol: acetic acid (3:1), stained with 0.5% crystal violet dye and the colonies were counted.

2.8. Real time RT-PCR analysis

MiR-224 levels in transfected cells were checked by real time RT-PCR (Reverse Transcription-Polymerase Chain Reaction) analysis using *RNU48* as a control house-keeping small RNA using Taqman assay (Applied Biosystems, ThermoFisher Scientific, Lafayette, CO, USA). *API5* expression levels were evaluated by real time RT-PCR analysis by SYBR Green assay using *GAPDH* as a house-keeping control gene (sequences of the primers used are given in Supplementary Table 1). $RQ = 2\text{^} - (Ct_{test} - Ct_{control}) \times 100$.

2.9. Analysis of the Cancer Genome Atlas (TCGA) expression profile data of GBM tumor tissues

Tertiary level expression data of GBM tumor tissues was downloaded from https://tcga-data.nci.nih.gov web site. For miR-224 and *API5* expression levels, Agilent miRNA 8×15 K array data and Affymetrix HT_HG U133A array data of 505 GBM tumor tissues, was used for the analysis. Distribution of miR-224 and *API5* expression levels by frequency distribution analysis, survival analysis by Kaplan–Meier method and statistical significance between the survival curves was estimated by Log rank test using Graph Pad Prism v 5.0 (GraphPad Software, La Jolla, CA).

Student's *t* test was performed to check for statistical significance of the difference in the performance of miRNA-transfected cells as compared to control siGLO (a non-targeting control siRNA) or pTRIPZ-NT control vector transfected cells.

3. Results

MiR-224 is not expressed in U87 MG cells while it is expressed in the range of RQ 0.2–0.4 in LN229 cells. In order to study the effect of miR-224 expression on growth of glioblastoma cells, U87 MG glioblastoma cell line was transduced with pTRIPZ lentiviral vector expressing miR-224 in a doxycycline inducible manner. Polyclonal population of U87 MG cells expressing pTRIPZ-miR-224 construct in a stable manner was selected in the presence of puromycin. Doxycycline induction of the polyclonal population transduced with pTRIPZ-miR-224 viral vector resulted in miR-224 expression in the range of RQ 25–50. In parallel, both U87 MG and LN229 cells were also transfected with synthetic miR-224 mimic at 25 nM concentration that brought about miR-224 expression in the range of RQ 5–20 and 5–10, respectively.

3.1. Effect of miR-224 expression on growth of glioblastoma cells

Doxycycline treatment resulted in marginal (15–25%) growth inhibition of U87 MG cells as judged by MTT assay (Fig. 1A). The polyclonal populations of U87 MG cells transduced with either control pTRIPZ-NT or pTRIPZ-miR-224 viral vector exhibited growth inhibition to similar extent on treatment with doxycycline. Thus, doxycycline induced miR-224 expression was not found to have any effect on growth of U87 MG cells. Further, in order to

study effect of miR-224 expression on anchorage-independent growth potential of U87MG cells, soft agar colony assay was performed. Doxycycline induction of miR-224 expression in the stable polyclonal population of U87 MG cells was found to bring about 30–40% reduction in the soft agar colony number. Transfection of U87MG cells with 25 nM miR-224 mimic was found to bring about 40–55% reduction in the number of soft agar colonies (Fig 1B and C).

3.2. Effect of miR-224 expression on clonogenic potential and radiation sensitivity

MiR-224 expression in U87 MG cells transfected with miR-224 mimic was found to reduce clonogenic potential of the cells by 42–50% as judged by the clonogenic assay (Fig. 2A and B). Irradiation at a dose of 6 Gy resulted in reduction of clonogenic potential of control siGLO transfected Daoy cells by 50–60% while Irradiation of miR-224 mimic transfected U87 MG cells resulted in reduction of clonogenic potential by 85–90%. Thus, miR-224 expression was found to bring about reduction in clonogenic potential by itself and further enhance the effect of radiation on clonogenic potential of U87MG cells.

Irradiation of control cell population, stably transduced with pTRIPZ-NT vector showed reduction in clonogenic potential by 50–55% at a dose of 6 Gy with or without doxycycline treatment (Fig. 2A and B). Doxycline induction of miR-224 expression was found to reduce clonogenic potential of the polyclonal population expressing pTRIPZ-miR-224 by 35–40%. On irradiation and doxycycline induction of miR-224 expression, the pTRIPZ-miR-224 population showed 80–85% reduction in clonogenic potential. Thus, miR-224 expression was found to increase in radiation sensitivity of U87 MG cells.

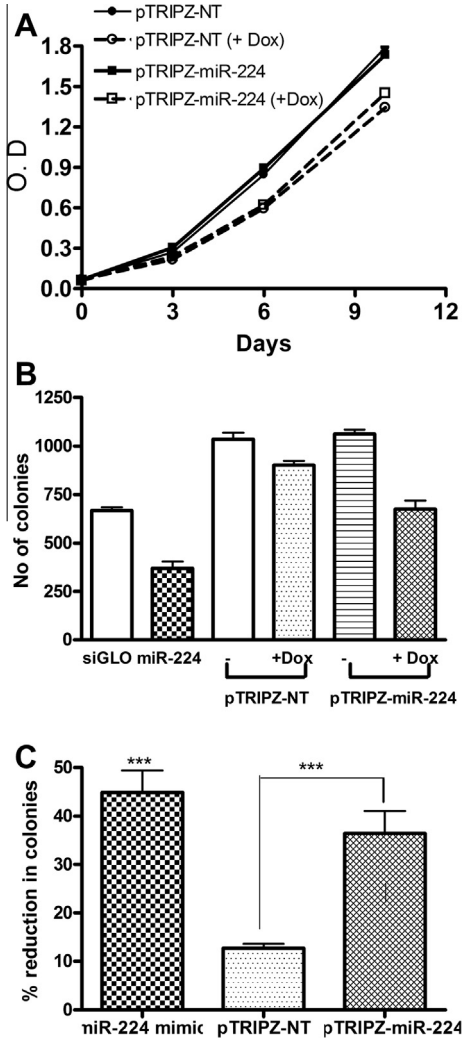

Fig. 1. Effect of miR-224 expression on growth of U87 MG cells studied by MTT assay. A. Growth of U87 MG cells transduced with pTRIPZ-NT or pTRIPZ-miR-224 vector was monitored over a period 9 days by MTT assay. *Y* axis indicates Optical Density (O.D.). B. Effect of miR-224 expression on anchorage independent growth potential of U87 MG cells studied by soft agar colony formation assay. *Y* axis indicates number of colonies obtained on seeding siGLO or miR-224 mimic transfected or pTRIPZ-NT or pTRIPZ-miR-224 transduced U87 MG cells. +Dox indicates induction with doxycycline. C. The percentage reduction in number of colonies obtained in soft agar assays performed with U87 MG cells before and after transient or stable miR-224 expression. The results are based on at least three independent soft agar assay experiments. All data points are presented as mean ± standard error (vertical bars).

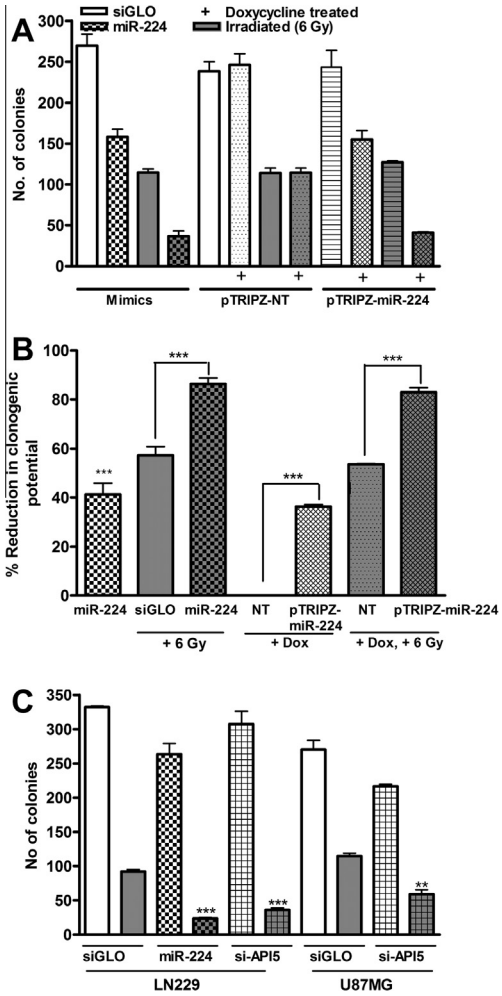

Fig. 2. Effect of miR-224 expression and siRNA mediated *API5* down-regulation on clonogenic potential A. *Y* axis indicates the number of colonies obtained after miR-224 expression using synthetic miRNA mimics and those obtained on plating the stable polyclonal populations of U87 MG cells transduced with pTRIPZ-NT or pTRIPZ-miR-224 vector before or after (+) induction with doxycycline. B. The percentage reduction in number of colonies obtained in clonogenic assays performed with U87 MG cells before and after transient or stable miR-224 expression and with or without irradiation. The results are based on at least three independent soft agar assay experiments. C. *Y* axis denotes the number of colonies obtained in a clonogenic assay performed on U87 MG and LN229 cells transiently transfected with control siGLO, miR-224 mimic or siRNA against *API5* (J-004375-07) with or without irradiation at a dose of 6 Gy. The numbers of colonies obtained on irradiation of the cells at a dose of 6 Gy are indicated by bars filled in grey shade. All data points are presented as mean ± standard error (vertical bars).

The effect of miR-224 expression was also analyzed on another glioblastoma cell line LN229. Clonogenic potential of miR-224 mimic transfected LN229 cells was found to be reduced by 20–25%. Irradiation of LN229 glioblastoma cells at a dose of 6 Gy was found to reduce clonogenic potential of the cells by 70–75% (Fig. 2C). MiR-224 expression was found to reduce clonogenic potential of LN229 cells on irradiation by 90–95%. Thus, miR-224 expression was found to enhance radiation sensitivity of LN229 glioblastoma cells as well.

3.3. MiR-224 mediated down-regulation of API-5 expression in glioblastoma cells

MiR-224 has been reported to bring about enhanced apoptosis of hepatocellular carcinoma cells by targeting apoptosis inhibitor 5 gene [9]. The expression levels of *API5* were evaluated in miR-224 expressing U87MG and LN229 cells by real time RT-PCR assay. U87MG and LN229 express *API5* at RQ in the range of 6.33 ± 1.07 (Fig. 3). *API5* expression levels in both the cell lines were found to be down-regulated by 55–65% on miR-224 expression. Reduction in clonogenic potential on miR-224 expression may therefore be a result of *API5* down-regulation. U87MG and LN229 cells were transiently transfected with an *API5* siRNA (J-004375-07) at 25 nM concentration. *API5* siRNA transfection resulted in 85–90% reduction in *API5* expression levels as judged by real time RT-PCR assay (Fig. 3).

3.4. Role of API5 in increase in radiation sensitivity of glioblastoma cells

Transfection of 25 nM *API5* siRNA ((J-004375-07) in U87 MG cells was found to reduce clonogenic potential of *API5* expression levels by 75–80% on irradiation at a dose of 6 Gy. Irradiation of control siGLO transfected cells brought about 50–60% reduction in clonogenic potential. Thus, siRNA mediated *API5* down-regulation was found to increase radiation sensitivity of U87MG cells. Similarly, siRNA mediated down-regulation of *API5* expression in LN229 cells was found to increase radiation sensitivity of LN229 cells as judged by reduction in clonogenic potential by 90–95% as compared to 70–75% reduction in siGLO transfected LN229 cells. Similar results were obtained using API5 siRNA (J-004375-06) (data not shown).

3.5. Correlation of miR-224 expression and overall survival of GBM patients

In order to investigate if miR-224 and *API5* expression levels in GBM tumor tissues influence treatment response and thereby overall survival of GBM patients, the Cancer Genome Atlas (TCGA) data on the expression profile of GBM tumor tissues was analyzed for possible correlation with overall survival of the GBM patients. The data on miR-224 expression levels in GBM tumor tissues assessed using Agilent 8 × 15 K miRNA arrays and that of *API5* expression levels assessed using Affymetrix U133A arrays was retrieved. Correlation of the miR-224 expression levels with overall survival of GBM patients 18–85 years of age was done by Kaplan–Meier analysis. The patients, who expired within the first month after surgery or those having follow-up data less than one month from surgery, were excluded from the analysis. Fig. 4A shows the histogram of miR-224 expression levels in 491 GBM patients. MiR-224 expression levels in GBM tumor tissues range from 5.63 to 8.89 with more than 78% of the values below 6.0. Thus, miR-224 expression in majority of the GBM tumor tissues is low. Log Rank test showed survival curves of the male GBM patients having miR-224 expression levels above 5.774 to be significantly different from those having lower expression levels (Fig. 4C). Male GBM patients having miR-224 expression levels above 5.774 were found to have better overall survival with the median survival duration of 435 days as compared to 300 days of those having lower miR-224 expression. *API5* expression levels in GBM patients were found to be in the range of 4.82–7.33 with median expression level of 6.661 (Fig. 4B). Kaplan–Meier survival curves of GBM patients having *API5* expression levels above or below the median expression level of 6.661 were found to be significantly ($p < 0.05$) different (Fig 4D). The median survival duration of GBM patients having lower *API5* expression was 469 days as compared to 383 days for those having higher *API5* expression.

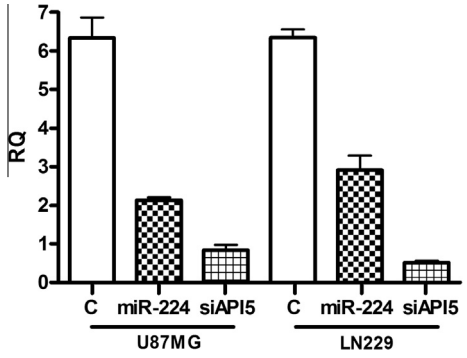

Fig. 3. *API5* expression levels in U87 MG and LN229 cells before and after transient transfection of control siGLO, miR-224 mimic or siRNA against *API5* (J-004375-07) as estimated by real time RT-PCR analysis using SYBR Green assay.

4. Discussion

In the present study, effect of miR-224 expression on growth characteristics of established glioblastoma cell lines U87 MG and LN229 was investigated. MiR-224 expression in the glioblastoma cell lines U87MG and LN229 was found to be absent or low. Analysis of the TCGA data showed miR-224 expression levels in majority of the GBM tumor tissues were also low, with the expression levels in the range of 5.5–6.0 in 427 out of 491 tumors analyzed. MiR-224 expression in U87MG cell line either in transient or stable manner was found to bring about 30–40% reduction in clonogenic potential. The effect of miR-224 expression on anchorage-independent growth potential as judged by the soft agar colony formation assay was found to be only marginally higher (30–55%) than the effect on the clonogenic potential indicating that the reduction in anchorage-independent growth is primarily due to the effect of miR-224 expression on clonogenic potential. MiR-224 expression was found to bring about 80–95% reduction in clonogenic potential on irradiation of U87 MG cells as well as LN229 cells. Thus, miR-224 expression was found to enhance radiation sensitivity of the two glioblastoma cell lines studied. MiR-224 expression has been reported to bring about apoptotic cell death of hepatocellular carcinoma, enhance activation induced cell death of Jurkat T cells and increase radiation sensitivity of medulloblastoma cells [6,9,10]. Under-expression of miR-224 has been reported in methotrexate resistant human colon cancer cells [11]. Thus, miR-224 expression enhancing radiation sensitivity of glioblastoma cells is in agreement with these observations on other established cancer cell lines.

MiR-224 expression has been reported to target Apoptosis Inhibitor 5 gene [9]. *API5* has been shown to suppress E2F transcription factor induced apoptosis [12]. MiR-224 expression in glioblastoma cells was found to decrease *API5* expression levels. Further, siRNA mediated down-regulation of *API5* was also found to reduce clonogenic potential of both U87MG and LN229 cells on irradiation. However, although *API5* siRNA brought about over

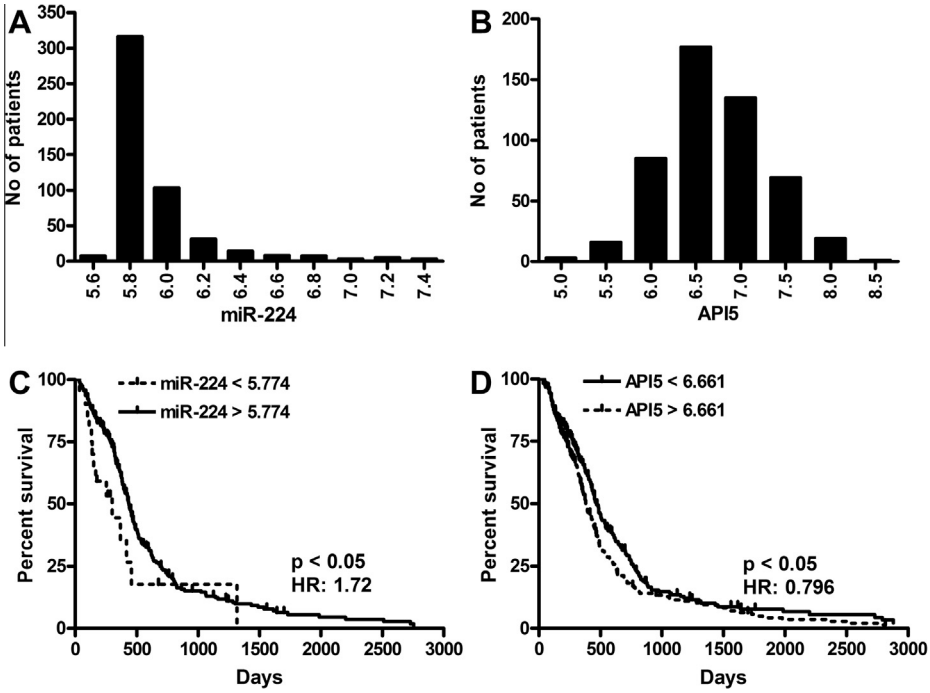

Fig. 4. Analysis of TCGA data on miR-224 and *API5* expression levels in GBM tumor tissues. A and C histograms show distribution of the miR-224 and *API5* expression levels in 505 GBM tumour tissues. B and D show Kaplan–Meier Survival analysis of 297 male GBM patients expressing miR-224 below or above 5.774 and 491 GBM patients expressing *API5* below or above the median level of 6.661. *p* Value indicates level of significant difference and HR indicates Hazard Ratio in the Kaplan–Meier survival curves estimated by Log Rank Test.

90% down-regulation in API mRNA levels as compared to 55–65% reduction on miR-224 expression, miR-224 expression was found to have higher radiation sensitizing effect on U87 MG cells (85–95% vs 75–80% in clonogenic potential) than *API5* siRNA. Therefore, targets other than *API5* may also be involved in mediating radiation sensitizing effect of miR-224.

Higher miR-224 expression was found to correlate with better survival in male GBM patients. This gender specific effect of miR-224 expression may be related to the fact that miR-224 is located on X chromosome. The present study wherein higher expression of miR-224 and lower expression of its target *API5* was found to correlate with better survival of GBM patients is in agreement with the lower miR-224 expression correlating with poor recurrence free survival of prostate cancer patients [13]. MiR-224 expression has been found to promote apoptosis of prostate cancer cells by targeting *TRIB1* gene [13,14]. MiR-224 expression levels have been reported to correlate inversely with tumor stage, nodal metastasis in colorectal cancer patients [15]. Up-regulation of miR-224 expression has been reported to correlate with absence of nodal metastasis, lower stages at diagnosis and higher biochemical cure in sporadic and familial medullary thyroid carcinomas [16]. Thus, a number of studies identify miR-224 expression to correlate with lower malignant potential and better survival rates.

On the other hand, up-regulation of miR-224 expression has been reported to correlate with higher grade of malignancy and poor survival in glioma patients in a study done on 108 glioma patients [17]. The study included both grade III and grade IV gliomas that differ significantly in their overall survival with the median survival duration of 3–5 years and 12–14 months for grade III astrocytic gliomas and grade IV GBMs, respectively. Therefore, higher miR-224 expression levels in GBM tumors in comparison with grade III gliomas would be interpreted as higher miR-224 expression levels correlating with poor survival. As stated before, majority (78%) of the GBM tumor tissues in the TCGA study were found to have low miR-224 expression. MiR-224 expression has also been reported to result in increased proliferation, migration, invasive potential of colorectal cancer cells and cervical cancer cells by targeting *SMAD4*, *HOXD10*, respectively [18,19]. Depending upon the expression of miR-224 target gens in the various cell types, effect of miR-224 expression may differ.

Radiation sensitizing effect of miR-224 on glioblastoma cells, indicates therapeutic potential of this miRNA in the treatment of the malignant glioblastoma that is refractory to the current treatment strategies. The effect of miR-224 on *in vivo* human GBM xenografts needs to be studied to further explore therapeutic potential of this miRNA. Further, radiation sensitizing effect of *API5* down-regulation in glioblastoma cells and correlation of higher *API5* expression with poorer survival of GBM patients suggests *API5* as a promising target to increase radiation sensitivity of glioblastomas. Identification of miR-224 targets other than *API5* that are instrumental in its radiation sensitizing effect is necessary for development of targeted treatment strategies that would enhance the radiation sensitizing effect and eliminate undesirable effect of miR-224, if any on migration, invasion of glioblastoma cells. In summary, the present study identified radiation sensitizing effect of miR-224 on glioblastoma cells, largely mediated by down-regulation of its target *API5*. Up-regulation of miR-224 and down-regulation of its target *API5* therefore appear as promising treatment options in combination with radiation for effective treatment of glioblastoma, the most aggressive brain tumor refractory to current treatment modalities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.095.

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